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

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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 colocated 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.

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CAPÍTULO 1

Genetic and molecular mechanisms of aluminum tolerance in plants

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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, cosegregating 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 Alactivated 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 nucleotidebinding 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.

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