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CARACTERIZAÇÃO E UTILIZAÇÃO DE GENES ENVOLVIDOS NA TOLERÂNCIA AO ALUMÍNIO TÓXICO EM MILHO

Geraldo Magela de Almeida Cançado

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Universidade Estadual de Campinas

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Caracterização e Utilização de Genes Envolvidos na Tolerância ao Alumínio Tóxico em Milho

Geraldo Magela de Almeida Cançado

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) GERALDO MAGELA DE ALMEIDA CANÇADO

e aprovada pela Comissão Julgadora.

Tese apresentada no Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do título de Doutor em Genética e Biologia Molecular, área de concentração Genética Vegetal e Melhoramento

Orientador: Prof. Dr. Marcelo Menossi

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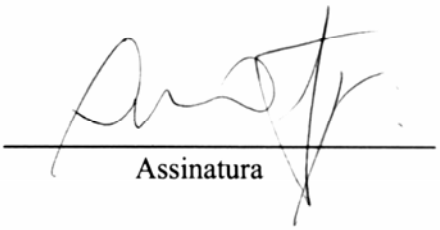

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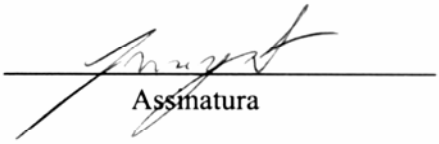

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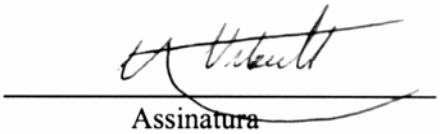

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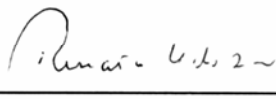

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Resumo

Caracterização e utilização de genes envolvidos na tolerância ao alumínio tóxico em milho

Solos ácidos são encontrados em todas as regiões do planeta e em grandes proporções nas regiões tropicais e subtropicais. Como conseqüência da acidez do solo, o alumínio (Al), metal mais abundante da crosta terrestre, torna-se solúvel e atinge concentrações tóxicas para a maioria das espécies de plantas cultivadas. O primeiro dano provocado pelo Al iônico é a redução do desenvolvimento radicular, causando distúrbios fisiológicos que acarretam na redução da produção.

Devido às limitações dos métodos convencionais de correção do pH do solo e a necessidade de longos períodos de tempo para o desenvolvimento de novas cultivares pelo melhoramento genético clássico, muita ênfase tem sido dada para a compreensão dos mecanismos de toxidez e de tolerância ao Al em plantas. Pois, com a aquisição destes conhecimentos, espera-se mais sucesso na obtenção de plantas tolerantes ao Al com o emprego da tecnologia de DNA recombinante e da seleção assistida por marcadores moleculares.

Neste trabalho utilizamos duas linhagens de milho contrastantes para tolerância ao Al: Cat100-6 (tolerante ao Al) e S1587-17 (sensível ao Al), com o objetivo de estudar as alterações na expressão gênica promovidas pelo estresse de Al no ápice radicular. A presente tese esta dividida em três linhas de pesquisa: *i*) identificação, clonagem e caracterização de um gene codificando uma enzima glutationa S-transferase e avaliação dos efeitos do Al na sua expressão gênica; *ii*) avaliação das alterações na expressão gênica em ápices de raízes de duas linhagens de milho quando submetidos ao estresse de Al, utilizando um sistema de hibridização heterólogo com ESTs (expressed sequence tags) de cana-de-açúcar; e *iii*) clonagem e caracterização em milho do gene *ALMT1*, pertencente a uma nova classe de proteína transportadora de moléculas orgânicas especificamente ativada pelo Al.

No trabalho com o gene *GST27*.2 observamos que este gene foi induzido pelo estresse provocado por Al e por Cd (cadmio) e que mutações que provocavam alterações na composição de aminoácidos da proteína poderiam promover alterações

na atividade e na especificidade desta enzima. Além disso, o gene *GST27.2* parece ser um novo alelo do gene *GST27* estando presente como cópia única no genoma das linhagens de milho estudadas.

Já no trabalho de avaliação da expressão gênica em larga escala, foram identificados 85 genes nos ápices radiculares das duas linhagens de milho cuja expressão foi diferencialmente alterada pela presença do Al. Embora alguns dos genes já tivessem sido descritos como responsivos ao Al, para a maior parte dos genes identificados neste trabalho, este foi o primeiro relato descrevendo seu envolvimento com o estresse de Al.

A clonagem em milho do gene homológo ao gene *ALMT1*, demonstrou que o milho também deve ter uma proteína de membrana presente em células do ápice radicular que pode estar envolvida com transporte de moléculas orgânicas. Embora a proteína não esteja envolvida com o transporte de malato, a mesma teve sua atividade melhorada pela presença do Al. Entretanto, o gene que codifica esta proteína é reprimido no tecido do ápice radicular das linhagens de milho tolerante e sensível ao Al, o que pode indicar a existência de regulação pós-transcricional.

Os resultados obtidos a partir destas três abordagens contribuíram para a compreensão dos mecanismos de tolerância e toxidez ao Al em raízes de milho. Futuramente, essas informações auxiliarão na escolha de genes mais apropriados para a criação de plantas geneticamente alteradas mais adaptadas a presença do Al no solo.

Abstract

Characterization and utilization of genes involved with aluminum tolerance in maize

Acid soils are found worldwide but most of them are located in tropical and sub-tropical regions. Aluminum (Al), the most abundant metal on the earth surface, becomes soluble in the soil solution as consequence of low pH in acid soils and achieves phytotoxic levels for most of the cultivated plant species. The first symptom of Al toxicity is the inhibition of the root growth that promote physiological disturbs reducing crop yield.

Because of limitations of correcting soil pH by liming and the time-consuming process of traditional plant breeding, the elucidation of the mechanisms involved with plant Al-tolerance and Al-toxicity has received more attention, since the production of genetically altered plants has emerged as an effective and fast strategy to the production of improved cultivars.

Two maize lines, Cat100-6 (Al-tolerant) and S1587-17 (Al-sensitive), were used in this study with the aim of understanding at the transcriptional level the alterations promoted by Al on the roots. The research was divided in three main sections: *i)* detection, cloning and characterization of a gene encoding a Glutathione S-transferase in maize and evaluation of Al effects on its expression; *ii)* Large-scale evaluation of gene expression in root tips of maize under Al stress using a heterologous system with Expressed Sequence Tags (ESTs) of sugarcane; and *iii)* cloning and characterization of the *ALMT1* gene in maize and evaluation of Al-effects on its activity.

In the first section was observed that Al and Cd-stress induced the *GST27*.2 gene. Two mutations present on the nucleotide chain of this gene promoted alteration on the amino acid compositions. These alterations might be responsible by alterations on the specificity and activity of the GST enzyme. Besides that, the *GST27.2* is a single copy gene in maize and seem to be a new allele of *GST27.*

In the section of large-scale gene expression evaluation were identified 85 genes in root tips of two Al-tolerant contrasting maize lines whose expression was altered by Al stress. Although several of the genes identified here were previously described as Al-

responsive in other works, to most of them this study is the first report about the involvement of these genes with Al stress.

The cloning of the *ALMT1* homologue in tissue from the root apex of maize shown that maize has a gene encoding a membrane protein that might be involved with organic molecules transport. Although the protein encoded by the maize homologue gene was not associated with malate transport the activity of this protein was stimulated by the presence of Al. Interestingly, the gene expression of the this gene was repressed by Al in the Al-tolerant and Al-sensitive genotype. This result might be an indicative of existence of posttranscriptional regulation.

The results accomplished with the experiments described here launched new light into the understanding of the Al-tolerance and Al-toxicity mechanisms in maize roots. Furthermore, the information presented here will contribute to a more accurate selection of genes that will be used to produce transgenic plants better adapted to soils with high Al concentration.

Introdução Geral

1. Origem e importância econômica da cultura do milho para o Brasil

Há indícios de que a origem do milho tenha sido no México, nas regiões de Tehuacán no estado de Puebla (MacNeish e Eubanks, 2000) e/ou Rio Balsas nos estados de Michoacán e Guerrero (Doebley, 1990). O milho é uma das culturas mais antigas do mundo, havendo indicações, através de escavações arqueológicas e geológicas, e através de medições por desintegração radioativa, de que é cultivado há pelo menos 6.000 anos (Piperno e Flannery, 2001). Logo depois do descobrimento da América Central e do Norte, o milho foi levado para a Europa, onde era cultivado em jardins, até que seu valor alimentício tornou-se conhecido. Passou então a ser plantado em escala comercial e difundiu-se desde a latitude de 58^{o} norte (União Soviética) até 40^{o} sul (Argentina) (Duarte, 2002).

Os parentes mais próximos do milho são os teosintes (Fig. 1), grupo de gramíneas anuais originárias do México e da Guatemala. Análises moleculares identificaram o teosinte (*Zea mays* ssp. *parviglumis*) como o precursor do milho. Apesar das divergências morfológicas entre o milho moderno e os teosintes, vários estudos indicam que algumas variedades de teosinte são citologicamente equivalentes ao milho, sendo capazes de formar híbridos férteis com este (revisado por Doebley, 2004). Ao que tudo indica o milho foi originado do teosinte através de seleção feita por civilizações indígenas (Beadle, 1978). Hoje são conhecidos cinco principais tipos de milho: Pipoca, Duro, Dentado, Farináceo e Doce.

Na evolução mundial de produção de milho, o Brasil tem destaque como terceiro maior produtor, superado apenas pelos Estados Unidos e pela China. No ano agrícola de 2005/06 a produção mundial ficou em torno de 684 milhões de toneladas, tendo sido produzido 282 milhões pelos Estados Unidos, 134 milhões pela China e 43 milhões pelo Brasil (USDA, 2006). Apesar de estar entre os três maiores produtores, o Brasil não se destaca entre os países com maior produtividade. Considerando que a produtividade média mundial está pouco acima de 4.000 kg/ha, nota-se que o Brasil está abaixo desta

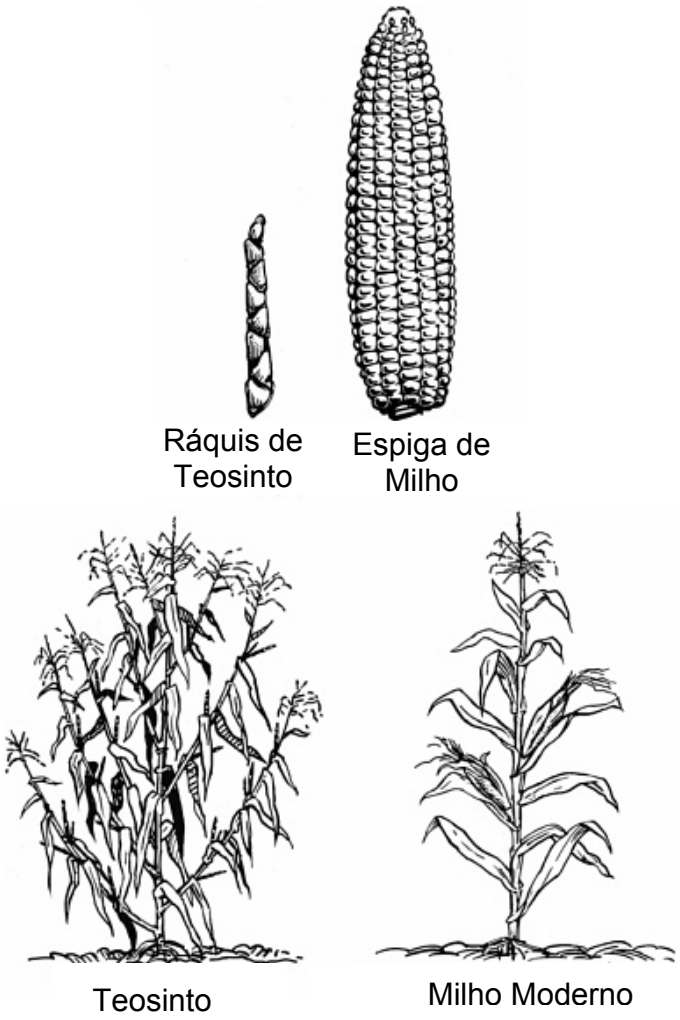

Figura 1. Comparação morfológica entre a inflorescência e a arquitetura da planta de teosinto e de milho moderno. Extraído de Joseph Henry Press. Mendel in the Kitchen: Scientist's View of Genetically Modified Food. 2004.

média. Porém, a produtividade brasileira tem crescido sistematicamente, passando de 1.870 kg/ha, em 1990, para 3.160 kg/ha, em 2005 (IBGE, 2006).

No cenário nacional, o milho é o segundo grão mais importante para a agricultura brasileira. No ano agrícola de 2005/06, sua produção correspondeu a 30,8% da produção total de grãos do país, só perdendo para a soja que representou 45,3% da produção nacional (IBGE, 2006). Embora o milho seja uma importante cultura para o agronegócio brasileiro, praticamente toda sua produção é consumida internamente, ao contrário da soja que concentra sua comercialização em mercados externos. A maior parcela dos grãos é destinada ao preparo de rações para alimentação animal, principalmente de aves e suínos, e para a produção de óleo comestível. Atualmente, uma crescente parcela da produção de milho tem sido destinada à fabricação de produtos para a alimentação humana direta, tais como farinhas, cereais matinais, salgadinhos e xaropes de dextrose, indicando uma maior adoção do milho como fonte de alimento pelos brasileiros. Um setor interessante onde a participação do milho tem crescido e deverá crescer bastante é na produção de plásticos biodegradáveis a partir de amido de milho (Da Róz, 2003).

A importância do milho ainda está relacionada ao aspecto social, pois grande parte dos produtores brasileiros de milho utiliza pouca tecnologia, não possui grandes extensões de terras, e cultiva para subsistência comercializando o excedente, fator que se reflete nas baixas produtividades médias. Pode-se, portanto, afirmar que há uma clara dualidade na produção de milho no Brasil: uma grande parcela de pequenos produtores que não estão envolvidos com a produção comercial e que atingem apenas baixos índices de produtividade, e uma pequena parcela de grandes produtores, com elevado índice de produtividade, cultivando extensas áreas de monocultura, com aplicação intensiva de tecnologia e elevado investimento de capital na produção de milho (Duarte, 2002).

Desta forma, a geração de tecnologias financiada pelo Poder Público deve levar em consideração não apenas a otimização do processo produtivo em sistemas com elevada aplicação de tecnologia, mas também favorecer o modelo praticado pela agricultura de subsistência, aumentando a disponibilidade de cultivares mais resistentes

a estresses bióticos e abióticos e com melhor desempenho em condições de baixa aplicação de insumos.

2. Solos ácidos e o alumínio solúvel

Solos com pH abaixo de 5,5 cobrem cerca de 30% da superfície terrestre não coberta pelos mares (Fig. 2), mais de 50% das terras potencialmente agricultáveis do mundo (Von Uexküll e Mutert, 1995) e mais de 64% da superfície da América do Sul (Bellon, 2001), caracterizando-se como um dos maiores fatores limitantes para produção agrícola na atualidade. As causas da acidez do solo têm origens naturais, devido ao material de origem e ao processo de intemperização e lixiviação de bases, e de ação antrópica, tais como utilização excessiva de fertilizantes amoniacais, exportação de bases do solo, tais como Ca^{2+} e Mg^{2+}, pelo cultivo excessivo dos solos e poluição industrial que promove o fenômeno de chuva ácida (Johnson et al., 1997; Samac e Tesfaye, 2003). Somente no Brasil, mais de $2x10^8$ ha de áreas potencialmente agricultáveis estão incluídas dentro do bioma Cerrado (Adámoli et al., 1985). Embora a região do Cerrado possua boas qualidades para a prática agrícola, tais como topografia relativamente plana, solos profundos e bem estruturados e distribuição de chuvas bem definida ao longo do ano, grande parcela dos solos do Cerrado são naturalmente ácidos (Goedert, 1983). Desta forma, a otimização da exploração dos solos do Cerrado brasileiro promoveria não só o aumento na produtividade agropecuária, como também refletiria positivamente na preservação de ecossistemas naturais, atualmente ameaçados pela emigração da agricultura para novas áreas.

Como conseqüência da acidez do solo, ocorre um aumento da solubilidade de alumínio (Al) a partir de aluminossilicatos e óxidos de Al, presentes nos minerais que constituem os solos brasileiros. Em valores de pH abaixo de 5,0, o Al existe como o octaedro hexahidratado $Al(H_2O)_6^{3+}$, que por convenção é denominado Al^{3+}. Embora existam ainda as formas desprotonadas $Al(OH)^{2+}$, $Al(OH)_2^{+}$ e o relativamente pouco solúvel $Al(OH)_3$, o Al^{3+} parece ser o íon mais fitotóxico para monocotiledôneas (Kochian, 1995).

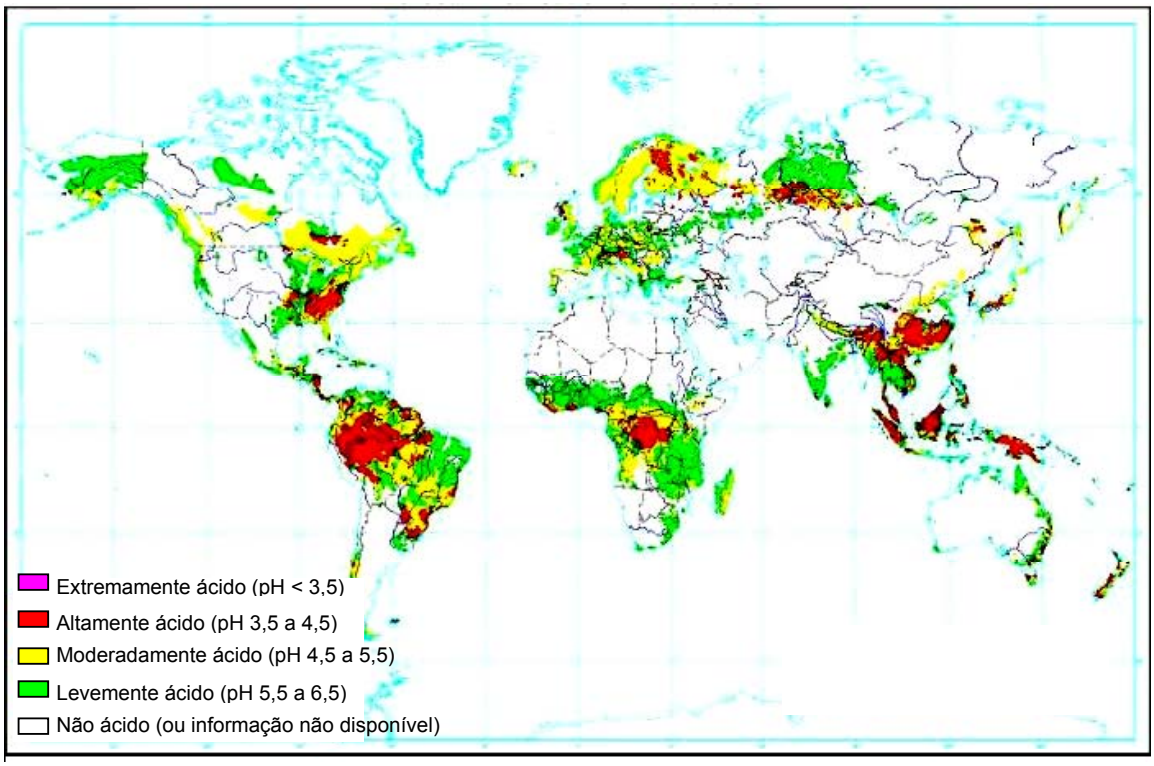

Figura 2. Distribuição global de solos ácidos. Modificado a partir de USDA, NRCS, World Soil Resources, Washington, D.C.

Comumente, a acidez do solo e os problemas advindos dela, tal como a toxidez de Al, são corrigidos através da prática de calagem, que consiste na aplicação e incorporação de calcário no solo. Embora a calagem possa eliminar os efeitos tóxicos do Al solúvel, para que isso ocorra com efetividade é necessário que o calcário seja aplicado no solo de forma uniforme e posteriormente seja bem incorporado. Apesar do custo do calcário ser baixo em relação a outros insumos agrícolas, o custo envolvido no seu transporte, aplicação e incorporação é elevado. Além disso, a incorporação do calcário em camadas profundas do solo é inviável, o que acaba favorecendo o desenvolvimento radicular apenas na camada superficial do solo, tornando as plantas mais suscetíveis a oscilações na disponibilidade de água no solo (Cançado et al., 2001).

Outra forma de se evitar os efeitos danosos do Al é pelo desenvolvimento de cultivares mais tolerantes à presença deste cátion. Em milho, a maioria das variedades comerciais é suscetível ao Al tóxico, sendo a utilização de genótipos tolerantes uma alternativa adequada para elevar a eficiência da cultura em regiões com limitação por acidez (Paterniani e Furlani, 2002).

3. Toxidez e tolerância ao alumínio em plantas

O primeiro sintoma de toxidez é a inibição da elongação da raiz, que ocorre após poucos minutos do início da exposição ao Al (Ryan et al., 1993). Este cátion, quando em contato com as raízes, promove rapidamente a paralisação do crescimento radicular, acabando por atrofiá-las em função da morte do meristema radicular. As plantas apresentam sintomas de deficiência de nutrientes, tais como fósforo, cálcio, magnésio, potássio e molibdênio, devido à interferência do Al nos processos de absorção, transporte e uso destes nutrientes, e se tornam mais suscetíveis ao estresse hídrico (Barcelo e Poschenrieder, 2002). Ao que tudo indica, o ápice radicular é o sítio primário da ação do Al (Bennet et al., 1987; Ryan et al., 1993), provavelmente mais em função do efeito do Al no processo de expansão das células do ápice do que no processo de divisão celular (Kochian 1995).

Embora o Al seja um metal sem atividade redox, ele é um poderoso pró-oxidante, pois é capaz de oxidação biológica tanto *in vitro* como *in vivo* (Exley, 2004). Devido a esta elevada reatividade do Al, há muitos alvos em potencial na célula para os efeitos tóxicos do Al. Kochian et al. (2004) em recente revisão sobre a toxidez de Al em plantas, aponta a parede celular, a superfície da membrana plasmática, o citoesqueleto e o núcleo celular como possíveis alvos para os efeitos tóxicos do Al. Estes autores ainda citam que o Al pode interagir de forma prejudicial com a homeostase do Ca^{2+} citossólico e desta forma, prejudicar todo o processo de sinalização celular.

Desde o início da década de 90, esforço considerável tem sido focado na elucidação dos mecanismos fisiológicos, bioquímicos e genéticos envolvidos com a tolerância ao Al em plantas. A partir destes esforços, foi sedimentada a idéia da existência de duas classes distintas de tolerância ao Al operando em plantas. A primeira classe seria a que envolve os processos de exclusão do Al do ápice radicular e a outra classe seria a que envolve mecanismos que permitem a planta tolerar o Al no simplasto das raízes e da parte aérea (Taylor, 1991). Ao longo dos últimos 20 anos, foram lançadas várias hipóteses para explicar os mecanismos de tolerância ao Al dentro das duas classes citadas anteriormente. No entanto, boa parte delas ainda permanece como especulativas. Provavelmente, mecanismos múltiplos de tolerância devem estar atuando conjuntamente em uma mesma espécie de planta, podendo o número e a intensidade de ação destes mecanismos variar entre as diferentes espécies (Kochian et al. 2004).

Um grande número de evidências tem indicado a exsudação de ácidos orgânicos pelas raízes como tendo um importante papel na detoxificação do Al externo à célula em raízes de várias espécies de plantas (Ma e Furukawa, 2003). Esse mecanismo já foi descrito até o momento para 17 espécies de plantas cultivadas (revisado por Mariano et al., 2005) e elevados níveis de exudação de ácidos di- e tricarboxílicos ativados pela presença do Al tem se correlacionado positivamente com a tolerância diferencial ao Al (Kochian et al., 2004).

Em plantas com capacidade de quelar e seqüestrar o Al internamente, compostos tais como catecois, compostos fenólicos e mesmo ácidos orgânicos ligam-

se ao Al inativando-o. Posteriormente, o complexo é armazenado em células especializadas, tais como células da epiderme foliar (Jensen et al., 2002; Watanabe e Osaki, 2002).

À medida que os mecanismos bioquímicos de tolerância ao Al vão sendo testados e validados pelos resultados da pesquisa, os genes codificando as enzimas ou as proteínas chaves desta rota bioquímica têm sido utilizados para produção de plantas geneticamente alteradas visando o aumento da tolerância ao Al. Como exemplos de sucesso desta abordagem, podemos citar o trabalho recente de Delhaize et al. (2004) com o gene *ALMT1* de trigo, que codifica uma proteína transportadora de malato ativada por Al. Estes autores demonstraram que plantas transgênicas de cevada superexpressando *ALMT1* são mais tolerantes ao Al do que plantas selvagens. Outro exemplo foi o trabalho desenvolvido por Basu et al. (2001) que produziu plantas de *Brassica napus* mais tolerantes ao Al devido a superexpressão do gene manganês superóxido dismutase (*MnSOD*) também de trigo.

Em milho, até a presente data, nenhum relato foi publicado sobre a produção de plantas transgênicas visando aumento da tolerância ao Al. Este fato é intrigante, já que o milho é uma cultura de grande importância econômica e social e é amplamente cultivado em solos ácidos. Duas hipóteses que poderiam explicar esta ausência de resultados para milho seriam a dificuldade de transformação genética e regeneração desta cultura, acrescida do limitado número de genótipos com resposta eficiente ao processo de transformação e regeneração. O híbrido de milho Hi-II (Armstrong et al., 1991) amplamente utilizado para transformação genética de milho, não foi severamente afetado pela presença de 222 μM de Al após 24 h de exposição quando comparado com dois genótipos de milho contrastantes para a tolerância ao Al. No entanto, após 3 dias de exposição ao Al, sofreu redução do crescimento radicular semelhante à linhagem de milho sensível ao Al (Fig. 1 e Fig. 2 em anexos).

4. Toxidez de alumínio e as linhagens de milho Cat100-6 e S1587-17

As linhagens de milho Cat100-6 e S1587-17 são os dois genótipos de milho mais bem caracterizados para resposta a toxidez de Al, com inúmeros artigos

publicados (Moon et al., 1997; Sibov et al., 1999; Jorge et al., 2001; Boscolo et al., 2003; Jorge e Menossi 2005; Cançado et al., 2005). Os dois genótipos fazem parte do Banco de Germoplasma do Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas. O genótipo Cat100-6 é uma linhagem proveniente do germoplasma Cat100. Este germoplasma é proveniente de uma raça autóctone de milho tipo duro, adaptada ao sul da Costa Atlântica do Brasil (Prioli, 1987). O prefixo Cat é proveniente da abreviação de Cateto, denominação utilizada para raça rústica proveniente de material selvagem, selecionada ao longo de várias gerações de cultivo por pequenos agricultores. O primeiro trabalho publicado com Cat100-6 e S1587-17 foi há quase uma década atrás, com a descrição por Moon et al. (1997) da obtenção da linhagem S1587-17 por meio de variação somaclonal em cultura de calos da linhagem tolerante ao Al, Cat100-6. Posteriormente, foi observado que o somaclone S1587-17 era sensível ao Al.

No final da década passada, Sibov et al. (1999) analisando a população segregante produzida a partir do cruzamento entre Cat100-6 e S1587-17, demonstraram que a tolerância ao Al observada na linhagem de milho Cat100-6 era controlada em grande parte, embora não totalmente, por dois genes mapeados no braço curto do cromossomo 6 e no braço curto do cromossomo 10. Foi demonstrado também que o *locus* no cromossomo 10 é aproximadamente 3 vezes mais efetivo que o *locus* no cromossomo 6 para explicar o caráter de tolerância ao Al na linhagem Cat100-6. Embora os genes presentes nos cromossomos 6 e 10 expliquem uma parcela da tolerância ao Al em Cat100-6, provavelmente existem outros genes de ação menor que desempenham papel importante na aquisição da tolerância ao Al nesta linhagem. Os autores ainda concluíram que apesar de S1587-17 ter sido proveniente de Cat100-6, a variabilidade genética observada para regiões de microssatélites entre as duas linhagens foi elevada.

Posteriormente, Jorge et al. (2001) observaram que a atividade da calmodulina não estava associada à tolerância ao Al e que esta molécula não é um sítio primário de ação do Al. Estes mesmos autores observaram que a linhagem tolerante ao Al exsudou cerca de 3,5 mais citrato do que a linhagem sensível, quando cultivadas em solução nutritiva com atividade de 16.2 μM de Al^{3+}.

Já Boscolo et al. (2003) observaram que para a linhagem sensível S1587-17, a presença de Al induzia a formação de espécies reativas de oxigênio e subseqüente oxidação de proteínas do extrato de pontas de raízes, o que não foi observado para Cat100-6. Aumento nas atividades das enzimas SOD e peroxidase também foram observados em maior nível para S1587-17, indicando a maior formação de radicais superóxidos e peróxido de hidrogênio nesta linhagem.

Recentemente, Jorge e Menossi (2005) estudando os efeitos de substâncias antagônicas aos canais de ânion e do La^{3+} no conteúdo de Al e na exsudação de citrato em ápices radiculares de S1587-17 e Cat100-6, observaram uma correlação inversa entre a atividade de canais de ânions, mensurada indiretamente, versus a exsudação de citrato e o conteúdo de Al.

Cat100-6 e S1587-17, mesmo não sendo linhagens isogênicas, têm sido um excelente modelo para estudar os efeitos da toxidez promovida pelo Al, dando sua parcela de contribuição para a elucidação da toxidez e tolerância ao Al em milho. Desta forma, com os resultados aqui apresentados, esperamos ter acrescentado informação relevante para a compreensão desta complexa rede de respostas desencadeadas pelo Al.

Objetivos

Objetivo Geral

Estudar os mecanismos genéticos e bioquímicos envolvidos na toxidez e na tolerância ao alumínio em duas linhagens de milho contrastantes para tolerância ao alumínio, Cat100-6 (tolerante) e S1587-17 (sensível).

Objetivos Específicos

I- Identificação de genes induzidos por alumínio em ápices radiculares de milho pela técnica de “differential display”;

II- Avaliação dos efeitos do alumínio na expressão gênica em ápices radiculares de milho pela técnica de arranjos de DNA;

III- Clonagem e caracterização do gene homologo de *ALMT1* (*aluminum-activated malate transporter*) em milho.

Capítulo I

Glutathione S-transferase and aluminum toxicity in maize

Glutationa S-transferase e toxidez por alumínio em milho

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Glutathione S-transferase and aluminum toxicity in maize

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Abstract. Aluminum (Al) toxicity induces changes in the expression of several genes, some of which are involved in plant responses to oxidative stress. Using mRNA differential display, we identified a maize Al-inducible cDNA encoding a glutathione S-transferase (GST). The gene was named *GST27.2* owing to its homology to the maize gene *GST27*, which is known to be induced by xenobiotics. *GST27.2* is present in the maize genome as a single copy and analysis of its expression pattern revealed that the gene is expressed mainly in the root tip. Expression was up-regulated in response to various Al and Cd concentrations in both Al-tolerant and Al-sensitive maize lines. Consistent with its role in plants, phylogenetic analysis of theta-type GSTs revealed that GST27.2 belongs to a group of proteins that respond to different stresses. Finally, structural analysis of the polypeptide chain indicates that the two amino acids that differ between GST27.2 and GST27 (E102K and P123L) could be responsible for alterations in activity and / or specificity. Together, these results suggest that GST27.2 may play an important part in plant defenses against Al toxicity.

Keywords: Al tolerance, Al toxicity, GST27.2, oxidative stress, *Zea mays*.

Introduction

Environmental stresses such as exposure to toxic metals, pathogen attack and xenobiotics can induce reactive oxygen species (ROS) (Alscher *et al*. 2002; Gratão *et al*. 2005). The action of ROS in plants includes stimulation of cell wall stiffening and apoptosis as a hypersensitive response (Lamb and Dixon 1997; Delledonne *et al*. 1998; Potikha *et al*. 1999; Grant and Loake 2000). Although ROS can help protect plants against stresses, when present in excess these oxidants can also cause oxidative damage to lipid bilayers, DNA and proteins (Sandermann 1994).

Glutathione S-transferases (GST, EC 2.5.1.18) are one of the major cellular detoxification enzymes protecting plants from oxidative damage. This multifunctional enzyme family catalyses the conjugation of various electrophiles with GSH (reduced glutathione), detoxifying both exogenously and endogenously derived toxic compounds (Ketterer and Meyer 1989). GSH is one of the cell's most important antioxidants, neutralising free radicals due to the high electron-donating capacity of its sulfhydryl (–SH) group (Lands *et al*. 1999). In addition, plant GSTs play a role in the cellular response to auxins and in the metabolism of plant secondary products such as anthocyanins and cinnamic acid (Bilang *et al*. 1993; Alfenito *et al*. 1998).

All plant GSTs have been previously classified as theta class enzymes (classification derived from the animal GST nomenclature), and were further subdivided into three types (I, II and III) on the basis of their gene architecture (Marrs 1996; Droog 1997). We have adopted the nomenclature suggested by Edwards *et al*. (2000) where the GST studied in this work is classified as a GST type theta. GST isoenzymes differ with respect to molecular mass, subunit composition (hetero or homodimeric) and type of substrate. Organisms usually express multiple GSTs, with specialised functions

Abbreviations used: GST, glutathione S-transferase; ROS, reactive oxygen species.

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determined by their substrate specifity and localisation (Wilce and Parker 1994). For example, Dean *et al*. (2003) found that four different GSTs from tau, phi and zeta classes showed different patterns of expression following infection of *Malva pusilla* leaves with the pathogen *Colletotrichum gloeosporioides*.

Native theta-type GSTs are homodimers composed of two 27-kD subunits and can conjugate GSH to chloroacetamide and S-triazine substrates but not to 1-chloro-2,4-dinitrobenzene (CDNB), a model GST substrate (Prade *et al*. 1998). Thus, theta-type GSTs seem to have a distinct detoxifying activity. Jepson *et al*. (1994) reported the isolation of a maize cDNA clone, *GST27*, that is inducible by a herbicide safener. *GST27* encodes the 27-kDa subunit present in both glutathione S-transferase isoforms zeta and theta, and was found to be constitutively expressed in roots, while no expression was detected in the aerial parts of the plant.

Each GST subunit contains an *N*-terminal α/β domain and a *C*-terminal α-helical domain. Each subunit has one kinetically independent active site (Mannervik and Danielson 1988) consisting of two distinct subsites: a glutathione binding site (G-site) and a binding pocket for hydrophobic substrates (H-site). The structure of a GST from *Arabidopsis thaliana* (Reinemer *et al*. 1996) showed that the serine residue (Ser11), highly conserved in most GSTs, could form a hydrogen bond with the glutathionyl sulfur atom. Both carboxyl moieties of GSH seem to be involved in the catalytic mechanism, acting as a proton acceptor and modulating the binding of the second substrate (Widersten *et al*. 1996).

Involvement of oxidative stress in Al toxicity has been suggested, although Al itself is not a transition metal and cannot catalyse redox reactions (Yamamoto *et al*. 2002). Al cations have a strong affinity to biomembranes and can induce them to stiffen (Deleers *et al*. 1986). Therefore, Al ions could act as indirect oxidative agents and induce further effects such as root growth inhibition and cell death, as has been observed in maize (Boscolo *et al*. 2003), barley (Pan *et al*. 2001), and wheat (Delisle *et al*. 2001). Several Al-inducible genes have been isolated from a range of plant species (Drummond *et al*. 2001). In *Arabidopsis* and cultured tobacco cells, Al induces the expression of several genes that are also induced by oxidative stress, including GSTs (Snowden and Gardner 1993; Richards *et al*. 1998; Ezaki *et al*. 2000). Furthermore, Ezaki *et al*. (2001) recently observed that transgenic *Arabidopsis* overexpressing GST and peroxidase (POX) were capable of diminishing the oxidative damage caused by Al stress.

We have investigated the molecular basis of Al toxicity and tolerance in maize using an mRNA differential display approach to identify Al-regulated genes in root tips. Here we present the characterisation of a cDNA encoding a GST. We have characterised the Al-induced accumulation of the GST transcript in two closely related maize germplasms with contrasting phenotypes for Al tolerance. Furthermore, amino acid sequences of GST27.2 homologues were obtained and used to generate a phylogenetic tree, indicating possible relationships among the members of the GST theta family. Additionally, an analysis of the GST27.2 protein structure was performed to interpret how small amino acid changes influence protein interactions when compared with another protein of the same class.

Materials and methods

Plant material, growing conditions and treatments

The maize (*Zea mays* L.) inbred lines Cat100-6 and S1587-17, tolerant and sensitive to Al, respectively (Moon *et al*. 1997), were obtained from the Centro de Biologia Molecular e Engenharia Genética germplasm collection (Universidade Estadual de Campinas, Campinas, Brazil). Seeds were surface-sterilised with 0.5% sodium hypochlorite for 20 min, rinsed four times with sterile water and germinated at 28°C. Three-day-old seedlings were grown in nutrient solution at pH 4.0 as described by Moon *et al*. (1997) with continuous aeration at 26°C under a 16-h photoperiod with a photon flux density of 70 $\mu mol\ m^{-2}\ s^{-1}$. Al was supplied as $AlK_3(SO_4)$ at concentrations indicated in the text. Cd treatments were performed with $CdSO_4$ under the conditions described above, but at pH 5.7. Root apex corresponds to the first 5 mm, the proximal region to the next 10 mm and the distal root region to the next 15 mm. Root length was measured at the beginning and after 24 h of growth in Al and Cd treatments in two independent experiments.

mRNA differential display

Differential display of mRNA was performed according to Ausubel *et al*. (1995) with minor modifications. DNAse-treated RNA (1 µg) was reverse-transcribed with SuperScript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD). First-strand cDNA was amplified with the same $T_{12}VN$ anchored primer and an arbitrary primer (10-mer from Operon, Huntsville, AL). Aliquots were heated at 80°C in loading dye containing formamide and separated in a 6% polyacrylamide sequencing gel. Gels were vacuum -dried at 80°C and autoradiographed overnight at –70°C on X-ray films (X-OMAT, Kodak, São Paulo, Brazil). Differentially displayed bands were excised and eluted in water. Eluted DNA was ethanol-precipitated, reamplified and TA-cloned into pMOSBlue (Amersham, Piscataway, NJ).

Isolation of the full-length GST27.2 *cDNA*

RACE-PCR (Frohman *et al*. 1988) was performed with the 5′ RACE System for Rapid Amplification of cDNA Ends Version 2.0 (Gibco-BRL). First-strand cDNA was synthesised from total RNA with an antisense primer, GST154 (5′-CCGTGGAGAAAGCAGC-3′). Excess primer was removed with a Microcon 30 column (Millipore, Billerica, MA) and the cDNA was polyC-tailed with Terminal Transferase (Gibco-BRL). PCR amplification was performed in two rounds with primers GST154 plus AAP (Gibco-BRL) and GST122 (5′-GTATAAAGAAAAGCAGGCACC-3′) plus AUAP (Gibco-BRL) Amplified fragments were TA-cloned into pGEM-T (Promega, Madison, WI) and sequenced in an ABI-PRISM 310 Genetic Analyzer (Perkin Elmer, Boston, MA).

DNA isolation and Southern-blot analysis

Genomic DNA was isolated from 2–3 g of maize leaves by a modified CTAB procedure (Hoisington *et al*. 1994). Southern analysis was performed with *Eco*RI-, *Bam*HI-, *Hin*dIII-, and *Xba*I-digested genomic

DNA. Digested samples (15 µg per lane) were separated in 0.8% (w/v) agarose gels in TAE buffer and transferred to nylon filters (Hybond-XL, GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The DNA was cross-linked to the membranes by baking at 90°C for 2 h. Membranes were pre-hybridised in a low-stringency hybridisation solution (30% formamide, 1 M NaCl, 0.5% SDS, 50 mM Tris pH 7.5, 1× Denhardt's solution, 125 µg mL^{-1} salmon sperm DNA) and hybridised overnight at 65°C in a solution containing 6× SSC, 5× Denhardt's solution, 0.2% SDS and 100 µg mL^{-1} denatured salmon sperm DNA (Sambrook *et al*. 1989). An insert corresponding to the last 249 bp of the *GST-27.2* cDNA was used to produce radioactive probes ([α-^{32}P]dCTP) by random priming, with the Ready-To-Go DNA Labelling Beads kit according to the manufacturer's instructions (GE Healthcare). Membranes were washed twice with 2× SSC and 0.1% SDS at room temperature for 15 min and twice with 0.1× SSC and 0.1% SDS at 60°C for 30 min. Washed membranes were exposed to image plates for 24 h, then scanned in a phosphorimager FLA 3000-G (Fujifilm, Tokyo, Japan).

RNA isolation and northern-blot analysis

Total RNA was isolated from roots and young shoots according to Logemann *et al*. (1987) with minor modifications. Root tips were frozen in liquid N_2 and ground in extraction buffer [8 M guanidine–HCl, 50 mM Tris–HCl pH 8.0, 20 mM EDTA pH 8.0 and 2% (v/v) 2-mercaptoethanol]. The mixture was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1 v/v/v) and centrifuged at 5000 *g* for 15 min. Aqueous phases were precipitated in ethanol and the pellets resuspended in DEPC-treated water. Total RNA samples (10 µg) were separated in MOPS–formaldehyde agarose gels. Ethidium bromide (200 µg mL^{-1}) was included in the loading buffer to confirm equal sample loading. RNA was transferred to nylon filters (Hybond-XL, GE Healthcare) according to the manufacturer's instructions. Probe radiolabelling was performed as described in *DNA isolation and Southern-blot analysis*. Membranes were prehybridised for 2 h at 42°C in a solution containing 6× SSC, 50% formamide, 5× Denhardt's solution, 0.2% SDS and 100 µg mL^{-1} denatured salmon sperm DNA, and hybridised overnight at 42°C in a solution containing 6× SSC, 50% formamide, 10% dextran sulfate and 0.2% SDS. Membranes were washed and exposed as described in *DNA isolation and Southern-blot analysis.*

Phylogenetic analysis

The GST amino acid sequence was extensively compared with proteins in the NCBI database and homology searches were performed using BLAST algorithm with *E* value cutoff of E^{-10} (Altschul *et al*. 1990). Only complete GST sequences were selected for further analysis.

An initial phylogenetic tree was generated using 118 sequences found to be similar to GST27.2. A multiple alignment of the amino acid sequences was obtained with the Clustal X software (Thompson *et al*. 1997) with gapped positions omitted from subsequent analyses. The phylogenetic tree was constructed by neighbour-joining (Saitou and Nei 1987) based on the proportion of different amino acid sites and was rooted with a GST tau. The reliability of this tree was assessed with 1000 bootstrap replications using MEGA (version 2.1) (Kumar *et al*. 1994). Subsequently, a more refined analysis limited to the GST theta sequence subgroup was carried out using only the most representative sequences related to regulation by stresses, redundancy and species diversity.

Modelling of GST27.2–lactoylglutathione complex

The structure of the GST-I–lactoylglutathione of *Zea mays* (PDB: 1AXD, http://www.rcsb.org/pdb/; verified 1 August 2005) was used to predict the 3-D structure of the GST27.2–lactoylglutathione complex. The atomic coordinates of the 1AXD structure, solved by Neuefeind *et al*. (1997*a*, *b*), were used as template in comparative modelling by satisfaction of spatial restraints (Sali and Blundell 1993) implemented in the MODELLER 6.2 software (Fiser *et al*. 2000). Twenty different models of the GST27.2–lactoylglutathione complex were generated in the modelling. The quality of the predicted fold was evaluated with the score of the variable target function method (Fiser *et al*. 2000) and the stereochemical quality of the five best scoring models was assessed by PROCHECK (Laskowski *et al*. 1993) at 2.0 Å resolution. The final model was selected based on the overall stereochemical quality.

To refine the molecular model of the GST27.2–lactoylglutathione structure, additional energy minimisation and equilibrating molecular dynamics simulations were carried out in the GROMACS 3.2 molecular dynamics package (Lindahl *et al*. 2001). In parallel, the Gromacs topology of the lactoylglutathione ligand was obtained in the PRODRG server (van Aalten *et al*. 1996). The protein model was submitted to a steepest-descent (s.d.) energy minimisation (5000 steeps) to remove bad van der Waals contacts.

Results

Isolation of a glutathione S-transferase gene

To investigate plant responses to Al stress we compared mRNA profiles in root tips of two maize germplasms, Cat100-6 (Al tolerant) and S1587-17 (Al sensitive), by differential display. A DNA fragment of ~250 bp with enhanced amplification upon 12 and 36 h of Al treatment was detected in both maize lines (Fig. 1). The fragment was extracted from the gel, cloned and sequenced. The nucleotide sequence showed 99% identity to *GST27* (accession U12679; Irzyk and Fuerst 1993; Irzyk *et al*. 1995), a gene that encodes a maize GST theta. This gene was named *GST27*.2.

The full-length cDNA was obtained by 5′ RACE with the primers GST154 and GST122 (see Material and methods). Two fragments of ~1 and 0.8 kb were obtained (not shown) and the larger one was cloned. The 3′ end of the sequence obtained overlapped with the 5′ end of the original fragment,

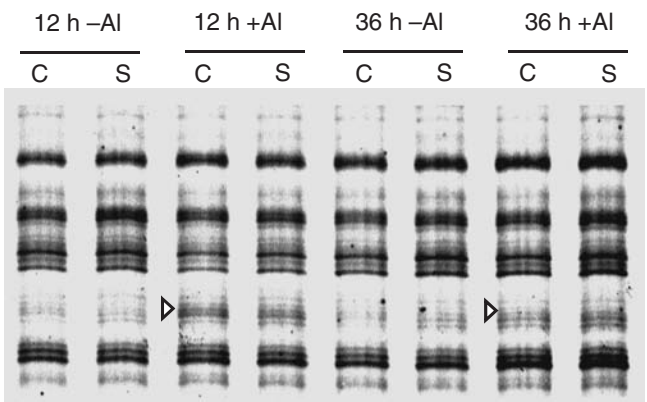


Fig. 1. mRNA differential display of maize roots treated with Al. Cat100-6 (C) and S1587-17 (S) seedlings were submitted to 0 (–Al) or 90 µM (+Al) in nutrient solution for 12 and 36 h. The arrow indicates a 250-bp band that was differentially amplified in Al-treated samples.

resulting in the full cDNA sequence shown in Fig. 2. The open reading frames from both lines were sequenced and no differences were found (not shown).

Genomic organisation of the GST27.2 *gene in maize*

To determine the number of copies of GST27.2, the same 3′ end fragment obtained by mRNA-DD was used as probe on a maize genomic DNA gel blot (Fig. 3). A single band was observed for most restriction enzymes, with the exception of the *Eco*RI digestion of Cat100-6 DNA. Another polymorphic fragment was detected in *Xba*I-digested DNA, indicating that the genomic organisation of *GST27.2* is different in these two genotypes. These results strongly suggest that a single-copy gene encodes *GST27.2*.

Accumulation of the maize GST27.2 *transcripts*

To analyse the expression profile of GST27.2, the same 3′ end fragment was used as a probe in RNA gel blots (Fig. 4*A*). The northern hybridisation studies confirmed the induction by Al observed in the mRNA differential display. The steady-state mRNA levels of the *GST27.2* gene along the roots and in shoots were also evaluated. Cat100-6 seedlings exposed to 90 μM Al for 24 h presented a gradient of transcript levels in the roots, with higher levels in the apex, while transcripts were hardly detectable in shoots (Fig. 4*B*).

The expression of *GST27.2* was also evaluated in root tips exposed to increasing doses of Al. A dose-response accumulation of *GST27.2* transcripts was observed for both genotypes after 24 h (Fig. 4*C*). Al induction of *GST27.2* transcripts levels was more evident at higher doses of Al. The Al-sensitive genotype also showed increased levels of transcripts in response to Al, although they were lower than those observed in the Al-tolerant Cat100-6. We previously showed that these Al concentrations caused inhibition of root growth in both maize lines, although Cat100-6 was less affected (Boscolo *et al*. 2003).

Cadmium (Cd) is another toxic metal that induces the activity of GST tau in maize leaves, as well as GST activity in *callus* protoplasts (Marrs and Walbot 1997). Thus, we evaluated the transcript levels of *GST27.2* in maize roots exposed to several Cd concentrations. Cd inhibited root growth in both lines. Inhibition was more pronounced in S1587-17 (Fig. 5*A*), similar to observations under Al treatment (Boscolo *et al*. 2003). Cd exposure resulted in strong accumulation of *GST27.2* transcripts, which was higher in the Al-tolerant Cat100-6 (Fig. 5*B*).

Phylogenetic analysis

Database searches for GST27.2 protein homologues were carried out and resulted in 118 complete amino acid sequences. These sequences were used to generate an initial phylogenetic tree for GSTs (data not shown). They were divided into three major groups, representing all classes of GSTs. The GST theta branch, where GST27.2

```
  1    acacacccat ccaattccag ctgctgatct tgatcctgca ccccgagccg tacacaagag
                                                              M  A   (02)
 61    ctagtcggta gaacttgcag gagcggagca gaactaagtg cagagaacag gacatatggc
        T  P  A   V  K  V   Y  G  W  A   I  S  P   F  V  S   R  A  L  L   (22)
121    tacgccggcg gtgaaggttt acgggtgggc tatctcgccg ttcgtatcgc gggctctgct
        A  L  E   E  A  G   V  D  Y  E   L  V  P   M  S  R   Q  D  G  D   (42)
181    ggccctggag gaggccggcg tcgactacga gctcgtcccc atgagccgcc aggacggcga
        H  R  R   P  E  H   L  A  R  N   P  F  G   K  V  P   V  L  E  D   (62)
241    ccaccgccgc ccggagcacc tcgccaggaa ccctttcggg aaggtgccgg tgctcgagga
        G  D  L   T  L  F   E  S  R  A   I  A  R   H  V  L   R  K  H  K   (82)
301    tggcgacctc acgctcttcg aatcacgtgc gatcgcgagg catgttctcc ggaagcacaa
        P  E  L   L  G  G   G  R  L  E   Q  T  A   M  V  D   V  W  L  K   (102)
361    gccggagctg ctgggcggcg gcaggctgga gcagacggcg atggtggacg tgtggctgaa
        V  E  A   H  Q  L   S  P  P  A   I  A  I   V  V  E   C  V  F  A   (122)
421    ggtggaggcc caccagctga gcccgccggc gatcgccatc gtggtggagt gcgtgttcgc
        L  F  L   G  R  E   R  N  Q  A   V  V  D   E  N  V   E  K  L  K   (142)
481    gctgttcctg ggccgcgagc gcaaccaggc ggtggtggac gagaacgtgg agaagctcaa
        K  V  L   E  V  Y   E  A  R  L   A  T  C   T  Y  L   A  G  D  F   (162)
541    gaaggtgctg gaggtgtacg aggcgcggct ggccacgtgc acgtacctcg ccggcgactt
        L  S  L   A  D  L   S  P  F  T   I  M  H   C  L  M   A  T  E  Y   (182)
601    cctcagcctc gccgacctca gccccttcac catcatgcac tgcctcatgg ccaccgagta
        A  A  L   V  H  A   L  P  H  V   S  A  W   W  Q  G   L  A  A  R   (202)
661    cgccgctctc gtccatgcgc tcccgcacgt cagcgcctgg tggcagggcc tcgccgcgcg
        P  A  A   N  K  V   A  Q  F  M   P  V  G   A  G  A   P  K  E  Q   (222)
721    cccggcggcc aacaaggtgg cgcagttcat gccggtcggc gccggagcgc ccaaggaaca
        E                                                             (223)
781    ggagtgacga tgaagcgatc gaagcgactt gtgttgttgt gcttgattag ttaattggaa
841    accttctcac tcatctagtc catcatggtg cctgcttttc tttatactat ttgtcttaat
901    tttgctgctt tctccacgga ataatagtag agattggaaa tgtaatgtat tttatcaaaa
961    tatattggat tttcaagcaa tatagtatgg ttttcgctgc caaaaaaaaa aaa
```

Fig. 2. Nucleotide and deduced amino acid sequences of Cat100-6 *GST27.2* cDNA. The numbers on the left and on the right (in brackets) indicate nucleotide and amino acid positions, respectively.

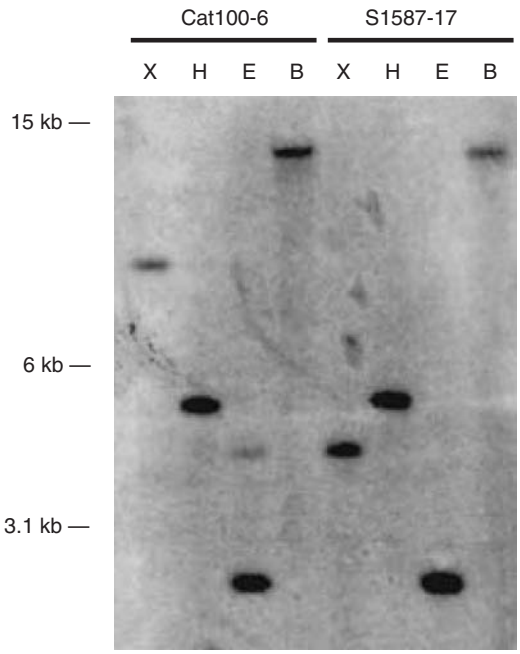


Fig. 3. Genomic organisation of *GST27.2*. Cat100-6 and S1587-17 genomic DNA was digested with *Xba*I (X), *Hin*dIII (H), *Eco*RI (E) and *Bam*HI (B) and probed with a fragment corresponding to the last 249 bp of *GST27.2*. The size (in kb) of molecular weight markers is indicated on the left.

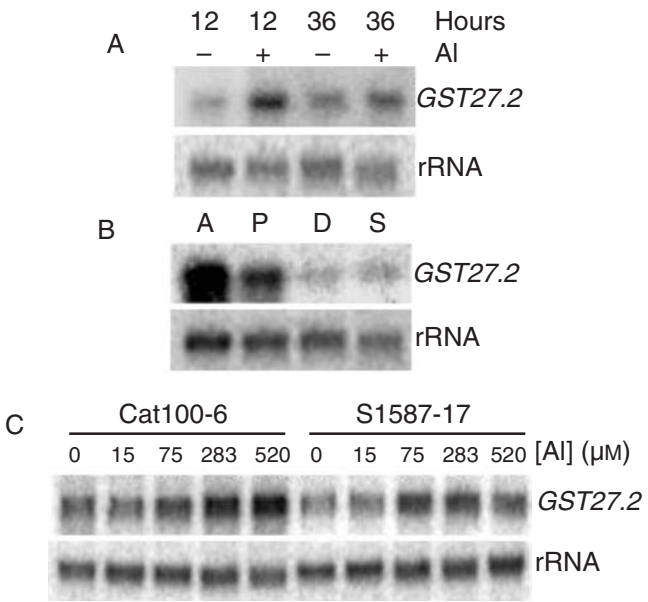


Fig. 4. Expression profile of *GST27.2* in RNA gel blots. (*A*) Total RNA from Cat100-6 root tips exposed to zero Al (–) or 90 μM Al (+) for 12 and 36 h. (*B*) Total RNA from the root apex [A], proximal root region [P], distal root region [D], and shoot [S] of 3-d-old Cat100-6 exposed to 90 μM Al for 24 h. (*C*) Total RNA from Cat100-6 and S1587-16 root tips exposed to 0, 25, 75, 283, and 520 μM Al for 24 h. Two independent experiments produced similar expression profiles (data not shown).

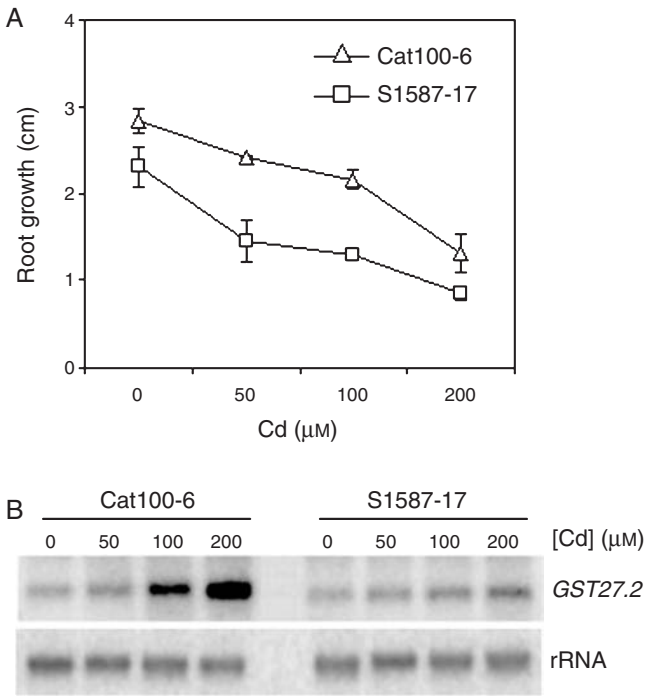
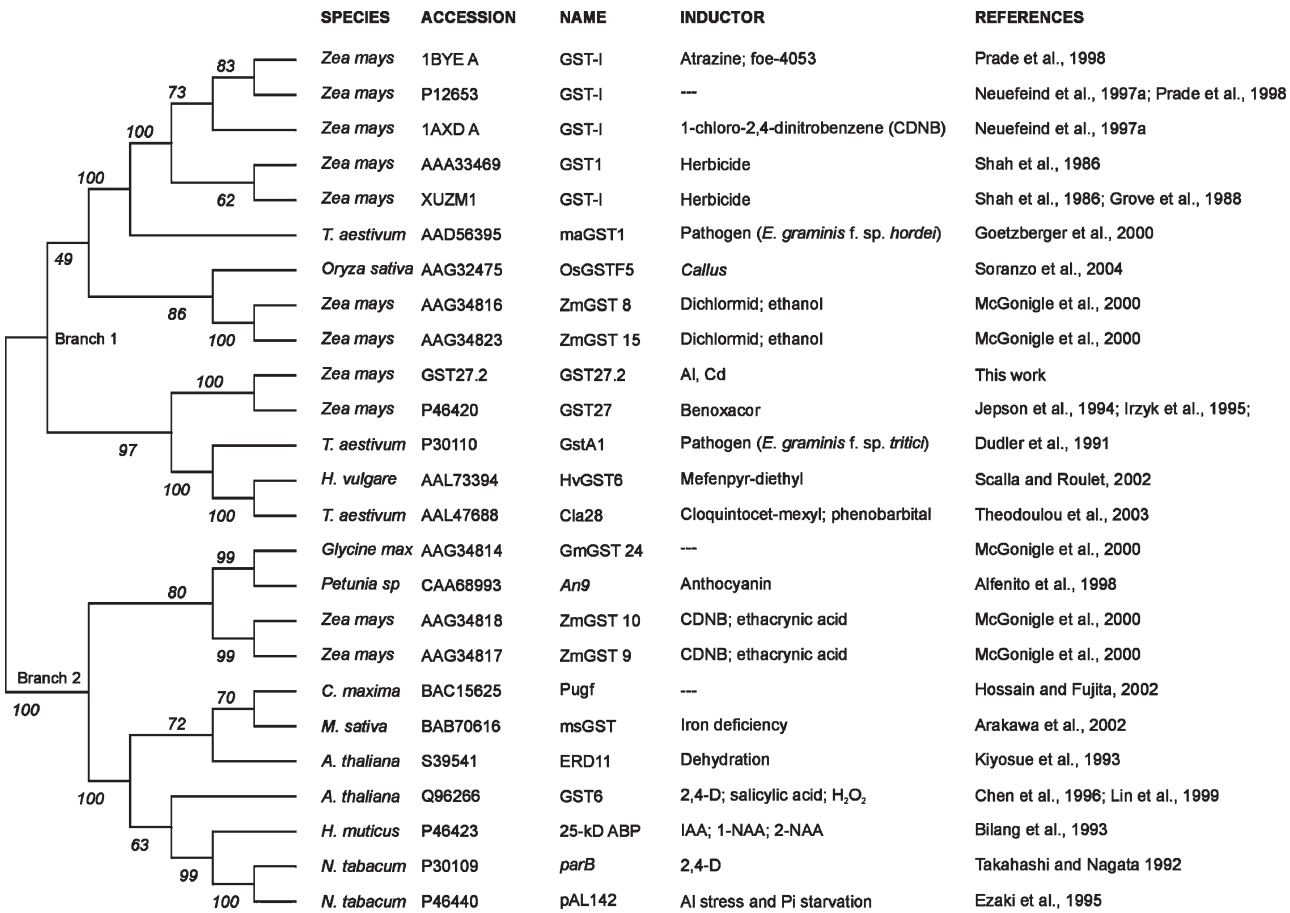


Fig. 5. Cd effect on maize plants. (*A*) Root growth dose-response curves for Cat100-6 (△) and S1587–17 (□) after 24 h of exposure to nutrient solution containing 0, 50, 100, and 200 μM Cd. The data are the means ± s.d. obtained from 30 replicates. (*B*) Total RNA from Cat100-6 and S1587-16 root tips exposed to Cd for 24 h probed with the *GST27.2* cDNA.

was situated, was thus used to create a new and reduced phylogenetic tree. Twenty-five complete proteins whose function has been characterised in the literature composed this new tree. Two distinct main branches were generated (Fig. 6). All branches contained GSTs previously tested for xenobiotics (herbicides and herbicide safeners) and abiotic / biotic stresses.

The first branch included GST27.2 and was composed of two subgroups, both composed exclusively of monocotyledonous GST sequences. In this branch, GST27.2 is the only one reported to be inducible by toxic metals. GST27.2 and GST27 were grouped very closely, as expected by their similarity. The proteins in this first subgroup have been extensively tested for herbicide detoxification, although some of them are induced by pathogen attack (Goetzberger *et al*. 2000). All genes in this branch have been reported to be induced by herbicides, with the exception of one sequence from wheat that was characterised under pathogen attack (Dudler *et al*. 1991; Mauch and Dudler 1993). The second main branch was also subdivided into two subgroups. The first included sequences of mono and dicotyledonous, and the second only of dicotyledonous. This main branch contained two GST sequences reported to be involved in the translocation of pigments and phytohormones.



SPECIES	ACCESSION	NAME	INDUCTOR	REFERENCES
Zea mays	1BYE A	GST-I	Atrazine; foe-4053	Prade et al., 1998
Zea mays	P12653	GST-I	---	Neuefeind et al., 1997a; Prade et al., 1998
Zea mays	1AXD A	GST-I	1-chloro-2,4-dinitrobenzene (CDNB)	Neuefeind et al., 1997a
Zea mays	AAA33469	GST1	Herbicide	Shah et al., 1986
Zea mays	XUZM1	GST-I	Herbicide	Shah et al., 1986; Grove et al., 1988
T. aestivum	AAD56395	maGST1	Pathogen (*E. graminis* f. sp. *hordei*)	Goetzberger et al., 2000
Oryza sativa	AAG32475	OsGSTF5	*Callus*	Soranzo et al., 2004
Zea mays	AAG34816	ZmGST 8	Dichlormid; ethanol	McGonigle et al., 2000
Zea mays	AAG34823	ZmGST 15	Dichlormid; ethanol	McGonigle et al., 2000
Zea mays	GST27.2	GST27.2	Al, Cd	This work
Zea mays	P46420	GST27	Benoxacor	Jepson et al., 1994; Irzyk et al., 1995;
T. aestivum	P30110	GstA1	Pathogen (*E. graminis* f. sp. *tritici*)	Dudler et al., 1991
H. vulgare	AAL73394	HvGST6	Mefenpyr-diethyl	Scalla and Roulet, 2002
T. aestivum	AAL47688	Cla28	Cloquintocet-mexyl; phenobarbital	Theodoulou et al., 2003
Glycine max	AAG34814	GmGST 24	---	McGonigle et al., 2000
Petunia sp	CAA68993	*An9*	Anthocyanin	Alfenito et al., 1998
Zea mays	AAG34818	ZmGST 10	CDNB; ethacrynic acid	McGonigle et al., 2000
Zea mays	AAG34817	ZmGST 9	CDNB; ethacrynic acid	McGonigle et al., 2000
C. maxima	BAC15625	Pugf	---	Hossain and Fujita, 2002
M. sativa	BAB70616	msGST	Iron deficiency	Arakawa et al., 2002
A. thaliana	S39541	ERD11	Dehydration	Kiyosue et al., 1993
A. thaliana	Q96266	GST6	2,4-D; salicylic acid; H_2O_2	Chen et al., 1996; Lin et al., 1999
H. muticus	P46423	25-kD ABP	IAA; 1-NAA; 2-NAA	Bilang et al., 1993
N. tabacum	P30109	*parB*	2,4-D	Takahashi and Nagata 1992
N. tabacum	P46440	pAL142	Al stress and Pi starvation	Ezaki et al., 1995

Fig. 6. Phylogenetic tree, based on the protein sequences of 25 plant type I GSTs. The length of the horizontal lines connecting the sequences is proportional to the estimated genetic distance between them.

GST27.2–lactoylglutathione model

Using the sequence alignment represented in Fig. 7*A*, the structure of GST I–lactoylglutathione (*Zea mays*, PDB 1AXD, Neuefeind *et al*. 1997*a*) was used to model the structure of the GST27.2–lactoylglutathione homodimer complex in comparative modelling (Fig. 7*B*). In the modelled structure no residues were found in disallowed regions of the Ramachandran plot and stereochemical parameters, checked in PROCHECK (Laskowski *et al*. 1993), were inside or better than expected at the 95% confidence level. Given the high degree of sequence identity and the overall stereochemical rating of the obtained GST27.2–lactoylglutathione complex model, the assumption that the structures of these complexes are nearly identical is valid for the purposes of the present analysis.

The lactoylglutathione binding site is formed by residues of the *N*-terminal α–β domain (highlighted in Fig. 7*A*) and important differences were found in the GST27.2 binding site, when compared with that reported in GST phi by Neuefeind *et al*. (1997*a*, *b*). These differences are exposed in Fig. 7*C*. The most important are the replacement of the Phe_{35} residue (GST phi complex, Neuefeind *et al*. 1997*b*) by Arg (GST27.2 model) and Gln_{13} by Phe, respectively (Fig. 7*C*). The replacement of these residues in the GST binding site imply different substrate binding properties.

Putative differences between GST27 and GST27.2 enzymes

As shown in the alignment (Fig. 7*A*), GST27 and GST27.2 enzymes differ only at residues 102 and 123. Although the $Pro–Leu_{123}$ substitution seems to be an equivalent substitution, the replacement of the Glu acidic residue by Lys at residue 102 implies the rearrangement of the inter-subunit contacts in the homodimer, and new contact possibilities for the carboxyl moiety of the lactoylglutathione ligand (Fig. 7*D*).

In our model the Lys_{102} residue is placed at the centre of the H3 helix and mediates the inter-subunit contacts in GST27.2 homodimer formation through a salt bridge with Glu_{69} and / or a hydrogen bond with Ser_{70} (Fig. 7*D*). It may be significant that the amino group of the Lys_{102} was found less than 3.7 Å from the lactoylglutathione carboxyl moiety,

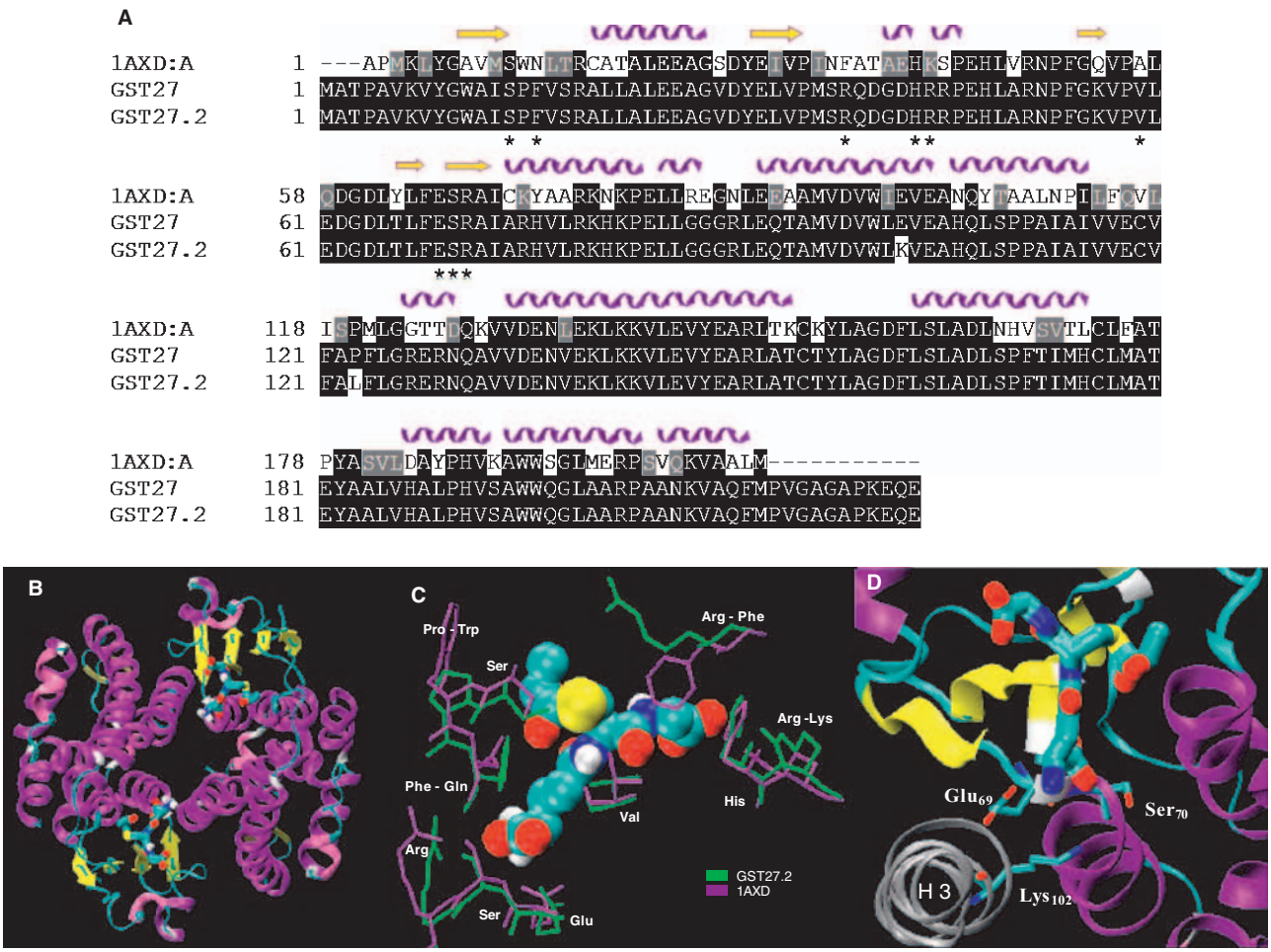


Fig. 7. (*A*) Amino acid sequence alignment of GST27.2, GST27 and GST-I used in modelling experiments (pdb: 1AXD, Neuefeind *et al.* 1997*a*). Identical residues are shaded in black, and lactoylglutathione-contacting residues are denoted by *. Secondary structure elements of the structure 1AXD are represented on the top of the alignment. (*B*) Representation of the modelled GST27.2-lactoylglutathione homodimer complex represented and coloured according to the secondary structure. The lactoylglutathione ligand is represented in CPK colour code. (*C*) Representation of the lactoylglutathione contacting residues in GST phi (represented by 1AXD structure in magenta), and in our GST27.2 model (represented in green). The lactoylglutathione ligand is represented in CPK colour code. (*D*) Representation of the H3 helix (represented in grey) and the Lys_{102} residue contacting the lactoylglutathione carboxyl moiety. The protein backbone is coloured according to the secondary structure and lactoylglutathione ligand, Glu_{69}, Ser_{70} and Lys_{102} residues are represented in CPK colour code.

indicating the possibilities of hydrogen bond formation. These evidences suggest that the Glu / Lys substitution at residue 102 may give rise to differences in substrate specificity and, as a consequence, the diversification of the mechanism of action of these proteins.

Discussion

This work describes the isolation and characterisation of an Al-inducible GST gene from maize, here named *GST27.2*. The *GST27.2* gene shares 99% nucleotide sequence identity with the *GST27* from maize and encodes a GST theta. To our knowledge, this is the first report of an Al-inducible gene from maize.

Glutathione S-transferases are encoded by a large multigene family identified in almost all organisms (Mannervik and Danielson 1988; Marrs 1996), and several classes of GSTs have been characterised. Genomic organisation analysis of *GST27.2* indicates that it is probably present as a single copy in maize and that it is polymorphic between the two maize lines studied, indicating the possible existence of at least two distinct alleles for the *GST27 locus*. Therefore, we have named this gene *GST27.2* based on the allele *GST27* described previously by Irzyk *et al.* (1995). Interestingly Grove *et al.* (1988) found that other maize GST genes, *GST phi* and *GST tau*, are present in single or low copy numbers in maize.

The expression analysis of *GST27.2* in different tissues indicates preferential expression in the roots. Higher levels of *GST27.2* transcripts in the root tips, when compared to other tissues in the presence of Al (Fig. 4*B*) suggest a tissue-specific response that could be due to coordinated regulation of *GST27.2* expression. This result agrees with previous studies in tobacco that revealed a *cis* element (*as-1*) and their *trans*-acting factors in the promoters of several GST genes, conferring on them tissue-specific activity (Klinedinst *et al*. 2000). Moreover, *in situ* hybridisation also demonstrated that these factors and GST genes are preferentially expressed in root tip meristems (Marrs 1996).

In all Al treatments *GST27.2* transcript levels were higher than those observed in the absence of Al in both maize lines (Fig. 4*A*, *C*). Genes encoding GSTs were also found to be induced by Al in *Arabidopsis* (Richards *et al*. 1998) and tobacco (Ezaki *et al*. 1995), but to our knowledge no comparison has been made between germplasms differing in Al tolerance. In this sense, it is interesting to note that Cat100-6 showed a greater increase in *GST27.2* transcript levels than S1587-17. A higher expression of *GST27.2* in Cat100-6 roots as a response to Al could decrease the accumulation of ROS, and consequently reduce the requirement for other enzymes involved in oxidative stress alleviation. Interestingly, Boscolo *et al*. (2003), working with the same genotypes, detected lower levels of peroxidases (POX) and superoxide dismutase (SOD) in Al-stressed Cat100-6 roots when compared with S1587-17. Devi *et al*. (2003) observed that Al-sensitive tobacco cells produced higher levels of MnSOD transcripts than Al-tolerant lines in response to Al, probably due to a higher accumulation of O_2^- in the sensitive cells. Ezaki *et al*. (2000, 2001) found that *Arabidopsis* plants transformed with a tobacco gene encoding a GST had a lower degree of root growth inhibition in the presence of Al, which was associated with lower levels of lipid peroxidation. Based on these findings we suggest that the GST-mediated detoxification system in Cat100-6 is more efficient than in S1587-17, contributing to the lower degree of protein oxidation observed in the Al-tolerant germplasm (Boscolo *et al*. 2003).

Both maize genotypes showed root growth inhibition and higher *GST27.2* transcript levels in response to Cd (Fig. 5), although Al-tolerant Cat100-6 plants presented a lower degree of root growth inhibition, which correlated with higher levels of *GST27.2* expression. GST genes have previously been reported to respond to Cd stress in maize and pea roots (Dixit *et al*. 2001; Marrs and Walbot 1997). These results reinforce the proposed role of GSTs as part of a more general detoxification system that is activated under different stresses (Mauch and Dudler 1993).

The phylogenetic analysis of GST theta produced two distinct branches (Fig. 6). It was not possible to associate functional data from the literature with the phylogenetic grouping, as no tendencies on activity or specificity were observed within the branches. This could, in part, be due to the fact that many more studies relate GSTs with herbicides and herbicides safeners than with abiotic stresses. In fact, this is the first report on the involvement of a GST theta in metal stress in maize. Moreover, our phylogenetic study increased the range of GST responses within the branch that includes GST27.2, as this cluster contained GSTs shown to participate only in xenobiotic detoxification and pathogen response.

The amino acid sequences of GST27.2 and GST27 are 99% identical (Fig. 7). Despite the low sequence identity (around 20%) between plant and animal GSTs, Reinemer *et al*. (1996) observed that these GSTs share significant structural homology and very similar topology. Since no crystal structure has been described for GST theta, the structure of GST phi (PDB 1AXD) was used for superposition. While the wild type protein GST27 contains a glutamic acid and a proline in positions 102 and 123, the mutant protein GST27.2 has a lysine and a leucine, respectively. Both changes are localised in the *C*-terminal α-helical domain positioned between residues 88 and 214 (Neuefeind *et al*. 1997*a*). The GST active site is located in the amino acid serine (amino acid 14 in GST27.2 and GST27). This residue forms a hydrogen bond with the glutathionyl sulfur atom stabilising the GSH as a thiolate anion that acts as a proton acceptor, modulating the binding of the second substrate, and it has been shown that mutation of this serine inactivates the enzyme (Board *et al*. 1995; Widersten *et al*. 1996).

With the purpose of showing that the mutated amino acids are spatially arranged, we used tertiary structure modelling to analyse topological aspects of GST theta (Fig. 7*B–D*). GST enzymes have very similar overall topology, but each structure exhibits unique features, particularly concerning the substrate recognition site (Neuefeind *et al*. 1997*a*). Consequently, the change of proline to leucine in a position spatially close to the serine in the active site may alter the interaction of the protein with GSH and / or its possible conjugation with toxic products. These variations in the amino acid composition could also affect the kinetics of enzyme–ligand formation. According to the *Arabidopsis* GST tertiary structure, intersubunit contacts between two GST homo or heteromers are predominantly mediated by hydrophobic residues (Neuefeind *et al*. 1997*a*) and, as related by Neuefeind *et al*. (1997*b*), the main hydrophobic interactions in GST involve residues in strand S4 (Leu_{64}, Phe_{65}), in helix H2 (Glu_{66}, Arg_{68}, Ala_{69}, Lys_{72}), and in helix H3 (Ala_{92}, Glu_{90}, Val_{94}, Glu_{99}, Val_{100}, Asn_{103}). The Glu_{99} of the helix H3 corresponds to the Glu_{102} of GST27. Thus, it is possible that substitution of Lys by Glu at residue 102 promotes alterations in the hydrophobic interactions of GST monomers as demonstrated by the modelling. These alterations can directly affect the enzyme activity or substrate specificity.

In summary, our data indicate that *GST27*.2 is possibly a new allele of *GST27*, which may present different activity and / or substrate specificity. The up-regulation of *GST27*.2 by Al and Cd indicates that this gene plays a role in metal-stress alleviation in maize roots.

Acknowledgments

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Capítulo II

Recent advances in gene expression data clustering: a case study with comparative results

Recentes avanços no agrupamento de dados de expressão gênica: estudo de um caso com comparações de resultados

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Recent advances in gene expression data clustering: a case study with comparative results

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ABSTRACT. Several advanced techniques have been proposed for data clustering and many of them have been applied to gene expression data, with partial success. The high dimensionality and the multitude of admissible perspectives for data analysis of gene expression require additional computational resources, such as hierarchical structures and dynamic allocation of resources. We present an immune-inspired hierarchical clustering device, called hierarchical artificial immune network (HaiNet), especially devoted to the analysis of gene expression data. This technique was applied to a newly generated data set, involving maize plants exposed to different aluminum concentrations. The performance of the algorithm was compared with that of a self-organizing map, which is commonly adopted to deal with gene expression data sets. More consistent and informative results were obtained with HaiNet.

Key words: Hierarchical clustering, Gene expression data, Artificial immune systems

INTRODUCTION

The capability to monitor the expression levels of genes on a genomic scale has led to a rapid evolution of molecular biology and functional genomics. By showing DNA transcriptional behavior under particular conditions, expression data give clues about the role of genes in biological processes of interest.

However, the huge amount of data produced by gene expression experiments must be preprocessed in order to reveal potentially useful information. In this preprocessing step, known as cluster analysis, the apparently arbitrary distribution of expression patterns may reveal groups of genes with high degrees of correlation in their expression profiles. As the result of this clustering, the data may expose a more comprehensible structure, which provides meaningful information for intuitive inspections.

Clustering gene expression data are especially challenging computational tasks. The data set is used to present complex characteristics like high dimensionality, low density, and high levels of noise and redundancy, thus preventing traditional computational tools, such as single-linkage hierarchical clustering, from giving satisfactory performance. This fact has motivated the application of several distinct and advanced techniques to the problem, including bio-inspired algorithms. Given that each technique presents its own peculiarities and manipulates the available data set by means of distinct methodologies, it is not possible to determine *a priori* the best choice among these possibilities.

This problem has led to an increasing interaction of people from biological and computational areas. On the one hand, computer scientists and engineers must provide efficient and generic techniques, capable of extracting the most relevant properties of the data set. Furthermore, it is very important that the software tools present a user-friendly interface, supplying intuitive output displays for biologists. On the other hand, biologists have to make it clear which specific properties of the data they are interested in, so that clustering analyses can be better planned and clustering tools can be designed for specific purposes.

We present an advanced clustering tool, named HaiNet (hierarchical artificial immune network) (Bezerra et al., 2004), specifically designed to deal with gene expression data. HaiNet is a powerful algorithm that uses ideas taken from the mammals' immune system, such as a population with a varying size, self-organizing interactions, and affinity maturation. The process somewhat resembles that of antibodies, recognizing invading antigens, with the antigens being associated with the original data set. A network of interconnected antibodies will be obtained with no specific neighborhood until the end of the self-organizing process. After that, a functional neighborhood is built by a minimal spanning tree. Each branch of the network is analyzed and those considered inconsistent are removed, leading to an automatic determination of the number of clusters. Additionally, HaiNet determines a hierarchical relation between clusters, which can be represented as a dendrogram. Larger clusters are initially detected using a small number of antibodies. By increasing the number of antibodies in the network, the representation becomes more and more specific, and refined clusters are then recursively determined.

We believe that the particular properties of HaiNet are especially suitable for gene expression data clustering. In most clustering techniques, the number of clusters is asked as input data. This is generally a serious drawback because no *a priori* information is available about the data structure, and consequently the user will arbitrarily set this value. HaiNet avoids this problem by performing an automatic determination of the number of clusters. In addition,

the hierarchical relation among clusters produced by the tool may favor the exploration of different degrees of correlation between genes. The specialist can choose which level of detail is more adequate for a given purpose and in a given application.

As an advanced clustering technique devoted to deal with bioinformatics problems, HaiNet is the result of a series of successful applications of immune-inspired algorithms. The first attempt involved solely the artificial immune network (aiNet), with no hierarchical resources (Bezerra and de Castro, 2003). Subsequently, the HaiNet was proposed (Bezerra et al., 2004) and validated by means of an already exhaustively investigated gene expression data set. Here we consider HaiNet as part of a broad research project, with newly generated data sets to be analyzed for the first time, and with alternative clustering approaches to be explored either in a competitive or a collaborative way.

As a case study, a data set was generated based on two strains of maize when exposed to different concentrations of aluminum ions. One strain is tolerant to aluminum, and the other is not. The ultimate purpose of this data analysis is to identify the genes responsible for the tolerance. So, the availability of a flexible and hierarchical clustering device will be an important stage in a complete genomic project. To better assess the performance of HaiNet, we compared the results obtained with those produced by a self-organizing map (SOM) (Kohonen, 1990). More specifically, we adopted as a testbed the approach proposed by Tamayo et al. (1999).

HaiNet

HaiNet is an extension of a clustering technique, called aiNet (de Castro and Von Zuben, 2000, 2001). The kernel of the learning algorithm is the same; however, HaiNet incorporates a hierarchical procedure that increases its potential.

aiNet clustering

The aiNet is a clustering technique well known in the artificial immune system community (de Castro and Von Zuben, 2000, 2001). It combines a preprocessing learning procedure, performed by an immune-based strategy, and a clustering partition step, which is achieved by the use of a minimal spanning tree. The aiNet extracts the most relevant characteristics from the input data by positioning its antibodies in the most representative portions of the data space, thus filtering out noise and redundancy. After that, the minimal spanning tree is built on the antibodies, and the edges of the tree are used as input to a clustering discrimination technique.

The aiNet learning procedure can be explained as follows. A random population of antibodies is initially created. The whole population is then presented to the input patterns, which are directly associated with antigens, and those antibodies that have a high affinity (low Euclidean distance, for example) with the antigens are selected to be cloned. Each antibody is cloned at a rate that is proportional to its affinity with the antigens, and the clones are then mutated at a rate inversely proportional to their affinity value. The aim is to produce antibodies that better recognize the antigens. This process, named clonal expansion, causes a growth in the population of antibodies. It is inspired by one of the most important theories in immunology: the clonal selection principle, originally proposed by Burnet (1959). After the expansion phase, those antibodies presenting an affinity with each other that is higher than a fixed threshold are removed,

thus eliminating the redundancy of the network. Again, this technique is inspired by immune theories, particularly the immune network theory suggested by Niels Jerne (1974). The whole process is then repeated, now with the remaining prototypes as the population of antibodies to be exposed to the antigens. After some iterations, the affinity maturation process leads to the convergence of the network. In our implementation of the aiNet learning algorithm, only the best-matching antibody is selected to be cloned, and the cloning process produces only one individual. The number of iterations used is 10. The computational cost of the aiNet is quadratic with the size of the input instance.

When the learning step is finished, the minimal spanning tree is constructed on the resulting antibodies, thus revealing the structure of the immune network. To extract the information stored in the tree, we use a local criterion proposed by Zahn (1971), which identifies the presence of clusters by evaluating the data distribution in the space. Each edge of the tree is analyzed in relation to its neighbors, and those considered inconsistent, i.e., with a length much greater than its immediate neighbors, are removed, leading to the data partition into clusters, also denoted natural clusters (Everitt, 1993). As a consequence, this criterion tends to preserve the inherent structure of the data set.

Hierarchy of networks

The aiNet can be extended to implement a hierarchical approach, which provides a topological structure of the correlation between clusters. This extension is denoted hierarchical aiNet, or simply HaiNet. Large clusters, representing more general differences within the data points, are initially identified. They are then analyzed individually with more constrained parameters, leading to more refined clusters, i.e., clusters with a higher level of similarity among components. This iterative process continues until no more natural clusters are identified.

To achieve this property, HaiNet makes use of a parameter called a suppression threshold (σ_s), which controls the level of refinement of the search. The suppression threshold is a value that determines the maximum similarity that two antibodies may have so that one does not recognize the other. It actuates in the network interaction phase of the aiNet learning procedure. Beyond this value, two antibodies are considered to recognize each other, and one of them must be suppressed to reduce the network redundancy. If σ_s is very high, the prototypes will represent the data set with a high degree of generality, and the number of antibodies in the network will be very small. As a result, the clustering device looks at the data set with gross eyes, and only large-scale divisions can be detected. When σ_s is low, antibodies are able to get closer to each other, leading to a more accurate representation of the data, with a larger number of prototypes. Under these circumstances, the minimal spanning tree can detect the presence of smaller divisions, revealing refined clusters with a higher density and specificity.

The hierarchical procedure consists in recursively running the aiNet, starting with a high suppression threshold. This parameter is then slowly reduced, and the aiNet is run again for each new value. Each value of σ_s corresponds to a novel hierarchical level. The algorithm can be summarized as follows:

1. Parameter definition: define the initial value for the suppression threshold σ_s.
2. aiNet learning: run the aiNet learning algorithm with the given σ_s.
3. Tree branching: each cluster detected by the minimal spanning tree, derived from the

obtained network of antibodies, generates an offspring network in the next level of the tree, i.e., a new branch of the tree. The clusters already detected will indicate the portion of the data set to be attributed to each newly generated branch.

4. Parameters' updating: reduce σ_s, e.g., by geometrically decreasing them by a factor α.
5. Offspring network evaluation: run each offspring network with the corresponding attributed portion of the data set.
6. Tree convergence: if the offspring network does not detect a novel cluster, the process is halted for that branch of the tree, and the tree expansion is completed at that branch. Each branch of the tree represents a cluster and a sequence of branches represents the hierarchy inherent to the data mapped into their corresponding clusters. Otherwise, while a given offspring network (branch) of the tree is still capable of identifying more than one cluster, return to Step 4 and the process continues until no new cluster can be identified in any active branch.

In step 6, the process is halted every time the suppression threshold is low enough so that each antibody of the network represents exactly one point of the data set.

MAIZE DATA

The data set consists of the expression levels of 187 genes of two strains of maize, Cat100-6 (Al-tolerant) and S1787-17 (Al-sensitive). The plants were exposed to different aluminum concentrations (Al ions), in a total of three experimental conditions: control (zero Al), 75Al (75 μM Al) and 283Al (283 μM Al). As both strains were put in the same matrix, the data set to be clustered assumes the form of a 187×6 matrix, in which the first three experiments correspond to Cat100-6 and the other three to S1787-17. Also, each gene was normalized by the mean of its attributes, thus all genes are in the same scale, and the shape of the expression profile becomes more important than the intensity of the values.

Aluminum is the most abundant metal on the earth surface, and most plants are sensitive to it. The toxic effect of aluminum inhibits plant growth, making most kinds of cultivations in soils with high concentrations of this metal impracticable. The main objective of the gene expression analysis is to find the genes involved in the tolerance of Cat100-6. This clustering strategy may elucidate mechanisms involved in the tolerance and in the toxicity of aluminum. This is a project carried out by the LGF (Laboratory of Functional Genome - http://cafe.cbmeg.unicamp.br).

COMPUTATIONAL ANALYSIS AND RESULTS

The self-organizing map

The implementation of the SOM proposed by Tamayo et al. (1999) needs the number of clusters as an input parameter. This is determined by the size of the bi-dimensional grid. Biologists made the choice of the grid size. By visual inspection they selected the grid that yields the most interesting patterns, and at the same time, a relative low deviation. The chosen grid was 5×6, i.e., 30 clusters (Figure 1).

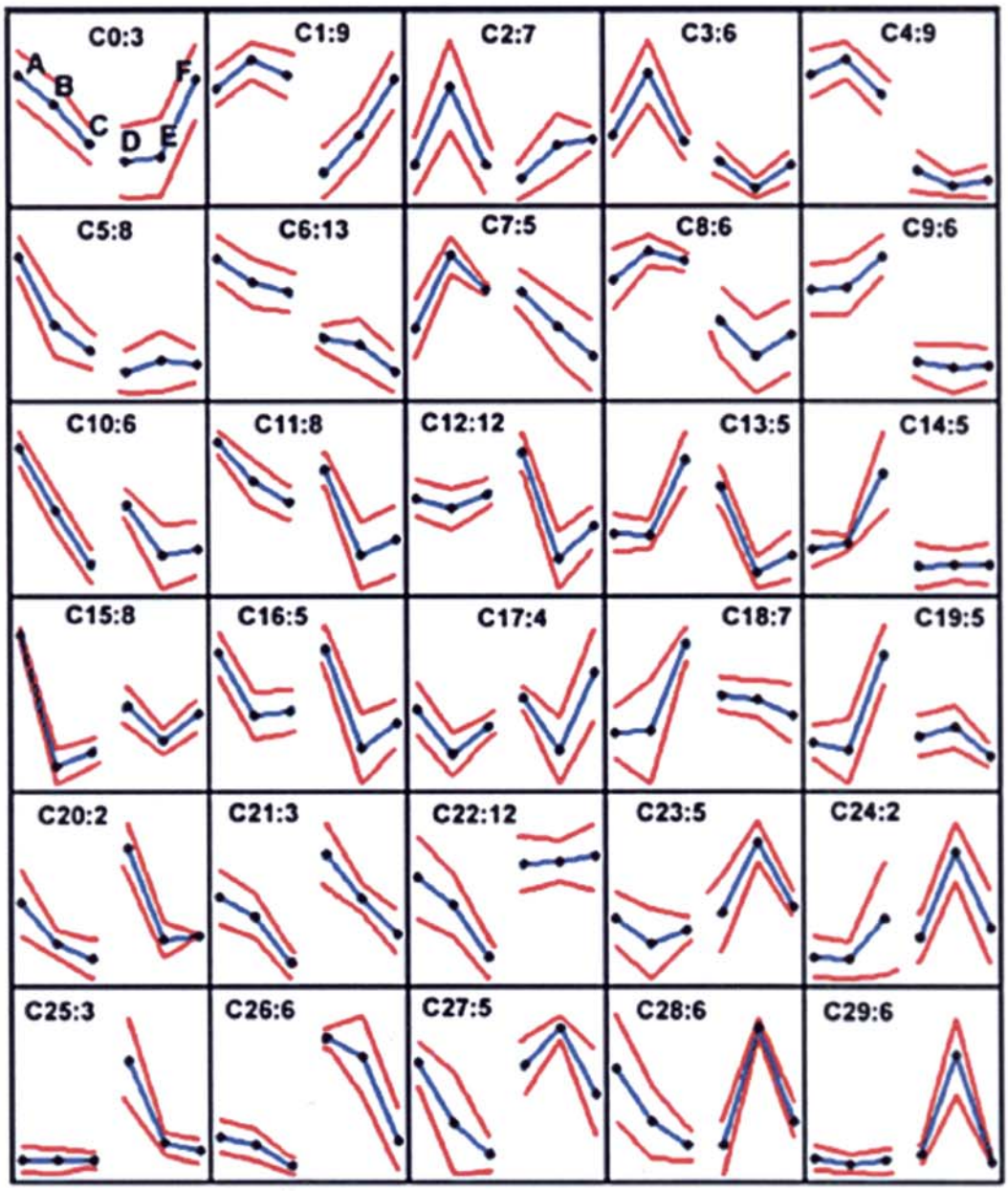

Figure 1. Self-organizing map output clusters, with the number of genes in each one. Letters A to F in cluster C0 indicate the experimental conditions.

The hierarchical aiNet (HaiNet)

The results produced by the HaiNet were considerably different from those of SOM.

The minimal spanning tree cutting criterion was capable of automatically detecting 40 clusters, and the content of the clusters are not necessarily consistent with the 30 clusters associated with the SOM strategy. The hierarchical tree that was obtained had six levels (Figure 2). Notice that there are some clusters with attached numbers. These numbers represent SOM clusters that are included into the corresponding HaiNet clusters.

A SOM cluster was considered to be inside of a HaiNet cluster if at least 80% of its genes were included. Only five clusters, C3, C7, C18, C25, and C29, were preserved by HaiNet. In the other cases, HaiNet found inherent subdivisions, most of them already in the initial hierarchical levels of the tree. This is an indication that some of the SOM clusters are not too stable, at least for this configuration. On the other hand, HaiNet found an interesting result concerning topology preservation of the maps. Note that there was no grouping in a same tree node of SOM clusters that were not connected by neighborhood links in the grid. This can be observed in clusters A and B. If, for example, cluster C25 were grouped within node A, this would characterize a topology violation of SOM, because the neuron corresponding to cluster C25 has no neighboring link with any SOM cluster inside A.

Furthermore, some clusters found by HaiNet, which present visible strong characteristic profiles, like G and H, are almost identical to the SOM clusters C29 and C25, respectively, as would be expected. Figure 3 shows these clusters side by side.

As these clusters were more specific in relation to the rest of the data set, they were already identified at the first level of the hierarchy, i.e., they were positioned quite far from the rest of the data set, so that the distance separating them from the other genes was perceivable, using a high value for σ_s.

Determining the number of clusters

The *a priori* definition of the number of clusters is a very risky practice. An incorrect estimate may distort the natural configuration of clusters. In our analysis, whenever SOM was designated to find only 30 clusters, natural clusters could be dissociated and put into two or more clusters in order to fit the restriction. A good example can be seen with clusters C5 and C28. They contain genes that visibly escape from the overall pattern (Figure 4). These genes together form a novel pattern, which was not identified by the SOM. However, HaiNet was capable of extracting this new profile, by removing the discrepant genes from both clusters, and better accommodating them into a novel cluster.

Note that two genes of C5 and one gene of C28 were put together in cluster DC. Also, the remaining genes of C28 were divided into clusters DA and DB, together with other similar genes of the data set. The remaining samples in C5 were also partitioned, but this takes place in another branch of the tree.

The results provided by HaiNet were found to be significantly better than those with SOM. The reason is that more refined clusters reduce the tedious work of biologists in filtering every cluster by visually detecting the genes that escape from the most representative patterns. However, it is fairly valid to say that SOM could have been used with a larger bi-dimensional grid. But, the number of clusters found would still not be automatic. Moreover, one can take advantage of the hierarchical relation produced by HaiNet, by traversing the correlation levels. A higher level, for example, is similar to an SOM executed using a smaller grid, but still with the same benefit of an automatically defined number of clusters.

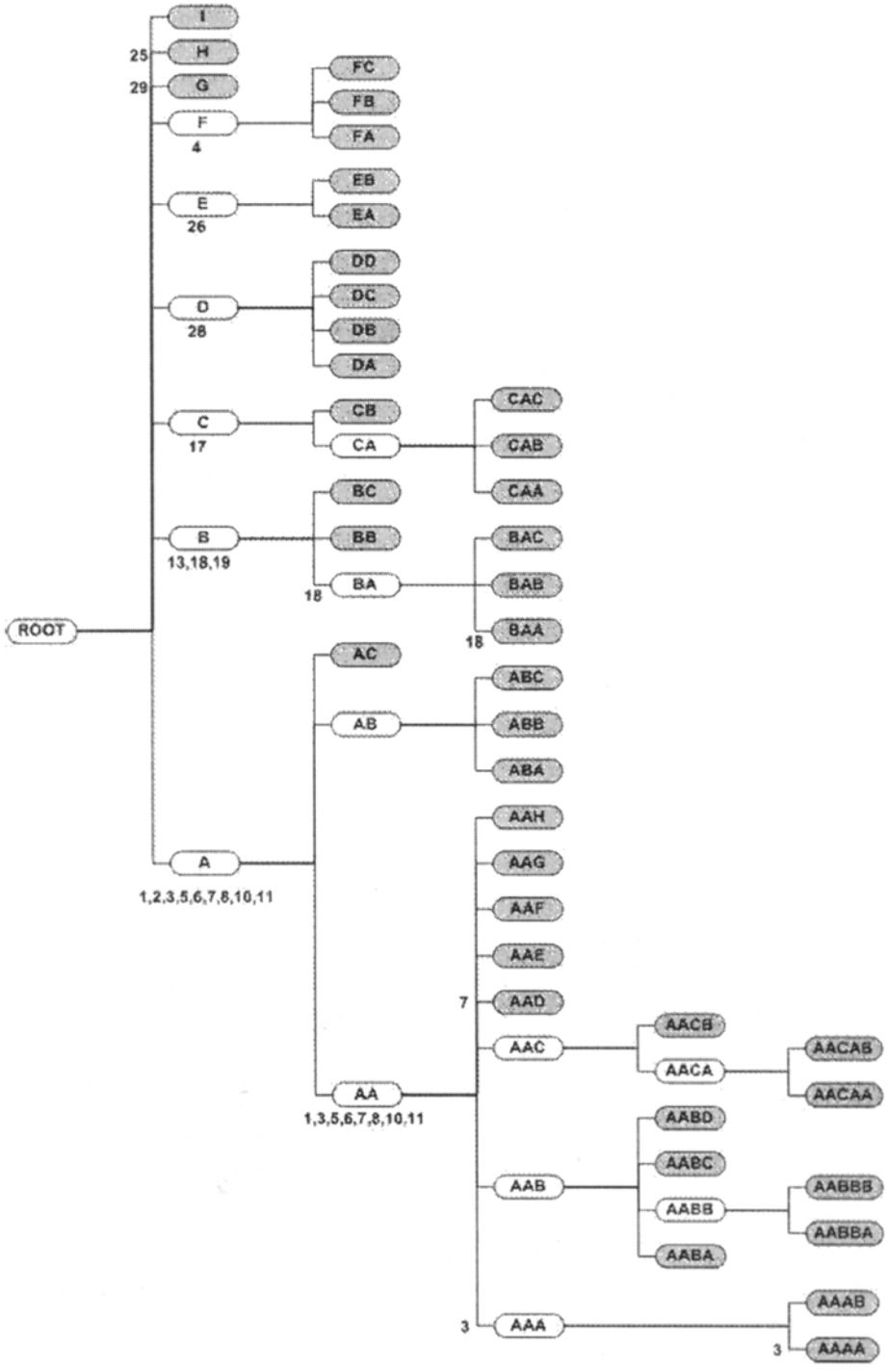

Figure 2. Hierarchical tree with six levels obtained by HaiNet. Numbers next to nodes indicate the self-organizing map clusters that are included.

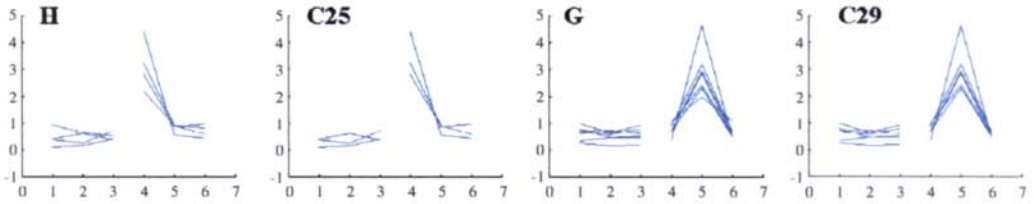

Figure 3. Clusters with the most evident characteristic patterns were identified by both techniques.

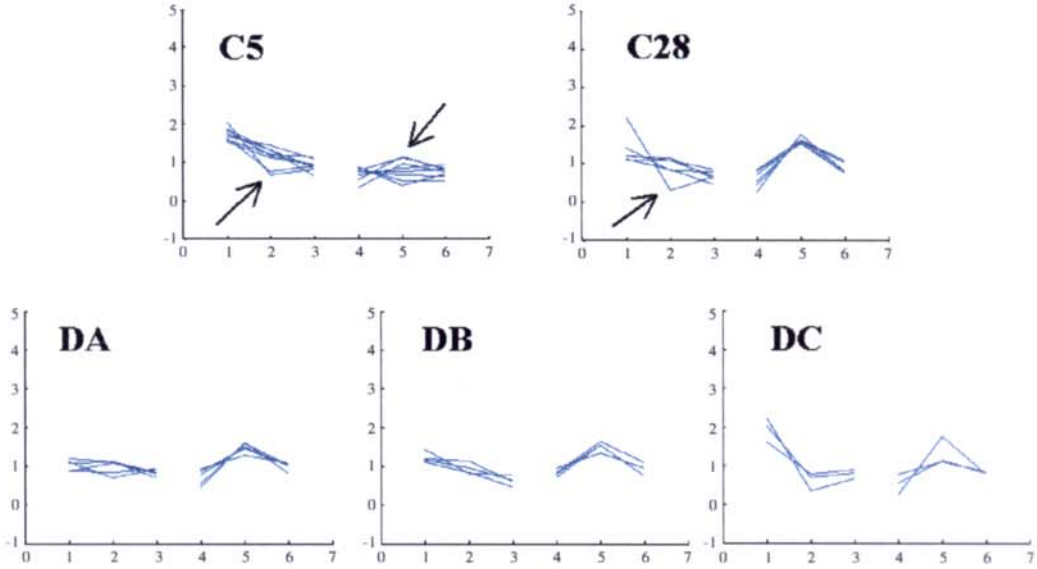

Figure 4. HaiNet put the discrepant genes in clusters C5 and C28 into a novel cluster, DC.

Exploring the hierarchical topology

In the hierarchical procedure explained in the "Hierarchy of networks" section, large-scale divisions are first detected, followed by more and more refined evaluations. In order to illustrate this property, Figure 5 gives prominence to one branch of the tree, concerning the expansion of cluster B. The intention is to demonstrate the fine-tuning process by detailing two descending hierarchical levels. A similar treatment could be adopted for the remaining branches of the tree.

Notice that cluster B presents a very noisy pattern. However, its genes are considerably distinct from the rest of the data set for this level of the hierarchy, being grouped to form an isolated cluster. As we descend to the second level of the branch, the most visible patterns within B are extracted, leading to clusters BA, BB and BC. Nevertheless, BA is still not good enough. By fine-tuning the HaiNet parameters, subdivisions in cluster BA are found, and the more refined clusters, BAA, BAB and BAC are thus identified.

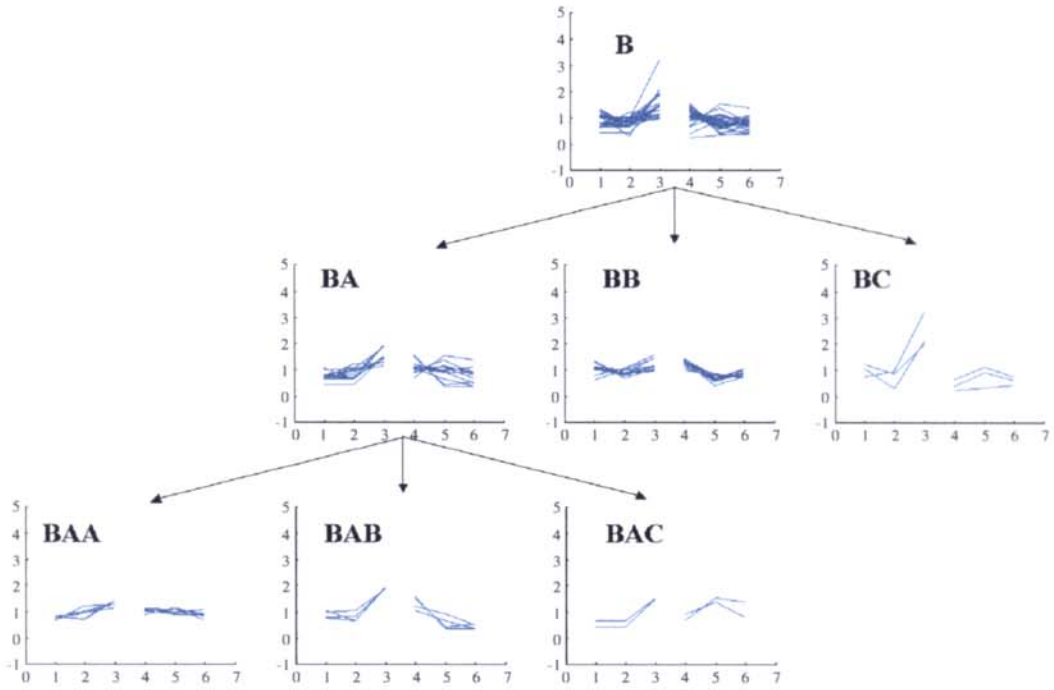

Figure 5. Detailed branch of the hierarchical tree concerning the expansion of cluster B.

CONCLUSION

We have presented a flexible and hierarchical clustering device for gene expression data analysis. HaiNet possesses desirable properties, including an automatic definition of the number of clusters and a hierarchical topology. Comparing HaiNet results with those produced with SOM, more consistent and informative outputs can be obtained with the former. This is a relevant aspect in the context of gene expression data analysis, mainly because the SOM approach is a well-disseminated clustering device for biologists.

As a demonstration of the practical applicability of HaiNet, we applied the algorithm to a data set concerning maize plants exposed to different aluminum ion concentrations. The results obtained will be further explored by biologists toward better understanding the genetic tolerance mechanisms of plants to aluminum.

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Capítulo III

Gene expression profiling of aluminum stress in maize roots

Padrão de expressão gênica do estresse de alumínio em raízes de milho

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Title: Gene expression profiling of aluminum stress in maize roots

Running Title: Genes modulated by Al in maize

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Number of figures: 4

Keywords: abiotic stress, heterologous hybridization, *Sacharum sp, Zea mays*

Abstract

To understand the mechanisms responsible for aluminum (Al) toxicity and tolerance in plants, a DNA macroarray approach was used to analyze Al-induced gene expression changes in the roots of two maize lines (Cat100-6, Al-tolerant, and S1587-17, Al-sensitive). Due to the high degree of conservation observed among plant species within the same family, we analyzed the expression profiling of maize genes using 2,304 sugarcane (*Saccharum* sp.) expressed sequence tags (ESTs) from different libraries. We have identified 85 genes in Al stressed maize root tips with significantly altered expression, demonstrating that cross-species hybridization is a suitable strategy for overall gene expression profiling. Among the ESTs up-regulated by Al, genes encoding for previously identified Al-induced proteins, such as phenyl ammonia-lyase, chitinase, Bowman-Birk proteinase inhibitor, and wali7, were identified. Several novel genes up- and down-regulated by Al-stress were also identified in Cat100-6 and S1587-17, providing new light upon the understanding of the Al-toxicity and -tolerance in plants.

Introduction

Aluminum (Al) toxicity is one of the major limiting factors to plant growth and development in many acid soils. Thus, the elucidation of the genetic and physiological basis of Al-stress responses is of great interest to agriculture around the world. Al toxicity problems are of enormous importance for maize production in developing countries located in tropical areas of Asia, Africa and Latin America. Most maize cultivars currently used are susceptible to toxic levels of Al in the soil. Therefore yield reductions up to 80% result from Al toxicity (Herrera-Estrella, 1999).

The most dramatic symptom of Al toxicity is the inhibition of root growth, which has become a widely accepted measure of Al stress in plants. Although Al toxicity primarily restricts root growth, given sufficient exposure, a myriad of different symptoms appear on both roots and shoots that are often mistaken with soil nutrient deficiencies. At the cellular level, Al has been shown to affect a large number of biochemical processes, including lipid peroxidation (Yamamoto *et al.*, 2001), inositol 1,4,5-triphosphate signaling transduction (Jones and Kochian, 1995), cytoplasmic calcium homeostasis (Zhang and Rengel, 1999), microtubules and actin organization in cell elongation (Blancaflor *et al.* 1998), and callose formation and deposition (Horst *et al.*, 1997).

Although Al is responsible for promoting serious metabolic dysfunctions, some plants have evolved Al tolerance mechanisms that enable them to grow in Al-toxic, acid soil environments (for a review, see Kochian et al, 2004; Ma *et al.*, 2001). The source of this tolerance is a genetic buffering capacity that permits plants to adapt to the most diverse terrestrial environments. Therefore, a comprehensive understanding of the molecular mechanisms underlying Al toxicity and tolerance in plants could provide important insights into the development of new varieties with improved Al-tolerance.

In fact, Al toxicity can promote profound changes in gene expression, altering the control of normal physiological processes. It has been repeatedly observed that Al induces transcription of several genes, including those significantly associated with pathogen-, wounding-, and oxidative stress-induced proteins (Cruz-Ortega and Ownby, 1993; Snowden and Gardner, 1993**;** Snowden *et al.*, 1995; Cruz-Ortega *et al.*, 1997; Hamel *et al.* 1998; Richards *et al*., 1998; Ezaki *et al.*, 2000, Watt 2003, Xiao *et al*., 2005). These studies have been restricted to wheat and *Arabidopsis*. In the case of maize, a crop with a wide range of Al tolerant germplasms, gene expression changes induced by Al stress are almost unknown, except for a gene encoding a glutathione S-transferase recently identified (Cançado *et al*., 2005).

In recent years, genomic approaches have been successfully used to examine global gene expression changes. Although the information derived from such studies undoubtedly provide a powerful means for studying the molecular mechanisms involved in biotic and abiotic stresses, the availability of cDNA sequences necessary to produce the arrays for large scale gene expression profiling remains a major limitation to several plants species. The use of heterologous hybridization is a well known tool to provide genetic information and it has been used in large scale gene expression profiling. cDNA arrays from *Pinus* have been used to assess gene expression in *Picea* and *Nicotiana* (Van Zyl *et al.* 2002) species. Renn *et al.* (2004) found that stringent statistical analysis can allow gene expression profiling using heterologous hybridization even in distantly related fish species.

Colinearity and synteny between related species have been largely studied through comparative mapping, specifically within the *Poaceae* family that contain many of the more important cereal crops. Several authors showed that the degree of cross-hybridization between maize and sugarcane oscillated from 68% to 97% (D'Hont et al. 1994; Da Silva et

al. 1993; Grivet et al. 1996; Asnaghi et al., 2000), indicating that sugarcane and maize could benefit from comparative analyses.

Taking into account the high degree of conservation among gene sequences from closely related species such as maize (*Zea mays* L.) and sugarcane (*Saccharum sp.*) (Kellogg, 2001) and the availability of filter DNA arrays containing genes from the sugarcane EST project (Nogueira *et al*., 2003), we decided to test whether these DNA arrays could be used to identify expression profiles in maize.

Therefore, if the heterologous hybridization between maize and sugarcane held true, more than forty thousands sugarcane genes identified by the SUCEST project would become a valuable resource to study gene expression in maize and other grasses with agronomic importance, whose genome data is not currently available.

In the present work, we have identified novel genes up- and down-regulated by Al stress in two Al-contrasting maize lines, giving new insights into the way tolerant and sensitive maize plants respond to Al. Additionally, we have demonstrated the suitability of the cross-species hybridization between maize and sugarcane as a large-scale expression profiling approach.

Material and methods

Plant material, seedling growth and Al-treatment

Maize (*Zea mays* L.) inbred lines Cat 100-6 (Al-tolerant) and S1587-17 (Al-sensitive) (Moon *et al.*, 1997), were obtained from the germoplasm collection of Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Campinas, Brazil. Plants were grown in the field and self-pollinated. The sugarcane (*Saccharum* sp.) genotypes growth conditions, and the SUCEST libraries production are described in Vettore *et al.* (2001).

Maize seeds were surface-sterilized with 70% (v/v) ethanol for 1 min, 0.5% (v/v) sodium hypochloride for 20 min, rinsed four times with sterile water and germinated at 28^{o}C between two layers of moist filter paper for 3 days. Seedlings were grown in nutrient solution at pH 4.2 continuously aerated as described by Jorge *et al.* (2001) and with 16 h of light at 80 to 100 μE $m^{-2}s^{-1}$ and temperatures of 26^{o}C during the day and 20^{o}C at night. The root growth inhibition (RGI) calculations were carried out according to Moon *et al.* (1997) and calculated as the percentage of the net root growth of Al treated seedlings in relation to the net root growth of Al untreated seedlings.

In macroarray experiments, 3 days-old seedlings of Cat100-6 and S1587-17 were exposed to 0 (control), 75 and 283 μM of $AlK_3(SO_4)_3$, corresponding to 15 and 50 μM of Al^{3+} activity for 24 h. Two additional concentrations of Al (15 and 520 μM, corresponding to 5 and 83 μM of Al^{3+} activity) were used for the RNA-gel blot analyses. The DNA macroarray experiment was independently replicated three times while RNA-gel blot experiments were independently replicated at least twice.

Macroarrays and Probe Preparation

Twenty-four 96-well plates containing EST plasmid clones were randomly sampled from the following sugarcane cDNA libraries: heat- and cold-treated and untreated *callus* (CL6), sugarcane plantlets infected *with Herbaspirillum rubrisubalbicans* (HR1) or *Gluconacetobacter diazotroficans* (AD1), and leaf roll tissue (LR1; Vettore *et al.*, 2001). Nylon filters containing EST plasmids were prepared as described in Nogueira *et al.* (2003). Three sets of filters were used, each one containing 768 ESTs, totaling 2,304 ESTs. Each EST was spotted twice on the same nylon filter and twice on the same spot to reduce experimental variation (Nogueira *et al.*, 2003).

RNA isolation and cDNA synthesis

Total RNA was isolated from roots according to Logemann *et al.* (1987) with minor modifications. Thirty root tips about 5 mm long from each treatment were excised, frozen in liquid nitrogen and grounded in extraction buffer (8 M Guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 2% [v/v] 2-mercaptoethanol). After extraction with 1 volume phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), the suspension was separated by centrifugation (5000 *g*, 15 min), the aqueous phase was recovered by ethanol precipitation and the pellet was resuspended in DEPC-treated water.

The cDNA probes were produced as described by Schummer *et al.* (1999) with minor modifications. About 30 μg of total RNA was reverse transcribed with Superscript II (Invitrogen, USA) using an oligo-dT18V (3μM) primer, with 3,000 Ci mmol-1[α-^{33}P]dCTP and unlabeled dATP, dGTP, and dTTP (1 mM each) for 20 min at 42^{o}C. Unlabeled dCTP was then added to a final concentration of 1 mM and the reaction continued for another 40 min. The cDNA probes were purified by using ProbeQuant G-50 microcolumns according

to the manufacturer's instructions (Amersham Biosciences, USA) and the radiolabeled probe intensity was normalized with the aid of a 1217 Rack Beta liquid scintillation counter (LKB Wallac, Finland).

Variation of the amount of spotted DNA was previously estimated by hybridizing the filters with an oligonucleotide probe that recognizes the sequence of the *Amp*^{r} gene of the pSPORT1 vector (Invitrogen, USA) as described in Nogueira *et al.* (2003). This probe was synthesized with the primers 5'-GTGGTCCTGCAACTTTATCCGC-3' and 5'-TAGACTGGATGGAGCGGATAA-3' in the presence of [α-^{33}P]dCTP, according to the protocol described by McPherson (http://www.tree.caltech.edu/protocols/overgo.html).

Macroarrays Analysis

Filters were initially hybridized with the oligo *Amp*^{r} probe. After hybridization and washing, the filters were exposed to imaging plates for 96 h and then scanned in a phosphorimager FLA3000-G (Fujifilm, Tokyo). Further, *Amp*^{r} probe was removed from the filters, hybridized with cDNA probes synthesized from the RNA samples and then washed as described by Schummer *et al.* (1997). The filters were sealed in plastic film, immediately exposed to imaging plates for 96 h, and scanned as above. Signal was quantified using Array Vision software (Imaging Research, St. Catherines, ON, Canada). Grids were predefined and manually adjusted to obtain optimal spot recognition, and spots were then quantified individually.

Filtered and normalized macroarray data were analyzed by the SAM (significance analysis of microarrays) software (Tusher *et al*., 2001) with parameters chosen in order to lead to conservative selections of differentially expressed genes. Treatment (75 μM and 283 μM of Al) comparisons against its control (absence of Al) were performed within each

maize genotype, and genotype comparisons were performed within each Al concentration. For all comparisons, SAM parameters were set as follows: minimum fold-change was set to 1.5 at least for one comparison and the delta value was chosen as the minimum value that leads to an estimated FDR (false discovery rate) threshold of 1% or less.

RNA- gel blot analysis

Samples of total RNA (10 μg) extracted from maize root tips were separated in 1% (w/v) formaldehyde-agarose gels. RNA blotting and filter hybridization were performed in hybridization solution containing 50% formamide at 42^{o}C for 18 h, according to Sambrook *et al.* (1989). After hybridization and washing, RNA filters were exposed to imaging plates for 24 h and then scanned in a phosphorimager. RNA filters were further hybridized with a 26S rRNA probe to confirm equal RNA loading.

Bioinformatics analysis

Comparative sequence analysis was performed with BLASTx and BLASTn algorithms (Altschul *et al.*, 1997) against GenBank database (http://www.ncbi.nlm.nih.gov). Matches were considered significant when *E* values were below 10^{-5} and PAM120 similarity scores were above 80 (Newman *et al.*, 1994). Data clustering of the gene expression profiles was performed using the algorithm HaiNet (Hierarchial Artificial Immune NETwork, Bezerra *et al.*, 2005), which provides an automatic detection of the optimized number of clusters and a hierarchical structure of the correlation among clusters. The average expression from the macroarray replicates was used for clustering the 85 genes previously selected by the SAM analysis. Before the clustering with HaiNet the expression

intensity from each treatment was normalized by the mean obtained from all treatments to set the sensitivity of expression into the same scale.

Results

Effect of Al on root growth of Cat100-6 and S1587-17

The percentage of root growth inhibition (RGI) for Cat100-6 (Al-tolerant) and S1587-17 (Al-sensitive) in complete nutrient solutions containing different concentrations of Al is shown in Fig. 1. Although Cat100-6 is tolerant to Al, both genotypes showed inhibition of the root growth under presence of high Al concentration after 24 h. The most evident effect of Al was observed with the two highest doses of Al. Although Al affected the root growth of Cat100-6 (reductions of almost 20 and 25% in the root growth in 283 and 520 μM Al, respectively), S1587-17 showed a much more pronounced root growth reduction. At the same Al doses, S1587-17 suffered reductions higher than 40 and 55%, respectively (Fig. 1).

Identification of Al-responsive genes using cross-species hybridization of macroarrays

Comparative maps among different grass species demonstrate that the information from one species can be extrapolated to other ones, for breeding, ecology, evolution and molecular biology purposes (Guimarães *et al*., 1997). The close relationship between maize and sugarcane (Bennetzen and Freeling, 1997; Draye *et al*., 2001) prompted us to test whether gene expression in maize roots under Al stress could be outlined using macroarrays containing sugarcane ESTs.

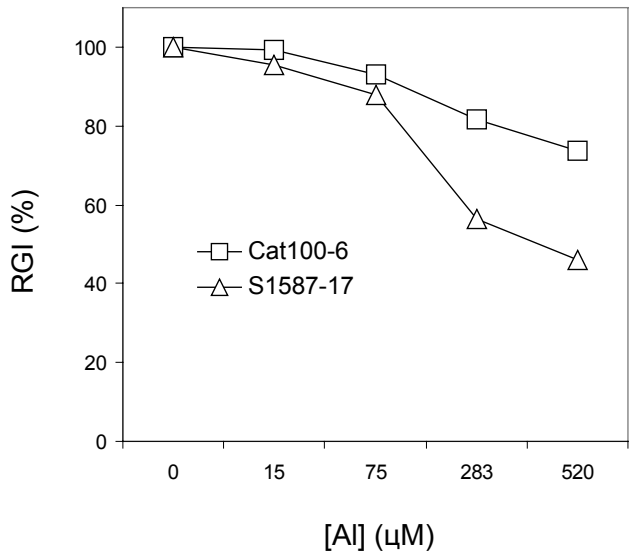


Figure 1. Dose–response curves for root growth inhibition (RGI) of Cat100-6 (square) and S1587-17 (triangle) after 24 of exposure to nutrient solution containing 0, 25, 75, 283, and 520 μM of Al (corresponding to 0, 5, 15, 50, and 83 μM of Al^{3+} activity).

Each nylon array containing sugarcane ESTs was tested with different sets of cDNA probes synthesized from maize total RNA from three independent experiments to verify reproducibility. The normalized signal intensity of each spot was determined after subtracting the local background intensity. Thus 85 sugarcane ESTs showing at least a 1.5-fold induction or repression were effectively selected, as shown in Table 1. As expected, the absolute-fold induction values were not identical among the biological samples, but the expression profiles were very similar, confirming the reproducibility of our macroarray data (data not shown). The worth of our macroarray experiment for screening genes whose expression is modulated by Al was demonstrated by the identification of several Al-inducible genes that had already been reported in other plant species, including maize.

The relevant biological functions of identified Al-responsive genes are shown in Table 1. The ESTs encoding known proteins represent a wide range of functions, including transcription, signalling, sugar metabolism, defense and development. These results suggest that several metabolic processes, including perception of stress signals and regulation of gene expression, were altered during Al stress in both Al-tolerant and Al-sensitive plants.

Among the 85 Al-responsive genes detected in our array data, 41 (48.2%) and 43 (50.5%) were up-regulated in Cat100-6 and S1587-17, respectively, noting that 15 (17.6%) were up-regulated in both genotypes (Table 2). This result shown that 81.2% of the all ESTs identified in this work had its expression up-regulated by Al. The number of ESTs down-regulated in Cat100-6 and S1587-17 were 8 (9.4%) and 9 (10.6%), respectively, totalising 18.8% of all ESTs significantly altered by Al (Table 2). The up-regulated

Table I. *Average expression ratios and sequence similarities of Al-responsive sugarcane ESTs*

				Ratios[a]					Ratios[b]	
Accession .	Class[c]	Blast Hit (E-value)	Description[d]	C75	C283	S75	S283	Cluster[e]	C/S 75	C/S 283
Induced in both Cat100-6 and S1587-17										
CA095811	III	JC5843 (2E-46)	Chitinase III	1.3	5.0	1.3	1.7	4	2.6	7.8
CA101236	VI	NP_922793 (2E-66)	Unknown protein	0.4	1.9	1.5	2.3	4	0.3	3.0
CA102633	VI	XP_469468 (E-129)	Unknown protein	0.7	1.9	3.4	2.5	4	0.9	3.0
CA098848	III	BAB63915 (0)	ERD protein	3.0	1.7	0.2	2.1	6	4.4	1.2
CA095754	III	1OM0A (E-119)	Xylanase inhibitor protein	1.5	1.7	3.1	1.4	7	1.9	4.5
CA102690	II	CAA27681 (0)	Alcohol dehydrogenase 1	0.7	2.3	1.5	1.3	12	0.3	1.0
CA096776	I	AAC69625 (E-115)	WD-40 protein	1.8	1.3	1.5	1.4	13	1.6	1.2
CA102731	II	AAF85966 (E-104)	Sucrose synthase	3.1	3.2	1.9	1.8	13	1.1	0.9
CA064600	III	BAA97804 (4E-46)	ß-Glucuronidase precursor	1.0	1.9	0.7	1.7	16	1.7	1.6
CA064719	III	BAB19963	ASR protein	4.9	6.6	1.7	2.3	16	1.4	2.2
CA095602	VI		No hit	1.4	2.2	1.3	1.5	18	1.2	2.1
CA095678	III	P81713 (2E-07)	Bowman-birk proteinase inhibitor	1.5	1.3	18.7	46.6	20	1.4	1.1
CA097100	VI		No hit	3.2	1.1	2.4	65.6	20	2.5	0.7
CA095919	I	P49625 (E-116)	60S Ribosomal protein L5	1.9	1.2	2.2	2.0	22	1.4	1.0
CA097212	II	2008300A (0)	Sucrose synthase	1.7	1.4	3.2	3.0	22	1.7	1.1
Induced only in Cat100-6										
CA102689	VI		No hit	0.7	1.7	0.9	0.5	1	0.3	1.3
CA120042	III	BAD14927 (0.0)	DAHP synthetase	2.9	4.9	1.1	1.1	1	0.3	1.0
CA064763	III	AAL40137 (0)	Phenylalanine ammonia-lyase	1.3	3.7	1.1	1.1	4	1.0	3.0
CA064602		CAA13177 (7E-62)	Cinnamyl alcohol dehydrogenase	3.0	1.6	0.7	0.8	5	3.4	1.9
CA097041	VI		No hit	1.9	1.1	0.5	0.6	5	3.3	2.0
CA064780	VI	NP_683323 (E-14)	Unknown protein	1.0	1.5	0.4	0.5	6	2.3	1.8
CA064608	II	CAB87248 (9E-33)	Glycerol 3-phosphate permease	2.3	2.7	0.8	1.4	7	5.9	3.8
CA064605	IV	AAB97114 (2E-89)	Small GTP-binding protein	2.3	1.7	0.9	0.3	10	1.2	3.0
CA064810	VI		No hit	1.7	1.5	0.8	0.8	10	1.4	1.3
CA095938	VI		No hit	1.6	1.2	0.7	0.6	10	1.9	1.5
CA096090	III	AAC49972 (2E-28)	Hypersensitive response protein	1.8	2.0	1.4	1.0	10	1.0	1.6
CA096097	III	NP_077728 (9E-67)	Putative ferredoxin reductase	1.8	1.7	1.0	0.8	10	1.3	1.5
CA064787	III	AAK71314 (E-102)	Papain-like cysteine peptidase	1.6	1.3	1.4	0.6	11	1.5	3.6
CA095663	I	P05621 (E-38)	Histone H2B.2	1.5	1.1	1.2	1.3	11	0.7	0.5
CA096797	VI	XP_467727 (7E-48)	Unknown protein	0.8	2.2	0.8	0.4	12	0.7	4.1
CA097462	I	NP_958815 (2E-14)	LUC7-Like protein	0.6	2.0	0.4	0.3	12	1.7	5.2
CA101237	III	Q40977 (4E-26)	Monodehydroascorbate reductase	1.0	2.3	1.3	1.0	12	0.5	2.0
CA097457	V	S72526 (0)	Vacuolar H^+-pyrophosphatase	1.9	1.5	1.2	1.4	13	1.3	0.8
CA064742	III	AAM75139 (5E-39)	Alkaline α-galactosidase	8.2	5.0	0.5	1.0	14	6.0	2.9
CA097200	VI		No hit	1.6	1.3	0.5	0.8	15	2.3	2.0
CA102716	III	AAC37416 (7E-50)	Wali7	1.1	1.6	1.2	1.0	15	1.1	2.1
CA096094	I	AAF76167 (E-174)	Nuclear cap-binding protein	1.2	1.9	1.0	0.7	18	0.9	2.0
CA097210	V	S52030 (E-24)	Oleosin 17	0.8	1.5	0.6	0.7	18	1.2	1.7
CA095623	III	CAD27730 (5E-41)	Xylanase inhibitor	0.9	1.8	1.0	1.3	19	0.7	1.4
CA095852	I	CAA58669 (2E-28)	Ribosomal protein S27	1.4	1.7	0.9	1.0	19	0.9	1.1
CA096135	I	AAG60059 (E-173)	Acetyltransferase-related protein	0.9	1.8	1.1	0.9	19	0.7	1.6

Table continues

				Ratios[a]					Ratios[b]	
Accession .	Class[c]	Blast Hit (E-value)	Description[d]	C75	C283	S75	S283	Cluster[e]	C/S 75	C/S 283
Induced only in S1587-17										
CA064736	V	AAB72111 (6E-57)	BP-80 vacuolar sorting receptor	0.9	0.9	3.4	0.8	2	0.1	0.7
CA102674	I	AAN15557 (4E-52)	ABI3-interacting protein 2	0.9	1.3	2.4	0.6	2	0.3	1.4
CA102687	VI	XP_475538 (8E-38)	Unknown protein	0.5	1.3	4.0	0.7	2	0.3	1.9
CA102772	II	CAA54609 (7E-75)	UTP-glucose glucosyltransferase	1.1	0.6	7.6	2.0	2	0.3	1.1
CA120045	I	NP_919951 (E-115)	Putative retroelement	0.4	0.5	9.1	0.8	2	0.0	0.4
CA101178	VI	AAF01557 (2E-28)	Unknown protein	1.5	1.1	1.8	0.9	3	0.3	0.6
CA097428	III	AAM64219 (2E-07)	Cadmium induced protein CdI19	0.9	0.5	2.1	1.0	8	0.7	0.8
CA097438	III	CAA31077 (8E-36)	ABA-inducible protein	0.9	0.4	1.7	1.2	8	0.5	0.4
CA101174	VI		No hit	0.6	0.5	2.9	1.6	8	0.6	1.0
CA095885	I	T52344 (2E-87)	OsNAC5 protein	0.3	0.6	1.3	2.8	9	0.7	0.7
CA097119	VI	CAE03862 (3E-91)	Unknown protein	1.0	1.1	1.8	1.3	11	0.6	0.9
CA097111	III	JC5845 (2E-84)	Chitinase III	1.1	1.0	1.5	1.0	14	5.5	5.4
CA064657	V	CAA45024 (5E-94)	Aspartate aminotransferase	0.9	0.8	4.5	2.9	17	1.3	1.5
CA064710	II	AAF70821 (0)	β-Galactosidase	0.3	0.3	3.8	1.4	17	0.7	0.9
CA096803	IV	XP_469542 (3E-99)	Putative ATP-binding protein	0.7	0.6	0.9	2.5	17	1.4	1.0
CA097066	VI	CAE05958 (E-129)	Unknown protein	1.2	1.1	0.9	2.1	17	2.2	1.1
CA097909	VI	XP_476424 (E-22)	Unknown protein	0.2	0.4	40.2	15.0	17	0.2	0.8
CA101149	I	T01996 (6E-45)	Nucleoid DNA-binding protein	0.8	0.4	4.0	5.0	17	2.0	0.5
CA064656	V	P23687 (E-156)	Prolyl oligopeptidase	1.2	1.2	1.8	2.3	20	1.3	0.8
CA096567	II	CAA39454 (0)	Enolase	1.3	0.6	1.6	2.7	20	1.4	0.5
CA096578	I	O15818 (E-105)	Putative eIF-3	0.9	0.6	1.3	1.6	20	1.0	0.6
CA102758	V	O13656 (6E-24)	Mitochondrial import receptor	1.0	0.8	1.2	2.6	20	1.1	0.4
CA119990	III	JC2510 (0)	β -Tubulin	1.1	0.9	1.6	2.1	20	1.3	0.9
CA064621	III	CAA55893 (E-140)	Putative imbibition protein	1.0	1.3	1.1	1.9	21	1.8	1.3
CA064717	VI		No hit	1.2	1.0	1.1	2.1	21	2.9	1.5
CA097411	I	AAC25599 (3E-10)	CRP1 protein	1.2	0.9	0.9	2.3	21	2.2	0.8
CA101244	III	NP_005856 (E-43)	Putative serine peptidase	1.0	0.7	0.8	1.9	21	3.9	1.3
CA119993	IV	CAA10660 (E-151)	Ca^{2+}-ATPase	1.1	1.1	1.8	2.2	22	1.6	1.5
Repressed in both Cat100-6 and S1587-17										
CA095925	II	AAG28503 (E-151)	Hexokinase	0.6	0.2	0.3	0.8	9	1.2	0.4
Repressed only in Cat100-6										
CA095622	VI		No hit	0.4	0.5	0.8	0.8	9	0.8	0.9
CA095948	VI	AAN41388 (2E-17)	Unknown protein	0.6	0.4	0.9	1.1	9	1.7	0.7
CA097085	VI		No hit	0.7	0.4	0.7	0.6	9	1.4	0.8
CA101148	I	T01996 (6E-45)	Nucleoid DNA-binding protein	0.8	0.4	1.3	1.5	9	1.6	0.8
CA095886	I	CAA63194 (E-155)	Ribonucleotide reductase R2	0.5	0.8	0.9	1.3	11	0.5	0.6
CA096779	I	P12629 (7E-60)	50S ribosomal protein L13	0.3	0.5	1.2	0.8	11	0.2	0.5
CA099555	I	CAB75508 (3E-14)	ABI3-interacting protein	0.5	0.6	1.5	1.1	17	0.7	1.2

Table continues

				Ratios[a]					Ratios[b]	
Accession .	Class[c]	Blast Hit (E-value)	Description[d]	C75	C283	S75	S283	Cluster[e]	C/S 75	C/S 283
Repressed only in S1587-17										
CA101271	III	AAM23263 (E-30)	DnaJ-like protein	1.0	0.6	1.1	0.3	3	0.4	0.9
CA095710	I	BAA95894 (2E-72)	Putative reverse transcriptase	1.0	0.7	0.5	0.2	5	4.0	6.5
CA096690	VI	BAB11002 (E-138)	Unknown protein	0.9	0.7	0.3	0.3	5	4.2	3.6
CA095910	V	AAF74980 (E-121)	Cystathionine b-lyase	1.1	0.9	0.3	0.7	6	2.6	1.0
CA097854	I	BAA31739 (2E-05)	COP1-Interacting protein 7	0.9	0.7	0.4	0.8	6	2.8	1.1
CA098865	I	AAT40500 (E-50)	Putative reverse transcriptase	0.9	1.2	0.5	0.8	6	1.7	1.3
CA064616	I	AAD45720 (2E-31)	Zinc finger protein	1.3	1.0	0.6	0.3	14	10.6	16.3
CA064644	V	AAM60860 (E-136)	Thiol-monophosphate biosynthesis	0.6	1.1	0.4	1.0	21	3.1	1.9

[a]Expression ratios of Al-treated intervals (75 and 283 μM of Al) in relation to its control (0 μM of Al): C75 = Cat100-6 under 75 μM of Al; C283 = Cat100-6 under 283 μM of Al; S75 = S1587-17 under 75 μM of Al; S283 = S1587-17 under 283 μM of Al. [b]Expression ratios of Cat100-6 in relation to S1587-17: C/S 75 = Cat100-6/S1587-17 under 75 μM of Al; C/S 283 = Cat100-6/S1587-17 under 283 μM of Al. [c]Classes dividing the genes in six general categories: I) Gene regulation; II) Sugar metabolism; III) Plant stress response; IV) Signal transduction; V) Other functions; and VI) Unknown and no hit proteins. [d]Description indicates the putative functions of the gene products expected from similarity sequences. [e]Clustering profile shown in Fig. 4.

Table 2. *Aluminum-regulated sugarcane ESTs identified by macroarray expression profiling*

Summary	Cat100-6		S1587-17		TOTAL	
	N°. of ESTs	% ESTs	N°. of ESTs	% ESTs	N°. of ESTs	% ESTs
Al-responsive ESTs						
Up-regulated ESTs	41	48.2	43	50.6	69	81.2
(Known Al-responsive[a])	(5)	(5.9)	(6)	(7.0)	(8)	(9.4)
(Other stress-responsive [b])	(17)	(20)	(14)	(16.5)	(25)	(29.4)
(Novel Al-responsive[c])	(19)	(22.3)	(23)	(27.1)	(36)	(42.3)
Down-regulated ESTs	8	9.4	9	10.6	16	18.8
Unknown ESTs						
Al up-regulated	4	4.6	7	8.1	9	10.4
Al down-regulated	1	1.2	1	1.2	2	2.3
No hit ESTs						
Al up-regulated	7	8.1	4	4.6	9	10.4
Al down-regulated	2	2.4	0	0	2	2.4

[a]ESTs homologous to previously described Al-induced genes. [b]ESTs homologous to previously described general-stress responsive genes. [c]ESTs encoding proteins that have not been described previously as Al-induced or other stress-induced.

sugarcane ESTs in Cat100-6 and S1587-17 were distributed in three main groups as described on the Table 2.

The first group contained ESTs encoding proteins previously described as Al-responsive, such as phenylalanine ammonia-lyase, wali7, Bowman-Birk proteinase inhibitor, and β-tubulin. The second group contained ESTs encoding proteins responsive to other biotic and abiotic stresses, but not reported as Al-responsive until now; this group included xylanase inhibitor protein, OsNAC5 protein, cadmium induced protein CdI19, and a ribosomal protein S27. The third group contained ESTs encoding unknown proteins, proteins with no hits in public databases, and proteins not reported as stress-responsive, such as nucleoid DNA-binding protein cnd41, mitochondrial import receptor tom40, BP-80 vacuolar sorting receptor, glycerol 3-phosphate permease, and histone H2B.2.

To estimate the relative contribution of Al-altered genes from each SUCEST library used in the array experiments, we calculated the number of Al-altered ESTs from each cDNA library, as shown in the Fig. 2. Interestingly, the library CL6 accounted for most of the Al-induced ESTs and almost all of the Al-repressed ESTs identified in Cat100-6 and S1587-17. The CL6 library was constructed from a mixture of heat- and cold- treated and untreated sugarcane *callus* tissues (Vettore *et al.*, 2001) and was therefore a good source of biotic and abiotic stress-related ESTs (Vettore *et al.*, 2003).

RNA-gel blotting Analysis

RNA-gel blotting analysis using total RNA were performed for specific genes in order to validate the macroarray data. Fig. 3A shows the expression profile of six genes with high similarity to: a phenylalanine ammonia-lyase (accession CA064763); an abscisic acid- and stress-inducible protein (CA064719); a protein homologous to B12D domain, described as

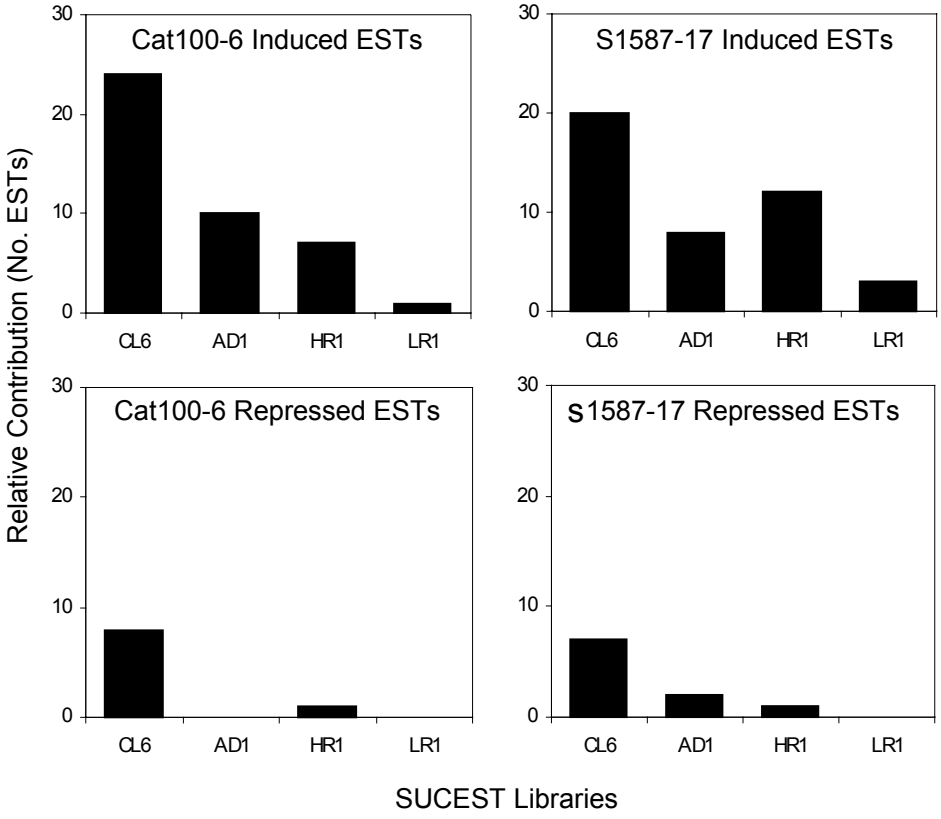


Figure 2. Relative contribution of the SUCEST libraries used in the macroarray experiments to identify Al-inducible genes. The values represent the percentage of normalized Al-altered genes identified from each cDNA library (see “Results”) in relation to the total number of ESTs identified as Al-altered in our arrays. CL6, Heat and cold-treated and untreated *callus*; AD1, sugarcane plantlets infected with *G. diazotroficans*; HR1, plantlets infected with *H. rubrisubalbicans*; and LR1, leaf roll tissue.

a pathogen inducible protein whose function remains unknown (CA097100); a chitinase class III (CA095811); a β-glucuronidase (sGUS) precursor (CA064600) that is involved in the initiation of H_2O_2 metabolism; and a hypothetical protein with no conserved domain (CA101236).

Phenylalanine ammonia-lyase, abscisic acid- and stress-inducible protein, protein homologous to B12D domain, and the chitinase class III, showed 85.8% (accession CO520609), 81.2% (BM499107), 41.6% (BE510590), and 52.0% (CF024252) identity with maize EST sequences from the Maize Genetics and Genomics Database (MaizeGDB, http://www.maizegdb.org/blast.php) respectively. Except CA064600 and CA101236 produced no significant hit to maize sequences. The Fig. 3B compares the gene expression profiles obtained from macroarrays and RNA-gel blotting. The absolute-fold induction or repression values from the RNA blots were close to those on the DNA-array, indicating a high consistency between the two data sets, although the relative intensity scale of radioactive probe was different between the methods due the emission differences between the radioisotope ^{33}P (used in the macroarray analysis) and the radioisotope ^{32}P (used in the RNA-blot analysis).

Data Clustering of Al-Responsive Genes

Clustering of the apparently arbitrary distribution of gene expression patterns might reveal groups of genes with strong correlations in their expression profiles, therefore exposing a more comprehensible structure that may provide meaningful information about the function of unknown genes.

The algorithm HaiNet was used to cluster the 85 genes previously selected by the SAM analysis. HaiNet automatically determined 22 clusters of genes based on their density

A

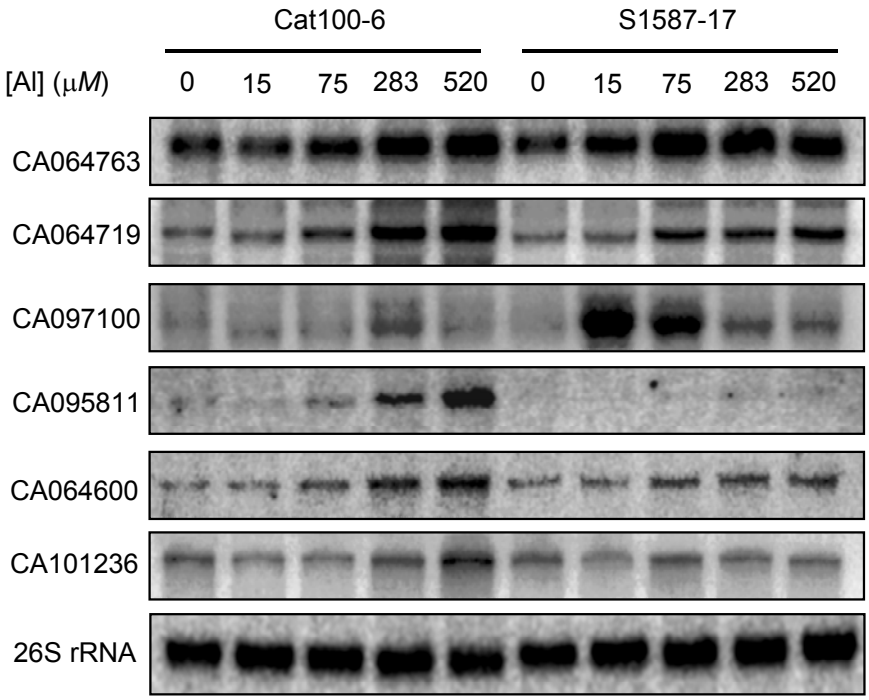
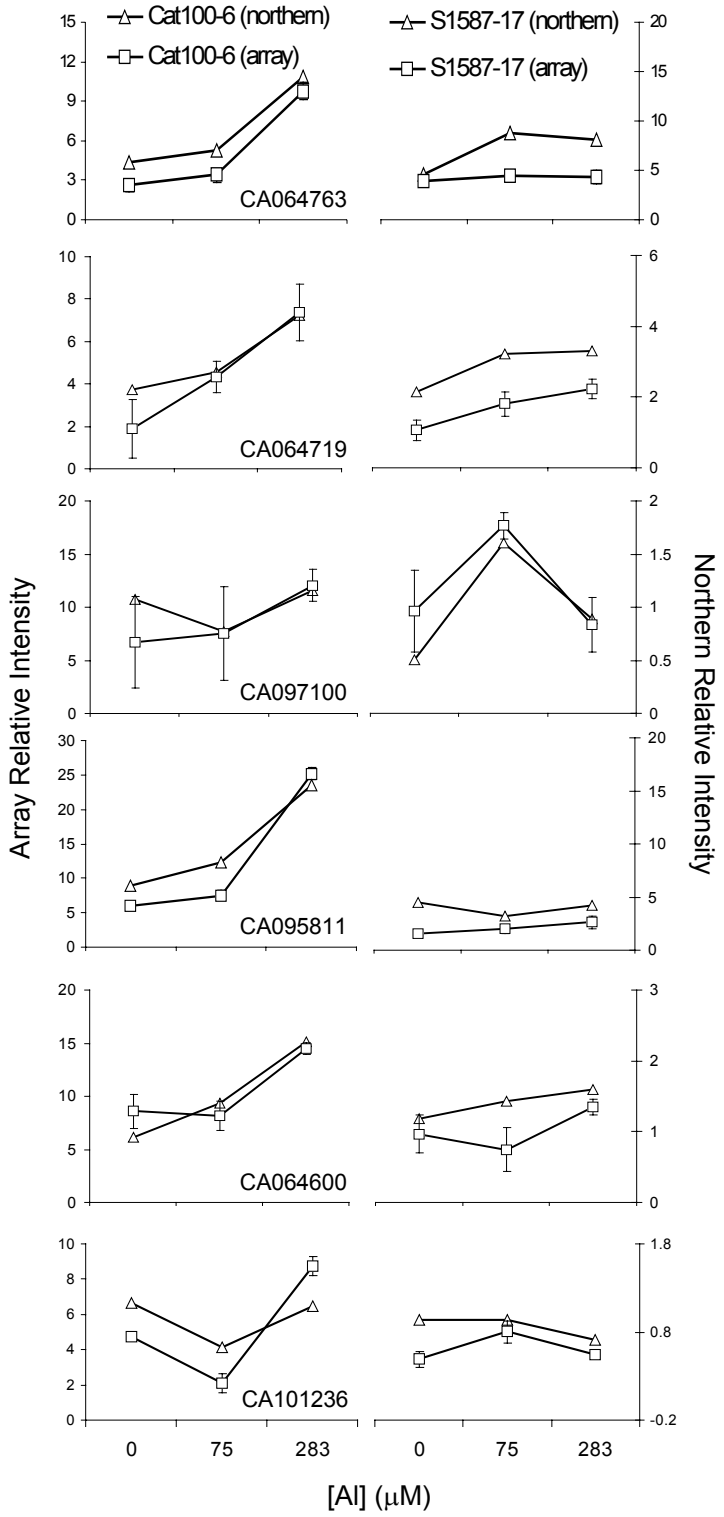


Figure 3. Comparison between EST macroarray and RNA-blot analysis for Al-altered maize cDNAs. (A) In RNA gel blots, each lane was loaded with 10 μg of total RNA isolated from root tips exposed to increasing Al concentrations. The RNA loading was monitored using a 26S rRNA as probe. (B) The graphs show the induction kinetics observed in the macroarrays (square) and RNA blots (triangle).

B


Cat100-6 (northern)
Cat100-6 (array)
S1587-17 (northern)
S1587-17 (array)
CA064763
CA064719
CA097100
CA095811
CA064600
CA101236
Array Relative Intensity
Northern Relative Intensity
0
75
283
[Al] (μM)

distribution. The cluster where each gene was positioned is indicated on the ninth column of Table 1. The algorithm also produced a hierarchical topology of the similarity relation among clusters based on the expression profile of each cluster (Fig. 4).

The most stable clusters were identified in the earliest level of the hierarchy and were positioned close to the base of the tree. Clusters 1, 2, and 3 are examples of this behaviour. Clusters 4, 5, 7, and 8 efficiently grouped genes involved in closely-related functions, such as plant defense (Table 1). It was found that many of the genes with unknown function or genes with no hit in the GenBank database were uniformly distributed among the 22 clusters, indicating no tendency of specific grouping. On the other hand, some of these unknown and no hit genes were located in clusters with genes involved in similar biochemical pathways, suggesting the involvement of them in known biochemical processes. This was the case for CA102633 and CA101236 ESTs, both unknown proteins that were located in the same cluster of chitinase III and phenylalanine ammonia-lyase (PAL), enzymes involved in plant defense mechanisms. The data clustering based on the absolute levels of transcripts in both maize lines also provided information about the interrelationships among the genes that were induced in Cat100-6 (Table 1 and Fig. 4).

Considering that the higher induction of a gene in the tolerant line in response to Al is not an adequate parameter to explain Al tolerance, since the unchanged levels of a transcript in the Al-sensitive line may be higher than in the Al-tolerant line. From those genes identified in the macroarrays experiments as induced by Al in both maize lines, five had at least three times more transcripts in the Al-tolerant line than in the sensitive one in the same Al treatment: CA095811 (encoding a chitinase III), CA095754 (xylanase inhibitor protein), CA098848 (ERD protein) and CA102633 and CA101236 (both unknown proteins). The most promising genes among those induced exclusively in Cat100-6 were CA064742

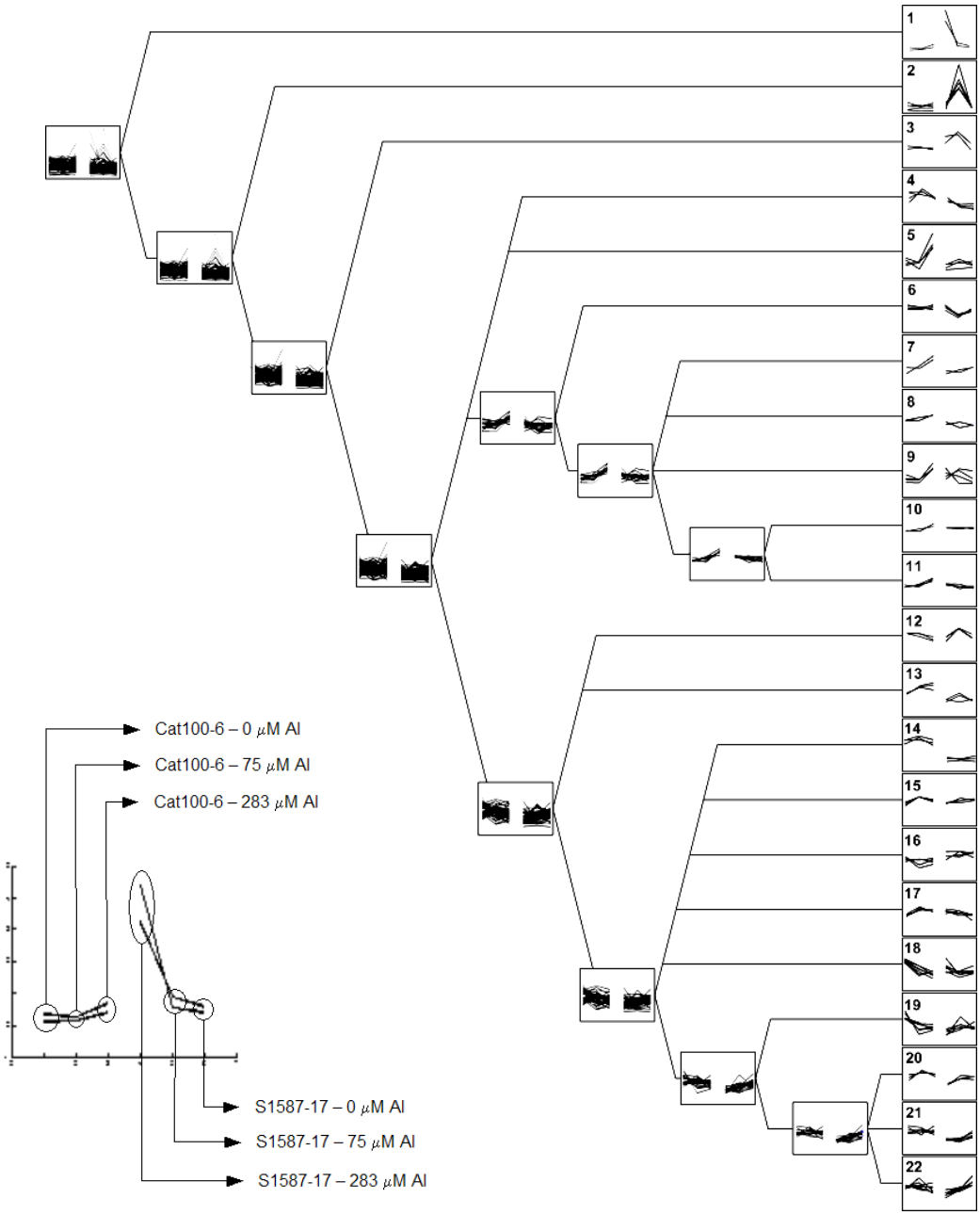


Figure 4. Hierarchical clustering of 85 ESTs selected in the macroarray analysis. The diagram on the base of left side shows the distribution of treatments (maize genotypes and Al concentrations) into of each cluster.

(encoding an alkaline α-galactosidase), CA064608 (glycerol 3-phosphate permease), CA097462 (LUC7-Like protein), CA096797 (unknown protein), CA064787 (papain-like cysteine peptidase), CA064602 (cinnamyl alcohol dehydrogenase), CA097041 (no hit), CA064605 (Small GTP-binding protein) and CA064763 (phenylalanine ammonia-lyase).

Discussion

Aluminum-induced root growth inhibition in maize

As a result of Al stress, the primary effect observed when plants are growing in phytotoxic levels of this metal is the inhibition of root growth (Foy *et al.* 1978; Ryan *et al.* 1993). The growth inhibition observed in roots of both Cat100-6 and S1587-17 was dependent on the Al concentration, although the toxic effect of Al to the root growth was almost twice more severe to S1587-17 than to Cat100-6 (Fig. 1). This result shows that high levels of Al might surpass the tolerance observed even for Al-tolerant genotypes. The root growth measurement under Al-stress obtained in this work agrees with previous results with the same maize genotypes (Moon *et al.*, 1997; Jorge *et al.*, 2001; Boscolo *et al.*, 2003). Twenty-four hours of Al-stress is shown to be enough time to promote visible alterations in the root phenotype of both maize lines, mainly in the concentrations of 283 and 520 μM Al (Fig. 1).

Cross-species hybridization of macroarrays

Maize and sugarcane are grasses that belong to the *Poaceae* family and to the same *Andropogoneae* tribe (Clayton & Renvoize, 1986). Recent comparative mapping information support that all grasses examined to date diverge from one common ancestral population of “grass alleles” (Freeling 2001). Comparative mapping within maize, sorghum and sugarcane has previously revealed the existence of high synteny (regions with homologous chromosomal segments) among these crops (Dufour *et al.*, 1997). Comparative mapping among different grass species demonstrate that the information from

one species can be extrapolated to other ones, for breeding, ecology, evolution and molecular biology purposes (Guimarães *et al.*, 1997).

Using large-scale gene expression profiling in which the tester (cDNA obtained from maize is used as probe) and the target (cDNA from sugarcane library is spotted on the nylon filters) were originated from related plant species, we identified 85 genes with expression significantly altered by Al (Table 1). The confirmation of the expression profiling using RNA-gel blots and the high quality signal produced by the heterologous hybridization of the sugarcane filters with maize probes (data not shown) for most of the 2,304 clones spotted on the filters are an indication that there is enough conservation of the nucleotide sequence between these two species to utilize comparative techniques. Thus, we have demonstrated that sugarcane EST macroarrays can used to analyse gene expression profiles in maize.

Effect of Al-stress in the gene expression of maize root tips

Because complex traits are polygenic and gene interactions are fundamental properties of these traits, taking advantage of a genomics approach to the study of gene expression improves the power to reveal the complex network of interrelated functional modules that are involved (Aubin-Horth *et al.*, 2005). Although the functional categorization of genes is not a simple task, since many genes seem to be involved in more than one biological process, we have tried to assemble the genes identified in this work into 6 main groups based on their putative function (Table 1). The first group is comprised of genes involved with transcriptional and post-transcriptional regulation. Genes encoding proteins and enzymes that participate in sugar metabolism and other sugar-dependent processes constitute the second group. The third group contains genes previously cited as stress-

responsive, including genes characterized as regulated by Al-stress. The fourth group includes genes involved with signal transduction pathways. The fifth group is composed of proteins whose known function does not match with any of the groups described above and the last group is formed of unknown proteins and genes with no significant hit in open access genetic sequence database. The real purpose of this classification was not to produce an exact categorization of all genes identified in this work, since many genes can be involved in more than one biological process, but coordinate the discussion about these genes into groups with biological function associated.

Several genes involved with the control of gene expression were up- or down-regulated by Al stress in both maize lines. The gene encoding the ribosomal protein S27 (accession CA095852) was up-regulated only in Cat100-6 plants growing in presence of Al. Interestingly, this protein displays DNA binding properties and is involved in response to DNA injury and degradation of damaged RNAs (Revenkova *et al.*, 1999). Another interesting observation was the up-regulation of a gene encoding an acetyltransferase (CA096135) in Cat100-6. This enzyme is responsible for acetylation of nucleosomal histones and, in general, there is a positive correlation between the histone acetylation state and transcriptional activity of genes (Bordoli *et al.*, 2001). Another gene involved in genetic regulation, up-regulated by Al in Cat100-6, encoded a H2B histone (CA095663). H2B is a target for posttranslational modification of chromatin topology, altering the regulation of gene expression (Henry *et al.*, 2003). This finding suggests that primary or secondary effects promoted by Al stress in root cells might be responsible for profound and complex alterations in mechanisms of genetic expression.

Fluctuations of other transcripts involved in gene regulation reinforce the conclusion that Al promotes important imbalances in the intricate network of gene expression. The

genes encoding the proteins CBP80 (Cap-Binding Protein 80Kda, CA096094) and LUC7 (lethal unless CBC, CA097462), both up-regulated in Cat100-6, are involved in mRNA processing. While CBP proteins are involved in the formation of the cap-binding complex (CBC) (Kmieciak *et al.*, 2002), LUC7 is required for the complex formation of pre-mRNA splicing machinery (Fortes *et al.*, 1999). A gene that was up-regulated in S1587-17 (CA095885) belongs to the family of NAC (NAM, ATAF1, and CUC2) domain proteins which are members of putative transcript factors found exclusively in plants and involved in development and recognition of environmental stimuli such as cold stress (Nogueira *et al.*, 2005), pathogen infection and wounding (Collinge and Boller, 2001).

Other important protein that participate in the control of gene expression is the ASR (ABA [abscisic acid]-, stress-, and ripening-induced, CA064719), involved in a common transduction pathway of sugar and ABA signaling (Çakir *et al.*, 2003). Additional Al responsive-genes encoding proteins involved in transcriptional regulation included AIB3 (ABA-insensitive, CA102674), which is known to be an activator of the expression of a large number of genes during embryo maturation (Kurup *et al.*, 2000) and WD-40 repeat proteins (CA096776), which are components of complexes involved in assembling and modification of chromatin (Kenzior and Folk, 1998).

The EST clone CA096797, up-regulated in Cat100-6 and down-regulated in S1587-17, encodes an unknown protein that has a bromo-adjacent homology (BAH) conserved domain containing a protein-protein interaction module specialized in gene silencing. Therefore, it might play an important role in DNA methylation, replication and transcriptional regulation (Callebaut *et al.*, 1999).

The expression profiles of genes involved with sugar metabolism also were affected by Al-stress. Although these genes are thought to have a main role in structural, storage,

and metabolic functions (Ruan *et al.*, 1997), currently it is well known that many of these genes have important roles in signal transduction processes. In plants, sugars not only function as metabolic resources and structural constituents of cells, but also act as important regulators of various processes associated with plant growth and development (Rolland *et al.*, 2002). In this work we can cite for example those genes encoding enolase (CA096567), sucrose synthase (CA102731 and CA097212), hexokinase (CA095925), alcohol dehydrogenase (CA102690), glycerol 3-phosphate permease (G3PP, CA064608), and UDP-glycosyltransferase (CA102772). The last gene was strongly up-regulated by Al in S1587-17 and the enzyme encoded this gene is not involved directly with energy metabolism but with the glycosylation of several molecules such as proteins and lipids, activating or inactivating these compounds (Ciereszko *et al.*, 2001). Probably, rapid alterations in the transcript level of this gene will promptly affect the cellular homeostasis.

Interestingly, some genes involved with sugar metabolism preferentially expressed in conditions of O_2 deprivation, such as enolase and alcohol dehydrogenase, respond to Al-stress. These enzymes are mainly expressed when plants switch their aerobic metabolism to a fermentative one during situations of hypoxia or anoxia. The increased expression of these genes by Al-stress might be an indication that Al affects the O_2 availability into the cell. In fact, Sugimoto *et al.* (2004) suggests that the increase of enolase expression in an Al-tolerant microorganism (*Penicillium chrysogenum*) might be involved with the extra energy supply required by cells under stress.

A gene encoding for the important enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP, CA120042), which is involved with the synthesis of aromatic amino acids, was induced in Cat100-6 up to four times, while in S1587-17 it remained constant (Table 1). The DAHP synthase catalyzes the first committed step in aromatic amino acid

biosynthesis which are precursors of a diverse array of plant secondary metabolites, including lignin, anthocyanic pigments, auxin, and antimicrobial phytoalexins (Keith *et al.*, 1991).

Monodehydroascorbate reductase (CA101237) was another enzyme whose gene was up-regulated by Al in the roots of Cat100-6. Monodehydroascorbate radicals are generated in plant cells enzymatically by the hydrogen peroxide scavenging enzyme, ascorbate peroxidase, and non-enzymatically via the univalent oxidation of ascorbate superoxide, hydroxyl, and various organic radicals. Regeneration of ascorbate, an important antioxidant (Murthy and Zilinskas, 1994), is achieved by monodehydroascorbate redutase (MDAR) using NAP(P)H as an electron donor. Moreover, Lukaszewski and Blevins (1996) reported that Al-stress resulted in a reduction of ascorbate concentration in root apices of squash (*Cucurbita pepo*), indicating that an increase on the transcript levels of MDAR in the root tips of Cat100-6 might be a response to the high levels of ascorbate oxidized by Al.

The CdI19 (CA097428) protein functions as a metallochaperone localized at plasma membrane, which directly binds Cd and increases upon heavy metal stress (Suzuki *et al.*, 2002). Although its expression remained constant in Cat100-6, this gene was up-regulated in S1587-17. Suzuki *et al.* (2002) showed that the overexpression of the CdI19 cDNA conferred Cd tolerance in transgenic *Arabidopsis*, suggesting that this protein plays an important role in the maintenance of heavy metal homeostasis and/or in the detoxification into the cell.

The gene encoding the β-tubulin protein (CA119990) was also up-regulated in S1587-17, while remaining unaltered in Cat100-6. This result might be explained by the fact that the root tip tissue was severely wounded in S1587-17, but not in Cat100-6 (data

not shown), perhaps because the cytoskeleton in Cat100-6 was less affected than in S1587-17. It was previously observed that microtubules play an important role in many cellular processes, such as cell division and elongation. β-tubulins, which are the basic components of microtubules, are encoded by a multigene family in eukaryotes and their nucleotide sequence are highly conserved in protein coding regions. The cytoskeleton in the root cells of maize is especially sensitive to Al (Sivaguru *et al.*, 2000; Sivaguru *et al.*, 1999, Elison *et al.*, 1998). MacDonald *et al.* (1987) reported that Al directly influenced tubulin polymerization *in vitro*, and Blancaflor *et al.* (1998) related Al-inducing root growth inhibition in maize as a function of the stabilization of microtubules in the central elongation zone of the root. According to Schwarzerová *et al.* (2002) the microtubular cytoskeleton is one of the early targets of Al cations.

In spite of the fact that the secretion of organic acid from roots has been cited as a probable mechanism involved with Al tolerance in maize (Jorge and Arruda, 1997), we have not identified genes involved with biosynthesis of organic acids whose expression was significantly altered by Al stress. For instance, ESTs corresponding to genes encoding four enzymes of the TCA cycle (aconitase, isocitrate dehydrogenase, fumarase and malate dehydrogenase) were present in the arrays but their transcript levels were unchanged (data no shown). This result agrees with Xiao *et al.* (2005) that were not able to find Al-induced genes involved with organic acids biosynthesis in wheat macroarrays. In fact, internal root organic acid concentration has no clear correlation with Al tolerance or root organic acids release in maize or in other plant species (revised by Mariano *et al*., 2005).

Gene expression associated to Al tolerance

Based upon the identification of several genes whose expression was modulated by Al-stress and whose functions are so diverse, it is clear that Al stress induces a very complex biochemical response in maize roots, triggering processes involved in defense, signalling and metabolic pathways. The clustering of the expression levels in each Al treatment allowed us to identify another set of genes that might have a closer relation to the tolerance observed in Cat100-6. Genes that have the same expression profiling and the same transcript level in both maize lines certainly cannot explain the differences observed in root growth, although there are post-translation levels of regulation such as translation that might have an important role. Clusters 4, 7, 10, 11 and 14 grouped the genes with the most interesting expression patterns.

Among the genes identified in the macroarrays experiments as induced by Al in both maize lines, five had at least three times more transcripts in the Al-tolerant line than in the sensitive one in the same Al treatment: CA095811 (encoding a chitinase III), CA095754 (xylanase inhibitor protein), CA098848 (ERD protein) and CA102633 and CA101236 (both unknown proteins).

The role of chitinases in plants is considered to be part of their defense mechanism against fungal pathogens that contain chitin as a structural component (Watanabe *et al.*, 1992). In addition, the increase in the transcription of chitinase genes or in chitinase activity may be induced by other external stimuli such as wounding, drought, cold, ozone, heavy metals, salinity and UV light (revised by Kasprzewska, 2003). This enzyme was also recently appointed as involved in Al-stress alleviation in plants of Norway Spruce (*Picea abies*) (Nagy *et al.*, 2004). Chitinases are also thought to play a role in growth and development by catalyzing the hydrolytic cleavage of arabinogalactan proteins (AGPs),

thus affecting cell-to-cell communication (Wiweger *et al.*, 2003). An increase in the level of chitinase transcripts and consequently in the level of the enzymatic activity might be involved with the developmental response of Cat100-6 roots when exposed to Al.

Two xylanase inhibitor genes originated from different assembled sequences (CA095754 and CA095754) were up-regulated by Al in Cat100-6. Xylanases are enzymes that promote the degradation of xylan, the most abundant natural polysaccharide after cellulose (Fierens *et al.*, 2003). Therefore, inhibitors of xylanases play an important role in plant defense by preventing enzymatic hydrolysis by microorganisms and predators (Juge *et al.*, 2004). As observed in lignin metabolism, inhibitors of xylanase genes might contribute to maintain cell wall strength as a response to Al-stress.

Abscisic acid (ABA) is an important plant hormone that helps plants to cope with several environmental stimuli (Zeevaart, 1999). ABA was demonstrated to increase the expression of several genes including genes from the group *ERD* (*e*arly-*r*esponsive to *d*ehydration, CA098848). These genes share similarity with HSP (heat shock proteins) from a variety of organisms and they are early-responsive to dehydration stress (Kiyosue *et al*., 1994). Although this gene was induced in Cat100-6 exposed to Al, its involvement with Al stress is unclear.

The most interesting genes among those induced exclusively in Cat100-6 were CA064742 (encoding an alkaline α-galactosidase), CA064608 (glycerol 3-phosphate permease), CA097462 (LUC7-Like protein), CA096797 (unknown protein), CA064787 (papain-like cysteine peptidase), CA064602 (cinnamyl alcohol dehydrogenase), CA097041 (no hit), CA064605 (Small GTP-binding protein) and CA064763 (phenylalanine ammonia-lyase).

The gene encoding an alkaline α-galactosidase (CA064742) might be involved in the signalization by the hormone methyl-jasmonate (MeJA). A homologue from rice has been implicated in leaf senescence and is supposed to act in the release of linolenic acid, which is the primary substrate for MeJA synthesis (Lee *et al*., 2004). This enzyme is also involved in the initial metabolism of sugars, mainly from raffinose family of oligosaccharides (Carmi *et al*., 2003). The balance and metabolism of these sugars have been associated previously with acquisition of cold and desiccation tolerance (Keller and Pharr, 1996).

Glycerol 3-phosphate permease (CA064608) is a protein that remains uncharacterized in plants but that seems to be important in transport of glycerol 3-phosphate (G3P), a molecule that plays a major role in glycolysis and phospholipid biosynthesis (Lemieux *et al.*, 2004). Glycerol 3-phosphate permeases can provide osmotic protection in yeast due to uptake of glycerol (Holst et al., 2000). Interestingly, the molecule G3P is also involved in the maintenance of membrane lipids integrity during chilling stress, which is also related to dehydration stress (Murata *et al*., 1992; Wolter *et al*., 1992). Together with the expression of other genes potentially related to dehydration, these data raise the feeling that Al might induce genetic responses similar in some stage to the responses trigged by dehydration.

The higher expression of LUC7 (CA097462), a gene involved in RNA splicing, and the papain-like cysteine peptidases (CA064787) suggest that these proteins might module specific RNA and protein targets to increase Cat100-6 responses to Al stress. In fact, papain-like cysteine peptidases have been involved in apoptosis and wounding stress (Funk *et al*., 2002). The putative RNA targets of LUC7 would certainly add an extra component to the complexity of plant responses to Al.

A remarkable feature in plant development is the ability to exhibit a number of adaptative and protective responses to environmental stresses. Two of these genes encoding to phenylalanine ammonia-lyase (PAL, CA064763) and cinnamyl alcohol dehydrogenase (CAD, CA064602), which are important enzymes involved with lignification processes, were strongly up-regulated in the presence of Al. Lignin is a heterogenous aromatic polymer produced in the phenylpropanoid pathway which also supplies intermediates for synthesis of phytoalexins, flavonoids and tannins (Whetten and Sederoff, 1995). Most of these compounds play important roles in the mechanisms of plant defense. CAD is an enzyme involved with catalysis reduction of various phenylpropenyl aldehyde derivatives that are converted to monolignols, the main precursors of lignins and lignans (Kim *et al*., 2004). The activation of the phenylpropanoid metabolism has been reported for various biotic or abiotic stresses such as wounding, pathogen attack, UV irradiation, heavy metals, and drought (Dixon and Paiva, 1995). Even for Al, the enzyme PAL has often been suggested as a constituent of the Al-stress alleviation process in plants (Hamel *et al.*, 1998; Snowden *et al.*, 1995; Snowden and Gardner, 1993). The enzyme PAL is supposed to play a beneficial role in detoxifying Al that has entered the symplasm by sequestration (Hamel *et al.*, 1998).

The small GTP-binding proteins (CA064605) are encoded by a multigene family and play a central role in modulation of cell growth, division, differentiation and morphogenesis, cytoskeletal organization, and vesicle trafficking (Yang and Watson, 1993) and play critical roles as signal transducers (Sano and Ohashi, 1995). The gene encoding a small GTP-binding protein strongly up-regulated in Cat100-6 in response to Al indicates that differences in the perception of Al might favour these plants in the activation of stress responses.

In conclusion, in this work we have increased our understanding of the genetic mechanisms triggered by Al stress in maize, revelling genes involved with several biological process. Most of these genes were up-regulated, showing that Al-stress is more associated with the induction of a complex genetic response in maize roots than with the repression of this response. Furthermore, the cross-species hybridization between maize and sugarcane shown to be effective to study of large-scale gene expression profiling in maize. A major challenge that remains is the evaluation of the real contribution of each responsive gene to the final phenotype of Al tolerance in Cat100-6.

Acknowledgments

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Capítulo IV

Cloning and characterization of an *ALMT1* homologue in maize

Clonagem e caracterização do homologo de *ALMT1* em milho

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Cloning and characterization of an *ALMT1* homologue in maize

Running title: A maize aluminum-induced transporter

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Summary

The toxic effect of aluminum (Al) in the root is one of the most serious problems for agriculture around the world. Exudation of Al-chelating molecules, such as low molecular weight organic acids, is an important Al-tolerance mechanism in several plant species. Therefore, genotypes with improved capacity to transport chelator molecules to rhizosphere will be less affected by Al. Initially, a detailed analysis of organic acid exudation and electrogenic activity in roots of two Al-contrasting maize genotypes was carried out to help understand the response of organic acid exudation and its association with transport activity at the level in these maize lines. Further, we have identified a maize homologue to *ALMT1* from wheat. *ALMT1* was recently reported as a malate transporter induced by Al and the maize homologue of *ALMT1* was named *MAIT* (Maize Aluminum Induced Transporter). The genetic characterization of *MAIT* showed that this gene is present as single copy in the maize genome and its expression profiling in roots of two Al-contrasting maize genotypes showed Al repressed it in both genotypes. The characterization of the MAIT protein in *Xenopus laevis* oocytes indicated that it is a membrane protein with transport activity induced by Al, although the protein does not seem to be a malate or citrate transporter. Although *MAIT* shares several similarities with *ALMT1*, its role in maize remains unclear.

Keywords: Aluminum toxicity; Aluminum tolerance; Root exudates; Transporter protein; Plasma membrane protein; *Zea mays*.

Introduction

Aluminum (Al) toxicity affects agricultural production on a worldwide scale, particularly in tropical latitudes (Rao et al., 1993). The effects of Al are related to acidity, because the low pH increases Al solubilization, consequently reduces crop productivity (Foy et al., 1978). Thus, acid soils limit crop yield in many developing countries where food production is critical (Kochian et al., 2004). The first notable effect of Al is the inhibition of the root elongation as a consequence of root apex disruption (Kochian 1995). Although Al-tolerant plants have been identified in several plant species the genetic and biochemical aspects behind the Al tolerance in plants remain obscure.

It is generally accepted that Al accumulates in root apices, including the root cap and the meristematic and elongation zones, and Al-tolerant genotypes accumulate less Al in root apices than sensitive ones, suggesting that Al exclusion mechanisms may be the key to Al resistance in plants (Collet et al., 2002). So far, several Al exclusion mechanisms have been proposed and the most accepted one is the exudation of Al-chelating organic acids that are thought to detoxify Al in the apical rhizosphere or, probably, in the apoplastic space of the root (Ryan et al., 2001; Matsumoto 2000).

Considerable evidence has been recently presented in the literature indicating that Al-dependent secretion of organic acids in several Al-tolerant plant cultivars is poorly associated with changes in internal organic acid content or with activities of enzymes that synthesize these acids in the root cells (Ryan et al., 1995a; Ryan et al., 1995b; Yang et al., 2001; Hayes and Ma, 2003; Piñeros et al., 2002). Therefore, the bottleneck of Al-tolerance acquisition will be on the ability of some genotypes to efficiently transport organic molecules present into the root cell to the rhizosphere.

As organic acids exist primarily as anions in the cytoplasm, the thermodynamics of organic acid efflux indicates that plasma membrane anion channels may mediate the organic

acid release activated by Al (Piñeros et al., 2002). Recently, Sasaki et al. (2004) have cloned from wheat roots a new class of a putative transport protein involved with malate release. This gene was closely linked to the genetic marker *Alt1* associated with Al tolerance phenotype in wheat (Delhaize et al., 1993). The gene was named *ALMT1* (aluminum-activated malate transporter) and its corresponding protein was recently confirmed as a plasma membrane protein (Yamaguchi et al., 2005). Electrophysiology data obtained from *Xenopus* oocytes synthesizing the ALMT1 protein showed an electrogenic transport activity different from control oocytes and its activity was specifically induced by Al (Sasaki et al., 2004). Further, Delhaize et al. (2004) showed that transgenic plants of barley overexpressing *ALMT1*, significantly increased the rate of root malate exudation and showed increased Al tolerance to the wild plants. However, until now the data are not conclusive about the involvement of this transporter with release of citrate as even other organic acids.

Since Al-tolerance in maize is associated with the rate of citrate release by roots (Jorge and Arruda, 1997; Kollmeier et al., 2001; Mariano and Keltjens, 2003; Pineros et al., 2005), we were concerned about the existence in maize of an analogous mechanism involving a homologue gene of *ALMT1*. In maize this mechanism would be involved with citrate instead of malate, considering that malate exudation does not respond to Al in maize (Jorge and Arruda, 1997; Piñeros et al., 2005). Therefore, in this work we have cloned a homologue of the wheat gene *ALMT1* from root apices of an Al-tolerant maize genotype. Furthermore, a detailed characterization of the maize homologue of *ALMT1* was carried throughout in oocytes to verify its properties as a transporter. Additionally, roots from two maize genotypes contrasting for Al tolerance were evaluated for organic acid exudation and electrogenic activity under Al-stress to help understand the involvement of the *ALMT1* maize homologue with Al-tolerance in maize.

Materials and Methods

Plant material and seedling growth

Five Al-contrasting maize (*Zea mays*) genotypes were used in this work. Two of these lines (Cat100-6 and Al237) were classified as Al-tolerant and three (S1587-17, L53 and B73) as Al-sensitive. Cat100-6 and S1587-17 were supplied by the Universidade Estadual de Campinas (Campinas, Brazil), L53 and Al237 by Embrapa Maize and Sorghum (Sete Lagoas, Brazil) and B73 by USDA-Cornell University (Ithaca, USA).

Seeds were surface sterilized in 0.5% (w/v) NaOCl for 15 min and then germinated in the dark (26^{o}C) for 3 days on filter paper saturated with distilled water. Secondary roots were trimmed and the seminal roots were inserted through polyethylene cups. The seedlings were covered with black polyethylene beads and the cups were placed into holes in the lids of containers that held 8 L of aerated nutrient solution (Magnavaca et al., 1987). The nutrient solution contained the following macronutrients (in millimoles): Ca, 3.53; K, 2.35; Mg, 0.85; N-NH_4, 1.3; N-NO_3, 10.86; P, 0.04; and S, 0.59, and micronutrients (in micromoles): B, 25; Cl, 596; Cu, 0.63; Fe-EDTA, 77; Mo, 0.83; Mn, 9.1; Zn, 2.3; and Na, 1.74. Seedlings were grown for 24 h in a growth chamber at 26^{o}C/23^{o}C (light/dark, 16h/8h) under light intensity of 550 μmol photons m^{-2} s^{-1}.

Excluding the experiments of root electrophysiology, all Al treatment were initiated after a 24-h period by replacing the control growing solution with identical solution that contained Al added as $AlK(SO_4)_2$ $12H_2O$ to the final concentration of 222 μM corresponding to 39 μM of Al^{3+} activity (estimated with GEOCHEM-PC speciation software, Parker et al., 1995). The pH of the control and treatment solutions was adjusted to 4.2 with 0.1 M HCl. Except for the root growth experiment where the seedlings were cultivated in nutrient solution for 5 days, all seedlings were exposed to the Al treatment for 24 or 48 h. In the experiment of gene expression, the Al

concentrations used were 0, 25, 75, 283, and 520 μM of Al, corresponding to 0, 5, 15, 50, and 85 μM of Al^{3+} activity and the period of treatment was 24 h.

For the membrane potential measurements, the maize seedlings were cultivated 24 h in solution of 200 μM of $CaCl_2$, pH 4.5. During the Al or La treatments the control solution was replaced by a solution with 200 μM of $CaCl_2$ plus 150 μM of Al Cl_3 or La Cl_3, pH 4.5.

Root growth measurements

The polyethylene cups containing the seedlings were gently removed from the nutrient solution and the length of the main root was measured with a ruler. Root growth measurements were taken prior to the Al treatment and during the following 5 days at 24-h intervals. The relative root growth (RRG) was calculated as: RRG = (RGR in Al solution/ RGR in control solution) and the RGR (root growth rate, RGR = final root length – initial root length) was expressed in millimeters per day.

Organic acid determination in root tissue and root exudates

Three types of experiments were designed to localize and characterize the Al-activated organic acid content and exudation: *i)* analysis of exudation by whole roots; *ii)* analysis of exudation by the first 10 mm of root tips of intact plants; and *iii)* analysis of organic acid concentration in tissues of 10 mm root tips.

Given the interference of some anions present in the complete nutrient solution, root exudates were collected after cultivation for 6 hours in a simple salt solution consisting of 4.3 mM $CaCl_2$, plus or minus $AlCl_3$ (to the activity of 39 μM of Al^{3+}), and pH adjusted to 4.2 with HCl. This Ca^{2+} concentration corresponded to the total divalent cation concentration in the full nutrient solution.

Root exudate samples were eluted in OnGuard-Ag chromatography column (DIONEX, Sunnyvale, CA) to remove the excess of Cl^-, followed by elution in cationic resin (Sigma-Aldrich

Co.) to remove the excess of cations. Subsequently, the samples were lyophilized and then ressuspended in 1 mL of background electrolyte solution consisted of 0.5 mM dodecyltrimetylammonium bromide, 7.5 mM salicylic acid, and 15 mM Tris, pH 9.5. Root tissue organic acids were extracted by homogenizing the first centimeter of 6 root apices in 500 μL of distilled and deionized water. Samples were centrifuged for 2 min at 14,000 RPM (Eppendorf Microcentrifuge 5415 R).

Organic acids in root exudates and root homogenates were analyzed with a capillary electrophoresis (CE) system (P/ACE 5510; Beckman Instruments, Fullerton, CA) interfaced via PACE 1.2.1 software (Beckman Instruments). The background electrolyte used for separation was similar to the described above. Organic acids were separated in a 67-cm capillary (75 μm I.D.) with a constant separation voltage of –28.5 KV at 25^{o}C. Prior to use the capillaries were pretreated by flushing with 0.1 N HCl for 5 min, followed by another 5 min of flushing with 0.1 N NaOH. Peaks were detected with a UV absorbance detector at a wavelength of 232 nm, and were first identified on the basis of their migration time, with subsequent confirmation by spiking samples with organic acids standards (Sigma-Aldrich Co.).

Membrane potential in maize roots

The seminal root of an intact maize plant was placed in a measuring chamber and gently fixed horizontally with small acrylic blocks and silicon grease. The root was allowed to stabilize for 5-10 min while approximately 4 mL/min of control solution (200 μM $CaCl_2$, pH 4.2) flowed through the chamber. Measurements of the electrical potential difference (V_m) across the root-cell membranes were recorded at two positions along the root. One position was directly on the root apex and the other position was 10 mm further back from the root apex (elongation zone). Borosilicate glass microelectrodes (Clark Electrochemical Instruments, Reading, UK) were filled with 3 M KCl, pH 2.0 and connected to an electrometer (FD 223, World Precision Instruments, Saratosa, FL, USA) via an Ag-AgCl half-cell and inserted into the root tissue with the help of a

hydraulic micromanipulator (Narishige, Tokyo, Japan). V_m was recorded continually with an amplifier and a data acquisition system (Digidata 1320A; Axon Instruments). The solution flow-rate across the chamber was maintained constant during the recording. Once a stable measurement of V_m was obtained, the control solution was replaced by an identical solution containing 150 μM of $AlCl_3$ or $LaCl_3$ with the same flow-rate. Measurements of at least 12 roots of different seedlings were made for each treatment.

ALMT1 maize homologue isolation, cloning and identification

Total RNA was extracted from root apices (10 mm) of Cat100-6 using the Guanidine-HCl protocol (Logemann et al., 1987). The total RNA was treated with DNase I (RNase-free, Invitrogen) and used to generate the first cDNA strand in a reverse transcriptase reaction with Superscript III (Invitrogen) at 50 ^{o}C for 40 min with Oligo$(dT)_{12-18}$ primer (Invitrogen). From the first strand of cDNA the *ALMT1* maize homologue was amplified using primers designed for the sequence of the *ALMT1-1* cDNA (accession AB081803) and the maize genomic sequence MAGI-92675 (http://maize.ece.iastate.edu), previously identified as homologue to *ALMT1*. The full-length sequence of the *ALMT1* maize homologue was amplified with 1 μL of Titanium Taq DNA polymerase (Clontech), 10x PCR buffer (40mM Tricine-KOH, pH 8.0, 16 mM KCl, 3.5 mM $MgCl_2$, 3.75 μg/mL BSA), 0.2 mM of each dNTPs, 10 μM of each of the specific oligonucleotide primers: forward primer 5'-ATGGAGATTGATGAGATGGAG-3' and reverse primer 5'-CTAGCTTTCTCAGCTCACAGC-3'. The Touch-down PCR reaction started with the predenaturation of the template at 95 °C for 2 min. Then, in the first 10 cycles, denaturing at 95 °C for 30 s and extension at 68 °C for 2 min, whereas annealing was undertaken for 30 s with a successive lowering of temperature from 65 °C to 55 °C at a ramp of 1 °C/cycle. In the subsequent 30 cycles, all parameters were kept unchanged, except for the annealing temperature that was kept at 55 °C. A final extension step at 68 °C for 10 min was included. The products obtained were electrophoresed in 1% (w/v) agarose gel and a 1400-bp fragment was

eluted, purified, cloned into pCRII-TOPO (Invitrogen), and fully sequenced. The nucleotide and theoretical amino acid sequences of the *ALMT1* maize homologue (named *MAIT*) were aligned with the *ALMT1-1* sequences using Matrix Blossum 62 (Henikoff and Henikoff, 1992) to measure the similarity. The *MAIT* sequence was deposited in the GenBank (accession DQ358745).

Southern blot analysis

Genomic DNA was extracted from shoots of four maize lines, two Al-tolerant (Cat100-6 and Al237) and two Al-sensitive (L53 and B73), by the CTAB protocol (Hoisington et al., 1994). Aliquots of 20 µg of genomic DNA were digested with *BamH* I, *EcoR* I, and *Hind* III and separated in 0.8% (w/v) agarose gel. The digested DNA was transferred to a nylon-filter (Hybond-XL, GE Healthcare) according to manufacture's instructions and then cross-linked on the filter by baking at 90 ^{o}C for 2 h. The probe was synthesized from the full sequence of *MAIT* and radioactively labeled with [α-^{32}P]dCTP, using the DNA labeling beads Ready-To-Go^{TM} (GE-Healthcare). The hybridization was carried out overnight at 65 ^{o}C in a solution of 5x SSC, 5x Denhardt's solution, 50 mM Sodium Phosphate pH 6.8, 1% SDS, and 100 µg mL^{-1} denatured salmon sperm DNA. The membrane was washed twice with 2x SSC and 0.1% SDS at room temperature for 15 min and twice with 0.1x SSC and 0.1% SDS at 65 ^{o}C for 30 min. Finally, the membrane was exposed to image plates for 24 h, and then scanned in a phosphorimager Storm Image System (GE-Healthcare).

Sub-cellular localization and classification of MAIT

We constructed plasmids for transient expression of *MAIT* tagged with *GFP* (green fluorescent protein). In the construction of plasmid pCAMBIA1302-*MAIT*::*GFP*, the coding frame of *MAIT* was amplified by PCR using the primer forward 5'-AGACTAGTATGGATATTGATCACGGCAG-3' and the primer reverse 5'-

TTACTAGTCAGATCTACCATCAAAATAACCACGTCAGGCAAAGG-3', to include the adaptors to the restriction site of *Spe*I (nucleotides underlined) and elimination of the stop codon from the *MAIT* sequence. After amplification, the *MAIT* fragment was cloned into the vector pCRII-TOPO (Invitrogen), digested with *Spe*I and linked in frame to the 5' end the *mgfp5* gene (Siemering *et al.*, 1996) present in the binary vector pCAMBIA-1302 (accession AF234298, http://www.cambia.org) previously digested with *Spe*I. The construction was fully sequenced to check the accuracy of *MAIT* sequence and to verify if *MAIT* was correctly in frame with *mgfp5*. The *MAIT*::*GFP* gene was driven by the cauliflower mosaic virus (CaMV) 35S promoter. The pCAMBIA-1302 vector was used as positive control.

A helium biolistic gene transformation system (EMBRAPA-CENARGEN, Brazil) was used to transiently transform onion (*Allium cepa*) epidermal cells with the *MAIT*::*GFP* fusion construct. Five micrograms of plasmid DNA purified on a column (Qiagen, USA) were precipitated onto 1.6 μm gold particles (Bio-Rad, USA) using 2.5 M $CaCl_2$ and 1 M spermidine (Sigma, USA). DNA-coated particles were rinsed with absolute ethanol, re-suspended in ethanol, and used to bombard the onion cells at 1,300 psi. The bombarded tissue was incubated in the dark at room temperature for 24 h until analysis.

Onion cells were also transformed with pCAMBIA-1302 vector, used as a positive control. The fluorescence photographs of onion cells were taken using a Nikon Eclipse 50i microscope equipped with a Nikon Digital camera DXM 1200F and a photodocumentation Nikon ACT-1 system. The light source was provided by a 50 W super high-pressure mercury lamp power supply.

The classification and prediction of the secondary structure of MAIT theoretical amino acid sequence was carried-out with the software SOSUI system (version 1.0/10, http://sosui.proteome.bio.tuat.ac.jp/) described by Hirokawa et al. (1998).

Semi-quantitative RT-PCR

Seedlings of Cat100-6 and S1587-17 were initially grown for 24 h in complete nutrient solution (Magnavaca et al., 1987), then transferred to identical solution with doses of 0, 25, 75, 283, and 520 μM of $AlK(SO_4)_2$ $12H_2O$, corresponding to 0, 5, 15, 50, and 85 μM of Al^{3+} activity. After 24 h of treatment the total RNA was extracted from 20 root apices with 10 mm of length using Guanidine-HCl method (Logemann et al., 1987). The samples were treated with DNAse I RNAse-free (Invitrogen) at room temperature for 30 min and 1μg of total RNA was used to synthesize of the first strand of cDNA. The reverse transcription reaction was carried out with SuperScript III (Invitrogen) at 50 ^oC for 40 min. Further, the samples were treated with RNAse H (Invitrogen) at 37 ^oC for 30 min.

The detection of *MAIT* was performed by PCR using two internal primers that amplified a fragment of 250-bp. The sequence of the forward primer was 5'-TGACCATCTTCATCCTCACCTT-3' and of the reverse primer 5'-GAACGTGGCGTTCTCTCT-3'. To each sample, 2 μL of the cDNA reaction was added to a PCR mixture containing 1 μL of Titanium Taq DNA polymerase (Clontech), 10x PCR buffer (40mM Tricine-KOH, pH 8.0, 16 mM KCl, 3.5 mM $MgCl_2$, 3.75 μg/mL BSA), 0.2 mM of each dNTPs, 20 μM of each specific oligonucleotide primer, and 4% DMSO. PCR amplification was set at 95 ^oC for 2 min followed by 45 cycles of denaturation at 95 ^oC for 30 s, annealing at 55 ^oC for 30 s and extension at 68 ^oC for 1 min. A final extension step at 68 ^oC for 5 min was included. The number of PCR cycles was optimized to avoid the saturated phase of PCR reaction. The amount of cDNA applied to each sample was monitored by the amplification of a *ribosomal 18S* gene whose expression was verified to be unaltered by Al stress. The optimal number of PCR cycles to amplify *ribosomal 18S* gene before saturation was 15.

Electrophysiology in oocytes

The *MAIT* gene was cloned into the translation plasmid T7TS (Krieg and Melton, 1984) which contains the 5' and 3' untranslated regions of the β-globin mRNA of *Xenopus laevis.* From the linearized construct, cRNA was transcribed *in vitro* with T7 RNA polymerase (Stratagene) according to the manufacture's instructions. Further, the cRNA was divided in aliquots of 1.5 µL and stored at –80 ^{o}C until injection.

The oocytes were extracted from *Xenopus laevis* as described in (Iverson et al., 1988). The oocytes were incubated in dark at room temperature during 1 h in 20 mL of OR-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) plus 20% (w/v) Collagenase-A (Sigma-Aldrich) with soft agitation. Further, the oocytes were washed twice in OR-2 solution and four times in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5). Then the oocytes were incubated in ND96 solution plus 2.5 mM Na-pyruvate, 50 µg mL^{-1} gentamycin, and 400 µg mL^{-1} of BSA in dark at 22^{o} C.

After 24 h of incubation, 50 ηL of *MAIT* cRNA were injected per oocytes, and an equal volume of water was injected to control oocytes. The oocytes were then incubated for 48 h in the same conditions described above to allow the translation of the MAIT cRNA. Two hours before the beginning of the electrophysiological measurements, 50 ηL of 100 mM of malate, 100 mM of citrate or water, pH 7.5, were injected into individual oocytes according to each treatment.

The two-electrode voltage clamp system was used to measure net currents across the oocyte membrane at different membrane voltages. The electrical potential difference across the membrane was clamped from –160 mV to +80 mV in 10-mV steps. The oocytes were first analyzed in a simple version of ND96 composed by 96 mM of NaCl, 1 mM of KCl and 0.8 mM $CaCl_2$, pH 4.5. The current recorded during the voltage stimuli was used to generate Voltage-Current Charts. After the current was stable, the control solution was replaced by a solution of

similar composition containing 100 μM of $AlCl_3$. In addition, a solution with 100 μM of $LaCl_3$ was used to check for specificity of Al response.

Finally, to check if the activity of MAIT was influenced by the presence of Cl^- ions present in the solution, the NaCl salt from simple ND96 solution was replaced by the same concentration of MES (2-morpholinoethanesulfonic acid monohydrate, Sigma-Aldrich) plus the addition of 100 μM of $LaCl_3$.

Results

Root growth of Cat100-6 and S1587-17

The root growth of Cat100-6, the Al-tolerant genotype, was almost not affected in the first day of cultivation in nutrient solution with 39 μM of Al^{3+} activity. However, in the same period and Al concentration, S1587-17, the Al-sensitive genotype, had about 40% of root growth reduction compared to its control (Fig 1A). In the following days, Cat100-6 showed a root growth reduction of around 20% that remained constant for the next four days. However, S1587-17 showed a strong inhibition of the root growth that culminated with complete paralyzation of root development at the end of the fifth day (Fig. 1A). Figure 1B shows that the roots of S1587-17 seedlings were severely damaged by 39 μM of Al^{3+} activity.

Organic acid concentration in root tissues and in root exudates

Due to the increase in sensitivity achieved by using capillary electrophoresis (CE) in organic acid determination (Piñeros et al., 2002), we decided to use this methodology to identify and quantify the organic acids present in root tissues and root exudates of Cat100-6 and S1587-17.

Initially the cellular concentration of organic acids was evaluated in root apices from Cat100-6 and S1587-17 seedlings. After 24 h of treatment in solution with 39 μM of Al^{3+} activity an increased concentration of citrate was observed to Cat100-6 and S1587-17, compared to seedlings cultivated in solution without Al for equivalent period (Fig. 2A).

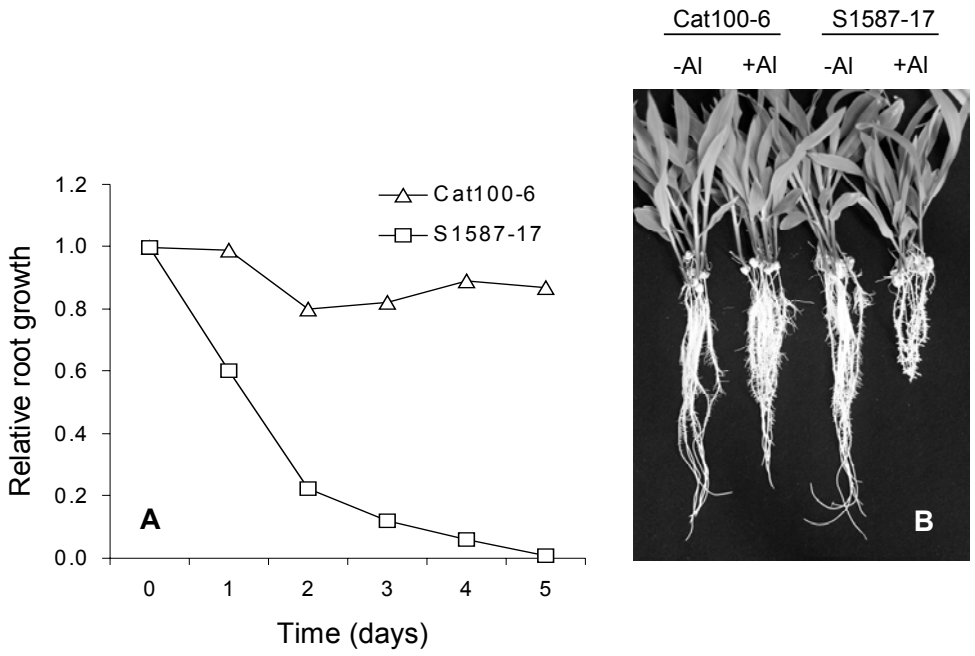

Figure 1. Effect of Al on root growth of Cat100-6 and S1587-17. A) Relative root growth of seedlings cultivated with or without 39 μM of Al^{3+} activity for 5 days, *n*=20; and B) Visual aspect of maize seedlings after 5 days of cultivation in control solution (-Al) or in solution with 39 μM of Al^{3+} activity (+Al).

The presence of Al increased the citrate content nearly four-fold (Fig. 2A), independently of the genotype. No difference was observed in the citrate concentration of root tissues between Cat100-6 and S1587-17 after 24 h of exposition neither without nor with Al (Fig. 2A). However, after 48 h in presence of Al, a reduced concentration of citrate in root apices of S1587-17 was observed when compared to Cat100-6 (Fig. 2A). It is worth to mention that after 24 h of Al exposure the root apices of S1587-17 were severely damaged by Al (supplementary data, Fig. 3) and therefore, the reduced concentration of citrate after 48 h probably reflects the loss of cellular content. In contrast to this result, the Al content in root tissues and the intensity of hematoxylin staining in root apices of S1587-17 were higher than in Cat100-6 (supplementary data, Fig. 4).

The exudation of citrate from the whole root revealed a much more interesting profile between genotypes and between Al levels. The amount of citrate exuded was almost not detectable in the treatments where Al was absent and several times larger when the roots were cultivated in solution with 39 μM of Al^{3+} activity (Fig 2B) in agreement with previous results of organic acid evaluation in maize (Jorge and Arruda, 1997; Piñeros et al., 2001; Piñeros et al., 2005). The differences between the roots of Cat100-6 and S1587-17 cultivated under Al stress were also notable because Cat100-6 exuded almost twice the amount of citrate exuded by S1587-17 after 24 and 48 h of Al exposition (Fig. 2B).

Since the root apex is believed to be the first target of Al, the mechanism of Al-stress alleviation is expected to be more active in this root region. Therefore we were interested in the evaluation of organic acid exudation only from root apices. A method was developed to collect the exudates only from the first 10 mm of roots from intact seedlings to avoid the wounding effect observed in excised roots (Collet et al., 2002). As expected, the exudation profile of citrate showed the same tendency observed with the whole root, although with lower rates of exudation observed with the apices. Once more it was observed a higher rate of citrate exudation in roots

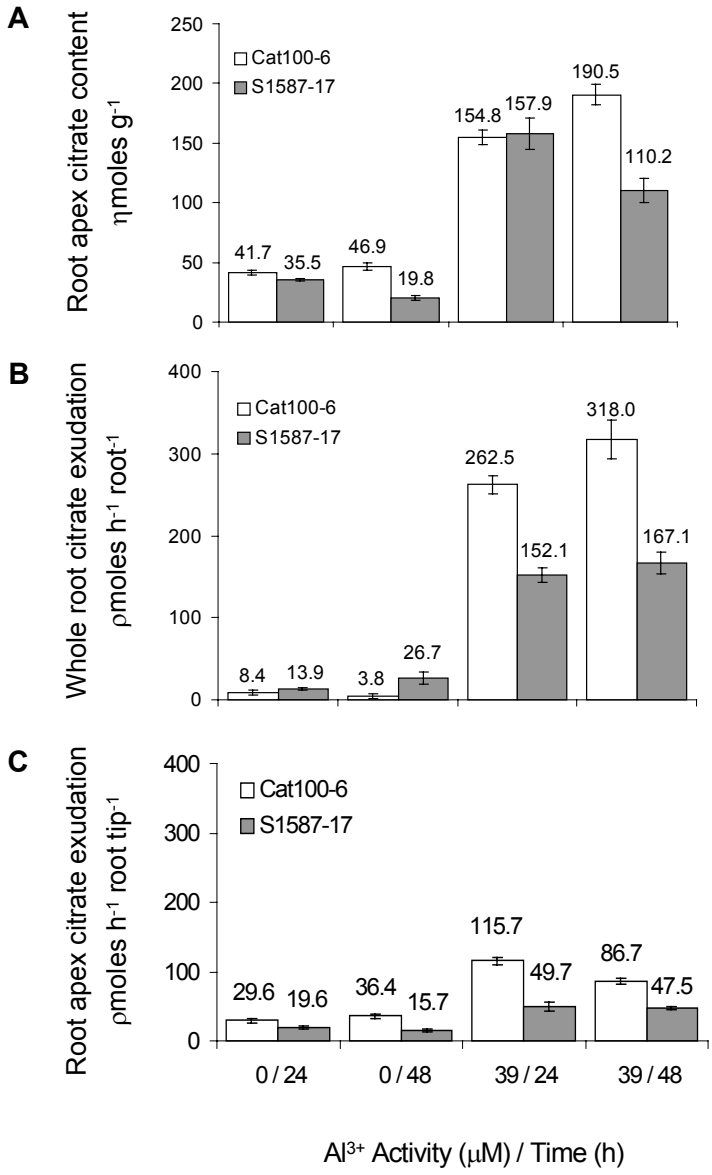

Figure 2. Citrate concentration in root tissues and citrate exudation of Cat100-6 and S1587-17. Seedlings were evaluated after 24 and 48 h of treatment in nutrient solution with and without 39 μM of Al^{3+} activity. The samples were collected during a period of 6 h of treatment. A) Citrate concentration in tissues from the first 10 mm of root apices; B) Citrate exudation by whole roots; and C) Citrate exudation by the first 10 mm of root apices. Bars correspond to mean ±SE, *n*=6.

subjected to Al stress (Fig. 2C) with Cat100-6 releasing at least twice more citrate than S1587-17 when exposed to Al (Fig. 2C).

A small increase of citrate concentration exuded by root apices when compared to whole roots was observed in the treatments without Al (Fig. 2C), which might be explained by the stress promoted during the experimental set up.

Another advantage of CE over the enzymatic procedure was the possibility of simultaneous detection of several compounds, such as malate and phosphate, on the same sample. However, the presence of these compounds in the root tissue content or in the root exudation showed no association to Al stress. The concentrations of malate and other organic acids detected were much smaller than the levels of citrate observed for all treatments (data not shown), confirming that citrate was the only compound whose exudation by roots of Cat100-6 responded to Al.

Resting membrane potential in Cat100-6 and S1587-17 roots

Since the efflux of organic acids through the plasma membrane is mediated by channels or transport proteins (Piñeros and Kochian, 2001), we were interested in the effects of Al on the resting membrane potential (RMP) of root cells. Given that the plasma membrane voltage varies as a function of flux of charged molecules across it, the measurement of membrane voltage alterations could provide an indirect but efficient insight about the effects of Al in the transport activity of root cells from Cat100-6 and S1587-17.

Although a depolarization was observed in root cells of both genotypes, this effect was larger and faster in Cat100-6 than in S1587-17 (supplementary data, Fig. 5). The average depolarization in cells of the root apex after exposition to Al is shown in the Figure 3A. The voltage directly recorded in cells from the root apex of Cat100-6 in the presence of Al was about 50% of the voltage recorded when Al was not present in the solution (Fig. 3A). In S1587-17 the voltage reduction observed in cells from the root apex under Al stress was smaller than 10% of

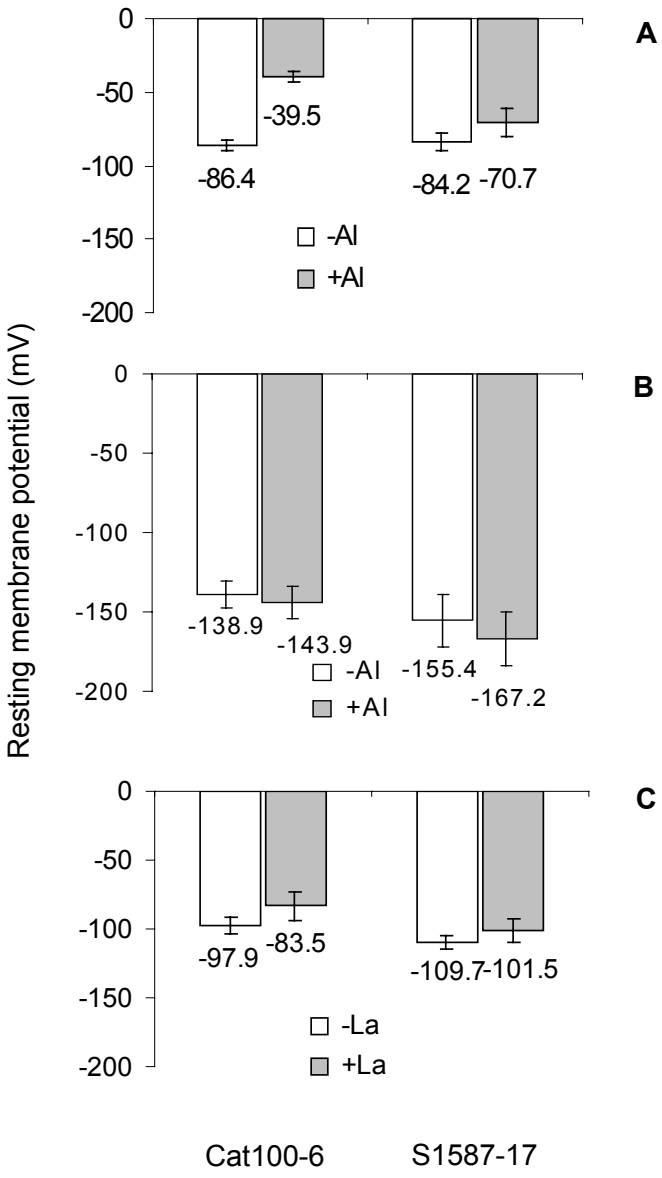

Figure 3. Resting membrane potential (RMP) of root cells from Cat100-6 and S1587-17. Effect of 150 μM of $AlCl_3$ on the RMP of cells from the root apex (A); at 10 mm back from the root apex (B); and effect of 150 μM of $LaCl_3$ on the RMP of cells from the root apex (C). The bars correspond to average ±SE, *n*=18 (A), *n* =22 (B), and *n* =12 (C).

cells not exposed to Al (Fig. 3A). When the voltage was recorded in cells from 10 mm back of the root apex, the differences observed between the presence and absence of Al were not detected any more, as well as the differences between Cat100-6 and S1587-17 (Fig. 3B).

Curiously, the voltage recorded in cells from 10 mm back from the root apex was two times higher than the voltage recorded in cells from the root apex (Fig. 3B). Possibly this effect was due the large volume of the vacuole in these cells, and consequently due the high number of transporters and channels in the tonoplast membrane. Further, to test if Al specifically induced the variations observed in the RMP of root apex or if it was an effect of the Cl^- ions supplied by the $AlCl_3$ salt, we repeated the experiment using $LaCl_3$ instead of $AlCl_3$, since this salt added the same amount of Cl^- ions to solution and because Lanthanum (La), similar to Al, is a metal with valence +3. The Figure 3C shows that the addition of La^{3+} was responsible by a small depolarization effect in Cat100-6 as well as in S1587-17, but in Cat100-6, the La effect was smaller than the effect observed in response to Al.

Cloning and characterization of the* ALMT1 *homologue in Cat100-6

The full-length *ALMT1* homogue in maize was cloned by RT-PCR from total RNA extracted of root apices of Cat100-6. The primers used to amplify the *ALMT1* maize homologue were designed using as template the *ALMT1-1* cDNA sequence (accession AB081803) and a genomic sequence (MAGI-92675) with identity to *ALMT1-1* cDNA. After reverse transcription, the first strand of cDNA was amplified and then a product with similar size of *ALMT1* sequence was purified and cloned. The sequencing of this gene showed that the *ALMT1* homologue in maize had 1,352 bp, 32 nucleotides smaller than the wheat *ALMT1*. This cDNA showed 79% nucleotide identity with *ALMT1-1*, while a 67% of identity at amino acid level was observed (Fig. 4). This *ALMT1* homologue in maize was named *MAIT* (Maize Aluminum-Induced Transporter) due to the electrophysiology results obtained from its corresponding protein in oocytes of *X. laevis* (see below).

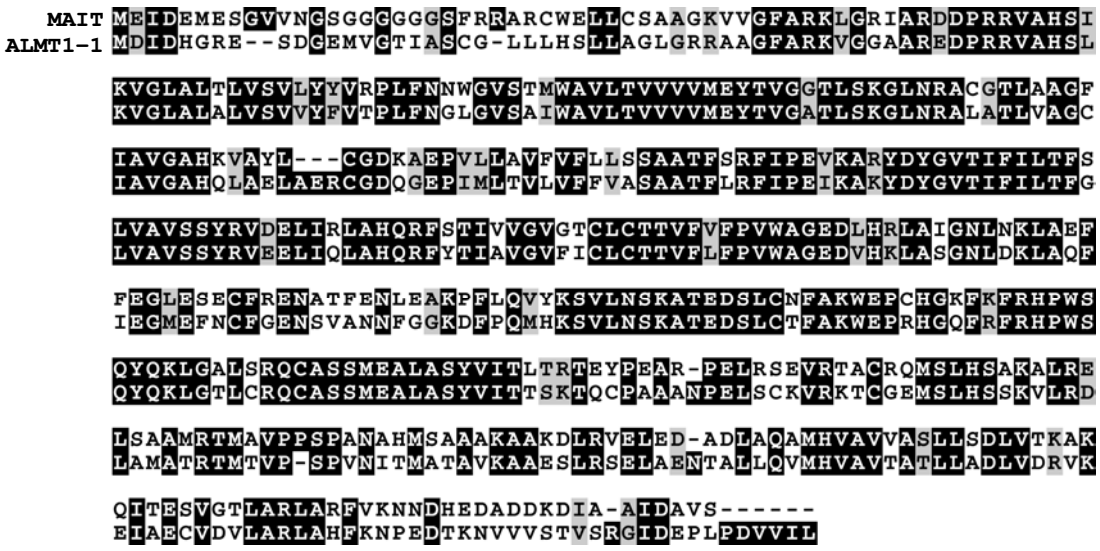

Figure 4. Alignment between the predict amino acid sequences of MAIT and ALMT1-1 (accession AB081803). The sequences presented 67% of identity at amino acid level (Blosum 62). Identical residues are shaded in black while residues with similar properties are shaded in gray. The broken lines represent gaps between the sequences of MAIT and ALMT1-1.

Secondary structure prediction and sub-cellular localization of MAIT

The classification and secondary structure prediction of MAIT was carried out using the SOSUI algorithm (Hirokawa et al. 1998; http://sosui.proteome.bio.tuat.ac.jp). Similarly to ALMT1, several transmembrane domains were identified in MAIT (supplementary data, Fig. 6). Consequently, MAIT was classified as a transmembrane protein. Other bioinformatic tools used in secondary structure prediction were applied and also identified transmembrane domains in MAIT.

Further, the *MAIT* gene was fusioned to the gene of a green fluorescent protein (GFP) into the pCAMBIA-1302 vector (CSIRO, Australia). This construct was used to transiently express the *MAIT::GFP* fusion in onion cells from epithelial tissue. The profile of fluorescence emitted by the MAIT attached to GFP was different from the cells transformed only with the pCAMBIA-1302 vector (positive control, Fig. 5). Onion cells expressing *MAIT::GFP* emitted fluorescence only on the cell edge close to the cell wall, typical of membrane proteins, while cells transformed only with pCAMBIA-1302 vector emitted fluorescence homogeneously around the cytoplasm (Fig. 5).

Genomic organization of* MAIT *gene in Al-contrasting maize genotypes

The number of copies and the existence of polymorphism among four Al-contrasting maize lines (Al-tolerant: Cat100-6 and Al237; Al-sensitive: L53 and B73) were evaluated using the cDNA of *MAIT* as probe in Southern blot analysis (Fig. 6A). The band profile observed in the genomic DNA of Cat100-6 and Al237 digested with the restriction enzyme *EcoR* I and *Hind* III, indicates that *MAIT* might be a single copy gene in maize. The polymorphism observed to *EcoR* I and *Hind* III digestion separated the genotypes into two groups similar to the Al tolerance classification based on root growth.

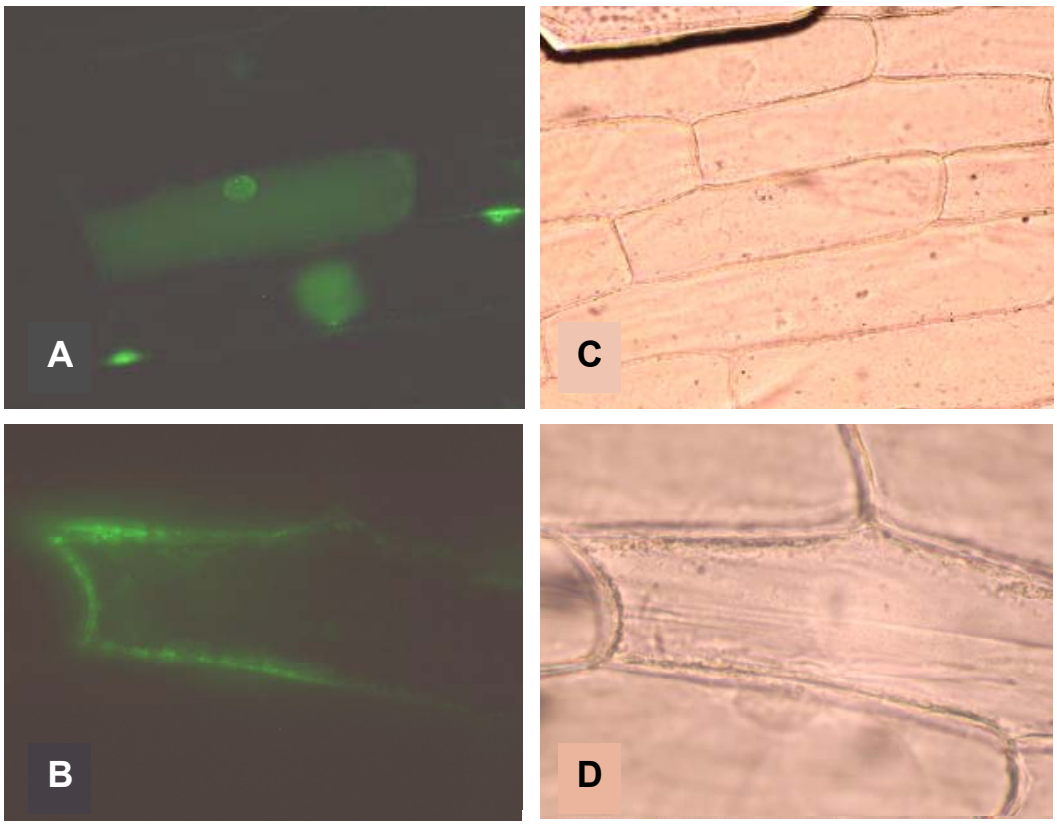

Figure 5. Sub-cellular localization of MAIT protein in epithelial onion cells. Cell expressing GFP under UV light (A); cell expressing GFP under white light (B); cell expressing MAIT::GFP under UV light (C); and cell expressing MAIT::GFP under white light (D).

The genomic DNA from Al-sensitive genotypes L53 and B73 digested with *EcoR* I and *Hind* III generated two bands while the genomic DNA of Al-tolerant Ca100-6 and Al237 digested with the same enzymes produced only a band (Fig. 6A). *BamH* I showed an identical restriction profile to the four genotypes (Fig. 6A).

Expression of* MAIT *in root tips of Cat100-6 and S1587-17

Because the primary target of Al toxicity is the root apex, the expression of *MAIT* was evaluated by semi-quantitative RT-PCR in this root region. The reason to use semi-quantitative RT-PCR was because Northern blot hybridizations failed to show any expression of the *MAIT* gene in roots and shoots (data not shown). Since this gene was cloned from total RNA of root apices of Cat100-6, its expression was expected to be detected at least in root apices of this genotype by RT-PCR. Therefore, to detect the expression of *MAIT*, two internal primers that amplified a *MAIT* fragment around 250 bp were used. To visualize the *MAIT* expression in root apices of Cat100-6 and S1587-17 subjected to different Al doses, 45 cycles of PCR were required prior to signal saturation (Fig. 6B).

Unexpectedly, the results showed that Al inhibited *MAIT* expression in both maize genotypes (Fig. 6B). When the Al concentration was increased to 283 and 520 μM of Al (corresponding to 50 and 85 μM of Al^{3+} activity, respectively), *MAIT* expression was almost undetected (Fig. 6B). On the other hand, the expression of *ribosomal 18S* gene, used as control to the amount of cDNA added to each reaction remained unaltered across all Al treatments in root apices of Cat100-6 and S1587-17 (Fig. 6B).

Electrophysiological measurements of the MAIT-mediated currents in* Xenopus laevis *oocytes

We investigated the activity of the MAIT protein using a heterologous approach with *X. laevis* oocytes. The two-electrode voltage clamp method was used to measure the net currents

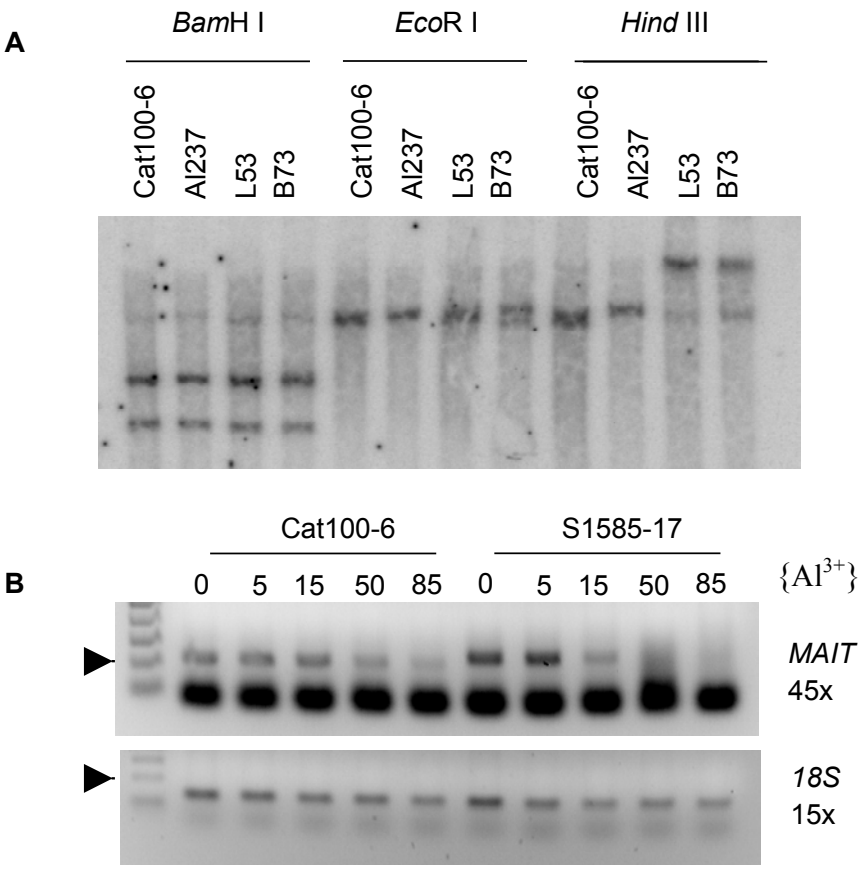

Figure 6. Genetic analysis of *MAIT* gene. A) Identification of polymorphism associated with *MAIT* in two Al-tolerant maize lines (Cat100-6 and Al237) and two Al-sensitive maize lines (L53 and B73). Genomic DNA was digested with the restriction enzymes *BamH* I, *EcoR* I, and *Hind* III. The nylon membrane was hybridized with a radioactive probe synthesized from the full sequence of *MAIT*; B) Expression profile of *MAIT* in root apices of Cat100-6 and S1587-17 exposed for 24 h to 0, 5, 15, 50, and 85 μM of Al^{3+} activity. The semi-quantitative RT-PCR was carried-out with 45 cycles of amplification of *MAIT* and 15 cycles to *ribosomal 18S*. *MAIT* amplification was done with two internal primers that amplified a fragment of 250 bp and *ribosomal 18S* was amplified with two internal primers that generated a fragment of 200 bp. The *ribosomal 18S* gene was used as a control of the amount of cDNA used in each amplification reaction. The experiment was replicated two times and the arrows indicate a 200 bp molecular mark. The bands on the bottom of *MAIT* and *18S* semi-quantitative RT-PCR are primers.

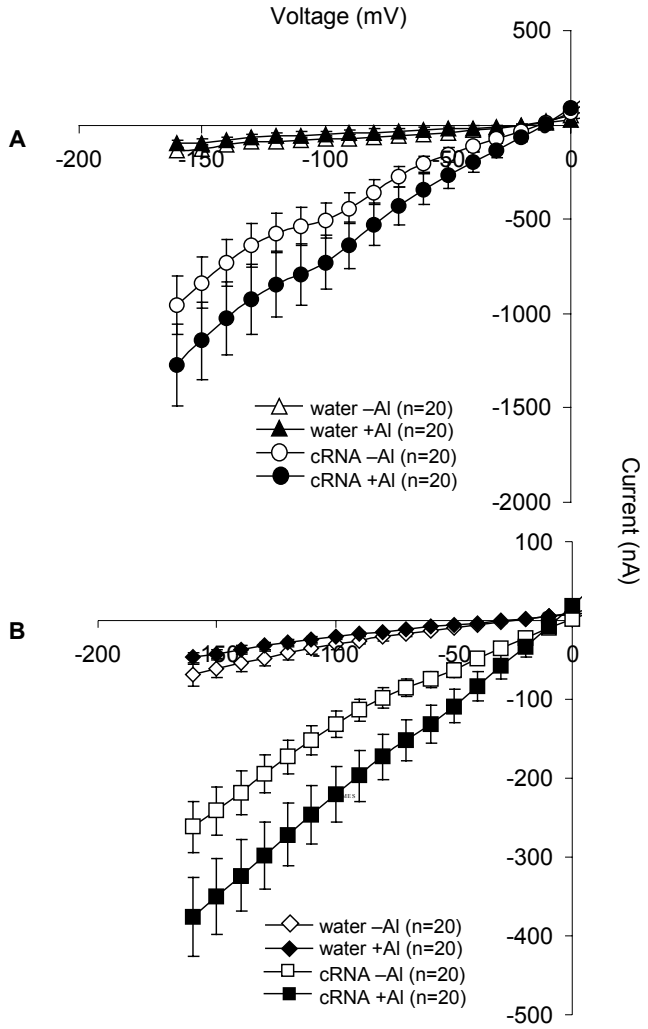

Figure 7. Current-voltage curves collected in oocytes of *X. laevis* injected with *MAIT* cRNA or water (negative control) evaluated in simple ND96 solution (A) and in MES + $LaCl_3$ solution (B). The current-voltage curves were initially collected from oocytes immersed in control solution: water injected oocytes in simple ND96 solution (white triangles); *MAIT* cRNA injected oocytes in simple ND96 solution (white circles); water injected oocytes in MES + $LaCl_3$ solution (white diamonds); and *MAIT* cRNA injected oocytes in MES + $LaCl_3$ solution (white squares). Further the oocytes were immersed in a solution with 100 μM $AlCl_3$: water injected oocytes in simple ND96 solution (dark triangles); *MAIT* cRNA injected oocytes in simple ND96 solution (dark circles); water injected oocytes in MES + $LaCl_3$ solution (dark diamonds); and *MAIT* cRNA injected oocytes in MES + $LaCl_3$ solution (dark squares). The symbols represent the mean ±SE.

in membranes of oocytes injected with *MAIT* cRNA and subjected to different treatments. Addition of $AlCl_3$ to the bathing solution (simple ND96), activated an inward current (consistent with anion efflux) in oocytes injected with the *MAIT* cRNA but not in control oocytes injected with water (Fig 7A). Interestingly, the injection of 1.5 ηL of 100 μM malate solution seems to have a small influence on the current recorded in the oocytes previously injected with *MAIT* (supplementary data, Fig. 7A). In addition, the injection of equal amount of 100 μM citrate solution had no effect on MAIT activity. In contrast to the absence or masking of citrate effect in oocytes expressing *MAIT*, an increase of endogenous negative currents was observed in control oocytes, indicating that citrate injection altered the electrogenic activity of control oocytes (data not shown). Since the current activities in control oocytes were altered by citrate injection, it is reasonable to think that the endogenous currents of the oocytes might negatively affect the recording of the currents promoted by MAIT.

We used La, a trivalent cation, to check if it was able to mimic the effects promoted by Al. La did not activate a similar Al response in oocytes injected with *MAIT* cRNA. In fact, La seems to have a blocking effect instead of activation effect on MAIT activity. These results showed that the MAIT activity is activated by Al but not by La.

To verify if the current produced by MAIT was originated from the influx of the large amount of Cl^- present in the simple ND96 solution, similar experiments were done replacing NaCl by 2-Morpholinoethanesulfonic acid (MES), an organic compound without Cl^- ions, and containing 100 μM of $LaCl_3$, used as a blocking agent of endogenous currents. The results were quite similar to the results obtained in simple ND96 solutions and once more the addition of 100 μM of $AlCl_3$ was responsible for activation of MAIT, although the currents recorded were around one third of the currents observed with simple ND96 solution (Fig. 7B). This indicates that the absence of large amounts of Cl^- ions or the presence of small amount of La, or both, were responsible for the reduction the net current across the plasma membrane. Similarly to the oocytes injected with *MAIT* cRNA and evaluated in simple ND96 solution, the oocytes injected

with malate evaluated in MES + La solution showed no increase in the activity of MAIT after the addition of Al (supplementary data, Fig. 7B). The citrate injection once more produced heterogeneous data even in control oocytes evaluated in MES + La solution (data not shown).

Discussion

Characterization of Al-tolerance in Cat100-6 and S1587-17

Before starting the cloning of *ALMT1* homologue in Cat100-6, a complete characterization of Cat100-6 and an Al-sensitive genotype, S1587-17, was carried out to achieve three objectives: *i*) to be sure that the maize genotypes used in this work were Al-contrasting to the experimental conditions used; *ii*) to identify the main organic acids associated with Al-tolerance in Cat100-6. *iii*) To analyze the effects of Al in the electrogenic activity of cells from roots of two Al-contrasting maize genotypes. This initial study was important to provide information about the putative association of organic acid exudation, root transport activity, and Al-tolerance behavior of the maize genotype used to clone the *ALMT1* homologue. The line S1587-15 was used because of its Al-sensitivity and because this line was generated from Cat100-6 (Moon et al., 1997). The results pointed toward the existence of an association between organic acid exudation and electrogenic activity at root level.

After the efficacy of the nutrient solution was confirmed to phenotypically discriminate Cat100-6 and S1587-17, new experiments were carried out to verify qualitatively and quantitatively the association of Al tolerance with the organic acid content and exudation in roots of Cat100-6 and S1587-17. For this proposes three different experiments were carried out to evaluate the organic acid concentration in root tissues and root exudates.

Although the CE analysis allowed the detection of several compounds, only citrate showed association with Al tolerance, in agreement with previous results obtained with maize (Pellet et al., 1995; Jorge and Arruda, 1997; Piñeros et al, 2002; Mariano and Keltjens, 2003; Piñeros et al, 2005). The concentration of malate in root tissues and in root exudates was very

low if compared to citrate. Additionally, malate did not show any clear tendency of induction by Al in Cat100-6 as well as in S1587-17 (data not shown), indicating this organic acid is not involved with Al-tolerance in Cat100-6. The knowledge of this fact was important, since malate is the organic acid associated to Al-tolerance in wheat and the ALMT1 protein of wheat was described as a malate transporter (Sasaki et al., 2004). Since citrate is the organic acid associated with the maize Al-tolerance, we expected that MAIT will be involved with citrate transport instead of malate. Besides malate, reduced concentrations of other organic acids such as aconitate and succinate and a high concentration of the anion phosphate were detected. However no one had its content or exudation stimulated by Al (data not shown).

Differently from those of other compounds, the exudation profiles of citrate seem to be linked to the phenotypic differences observed in Cat100-6 and S1587-17 roots when exposed to Al. This result indicated a possible involvement of citrate with the Al-tolerance of Cat100-6. Since the concentrations of citrate in roots of Cat100-6 and S1587-17 were not different at least after 24 h of Al-exposition, an improved citrate transport might explain the enhanced exudation of citrate in roots of Cat100-6, suggesting the existence of transporters or channels involved with transport of this molecule.

The concentration of citrate in root tissues of Cat100-6 and S1587-17 increased in the presence of Al although the differences between genotypes were not evident after 24 h of exposition. Only after 48 h of exposure to Al a reduction on the level of citrate in root tissues of S1587-17 was observed. However, the causes of this reduction in citrate concentration in S1587-17 might be the metabolic damage promoted by Al in the root apices, disrupting the synthesis of this compound, or the extravasation of cellular content from ruptured cells.

Similarly to the citrate detected in the root tissues, the amount of citrate exuded by whole roots in the control treatment was much smaller than the concentration detected when Al was present. Al stimulated the citrate exudation almost 10-fold in both genotypes and the concentrations of citrate exuded by Cat100-6 roots were almost two times higher than the citrate

exuded by S1587-17 roots. These data indicate the existence of an association between citrate exudation and Al-tolerance phenotype.

Because the root apex is the main target of Al-toxicity we were interested in the organic acid exuded only in this region. Therefore the exudates were collected only from the first 10 mm of the root apex of intact seedlings to avoid the interference of wounding effects observed in excised roots (Collet et al., 2002). The presence of Al promoted an increase on citrate release in root apices of Cat100-6 and S1587-17 very similar to the profile observed previously to exudation in whole roots after 24 h and 48 h of Al exposition. Again the concentration of citrate exuded was almost two times higher in Cat100-6 than in S1587-17 (Fig. 2B), confirming that either in complete roots or root apices, Cat100-6 always exuded more citrate than S1587-17 in response to Al. However, the concentration of citrate exuded by the root apices were around half of the concentration exuded by the whole roots indicating that the contribution of other root segments or even lateral roots (present in the whole roots) are important. Interestingly, the concentration of citrate detected in control treatments was slight superior to the concentration observed in whole roots. One explanation for that may be the stress imposed on the roots during the experimental set up. The results obtained by the exudation experiments were similar to the results described by other authors working with different maize genotypes (Pellet et al., 1995; Jorge and Arruda, 1997; Piñeros et al., 2002; Mariano and Keltjens, 2003; Piñeros et al., 2005) that also concluded that citrate was the main organic acid associated with Al response in maize.

Effects of Al on membrane potential of Cat100-6 and S1587-17 roots

Since the release of citric acid was associated to the differential Al tolerance observed between Cat100-6 and S1587-17 and because Al tolerance in maize seem to be associated with organic acid transport rather than synthesis (Piñeros et al., 2005) we were interested in investigating effects of Al on membrane potential of root-cells, since this could explain the

increase of citrate exudation in Cat100-6. The recording of the resting membrane potential (RMP) was the way to observe the Al-effects in the electrogenic activity of the plasma membrane of root cells, since the RMP might provide an indirect information about the transport of charged molecules taking place in root cells of Cat100-6 and S1587-17.

The depolarization of plasma membranes can be interpreted in different ways. The first explanation is the blocking of Ca^{2+} and K^{+} channels induced by Al^{3+} (Piñeros and Tester, 1993; Gassmann and Schroeder, 1994) or inhibition of the H^{+}-ATPase drive force (Ahn et al., 2002). Although the blocking of transporters and channels might be a reasonable effect of Al, it does not explain the hypothesis of existence of an inducible ion transport system in Cat100-6 roots, since the depolarization observed in Cat100-6 was larger than the depolarization observed in S1587-17. In contrast, the efflux of anions, such as Cl^{-} and ionized organic acids, from the cell would promote a membrane depolarization if the flow of charge outward the cell is not balanced by other ions such as K^{+} (Wherrett et al., 2005).

These data are in agreement with those from Papernik and Kochian (1997) that evaluated two Al-contrasting wheat genotypes in a similar experiment and found that the Al-tolerant genotype showed higher depolarization than the sensitive one. These authors formulated two hypotheses to explain this result: *i*) the depolarization was induced by a large efflux of anions such as $malate^{-2}$ that altered the equilibrium of charges on the plasma membrane, and *ii*) the presence of Al might block the ion flux across the plasma membrane and as consequence of the depolarization, a signal would be generated to activate specific transporters, such as transporters of organic acid anions. These hypotheses were recently evaluated and partially confirmed in two Al-contrasting wheat genotypes studied by Wherrett et al. (2005). Regardless of the fact that in maize is not clear why Al promote a higher depolarization in Cat100-6 than in S1587-17, the origin of this effect would be the same of wheat.

To verify if the Al effect on the RMP was restricted only to cells from the root apex we evaluated cells situated 10 mm back from the root apex, and the differences detected between Cat100-6 and S1587-17 were not observed anymore. Besides that, the RMP of these cells was almost twice superior to those of the root apex cells, indicating that the magnitude and/or category of electrogenic transport taking place in these cells was different from the cells of root apex. A reasonable explanation is the morphological differences between these cells, mainly because the existence of a large vacuole and presence of several channels in the tonoplast membrane. Alternatively the addition of Al produced no substantial depolarization in the RMP of Cat100-6 as well as in S1587-17, showing that Al did not affected the electrogenic activity of these cells. This result shows spatial differences in the response to Al in different root zones. Furthermore, the replacement of Al by La showed that La was responsible for a slight depolarization in Cat100-6 and S1587-17. The level of depolarization in S1587-17 was similar to depolarization caused by Al but the depolarization in Cat100-6 was insignificant if compared to depolarization caused by Al. This is an indication that Al has a distinct effect on the RMP of Cat100-6 that might directly reflect on the efflux of charged molecules in the roots of this genotype.

***The maize homologue of* ALMT1**

The results of organic acid exudation and the differences on the RMP in roots cells of Cat100-6 indicated the involvement of citrate in alleviation of Al toxicity and the existence of a differential transport activity in this genotype. We then decide to investigate if Cat100-6 had a homologue of the Al activated transporter recently described in wheat (Sasaki et al., 2004). Therefore, using the nucleotide sequence of *ALMT1-1* and a maize genomic sequence with identity to *ALMT1* we were able to clone the full cDNA sequence of a maize gene corresponding to *ALMT1*. The alignment of its nucleotide and amino acid sequences with *ALMT1-1* showed elevated identity, showing this gene is a homologue of *ALMT1*. The maize homologue of *ALMT1*

was named *MAIT* (Maize Aluminum Induced Transporter) due to its electrophysiological properties, considering that unlike ALMT1 (Sasaki et al., 2004), the MAIT protein is not exclusively involved with malate transport.

The restriction analysis of genomic DNA indicates that *MAIT* is possibly a single copy gene in maize. This result is different from the result obtained in RFLP analysis with wheat genomic DNA that pointed a more complex band profile to *ALMT1*, indicating the existence of multiple copies of this gene (Sasaki et al., 2004). In addition, the digestion with *EcoR* I and *Hind* III produced a polymorphism to *MAIT* that seem to be associated with the Al-tolerance phenotype, at least to the maize lines evaluated. To confirm if this gene or regions flanking it might be associated with the Al tolerance trait, analysis of recombinant inbred maize lines derived from the cross between Al-contrasting progenitors will be evaluated by RFLP.

The transient expression of *MAIT*::*GFP* in epithelial onion cells showed that MAIT is certainly located in the plasma membrane. Corroborating with this result, the secondary structure prediction of MAIT showed that it is a transmembrane protein containing several alpha-helices domains in its secondary structure. Besides these results, Yamaguchi et al. (2005) showed results of sub-cellular localization for ALMT1 using GFP very similar to the results obtained for MAIT. Since MAIT and ALMT1 have high degree of identity, it is reasonable to expect that these proteins have secondary and even tertiary structure closely related.

Interestingly, the *MAIT* expression in maize roots was difficult to detect. The usual techniques based in hybridization failed to show any signal of *MAIT* expression in root and shoots of maize. Since this kind of technique detects the gene expression based on the total number of mRNA molecules in a sample, the reduced number of *MAIT* transcripts might difficult its detection. From the premise that the number of *MAIT* mRNA molecules was not enough to be detected by hybridization approaches, we decided to check the expression of *MAIT* in the same way that its full cDNA was detected: by reverse transcriptase reaction followed by PCR amplification (semi-quantitative RT-PCR). Using this approach the expression of *MAIT* in root

apices of Cat100-6 and S1587-17 was detected and showed a substantial repression in root apices of both genotypes in response to increase of Al concentration. Therefore it is difficult to visualize the main role of this gene in Al-tolerance acquisition in Cat100-6, since Al represses it in the Al activities tested. MAIT seems to be activated by Al and its regulation might happen in post-transcriptional level rather than in the transcription level. Consequently, a basal level of this protein on the plasma membrane would be enough to respond to Al, since that the electrophysiological data showed that MAIT can be induced by Al. Besides that, MAIT proteins might have a long turnover rate on the plasma membrane reducing the need of an inducible expression. The quantification of MAIT protein in plasma membrane of root apices to determine the steady-state of MAIT protein under different Al activities would help to confirm this hypothesis.

Electrophysiological properties of MAIT

To show that the protein encoded by MAIT was involved with ion transport across the plasma membrane we used a heterologous approach. The MAIT electrogenic activity was evaluated in plasma membrane of *Xenopus laevis* oocytes. The use of *X. laevis* oocytes presented several advantages over using a plant system. The most relevant were low complexity of working with a large cell, the reduced magnitude of endogenous currents, and the elevated reproducibility.

Initially, the oocytes expressing *MAIT* were evaluated in a solution of simple composition containing high amounts of NaCl. The results showed a very strong current activity in these oocytes when negative voltages were applied on the membrane. The currents recorded were at least five times superior to the currents previously detected with negative voltages applied in oocytes expressing *ALMT1* (Sasaki et al., 2004). While the oocytes expressing *MAIT* produced currents lower than –1,000 mA (to an applied voltage of -100 mV) the control oocytes produced currents around -50 mA to the same voltage. This result clearly showed that MAIT promoted

deep alterations on the electrogenic activity of the transformed oocytes. When the control solution was replaced by a solution with 100 μM of Al an increase of the negative currents was observed (Fig. 7A) showing an activation effect of this cation on the transport activity of the protein.

Further, we replaced the Al solution by an identical solution containing La instead of Al to verify if the activation promoted by Al might be mimicked by a cation with similar properties to Al. The results revealed that La promoted a blocking of the currents in oocytes expressing MAIT and the same was observed in control oocytes (data not shown). Further, La was regularly used in the MES solution to eliminate any interference of endogenous channels in the oocytes.

Interestingly, oocytes transformed with *MAIT* and subsequently injected with malate solution showed small responses to the addition of Al, although the currents recorded remained high at different voltages applied (supplementary data, Fig. 3A). In this aspect, MAIT seem to be different of ALMT1, considering that the last one was described as specifically involved with malate transport (Sasaki et al., 2004). Considering that MAIT response to malate injection was smaller than the response observed to ALMT1, we tried citrate injection, since citrate was the main organic acid exuded by Cat100-6 roots. The injection of 1.5 ηL of 100 μM of citrate solution (at physiological pH of 7.5) in oocytes, injected or not with *MAIT* cRNA, promoted an activation of endogenous currents even in the control oocytes showing a citrate effect in the endogenous transport of the oocytes. Consequently, this citrate effect made an accurate evaluation of the MAIT activity difficult, since the control oocytes showed currents higher than the usual after citrate injection. Therefore, using this model, we were not able to verify the effect of citrate over the MAIT activity.

Because the simple ND96 solution used had a high amount of Cl^- anions, we tried to avoid the influence of this anion on the transport activity by replacing NaCl by MES. In addition, we added 100 μM of $LaCl_3$ in this solution based in the previous information that this trivalent cation was responsible for decreasing the electrogenic activity on the oocytes membrane. Using

this solution, we evaluated oocytes injected with citrate expecting to eliminate or reducing the interferences observed in the control oocytes evaluated in simple ND96 solution. However, the control oocytes injected with citrate maintained the same irregular and elevated background currents, showing it was not influenced by the Cl^- or even alleviated by La. Despite the fact that oocytes expressing *MAIT* and injected with citrate showed irregular currents, we were not able to distinguish the MAIT activity from the endogenous electrogenic activity.

In addition, the evaluation of oocytes expressing *MAIT* but not injected with malate or citrate, produced a current activation by Al similar to the results obtained in simple ND96 solution. We have detected a reduction in the magnitude of the currents recorded in the oocytes expressing *MAIT*, indicating that the presence of La^{3+} or the decrease of Cl^- concentration, or both, negatively influenced the activity of MAIT, consequently reducing its activity. If the absence of the ion Cl^- was the responsible for this effect it is an indicative of the influx of this ion into the oocytes promoting the efflux of other molecules from the oocytes cytosol to balance the charges. If this effect is due to the presence of La, it might be explained by the blocking effect of this cation.

Concluding, although the role of MAIT in transport of citrate or malate was not elucidated, the results obtained clearly showed that Al stimulated the MAIT activity, mainly when the oocytes were injected only with *MAIT* cRNA in ND96 as well as in MES + La. Another important point is that the activity of MAIT seems to be stronger than the activity of ALMT1 recorded by Sasaki et al. (2004) using the same experimental procedures.

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Conclusões Finais

Durante o desenvolvimento deste trabalho, três linhas distintas de pesquisa foram desenvolvidas. Inicialmente através da técnica de "differential display" foi identificado e descrito uma nova versão do gene codificando a enzima glutationa S-transferase (GST) do tipo Teta (θ), denominado *GST27.2*. Esse gene foi induzido em ápices radiculares da linhagem Cat100-6 quando cultivados na presença de Al. Como esta enzima está diretamente envolvida com o metabolismo do estresse oxidativo, a clonagem de *GST27.2* e sua caracterização foram importantes para demonstrar a associação da toxidez provocada pelo Al com o metabolismo de espécies reativas de oxigênio em milho. Posteriormente, por meio de modelagem a partir da estrutura terciária determinada por cristalografia de uma GST do tipo Fi (ϕ), foi possível observar que as duas mutações presentes em *GST27.2* ocasionavam alterações de aminoácidos que podem refletir na atividade ou especificidade da enzima. Adicionalmente, este foi o primeiro relato demonstrando alterações na expressão de um gene codificando uma GST promovido pelo estresse de Al.

Embora a técnica de “differential display” tenha se mostrado eficiente na identificação de pelo menos um gene cuja expressão foi alterada pelo estresse de Al, observamos uma alta proporção de falso-positivos. Já a utilização de técnicas como a de arranjos de DNA que são mais sensíveis e robustas, permitem um estudo mais eficiente da expressão gênica global. Desta forma, como permanecia o interesse de estudar em maior detalhes os efeitos do estresse de Al na resposta da expressão gênica em milho, foi utilizada a técnica de macroarranjos de DNA (“macroarrays”) para avaliar a expressão gênica de forma estatisticamente comparativa entre ápices radiculares das linhagens de milho Cat100-6 e S1587-17. As membranas de arranjo foram construídas com 2.304 “expressed sequence tags” (ESTs) escolhidos ao acaso em diferentes bibliotecas de ESTs de cana-de-açúcar do projeto SUCEST.

O primeiro resultado positivo desta abordagem foi a validação do modelo de hibridização heteróloga entre as seqüências de nucleotídeos de milho e de cana-de-açúcar, o que viabiliza a utilização de mais de 40 mil seqüências de ESTs do projeto SUCEST em outros estudos de transcriptoma em milho. Posteriormente, após a

filtragem e análise estatística dos dados da expressão, foram selecionados 85 genes em ambas linhagens de milho, cuja expressão gênica foi significativamente alterada pelo estresse de Al. Embora alguns dos genes identificados já tivessem sido descritos na literatura como induzidos por Al, para uma significativa parcela destes 85 genes, este trabalho foi o primeiro relato descrevendo seu envolvimento com o estresse de Al.

Da mesma forma, este é o primeiro relato em milho sobre as alterações na expressão gênica em larga escala em resposta ao estresse provocado pelo Al. Portanto, há expectativa que estes resultados norteiem futuras investigações envolvendo milho e estresse de Al.

A terceira e última etapa do trabalho foi desenvolvida nos laboratórios do USDA/Cornell University, sob a orientação do Dr. Leon V. Kochian e com o apoio financeiro da agência CAPES. Devido à exsudação diferencial de citrato induzida por Al entre as raízes de Cat100-6 e S1587-17 e devido a recente descoberta de um novo transportador de ácido orgânico em raízes de trigo, despertou-se o interesse em estudar os processos de transporte de ácidos orgânicos na membrana plasmática de células da raiz de milho. Nesta etapa do projeto verificamos em milho, mais especificamente na linhagem Cat100-6, a existência de um gene homólogo para o gene de trigo *ALMT1*, que codifica uma proteína de membrana possivelmente envolvida com o transporte de ácidos orgânicos e ativada por Al. O gene correspondente em milho foi denominado de *MAIT* (maize aluminum induced transporter) devido à confirmação de sua ativação pela presença de Al. As análises da atividade da proteína codificada por *MAIT* demonstraram que embora a proteína responda a presença de Al, seu envolvimento com o transporte de ácidos orgânicos na raiz ainda não é claro, principalmente para citrato, o ácido orgânico cuja exsudação responde ao Al em raízes de milho.

Os resultados aqui apresentados contribuíram para a compreensão dos efeitos do Al em milho. Como perspectivas futuras, e necessária a aplicação de novas abordagens para verificar se os genes aqui descritos possuem um papel preponderante no fenótipo de tolerância ao Al em milho. Para *GST27.2* e *MAIT*, projetos que visam à superexpressão destes genes em milho estão em andamento para verificar o envolvimento dos mesmos com a redução do estresse provocado pelo Al. Para os genes identificados no trabalho de arranjos de DNA, novas análises de expressão

gênica e atividade enzimática em diferentes condições de estresse de Al, serão testadas para genes candidatos que podem estar envolvidos diretamente com a resposta da planta ao estresse provocado pelo Al. Posteriormente, a superexpressão destes genes em milho ou em plantas modelos, tais como tabaco e Arabidopsis, também será realizada com o intuito de avaliar os efeitos destes genes no fenótipo de tolerância ao Al.

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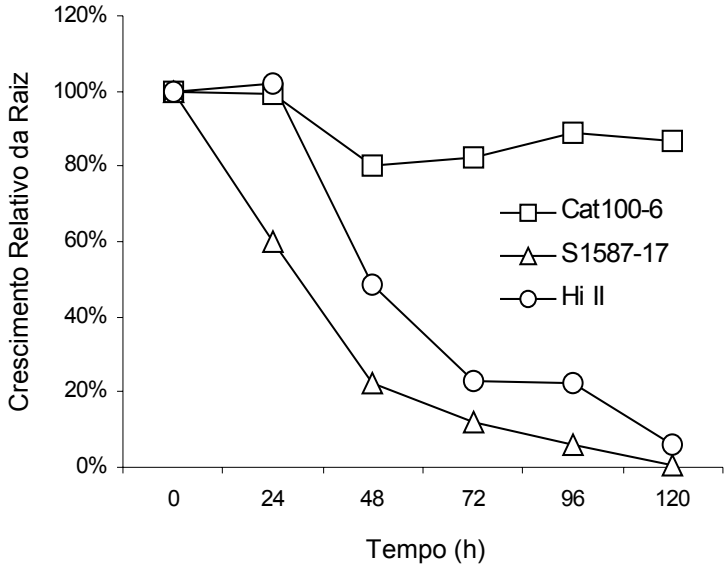

Figura 1. Crescimento relativo da raiz seminal de Cat100-6, S1587-17 e Hi-II, calculado após 24, 48, 72, 96 e 120 horas de cultivo em solução nutritiva (Magnavaca et al., 1987) sem Al e com atividade de 39 μM de Al^{3+}, *n* = 20.

Figura 2. Aspecto visual das raízes de Cat100-6, S1587-17 e Hi-II após 5 dias de cultivo em solução nutritiva (Magnavaca et al., 1987) sem Al (-Al) e com atividade de 39 μM de Al^{3+} (+Al).

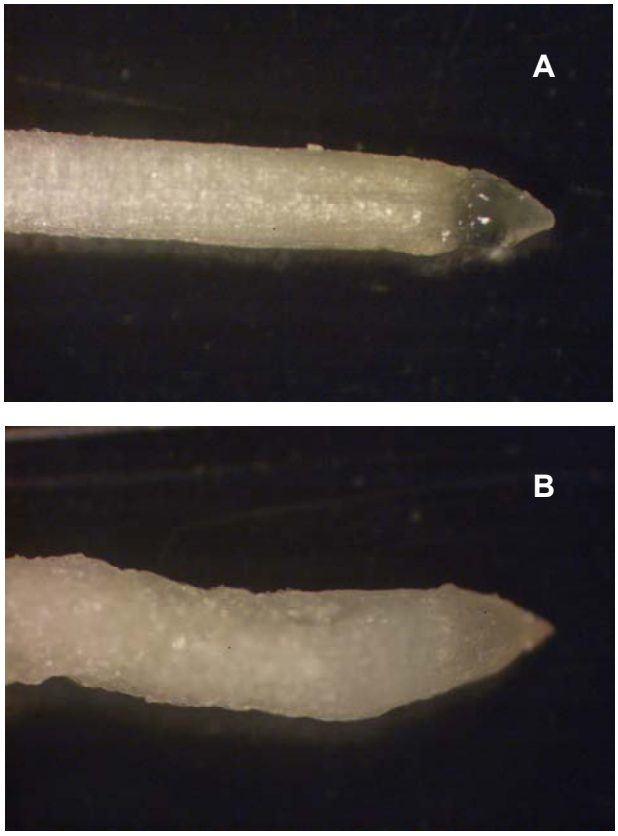

Figure 3. Visual aspect of Cat100-6 (A) and S1587-17 (B) root apex after 24 h of exposition in nutrient solution (Magnavaca et al. 1987), containing 39 μM of Al^{3+} activity.

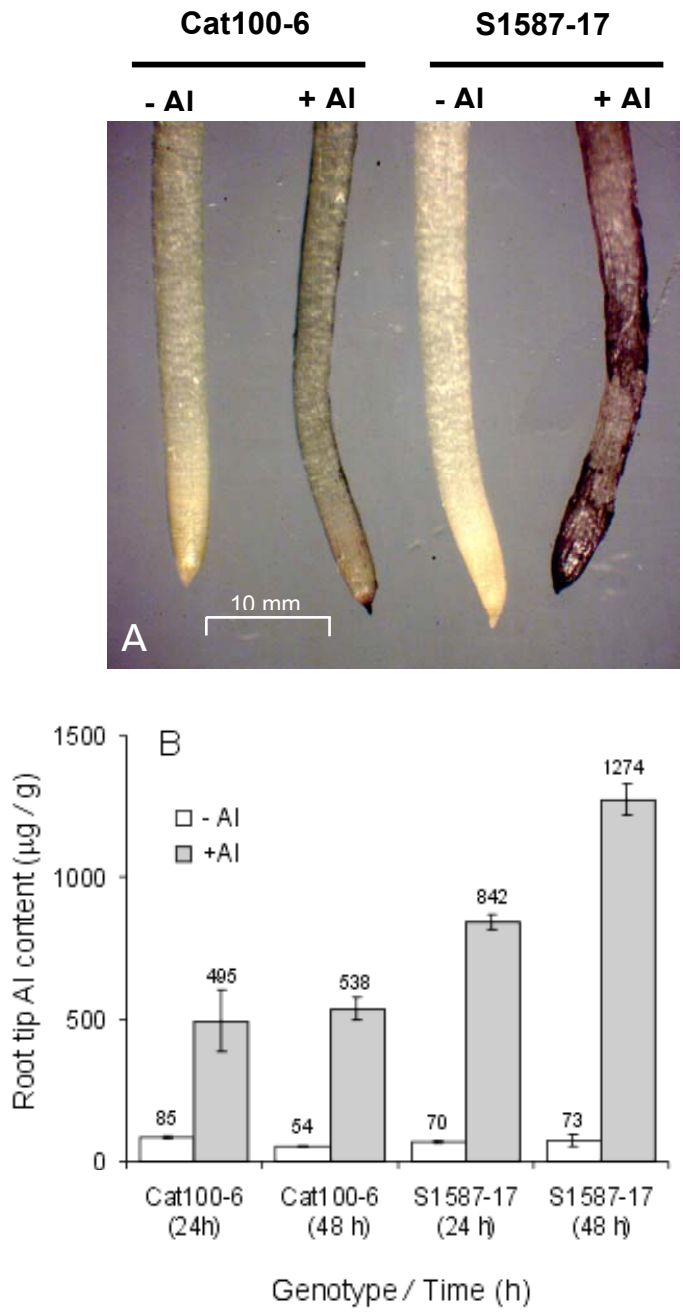

Figure 4. Aluminum accumulation in root apices of Cat100-6 and S1587-17. A) Hematoxylin staining of Cat100-6 and S1587-15 roots after 24 h of cultivation in nutrient solution (Magnavaca et al., 1987) without and with 39 μM of Al^{3+} activity. B) Aluminum concentration in root apices (10 mm) tissues from Cat100-6 and S1587-17, after 24 h and 48 h of cultivation in nutrient solution (Magnavaca et al., 1987) without and with 39 μM of Al^{3+} activity. The bars correspond to the average ±SE, *n*=6. The hematoxylin staining and the method of Al content evaluation used are described in Cançado et al. (1999) and Piñeros et al. (2005), respectivelly.

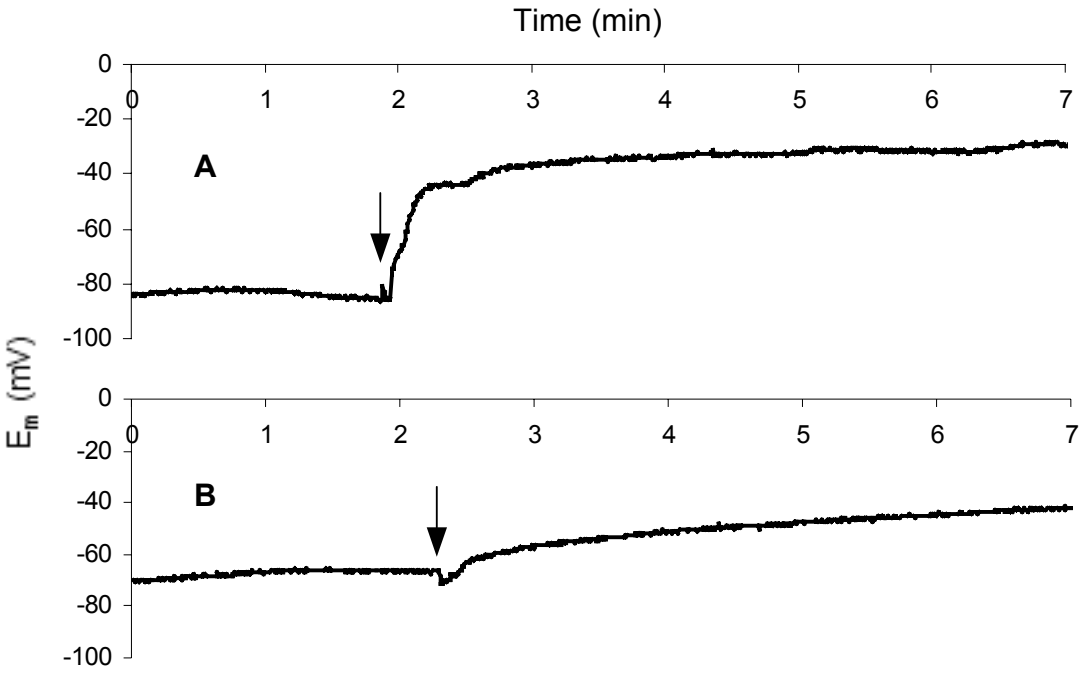

Figure 5. Measurement of the resting membrane potential (E_m) of a single cell from the root apex of Cat100-6 (A) and S1587-17 (B). The arrows indicate the substitution of the control solution (200 μM $CaCl_2$) by the treatment solution (200 μM $CaCl_2$ and 150 μM $AlCl_3$).

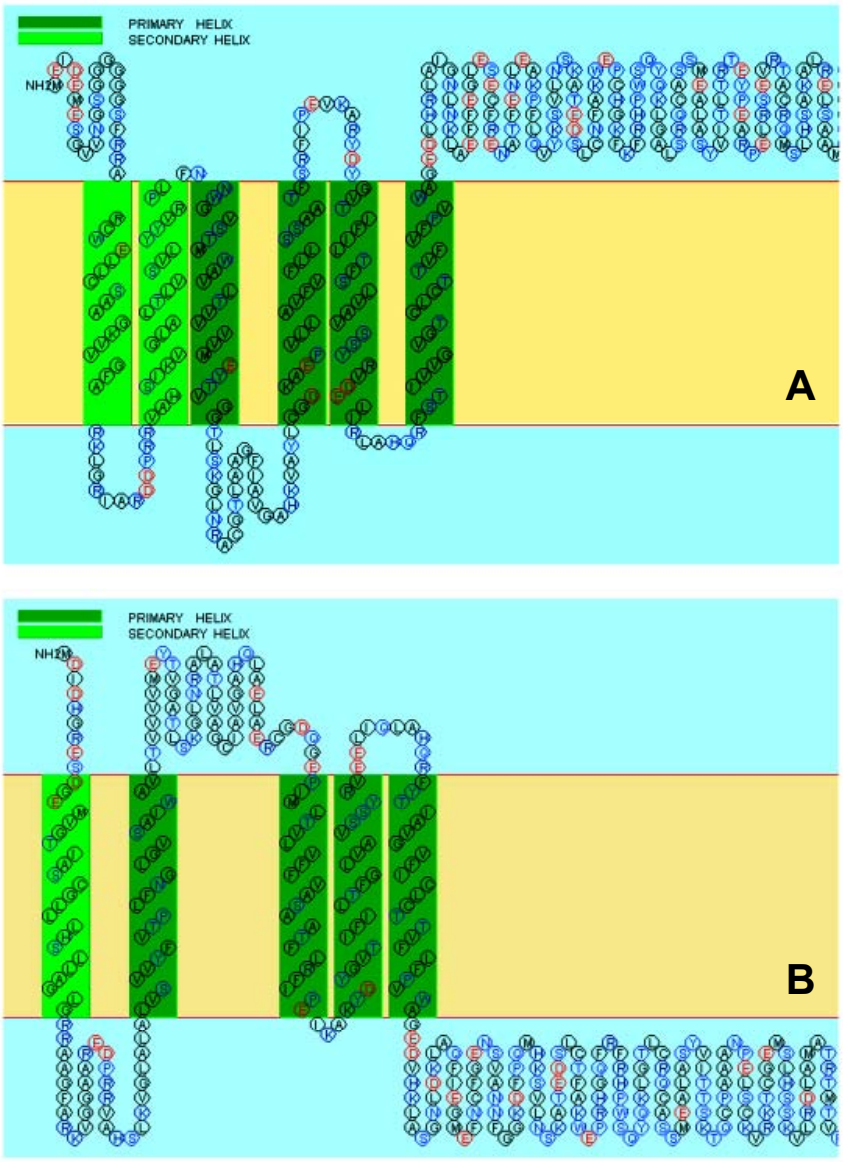

Figure 6. Secondary structure prediction of MAIT (A) and ALMT1-1 (B) using the SOSUI algorithm (http://sosui.proteome.bio.tuat.ac.jp).

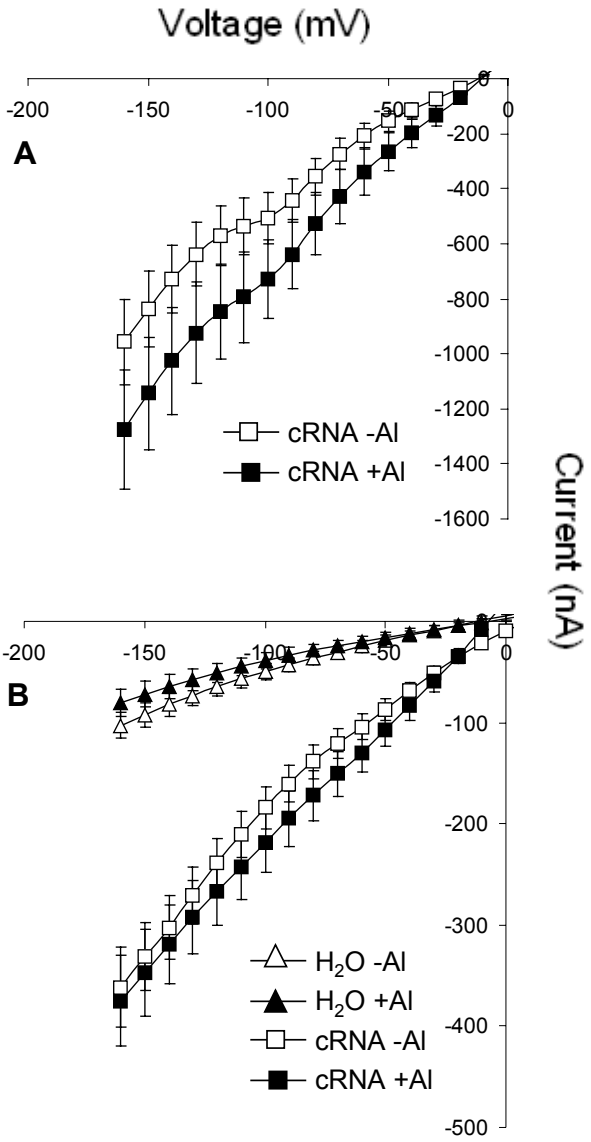

Figure 7. Current-voltage curves collected in oocytes of *X. laevis.* A) Oocytes injected with *MAIT* cRNA and 1,5 ηL of 100 mM malate pH 7,5 and evaluated in ND96 control solution (white squares) and ND96 solution plus 100 μM of $AlCl_3$ (black squares). B) Oocytes injected with water (triangles) and with *MAIT* cRNA (squares). The oocytes were injected with 1,5 ηL of 100 mM malate pH 7,5 and evaluated in MES-La^{3+} control solution (white symbols) and MES-La^{3+} plus 100 μM of $AlCl_3$ (black symbols).

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Errata

Na página 29, segunda linha do primeiro paragráfo da sessão MAIZE DATA, a denominação correta é S1587-17 e não S1787-17.

Na página 30, na legenda da Figura 1, incluir: A) Cat100-6 in control solution; B) Cat100-6 in 75 μM of Al; C) Cat100-6 in 283 μM of Al; D) S1587-17 in control solution; E) S1587-17 in 75 μM of Al; and F) S1587-17 in 283 μM of Al.