UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

TESE DE DOUTORADO

ABORDAGENS GENÉTICO-GENÔMICAS PARA IDENTIFICAÇÃO E VALIDAÇÃO DE QTLs DE TOLERÂNCIA AO ALUMÍNIO EM MILHO

ORIENTADO: CHRISTIANO COSTA SIMÕES

ORIENTADORA: CLAUDIA TEIXEIRA GUIMARÃES

BELO HORIZONTE

Julho – 2012

CHRISTIANO COSTA SIMÕES

ABORDAGENS GENÉTICO-GENÔMICAS PARA IDENTIFICAÇÃO E VALIDAÇÃO DE QTLs DE TOLERÂNCIA AO ALUMÍNIO EM MILHO

Tese apresentada ao Programa de Pós-Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Genética.

Área de concentração: Genômica e Bioinformática.

Orientadora: Cláudia Teixeira Guimarães.
Universidade Federal de Minas Gerais.

Co-orientador: Jurandir Vieira de Magalhães.
Universidade Federal de Minas Gerais.

Belo Horizonte

2012

Simões, Christiano Costa
Abordagens genético-genômicas para identificação e validação de QTLs de tolerância ao alumínio em milho. [manuscrito] / Christiano Costa Simões. – 2012.
46 f. : il ; 29,5 cm.

Orientadora: Cláudia Teixeira Guimarães. Co-orientador: Jurandir Vieira de Magalhães.

Tese (doutorado) – Universidade Federal de Minas Gerais, Departamento de Biologia Geral.

1. Genética quantitativa - Teses. 2. Mapeamento cromossômico – Teses. 3. Genética – Teses. 4. Milho – Genética – Teses. 5. Marcadores genéticos – Teses. 6. Genômica – Teses. 7. Bioinformática – Teses. 8. Técnicas de genotipagem. 9. Fatores abióticos. I. Guimarães, Cláudia Teixeira. II. Magalhães, Jurandir Vieira de. III. Universidade Federal de Minas Gerais. Departamento de Biologia Geral. IV. Título.

CDU: 577.2:575

Pós-Graduação em Genética
Departamento de Biologia Geral, ICB
Universidade Federal de Minas Gerais
Av. Antônio Carlos, 6627 - C.P. 486 - Pampulha - 31270-901 - Belo Horizonte - MG
e-mail: pg-gen@icb.ufmg.br FAX: (+31) - 3409-2570

"Abordagens Genético-Genômicas para Identificação e Validação de QTLs de Tolerância ao Alumínio em Milho"

Christiano Costa Simões

Tese aprovada pela banca examinadora constituída pelos Professores:

Cláudia Teixeira Guimarães - Orientadora
EMBRAPA

Jurandir Vieira de Magalhães - Co-orientador
EMBRAPA

Eduardo Martin Tarazona Santos
UFMG

Everaldo Gonçalves de Barros
UFV

Maria Raquel Carvalho
UFMG

Roberto Williams Noda
EMBRAPA

Belo Horizonte, 22 de junho de 2012.

AGRADECIMENTOS

À minha família: Athos, Maria, Luciano, Danilo, Edith, Cíntia, Thiago, Gabi, Marina e Pepê.

À Daniela, pelo seu amor, carinho, atenção compreensão e ajuda.

À Claudia Guimarães, pela orientação em todas as áreas da minha vida, apoio e amizade.

À Universidade Federal de Minas Gerais, em especial ao departamento de genética pela oportunidade, compreensão e respeito.

Aos amigos: Bira, Carlos, Célio, Gabriel, Bana, Miguel, Janaína, Bárbara, Juninho, Michel e Marlos por tornarem prazerosa a dura tarefa do doutorado.

A todos da Embrapa Milho e Sorgo, em especial ao NBA, por formarem um ambiente agradável de trabalho.

A todos que contribuíram de forma direta ou indireta para uma vitória na minha vida.

Aos órgãos financiadores: CNPq, FAPEMIG, McKnight, IFS, GCP, CAPES, Embrapa.

ÍNDICE

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS

^{o}C	Grau centígrado
µM	Micromolar
µmol	Micromol
AAA	Formas alélicas dos locos marcadores ZmMATE1, umc1018 e ZmMATE2 derivadas da linhagem Cateto Al237
ALMT	Aluminum-activated malate transporters
ALS	Aluminum sensitive
Alt_{SB}	Loco de tolerância ao Al em sorgo
ART1	Aluminum resistance transcription factor 1
AtALMT1	*Arabidopsis thaliana* ALMT
AtMATE	*Arabidopsis thaliana* MATE
BBB	Formas alélicas dos locos marcadores ZmMATE1, umc1018 e ZmMATE2 derivadas da linhagem L53
BnALMT	*Brassica napus* ALMT
CI	Confidence interval
GBS	Genotyping-by-sequencing – genotipagem por sequenciamento
GLM	Generalized Linear Model
h	Horas
HvAACT1	*Hordeum vulgare* MATE
HvALMT1	*Hordeum vulgare* ALMT
MATE	multidrug and toxic compound extrusion
Mb	Mega bases - Milhões de pares de base
MIM	Multiple interval mapping
NILs	*Near Isogenic Lines* – linhagens semi-isogênicas
Nramp	Natural resistance-associated macrophage protein
Nrat1	Nramp aluminum transporter 1
NRG	Net root growth – crescimento líquido da raiz
OsFRDL4	*Oryza sativa* MATE
QTL	Quantitative trait loci – loco de característica quantitativa
RIL	Recombinant Inbred Line – linhagens recombinantes endogâmicas
RNRG	Relative net root growth – crescimento relativo da raiz
R^2	Coeficiente de determinação
SNP	Single nucleotide polymorphism – polimorfismo de nucleotídeo único
SbMATE	Sorghum MATE

ScALMT1	*Secale cerale* ALMT.
ScFRDL2	*Secale cerale* MATE
SSR	Single sequence repeat
STAR	Sensitive to Al rhizotoxicity
STOP1	Sensitive to proton rhizotoxicity 1
TaALMT1	*Triticum aestivum* ALMT
TaMATE1	*Triticum aestivum* MATE
ZmASL	*Zea mays* Alt_{SB}-like
ZmMATE	*Zea mays* MATE
ZmALMT	*Zea mays* ALMT

RESUMO

O Al tóxico é um dos principais fatores limitantes ao crescimento radicular em solos ácidos, acarretando sérias limitações á produção agrícola. Estes solos estão amplamente distribuídos nas regiões tropicais e subtropicais, englobando cerca de 50% das terras agricultáveis no planeta. O desenvolvimento de genótipos tolerantes ao Al é uma alternativa sustentável para superar as limitações causadas pelos solos ácidos. A tolerância ao Al em milho é uma característica complexa, envolvendo possivelmente múltiplos genes e mecanismos, que não estão bem compreendidos até o momento. No presente trabalho, foram avaliados 36.147 marcadores SNPs gerados pela técnica de genotipagem por sequenciamento (GBS), 39 SSRs e três genes candidatos em uma população de RILs derivada do cruzamento entre duas linhagens altamente contrastantes quanto à tolerância ao Al. A associação entre marcadores e fenótipo foi realizada por meio de modelos lineares generalizados (GLM) e do mapeamento de QTLs por intervalos múltiplos (MIM), sendo identificados oito QTLs nos cromossomos 2, 3, 4, 5, 6 e 8. Um QTL de efeito maior, explicando 22% da variância genotípica da tolerância ao Al, foi identificado no cromossomo 6 (bin 6.00), corroborando com resultados previamente publicados. Nessa região, além do gene candidato *ZmMATE1*, dois QTLs de expressão (eQTL) foram mapeados flanqueando o gene alvo, sendo considerados como *cis* eQTLs. Essa região genômica foi transferida para linhagens semi-isogênicas de milho, resultando em um aumento de duas vezes na tolerância ao Al, associados com um aumento na expressão do *ZmMATE1*. Tais resultados validam o QTL6 como uma região genômica capaz de aumentar a tolerância ao Al em milho. O gene candidato *ZmMATE2* foi co-localizado com o segundo QTL de maior efeito para a tolerância ao Al, mas não foi validado nas linhagens semi-isogências de milho. No entanto, um *trans* eQTL explicando 24% da variação genotípica da expressão do *ZmMATE2* foi mapeado no cromossomo 3. O uso de uma elevada densidade de marcadores permitiu um aumento considerável na precisão dos QTLs identificados, cujos intervalos de confiança foram restringidos entre 1,7 e 31,7 Mb. A integração entre informações do mapa genético com distância física foi possível devido ao alinhamento das sequências dos SNP no genoma de referência do milho, outra grande vantagem dos marcadores baseados na técnica de GBS. Assim, os resultados gerados contribuem com informações relevantes que podem ser aplicadas diretamente no melhoramento assistido visando o desenvolvimento de genótipos de milho mais tolerantes ao Al. Adicionalmente, as demais regiões de QTLs associadas com buscas *in silico* possibilitaram elencar novos genes candidatos, que poderão ser alvos para estudos avançados contribuindo para uma melhor compreensão dos mecanismos e genes envolvidos na tolerância ao Al em milho.

ABSTRACT

The Al toxicity is a major constraint for root growth on acid soils, leading to serious limitations in crop production. These soils are widely distributed in tropical and subtropical regions, comprising about 50% of arable land on the planet. The development of Al tolerant genotypes is a sustainable alternative to overcome the limitations caused by acid soils. Aluminum tolerance is a complex trait in maize, possibly involving multiple genes and mechanisms that are still not well understood. In our current work, 36,147 SNPs based on genotyping-by-sequencing technology (GBS), 39 SSRs and three candidate genes were assessed in a population of RILs derived from a cross between two maize lines highly contrasting for Al tolerance. Marker-trait associations were performed using generalized linear models (GLM) and multiple interval mapping (MIM), been identified eight QTL on chromosomes 2, 3, 4, 5, 6 and 8. A major QTL, explaining 22% of the genotypic variance of Al tolerance, was mapped on chromosome 6 (bin 6.00), confirming previously published results. The candidate gene *ZmMATE1* was mapped in this region, as well as two expression QTL (eQTL) flanking the target gene, which were considered as *cis* eQTL. This genomic region was transferred to maize near-isogenic line, resulting in a two-fold increase of Al tolerance associated with an enhanced *ZmMATE1* expression. These results validated the QTL6 as capable to improve Al tolerance in maize. The candidate gene *ZmMATE2* was co-located with the Al tolerance QTL5.1, but it was not validated in the lines the maize NILs. However, a *trans* eQTL explaining 24% of genotypic variation of *ZmMATE2* expression was mapped to chromosome 3. A high density of GBS-based markers allowed a considerable precision improvement of QTL identified, whose confidence intervals were restricted from 1.7 to 31.7 Mb. Integration of genetic map information with physical genomic position was possible due to the alignment of SNP sequences in the reference maize genome, which is another great advantage of these GBS-based markers. Thus, the results generated here can be directly applied on marker-assisted breeding to develop maize genotypes with improved Al tolerance. Moreover, the other QTL regions combined with in silico search allowed to select new candidate genes to be target for advanced studies, which can contribute to a better understanding of the mechanisms and genes involved in maize Al tolerance.

INTRODUÇÃO

O milho é uma cultura mundialmente disseminada, sendo principalmente utilizada na alimentação animal e humana. No Brasil, a produção estimada de milho é de 67.8 milhões de toneladas para a safra 2011/2012, ranqueando o Brasil como terceiro maior produtor mundial de milho (CONAB, 2012). Apesar do grande volume produzido, a produtividade média do milho no Brasil ainda é baixa, sendo comparável com a de países da África e da América Latina, que também possuem solos ácidos (FAO, 2012).

Os solos ácidos estão presentes em aproximadamente 50% das terras cultiváveis do mundo e apresentam importantes entraves à produção mundial de alimentos (von Uexküll e Mutert, 1995). Nesses solos, onde o pH normalmente está abaixo de 5,0, o alumínio encontra-se na forma solúvel Al^{+3}, que possui ação citotóxica, inibindo o crescimento radicular (Kochian et al., 2004). As anomalias e os danos causados ao sistema radicular restringem o volume de solo explorado, resultando em prejuízos na absorção de nutrientes e no aproveitamento da água do solo (Kochian et al., 2004). Assim, os efeitos causados pelo Al culminam em uma maior sensibilidade ao estresse de seca e em perdas na produtividade das culturas.

A aplicação do calcário é frequentemente utilizada para aumentar o pH dos solos ácidos, visando neutralizar a toxidez do alumínio (Al) solúvel. Porém, a calagem não é efetiva para neutralização do Al nas camadas abaixo de 20 cm, limitando o aprofundamento das raízes (Foy et al., 1984). Além disso, o uso do calcário implica em aumento nos custos de produção e, em algumas regiões, o acesso ao insumo é escasso, limitando a produção agrícola nesses locais. Adicionalmente, Ciotta et al. (2002) verificaram a acidificação de um solo cultivado por 21 anos sob condições de plantio direto com as culturas de trigo, soja, arroz e pastagem. Como o plantio direto é uma prática agrícola amplamente utilizada no Brasil, é possível que a acidez dos solos agricultáveis seja intensificada, necessitando de estratégias adicionais para manter e elevar os níveis de produtividade agrícola. Assim, uma alternativa sustentável para atingir tais objetivos é o desenvolvimento de cultivares tolerantes ao Al. Para isso, a identificação de fatores genéticos que controlam a tolerância ao Al torna-se fundamental para auxiliar programas de melhoramento visando à geração de cultivares mais adaptados ao cultivo em solos ácidos.

Dentre os métodos descritos para avaliar a tolerância ao Al em plantas, os ensaios em solução nutritiva foram propostos por Aniol (1984) e são os mais utilizados em estudos moleculares e fisiológicos, uma vez que o efeito do Al pode ser avaliado independentemente de outros fatores. O índice fenotípico normalmente utilizado é a inibição do crescimento radicular em função da presença de níveis tóxicos de Al. Nessas condições, as plantas sensíveis sofrem uma maior inibição do crescimento radicular em comparação com as

plantas tolerantes. Como existe uma variabilidade intrínseca ao desenvolvimento das raízes, é importante que a medida do crescimento radicular sob estresse de Al seja em relação ao crescimento da raiz sem Al, também conhecido como crescimento relativo. Parentoni et al. (2003) demonstraram a importância da utilização de experimentos controle (sem Al) para corrigir o crescimento da raízes sob níveis tóxicos de Al em milho. Esses autores, utilizando nove linhagens de milho com diferentes níveis de tolerância ao Al, verificaram o aumento da relação entre efeitos aditivos e não aditivos de 0,50 para 2,67, com o uso do crescimento relativo em comparação ao crescimento líquido com Al.

A tolerância ao Al em milho é uma característica de herança quantitativa (Magnavaca et al., 1987; Pandey et al., 1994; Lima et al., 1995) e estudos de mapeamento de QTLs usando crescimento relativo das raízes como índice fenotípico, resultaram na identificação de duas a cinco regiões genômicas associadas com a tolerância ao Al nos cromossomos 2, 4, 5, 6, 8 e 10 de milho (Sibov et al., 1999; Ninamango-Cárdenas et al., 2003; Conceição et al., 2009). Entretanto, em função da baixa saturação de marcadores, poucas regiões podem ser consideradas coincidentes entre esses estudos, além de englobarem grandes distâncias físicas, inviabilizando uma busca mais direcionada por genes candidatos.

Uma tentativa de integrar informações sobre genes diferencialmente expressos e QTLs de tolerância ao Al em milho foi realizada por Mattiello et al. (2012), mas uma vasta lista de genes candidatos foi obtida, o que requer estudos adicionais para uma caracterização detalhada da função desses genes. Um número bem menor de genes candidatos foi obtido por meio da combinação entre a análise de ligação e o mapeamento associativo (Krill et al., 2010), onde apenas quatro candidatos foram selecionados como possivelmente associados com a tolerância ao Al em milho. Dentre eles, o gene *ZmALMT2* foi caracterizado como um transportador de membrana, mas não foi relacionado com a tolerância ao Al em milho (Ligaba et al., 2012). Já a integração do mapeamento de QTLs com estudos funcionais resultou na identificação do gene *ZmMATE1* co-localizado com um QTL de efeito maior para a tolerância ao Al no cromossomo 6 de milho (Maron et al., 2010). O gene *ZmMATE1* codifica uma proteína transmembrana que media a exsudação de citrato no ápice radicular em milho. A super-expressão desse gene em Arabidopsis resultou em um aumento na exsudação de citrato associado com uma maior tolerância ao Al (Maron et al., 2010). Esse resultado genético corrobora com estudos fisiológicos, que apontam a exsudação de citrato pelas raízes como o principal mecanismo de tolerância ao Al em milho (Piñeros et al., 2002). No entanto, Piñeros et al. (2005) sugerem a existência de outros mecanismos complementares para tolerância ao Al nessa espécie.

O milho tem o genoma relativamente grande (2500 Mb) quando comparado com o do arroz (450 Mb) (Messing et al., 2004), aproximando-se ao tamanho próximo do genoma humano (2900 Mb) (Venter et al., 2001). Os 10 cromossomos do milho são estruturalmente

diversos e estão submetidos a mudanças dinâmicas na composição de sua cromatina, onde foram preditos mais de 32.000 genes (Schnable et al., 2009), enquanto que no genoma humano há predição de cerca de 27.000 genes ao longo dos 23 cromossomos (Venter et al., 2001). Consequentemente, a identificação de genes envolvidos com a tolerância ao Al em milho consiste em uma tarefa complexa que necessita da integração de informações como mapeamento de QTLs, sequenciamento, estudos de expressão gênica em larga escala e análise comparativa de genomas. Considerando a complexidade do genoma do milho, as evidências do envolvimento de múltiplos genes e mecanismos na tolerância ao Al tóxico e a deficiência no entendimento sobre genes/QTLs que controlam essa característica, torna-se estratégico o delineamento de estratégias genético-moleculares que permitam desvendar o controle genético da tolerância ao Al em milho. Além do conhecimento gerado sobre QTLs e genes candidatos, tais informações são fundamentais para subsidiar programas de melhoramento visando o aumento dos patamares de tolerância ao Al em milho, com potencial impacto na produtividade de grãos em solos ácidos.

Assim, o presente trabalho foi estruturado em dois capítulos. O capítulo 1 apresenta uma revisão enfocando genes e mecanismos envolvidos com a tolerância ao Al em plantas, que foi aceito para publicação na revista *Genetics and Molecular Research*. Já o capítulo 2 descreve o mapeamento de QTLs com uma alta densidade de marcadores moleculares integrado com análises de expressão gênica e de bioinformática visando à identificação de genes candidatos e de QTLs associados com a tolerância ao Al em milho. O artigo será submetido para publicação na revista *BMC Genomics*. No entanto, as figuras e as tabelas serão apresentadas à medida que forem citadas no texto para facilitar a leitura.

OBJETIVOS

- Mapear QTLs e genes candidatos associados com a tolerância ao Al em milho.
- Avaliar o perfil de expressão de genes candidatos previamente mapeados nas regiões de QTLs.
- Validar QTLs de tolerância ao Al em linhagens semi-isogênicas.
- Buscar genes candidatos nas regiões de QTLs associados com tolerância ao Al.

REFERÊNCIAS BIBLIOGRÁFICAS

ANIOL, A.; GUSTAFSON, J. P. Chromosome location of genes controlling aluminum tolerance in wheat, rye and triticale. **Canadian Journal of Genetics and Cytology**, 26: 701-705, 1984.

CIOTTA M. N.; BAYER C.; ERNANI P. R.; FONTOURA S. M. V.; ALBUQUERQUE J. A.; WOBETO C. Acidificação de um latossolo sob plantio direto**. Revista Brasileira de Ciência do Solo**, 4:1055-1064, 2002.

CONAB: Companhia Nacional de Abastecimento. Brasília: CONAB, 2012. Disponível em <http://www.conab.gov.br/conabweb/> Acesso em 05 jun 2012.

CONCEIÇÃO, L.D.H.C.S.; TESSELE C, BARBOSA NETO JF: **Diallel analysis and mapping of aluminum tolerance in corn inbred lines.** *Maydica* 2009, **54:**51-64.

FAO Statistical Database. FAO, 2012. Disponível em <http://faostat.fao.org/default.aspx> Acesso em: 20 de fevereiro de 2012.

FOY, C. D. Physiological effects of hydrogen, aluminum and manganese toxicities in acid soil. In: ADAMS, F. (Ed.). **Soil acidity and liming**, 12: 57-97, 1984.

KOCHIAN, L. V.; HOEKENGA, A. O.; PIÑEROS, M. A. How do plants tolerate acid soils? Mechanism of aluminum tolerance and phosphorus efficiency**. Annual Reviews Plant Molecular Biology,** 55: 459-493, 2004.

KRILL AM, KIRST M, KOCHIAN LV, BUCKLER ES, HOEKENGA OA: Association and linkage analysis of aluminum tolerance genes in maize. **Plos One** 2010, 5: 1-11.

LIGABA A, MARONL, SHAFF J, KOCHIAN L, PIÑEROS M: Maize ZmALMT2 is a root anion transporter that mediates constitutive root malate efflux. **Plant Cell Environment**, in press. 2012

LIMA M, MIRANDA FILHO JB, FURLANI PR: Diallel cross among inbred lines of maize differing in aluminum tolerance. **Brazilian Journal of Genetics**, 4:579-584. 1995.

MAGNAVACA R, GRAMINER CD, CLARK, RB: **Inheritance of aluminum tolerance in maize**. In: Genetic aspects of plant mineral nutrition. Ed. Gabeiman HW, Lougnmam BC. Dordrecht: Martinus Nijhoff; 1987:201-212.

MARON LG, PIÑEROS MA, GUIMARÃES CT, MAGALHÃES JV, PLEIMAN JK, MAO C, SHAF J, BELICUAS SN, KOCHIAN LV: Two functionally distinct members of the MATE (multidrug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. **Plant Journal**, 61:728-740. 2010.

MATTIELLO, L.; SILVA, F.R.; MENOSSI, M. Linking microarray data to QTLs highlights new genes related to Al tolerance in maize. **Plant Science**, 191-192: 8-15, 2012.

NINAMANGO-CÁRDENAS F, GUIMARÃES CT, MARTINS PR, PARENTONI SN, CARNEIRO NP, LOPES MA, MORO JR, PAIVA E: Mapping QTLs for aluminum tolerance in maize. **Euphytica**, 130: 23-232. 2003.

PANDEY S, MAGNAVACA R, BAHIA FILHO AFC, DUGUE-VARGES J, VINASCO LE: Genetics of tolerance to soil acidity in tropical maize. **Crop Science**, 34:1511-1514. 1994.

PARENTONI, S. N.; ALVES, V. M. C.; OLIVEIRA, A. C.; MAGALHÃES, J. V.; COELHO, A. M.; SCHAFFERT, R. E.; GUIMARAES, C. T.; GAMA, E. E. G. E.; GODOY, C.L.; SANTOS, M. X.; GUIMARAES, P. E. O.; PACHECO, C. A. P.; MEIRELLES, W. F.; RIBEIRO, P. H. E.; PITTA, G.; BAHIA FILHO, A. F. C.. Genetics of aluminum tolerance in maize evaluated in nutrient solution with and without control experiments. In: **International Symposium on Plant Breeding**, 60-61, 2003.

SCHNABLE, P. S., et al. The B73 Maize Genome: Complexity,Diversity, and Dynamics. **Science**, v.326, p.112-115, 2009.

SIBOV ST, GASPAR MJ, OTTOBONI LMM, ARRUDA P, SOUZA AP: Two genes controlling aluminum tolerance in maize: genetic and molecular mapping analyses. **Genome**, 42:475-482. 1999.

VENTER, J. C., et al. The sequence of the human genome. **Science**, v.291, p1304-1351, 2001.

VONUEXKÜLL H R, MUTERT E: Global extent, development and economic impact of acid soils. **Plant Soil**, 71:1-15. 1995.

CAPÍTULO 1

Genetic and molecular mechanisms of aluminum tolerance in plants

C.C. Simões[1], J.O. Melo[1], J.V. Magalhaes[2] and C.T. Guimarães[2]

[1]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[2]Núcleo de Biologia Aplicada, Embrapa Milho e Sorgo, Sete Lagoas, MG, Brasil

C.C. Simões and J.O. Melo contributed equally as first authors

Corresponding author: C.T. Guimarães; E-mail: claudia@cnpms.embrapa.br

Running title: Mechanisms of aluminum tolerance in plants

ABSTRACT

Aluminum toxicity restricts root growth and agricultural yield in acid soils, which constitute approximately 40% of the potentially arable lands worldwide. The two main mechanisms of aluminum tolerance in plants are internal detoxification of Al and its exclusion from root cells. Genes encoding membrane transporters and accessory transcription factors, as well as *cis*-elements that enhance gene expression, are involved in Al tolerance in plants; thus studies of these genes and accessory factors should be the focus of molecular breeding efforts aimed at improving Al tolerance in crops. In this review, we describe the main genetic and molecular studies that led to the identification and cloning of genes associated with Al tolerance in plants. We include recent findings on the regulation of genes associated with Al tolerance. Understanding the genetic, molecular, and physiological aspects of Al tolerance in plants is important for generating cultivars adapted to acid soils, thereby contributing to food security worldwide.

Key words: Aluminum; Tolerance; Organic acids; QTL; Regulatory factors

INTRODUCTION

Aluminum (Al) toxicity in acid soils is an important abiotic stress factor that reduces crop yield (Ma et al., 2001). In addition to the extensive distribution of acid soils in tropical and subtropical regions, especially in regions where food supply is more tenuous, agricultural activities can also lead to soil acidification (Ciotta et al., 2002). Under acidic conditions, Al is

released from soil minerals in ionic forms such as $Al(OH)_2^+$, $Al(OH)^{2+}$, and $Al(H_2O)_6^{3+}$, with the last species being commonly known as Al^{3+} (Kinraide et al., 1992). Low soil pH allows for the solubilization and release of Al3+ ions into the rhizosphere, causing a highly toxic effect on sensitive plants (Delhaize and Ryan, 1995).

The effect of Al on plant metabolic processes can be observed within minutes after the onset of the stress syndrome, and is followed by secondary effects that occur at later stages (Kochian, 1995). In the cell wall, Al may bind to carboxyl and phosphate groups due to its high affinity for electron donors such as oxygen (Dale and Sutcliffe, 1986), causing structural changes and compromising cell wall expansion (Ma et al., 2004a). Therefore, Al toxicity causes severe damage to root systems, which consequently reduces water and mineral nutrient uptake, thus limiting agricultural yield (Ma et al., 2001; Kochian et al., 2004).

MECHANISMS OF ALUMINUM TOLERANCE

Mechanisms of Al tolerance are classified as those that prevent Al ions from entering the root apical cells (i.e., apoplastic mechanisms) or that detoxify internal Al (i.e., symplastic mechanisms) (Kochian, 1995; Kochian et al., 2004). In symplastic mechanisms, Al enters the cytoplasm and is detoxified once inside the cell by complexation with organic compounds (Kochian, 1995). Several compounds can form stable complexes with Al inside the cell, including organic acids such as citrate, oxalate, and malate (Foy, 1988; Taylor, 1988, Ma and Miyasaka, 1998) and proteins (Suhayda and Haug, 1985). Free Al3+ or Al complexes with chelating agents can be transported to cell vacuoles, where they are stored without causing toxicity (Kochian et al., 2004).

Apoplastic mechanisms are also known as Al exclusion mechanisms. The following Al exclusion mechanisms have been reported: release of phenolic compounds (Ofei-Manu et al., 2001), mucilage formation (Miyasaka and Hawes, 2001), "pH barrier" resulting from increased pH in the rhizosphere (Degenhardt et al., 1998) and organic acid exudation (Delhaize et al., 1993; Pellet et al., 1995; Sasaki et al., 2004; Magalhaes et al., 2007). Roots of several plant species secrete organic acids in response to Al, which are mediated by membrane transporters, resulting in the formation of non-toxic complexes with the metal. Thus, this mechanism prevents Al from crossing the plasma membrane into the symplast.

Although organic acid exudation is a conserved Al tolerance mechanism being present in different plant species, there are species-specific peculiarities worth noting. The Al-activated mechanism of malate exudation is well described in wheat (Delhaize et al., 1993; Sasaki et al., 2004), Arabidopsis (Hoekenga et al., 2006), Brassica napus (Ligaba et al., 2006), and rye (Collins et al., 2008), whereas the mechanism of Al tolerance in maize, soybean, sorghum, and barley involves mainly citrate release (Pellet et al., 1995; Yang et al., 2000; Magalhaes et al., 2007; Furukawa et al., 2007; Maron et al., 2010).

Nevertheless, co-occurrence of different Al tolerance mechanisms has been reported in some species. In maize, root citrate (Piñeros et al., 2002) and oxalate (Kidd et al., 2001) exudation are likely involved in Al tolerance. However, Piñeros et al. (2005) observed a low correlation between citrate exudation and Al tolerance in maize, suggesting that this species has other complementary mechanisms enabling them to tolerate Al. In addition to malate, citrate exudation has also been reported to contribute to Al tolerance in wheat, Arabidopsis, and rye (Ryan et al., 2009; Liu et al., 2009; Yokosho et al., 2010). In rice, citrate exudation (Yokosho et al., 2011) as well as symplastic mechanisms are likely to contribute to the extreme Al tolerance in this species (Huang et al., 2009).

ALUMINUM TOLERANCE GENES

The ALMT family

The genetic control of the Al tolerance mechanism based on malate exudation is due to the action of genes encoding aluminum-activated malate transporters in the ALMT family. The first Al tolerance gene to be cloned in plants was designated *TaALMT1*, which encodes a transporter protein involved in malate exudation from root apices and is responsible for Al tolerance in wheat (Sasaki et al., 2004). *TaALMT1* was mapped to chromosome 4DL, co-segregating with a major Al tolerance QTL identified in different wheat populations (Raman et al., 2005; Ma et al., 2005). Based on its location, *TaALMT1* possibly corresponds to the previously mapped Al tolerance loci, *Alt2* (Luo and Dvorák, 1996) and Alt_{BH} (Ried and Anderson, 1996).

A large number of *ALMT* members were implicated in malate exudation and Al tolerance in Arabidopsis (*AtALMT1*, Hoekenga et al., 2006), rapeseed (*BnALMT1* and *BnALMT2*, Ligaba et al., 2006), rye (*ScALMT1*, Collins et al., 2008), and barley (*HvALMT1*, Gruber et al., 2010). In contrast, *ZmALMT1* (Piñeros et al., 2008) and *ZmALMT2* (Ligaba et al., 2012), two members in the ALMT family, were not found to be associated with maize Al tolerance.

The MATE family

Members of the multidrug and toxic compound extrusion (MATE) family have been associated with several cellular processes, including Al tolerance. Al tolerance in sorghum relies mostly on the Alt_{SB} locus, which has a major phenotypic effect and has been mapped to sorghum chromosome 3 (Magalhaes et al., 2004). The *SbMATE* gene mediates Al-activated citrate exudation from root apices and underlies the Al tolerant locus, Alt_{SB} (Magalhaes et al., 2007). *SbMATE* expression is induced with time of exposure to Al and is higher in the root apex compared to the rest of the root (Magalhaes et al., 2007). As in sorghum, Al tolerance in barley is related to citrate efflux mediated by *HvAACT1*, which also

belongs to the MATE family and is highly expressed in roots of Al-tolerant barley genotypes (Furukawa et al., 2007). QTL mapping in this species has shown that *HvAACT1* is located on chromosome 4H, co-localizing with a major QTL that explains more than 50% of the phenotypic variation in Al-activated citrate exudation (Ma et al., 2004b).

Functional MATE homologs associated with Al tolerance were also identified in Arabidopsis (*AtMATE*; Liu et al., 2009), wheat (*TaMATE1*; Ryan et al., 2009), rye (*ScFRDL2*; Yokosho et al., 2010), and rice (*OsFRDL4*, Yokosho et al., 2011). Some of these genes are located near Al tolerance QTL, such as *OsFRDL4*, which co-localizes with a QTL on chromosome 1 that was detected in different studies (Yokosho et al., 2011). A major Al tolerance QTL explaining 49% of the phenotypic variation was mapped to wheat chromosome 3BL (Navakode et al., 2009), which possibly harbors *TaMATE1*. According to the authors, this is supported by the fact that this region in wheat is synthenic to sorghum chromosome 3 and rice chromosome 1, where Al tolerance MATE members were located (Navakode et al., 2009).

Two maize MATEs, *ZmMATE1* and *ZmMATE2*, were co-localized with two major Al tolerance QTL on maize chromosomes 6 and 5, respectively (Maron et al., 2010). *ZmMATE1* encodes a transmembrane protein that is highly similar to SbMATE, and its overexpression in Arabidopsis results in increased citrate exudation as well as higher Al tolerance (Maron et al., 2010). Al tolerance QTL were mapped to this genomic region of chromosome 6 in two other studies using different mapping populations (Sibov et al., 1999; Ninamango-Cárdenas et al., 2003). In contrast, *ZmMATE2* expression, which was not induced by Al, was similar between Al-tolerant and Al-sensitive genotypes (Maron et al., 2010). In addition, association between ZmMATE2 and citrate exudation has not been found, raising questions to a possible role for *ZmMATE2* in maize Al tolerance.

ATP-binding cassette (ABC) transporter family

In addition to genes encoding organic acid transporters, other genes have been associated with Al tolerance in plants. Two genes encoding ATP-binding cassette (ABC) transporters, *ALS3* and *ALS1*, were associated with Al tolerance in Arabidopsis (Larsen et al., 2005; 2007). *ALS1* is primarily expressed in the root apex and vascular tissues, and ALS1 is present in vacuolar membranes (Larsen et al., 2007). *ALS3* is expressed in different organs but mainly in leaf hydathodes and phloem, whereas ALS3 is localized to the plasma membrane (Larsen et al., 2005). Knockout mutants of both genes caused Al hypersensitivity but their overexpression in Arabidopsis did not improve Al tolerance. ALS1 and ALS3 have been hypothesized to act in the intracellular redistribution of Al, keeping this metal away from sensitive tissues (Larsen et al., 2005; 2007).

In rice, sensitive to aluminum rhizotoxicity genes 1 and 2 (*STAR1* and *STAR2*) were identified and the fact that knocking out either *star1* or *star2* resulted in Al hypersensitivity suggested their function in Al tolerance (Huang et al., 2009). *STAR1* encodes a nucleotide-binding domain, whereas *STAR2* encodes a transmembrane domain of a bacterial-type ABC transporter, which is involved in UDP-glucose transport (Huang et al., 2009). *STAR1* and *STAR2* are primarily expressed in the roots and specifically induced by Al, and the proteins encoded by these genes form a complex that localizes to cytosolic vesicles membranes. Although the mechanism triggered by this transporter is not yet completely understood, the authors suggest that UDP-glucose may be involved in cell wall modifications, reducing the toxic effects of Al (Huang et al., 2009). Recently, a half-size ABC transporter encoded by OsALS1 was functionally characterized as responsible for Al sequestration into vacuole, which is required for internal detoxification of this metal in rice (Huang et al., 2012).

Nramp family

Recently, the Nramp aluminum transporter 1 (*Nrat1*) was found to be associated with Al tolerance in rice. *Nrat1* belongs to the natural resistance-associated macrophage protein (Nramp) transporter family (Xia et al., 2010). Nramp proteins are conserved in different species and are involved in divalent ion transport (Courville et al., 2006; Xia et al., 2010). Nrat1 is a transporter located in the plasma membrane of root apical cells, exhibiting transport activity for Al^{3+}, but not for divalent metals or the Al-citrate complex. *Nrat1* expression is induced by Al and is root-specific, occurring in all root cells, except for the epidermis. Knockout lines for *Nrat1* exhibited higher Al sensitivity, higher Al accumulation in the cell wall, and lower Al concentration in root cells in the presence of Al^{3+} (Xia et al., 2010). Such findings led the authors to suggest that Nrat1 controls intracellular Al^{3+} uptake, with subsequent detoxification via transport and Al accumulation into cell vacuoles, possibly mediated by OsALS1 (Huang et al., 2012).

REGULATION OF ALUMINUM TOLERANCE GENE EXPRESSION

Due to the close relationship between allelic variation for Al tolerance and the expression of Al tolerance genes, efforts are underway to validate the molecular nature of regulatory factors involved in Al tolerance. In Arabidopsis, the sensitive to proton rhizotoxicity 1 (*STOP1*) gene was identified, which encodes a transcription factor involved in Al tolerance (Iuchi et al., 2007; Liu et al., 2009; Sawaki et al., 2009). Initially, Iuchi et al. (2007) described a proton-sensitive Arabidopsis mutant, where a recessive mutation was detected in a gene encoding a Cys_2-His_2 transcription factor. The stop1 mutant showed reduced root growth under low pH conditions and under Al toxicity. Interestingly, these phenotypes were associated with inhibited *AtALMT1* gene expression and malate exudation after Al treatment

(Iuchi et al., 2007). Microarray analyses of *stop1* indicated that multiple genes possibly involved in Al tolerance are co-regulated by STOP1 (Sawaki et al., 2009). Among those genes is ALS3 (Larsen et al., 2005), which was repressed in the *stop1* mutant (Sawaki et al., 2009). Additional studies indicated that STOP1 is also necessary for *AtMATE* expression and Al-activated citrate exudation in Arabidopsis. Therefore, although both *AtALMT1* and *AtMATE* genes act independently to confer aluminum tolerance in Arabidopsis, the STOP1 transcription factor represents a transcriptional link between them (Liu et al., 2009).

The Al resistance transcription factor 1, *ART1*, is a rice homologue of *AtSTOP1* that regulates the expression of several genes related to rice Al tolerance, such as *STAR1* and *STAR2* (Yamaji et al., 2009), *Nrat1* (Xia et al., 2010), *OsFRDL4* (Yokosho et al., 2011) and *OsALS1* (Huang et al., 2012).

Cis-elements are located in non-coding regions along the DNA sequence, near or far from the target gene and influence gene expression (von Korff et al., 2009). *Cis*-acting regulatory sequences, such as polymorphisms within introns, and modified promoter regions, may influence aluminum tolerance in plants.

In sorghum, the coding region of the aluminum tolerance gene, *SbMATE*, was identical between Al-tolerant and Al-sensitive genotypes, with polymorphisms being found in the second intron of *SbMATE*. Furthermore, a tourist-like miniature inverted repeat transposable element (MITE) transposon was detected in the promoter region, and the number of repeats was positively correlated with Al tolerance (Magalhaes et al., 2007). It was then suggested that the causative mutations underlying aluminum tolerance may have a regulatory nature (Magalhaes et al., 2007).

The *TaALMT1* coding region is conserved between Al-tolerant and Al-sensitive lines (Raman et al., 2005). In turn, a 160-bp transposon and eight SNPs were detected downstream of *ALMT1*, but allelic variation at these loci was not correlated with aluminum tolerance. However, blocks of tandemly repeated sequences that were duplicated or triplicated were found in genomic regions upstream of the *ALMT1* coding region (Sasaki et al., 2006). In general, high *ALMT1* gene expression and Al tolerance were correlated with the number of repeats. Subsequently, transgenic plants containing different *TaALMT1* promoter alleles were shown to enhance gene expression (Ryan et al., 2010).

An important *cis* element for binding the ART1 transcription factor was identified in the STAR1 promoter region, which confers aluminum tolerance in rice (Huang et al., 2009; Yamaji et al., 2009; Tsutsui et al., 2011). This element consists of the sequence GGN(T/g/a/C)V(C/A/g)S(C/G), located upstream of the *STAR1* start codon. Moreover, this element was found in the promoter region of 29 of the 31 genes regulated by ART1 (Yamaji et al., 2009; Tsutsui et al., 2011), including *STAR2*, *Nrat1* (Tsutsui et al., 2011) and *OsFRDL4* (Yokosho et al., 2011), which are all involved in rice Al tolerance. In the *STAR2*

promoter, two copies of this element were identified, in addition to three copies in the Nrat1 (Tsutsui et al., 2011) promoter region.

More recently, a distinct mechanism for regulating *HvAACT1* expression was presented in barley (Fujii et al., 2012). An insertion of 1-kb sequence at 6 kb upstream from the *HvAACT1* coding region added multiple transcriptional start sites, enhancing this gene expression in the root tips. The modified *HvAACT1* expression pattern resulted in a superior Al-induced citrate exudation that consequently improved Al tolerance in barley (Fujii et al., 2012).

CONCLUSION

Plants have developed several mechanisms to overcome the limitations imposed by Al toxicity. Despite a prevalence of mechanisms involving organic acid exudation, symplastic mechanisms also play a role in Al tolerance in plants. In some species, Al tolerance is a genetically complex trait, where different tolerance mechanisms coexist. The involvement of multiple mechanisms is apparently independent of the level of tolerance intrinsic to each species, occurring in both comparatively Al-sensitive species, such as Arabidopsis, and highly Al-tolerant species, such as rice. Molecular and genetic studies have contributed to the identification of genes associated with Al tolerance. Those genes include membrane transporters of the ALMT, MATE, and ABC families, and functional homologs of these transporter genes are found in different species. Transcriptional factors and cis-elements are highly involved in the expression of Al tolerance genes. Integrating information about QTL, genes, and mechanisms involved in Al tolerance allows for a broad understanding of this trait across different plant species. Pyramiding of these genes and tolerance mechanisms by marker-assisted introgression of superior alleles or via genetic transformation may significantly contribute to the development of highly Al tolerant cultivars by molecular breeding, which should contribute to crop production on acid soils.

ACKNOWLEDGMENTS

Research supported by the Minas Gerais Research Foundation (FAPEMIG), National Council for Scientific and Technological Development (CNPq), Coordination of Improvement of Higher Education Personnel (CAPES), Generation Challenge Program, McKnight Foundation Collaborative Crop Research Program and Brazilian Agricultural Research Corporation (EMBRAPA).

REFERENCES

Ciotta MN, Bayer C, Ernani PR, Fontoura SMV, et al. (2002). Acidificação de um latossolo sob plantio direto. Rev. Bras. Ciênc. Solo 4: 1055-1064.

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

Courville P, Chaloupka R and Cellier MFM (2006). Recent progress in structure-function analyses of Nramp proton-dependent metal-ion transporters. Cell 978: 960-978.

Dale JE and Sutcliffe JF (1986). Water relations of plant cells. Plant Physiol. 9: 1-48.

Degenhardt J, Larsen PB, Howell SH and Kochian LV (1998). Aluminum resistance in the Arabidopsis mutant alr-104 is caused by an aluminum-induced increase in rhizosphere pH. Plant Physiol. 117: 19-27.

Delhaize E and Ryan PR (1995). Aluminum toxicity and tolerance in plants. Plant Physiol. 107: 315-321.

Delhaize E, Craig S, Beaton CD, Bennet RJ, et al. (1993). Aluminum tolerance in wheat (Triticum aestivum L.) I. Uptake and distribution of aluminum in root apices. Plant Physiol. 103: 685-693.

Foy CD (1988). Plant adaptation to acid aluminum-toxic soils. Commun. Soil Sci. Plant Anal. 19: 959-987.

Fujii M, Yokosho K, Yamaji N, Saisho D, et al. (2012) Acquisition of aluminium tolerance by modification of a single gene in barley. Nature Commun. 3: 713.

Furukawa J, Yamaji N, Wang H, Mitani N, et al. (2007). An aluminum-activated citrate transporter in barley. Plant Cell Physiol. 48: 1081-1091.

Gruber BD, Ryan PR, Richardson AE, Tyerman SD, et al. (2010). HvALMT1 from barley is involved in the transport of organic anions. J. Exp. Bot. 61: 1455-1467.

Hoekenga OA, Maron LG, Piñeros MA, Cançado GMA, et al. (2006). AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. Proc. Natl. Acad. Sci. USA 103: 9738-9743.

Huang CF, Yamaji N, Chen Z and Ma JF (2012). A tonoplast-localized half-size ABC transporter is required for internal detoxification of aluminum in rice. Plant J. 69: 857-867.

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

Iuchi S, Koyama H, Iuchi A, Kobayashi Y, et al. (2007). Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. Proc. Natl. Acad. Sci. USA 104: 9900-9905.

Kidd PS, Llugany M, Poschenrieder C, Gunse B, et al. (2001). The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (Zea mays L.). J. Exp. Bot. 52: 1339-1352.

Kinraide TB, Ryan PR and Kochian LV (1992). Interactive effects of Al, H, and other cations on root elongation considered in terms of cell-surface electrical potential. Plant Physiol. 99: 1461-1468.

Kochian LV (1995). Cellular mechanism of aluminum toxicity and resistance in plants. Annu. Rev. Plant Physiol. 46: 237-260.

Kochian LV, Hoekenga AO and Piñeros MA (2004). How do plants tolerate acid soils? Mechanism of aluminum tolerance and phosphorus efficiency. Annu. Rev. Plant Mol. Biol. 55: 459-493.

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

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

Ligaba A, Katsuhara M, Ryan PR, Shibasaka M, et al. (2006). The BnALMT1 and BnALMT2 genes from rape encode aluminum-activated malate transporters that enhance the aluminum resistance of plant cells. Plant Physiol. 142: 1294-1303.

Ligaba A, Maron LG, Shaff J, Kochian LV, et al. (2012). Maize ZmALMT2 is a root anion transporter that mediates constitutive root malate efflux. Plant Cell Environ. 35: 1185-1200.

Liu J, Magalhaes JV, Shaff J and Kochian LV (2009). Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance. Plant J. 57: 389-399.

Luo MC and Dvorák J (1996). Molecular mapping of an aluminum tolerance locus on chromosome 4D of Chinese spring wheat. Euphytica 91: 31-35.

Ma HX, Bai GH, Carver BF and Zhou LL (2005). Molecular mapping of a quantitative trait locus for aluminum tolerance in wheat cultivar Atlas 66. Theor. Appl. Genet. 112: 51-57.

Ma JF, Nagao S, Sato K, Ito H, et al. (2004b). Molecular mapping of a gene responsible for Al-activated secretion of citrate in barley. J. Exp. Bot. 55: 1335-1341.

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

Ma JF, Shen R, Nagao S and Tanimoto E (2004a). Aluminum targets elongating by reducing cell wall extensibility in wheat roots. Plant Cell Physiol. 45: 583-589.

Ma Z and Miyasaka SC (1998). Oxalate exudation by taro in response to Al. Plant Physiol. 118: 861-865.

Magalhaes JV, Garvin DF, Wang Y, Sorrells ME, et al. (2004). Comparative mapping of a major aluminum tolerance gene in sorghum and other species in the Poaceae. Genetics 167: 1905-1914.

Magalhaes JV, Liu J, Guimarães CT, Lana UGP, Alves VMC, et al. (2007). A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nat. Genet. 39: 1156-1161.

Maron LG, Piñeros MA, Guimarães CT, Magalhaes JV, et al. (2010). Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. Plant J. 61: 728-740.

Miyasaka S and Hawes MC (2001). Possible role of root border cells in detection and avoidance of aluminum toxicity. Plant Physiol. 125: 1978-1987.

Navakode S, Weidner A, Lohwasser U, Röder MS, et al. (2009). Molecular mapping of quantitative trait loci (QTLs) controlling aluminium tolerance in bread wheat. Euphytica, 166: 283-290.

Ninamango-Cárdenas FE, Guimarães CT, Martins PR, Parentoni SN, et al. (2003). Mapping QTLs for aluminum tolerance in maize. Euphytica 130: 223-232.

Ofei-Manu P, Wagatsuma T, Ishikawa S and Tawaraya K (2001). The plasma membrane strength of the root tip cells and root phenolic compounds are correlated with Al tolerance in several common woody plants. Soil Sci. Plant Nutr. 47: 359-375.

Pellet DM, Grunes DL and Kochian LV (1995). Organic acid exudation as an aluminum-tolerance mechanism in maize (Zea mays L.). Planta 196: 788-795.

Piñeros MA, Cançado GMA, Maron LG, Lyi SM, et al. (2008). Not all ALMT1-type transporters mediate aluminum-activated organic acid responses: the case of ZmALMT1 - an anion-selective transporter. Plant J. 53: 352-367.

Piñeros MA, Magalhaes JV, Alves VMC and Kochian LV (2002). The physiology and biophysics of an aluminum tolerance mechanism based on root citrate exudation in maize. Plant Physiol. 129: 1194-1206

Piñeros MA, Shaff AE, Manslank HS, Alves VMC, et al. (2005). Aluminum resistance in maize cannot be solely explained by root organic acid exudation. A comparative physiological study. Plant Physiol. 137: 231-241.

Raman H, Zhang K, Cakir M, Appels R, et al. (2005). Molecular characterization and mapping of ALMT1, the aluminium-tolerance gene of bread wheat (Triticum aestivum L.). Genome 791: 781-791.

Ried CR and Anderson JA (1996). Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci. 36: 905-909.

Ryan PR, Raman H, Gupta S, Horst WJ, et al. (2009). A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. Plant Physiol. 149: 340-51.

Ryan PR, Raman H, Gupta S, Sasaki T, et al. (2010). The multiple origins of aluminium resistance in hexaploid wheat include Aegilops tauschii and more recent cis mutations to TaALMT1. Plant J. 64: 446-455.

Sasaki T, Ryan PR, Delhaize E, Hebb DM, et al. (2006). Sequence upstream of the wheat (Triticum aestivum L.) ALMT1 gene and its relationship to aluminum resistance. Plant Cell Physiol. 47: 1343-1354.

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

Sawaki Y, Iuchi S, Kobayashi Y, Kobayashi Y, et al. (2009). STOP1 regulates multiple genes that protect arabidopsis from proton and aluminum toxicities. Plant Physiol. 150: 281-294.

Sibov ST, Gaspar M, Silva MJ, Ottoboni LM, et al. (1999). Two genes control aluminum tolerance in maize: genetic and molecular mapping analyses. Genome 42: 1-8.

Suhayda CG and Haug A (1985). Citrate chelation as a potential mechanism against aluminum toxicity in cells - The role of calmodulin. Can. J. Biochem. Cell Biol. 63: 1167-1175.

Taylor GJ (1988). The physiology of aluminum tolerance. Commun. Soil Sci. Plant Anal. 19: 7-12.

Tsutsui T, Yamaji N and Feng Ma J (2011). Identification of a cis-acting element of ART1, a C2H2-type zinc-finger transcription factor for aluminum tolerance in rice. Plant Physiol. 156: 925-931.

von Korff M, Radovic S, Choumane W, Stamati K, et al. (2009). Asymmetric allele-specific expression in relation to developmental variation and drought stress in barley hybrids. Plant J. 59: 14-26.

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

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

Yang ZM, Sivaguru M, Horst WJ and Matsumoto H (2000). Aluminum tolerance in achieved by exudation of citric acid from roots of soybean (Glycine max). Physiol. Plant. 110: 72-77.

Yokosho K, Yamaji N and Ma JF (2010). Isolation and characterization of two MATE genes in rye. Funct. Plant Biol. 37: 296-303.

Yokosho K, Yamaji N and Ma JF (2011). An Al-inducible MATE gene is involved in external detoxification of Al in rice. Plant J. 68: 1061-1069.

CAPÍTULO 2

GENOMIC DISSECTION AND VALIDATION OF ALUMINUM TOLERANCE QTL IN MAIZE

Claudia Teixeira Guimarães[1], Christiano Costa Simões[2], Jurandir Vieira de Magalhães[1], Lyza Gontow Maron[3], Sílvia Neto Jardim-Belicuas[1], Lauro José Moreira Guimarães[1], Vera Maria Carvalho Alves[1], Edilson Paiva[1], Carlos Fasane da Silva Tinoco[4], Ubiraci Gomes de Paula Lana[1,2], Roberto Williams Noda[1]; Leon V. Kochian[3]

[1] Embrapa Milho e Sorgo, Rod. MG 424, Sete Lagoas, MG, Brasil

[2] Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

[3] Robert W. Holley Center for Agriculture and Health, USDA – ARS, Cornell University, Ithaca, NY, USA

[4] Departametno de Biologia, Centro Universitário UNIFEMM, Sete Lagoas, MG, Brasil

ABSTRACT

Aluminum (Al) toxicity is an important limitation to food security in the tropics and subtropics. On acid soils, the high Al saturation limits root development, and consequently the exploitation of water and nutrient uptake. In maize, Al tolerance is quantitatively inherited, involving multiple genes, not well described so far. In our work, we tested 36,147 GBS-based markers and three candidate genes in a highly contrasting RILs population to dissect the genetic complexity of Al tolerance in maize. Two different statistical approaches, generalized linear model and multiple interval mapping, provided a complementary detection of eight genomic regions highly associated with Al tolerance with a significant improvement on the genetic precision. Among those, the main Al tolerance QTL6, explaining 22% of the genetic variance, was able to increase Al tolerance in maize NILs. This genomic region harbors the *ZmMATE1* and two *cis* eQTL explaining close to 80% of this candidate gene expression profile. NILs introgressed with the QTL6 presented a two-fold increase in Al tolerance followed by a superior *ZmMATE1* expression when compared to the recurrent Al-sensitive line, providing additional support for the *ZmMATE1* role in maize Al tolerance. We also offered well defined genomic regions to search for novel candidate genes for Al tolerance in maize. Thus, our data expand upon the current knowledge, offering a major target for molecular breeding and opening an avenue for future studies to fully understand Al tolerance in maize.

BACKGROUND

Aluminum (Al) toxicity is the major constraint for crop production on acidic soils, which comprise over 50% of the world's potentially arable lands (von Uexküll & Mutert, 1995), mostly found in tropics and subtropics. Because approximately 20% of the maize grown worldwide is cultivated on acid soils, Al toxicity is a major threat to food security worldwide. On acid soils, Al is solubilized into highly rhizotoxic ionic forms that inhibit root growth, reducing water and nutrient uptake (Kochian, 1995). Even though agronomic practices such as liming can ameliorate soil acidity, these technologies are not readily available for a large number of small-scale farmers that depend on agriculture as their main source of income and food. Additionally, agricultural activities can lead to soil acidification (Ciotta et al., 2002).

Plants have developed two major Al tolerance mechanisms that either prevent Al from entering the root (i.e. Al exclusion) or neutralize internal Al. Al exclusion by organic acids released from the roots is currently proposed as the main physiological mechanism of Al tolerance, which involves the chelation of toxic forms of Al producing stable, non-toxic complexes (Kochian et al., 2004). In maize, Al tolerance is associated with root citrate release (Pellet et al., 1995; Piñeros et al., 2002), although other mechanisms are likely to play a role in maize Al tolerance (Piñeros et al., 2005). Physiological evidences are in agreement with the typical quantitative nature of maize Al tolerance (Magnavaca et al., 1987; Pandey et al., 1994; Lima et al., 1995), probably involving multiple genes and mechanisms.

The genetic control of the Al tolerance mechanism based on organic acid exudation has been attributed to the action of two different gene families, aluminum-activated malate transporter (*ALMT*) and multidrug and toxic compound extrusion (*MATE*). The first Al tolerance gene was *TaALMT1*, which encodes an ALMT membrane transporter responsible by the Al-activated malate efflux from root apices in wheat (Sasaki et al., 2004). Functional *ALMT1* homologs were further identified in Arabidopsis (Hoekenga et al., 2006), barley (Furukawa et al., 2007), and rye (Yokosho et al., 2010). The involvement of MATE transporters in Al tolerance was reported in sorghum and barley, with the cloning and characterization of *SbMATE* (Magalhaes et al., 2007) and *HvAACT1* (Furukawa et al., 2007), respectively. These MATE transporters are involved on citrate exudation activated by Al in root apex, and are major determinants of both sorghum and barley Al tolerance. *MATE* genes were subsequently associated with Al tolerance in other species, including Arabidopsis (*AtMATE* - Liu et al., 2009), wheat (*TaMATE1* - Ryan et al., 2009), rye (*ScFRDL2* - Yokosho et al., 2010), and rice (*OsFRDL4* - Yokosho et al., 2011). Indeed, most of these genes were co-localized with Al tolerance quantitative trait loci (QTL) previously mapped in their respective species (Hoekenga et al., 2003; Magalhaes et al., 2004; Ma et al., 2004; 2005; Raman et al., 2005; Navakode et al., 2009; Yokosho et al., 2011). Thus, Al

exclusion driven by citrate and malate exudation is rather ubiquitous Al tolerance mechanism in grasses.

However, other genes are also related to Al tolerance in plants. *STOP1*, a Cys2-His2 zinc finger transcription factor, was shown to regulate the expression of both *AtMATE* and *AtALMT* (Iuchi et al., 2007; Liu et al., 2009; Sawaki et al., 2009) in Arabidopsis. A rice homolog of *STOP1*, *ART1*, also regulates the expression of multiple genes possibly involved in rice Al tolerance (Yamaji et al., 2009). ATP binding cassette (ABC) transporters were associated with Al tolerance in Arabidopsis (ALS3 - Larsen et al., 2005 and ALS1 - Larsen et al., 2007) and rice (START1 and STAR2 - Huang et al., 2009; 2010). Biochemical and molecular characterization of ALS1 and ALS3 suggest that these transporters are important components of intracellular redistribution of Al in Arabidopsis, keeping this metal away from the more sensitive tissues (Larsen et al., 2005; 2007). In rice, STAR1 interacts with STAR2 to form an ABC transporter responsible for UDP-glucose efflux, which may be involved in cell wall modifications (Huang et al., 2009). Nramp aluminum transporter 1 (*Nrat1*) was shown to encode an Al^{3+} transporter localized at plasma membrane, which can be required for internal Al detoxification in rice (Xia et al., 2010). Although the mechanisms triggered by these transporters are not completely understood, the recent finds suggest that internal detoxification of Al is a complementary mechanism of Al tolerance that may also be present in other grasses.

In maize, the few QTL studies reported two to six genomic regions associated with Al tolerance (Sibov et al., 1999; Ninamango-Cárdenas et al., 2003; Conceição et al., 2009). Lately, two maize homologs of sorghum *SbMATE*, *ZmMATE1* and *ZmMATE2*, were co-localized with Al tolerance QTL on chromosomes 6 and 5, respectively (Maron et al., 2010). These two QTL were consistently detected in two of the previous QTL studies. *ZmMATE1* was shown to mediate Al-induced citrate exudation in maize and improved Al tolerance in transgenic Arabidopsis, whereas the role of *ZmMATE2* in Al tolerance remained unclear (Maron et al., 2010). A combination of association and linkage analyses ranked four candidate genes as targets for future Al tolerance studies (Krill et al., 2010). Of these candidate genes, only *ZmALMT2* was further characterized, but the functional analyses did not support its role in maize Al tolerance (Ligaba et al., 2012). So far, little knowledge on genetic factors underlying maize Al tolerance is available when compared with other grasses.

Here we tested marker-trait associations with unprecedented marker density as provided by next-generation sequencing (Elshire et al., 2011) to provide the most detailed description so far of the genetic complexity underlying maize Al tolerance. Our analysis included 36,147 SNPs that were used to scan the maize genome for regions associated with Al tolerance using a general linear model with Al-inhibition of root growth data as well as QTL mapping. Seven genomic regions were found to be highly associated with Al tolerance,

which were either confirmed in previous QTL studies or newly detected ones. Our study also revealed that the vast majority of the *ZmMATE1* expression mapped to the major Al tolerance QTL on chromosome 6, where *ZmMATE1* is co-localized. Finally, near-isogenic lines generated by marker assisted introgression of the ZmMATE1 QTL presented a two-fold increase in Al tolerance and higher *ZmMATE1* expression compared to the Al-sensitive parental line, providing further support that *ZmMATE1* is an important determinant of maize Al tolerance.

RESULTS

Al tolerance on maize elite tropical lines

Thirty-six tropical maize inbred lines and the temperate line, B73, were characterized for Al tolerance, based on relative net root growth (RNRG) in nutrient solution at {39} µM activity of Al^{3+}. The lines in the panel varied from Al-sensitive (RNRG < 0.30) to highly tolerant (RNRG > 0.80), with the parents of the RIL population, Cateto Al237 and L53, showing ~1.00 and 0.22 of RNRG, respectively (Figure 1). The criteria to classify the lines as Al-sensitive and highly Al-tolerant followed the definition used for sorghum (Caniato et al., 2011). The inbred lines Cateto Al237 and L1154 showed genotypic mean of RNRG exceeding 0.80, being thus highly Al-tolerant, whereas nine lines were considered as Al sensitive, with RNRG values less than 0.30 (Figure 1). Most of the lines showed RNRG ranging from 0.30 to 0.80, being intermediately Al tolerant (Figure 1). The mixed model analysis presented an accuracy of 0.97, indicating a high reliability of the phenotypic evaluation of these lines.

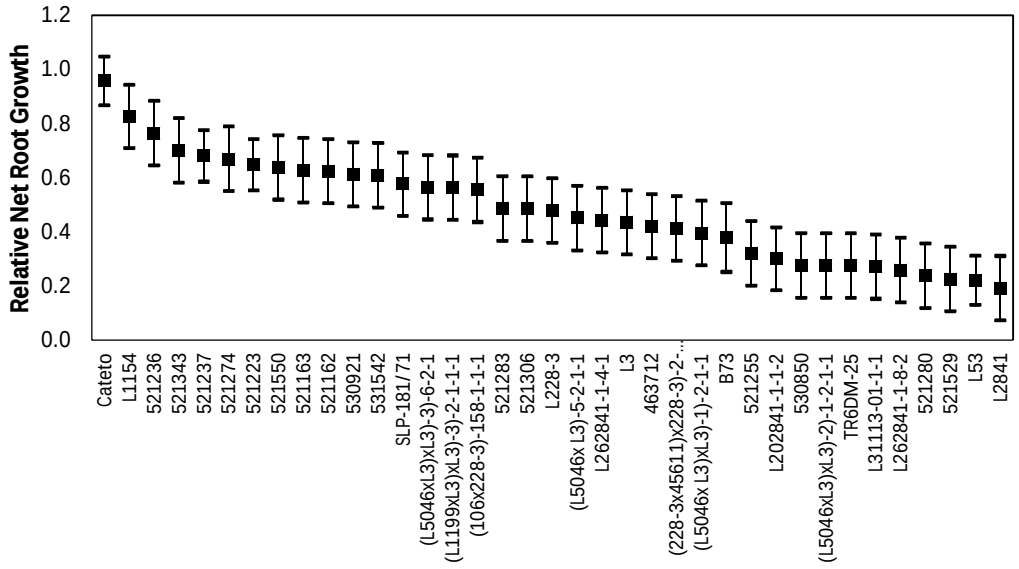


Figure 1. Aluminum tolerance of maize inbred lines measured as relative net root growth (RNRG) after five days in nutrient solution containing {39} µM activity of Al^{3+}, including 36 lines from Brazil and the temperate line B73.

Multiple genomic regions associated with Al tolerance

Approximately 8% of the 458,255 SNPs genotyped by GBS in a RIL population, showed minimum allelic frequency of 0.4 and less than 20% of missing data, and were selected for further statistical analyses. The final genetic map comprised 1,106 SNP markers, 39 SSRs and the candidate genes, *ZmMATE1*, *ZmMATE2* and *ZmASL*, covering 2,508.2 cM with a mean distance between adjacent markers of 2.2 cM. Two gaps of 26 and 22 cM remained on chromosomes 1 and 10, respectively. These gaps represented a physical distance around 30 Mb, indicating that even with more than 36,000 SNPs some regions had a low sequencing coverage.

Variance analyses of phenotypic traits were highly significant, with the heritability estimates of 93.6 and 92.2% for RNRG and for net root growth (NRG) without Al, respectively. The coefficient of variation was below 10% for these traits, indicating high experimental precision.

Using multiple interval mapping (MIM) six Al tolerance QTL were detected on chromosomes 2, 3, 5, 6 and 8, jointly explaining approximately 65% of the total genetic variance (Table 1). Two major QTL were mapped on chromosomes 5 (QTL5.1) and 6 (QTL6), explaining ~14% and ~22% of the genetic variance of Al tolerance. These QTL were co-localized with the candidate genes *ZmMATE2* and *ZmMATE1*, respectively, confirming previous results (Maron et al., 2010). Additionally, four minor effects QTL were detected,

explaining 6% to 8% of the genetic variance for RNRG. Four QTL presented positive effects, and one minor QTL had negative effect, indicating that alleles increasing Al tolerance were mostly derived from the Al tolerant parent, but L53 also presented a minor contribution. The confidence interval of the Al tolerance QTL ranged from 10 to 30 cM, which were flanked by markers physically spaced from 1.7 to 31.7 Mb. The correlation between genetic and physical distances was possible due to the alignment of GBS-based SNPs to the reference maize genome.

Table 1. Aluminum tolerance QTL evaluated based on Relative Net Root Growth in a population of 118 maize RILs using multiple interval mapping. The position of each QTL was determined in cM as a higher likelihood of odds (LOD) peak. Marker identification indicates the chromosome location and the physical position of the SNP. Confidence interval (CI) was determined as -2 LOD values.

QTL	Bin	Markers	Position	LOD	Effect	R^2 (%)	CI
QTL2	2.07	S2_185399444	191.5	3.62	0.0463	7.7	S2_179458281-S2_199180856
QTL3	3.06	S3_186794084	165.4	5.04	0.0513	8.1	S3_184851661-S3_189804136
QTL5.1	5.03	*ZmMATE2*	83.8	5.66	0.0572	14.5	S5_17623310-S5_37256357
QTL5.2	5.06	S5_204229846	227.4	4.04	-0.0515	6.1	S5_200649355-S5_205792028
QTL6	6.00	*ZmMATE1*	17.2	8.15	0.0699	22.1	S6_4434544-S6_6077188
QTL8	8.03	S8_92966949	80.6	5.09	0.0519	6.1	S8_74529328-S8_106236514
R^2 Total						64.5	

Analysis of marker data and RNRG by means of a generalized linear model (GLM) with 36,147 SNPs detected five independent genomic regions significantly associated with Al tolerance on maize chromosomes 2, 4, 5 and 6 (Table 2). Three of these SNPs (S2_188406689, S5_26362150 and S6_5510425) were coincident with the confidence interval of the Al tolerance QTL2, QTL5.1 and QTL6, detected by MIM. The SNP S5_159332054 is predicted in a position distinct of the two other Al tolerance QTL mapped on chromosome 5. Additionally, S4_65674165 represented a genomic region at chromosome

4 detected only by GLM. In summary, a total of eight genomic regions were associated with Al tolerance by either MIM or GLM, being three of them coincident between both statistical approaches.

Table 2. SNP markers associated with aluminum tolerance using general linear model at significance level determined by 1,000 permutations at $p < 0.05$. SNPs in bold were located close to Al tolerance QTL. Markers in bold are coincident with the Al tolerance QTL.

Marker	Bin	F	probability	perm	Effect	R^2 (%)
S2_188406689	2.07	25.7578	1.57E-06	0.001	0.1416	18.8
S4_65674165	4.04	17.9618	5.22E-05	0.013	0.1352	14.9
S5_26362150	5.03	26.9437	9.56E-07	0.001	0.1466	19.5
S5_159332054	5.05	21.7904	8.86E-05	0.005	0.1495	16.9
S6_5510425	6.00	30.7640	2.31E-07	0.001	0.1620	23.2

In order to confirm that Al tolerance QTL were not influenced by genetic variation for root growth unrelated to Al tolerance (i.e. assessed in control conditions) MIM and GLM were undertaken using NRG without Al. Four genomic regions located on chromosomes 1, 3, 4 and 6 were consistently associated with NRG using MIM (Table 3) and GLM (Supplementary Table S1). Among those, only the NRG QTL mapped on chromosome 3 was coincident with the confidence interval on Al tolerance QTL3. Then, it can be contributed that the majority of the Al tolerance was not influenced by the segregation for root growth.

Table 3. QTLs associated with net root growth in nutrient solution without aluminum using multiple interval mapping**.** The position of each QTL was determined in cM as a higher likelihood of odds (LOD) peak. Marker identification indicates the chromosome location and the physical position of the SNP.

Bin	Markers	Position	LOD	Effect	R^2 (%)
1.04	S1_67410866	138.1998	5.50	-7.2940	11.4
3.06	S3_188806451	171.2541	7.59	-8.4451	18.1
4.06	S4_164097531	268.0593	1.54	-3.8021	7.2
6.06	S6_153773010	157.2291	7.33	-8.6334	18.1
R^2 Total					54.8

Supplementary Table S1. SNP markers associated with root growth in nutrient solution without aluminum using general linear model at significance level determined by 1,000 permutations at $p < 0.05$.

Marker	Bin	F	probability	perm	Effect	R^2
S1_64014376	1.04	18.5464	3.51E-05	0.019	-15.7665	13.9
S3_186210759	3.06	37.3399	1.64E-08	0.001	-20.5397	25.9
S4_173373135	4.07	29.5125	3.23E-07	0.001	-19.3054	20.7
S6_158279509	6.06	33.6825	6.14E-08	0.001	-20.0425	23.1

Improved precision on QTL detection

The major Al tolerance QTL6 was mapped in a confidence interval of 10 cM, which was saturated by four SNPs as well as the *ZmMATE1*. This region was predicted to span a physical interval of 1.7 Mb, considering the alignment of the SNPs on the reference genome sequence. Based on this inference, the SNP mostly associated with Al tolerance by GLM (S6_5510425) was putatively located at ~0.3 Mb of the *ZmMATE1*, which is predicted at 5.8 Mb on the chromosome 6. Previously, this QTL was mapped in a 25 cM interval, including the *ZmMATE1* and two flanking SSR markers (Maron et al., 2010).

The second most important Al tolerance QTL on chromosome 5 was comprised by six markers in 13 cM confidence interval. Based on predicted position of the flanking SNPs, this region would be spanning around 20 Mb. This QTL was also detected by Maron et al. (2010), including three markers in a confidence interval of 18 cM.

Mapping the expression of ZmMATE1* and *ZmMATE2

Out of the three candidate genes mapped herein, *ZmMATE1* and *ZmMATE2* were co-localized with the Al tolerance QTL, being further investigated for their expression pattern in the RILs population. Two expression quantitative trait loci (eQTL) for *ZmMATE1* were detected flanking this gene on chromosome 6, explaining almost 80% of its expression (Table 4).

Table 4. Expressed QTLs of ZmMATE1 mapped in the RILs population by multiple interval mapping. The position of each eQTL was determined in cM as a higher likelihood of odds (LOD) peak. Marker identification indicates the chromosome location and the physical position of the SNP. Confidence interval (CI) was determined as -2 LOD values.

Bin	Markers	Position	LOD	Effect	R^2 (%)	CI
2.03	S2_23118298	90.3817	2.71	0.2348	2.0	S2_23118298-S2_26136230
6.00	S6_3835286	12.2355	3.60	0.8695	46.6	S6_2591236-S6_4434544
6.00	S6_6077188	22.3553	3.08	0.6506	34.4	S6_5160638-S6_6901831
R^2 Total					82.9	

These regions could be considered as *cis* eQTL due to their close location to the target gene, which were co-localized with the Al tolerance QTL6 (Figure 2). An additional minor eQTL was mapped on chromosome 2 (Table 4).

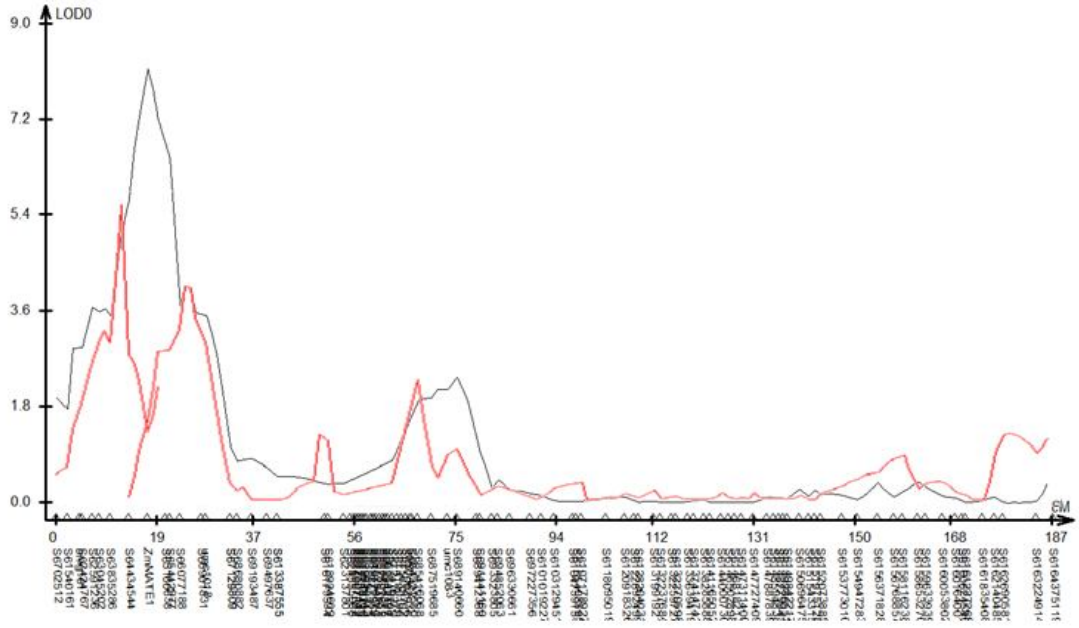


Figure 2. Al tolerance (black line) and *ZmMATE1* expression (red line) QTL were mapped on maize chromosome 6, in the region where the candidate gene *ZmMATE1* was located. Each LOD peak with higher value indicates one individual QTL.

Both eQTL on chromosome 6 seem to be important to control the *ZmMATE1* expression, once the RILs carrying either one of these eQTL derived from Cateto Al237 presented as high *ZmMATE1* expression as the RILs presenting both eQTL from Cateto Al237. Additionally, the *ZmMATE1* expression decreased significantly in the RILs carrying both eQTL from L53 (Figure 3).

For the candidate gene *ZmMATE2*, three eQTL were mapped on chromosomes 3 and 7, explaining 38.0% of this gene expression (Table 5). All eQTL were located on different chromosomes from where the *ZmMATE2* was mapped, which was on chromosome 5. The main effect eQTL explained 23.8% of the *ZmMATE2* expression, being considered as *trans* eQTL.

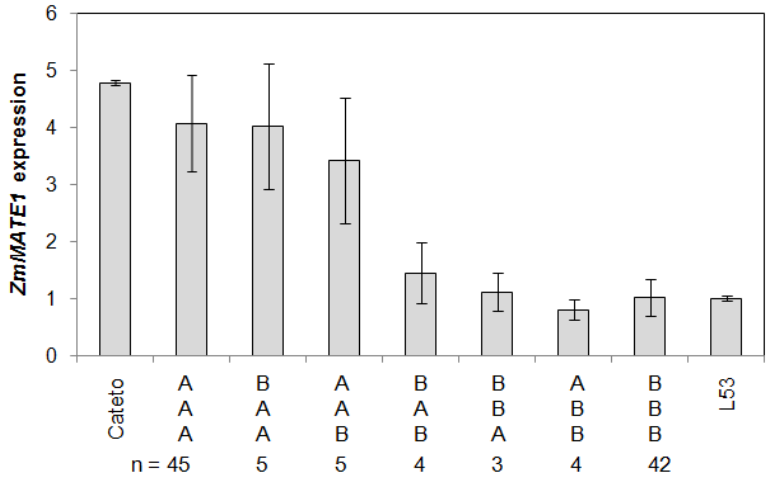


Figure 3. *ZmMATE1* relative expression profile among groups of RILs with contrasting genotypes for the *ZmMATE1* and for the eQTL of this gene. The letter **A** means that the allele was derived from Cateto Al237 and **B**, from L53, in a sequential order represented by the markers S6_3835286, *ZmMATE1* and umc1018. *n* is the number of RILs in each group and the bars are the standard deviation. *ZmMATE1* expression in the parental lines, CatetoAl237 and L53, were considered as controls.

Table 5. Expressed QTL of *ZmMATE2* mapped in the RILs population by multiple interval mapping. The position of each eQTL was determined in cM as a higher likelihood of odds (LOD) peak. Marker identification indicates the chromosome location and the physical position of the SNP.

Bin	Markers	Position	LOD	Effect	R^2 (%)
3.06	S3_175937355	134.9255	1.74	-0.1158	7.7
3.07	S3_201230364	197.4535	4.56	0.1990	23.8
7.06	S7_174537726	240.3150	1.46	-0.1039	6.4
R^2 Total					38.0

Validation of Al tolerance QTL6

Two sets of near-isogenic lines (NILs) developed by marker-assisted backcrosses were used to validate the two major Al tolerance QTL. NILs carrying the QTL6 alleles derived from Cateto Al237 presented a two-fold increase in Al tolerance in comparison with the recurrent line L53 (Figure 4). This region was flanked by the ZmMATE1 and umc1018 markers. These NILs were as tolerant as their donor RIL-84, which still had 50% of the Cateto Al237 genome. In addition, the NILs for QTL6 showed a *ZmMATE1* expression similar to the RIL-84 and Cateto Al237 (Figure 5). In contrast, the NILs for the *ZmMATE2* were as Al sensitive as L53, indicating that the *ZmMATE2* alone was not able to improve the Al tolerance in the recurrent L53.

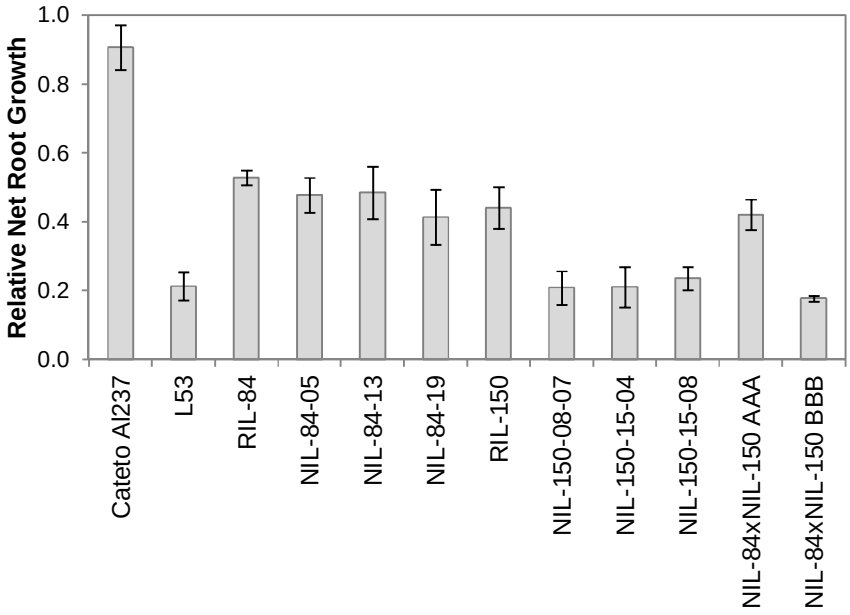


Figure 4. Aluminum tolerance measured as relative seminal root growth of NILs, their parental lines, and respective donor RILs in nutrient solution with {39} uM Al activity. Two different NILs were crossed followed by a selfing cycle to obtain the segregating plants that were selected based on the presence of markers *ZmMATE1*, umc1018 and *ZmMATE2*. The alleles derived from Cateto Al237 were identified as AAA for each marker, respectively, whereas the alleles derived from L53 were identified as BBB.

When the two sets of NILs were crossed and selfed, individuals carrying homozygous alleles for both QTL were also evaluated for Al tolerance. These results confirmed that individuals carrying both QTL6 and *ZmMATE2* from Cateto Al237 were as Al tolerant as the NILs carrying only the QTL6. Moreover, the individuals containing L53 alleles for both regions were as Al sensitive as the recurrent line L53 (Figure 4).

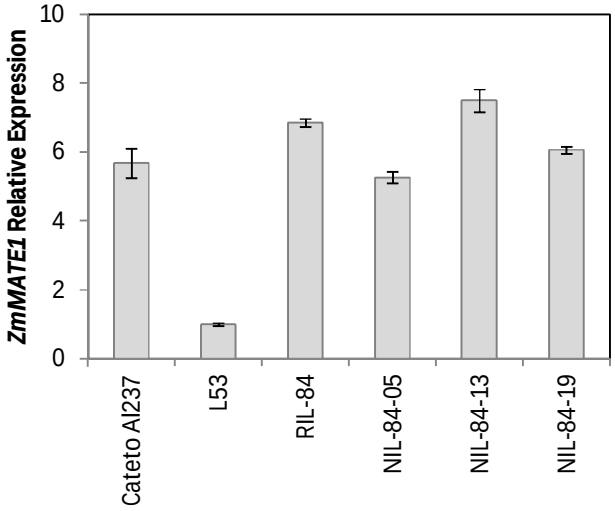


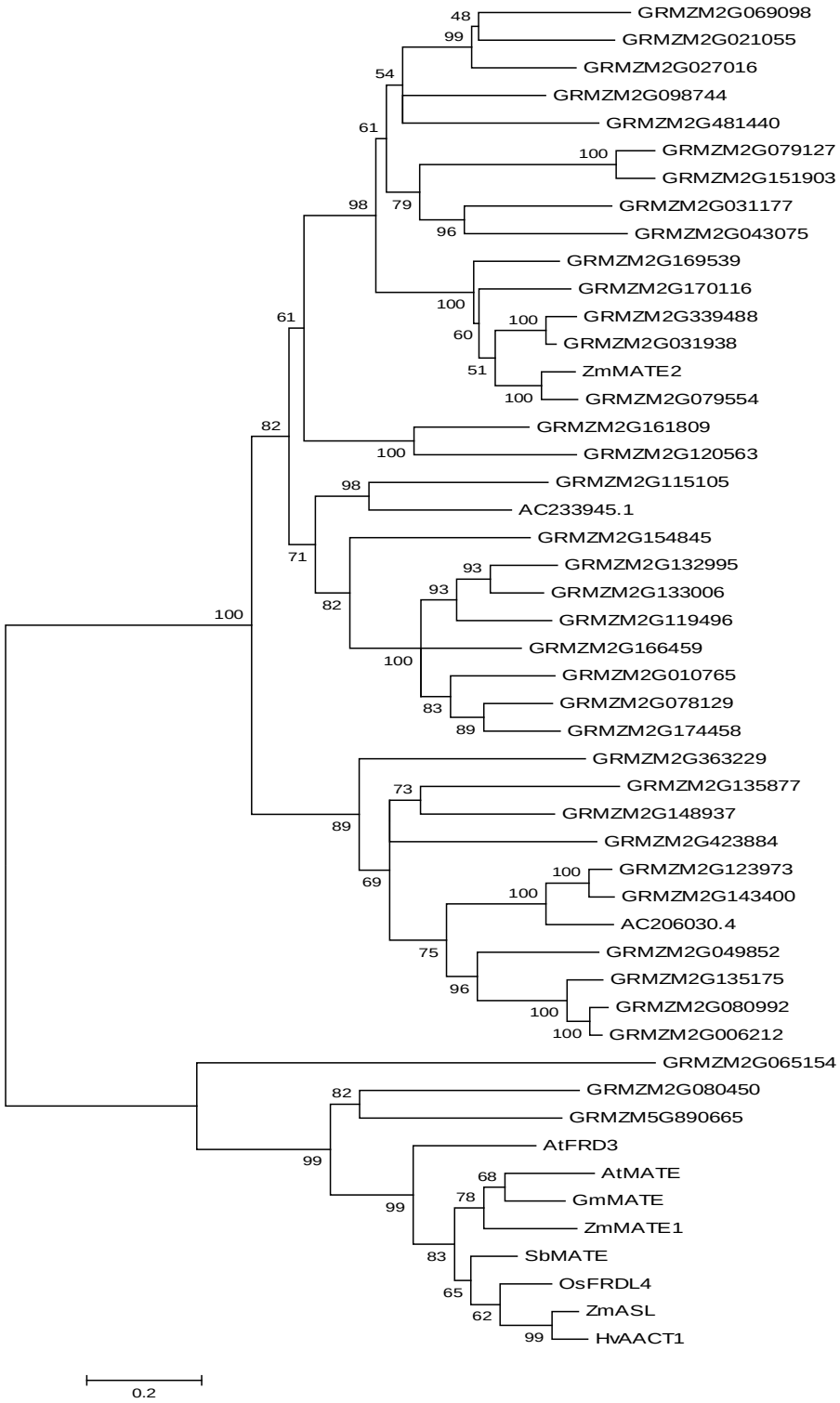
Figure 5. *ZmMATE1* relative expression of the NILs developed for the QTL6, including the donor RIL-84, the recurrent L53 and the Cateto Al237, as the parental lines.

Candidate genes predicted within Al tolerance QTL

Considering the importance of *MATE* genes in Al tolerance, a phylogenetic analysis was performed using all 43 *MATE* members predicted in the maize genome. Two major clusters were formed, one of them including five maize members as well as functional MATEs associated with Al tolerance in different plant species (Supplementary Figure S1). This group included *ZmMATE1*, *ZmASL*, and three other predicted members, sharing 33.0 to 73.9% of amino acids sequence similarity with SbMATE.

Using the Al tolerance gene *TaALMT1* from wheat (Sasaki et al., 2004) as query, we found the member GRMZM2G439638 with 25.7% of sequence similarity located at 98.8 Mb on chromosome 8, which is within the Al tolerance QTL8 confidence interval.

A predicted gene GRMZM2G069198 sharing 66.0% of amino acid sequence similarity with the Nrat1 from rice is located at 43.1 Mb, and GRMZM2G432480 with 84.9% of similarity with the STAR1 is predicted at 52.9 Mb, both on chromosome 5. Even outside of the Al tolerance QTL5.1 confidence interval, these genes were recently associated with Al tolerance in rice (Huang et al., 2009; Xia et al., 2010) and should be further investigated.



Supplementary Figure S1. Maximum likelihood phylogenetic tree of maize MATE members and MATE functionally characterized as citrate transporters in other plant species constructed based on amino acid sequences. Numbers in the nods indicate bootstrap values calculated using 500 resampling.

DISCUSSION

Germplasm characterization is an important start point to identify new sources of tolerance and to select contrasting parental genotypes. Phenotyping a relatively small group of maize tropical elite lines revealed a considerably low frequency of Al sensitive genotypes (24%) and a high proportion of lines with intermediate Al tolerance (70%). The maize lines developed by Embrapa at last ten years were mainly grouped within the intermediate and high Al tolerant genotypes. This tendency enforces the importance of selection to improve Al tolerance in maize, which is also in agreement with the high heritability of this trait. Other hypothesis for the relative high frequency of Al tolerance in maize than in sorghum is the genetic complexity of this trait in maize (Magnavaca et al., 1987; Pandey et al., 1994; Ninamango-Cárdenas et al., 2003). In contrast, the source of Al tolerance in sorghum relies mostly on the *Alt*$_{SB}$ locus (Caniato et al., 2007; 2011). On the other hand, only 6% of the maize lines were considered highly Al tolerant, similarly of what was found for sorghum (Caniato et al., 2011). These data suggest that even with a considerable proportion of intermediate Al tolerance, genotypes highly Al tolerant are still rare among breeding material, requiring additional efforts to improve this trait.

In this work, the complexity of Al tolerance in maize was dissected using a great number (>36,000) of markers generated by genotyping-by-sequencing (GBS) and putative candidate genes in a RILs population. We first presented an application of GBS-based markers to improve the resolution of marker-trait association.

Among the advantages of the markers generated by GBS, their random distribution along the genome improved the saturation of target QTL regions and narrowed down their confidence interval. Another great benefit of GBS-based SNP was the alignment of their sequences in a reference genome, bridging genetic maps to physical genomic position. The improved accuracy by the GBS was highlighted for the region where the candidate gene *ZmMATE1* was mapped. One SNP was highly associated to Al tolerance at 0.3 Mb of the candidate gene by GLM, whereas the Al tolerance QTL6 spanned close to 1.7 Mb of confidence interval. This precision was much higher than the previous study using only SSR markers (Maron et al., 2010). Additionally, the coefficient of determination (R^2) calculated by both procedures were very similar, 23.2% for GLM (S6_5510425) and 22.1% for MIM (QTL6) models, confirming the reliability of both analyses, especially for genomic regions with major effects. Thus, GLM and MIM were consistent on improving the accuracy of detection of Al tolerance QTL/candidate gene in maize.

However, the QTL effects estimated by these statistical models were somehow discrepant for the RNRG phenotypic index on the chromosome 6, being 0.16 for GLM and 0.07 for MIM. This QTL effect measured in the NILs was close to 0.3, which could be

considered the observed effect due to the homogeneous genetic background. One reason to justify the superior effect observed in the NILs would be that this QTL may harbor genetic factor(s) enhancing *ZmMATE1* expression that could also induce the expression of other Al tolerance genes present in the Al sensitive line. Indeed, the Al tolerance QTL5.2 presented negative effect, indicating that L53 also carry alleles able to improve Al tolerance.

Considering the consistency of the results, GLM would be recommended to process a high-throughput molecular data generated by GBS due to the simplicity of the analysis. The lack of genetic order of the markers could be overcame by the physical predicted position of the SNPs. On the other hand, the additional effort to construct a linkage map required in MIM model provided complementary information such as estimates of the total genetic variance explained by all QTL and the putative QTL interactions. Therefore, we recommend the QTL mapping and GLM analysis.

The major Al tolerance QTL6 harbored the *ZmMATE1* as well as two *cis* eQTL, which were equally important for the *ZmMATE1* expression and extended from 2.6 to 6.9 Mb. This genomic region was searched for a putative homolog to *STOP1* and *ART1*, transcription factors controlling MATE members in Arabidopsis (Liu et al., 2009) and rice (Yokosho et al., 2011) but no candidate genes were found. Recently, a transposable element (Hopscotch) inserted at approximately 58 - 69 kb upstream of *teosinte branched1* (*tb1*) acted as an enhancer of this gene expression and to partially explained the increasing apical dominance in maize (Studer et al., 2011). Transposable elements are widespread in the maize genome and can be a possible target for future studies to better understand the control of *ZmMATE1* expression. The QTL6, including the *ZmMATE1* and the factor(s) controlling most of its expression, improved the Al tolerance when transferred to an Al sensitive maize line. Thus, this QTL could be exploited to improve Al tolerance in maize aiming to develop genotypes more adapted to acid soils.

Genomic regions detected at bins 3.06, 5.05 and 5.06 were associated with Al tolerance for the first time, and could be searched for new candidate genes. The Al tolerance QTL8 can be considered coincident with QTL and markers previously associated with Al tolerance (Ninamango-Cárdenas et al., 2003; Conceição et al., 2009). A putative *ALMT* member (GRMZM2G439638) was identified within the QTL8 confidence interval. *ALMT* has been functionally responsible for Al tolerance in wheat (Sasaki et al., 2004), Arabidopsis (Hoekenga et al., 2006), barley (Furukawa et al., 2007), and rye (Yokosho et al., 2010). However, ZmALMT1 and ZmALMT2 were characterized as anion transporters but not associated with Al tolerance in maize (Piñeros et al., 2008; Ligaba et al., 2012, respectively). Therefore, this *ALMT* member can be a novel candidate gene to be exploited as responsible for an additional Al tolerance mechanism in maize.

Within the second main Al tolerance QTL5.1, the *ZmMATE2* was not validated as a candidate gene underlying this QTL, but other candidate genes were predicted near to this region. GRMZM2G069198 is a putative homolog of the rice *Nrat1* that encodes an Nramp aluminum transporter acting in the Al uptake into the cell (Xia et al., 2010). This physiological mechanism was never proposed in maize, but an additional evidence supporting this hypothesis was found in a global expression profile experiment using microarray, where the oligoMZ00052211, representing the predicted gene GRMZM2G069198, was induced by Al in root tips in the Al tolerant Cateto100-6 (Maron et al., 2008).

Other candidate gene GRMZM2G432480, highly similar to *STAR1*, was also found in the vicinity of the Al tolerance QTL5.1. In rice, *STAR1* encodes the nucleotide binding domain of an ATP binding cassette (ABC) transporter complex responsible to the UDP-glucose efflux to apoplast (Huang et al., 2010). The UDP-glucose can modify the cell wall by glycosylation making a protective outer layer as mucilage hindering Al entrance in the cell (Huang et al., 2010). Indeed, cytoplasmatic UDP-glucose is a substrate for UDP-glycosyltransferases, a key enzyme in the biosynthesis of the benzoxazinoid acids (Frey et al., 2009). This pathway in maize ends with the production of two important benzoxazinoid acids (DIMBOA-Glc and DIBOA-Glc) that are transported to the vacuole where they may act as chelator (Frey et al., 2009). Interestingly, UDP-glycosyltransferase, represented by the oligoMZ00044436 in a microarray experiment, was induced by Al treatment in root apices of a maize Al tolerant line (Maron et al., 2008). The UDP-glycosyltransferase gene was also reported to be induced in the Al sensitive maize line S1587-17 (Cançado et al., 2008). Thus, it could be suggested that the UDP-glucose brought to the cytoplasm by the ABC transporter is somehow involved in the internal chelation of the toxic Al. Further experimental studies are certainly required to investigate these hypotheses. However, a combination of in silico information supports physiological evidences that other mechanisms besides organic acid exudation are involved in maize Al tolerance (Piñeros et al., 2005).

CONCLUSIONS

Genome scanning using a high density of GBS-based markers in a RILs population developed using extremely contrasting lines was able to confirm two major Al tolerance QTL previously identified and to detect novel genomic regions contributing with minor effects on this trait. We also validated the principal Al tolerance QTL6, providing additional support that *ZmMATE1* is the candidate gene responsible for improving Al tolerance in maize. This QTL can be transferred by molecular breeding into elite maize line, aiming to develop cultivars with superior Al tolerance. Finally, we combined genetic and in silico information to rise new

insights about genes and mechanisms that can be evaluated to better understand Al tolerance in maize.

MATERIAL AND METHODS

Genetic material

We first characterized for Al tolerance 36 tropical maize inbred lines developed by the maize breeding program of Embrapa Maize and Sorghum, Sete Lagoas, Brazil, as well as the temperate line B73. The line names starting with numbers or parenthesis are elite lines developed in the last ten years, whereas lines starting with "L" were developed in early 90^{ths}.

For marker-trait association and QTL mapping a population composed by 118 recombinant inbred lines derived from a cross between Cateto Al 237 and L53 was used. The parental lines have been widely characterized as contrasting for Al tolerance, being Cateto Al 237 highly Al-tolerant and L53 Al-sensitive.

Aluminum tolerance evaluation in nutrient solution

The experiment was performed in a growth chamber at 27/20^{o}C day/night temperatures, light intensity of 330 µmol photons m^{-2} s^{-1} and 12-h photoperiod. Maize seeds were disinfested with 0.5% (w/v) NaOCl for 5 minutes, thoroughly rinsed in deionized water and germinated for four days on moistened germination paper rolls. Seedlings were transferred to polyethylene cups placed into containers filled with 8.5 l of nutrient solution described by Magnavaca et al. (1987) at pH 4.0 under continuous aeration, and acclimatized in full nutrient solution for 24 h. After measurements of the initial seminal root length (ISRL) of all seedlings, half of them were replaced with nutrient solutions with or without {39} µM Al^{3+} activity supplied with $KAl(SO_4)_2$. Free Al^{3+} activities (number in brackets) were calculated using GEOCHEM-EZ speciation software (Shaff et al., 2010). The solution pH was adjusted and monitored to 4.0 using 1 M NaOH. After growing for five days, the final seminal root length (FSRL) was measured for each plant. Aluminum tolerance was evaluated as Relative Net Root Growth (RNRG) measured as net root growth in nutrient solution containing {39} µM Al^{3+} (FSRL – ISRL) divided by the net root growth in control condition, without Al.

Statistical analysis of the phenotypic data

To characterize Al tolerance of maize lines, statistical analysis was carried out in groups of experiments using the incomplete blocks design considering genotypes and blocks as random effects, and experiments as fixed effects. This analysis allows the adjustment of genotypic values (u + g) eliminating the influences of fixed factors, which were the experimental means. Two lines, L53 and Cateto Al237, were used as common checks in each experiment, which was carried out with three replicates and seven plants per plot. The linear mixed model methodology was implemented by the software SELEGEN-REML/BLUP (Resende, 2007) following the model:

$y = Xt + Zg + Wb + e$, where:

y: vector of evaluated trait;

t: vector of experimental effects (fixed);

g: vector of genotypic effects (random);

b: vector of block effects (random);

e: vector of errors (random);

X: incidency matrix of experimental effects;

Z: incidency matrix of genotypic effects;

W: incidency matrix of block effects.

Confidence intervals (CI) were estimated using the standard error of the predicted genotypic values (SEP) assuming no missing plots, which corresponded to the square root of predicted error variance (PEV), according to the expression: IC = (u+g) ± t x SEP, where: $t_{(0.95)}$ = 1.96, value of Student distribution at 95% of interference confidence. No overlapping of the confidence intervals indicates statistical differences of predicted genotypic effects in multiple comparisons.

For QTL mapping, the RNRG was used as phenotypic index to evaluate Al tolerance and the net root growth (NRG) in nutrient solution without Al was used to measure the intrinsic root growth. The experiment was carried out in a complete randomized design with two replicates and seven plants per plot. Variance analyses were performed using Genes software (Cruz, 2006) to check the heritability and coefficients of genetic and experimental variation.

Molecular markers

Leaf DNA was extracted from the parental lines and from the 118 RILs using the CTAB protocol described by Saghai-Maroof et al. (1984). Each line was represented by 10 to

12 individuals. PCR reactions for SSR markers were performed according to Ninamango-Cárdenas et al. (2003) and the amplified fragments were resolved in 10% (w/v) polyacrylamide gels loaded in TBE buffer. After two hours of electrophoresis at 200 V, gels were incubated under constant stirring for 15 minutes in fixative solution [ethanol 10% (v/v), acetic acid 0.5% (v/v)], and 15 minutes in a silver nitrate 0.2% (w/v) solution followed by the developer solution [NaOH 3% (w/v), formaldehyde 0.18% (v/v)] until bands appeared. Gel images were photographed under white light in Gel Logic 200 image system (Kodak). Primer sequences and genomic location of the SSRs were obtained at Maize Genetics and Genomics Database (http://www.maizegdb.org/).

STS markers for candidate genes were based on sequences alignment from genomic fragments of the parental lines. *ZmMATE1* and ZmMATE2 were genotyped according to Maron et al. (2010). For *ZmASL*, primers 5´-CCGGCACAGCAGTATCAAC-3´ and 5´-TTGCTTTCCCCGATAGAGAA-3´ were designed based on reference sequences obtained from the Maize Assembled Genomic Island (MAGI) Database (Fu et al. 2005). The polymorphism of 25 pb indel was evaluated on 1% (w/v) agarose gels stained with ethidium bromide.

SNPs were generated using the genotyping-by-sequence technology as described by Elshire et al. (2011). Detailed documentation and protocols can be found at http://www.maizegenetics.net/Table/Genotyping-By-Sequencing/. Genomic library quality was assessed on an Experion/Automated Electrophoresis station (BioRad). Single-end sequencing of one 96-plex library per flowcell channel was performed on a Genome Analyzer II (Illumina). Sequencing reads of 86 bp were extracted from unfiltered qseq files, since the filtering process that produces fastq files can discard usable reads that align perfectly to the reference genome for at least 64 bases (Elshire et al. 2011).

Marker-trait association, linkage map construction and QTL mapping

A total of 458,255 SNPs were detected in the population, which was filtered using a minimum allelic frequency of 0.4 and 20% of missing data. After filtering, 36,147 SNPs (7.9%) were computed for missing data and tested with the phenotypic traits using the generalized linear model (GLM) with a significance threshold calculated by 1,000 permutations at $P< 0.05$. SNPs filtering and marker-trait association were performed using TASSEL software version 3.0.83 (Bradbury et al., 2007). SNPs showing the least association probability were selected in a physical interval of up to 50 Mb.

For genetic map construction, different procedures were implemented manually to filter non-informative markers. Initially, SNPs showing expected segregation ($P < 0.05$) were ordered using a LOD threshold of 5 and maximum distance of 30 cM with the Kosambi

mapping function (Kosambi 1944) by a combination of the computational packages MapMaker/EXP 3.0 (Lander et al., 1987) and OneMap (Margarido et al., 2011). Physical position of SNPs based on the reference maize genome (Elshire et al. 2011) was used to consolidate their order and assignment to chromosomes. Then, SSR and the candidate genes were added to the map accepting the best order given by the multi-point estimates of likelihood of odds. Markers linked by less than 0.5 cM were discarded from the final map.

QTL mapping was performed using multiple interval mapping proposed by Kao et al. (1999) and implemented by QTL Cartographer 2.5 for Windows (Wang et al., 2007). The final MIM model was selected using forward search method followed by several rounds of adjustments of main effect QTL and QTL interactions based on the Bayesian Information Criteria (BIC). MIM estimates the position, effects and proportion of the genetic variance contributed by individual QTL as well as the broad sense heritability, which can be approximated by the total R^2 of the MIM model. Confidence intervals were established as two LOD support interval (Lander & Botstein, 1989)

Development of NILs for the two major Al tolerance QTL

RIL-150 and RIL-84 were selected based on the presence of each individual QTL and the percentage of recurrent genome close to 50%. These lines were submitted to two cycles marker-assisted backcrosses with L53 and selfed, reaching the BC2F3 generation. For QTL on chromosome 6, the foreground selection was based on the markers *ZmMATE1* and umc1018, whereas the QTL on chromosome 5 was selected using only the *ZmMATE2* marker. The background selection was performed using around 20 SSR markers randomly distributed along the genome. The selected NILs presented on average 97% of the L53 genome, except for each target QTL/candidate gene region.

Additionally, the NILs for each QTL were crossed and selfed twice. The individuals carrying homozygous alleles for both QTL derived from Cateto Al237 or from L53 were selected using the same marker combinations.

Expression pattern of ZmMATE1 and ZmMATE2

Expression of both candidate genes was evaluated in the RILs population, NILs and their parental lines L53 and Cateto Al237. Total RNA was extracted using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions, from root tips submitted to 12 hours of {39} µM of Al^{3+} activity, following the same experimental conditions in nutrient

solution described before. First-strand cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). *ZmMATE1* expression was determined using a custom-designed TaqMan assay consisting of forward primer 5'-CACCCGCTTAGCGTATTCCT-3', reverse primer 5'-GCACCGCGATCCTCATGAT-3' and probe 5'-TCTGAATGCGAGCCTCG-3'. A predesigned TaqMan assay for Eukaryotic 18S (Applied Biosystems) was used as endogenous control. Quantitative real-time PCR was conducted in a 7900HT Sequence Detection System (Applied Biosystems). *ZmMATE2* expression profile was evaluated using TaqMan assay according to Maron et al. (2010) in an ABI 7500 Fast. Each biological sample was composed by 21 root tips of 1 cm, and all reactions were performed in triplicates for the target gene and the control. The Al-sensitive parent L53 was set as calibrator sample. Raw data were collected in RQ Manager (Applied Biossystems) and the relative expression levels were calculated using the ddCT method.

Search for candidate genes

To identify candidate genes in maize we used the protein sequences of the genes involved in Al tolerance in other species such as *SbMATE*, *TaALMT1,Nrat1*, *ART1*, *STOP1*, *STAR1* and *STAR2*. BLAST searches were performed in the Phytozome (www.phytozome.net) and MaizeSequence (www.maizesequence.org) databases. Maize predicted genes showing high similarity with the query and localized within the Al tolerance genomic regions were selected. Percentage of sequences similarities were obtained using the T-Coffee alignment with the default settings (http://www.ebi.ac.uk/Tools/).

Phylogenetic studies of MATE genes in maize genome

Predicted MATE members in maize were identified by sequence similarity analysis using the SbMATE amino acid sequence as reported above. However, serial rounds of searches using other maize best hits as query were carried out. The Peptide Homologs tool in Phytozome was used to confirm that all predicted maize MATEs were identified.

Amino acid sequences of maize MATEs as well as the MATE genes previously identified as Al tolerance genes in other crops as sorghum (*SbMATE*), Arabidopsis (*AtMATE* and *AtFRD3*), rice (*OsFRDL4*), barley (*HvAACT1*), and wheat (*TaMATE*) were aligned using the Advanced M-COFFEE package available at T-COFFEE (www.tcoffee.org). Phylogenetic tree was constructed using maximum parsimony and 500 bootstraps with MEGA5 (www.megasoftware.net).

REFERENCES

Bradbury, PJ, Zhang Z, Kroon DE, Casstevens TM, Ramdoss Y, Buckler ES:.TASSEL, software for association mapping of complex traits in diversesamples.*Bioinformatics* 2007, 23: 2633-2635.

Cançado GMA, Nogueira FTS, Camargo SR, Drummond RD, Jorge RA, Menossi M: Gene expression profiling in maize roots under aluminum stress. Biol Plantarum 2008, 52:475-485.

Caniato FF, Guimarães CT, Schaffert RE, Alves VMC, Kochian LV, Borém A, Klein PE, Magalhães JV:. Genetic diversity for aluminum tolerance in sorghum.Theor Appl Genet2007, 85: 863-876.

Caniato FF, Guimarães CT, Hamblin M, Billot C, Rami J-F, Hufnagel B,Kochian LV, Liu J, Garcia AA, Hash CT, Ramu P, Mitchell S, Kresovich S, Oliveira AC, Avellar G, Borém A, Glaszmann JC, Schaffert RE, Magalhães JV: The Relationship between Population Structure and Aluminum Tolerance in Cultivated Sorghum.Plos One 2011, 6: 1-14.

Conceição LDHCS, TESSELE C, BARBOSA NETO JF: Diallel analysis and mapping of aluminum tolerance in corn inbred lines.Maydica 2009, 54:51-64.

Cruz C D: Programa Genes - Biometria. 1. ed. Viçosa, MG: Editora UFV, 2006. v. 1. 382 p.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler E, Mitchell SE: A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species.Plos One 2011, 6:1-10.

Frey M, Schullehner K, Dick R, Fiesselmann A, Gierl A: Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Phytochemistry 2009, 70:1645-1651.

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

Hoekenga OA, Maron LG, Piñeros MA, Cançado GMA, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T, Matsumoto H, Yamamoto Y, Koyama H, Kochian L V: AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. Proc Natl Sci 2006, 103: 9738-9743.

Huang CF, Yamaji N, Ma JF: Knockout of a bacterial-type ATP-binding cassette transporter gene, AtSTAR1, results in increased aluminum sensitivity in Arabidopsis. Plant Physiol2010, 153:1669-1677.

Hoekenga OA, Vision TJ, Shaff JE, Monforte AJ, Lee GP, Howell SH, et al. (2003). Identification and Characterization of Aluminum Tolerance Loci in Arabidopsis (Landsbergerecta x Columbia) by Quantitative Trait Locus Mapping.A Physiologically Simple But Genetically Complex Trait.Plant Physiol. 132: 936-948.

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

Iuchi S, Koyama H, Iuchi A, Kobayashi Y, Kitabayashi S, Kobayashi Y, Ikka T, Hyrayama T, Shinozaki K, Kobayashi M:. Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. Proc Natl Sci 2007, 104:9900-9905.

Kao, C H, Zeng Z B, Teasdale RD: Multiple interval mapping for quantitative trait loci. Genetics 1999, 132:1203-1216.

Kochian LV:Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Biol1995, 46: 237-260.

Kochian LV, Hoekenga OA, Piñeros MA: How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency.Annu Rev Plant Biol2004, 55:459-493.

Krill AM, Kirst M, Kochian LV, Buckler ES, Hoekenga OA: Association and linkage analysis of aluminum tolerance genes in maize. Plos One 2010, 5: 1-11.

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L: MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1987, 1:174-81

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

Ligaba A, MaronL, Shaff J, Kochian L, Piñeros M: Maize ZmALMT2 is a root anion transporter that mediates constitutive root malate efflux. Plant Cell Environ 2012,in press.

Lima M, Miranda Filho JB, Furlani PR: Diallel cross among inbred lines of maize differing in aluminum tolerance. Brazil J Genet 1995, 4:579-584.

Liu J, Magalhães JV, Shaff J, Kochian LV: Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance. Plant J 2009, 57:389-399.

Ma JF, Nagao S, Sato K, Ito H, Furukawa J and Takeda K (2004b).Molecular mapping of a gene responsible for Al-activated secretion of citrate in barley.J. Exp. Bot. 55: 1335-1341.

Ma HX, Bai GH, Carver BF and Zhou LL (2005).Molecular mapping of a quantitative trait locus for aluminum tolerance in wheat cultivar Atlas 66.Theor. Appl. Genet. 112: 51-57.

Magalhaes JV, Garvin DF, Wang Y, Sorrells ME, Klein PE, Schaffert RE, Li L, Kochian LV (2004). Comparative mapping of a major aluminum tolerance gene in sorghum and other species in the Poaceae.Genetics. 167: 1905-1914.

Navakode S, Weidner A, Lohwasser U, Röder MS, Börner A (2009). Molecular mapping of quantitative trait loci (QTLs) controlling aluminium tolerance in bread wheat. Euphytica, 166: 283–290.

Magalhães JV, Liu J, Guimaraes CT, Lana UGP, Alves VMC., Wang Y, Schaffert RE, Hoekenga OA, Piñeros MA, Shaff JE, Klein PE, Carneiro NP, Coelho CM, Trick H N Kochian LV: A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nature Genet 2007, 39:1156-1161.

Magnavaca R, Graminer CD, Clark, RB: Inheritance of aluminum tolerance in maize. In Genetic aspects of plant mineral nutrition. Edited by Gabeiman HW, Lougnmam BC. Dordrecht: Martinus Nijhoff; 1987:201-212.

Margarido GRA, Mollinari M, Garcia AAF, (2011). OneMap Tutorial: Software for constructing genetic maps in experimental crosses. Department of Genetics, ESALQ, USP p.96.

Maron LG, Piñeros MA, Guimarães CT, Magalhães JV, Pleiman JK, Mao C, Shaf J, Belicuas SN, Kochian LV: Two functionally distinct members of the MATE (multidrug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. Plant J 2010, 61:728-740.

Maron LG, Kirst M, Mao C, Milner MJ, Menossi M, Kochian LV: Transcriptional profiling of aluminum toxicity and tolerance responses in maize roots. New Phytol 2008, 179:116-128.

Ninamango-Cárdenas F, Guimarães CT, Martins PR, Parentoni SN, Carneiro NP, Lopes MA, Moro JR, Paiva E: Mapping QTLs for aluminum tolerance in maize. Euphytica 2003, 130: 23-232.

Pandey S, Magnavaca R, Bahia Filho AFC, Dugue-Varges J, Vinasco LE:Genetics of tolerance to soil acidity in tropical maize. Crop Sci 1994, 34:1511-1514.

Pellet DM, Grunes DL, Kochian LV: Organic acid exudation as an aluminium-tolerance mechanism in maize (Zea mays L.). Planta 1995, 196:788-795.

Piñeros MA, Shaff JE, Manslank HS, Alves VM, Kochian LV: Aluminum Resistance in Maize Cannot be Solely Explained by Root Organic Acid Exudation. A Comparative Physiological Study.Plant Physiol 2005, 137:231-241.

Piñeros MA, Cancado GMA, Maron LG, Lyi SM, Menossi M, Kochian LV: Not all ALMT1-type transporters mediate aluminum-activated organic acid responses: the case of ZmALMT1, an anion-selective transporter. Plant J 2008, 53:352-367..

Poland JA, Brown PJ, Sorrells ME, , Jannink JL: Development of High-Density Genetic Maps for Barley and Wheat Using a Novel Two-Enzyme Genotyping-by-Sequencing Approach. Plos One 2012, 7:1-8.

Raman H, Zhang K, Cakir M, Appels R, Garvin DF, Maron LG, et al. (2005). Molecular characterization and mapping of ALMT1, the aluminium-tolerance gene of bread wheat (*Triticum aestivum* L .). Genome, 791: 781-791.

Resende MDV (2007) Software Selegen – REML/BLUP: sistema estatístico e seleção genética computadorizada via modelos lineares mistos. Embrapa Florestas, Colombo, 350p.

Ryan PR, Raman H, Gupta S, Horst WJ, Delhaize E: A second mechanism for aluminium resistance in wheat relies on the constitutive efflux of citrate from roots. Plant Physiol2009, 149:340-351.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW: Ribosomal DNA spacer length polymorphism in barley: mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci 1984, 81: 8014-8018.

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

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

Shaff JE, Schultz BA, Craft EJ, Clark RT, Kochian LV: GEOCHEM-EZ: a chemical speciation program with greater power and flexibility. Plant Soil 2010, 330:207-214

Sibov ST, Gaspar MJ, Ottoboni LMM, Arruda P, Souza AP: Two genes controlling aluminum tolerance in maize: genetic and molecular mapping analyses. Genome 1999, 42:475-482.

Studer A, Zhao Q, Ross-Ibarra J, Doebley J: Identification of a functional transposon insertion in the maize domestication gene tb1. Nature Genet 2011, 43:1160-1165.

vonUexküll H R, Mutert E: Global extent, development and economic impact of acid soils. Plant Soil 1995, 71:1-15.

Wang S, Basten CJ, Zeng Z: Windows QTL Cartographer 2.5. N.C. State University, Bioinformatics Research Center, 2007, USA.

Xia J, Yamaji N, Kasai T, Ma JF: Plasma membrane-localized transporter for aluminum in rice. Proc Natl Sci 2010 107:18381-18385.

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

Yokosho K, Yamaji N, Ma JF: Isolation and characterization of two MATE genes in rye. Func Plant Biol 2010, 37:296-303.

Yokosho K, Yamaji N, Ma JF: An Al-inducible MATE gene is involved in external detoxification of Al in rice. Plant J 2011, 68:1061-1069.