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"FISIOLOGIA E TRANSCRIPTOMA DE MILHO CULTIVADO EM SOLO ÁCIDO"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Genética e Melhoramento Vegetal.

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Dedico essa Tese aos meus pais, Jorge e Jurema,
e ao meu irmão Henrique.

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but rather, "hmm.... that's funny...."

Isaac Asimov

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RESUMO

A presença do alumínio (Al) em solos ácidos é o principal fator limitante da produtividade agrícola no Brasil e no mundo. A resposta desenvolvida pelas plantas contra o Al é complexa e a identificação de genes responsivos após a exposição ao íon através de técnicas de análise em larga escala, como microarrays, pode facilitar a sua compreensão. Este projeto possui como objetivo ampliar o conhecimento sobre a fisiologia e a regulação gênica de raízes e folhas utilizando genótipos contrastantes de milho (Cat100-6 (Al-tolerante) e S1587-17 (Al-sensível)) cultivadas em solo ácido com concentração fitotóxica de Al.

As linhagens de milho Cat100-6 e S1587-17 foram cultivadas por um ou três dias em solo ácido (pH 4,1) ou solo corrigido com $Ca(OH)_2$ (pH 5,5). O genótipo S1587-17 apresentou uma maior inibição do crescimento radicular, resultado este altamente correlacionado com a acumulação de Al nos ápices radiculares e deposição de calose. Os dados fisiológicos confirmam a discriminação entre as duas linhagens em solo, abrindo perspectivas para entender pela primeira vez a base molecular das alterações das plantas em condições próximas à realidade de campo.

O transcriptoma de raízes possibilitou a identificação possíveis candidatos a tolerância ao Al. Adicionalmente, com um experimento de hidroponia separamos as variáveis pH e presença de Al, ambas condições diferenciais do tratamento com solo. Identificamos, entre os candidatos, genes responsivos pela presença do Al e não pela acidez delimitando assim os genes com possíveis papéis na tolerância ao Al presente no solo ácido a apenas três: retinol desidrogenase, um fator de transcrição WRKY e uma proteína desconhecida. Esses resultados permitem concluir que o cultivo em solo é diferencial em relação à hidroponia, e outros fatores que apenas presentes

no substrato solo podem provocar a indução de alguns genes. Diversas vias metabólicas são afetadas na linhagem sensível pelo tratamento em solo ácido e podem estar envolvidas na inibição radicular como a produção de lignina, celulose e calose e a síntese de etileno e auxina.

O mapeamento nos cromossomos dos genes identificados pelo experimento de microarray das raízes de milho permitiu a identificação de genes localizados dentro de QTLs de milho previamente descritos na literatura como responsáveis pelo fenótipo tolerante. Diante esse resultado, podemos especular o papel de genes como uma proteína ligadora de RNA, uma inibidora de proteases e ciclinas na tolerância ao Al contido no solo ácido.

Pela primeira vez na literatura, o transcriptoma de folhas coletadas após três dias de cultivo em solo ácido ou solo corrigido foi obtido com o uso de microarrays da Affymetrix. Essa análise indicou profundas alterações na Cat100-6, em contraposição à ausência de alteração significativa nas folhas na S1587-17. Genes referentes à fotossíntese e a fotorrespiração foram regulados negativamente pelo tratamento em solo ácido no genótipo tolerante. Contudo, o ciclo do ácido cítrico está ativado indicando uma putativa participação da produção de ácidos orgânicos nas folhas na resposta ao Al.

ABSTRACT

The presence of aluminum (Al) is the main factor limiting crops yield in Brazil and worldwide. The plant responses developed against this ion are complex and the identification of responsive genes after exposure to the ion with the use of a large scale technique, such as microarrays, can facilitate its comprehension. This project aimed to amplify the knowledge about physiology and gene expression regulation of roots and leaves associated towards Al resistance using contrasting maize genotypes (Cat100-6 (Al-tolerant) and S1587-17 (Al-sensitive) cultivated in acid soil containing phytotoxic concentrations of Al.

Maize lines Cat100-6 and S1587-17 were cultivated for one or three days in acid soil (pH 4,1) or limed soil with $Ca(OH)_2$ (pH 5,5). The genotype S1587-17 presented a higher root growth inhibition, which is highly correlated with Al accumulation in the root apexes and callose deposition. The physiological data confirms the discrimination of the two maize lines cultivated in soil, opening perspective to understand for the first time the molecular bases of alterations in plants on a closer condition to the field.

Transcriptome from roots made possible the identification of possible tolerance candidates and genes constitutively expressed genes in the tolerant line. Additionally, throw a hydroponic experiment we splited the variables pH and Al presence, both differential conditions between soil treatments. It was possible to identify, among the candidates, genes responsive in the presence of Al in acid soil rather than acidity limiting genes with a possible roles in Al present in the acid soil tolerance to only three: retinol dehydrogenase, the transcription factor WRKY and an unknown protein. These results allow the conclusion that the soil culture is different in relation to hydropony, and other factors present only in soil substrate could provoke the induction of some

genes. Several metabolic pathways were affected in the sensitive line after acid soil growth and could be involved on root growth inhibition such as lignin, cellulose and callose production and ethylene and auxin synthesis.

The mapping of the identified genes through the microarray experiments into the chromosomes allowed the identification of genes localized into maize QTLs previously reported in the literature as responsible for the tolerant phenotype. Facing these results, we can speculate the role of these genes such as a RNA binding protein, a protease inhibitor, and cyclines in the Al present in the acid soil tolerance.

For the first time in literature, the transcriptome of leaves collected after three days in culture with acid soil or limed soil with the Affymetrix microarrays. This analysis indicated great alterations in Cat100-6, meanwhile S1587-17 showed no significative alteration. Genes related to photosynthesis and photorespiration were down-regulated due acid soil treatment in the tolerant genotype. However, citric acid cycle was activated indicating the putative partitipation of organic acids produced in the leaves in thr Al response.

INTRODUÇÃO GERAL

Solos ácidos e toxidez do Al

Estresses abióticos são variáveis físicas do meio ambiente que podem afetar negativamente o crescimento vegetal como baixas ou altas temperaturas, salinidade, seca, alagamento, alta radiação, falta ou excesso de nutrientes, alto ou baixo pH e existência de metais pesados no meio de crescimento. A existência dos solos ácidos é um dos fatores que mais comprometem a produção de vários tipos de lavouras, especialmente as de cereais. A baixa produção em solos ácidos é uma combinação de baixo pH, toxicidade por alumínio (Al), manganês (Mn) e ferro (Fe) e deficiências em nitrogênio (N), fósforo (P), cálcio (Ca) e magnésio (Mg) (Kochian et al., 2004).

O primeiro artigo relacionado acidez do solo, áreas de baixa produtividade e toxicidade do Al foi publicado por Hartwell e Pember em 1918 e desde então diversos estudos tentam desvendar a complexa resposta das plantas contra estresses típicos desses solos. Cerca de 30% dos solos do mundo são ácidos sendo que os trópicos e subtrópicos correspondem a 60% deste tipo de (Figura 1), devido principalmente à ação prolongada de um clima quente e úmido (Kochian et al., 2004). Mas em outros locais do mundo, como EUA e diversos países da Europa, a acidificação do solo tem se tornado um problema sério devido à chuva ácida, a remoção da cobertura natural da área de produção e principalmente a utilização de fertilizantes nitrogenados amoniacais (Johnson et al., 1997). Aproximadamente 20% do milho plantado no mundo estão cultivados em áreas de solo ácido, sendo que este cereal é responsável por 70% da ingestão de calorias por humanos no mundo (Chandler e Brendel, 2002).

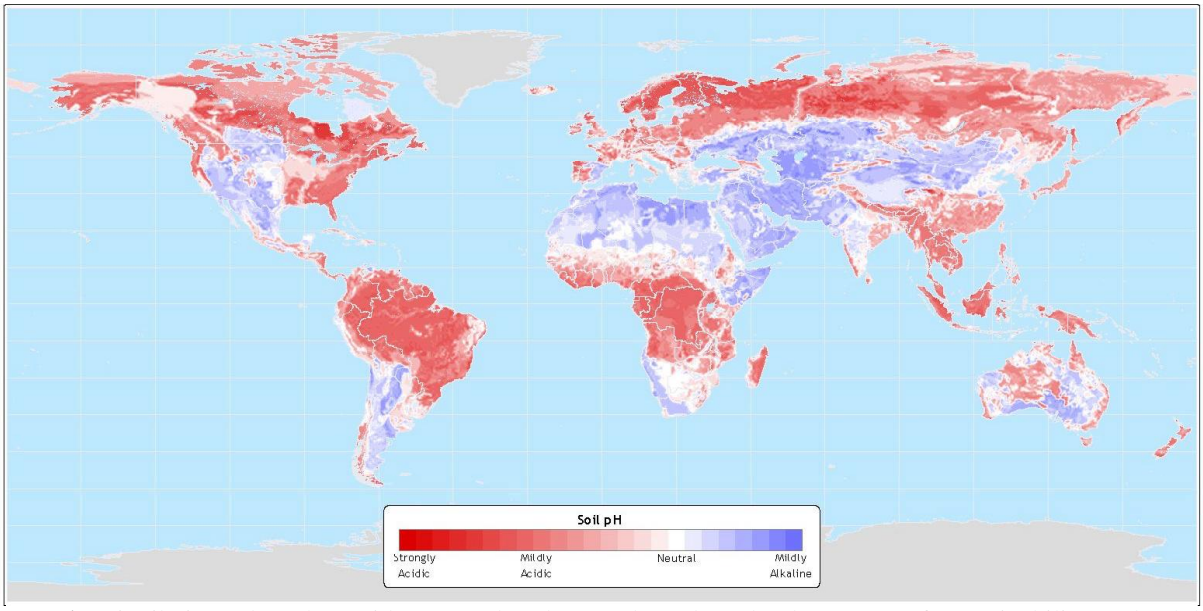

Figura 1. Distribuição de solos ácidos ao redor do mundo (adaptado de Center of Sustainability and Global Environment – University of Wisconsin – Madson, com dados extraídos do IGBP- DIS Global Soils Dataset, 1998).

Um dos principais problemas desencadeados pelo baixo pH do solo é a fitotoxicidade do Al. O Al é o componente principal de uma ampla variedade de minerais primários e secundários (Sommers e Lindsay, 1979). Em pH > 5,5 o Al está predominantemente na forma de gibsita ($Al(OH)_3$). Em pH mais baixo o composto é solubilizado liberando a forma trivalente (Al^{3+}) que é tóxica às plantas e pode interferir severamente no desenvolvimento vegetal. A calagem é o método mais empregado para a correção da acidez do solo, mas os compostos utilizados (como o $CaCO_3$ e $Ca(OH)_2$) apresentam baixa mobilidade no solo, não sendo portanto eficientes na neutralização do pH no subsolo, havendo também um encarecimento da produção.

O sintoma mais evidente da toxidez do Al é a inibição do crescimento radicular, sendo este o parâmetro mais utilizado para avaliar a injúria por Al. Adicionalmente, a quantidade de calose também é considerada um marcador fisiológico sensível e um indicador de diferença genotípica na sensibilidade ao Al de milho (Collet et al., 2002), trigo (Zhang et al., 1994) e soja (Wissemeier et al., 1995). O estudo de Wissemeier e colaboradores (1998) avaliou o efeito de Al no crescimento e na deposição de calose em raízes de *Picea abies* (L.) Karst cultivadas no campo

com solo alterado por irrigação normal ou ácida (simulação de chuva ácida). Foi verificada uma correlação positiva entre a quantidade de calose e a concentração de alumínio livre no solo. Tendo em vista que o sítio primário de ação fitotóxica deste íon localiza-se na parte distal da zona de transição do ápice das raízes (Ryan et al., 1993; Sivaguru e Horst, 1998), o estudo do mecanismo de toxidez do Al, bem como dos mecanismos de defesa da planta, tem sido focado na interação do íon com componentes celulares do ápice radicular.

Diversos estudos buscando o motivo da inibição do crescimento radicular caudado pelo Al, permitiram a formulação de várias hipóteses. O Al pode interferir na homeostase de Ca no simplasto através da alteração de seu fluxo através da membrana plasmática. Essa alteração pode prevenir o aumento da atividade do Ca citosólico, necessária para a formação do fuso durante a prófase e iniciar transição metáfase/anáfase, comprometendo assim a divisão celular (Rengel, 1992). Outro estudo que demonstra o papel da assimilação de Ca e a sensibilidade ao Al é Huang et al. (1992). Em trigo a assimilação de Ca é inibida imediatamente e drasticamente quando exposta ao Al em um genótipo sensível (Scout66). Por outro lado o genótipo Atlas66 (tolerante ao Al) demonstrou quase nenhum efeito na cinética da assimilação de Ca pelas raízes (Huang et al., 1992). Esse estudo indica que a interrupção do transporte de Ca no ápice radicular compõe um mecanismo de fitotoxicidade de Al importante em trigo e a tolerância diferencial apresentada pelos dois genótipos pode estar associada com a habilidade do sistema transporte de Ca nas células do ápice radicular resistir aos efeitos fitotóxicos do Al.

Sun et al. (2007) demonstraram que o aumento da atividade da ACC oxidase e conseqüente aumento da produção de etileno pode ser a causa principal de inibição radicular induzida pela exposição ao Al de plântulas de *Lotus japonica* e *Medicago truncatula*.

Yang et al. (2008) evidenciaram que um genótipo sensível de arroz possuía maior conteúdo de Al ligado à parede celular quando comparada com a genótipo tolerante, dessa forma, os

autores concluíram que a inibição do crescimento radicular pode ser resultado do dano apoplástico induzido pela ligação do Al. O grau de metilação da pectina presente nas paredes celulares vegetais é um fator determinante para a ligação do Al aos grupos carboxílicos livres e assim proporcionar a inibição do crescimento radicular (Schmohl e Horst, 2000; Blamey et al., 1990; Grauer e Horst, 1992).

Várias hipóteses para explicar os mecanismos de resistência ao Al foram desenvolvidas e podem ser divididas em dois grupos: mecanismos de exclusão, os quais ocorreriam no exterior da célula, e mecanismos de tolerância, que ocorreriam no interior da célula (Kochian et al., 2004).

Os mecanismos de exclusão impediriam a entrada do Al na célula. Esses mecanismos incluem: imobilização do Al na parede celular, permeabilidade seletiva da membrana plasmática, formação de uma barreira de pH na rizosfera, efluxo de Al e exsudação de quelantes como ácidos orgânicos e compostos fenólicos (Taylor, 1988; Wenzl et al., 2001; Kochian et al., 2004; Tolrà et al., 2005). Já nos mecanismos de tolerância, o Al entraria no simplasto e sofreria imobilização por proteínas quelantes, compartimentalização no vacúolo ou detoxificação pela ação de proteínas cujos genes são responsivos ao Al (Taylor, 1988; Kochian, 1995; Delhaize e Ryan, 1995).

Dentre todos os mecanismos propostos, a resistência ao Al mediada pela exsudação de ácidos orgânicos é o mais aceito atualmente. Desde o primeiro relato da indução da secreção de malato induzido por Al em trigo (Kitagawa et al., 1986), diversos grupos identificaram esse tipo de mecanismo em diferentes espécies vegetais. Em feijão a resistência envolve efluxo de ácido cítrico (Miyasaka et al., 1991), em trigo está correlacionada com o efluxo de malato (Delhaize et al., 1993) e raízes de milho de genótipo tolerante exsudam mais citrato que as raízes de genótipo sensível (Pellet et al., 1995; Jorge e Arruda, 1997; Jorge et al., 2001; Mariano e Keltjens, 2003).

Existem dois padrões para a exsudação de ácidos orgânicos (Ma, 2000). No padrão I a

secreção ocorre imediatamente após a exposição ao Al, em outras palavras, neste padrão há a ativação de canais aniônicos previamente existentes na membrana plasmática das células radiculares. O padrão II é caracteriza-se pela demora da secreção de ácidos orgânicos de até horas após a exposição ao Al. Essa característica pode estar associada com algum mecanismo de tolerância, ou seja, alguns genes devem ser induzidos após a penetração do Al no simplasto.

A exsudação de ácidos orgânicos não pode explicar todos os níveis de resistência entre os genótipos de milho, principalmente já que genótipos sensíveis e tolerantes exsudam a mesma quantidade de citrato após a exposição ao Al (Piñeros et al., 2005). Um estudo do nosso grupo demonstrou que mesmo quando a taxa de liberação de citrato das variedades contrastantes para a tolerância ao Al é igual, a variedade tolerante (Cat100-6) acumula menos Al do que a variedade sensível (S1587-17) indicando que existem outros mecanismos de resistência ao Al operantes na Cat100-6 (Jorge e Menossi, 2005). Dados similares foram observados por Piñeros et al. (2005) em milho e Zeng et al. (2005) em trigo, sendo que neste último a imobilização e detoxificação do Al pelo fósforo nos tecidos radiculares também fazem parte do complexo sistema de resistência ao Al. Recentemente, Maron et al. (2008) demonstraram que a tolerância em milho não está associada com o aumento da expressão de genes responsáveis pela biossíntese de ácidos orgânicos, mas sim, está envolvido com a expressão diferencial de seus transportadores.

Por outro lado, Wenzl et al. (2001) sugeriram que outras estratégias fisiológicas como baixa permeabilidade da membrana plasmática ao Al e extrusão ativa do Al do simplasto poderiam ser responsáveis pelo alto nível de resistência da gramínea *Brachiaria decumbens* (Staff cv Basilesk). Poschenrieder et al. (2005) demonstrou que em milho, os benzoxiazinóides (Bx) podem evitar os efeitos fitotóxicos do Al. O genótipo de milho tolerante utilizado apresentou níveis mais altos de Bx em seus ápices radiculares quando comparados com o genótipo sensível. Adicionalmente, a adição de DIMBOA na meio protegeu os ápices do

genótipo sensível do Al em um ensaio *in vitro*. Compostos fenólicos do tipo flavonóides como cataquina e quercitina também podem estar envolvidos com a proteção contra Al, já que a variedade tolerante de milho, quando pré-tratada com Si antes da exposição ao Al, exsudou cerca de 15 vezes mais compostos fenólicos do que plantas não pré-tratadas com Si, demonstrando o efeito de proteção do Si em relação ao Al (Kidd et al., 2001).

Assim sendo, existem vários mecanismos adicionais à liberação de ácidos orgânicos que contribuem para a resistência do milho ao Al.

Estudos com as variedades Cat100-6 e S1587-17

A cultura do milho apresenta grande diversidade genética em relação à resistência ao Al (Granados et al., 1993; Duque-Vargas et al., 1994). A linhagem S1587-17 foi obtida a partir da variação somaclonal da variedade Cat100-6 regenerada de cultura de calos do tipo I (Moon et al., 1997). De aproximadamente 2000 plantas regeneradas da cultura de tecidos a linhagem denominada S1587-17 apresentou uma severa inibição do crescimento radicular (raízes principais e laterais), além do inchaço na ponta da raiz, quando desafiada com uma atividade de Al de $40x10^{-6}$ por 7 dias.

O cruzamento entre Cat100-6 e S1587-17 e subseqüente análise da progênie F1 demonstrou que os heterozigotos comportavam-se como o parental tolerante quando submetidos ao tratamento hidropônico com atividade de Al de até $40x10^{-6}$ e apresentavam fenótipos intermediários entre os dois parentais quando tratados com atividades mais altas. A análise de segregação da geração F2 e de retrocruzamentos com a variedade sensível, através da análise morfológica quando desafiadas com a atividade de Al de $30x10^{-6}$, revelou uma taxa de segregação mendeliana típica de 3:1 e 1:1 (tolerante : sensível), respectivamente, indicando que a tolerância, entre essas duas variedades, pode ser controlada por um único gene nuclear,

semidominate, denominado *Alm1*. Além disso, o mesmo estudo de Moon e colaboradores (1997) verificaram que cortes histológicos dos ápices radiculares da linhagem S1587-17 desafiados com Al, corados com hematoxilina, apresentaram uma destruição progressiva da sua estrutura indicando que o mecanismo operante na variedade Cat100-6 pode ser a exclusão do íon fitotóxico.

Em contrapartida, Sibov et al. (1999), utilizando o método BSA (*Bulk Segregant Analysis*) e RFLP (*Random Fragment Length Polimorfism*), concluiram que a característica de tolerância da Cat100-6 é controlada por dois genes, sendo que, um apresenta um efeito fenotípico maior que o outro. Além disso, foram localizados dois loci ligados à tolerância, um denominado *Alm1* e localizado no cromossomo 10, e o outro, denominado *Alm2* localizado no cromossomo 6.

O estudo realizado por Jorge e colaboradores (2001) evidenciou, através da coloração por hematoxilina, uma diminuição da quantidade de Al adsorvida pelas raízes da Ca100-6 após 24 horas de tratamento, similar ao que foi observado em outras variedades de milho (Pellet et al., 1995; Jorge e Arruda, 1997), indicando a existência de uma mecanismo de exclusão. Foi observado também que as duas variedades liberam malato de forma dose-dependente até 48h de tratamento (com atividade de Al^{3+} de $21x10^{-6}$) em contraste a liberação de citrato foi bem diferente nas duas variedades, sendo que a sua velocidade de liberação foi constante nas concentrações de Al testadas, mas a linhagem Cat100-6 liberou cerca de 3,5 vezes mais desse ácido orgânico que a linhagem S1587-17. Ainda no mesmo estudo, constatou-se que a calmodulina não está envolvida na tolerância ao Al, pois a taxa de exsudação do citrato não foi alterada na presença de inibidores da atividade da calmodulina.

Outro estudo realizado por nosso grupo correlacionou a quantidade de Al presente nos ápices radiculares e a exsudação de ácidos orgânicos (Jorge e Menossi, 2005). A variedade Cat100-6 quando tratada com antagonistas de canais iônicos liberou menos citrato e

conseqüentemente houve um aumento na quantidade de Al adsorvido em seus ápices radiculares. Entretanto, como mencionado anteriormente, a acumulação de Al na Cat100-6 foi menor que na S1587-17 quando a taxa de liberação de citrato era a mesma entre elas, indicando que existe outro mecanismo de resistência ao Al operando na variedade tolerante. A presença de La^{3+} (um bloqueador de canais catiônicos) não modificou a taxa de liberação de citrato, contudo, a quantidade de Al nos ápices radiculares era reduzido em até 70%, e essa redução é dose dependente, indicando que a liberação de ácidos orgânicos é insuficiente para a formação de complexos com todo o Al extracelular e até mesmo pequenas atividades de Al são capazes de interagir com esse canais e ativar a sua abertura.

No trabalho de Boscolo et al. (2003) as duas variedades foram utilizadas para investigar o efeito do Al na indução do estresse oxidativo. Foi observado que o Al induz, de forma dose-dependente e tempo-dependente, a formação de espécies reativas de oxigênio (ROS) e subseqüente oxidação de proteínas na linhagem S1587-17, mas não na linhagem Cat100-6. Essa oxidação de proteínas é posterior a inibição do crescimento radicular, indicando que o estresse oxidativo não é a causa primária para a inibição do crescimento radicular, mas provoca morte celular no ápice radicular da variedade sensível. Além disso, foi evidenciado que a peroxidação de lipídeos não é induzida por Al, indicando que os lipídeos não são o alvo celular primário do estresse oxidativo.

Recentemente, Piñeros et al. (2008) investigaram os efeitos no potencial de membrana de repouso das variedades Cat100-6 e S1587-17 quando desafiadas com 39µM de Al. Foi verificada uma despolarização de 50% (tratamento em relação ao controle) na Cat100-6 enquanto que o efeito foi de 10% na S1587-17. Esse efeito também foi específico ao Al, pois os resultados não foram reproduzidos pelo tratamento com o análogo La^{3+}. No mesmo estudo o gene *ZmALMT1* foi clonado a partir das duas variedades. Esse gene é ortólogo aos genes *ALMT1* de aveia e

AtALMT1 de Arabidopsis, sendo que, ambos já conferiram resistência ao Al em plantas transgênicas (Sasaki et al., 2004; Delhaize et al., 2004; Hoekenga et al., 2006). A partir da fusão da proteína ZmALMT1 com GFP foi determinado que a proteína de milho localiza-se na membrana plasmática. A análise electrofisiológica em oócitos de *Xenopus laevis* indicou que o gene *ZmALMT1* é um transportador seletivo de íons e não um transportador específico de ácido orgânico com o qual é sugerido que ele não está envolvido com a exsudação de citrato induzido por Al. Assim sendo, o principal transportador de citrato em resposta ao Al em milho ainda está por ser identificado.

Todos esses trabalhos demonstram que as variedades S1587-17 e Cat100-6 são bons modelos de estudo para evidenciar as alterações fisiológicas e transcripcionais acarretadas pelo tratamento com Al fitotóxico.

Análise da hidroponia como modelo experimental para avaliação da tolerância ao Al.

O ápice radicular é o sítio primário de ação do Al, já que o tratamento com o íon nos 2-3 mm iniciais da raiz é suficiente para inibir o crescimento radicular, algo que não acontece com tratamento nas demais regiões (Ryan et al., 1993). O ápice radicular é composto de diversos tipos celulares, cada um contendo uma função bem definida (Figura 2).

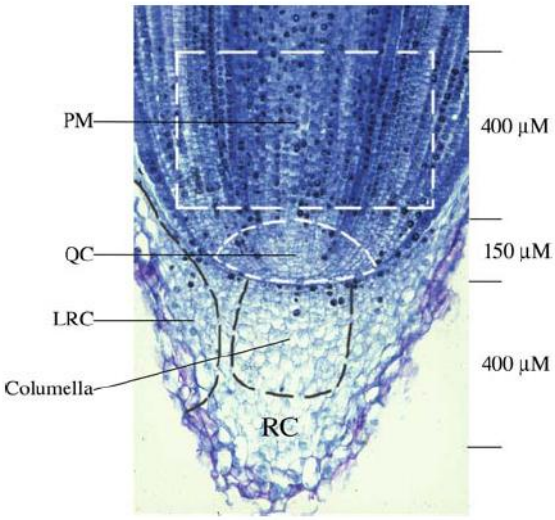

Figura 2. Diferentes regiões do ápice radicular. PM: meristema proximal; QC: centro quiescente; LRC: cap radicular lateral; RC: coifa (*cap*) radicular. À direita comprimento da região em µm. Figura extraída de Jiang et al. (2006).

As células da lateral do ápice radicular destacam-se e se distanciam da raiz e modificam suas funções de acordo com a sua posição até se desprenderem totalmente e assim serem denominadas células da borda. Essas células possuem um perfil de expressão bastante peculiar e muitas de suas proteínas são excretadas para o meio (Birgham et al., 1995) e são responsáveis pela interface entre o solo e a raiz, sendo as primeiras a perceberem modificações no solo (Baluska et al., 1996). Elas são as responsáveis por degradar o amido e sintetizar polissacarídeos de alto peso molecular que são ativamente secretados para fora da célula, através do complexo de Golgi, formando a mucilagem ao redor do ápice radicular ou coifa radicular. A mucilagem, as células da borda e seus exsudados correspondem a 98% do material liberado por raízes de milho saudáveis e são finamente regulados em resposta a sinais ambientais e endógenos (Hawes et al., 2000).

A coifa radicular está envolvida com a percepção de estímulos ambientais como luz, umidade e disponibilidade nutricional além de ser essencial para a manutenção da taxa de crescimento da raiz e para a formação de raízes laterais (Tsugeki e Federoff, 1999; Eapen et al.,

2003). A mucilagem tem como funções a lubrificação da raiz para diminuir o atrito com o solo, a retenção de água que impede a dissecação da raiz, a agregação do solo na rizosfera e a proteção contra elementos tóxicos como Al e Cd (Horst et al., 1982; Morel et al., 1986; Uren e Reisenauer; 1988; Morel et al., 1991; McCully e Sealey, 1996). Horst et al. (1982) demonstraram que 50% do Al total nos ápices radiculares de *Vigna unguiculata* estavam seqüestrados pela mucilagem e que sua remoção tornava as raízes mais sensíveis ao Al, pois a entrada do íon no tecido radicular era facilitada. Archambauldt et al. (1996) verificaram que em genótipos de trigo sensíveis ao Al a produção de mucilagem foi inibida mais rapidamente pela presença do íon quando comparada com genótipos resistentes.

A avaliação da variabilidade genotípica natural e de plantas transgênicas quanto à resistência ao Al é comumente feita em plântulas cultivadas em solução nutritiva aquosa (hidroponia) (Spehar, 1994; Wenzl et al., 2003; Delhaize et al., 2003; Anoop et al., 2003; Sasaki et al., 2004; Delhaize et al., 2004). Diversos estudos sobre métodos de distinção entre genótipos para programas de melhoramento procuram correlações entre avaliações de plantas cultivadas em hidroponia, em vasos com solo e no campo. Sartain e Kamprath (1978) e Noble et al. (1987) compararam o crescimento de plantas cultivadas em solo e em solução nutritiva aquosa e não verificaram correlação significativa entre os resultados. Baier et al. (1995) encontraram correlação positiva entre o comprimento de raízes desenvolvidas em solução e peso seco de raízes desenvolvidas em solo de 43 genótipos de aveia. Em milho, a distinção entre genótipos resistentes e sensíveis também foi possível, corroborando com os resultados de hidroponia (Urrea-Gomez et al., 1996). Vale ressaltar que nesses estudos apenas um tratamento é utilizado, ou seja, apenas o solo ácido sem que haja um controle com solo corrigido. Entretanto, alguns estudos demonstraram a eficiência da utilização de solo ácido e solo corrigido para avaliar o fenótipo com relação à tolerância ao Al em triticale, *Arabidopsis thaliana* e cevada (Ma et al.,

2000a; Kobayashi et al., 2005; Delhaize et al., 2009).

Não obstante, a utilização da hidroponia é criticada por não proporcionar condições para desenvolvimento da rizosfera (Marschener, 1991; Miyasaka e Hawes, 2001). A rizosfera é a camada de solo sob influência direta dos processos radiculares (ex. absorção de nutrientes, liberação de compostos) e que contém uma mistura complexa de microorganismos, células da borda e mucilagem (Miyasaka e Hawes, 2001). Marschener (1991) defende que na rizosfera desenvolvem-se condições locais específicas no que se diz respeito a pH, concentração de Al e exsudados radiculares. Em solução, a população de células da borda, a mucilagem da coifa radicular e outros materiais secretados pelas raízes são removidos da periferia do ápice radicular assim que são produzidas (Miyasaka e Hawes, 2001).

Um estudo de Miyasaka e Hawes (2001) atestou que plântulas de feijão com genótipos contrastantes para resistência ao Al suportavam maiores concentrações do íon quando cultivadas em agarose em comparação ao cultivo hidropônico. Os autores do estudo indicam a manutenção da estrutura espacial dos componentes da raiz, principalmente da mucilagem, como a responsável pela manutenção do crescimento radicular mesmo em altas doses de Al. Sendo assim, as células do ápice radicular trabalham em conjunto para a manutenção do crescimento radicular e a sinalização entre elas depende da manutenção da arquitetura do sistema e a hidroponia pode afetar a estabilidade dos componentes do ápice que protegem e sinalizam o tipo de estresse enfrentado durante o crescimento da raiz e a remoção dessas camadas protetoras possibilita que concentrações altíssimas de Al entrem em contato com as raízes provocando danos tão intensos que poderíamos passar a identificar respostas constitutivas de injúria, e não mais específicas ao estresse. Entretanto, Li et al. (2000) observaram que a inibição do crescimento de raízes de milho causada pelo Al era independente da presença ou da ausência de mucilagem nos ápices radiculares. Contudo, vale ressaltar que este ensaio foi executado em cultivo hidropônico, cuja

utilização pode afetar as propriedades da mucilagem, alterando sua ação protetora.

Baseado nestas observações, os estudos em hidroponia apresentam grande utilizadade para discriminar fenótipos de tolerância e susceptibilidade, bem como para análises de expressão gênica. No entanto, os estudos com genótipos de milho sensíveis e tolerantes ao Al quando cultivados em solo (sistema bem mais próximo à realidade do campo) certamente fornecerá informações importantes e relevantes para a compreensão da base genética e bioquímica da resistência ao íon. Como perspectiva a médio e longo prazo, esse conhecimento tem o potencial de ampliar as chances do cultivo agrícola em áreas de baixa fertilidade e com maior sustentabilidade, graças a uma possível redução do uso de insumos agrícolas e manejo do solo.

Identificação de genes induzidos por Al

A identificação de genes induzidos por Al indica influência em uma ampla gama de funções biológicas. Dentre os genes identificados como responsivos ao Al podemos citar fenilalanina amonia-liase, inibidores de proteinases e uma proteína semelhante às metalotioneínas (Snowden e Gardner, 1993), β-glucanase e uma proteína de citoesqueleto semelhante às fimbrinas (Cruz-Ortega et al. 1997), duas glutationa-S-transferases (Ezaki et al., 1995; Cançado et al., 2005), peroxidase (Ezaki et al., 1996), ATPase vacuolar e mitocondrial (Hamilton et al., 2001), receptores kinase (Sivaguru et al., 2003) fator de transcrição da família ART1 (Yamaji et al., 2009) e membros da família MATE (Maron et al., 2008; Maron et al., 2010).

O estresse oxidativo é observado em diversos estresses abióticos (Kochian et al., 1995) e assim, a indução de genes relacionados a produção ou combate de espécies reativas de oxigênio foram observadas após o tratamento com Al em diversas espécies vegetais como soja (Cakmak e

Horst, 1991), ervilha (Yamamoto et al., 2001), tabaco (Ikegawa et al., 2000), *Arabidopsis thaliana* (Sugimoto e Sakamoto, 1997; Richards et al., 1998), trigo (Snowden e Gardner, 1993; Cruz-Ortega et al., 1997; Hamel et al., 1998), cevada (Tamás et al., 2006), alho (Achary et al., 2008) e batata (Tabaldi et al., 2009). Boscolo et al. (2003) detectaram que em milho ocorre oxidação de proteínas em presença de Al, algo até então desconhecido em plantas, e que essa oxidação ocorre após a inibição do crescimento radicular, indicando que existem outros alvos da toxidez desse íon.

Vários desses genes induzidos por Al já foram avaliados em plantas transgênicas, conferindo diversos níveis de tolerância ao Al, como revisado por Mariano et al. (2005). É importante destacar que existem poucos relatos de avaliação da tolerância em solo ácido e solo corrigido (Sasaki et al., 2004; Delhaize et al., 2009) já que todos os demais genes foram avaliados em solução hidropônica, que difere consideravelmente da situação real de campo.

A tecnologia de chips ou microarrays de DNA tem sido amplamente utilizada para monitorar as alterações da expressão gênica global nos mais diversos organismos (Kawasaki et al. 2001; Seki et al. 2002; Ozturk et al. 2002). A empresa Affymetrix comercializa chips contendo 17.555 oligonucleotídeos para avaliar cerca de 14.850 transcritos de milho que representam 13.339 genes. As seqüências utilizadas para o microarray foram selecionadas do GenBank do NCBI (dados até 29 de setembro de 2004) e do *Zea mays* UniGene Build 42 (dados até 23 de julho de 2004). Esses bancos de dados representam cerca de 100 cultivares sendo que os mais representados são as linhagens B73, Ohio43, W22, W23, W64A e Black Mexican Sweet (http://www.affymetrix.com/products/arrays/specific/maize.affx). Cada oligonucleotídeo de 25 bases é desenhado para ser capaz de distinguir uma seqüência dentre bilhões de seqüências similares, permitindo identificar apenas um único transcrito e assim rejeitar alvos que não são idênticos, com o qual é possível distinguir os níveis de expressão entre genes de uma mesma

família multigênica. Além disso, para cada transcrito são desenhados 15 oligonucleotídeos dispostos de forma aleatória no array permitindo que mesmo com erros experimentais toda a informação sobre um gene não seja perdida. Para cada seqüência alvo desenhada, o array apresenta uma seqüência *mismatch* que contem um único nucleotídeo trocado no meio da seqüência servindo para a medição de hibridização inespecífica e assim medir a fluorescência do pareamento perfeito com mais credibilidade. Adicionalmente, a química da hibridação está muito bem estabelecida, com kits e controles para as diversas etapas do processo.

Che et al. (2002) utilizando o chip da Affymetrix verificaram que existe variação do perfil de expressão em diferentes estágios de desenvolvimento de caules regenerados de raízes de *Arabidopsis thaliana* em meio de cultura. Cheong et al. (2002) estudaram o efeito dos ferimentos no padrão de expressão de mRNA e observaram que 20% dos genes diferencialmente expressos codificavam proteínas que apresentavam funções na transdução de sinal e regulação gênica. Com essa técnica foi possível verificar as diferenças no perfil de expressão de células vegetais nas diferentes fases da cultura de tecido de milho (Che et al. 2006) e em *Arabidopsis thaliana* (Pinschke et al. 2006). Jiang et al. (2006) verificaram grande variação do perfil de expressão gênica nas três principais porções do ápice radicular (meristema proximal, centro quiescente, e coifa radicular) em milho utilizando chip de oligonucleotídeos de arroz identificando genes centrais para a função da coifa radicular. Outro estudo em milho verificou que genótipos mutantes para a produção e acumulação de zeínas em endosperma (mutações opaco) apresentavam alterações gênicas pleiotrópicas quando comparadas com mutantes para a síntese de amido (Hunter et al., 2002).

A utilização de linhagens contrastantes para um determinado estresse é importante durante o processo de identificação de genes responsáveis pela resistência como demonstrado por Walia et al. (2005) que identificaram diversos genes possivelmente envolvidos na resposta à salinidade,

já que os dois genótipos de arroz contrastantes apresentaram padrões de indução bem distintos. Loreti et al. (2005) utilizaram o array de genoma completo ATH1 da Affymetrix para caracterizar genes induzidos por anóxia em *A. thaliana*. Já foram executados estudos visando descobrir genes potencialmente importantes para a resistência a estresses como submersão, frio, salinidade, seca e dissecação utilizando linhagens sensíveis e tolerantes (Zhang et al. 2006; Wong et al. 2006; Buitink et al. 2006).

A elucidação em larga escala de genes envolvidos com a tolerância ao alumínio foi atestada em alguns estudos. Guo e colaboradores (2007) utilizando linhagens isogênicas de trigo contrastantes quanto à resistência ao Al, identificaram 28 genes diferencialmente expressos como transportador de malato, β-glucosidase, lectina e histidina kinase. Kumari et al. (2008) verificaram que mais transcritos de *A. thaliana* eram responsivos ao Al (25 μM de Al) durante longos tratamentos (48h – 1114 genes) quando comparado com o tratamento de curta duração (6h – 401 genes). A exposição ao Al induziu a expressão de diversas proteínas ribossomais, peptidades e proteinas fosfatases (principalmente após 48h de tratamento). Outro dado interessante foi a falta da detecção de enzimas do ciclo do ácido tricarboxílico (exceto a malato desidrogenase) sugerindo que a síntese de ânions orgânicos podem não ser regulados pela exposição ao Al. Além disso, receptores de membrana e fatores de transcrição também foram identificados.

Recentemente, Maron e colaboradores (2008) investigaram o efeito do Al no perfil transcripcional de duas variedades contrastantes de milho (a variedade tolerante é a mesma utilizada no presente trabalho) quando submetidas a tratamentos de curto período (0, 2, 6 e 24h) através dos microarrays fornecidos pelo Maize Oligonucleotide Array Project (www.maizearray.org). Os genes diferencialmente expressos foram divididos em grupos de acordo com o seu papel fisiológico. Dentre os genes relacionados com a estrutura e composição

da parede celular, a pectina metilase foi mais expressa nos tratamentos de 2h nos dois genótipos sendo que o aumento foi mais significante na variedade sensível e pode estar relacionado com o maior acúmulo de Al na parede. A laccase ou difenol oxidase, responsáveis pela biossíntese de lignina, também foi mais expressa na variedade sensível, indicando que a deposição de lignina em virtude da exposição ao Al pode causar a inibição do crescimento radicular.

Dos genes relacionados com estresse oxidativo, a glutationa S-transferase foi superexpressa nos dois genótipos ou de forma transiente na variedade sensível. Já a superóxido dismutase foi transientemente expressa na variedade tolerante. A ferritina 1 e tioredoxina h genes relacionados com a proteção do dano oxidativo, foram mais expressos na variedade sensível mostrando que a tolerância da Cat100-6 ocorre antes do estresse oxidativo, provavelmente devido à algum mecanismo de exclusão. Também foi identificado, na variedade Cat100-6, a expressão genes que respondem a baixo teor de fosfato, como as fosfatases ácidas, indicando que a tolerância também pode estar relacionada com a imobilização do Al pelo fosfato. Não houve modificação na expressão de genes relacionados com a síntese de ácidos orgânicos, mas o padrão de expressão de transportadores de ácidos orgânicos está associado à maior tolerância ao Al em Cat100-6.

Cabe destacar que todos os estudos de expressão gênica foram feitos em hidroponia. A proposta deste estudo envolve a utilização da técnica de chip de oligonucleotideos possibilitará a comparação do perfil transcripcional entre variedades sensíveis e resistentes de milho (cultivadas em solo) e certamente revelará uma visão mais próxima da realidade e que, futuramente, através de ensaios em plantas transgênicas, (superexpressão, produção de *knockout*), podem ser úteis na produção de plantas mais tolerantes ao Al.

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OBJETIVOS

O presente trabalho tem como objetivos:

- Avaliar os efeitos do cultivo em solo ácido na estrutura e fisiologia de raizes de milho de genótipos contrastentes para a tolerancia ao Al;
- Determinar o padrão de expressão global de transcritos das raízes e folhas de milho de genótipos contrastantes para a tolerância ao Al cultivadas em solo ácido;
- Integrar respostas fisiológicas com os perfis de expressão.

APRESENTAÇÃO DO TRABALHO

O presente trabalho está dividido em três Capítulos.

Capítulo I – Manuscrito do artigo submetido à BMC Plant Biology intitulado: "Transcriptional profile of maize roots under acid soil growth".

Capítulo II – Localização cromossômica e análise de QTL dos genes diferencialmente expressos nas raízes de milho submetidas ao cultivo em solo ácido.

Capítulo III – Manuscrito do artigo intitulado: "Transcriptional profile of maize leaves under acid soil growth".

CAPÍTULO I

Transcriptional profile of maize roots under acid soil growth

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BMC Plant Biology, Submetido

Transcriptional profile of maize roots under acid soil growth

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Running title: Transcriptional profile of maize roots under acid soil growth

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Supplementary Materials: All the supplementary data are available in the annexed CD.

Abstract

Background

Aluminum (Al) toxicity is one of the most important yield-limiting factors of many crops worldwide. The primary symptom of Al toxicity syndrome is the inhibition of root growth leading to poor water and nutrient absorption. Al tolerance has been extensively studied using hydroponic experiments. However, unlike soil conditions, this method does not address all of the components that are necessary for proper root growth and development. In the present study, we grew two maize genotypes with contrasting tolerance to Al in soil containing toxic levels of Al and then compared their transcriptomic responses.

Results

When grown in acid soil containing toxic levels of Al, the Al-sensitive genotype (S1587-17) showed greater root growth inhibition, more Al accumulation and more callose deposition in root tips than did the tolerant genotype (Cat100-6). Transcriptome profiling showed a higher number of genes differentially expressed in S1587-17 grown in acid soil, probably due to secondary effects of Al toxicity. Genes involved in the biosynthesis of organic acids, which are frequently associated with an Al tolerance response, were not differentially regulated in both genotypes after acid soil exposure. However, genes related to the biosynthesis of auxin, ethylene and lignin were up-regulated in the Al-sensitive genotype, indicating that these pathways might be associated with root growth inhibition. By comparing the two maize lines, we were able to discover genes up-regulated only in the Al-tolerant line that also presented higher absolute levels than those observed in the Al-sensitive line. These genes encoded a lipase hydrolase, a retinol dehydrogenase, a glycine-rich protein, a member of the WRKY transcriptional family and two unknown proteins.

Conclusions

This work provides the first characterization of the physiological and transcriptional responses of maize roots when grown in acid soil containing toxic levels of Al. The transcriptome profiles highlighted several pathways that are related to Al toxicity and tolerance during growth in acid soil. We found several genes that were not found in previous studies using hydroponic experiments, increasing our understanding of plant responses to acid soil. The use of two germplasms with markedly different Al tolerances allowed the identification of genes that are a valuable tool for assessing the mechanisms of Al tolerance in maize in acid soil.

Background

Acid soils are the most important cause of low yield for many crops [1]. About 30% of the world's soils are acidic, and 60% of them are in tropical and subtropical areas associated with long periods of hot and moist weather [1]. Soil acidification is an increasing problem in the United States and Europe because of acid rain, removal of natural plant coverage from large production areas and the use of ammonium-based fertilizers [2]. One of the major problems caused by soil acidification is aluminum (Al^{3+}) phytotoxicity. Al^{3+} is the principal component of mineral soils and is present in a wide range of primary and secondary minerals [3]. In soils with pH above 5, Al^{3+} is precipitated predominately in gibsit form ($Al(OH)_3$) and has no phytotoxic effect. At lower pH, $Al(OH)_3$ is solubilized and Al^{3+} is released.

The most evident symptom of Al toxicity is the inhibition of root growth. In maize root tips, Al induces a rapid change in cell number and positioning [4], and recent evidence suggests that DNA damage and interference with cell-cycle progression and cell differentiation are the primary causes of root growth inhibition due to Al toxicity [5]. Other reported effects of Al exposure are the disruption of Ca^{2+} homeostasis [6, 7], increased ACC oxidase activity with a

consequent increase in ethylene production [8], Al binding to cell wall polysaccharides [9,10] and reduced membrane fluidity [11].

To cope with Al stress, plants activate exclusion and tolerance mechanisms [1]. Exclusion mechanisms take place outside the roots and prevent the entry of Al into the cell. These mechanisms include cell wall Al immobilization, increased selective permeability of the plasma membrane, rhizosphere pH barrier formation and quelling by exudates such as organic acids and phenolic compounds [1, 12-15]. Tolerance mechanisms are active after Al enters the cell – Al ions can be quelled in the cytosol, compartmentalized inside the vacuole or proteins that bind directly to Al may become highly expressed [12, 16, 17].

Among all of the proposed mechanisms, organic acid release is the most well-characterized resistance strategy used by plants. Since the first report demonstrating Al-induced malate secretion in wheat [18], several research groups have observed that organic acid exudation is higher in tolerant than sensitive genotypes in species such as snap beans [19], wheat [20] and maize [21-24]. However, in maize and wheat, organic acid release does not correlate with resistance in all genotypes, indicating that other mechanisms, such as active Al exclusion, may also play a relevant role [25-27]. Similarly, Maron et al. [28] and Kumari et al. [29] recently demonstrated that tolerance in maize and Arabidopsis is not associated with increased expression of genes encoding enzymes responsible for organic acid biosynthesis, but rather with differential expression of their transporters.

The identification of genes related to Al tolerance has indicated that a plethora of biological functions are influenced by this ion. With the advent of cDNA arrays, the evaluation of global gene expression changes in response to Al stress allowed the identification of a broader number of genes that are modulated by this ion [28-34]. Guo and colleagues [34] used isogenic lines of wheat with differential tolerance to Al and identified 28 differentially expressed genes,

including malate transporters, a β-glucosidase, lectin and a histidine kinase. Kumari et al. [29] reported that exposure to Al induces several ribosomal protein genes, peptidases and phosphatases. Maron et al. [28] compared gene expression in two maize genotypes with contrasting Al tolerance and found that several genes involved in processes such as cell wall remodeling, response to oxidative stress and Pi starvation were differentially regulated.

While the identification of genes related to Al stress has led to a greater understanding of plant responses to this ion, these studies have been conducted mostly using hydroponic culture. This growth condition may not adequately mimic the soil environment with respect to rhizosphere development [35, 36], which involves a complex mixture of microorganisms, border cells and mucilage [36]. Several other studies have addressed the role of mucilage in the detection and avoidance of Al toxicity. Horst et al. [37] demonstrated that 50% of all Al in root apexes of *Vigna unguiculata* is sequestered by the mucilage layer, and its removal increases root sensitivity to Al [37]. Similarly, Archambauldt et al. [38] found that mucilage production by sensitive varieties of wheat was inhibited more rapidly than that by tolerant varieties when exposed to phytotoxic levels of Al [38]. Similarly, Miyasaka and Hawes [36] found evidence that in snap beans, border cells are involved in detecting and avoiding Al toxicity. By contrast, Li et al. [39] observed that root growth inhibition in maize was not affected by the removal of root mucilage. These findings indicate that different species, or even different varieties of the same species, can present distinct resistance and/or tolerance mechanisms. Therefore, evaluating Al tolerance in conditions that are similar to those in the field may provide a better understanding of the mechanisms required to avoid Al toxicity.

Here we present an analysis of transcriptome changes in two maize varieties with contrasting levels of Al tolerance, using acid soil as the growth substrate. Our analysis identified genes in several metabolic pathways whose expression was modified when plants were growth in

acid soil. While we found some Al-responsive genes previously identified in studies carried out in hydroponic growth conditions, growth in acid soil clearly also triggered a new suite of physiological and transcriptional responses not previously reported. Taken together, our results offer a more complete picture of the transcriptomic changes imposed by acid soils, and they may lead to the discovery of novel genes involved in Al tolerance.

Results

Physiology of maize seedlings grown in acid soil

Most recent studies aiming to characterize plant transcriptomic or proteomic responses to Al exposure have used hydroponic culture [28, 29, 40]. In the present study, soil was used as the substrate to better mimic field conditions and to allow the maintenance of all root apex components and root architecture. Plants were grown in Dark Red Latossol (pH 4.1) with an Al content of 10 $mmol_c/dm^3$. As a control, the same soil was used, but with pH corrected to 5.5 (see Materials and Methods). Two lines used in previous studies that evaluated Al tolerance in hydroponic growth conditions were characterized: Cat100-6 (Al-tolerant) and S1587-17 (Al-sensitive) [25, 28, 33, 41-44]. S1587-17 is a somaclonal variant regenerated from a callus culture of Cat100-6 [42]. Relative root growth (RRG) was measured after one and three days of growth in soil. The Al-sensitive plants were severely affected by acid soil at both time points, while the Al-tolerant plants were affected only after three days and to a significantly lower extent (Figure 1). Both maize genotypes had higher levels of callose when grown in acid soil, a response typically correlated with Al stress [45-47]. However, the increase in callose content was significantly higher in the Al-sensitive line (Figure 2). To investigate whether the root inhibition and callose accumulation were due to Al phytotoxicity associated with acid soil, the Al absorbed by root tips was quantified after one and three days of soil exposure. Figure 3 demonstrates that the sensitive

line S1587-17 had significantly higher amounts of Al than the Al-tolerant variety. These results indicate that these maize varieties have different physiological responses to acid soil and that these responses are most likely due to the presence of Al. Nevertheless, we cannot exclude the possibility that pH also contributes to plant responses during acid soil exposure.

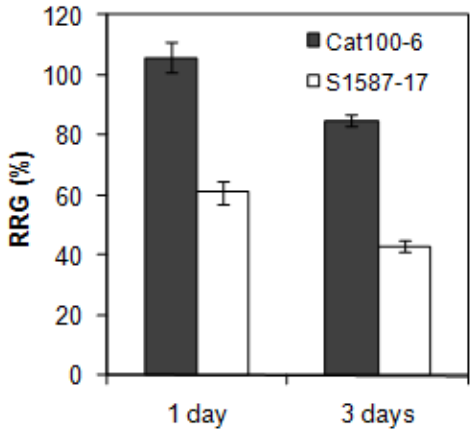

Figure 1. Effect of acid soil saturated with Al^{3+} on maize root growth. Plants were grown in acid (pH 4.2) or control (pH 5.5) soil for one or three days. The growth is relative to the control soil (pH 5.5). Vertical error bars represent mean ± SE (n=20). The difference between the two lines in each treatment was significant at $p < 0.05$.

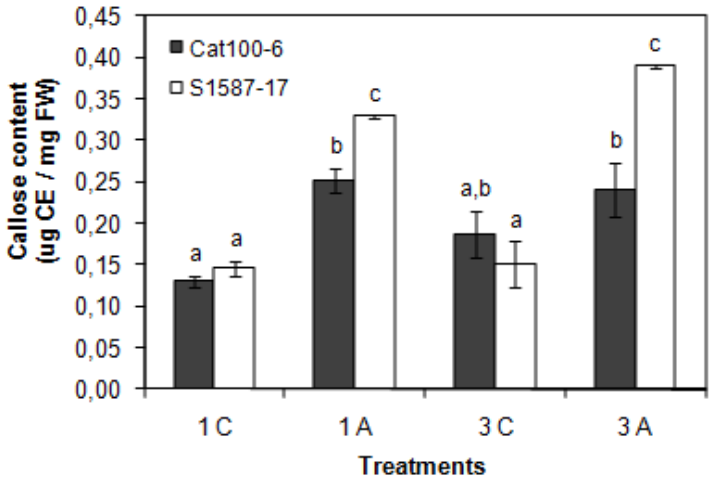

Figure 2. Acid soil-induced callose deposition in root tips. Each bar represents the callose content of root apexes grown on acid soil or control soil. C: control soil; A: acid soil; one or three days of treatment. Each quantification refers to the mean ± SD (n=20). The experiment was done in duplicate. Means with different letters are significantly different (p<0.05) from each other.

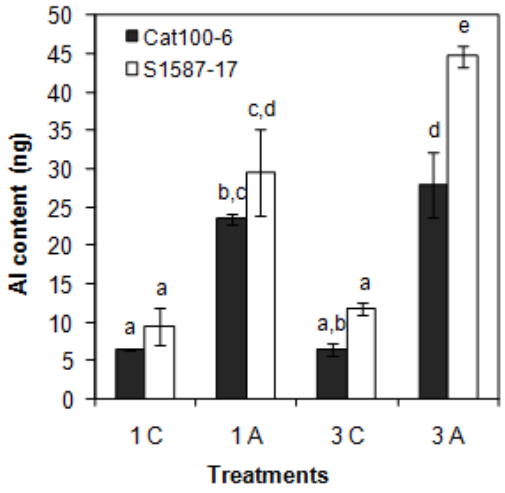

Figure 3. Al^{3+} quantification in soil grown maize seedlings. The experiment was done using the first 5 mm of 10 root tips. C: control soil; A: acid soil; one or three days of treatment. Bars refer to the mean ± SD of the Al content of 10 root tips (n= 2). The means with different letters are significantly different ($p<0.05$) from each other.

Gene Expression Profiling

The Affymetrix GeneChip® Maize Genome Array was used to evaluate the transcriptional response of the two contrasting maize genotypes to growth in acid soil. Analysis of variance (ANOVA) was used to dissect the transcriptional responses associated with the individual maize lines (independent of soil treatment or time of exposure), time of collection (1 and 3 days), soil type (control or acid soil treated) and all possible interactions (see Materials and Methods). In the Al-tolerant line (Cat100-6) exposed to acid soil for one day, only eight genes were differentially expressed compared to plants grown in control soil (Figure 4A). The number of differentially regulated genes increased (59) after three days of treatment (Figure 4B). However, the Al-sensitive maize line showed a significantly higher number of differentially expressed genes. On the first day, 339 genes were differentially regulated (Figure 4C), while 776 were affected by the treatment on the third day (Figure 4D). The genes that were differentially regulated under all conditions are described in the Supplemental Material (Tables S1-S4), and the number of genes up- or down-regulated in each genotype and at each time point is shown in Figure 5.

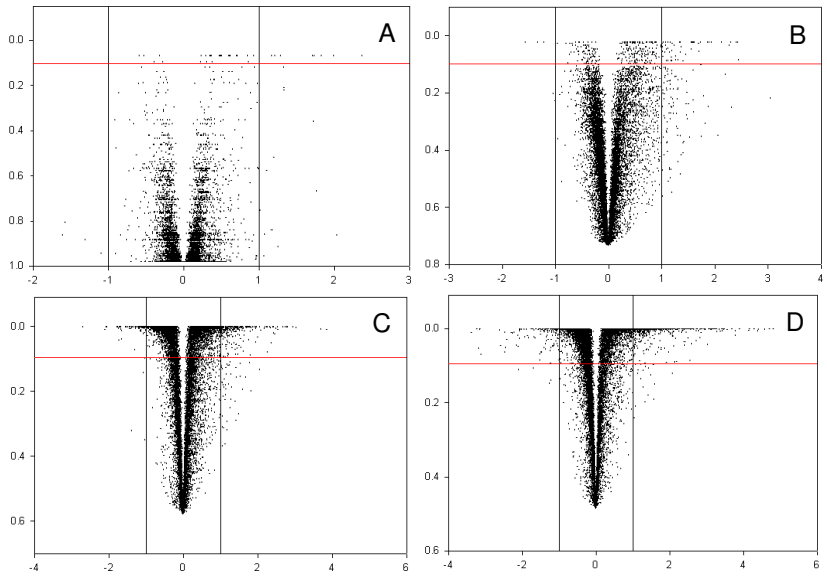

Figure 4. Volcano plots representing interactions of various effects. Estimates were calculated as the difference between the least-square means for each comparison (x-axis). Estimates equal to zero represent no expression change and estimates different from zero represent gene expression modifications. A: interaction effect between genotype, treatment and time for Cat100-6 one day; B: interaction effect between genotype, treatment and time Cat100-6 three days; C: interaction effect between genotype, treatment and time S1587-17 one day; D: interaction effect between genotype, treatment and time S1587-17 three days. The red line represents an FDR of 10%, and consequently data points above this line represent significant observations (the y-axis represents Qvalues). Note that the Estimate axis is different for each plot.

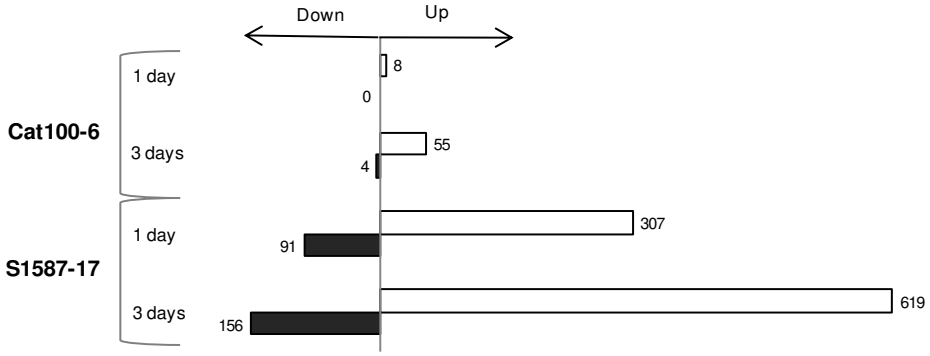

Figure 5. Diagram representing the number of genes differentially expressed in each genotype at each time point.

Figure 6 depicts the number of genes differentially regulated between acid and control soil conditions that are unique or shared between S1587-17 and Cat100-6 at each time point. All of the eight genes differentially regulated on day 1 in Cat100-6 were up-regulated, and they were also detected after three days of acid soil exposure. Only two genes were exclusively detected in the

Al-tolerant line: Zm.10003.1.A1_at (no hit) and Zm.19066.1.S1_at (glutamate decarboxylase). Five genes were differentially expressed in both S1587-17 and Cat100-6 at one and three days: Zm.125.1.S1_at (nitrate reductase), Zm.17306.1.A1_at (multidrug resistance protein / phosphate import ATP-binding protein pstB 1), Zm.11852.1.A1_a_at (no hit), Zm.13437.2.S1_at (fructose-bisphosphate 1-phosphohydrolase) and Zm1416.1.S1_at (mitochondrial 2-oxoglutarate / malate carrier protein).

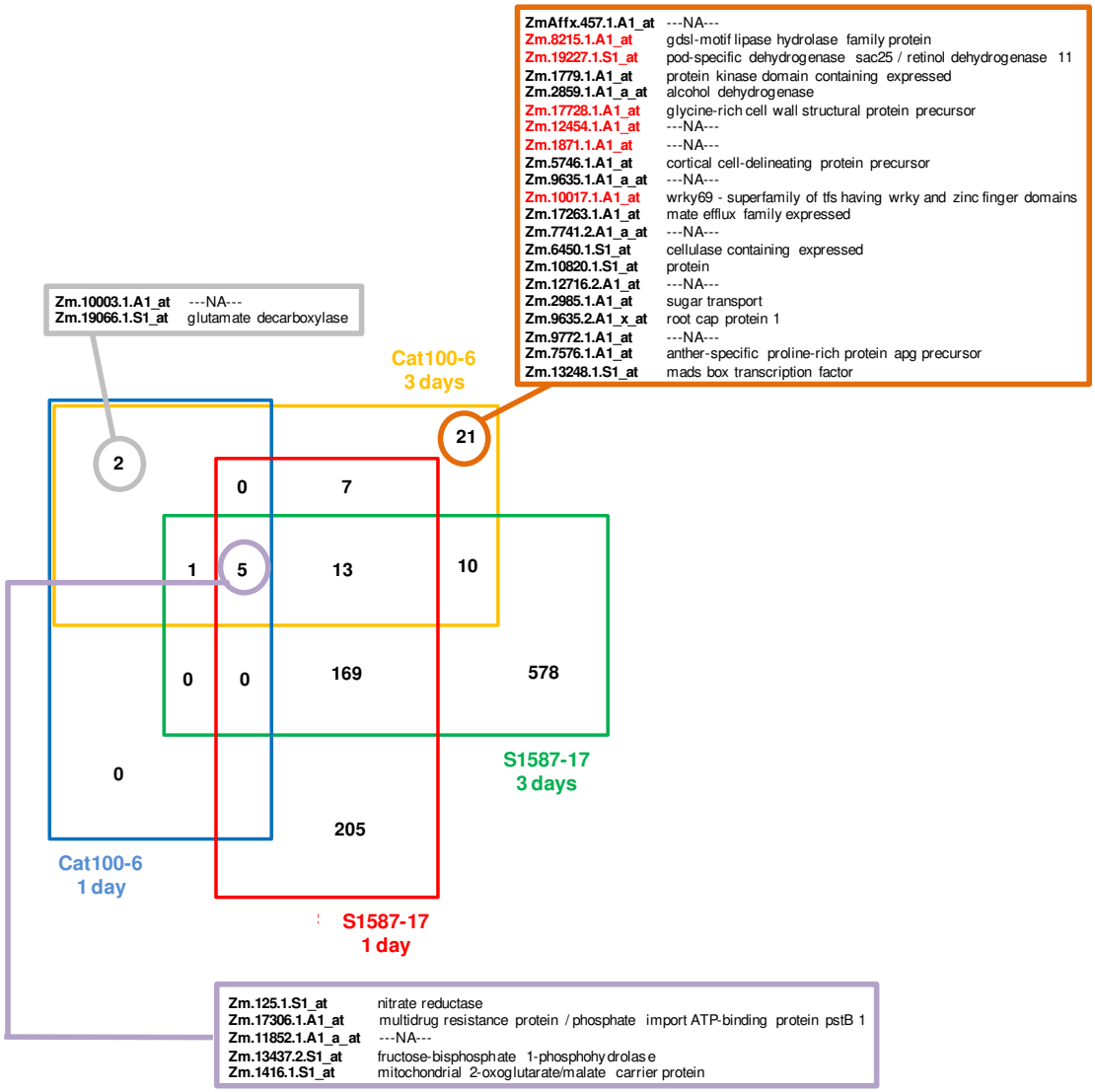

Figure 6. Multiple comparison Venn diagram. Each quarter represents a list of IDs of genes differentially expressed in the comparison between acid and control soils for each maize variety. The boxes indicate the Affymetrix ID and the annotation of the genes identified only in Cat100-6 (both time points – grey box), in all genotypes and time points (purple box) and genes identified only in Cat100-6 after three days (orange box - the genes identified in red are those that also presented significantly higher expression values in Cat100-6 in the comparison between genotypes after three days in acid soil).

To further evaluate the quantitative extent by which genes were differentially regulated between the two maize lines, the difference in the least-square means estimates (DEs) of gene expression levels between Cat100-6 and S1587-17 grown in acid soil were calculated at each time point (e.g., Cat100-6 in acid soil for three days versus S1587-17 in acid soil for three days), and the statistical significance was assessed. We aimed to identify genes induced by acid soil in Cat100-6 that also had significantly higher absolute levels than in S1587-17. We observed that none of the genes differentially expressed in Cat100-6 after one day of acid soil treatment presented significantly higher expression levels than in S1587-17 under the same conditions. However, eight genes out of the 59 differentially expressed in Cat100-6 after three days were also significantly more highly expressed relative to S1587-17 growing in acid soil for three days. Six of these genes are in the group of 21 exclusively identified as differentially regulated in Cat100-6 after three days (Figure 6), while the other two were also differentially expressed in S1587-17 after three days (Zm. 3371.1.A1_at – O-methyltransferase and Zm.8742.1.A1_at – unknown protein).

Due to the large number of genes identified in the Al-sensitive maize, a functional analysis was performed with Gene Ontologies to help identify the pathways affected by the toxicity imposed by acid soil. Most of the genes up-regulated on the first day are involved in lipid metabolism, oxidative stress responses and cell wall metabolism (Figure S2A – Supplemental Material 1). Most of the genes down-regulated on the first day encode proteins involved in DNA packaging and cell cycle (Figure S2B – Supplemental Material 1). Most of the genes up-regulated on the third day are involved in cell wall metabolism, oxidative stress responses and anionic transport (Figure S3A – Supplemental Material 1). On the other hand, most of the repressed genes are involved in protein metabolism (Figure S3B – Supplemental Material 1).

Validation of gene expression profiles using qPCR

To validate the microarray results, eleven differentially expressed genes were selected and real-time qPCR was performed (Figure 7). This validation was done with two independent biological replicates (different from the replicates used for the microarray experiment). A significant correlation between the two data sets was observed ($R^2 = 0.8812$).

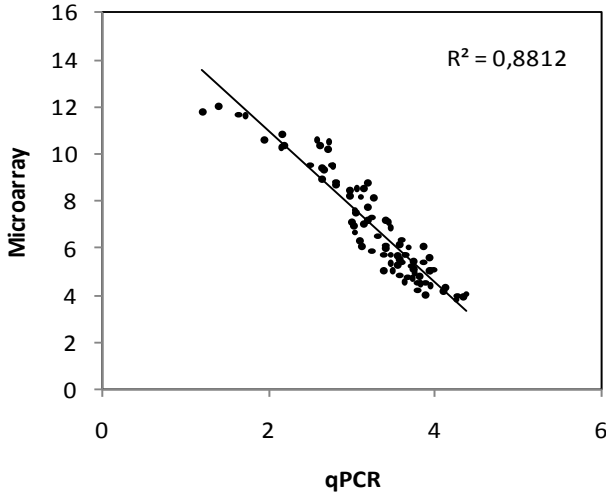

Figure 7. Real-time qPCR validation of the microarray results. The qPCR data were log2 transformed and plotted against the microarray data (least-square means). The correlation is negative because in the qPCR data, the more the gene is expressed the lower is its Ct value.

Comparison to gene expression response to Al treatment in hydroponic culture

The soil treatment used in this work has two major variables that must be considered, the presence of phytotoxic Al and the pH. However, it is not possible to separate these properties in the soil or even use a different acid soil with no Al because diversity in the physical and chemical characteristics would affect the results. Therefore, hydroponic culture has been used to evaluate the effects of pH and Al levels on the expression of selected genes. Six out of the eight acid soil-induced genes from Cat100-6 that also had absolute levels higher than those of S1587-17 were used in an experiment consisting of three treatments: pH 5.5; pH 4.2 with no Al and pH 4.2 with 36 µM Al. The effect on gene expression in Cat100-6 seedlings is illustrated in Figure 8A (pH-

effect, using pH 5.5 as the reference). The relative expression in seedlings grown in pH 4.2 with Al relative to pH 4.2 without Al (Al-effect) is shown in Figure 8B. Genes such as Zm.8215.1.A1_at (GDSL-motif lipase hydrolase family protein), Zm.17728.1.A1_at (glycine-rich cell wall structural protein precursor) and Zm.12454.1.A1_at (protein with unknown function) showed no significant differential regulation under treatment with pH 4.2 in the presence or absence of Al or between pH 4.2 and pH 5.5 treatments , suggesting significant differences between the gene expression profiles from the hydroponic and soil experiments. However, Zm.19227.1.S1_at (Pod-specific dehydrogenase SAC25 / retinol dehydrogenase 11), Zm.1871.1.A1_at (protein with unknown function) and Zm.10017.1.A1_at (WRKY 69 transcription factor) were up-regulated in the presence of Al, indicating that Al and not pH was the main factor behind their induction in the soil treatment.

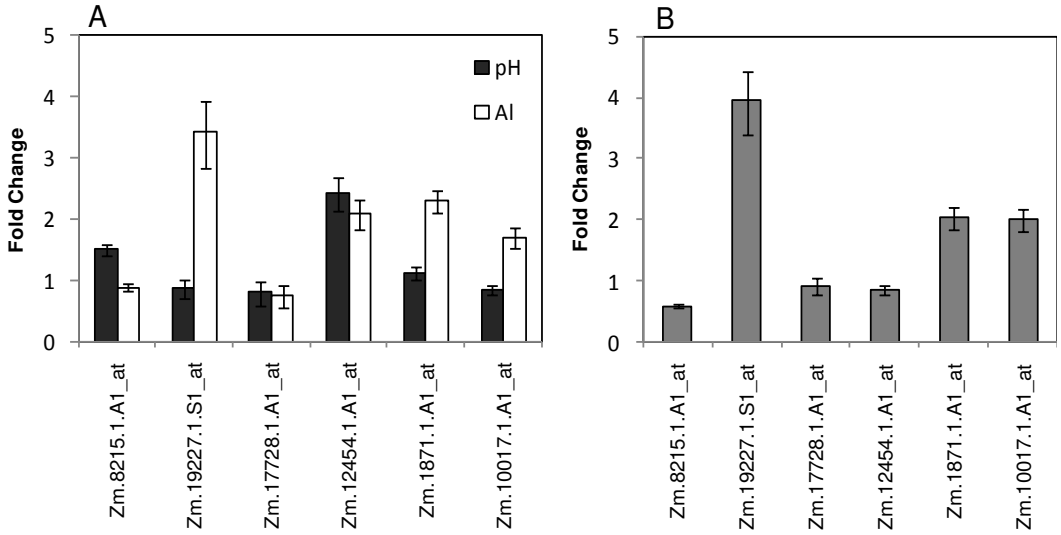

Figure 8. Expression of selected genes after hydroponic culture. (A) Data show the relative expression (in fold change) in relation to pH 5.5; (B) Data show the relative expression (in fold change) of the treatment with pH 4.2 plus Al versus pH 4.2 without Al. The results are from three independent biological replicates.

To further highlight the different responses of maize when grown in hydroponics versus soil, we compared the target sequences from the Affymetrix platform used in this work with the target sequences from Maron et al. [28]. We observed that only a fraction of the genes modulated

by Al in Cat100-6 grown in the hydroponic experiment were also modulated in acid soil-grown plants: of the 59 genes differentially expressed in Cat100-6 grown in soil, only six were also found in the hydroponic assay. We also compared our data from S1587-17 with the data from the Al-sensitive line L53 because in the work of Maron et al., [28] S1587-17 was not used. In this case, the differences were even higher because only 34 out 952 genes modulated in S1587-17 were common to both gene sets. A complete list of the genes found in both experiments is shown in Table S5.

Discussion

Physiology of maize roots grown in acid soil

Plant tolerance to Al is usually evaluated using hydroponic culture [48-53], but conflicting results may be obtained when compared to soil conditions [54, 55]. Even when Al tolerance is consistent between hydroponic and soil conditions [56-60], plant responses may differ in the two growth systems. To address this limitation, we evaluated maize tolerance to acid soil containing 10 $mmol_c/dm^3$ (equivalent to 90 ppm) of Al and at pH 4.2. These conditions are within the range of previous studies and are sufficient to elicit a phytotoxic response [35, 61-63], allowing phenotypic discrimination between two maize lines with distinct levels of resistance to Al, Cat100-6 (tolerant) and S1587-17 (sensitive). As shown in previous studies using these and other maize lines [25, 28, 39-42, 46], Cat100-6 accumulated less Al in its root tips when grown in acid soil when compared to S1587-17. The amount of Al absorbed by root tips is indicative of the sensitivity of plants to this abiotic stress [13, 64, 65], presumably because genotypes that accumulate less Al in their root apexes have a more efficient exclusion mechanism. However, after prolonged exposure (3 – 5 days), the amount of Al in Cat100-6 roots continued to increase (although at a lower rate than in S1587-17), in disagreement with previous studies that reported a

reduction in Al accumulation in root tips after 24 hours in hydroponics [28, 41]. Therefore, the exclusion mechanism of Cat100-6 appears to have less activity in soil than in hydroponic conditions. Nonetheless, Al-induced root growth inhibition and callose formation were markedly more limited in Cat100-6 than in S1587-17.

Gene expression profiles of an Al-tolerant and an Al-sensitive maize line

The abiotic stress caused by the toxicity of acid soil was clearly reflected in the gene expression profiles. The number of genes differentially regulated between control and treated (acid soil) plants increased in both Cat100-6 and S1587-17 when they were exposed to the stress for an extended period. It is worth mentioning that these changes observed in the transcriptome certainly reflect both direct and indirect effects of the stress caused by acid soil. In the field, where roots continuously grow and explore different soil regions, this is also certainly true. Interestingly, a smaller fraction of the transcriptome responded to acid soil stress in the resistant line Cat100-6 than in S1587-17, similarly to previous reports using Al and hydroponics [28, 33, 66]. Therefore, part of the resistance of Cat100-6 (and Al-tolerant plants generally) may involve a mechanism that limits major disturbances to plant function and thereby avoids a cascade of detrimental downstream effects.

Genes that are modulated by a stress are natural candidates for explaining the defenses activated by plants. By using contrasting genotypes it is possible to narrow the scope to genes with a higher probability of playing major roles in the plant response. Therefore, we selected eight genes that were more highly expressed in Cat100-6 than in S1587-17 under acid soil conditions. Six of these genes were also found to be induced by acid soil only in Cat100-6, while two were also induced in S1587-17, although to a lesser degree. We further characterized those six genes that were specific to Cat100-6, which encoded a GDSL-motif lipase hydrolase family protein, a

pod-specific dehydrogenase / retinol dehydrogenase 11, a glycine-rich protein (GRP), a member of the WRKY transcriptional factor family and two unknown proteins. However, the hydroponic experiment demonstrated that only three of these genes have interesting expression patterns (up-regulated during Al treatment – Figure 8). Although further work will be needed to assess the individual contribution of each gene to acid soil tolerance, these data give insight into the strategies used by plants in fields where this stress takes place.

Zm.19227.1.S1_at (Pod-specific dehydrogenase / retinol dehydrogenase 11) was first described as a gene involved in rape oilseed pod development [67]. Members of this family may function as a bridging molecule between the nutritional signaling pathway and the hormone biosynthesis pathway in Arabidopsis [68]. This member is associated with ABA production and is critical for growth and development, and also for plant responses to stress via glucose signaling [68].

The WRKY family of transcription factors was first identified in plants and presents a high number of members [69]. About 70 members have been indentified in Arabidopsis, and several of them are induced in response to wounding, pathogen infection and abiotic stresses such as drought, cold and salinity [31, 69-71]. Our microarray experiment indentified Zm.10017.1.A1_at (WRKY69) as differentially expressed in Cat100-6 after three days of acid soil treatment and also as presenting higher expression than in the Al-sensitive S1587-17 genotype. Hydroponic gene expression experiments also demonstrated that Al induces the expression of this gene. Kumari and colleagues [29] identified two WRKY family members as being down-regulated after exposure of Arabidopsis to Al. Meanwhile, Goodwin and Sutter [72], also studying Arabidopsis, identified WRKY 33 as up-regulated due to Al treatment. An additional study identified another WRKY member as up-regulated due to Al and Cd stress. Using the same tolerant variety (Cat100-6) Maron et al. [28] identified two WRKY family members after 6 h of Al treatment. These results

suggest that this transcription factor may be involved in the regulation of other genes that contribute to acid soil tolerance in plants.

Zm.1871.1.A1_at is a protein with unknown function that was up-regulated in acid soil and in hydroponics, indicating that Al rather than low pH is the inducer. Interestingly, this protein has a conserved domain typical of methyltransferases (MTase), which are responsible for methylation of several cellular components such as DNA, RNA, proteins and also small molecules [73]. These enzymes may also play important roles in disease resistance, growth and development [74]. Studies with rice [75], Arabidopsis [76] and tomato [40] have also identified members of this family as up-regulated after Al treatment. This is the first study to detect a potential role in acid soil tolerance for this maize protein.

A comparison of the transcriptional profile of roots grown in soil (this work) with that of roots grown hydroponically [28] showed only a minor overlap between these two growth systems. Although such comparisons are difficult because of differences in the chip platforms, conditions in different laboratories and other aspects, it strongly suggest that root responses in acid soil differ at least in part from those observed in hydroponic experiments. However, several pathways that are affected by Al in hydroponics were also observed in acid soil grown plants, as discussed below.

Organic acid biosynthesis

Of particular interest are genes involved in organic acid biosynthesis, which can protect the plant from deleterious effects of Al by binding to it after being secreted by root apexes [77]. However, only one gene belonging to an organic acid biosynthesis pathway was identified as down-regulated in S1587-17 after a three-day treatment (citrate synthase – Zm.15069.1.A1_at). Previous studies have suggested that Cat100-6 activates pre-existing anionic channels after exposure to Al but prior to activation of the organic acid biosynthesis pathways [23, 25]. It has

also been observed that the levels of citrate exudation induced by Al in Cat100-6 roots is higher than in other Al-sensitive genotypes (such as L53), but it stays constant over time [28], although no correlation between organic acid exudation and Al-alteration of genes of the organic acid biosynthetic pathway has been observed in maize [28, 33].

Oxidative stress in soil grown plants

Plant cells normally produce reactive oxygen species (ROS) due to cellular processes that result in reduction of oxygen molecules. Plants have several enzymes capable of producing ROS and others that fight ROS to avoid cellular damage. Al toxicity can lead to an imbalance that results in oxidative stress and increases in the activity of enzymes and genes that reduce ROS effects, as previously observed in maize [28, 33, 41, 78, 79] and other plant species [80-84]. Specifically in the case of the two maize lines used in this work, a previous study indicates that S1587-17 produces higher levels of ROS under Al stress, while ROS production remains constant in Cat100-6 [41]. Expectedly, genes involved in ROS production such as an oxalate oxidase (Zm.503.1.A1_at) and four germins (Zm.1315.1.A1_at; Zm.2525.1.A1_at; Zm.842.1.A1_at and Zm.9049.1.A1_x_at) were up-regulated in S1587-17 after acid soil treatment. In contrast, the up-regulation of genes implicated in the production of ROS was not detected in the Al-tolerant genotype Cat100-6 under stress.

Higher expression of oxalate oxidases in S1587-17 was also correlated with the up-regulation of peroxidases in the Al-sensitive genotype. A gene encoding a glutathione peroxidase (Zm.6103.1.A1_a_at) was up-regulated in the Al-sensitive line, confirming the data obtained by Boscolo et al. [41], who found higher levels of this enzyme in S1787-17 under Al stress. In fact, more ROS scavenging genes were differentially expressed in S1587-17 than in Cat100-6, possibly reflecting a response to the up-regulation of ROS-producing genes. These data are in agreement

with those from Maron et al. [28] suggesting that Cat100-6 has mechanisms that act before the oxidative stress takes place. However, none of the genes encoding superoxide dismutase were identified as up-regulated in S1787-17, in contrast to the induction of this enzyme [41] and transcriptional up-regulation detected previously in hydroponics [28].

The degree of ROS production and the enzymes involved in their metabolism may partially explain the differences in root growth detected in S1587-17 and Cat100-6 in acid soil. Together with oxalate oxidase, peroxidases act to remodel the cell wall during development and stress responses [85, 86]. Our results indicate that the elevated number of peroxidases up-regulated in S1587-17 may be one of the causes of the root inhibition in this genotype, either by increasing ROS production or by changing the cell wall structure. On the other hand, Cat100-6 appears to be more effective at preventing ROS generation. This is supported by the smaller number of genes contributing to ROS production compared to the number observed in hydroponic culture [28, 33] and also by the constitutive (i.e., independent of soil type) expression of ROS scavenging genes (such as GST) in Cat100-6.

The phenylpropanoid pathway is activated in Al-sensitive maize plants

The higher expression of peroxidases in S171587-17 in acid soil was also correlated with an increase in the expression of several genes implicated in the synthesis of monolignols. Genes related to lignin biosynthesis have often been identified as responding to Al stress in monocots [28, 33, 87, 88], and higher lignin deposition has been associated with root growth inhibition in Al-sensitive wheat genotypes [87]. The phenylpropanoid pathway is the last biochemical step in the production of monolignols and the lignin polymer. The up-regulation of genes in the shikimate pathway, including shikimate kinases (Zm.3954.1.A1_at and Zm.10310.1.A1_at, are up-regulated in S1587-17 / 3 days) and chorismate mutase (up-regulated in S1587-17, Zm.9783.1.A1_at and

Zm.10652.1.S1_at, and Zm.9867.1.A1_at constitutively expressed in Cat100-6) might increase the production of phenylalanine, the precursor for the phenylpropanoid pathway. Cinnamoyl-reductase (Zm.3297.1.A1_at) and several O-methyltransferases were up-regulated at days 1 and 3 in the Al-sensitive line, indicating that S1587-17 might accumulate lignin, reducing root growth. Similarly, several genes related to callose biosynthesis were up-regulated in the Al-sensitive line at both time points (Zm.16347.1.A1_at – beta-glucan binding protein; Zm.14573.1.S1_at – glucan endo-beta-glucosidase 7 precursor; Zm.5768.1.A1_at – beta-glucanase precursor and Zm.12098.1.A1_at – endo-1,3;1,4-beta-d-glucanase precursor), corroborating the physiological data that show higher levels of callose in S1587-17.

Insights into hormonal responses to acid soil

Al has also been shown to impact root growth by modifying the levels of phytohormones such as auxin [89] and ethylene [8]. Genes encoding enzymes involved in auxin biosynthesis such as IAA amidohydrolase (Zm.3056.1.A1_at) and anthranilate phosphoribosyltransferase (Zm.1556.1.A1_at) were up-regulated in the root apex of the sensitive line S1587-17 when under acid soil stress, while the auxin-degrading enzyme indole-3-acetate beta-glucosyltransferase (Zm.18805.1.A1_at) was down-regulated after three days of acid soil exposure. Although auxin can induce new root formation, higher concentrations inhibit root elongation and enhance adventitious root formation. The genotype S1587-17 grown in acid soil developed significantly more lateral roots compared to plants grown under control conditions (data not shown). In coordination with the up-regulation of auxin-responsive genes (Zm.16990.1.S1_at, Zm.255.1.A1_at and Zm.5214.1.S1_at), its F-box receptor [90] was also up-regulated in S1587-17 (Zm.15393.1.S1_at). This might indicate a compensatory mechanism of primary root inhibition and lateral root stimulation to avoid nutrient and water deficiencies.

The transcriptome response of the Al-susceptible line S1587-17 to acid soil treatment also involved the up-regulation of two ACC oxidases (Zm.18900.1.S1_at and Zm.7909.1.A1_at), suggesting activation of the ethylene production pathway. The phytohormone ethylene mediates root growth inhibition [91, 92], and treatment with inhibitors of ethylene perception increases root elongation [93]. Therefore, increased ethylene production is involved in root growth inhibition in the Al-sensitive genotype, and this response might be modulated not only by the germplasm but also by the culture conditions.

Conclusion

In this study we have characterized the transcriptomic changes of maize roots growing in acid soil containing toxic levels of Al. Our data highlighted several metabolic pathways that are challenged due to the stress caused by acid soil, including those involved with ROS production and detoxification, cell wall structure and hormone biosynthesis. Several genes previously reported as up-regulated by Al treatment in hydroponic experiments were also identified in acid soil grown plants. Most interestingly, we found genes that provide interesting clues to the mechanisms underlying the acid soil tolerance of an Al-tolerant maize line. These genes encode a GDSL-motif lipase hydrolase family protein, a pod-specific dehydrogenase / retinol dehydrogenase 11, GRP, WRKY and two proteins of unknown function. Taken together, these data provide a better understanding of the basis of Al toxicity and tolerance in acid soils.

Materials and Methods

Plant material and growth conditions

Seeds from the tropical maize (*Zea mays* L.) inbred lines Cat100-6 and S1587-17 were geminated for two days in moist filter paper. Seedlings with similar initial root length were transferred to 0.5-

liter plastic pots with 1 kg of soil (with 15% water – mL/Kg). Each bag received 20 seedlings, which were grown in a growth chamber at 26°C (light: dark, 16:8h). Bags were weighed twice daily and the weight was completed with distilled water to maintain the humidity at 15%.

Plants were grown in a Dark Red Latossol sieved through a 4-mm mesh. Soil analysis indicted a pH of 4.1 and Al content of 10 $mmol_c/dm^3$ (referred to as the acid soil treatment). Fertilization was applied to avoid nutritional stress and consisted of the following nutrients (mg/Kg of soil): 56 of N; 38.75 of P; 78 of K; 32 of S; 60 of Mg; 0.5 of B; 0.5 of Cu; 0.01 of Mo; 1.0 of Zn. The soil used in the control treatment was incubated with 0.8 g of $Ca(OH)_2$ per Kg for one week prior the fertilization and the same amount of nutrient was added to the acid soil. The incubation with $Ca(OH)_2$ increased the pH to pH 5.5 and the presence of free Al was no longer detected. The acid soil also received a correction for Ca through the addition of a $CaCl_2$ solution to compensate for the $Ca(OH)_2$ added to the control soil. The soil was thoroughly mixed to reduce natural variability of the physical and chemical properties and to ensure homogeneous fertilization.

Relative root growth (RRG)

Before transferring the seeds to soil, the initial root length of each seedling was measured. After each growth period (1 and 3 days), the pots were cut and the soil was gently removed to expose the roots. Each root was washed in running water to remove the excess soil and the root length was measured. Root growth (RG) was calculated as the final length (after growth in soil) minus the initial length. The relative root growth (RRG) of each maize line was calculated as the RG of all the seedlings grown in acid soil divided by the mean RG of all the seedlings grown in control soil times 100.

Aluminum quantification

Al quantification was carried out as described by Bloom et al. [94] after the roots were washed in acidified water (pH 4.0). Measurements were performed in a spectrofluorometer (ISS PCI Photon Counting Spectrofluorometer) with lamp intensity of 10 A, emission and excitation gap of 2 mm. The excitation wavelength was 390 nm and the emission wavelength was 497 nm. Each sample was measured 10 times with a quartz cuvette (optical length of 1 cm). A standard curve was made with serial dilutions of $AlCl_3$.

Callose quantification

Callose content was quantified as described by Jones et al. [95] with modifications. Ten root apexes were fixed in formalin. After 48 h, the solution was replaced with 200 μL of NaOH (1 M) and the root tips were disrupted with the use of a pistile. After 24 h, an additional 800 μL of NaOH (1 M) was added to each sample and they were placed in a water bath at 80°C for 15 minutes. The samples were rapidly centrifuged at 1000 g after cooling off. A total of 400 μL of the upper phase was transferred to a new tube and 800 μL of aniline blue solution (0.1% - w/v), 420 μL of HCl (1 M) and 1,180 μL of glycine/NaOH buffer (pH 9.5) were added, and they were incubated at 80°C for 20 minutes. Callose content was quantified in a spectrofluorometer, as described above, but with an excitation wavelength of 385 nm and an emission wavelength of 485 nm. Each sample was read 10 times with a quartz cuvette (optical length of 1 cm). A standard curve was made with serial dilutions of curdlan solution. The amount of Al-induced callose deposition was calculated as the quantity in the acid soil treatment minus the quantity in the control soil treatment.

RNA extraction

RNA was extracted with an RNeasy Plant Mini Kit (Qiagen, Valencia, USA). The RNA was evaluated in an agarose/formaldehyde gel, quantified in a spectrophotometer and stored at -80°C.

Microarray hybridization and analysis

For the microarray experiment, three independent replicates were used, for a total of 24 samples. Two micrograms of each RNA sample was processed and hybridized to the Affymetrix GeneChip® Maize Genome Array as described by the manufacturer's protocol. The hybridization, staining, washing and scanning were performed at Laboratório Nacional de Luz Sincrontron (LNLS), Campinas, SP, Brazil, with the use of the Command Console Software (Affymetrix, USA). The data were normalized with the RMA method, $\log_2$ transformed and loaded into SAS (SAS Institute, USA) to perform the contrasts. A one-way analysis of variance (ANOVA) was used to separate the contribution of each effect on the expression level of a given gene. The model used was: $y_{ikl} = \mu + G_i + Ta_k + Te_l + (G \times Ta)_{ik} + (G \times Te)_{il} + (G \times Ta \times Te)_{ikl} + \xi_{ikl}$ where μ is the sample mean, G_i represents the genotype effect for the i^{th} genotype (e.g., Cat100-6 or S1587-17) (df = 1), Ta_k is the effect of the k^{th} Treatment (e.g., acid soil or control soil) (df = 1), Te_l is the effect of l^{th} time point (e.g., 1 or 3 days) (df = 1), $(G \times Ta)_{ik}$ is the effect of interaction between genotype and treatment (df = 1), $(G \times Te)_{il}$ is the effect of interaction between genotype and time point (df = 1), $(G \times Ta \times Te)_{ikl}$ is the effect of interaction between genotype, treatment and time (gl = 1) and ξ_{ikl} is the residual error. Least-square means for each gene in each sample were generated and differential estimates (DE) of expression were calculated as the difference between least-square means for each of the terms in the model. DE values were calculated between the acid and control soil treatments and also between genotypes. The false discovery rate (FDR) was set to 10% to control Type I errors [96]. Q values were calculated from *P*-values using the software Q-

value from the R platform [97]. Only the genes with DE above 1 were further analyzed. The list of differentially expressed genes was annotated with the use of Blast2GO software (www.blast2go.org) [98-100] using default settings.

Hydroponic culture

To validate the microarray data and to separate the effect of pH from that of Al, a hydroponic experiment was performed. The basic solution consisted of 0.5 mM $CaCl_2$; 0.125 mM $MgCl_2$; 1 mM KCl; 1 mM NH_4NO_3. This basic solution was divided into two portions and their pHs were corrected to 5.5 (control solution) and 4.2. The solution with the pH of 4.2 was again divided in two and one portion received 36 µM $AlCl_3$. This Al concentration yielded the same RRG as was obtained with soil treatment (data not shown). The plants were grown in a growth chamber at 26°C (light: dark, 16:8h) with constant solution aeration.

Real Time qPCR

To validate the microarray results, RNA from two additional independent replicates was treated with DNase I Amplification Grade (Invitrogen, USA) and cDNA was synthesized from 2 µg of RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA). Real-time qPCR for eleven genes identified as differentially regulated in at least one of the experimental conditions was performed with an ABI 7500 (Applied Biosystems, USA) using Sybr Green I PCR Master Mix (Applied Biosystems, USA). The primers were designed using Primer Express 2.0 software (Supplemental Material 2 - Table S6). The efficiency of each pair was tested with a relative standard curve experiment. The maize tubulin gene (Zm.6045.1.A1_s_at) was used as an endogenous control. As the efficiency of all the primers was near 100%, the relative expression was calculated by the ΔΔCt method. For microarray validation, the ΔCt values were calculated for

each gene in each sample, $\log_2$ transformed and plotted against its corresponding least-square means data from the microarray. For the hydroponic experiment, the ΔΔCt values were calculated relative to the ΔCt from the pH 5.5 treatment.

Authors' contributions

LM designed the experiments; performed the microarray hybridizations, data analysis and interpretation; and drafted the manuscript. MK evaluated the data from the microarrays and edited the manuscript. FRS helped with the comparison of the data from the acid soil and hydroponic experiments. RAJ helped with the experiments in acid soil and interpretation of the data. MM designed the experimental approach, led and coordinated the project and edited the manuscript. All authors have read and approved the final manuscript.

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Description of Additional Files

Supplemental Material 1

Figure S1. Root phenotype under control (first and third row) and acid soil (second and fourth row) for 1 day treatment (A), 3 days treatment (B).

Figure S2. Function analysis of genes differentially expressed in S1587-17 after 1 day of treatment in acid soil. A: Up-regulated; B: Down-regulated. All the genes that didn't present Gene Onthologies were removed from the analysis.

Figure S3. Function analysis of genes differentially expressed in S1587-17 after 3 days of treatment in acid soil. A: Up-regulated; B: Down-regulated. All the genes that didn't present Gene Onthologies were removed from the analysis.

Supplemental Material 2

Table S1: Genes differentially expressed in Cat100-6 after one day of acid soil treatment. FDR = 10% and DE ≤ 1.

Table S2: Genes differentially expressed in Cat100-6 after three days of acid soil treatment. FDR = 10% and DE ≤ 1.

Table S3: Genes differentially expressed in S1587-17 after one day of acid soil treatment. FDR = 10% and DE ≤ 1.

Table S4: Genes differentially expressed in S1587-17 after three days of acid soil treatment. FDR = 10% and DE ≤ 1.

Table S5: Genes that were differentially expressed in Cat100-6 grown both in soil (this work) and hydroponics [27] and in S1587-17 grown in soil (this work) and L53 in hydroponics [27].

Table S6: Primers designed for Real Time qPCR.

CAPÍTULO II

Análise de QTL dos Genes Identificados no Transcriptoma de Raízes

A cultura do milho possui uma grande variabilidade genética para a tolerância ao Al (Rhue et al., 1978; Lopes et al., 1987; Sibov et al., 1999; Ninamango-Cárdenas et al., 2003) que pode ser utilizada para desenvolver genótipos tolerantes ao íon fitotóxico através do melhoramento clássico (Foy et al., 1978; Sephar, 1994), auxiliada pela identificação de marcadores moleculares relacionados com a tolerância ao Al. O princípio básico dessa técnica é a identificação de regiões cromossômicas através de análises de QTL (*Quantitative Trait Loci*). A localização de genes identificados como resposivos ao Al experimentos transcriptoma pode contribuir na identificação genes localizados em QTLs já conhecidos como contribuintes para a tolerância ao Al ao exemplo de Maron e colaboradores (2010) que após a identificação de genes diferencialmente expressos em raízes de milho expostas ao Al (Maron et al., 2008), alguns genes foram escolhidos e mapeados em QTLs previamente conhecidos em milho.

O primeiro passo foi a escolha das listas de genes, identificadas no Capítulo I, que seriam utilizados para a localização. Para isso partimos do princípio que para um gene ser considerado um possível integrante da resposta ao íon fitotóxico ele não precisaria apenas ser diferencialmente expresso na variedade tolerante de milho submetida a solo ácido em contraste com o solo controle, mas precisaria ser diferencialmente expresso na Cat100-6 em contraste com a S1587-17. Como forma de expemplificar, a Figura 1A representa um gene diferencialmente expresso na Cat100-6 no solo ácido em contraste com o solo controle por 3 dias, mas a S1587-17 apresentou valores de expressão estatisticamente maiores que a Cat100-6 em todos as situações, portanto esse tipo de gene não foi considerado um bom candidato para a tolerância.

Assim, mapeamos as seqüências identificadas em raízes de milho como:

- Genes Candidatos à tolerância, ou seja, aqueles que foram diferencialmente expressos na variedade Cat100-6 após 3 dias de cultivo em solo ácido em contraste com o mesmo genótipo submetido ao solo corrigido por 3 dias e que também possuíram valores de expressão mais altos na variedade tolerante em contraste com a variedade sensível no tratamento com solo ácido por 3 dias (FDR 10%; Estimativa Diferencial ≥ 1; total de 6 identificadores da Affymetrix; Figura 1B);

- Genes diferencialmente expressos no genótipo S1587-17 após o cultivo em solo ácido por 1 dia em contraste com o mesmo genótipo submetido ao solo corrigido por 1 dia, mas que apresentaram valores de expressão significativamente mais altos na Cat100-6 em contraste com a S1587-17 no cultivo em solo ácido por 1 dia (FDR 10%; Estimativa Diferencial ≥ 1; total de 58 identificadores da Affymetrix; Figura 1C);

- Genes diferencialmente expressos no genótipo S1587-17 após o cultivo em solo ácido por 3 dias em contraste o mesmo genótipo submetido ao solo corrigido por 3 dias, mas que apresentaram valores de expressão significativamente mais altos na Cat100-6 em contraste com a S1587-17 no cultivo em solo ácido por 3 dias (FDR 10%; Estimativa Diferencial ≥ 1; total de 75 identificadores de Affymetrix; Figura 1D);

- Genes Constitutivos: aqueles que apresentaram valores de expressão significativamente maiores na Cat100-6 em todos os contrastes entre genótipos (FDR 10%; Estimativa Diferencial ≥ 1), ou seja, são necessariamente modulados pelo tratamento com o solo ácido, mas o genótipo tolerante apresenta valores de expressão desses genes sempre maiores que o genótipo sensível, independente do tratamento e do tempo amostral, o que pode indicar genes de tolerância constitutivos (total de 156 identificadores da Affymetrix; Figura 1E).

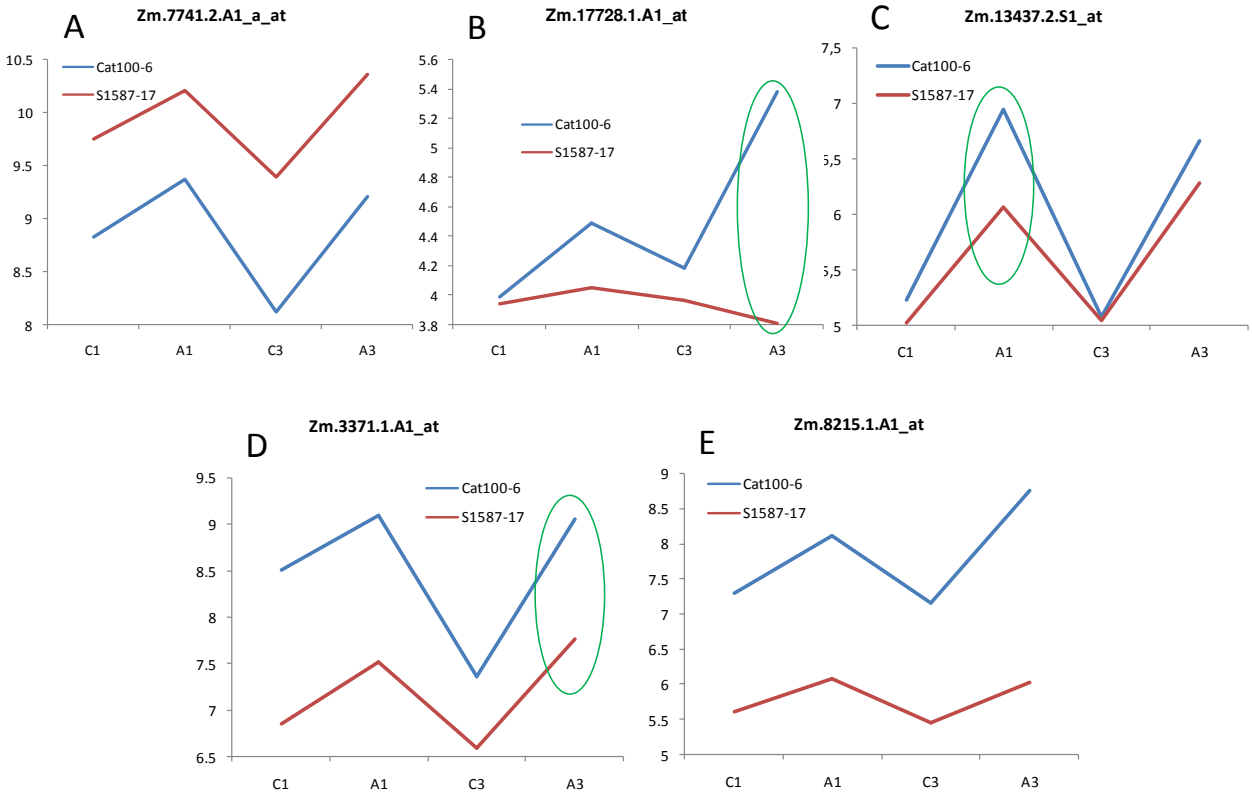


Figura 1. Exemplificação das listas de genes adotadas para a localização cromossômica. (A) Gene diferencialmente expresso na Cat100-6 e na S1587-17 mas que os valores absolutos são maiores na S1587-17, não sendo assim, um bom candidato a tolerância. (B) Gene considerado candidato, já que além de ser diferencialmente expresso na Cat100-6 após 3 dias de tratamento no solo ácido em contraste ao controle, ele também possui expressão diferencial em contraste com o genótipo S1587-17. (C) Gene diferencialmente expresso na S1587-17 após um dia de tratamento, mas que apresentou valores de expressão significativamente maiores na Cat100-6 após o tratamento com solo ácido por um dia no contraste entre genótipos. (D) Gene diferencialmente expresso na S1587-17 após três dias de tratamento, mas que apresentou valores de expressão significativamente maiores na Cat100-6 após o tratamento com solo ácido por três dias no contraste entre genótipos. (E) Exemplo de gene considerado constitutivo na Ca100-6. C1: tratamento com solo controle por um dia; A1: tratamento com solo ácido por um dia; C3: tratamento com solo controle por três dias; A3: tratamento com solo ácido por três dias. O eixo y representa o valor de expressão em valores arbitrários.

As sequências alvo da Affymetrix de cada um dos genes foram localizadas nos cromossomos de milho utilizando o banco de dados Gramene (Liang et al., 2008) através da homologia entre as sequencias alvo da Affymetrix e as sequencias dos cromossomos. Os resultados estão expostos na Figura 2.

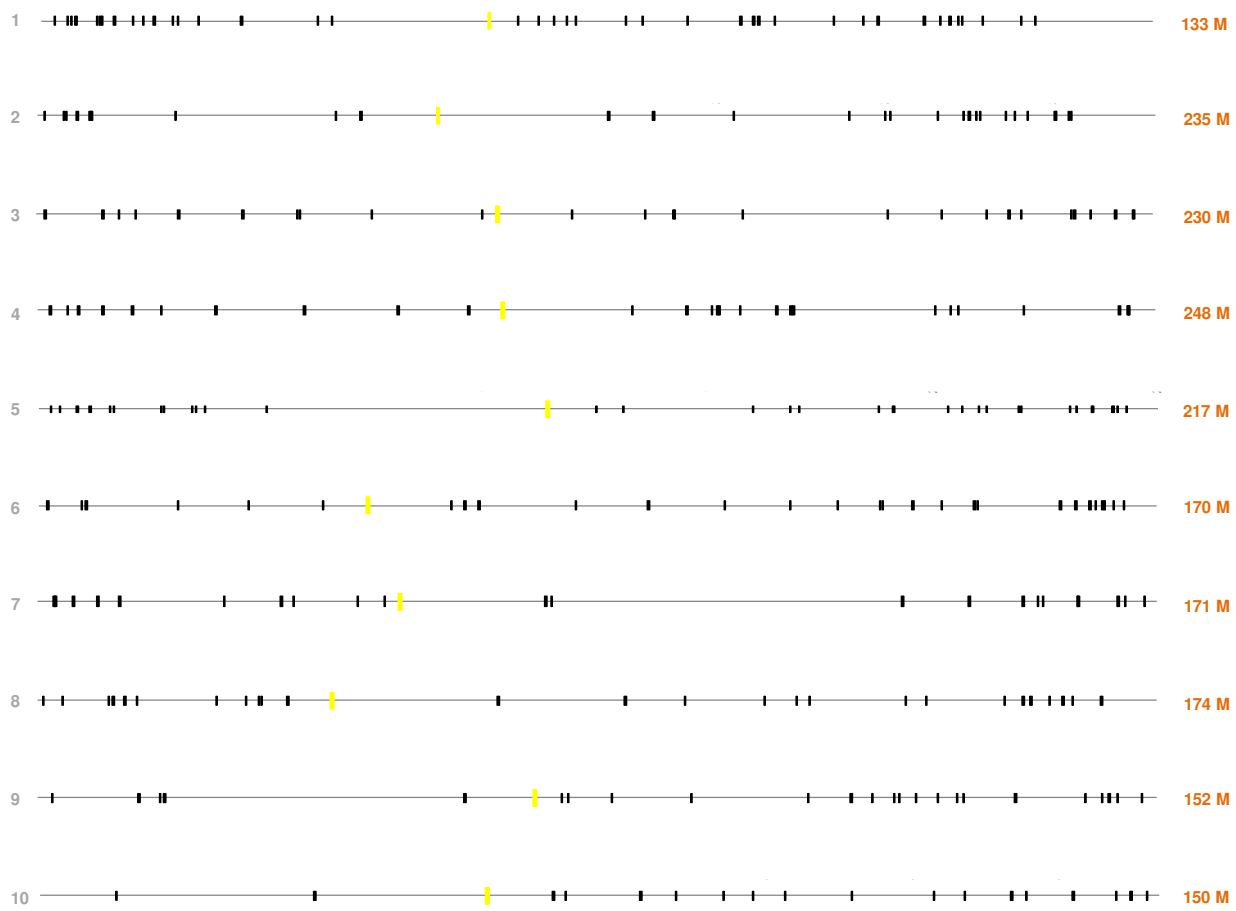


Figura 2. Localização dos genes identificados por microarray nos cromossomos de milho. À esquerda está identificação numérica do cromossomo. À direita está anotado o tamanho aproximado de cada cromossomo (em milhões de pares de base). Os traços amarelos representam a posição aproximada dos centrômeros e os traços pretos representam a localização dos genes identificados pela análise de microarray.

Podemos verificar que o número total de genes mapeados em cada cromossomo não difere muito, mas a contribuição de cada lista para o total é bastante distinta (Figura 3).

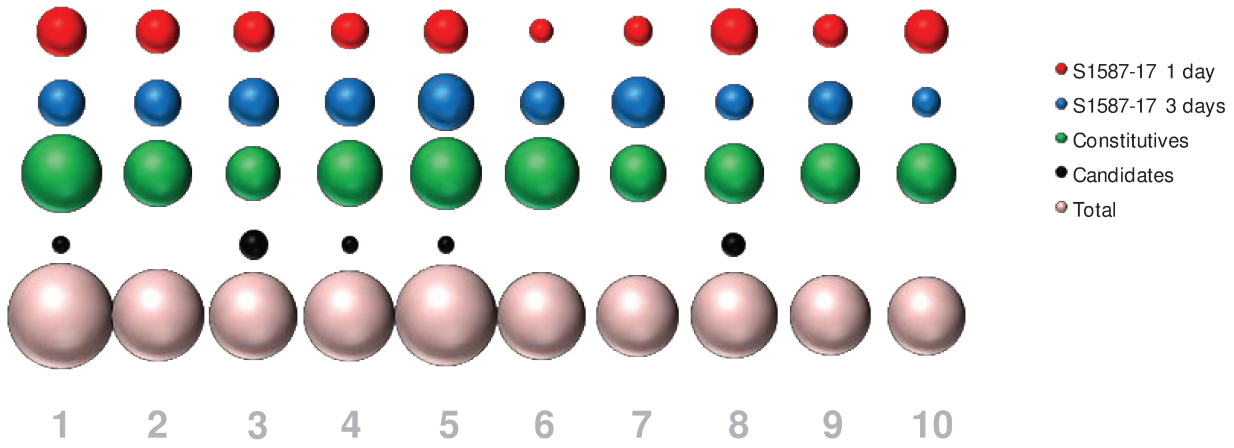


Figura 3. Contribuição de cada lista de genes diferencialmente expressos no total de genes identificados em cada cromossomo. O diâmetro de cada bolha é proporcional ao número de genes. Em cinza abaixo das bolhas está a identificação numérica de cada cromossomo.

Para identificar quais genes estão dentro de QTLs já descritoss para a tolerância ao Al em milho verificamos a posição dos marcadores delimitantes de QTLs descritos em artigos publicados (através do banco de dados MaizeGDB – www.maizegdb.org) e comparamos com as posições dos genes das listas de genes diferencialmente expressos das listas descritas anteriormente.

O estudo conduzido por Moon et al. (1997) analisou a geração F2 do cruzamento entre os genótipos Cat100-6 e S1587-17 e sugeriu que a tolerância ao Al entre elas seria controlado por um único gene dominante denominado *Alm1*. Contudo, o estudo de Sibov et al. (1999) identificaram dois locus distintos, um no cromossomo 6 denominado *Alm2* e outro no cromossomo 10, o já identificado *Alm1*. O estudo de Ninamango-Cárdenas et al. (2003) através da análise da progênie do cruzamento entre as variedades de milho L53 (Al-sensível) e L1327 (Al-tolerante) identificou um total de 5 QTLs nos cromossomos 2, 6 (com sobreposição ao trabalho de Sibov et al., 1999) e cromossomo 8.

Na Figura 4 está diagramado a posição dos genes identificados em nosso estudo em relação ao QTL_1 identificado no cromossomo 2 por Ninamango-Cárdenas et al. (2003).

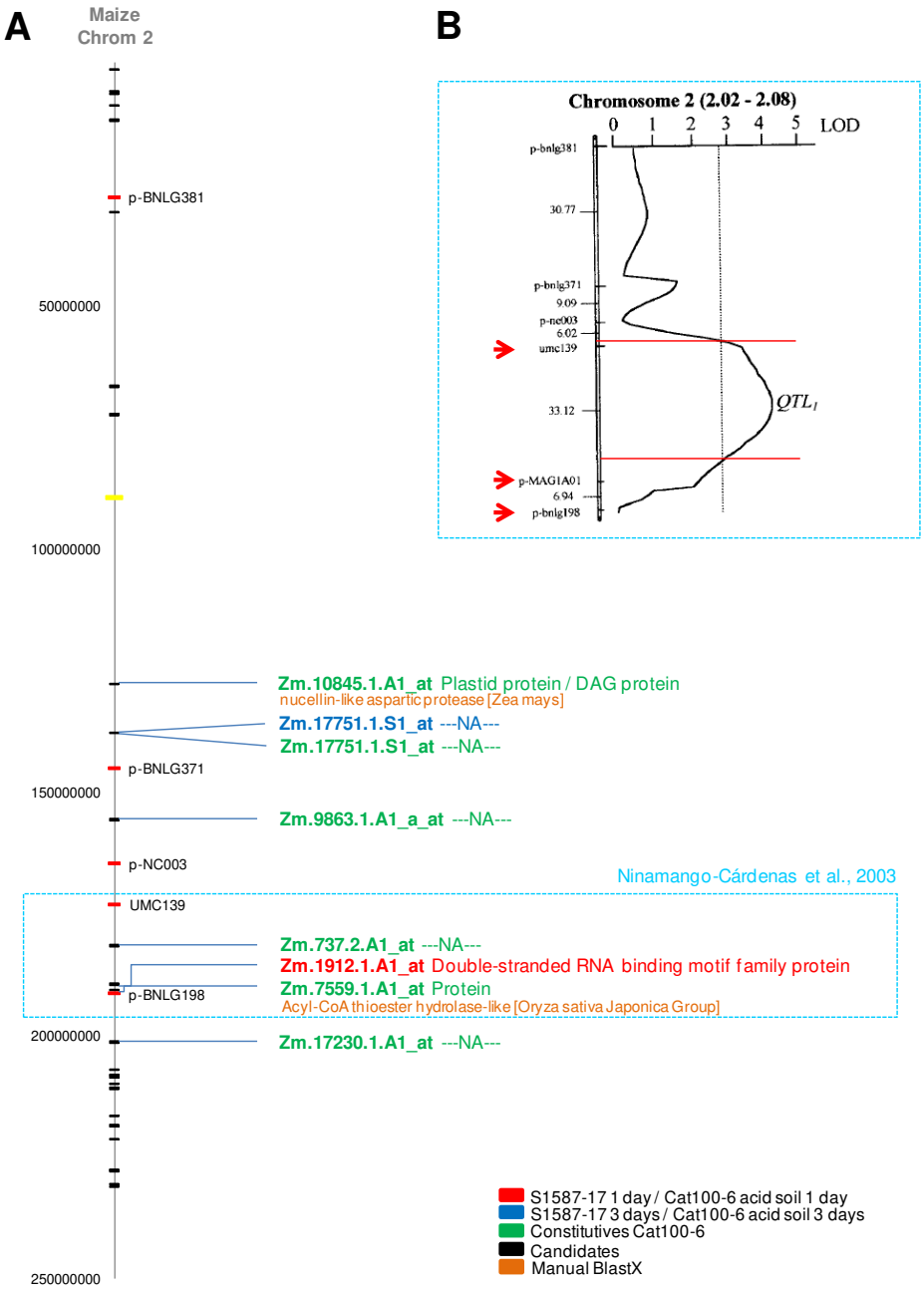


Figura 4. Representação da posição dos marcadores que identificam o QTL_1. A: Representação esquemática do cromossomo 2 de milho. À esquerda está a posição em pares de base. O traço amarelo representa a posição do centrômero; os traços vermelhos representam as posições dos marcadores identificados e em preto as posições dos genes identificados por microarray. As cores de cada gene identificado representa a lista da qual esse gene foi obtido, caso haja outra anotação em laranja, significa uma anotação adicional obtida por BlastX manual. B: Gráfico retirado do trabalho de Ninamango-Cárdenas et al. (2003) identificando a região entre os marcadores que promove a maior contribuição para a tolerância (identificados com setas vermelhas).

A maioria dos genes nesta região não possuem função conhecida. Entretanto, uma proteína ligadora de RNA dupla fita e uma *acyl-Coa thioester hydrolase* foram localizadas dentro do QTL_1. Proteínas ligadoras de RNA dupla fita podem fazer parte da supressão da expressão de transgenes assim como a expressão do gene endógeno, denominado de co-supressão (Napoli et al., 1990; van der Kroll et al., 1990). Guo e Kemphues (1995) iniciaram os estudos com células de animais no intuito de desvendar dar as bases moleculares do silenciamento, mas foi Fire e colaboradores (1998) que descobriram a potencialidade da formação do RNA de dupla fita no silenciamento gênico. Neste contexto, a identificação de uma proteína ligadora de RNA dupla fita pode ser uma indicação que mecanismos de silenciamento gênico por miRNAs podem estar atuantes em genes alvo que contribuem para a tolerância ao Al.

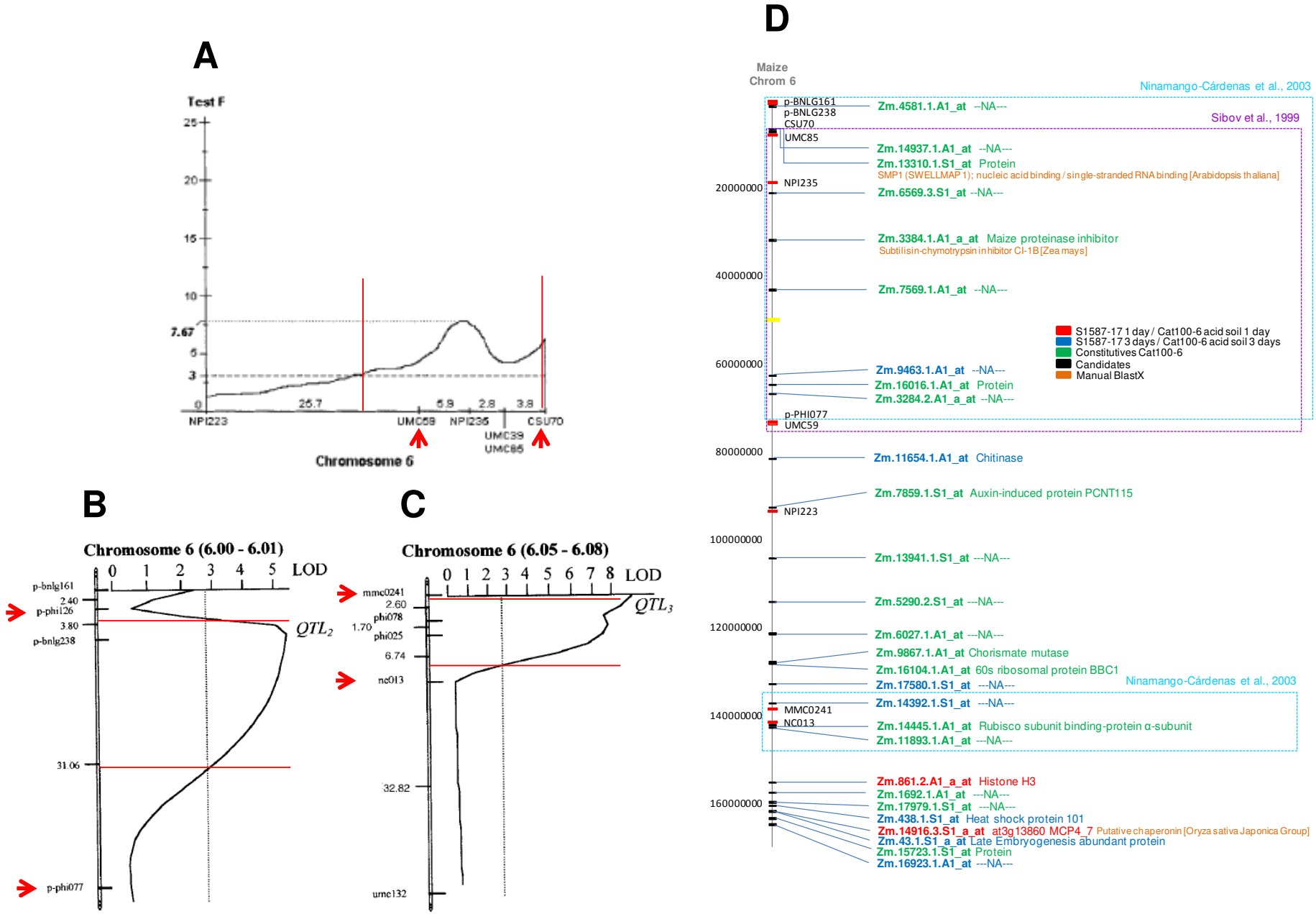
O BlastX efetuado individualmente para as sequências identificadas como pertencentes ao QTL_1 demonstrou que uma sequência que antes não havia sido identificada apresentou semelhança significantiva (E-value < 10^{-5}) com uma *acyl-Coa thioester hydrolases* de arroz (também denominadas *acyl-Coa thioesterases*). A primeira *acyl-Coa thioesterase* de plantas foi clonada de *Arabidopsis thaliana* e é uma provável enzima ativa no peroxissomo (Tilton et al., 2004). Essas enzimas podem estar relacionadas com a produção de ácidos graxos ou na β-oxidação de lipídeos, mas a sua verdadeira função fisiológica permanece desconhecida (Tilton et al., 2000).

O cromossomo 6 de milho é o que possui a maior quantidade de QTLs identificados como importantes para a tolerância ao Al. Ambos trabalhos de Sibov et al. (1999) e Ninamango-Cárdenas et al. (2003) identificaram uma região no braço curto do cromossomo 6, *Alm2* e QTL_2 respectivamente (Figura 5). Adicionalmente o estudo de Ninamango-Cárdenas et al. (2003) identificou o QTL_3 no braço longo do mesmo cromossomo. Como no caso do cromossomo 2, a maioria dos genes identificados por microarray que se encontram nesses QTLs possuem função

desconhecida.

Uma proteína constitutiva da Cat100-6 que na primeira anotação automática (através do programa Blast2GO) não apresentou identidade com nenhuma proteína conhecida, ao fazer nova análise com BlastX mostrou uma identidade com o gene *SMP1* (*Swellmap1*) que refere-se a uma proteína ligadora de ácidos nucléicos como RNA em fita simples. Um mutante *knockout* de Arabidopsis para esse gene possui um reduzido tamanho foliar e número de células, defeito este que é compensado por um tamanho celular final maior (Clay e Nelson, 2005). A falta da expressão do gene causa, aparentemente, uma parada precoce na proliferação celular no primórdio foliar reduzindo a taxa de divisão celular. Além disso, esse gene está envolvido da seleção do sítio de *splicing* na região 3'. A expressão do *SMP1* está envolvida com regiões de proliferação celular e a superexpressão do mesmo também causou a redução do tamanho foliar, mas o número de células final foi aumentado (Clay e Nelson, 2005). Apesar desse gene não ter sido estudado profundamente em raízes até o momento, podemos supor que a expressão constitutiva desse gene na Cat100-6 possa favorecer a permanência da divisão células mesmo quando exposto a solo ácido com concentrações fitotóxicas de Al.

Figura 5. Representação da posição dos marcadores que identificam como *Alm2*, QTL_2 e QTL_3. A: Gráfico retirado do trabalho de Sibov et al. (1999) identificado a região entre os marcadores que promove a maior contribuição para a tolerância denominado *Alm2* (identificados com setas vermelhas). B: Gráfico retirado do trabalho de Ninamango-Cárdenas et al. (2003) identificando o QTL_2, os marcadores identificados por setas vermelhas indicam a região cromossômica com maior contribuição para a tolerância ao Al. C: Gráfico retirado do trabalho de Ninamango-Cárdenas et al. (2003) identificando o QTL_3, os marcadores identificados por setas vermelhas indicam a região cromossômica com maior contribuição para a tolerância ao Al. D: Representação esquemática do cromossomo 6 de milho. À esquerda está a posição em pares de base. O traço amarelo representa a posição do centrômero; os traços vermelhos representam as posições dos marcadores identificados e em preto as posições dos genes identificados por microarray. As cores de cada gene identificado representa a lista da qual esse gene foi obtido. A anotação em laranja indica uma anotação adicional obtida por BlastX manual. A área delimitada por um quadrado azul represeta o QLT_2 e QTL_3; a área destacada em roxo delimita a região do *Alm2*.


A
Test F
25
20
15
10
7.67
5
3
0
25.7
5.8
2.8
3.8
NPI223
UMC59
NPI235
UMC39
UMC85
CSU70
Chromosome 6
B
Chromosome 6 (6.00 - 6.01)
0 1 2 3 4 5 LOD
p-bnlg161
2.40
p-phi126
3.80
p-bnlg238
31.06
p-phi077
QTL2
C
Chromosome 6 (6.05 - 6.08)
0 1 2 3 4 5 6 7 8 LOD
mmc0241
2.60
phi078
1.70
phi025
6.74
nc013
32.82
umc132
QTL3
D
Maize
Chrom 6
Ninamango-Cárdenas et al., 2003
Sibov et al., 1999
p-BNLG161
p-BNLG238
CSU70
UMC85
Zm.4581.1.A1_at --NA---
Zm.14937.1.A1_at --NA---
Zm.13310.1.S1_at Protein
SMP1 (SWELLMAP 1); nucleic acid binding / single-stranded RNA binding [Arabidopsis thaliana]
NPI235
20000000
Zm.6569.3.S1_at --NA---
Zm.3384.1.A1_a_at Maize proteinase inhibitor
Subtilisin-chymotrypsin inhibitor CI-1B [Zea mays]
40000000
Zm.7569.1.A1_at --NA---
S1587-17 1 day / Cat100-6 acid soil 1 day
S1587-17 3 days / Cat100-6 acid soil 3 days
Constitutives Cat100-6
Candidates
Manual BlastX
60000000
Zm.9463.1.A1_at --NA---
Zm.16016.1.A1_at Protein
Zm.3284.2.A1_a_at --NA---
p-PHI077
UMC59
80000000
Zm.11654.1.A1_at Chitinase
Zm.7859.1.S1_at Auxin-induced protein PCNT115
NPI223
100000000
Zm.13941.1.S1_at ---NA---
Zm.5290.2.S1_at ---NA---
120000000
Zm.6027.1.A1_at ---NA---
Zm.9867.1.A1_at Chorismate mutase
Zm.16104.1.A1_at 60s ribosomal protein BBC1
Zm.17580.1.S1_at ---NA---
Ninamango-Cárdenas et al., 2003
Zm.14392.1.S1_at ---NA---
MMC0241
NC013
140000000
Zm.14445.1.A1_at Rubisco subunit binding-protein α-subunit
Zm.11893.1.A1_at ---NA---
Zm.861.2.A1_a_at Histone H3
Zm.1692.1.A1_at ---NA---
Zm.17979.1.S1_at ---NA---
Zm.438.1.S1_at Heat shock protein 101
160000000
Zm.14916.3.S1_a_at at3g13860 MCP4_7 Putative chaperonin [Oryza sativa Japonica Group]
Zm.43.1.S1_a_at Late Embryogenesis abundant protein
Zm.15723.1.S1_at Protein
Zm.16923.1.A1_at ---NA---

Outro gene identificado no QTL_2 (Ninamango-Cárdenas et al. 2003) e *Alm2* (Sibov et al., 1999) foi um inibidor de proteinase do tipo *subtilisin-chymotrypsin*. A maquinaria de *turnover* de proteínas possui três elementos principais: as proteases, suas proteínas alvo para degradação e os inibidores das proteases (Lopez-Otin e Overall, 2002). Inibidores de proteases constituem um grupo complexo em plantas e apresentam funções bastante diversificadas na regulação da atividade proteolítica de suas proteínas alvo (Leung et al., 2000). Inibidores de proteases também podem estar envolvidos na resposta de diversos estresses. Uma *cysteine-protease inhibitor* possui seus níveis de expressão induzidos quando plantas de soja são submetidas a ferimentos ou tratamento com metil-jasmonato (Botella et al., 1996). Inibidores de proteases da família Kunitz foram identificados em plantas de rabanete expostas a estresse salino (Lopez et al., 1994) e em plantas de *Arabidopsis thaliana* submetidas a seca (Gosti et al., 1995). Em tabaco, um inibidor de protease do tipo II foi rapidamente ativado por patógenos e ferimentos (Choi et al., 2000). Um estudo mais detalhado em arroz demonstrou que um inibidor de *chymotrypsin* foi responsivo a estresses abióticos como seca e estresse salino, além disso, a superexpressão do gene *OCPII* em arroz aumentou significativamente a tolerância a seca e estresse salino (Huang et al., 2007). Esses estudos demonstram que esse gene, expresso constitutivamente na variedade Cat100-6, pode fazer parte do sistema de tolerância a solo ácido por controlar a atividade de proteases.

O QTL_3, identificado Ninamango-Cárdenas e colaboradores (2003) também foi localizado no cromossomo 6 de milho. O único gene dentro desse QTL que obtivemos a anotação foi uma subunidade α da Rubisco. Até o momento não existem estudos indicando que a expressão da Rubisco em raízes pode estar relacionada tolerância a algum estresse abiótico.

O trabalho de Ninamango-Cárdenas e colaboradores (2003) identificou outros dois QTLs localizados no cromossomo 8 (Figura 6).

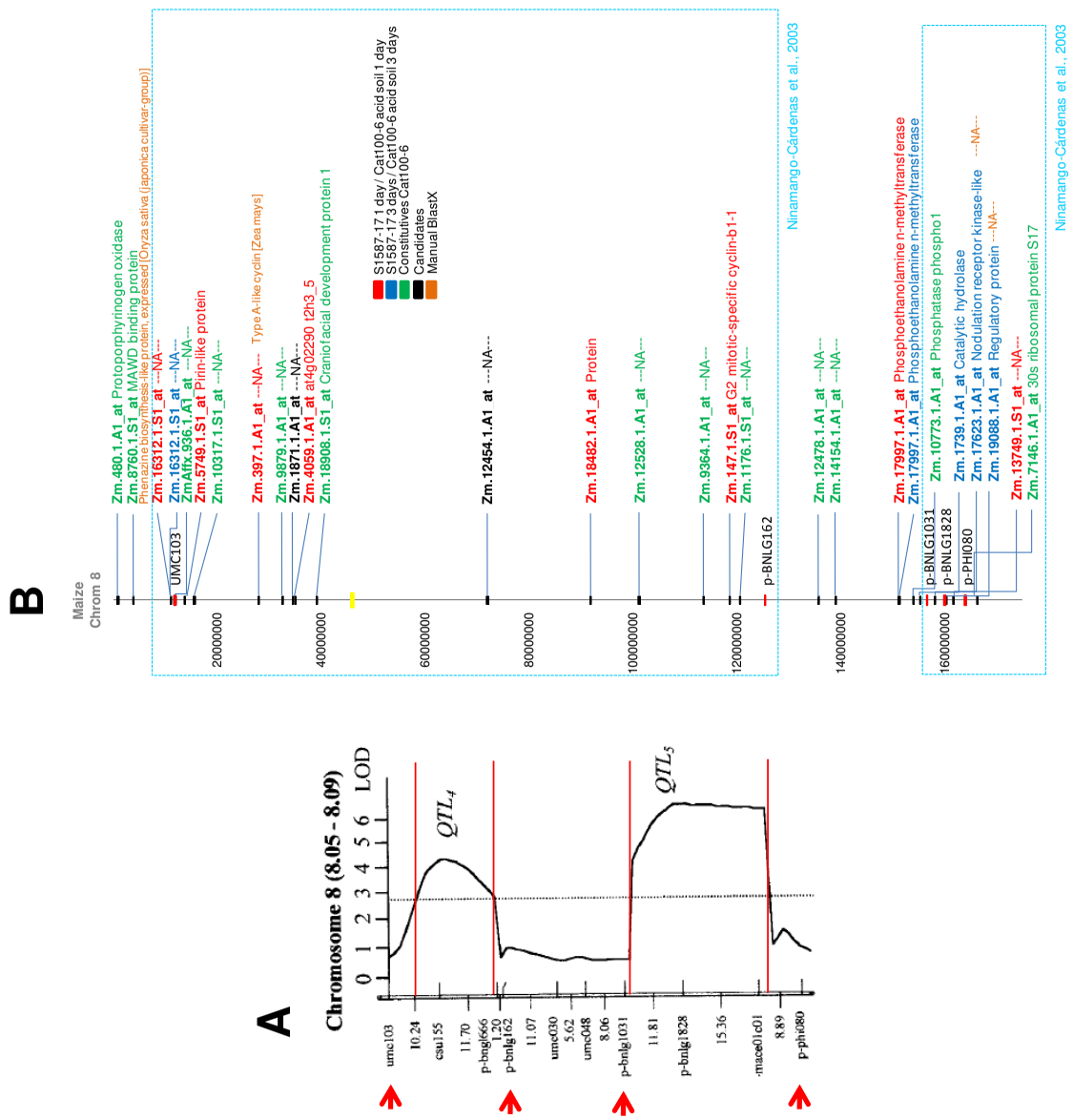


Figura 6. Representação da posição dos marcadores do QTL_4 e QTL_5. A: Gráfico retirado do trabalho de Ninamango-Cárdenas et al. (2003) identificando o QTL_4 e QTL_5, os marcadores identificados por setas vermelhas indicam a região cromossômica com maior contribuição para a tolerância ao Al. B: Representação esquemática do cromossomo 8 de milho. À esquerda está a posição em pares de base. O traço amarelo representa a posição do centrômero; os traços vermelhos representam as posições dos marcadores identificados e em preto as posições dos genes identificados por microarray. As cores de cada gene identificado representa a lista da qual esse gene foi obtido, caso haja outra anotação em laranja, significa uma anotação adicional obtida por BlastX manual.

O gene de uma proteína *pirin-like* foi identificado dentro do QTL_4. Em um experimento de duplo-híbrido foi demonstrado que a proteína GPA1, responsável pelo controle de inúmeros processos celulares, interage com a proteína AtPirin1 (Lapik et al., 2003). Além disso, dois mutantes para o gene *AtPirin1* apresentaram baixa taxa de germinação das sementes (sem sofrer

estratificação), hipersensibilidade ao ABA e iniciação prematura da floração (Lapik et al., 2003). Em células em suspensão de tomate o gene *Le-pirin* foi altamente expresso quando tratadas com um indutor de morte celular (Orzaez et al., 2001). Além disso, o mesmo trabalho apontou que folhas senescentes de tabaco apresentam maior expressão desse gene comparado com folhas jovens. Esse gene é um ortólogo da proteína PIRIN de humanos e esta tem a capacidade de se ligar ao fator de transcrição humano NE-κB (Dechend et al., 1999), que está envolvido na via de sinalização TNF que é responsável pelas alterações celulares observadas durante a apoptose. Assim que é ativado, o fator de transcrição NE-κB promove a transcrição de genes anti-apoptóticos (Foo e Nolan, 1999). Boscolo et al. (2003) demonstraram que as células do ápice radicular da variedade sensível de milho é bastante acometida pela morte celular após a exposição ao Al. Em contrapartida, a variedade tolerante não apresentou morte celular detectável quando exposta ao Al com o qual podemos especular a possível contribuição do gene *pirin-like* na ativação de genes anti-apoptóticos e assim minimizar a morte celular na Cat100-6 perante o tratamento com Al.

Dois genes anotados como ciclinas também foram localizados na região do QTL_4 (Zm.397.1.A1_at e Zm.141.1.S1_at). São genes que controlam o progresso geral do ciclo celular (Criqui et al., 2002). Cliclinas do tipo B já foram identificadas em uma gama de espécies vegetais, como foi revisado por Renaudin et al. (1996), contudo, sua função exata na manutenção do progresso do ciclo celular ainda permanece desconhecida. A expressão da ciclina B1 de Arabidopsis acelerou a divisão das células radiculares indicando que esse gene pode ser um fator limitante para a divisão celular (Doerner et al., 1996a; Doerner et al., 1996b). Sendo assim, podemos especular que a identificação de ciclinas dentro de QTLs previamente identificados como importantes para a tolerância ao Al, indicaria que a manutenção da divisão celular é um mecanismo fundamental para superar as dificuldades impostas pela presença de Al fitotóxico.

Dois genes candidatos com função desconhecida foram localizados no QTL_4 entre eles o gene Zm.1871.1.A1_at que apresentou padrão de expressão relacionado com Al no experimento em hidroponia. Este gene apresenta um domínio conservado típico de metiltransferases e é a primeira vez que um gene com esse domínio é relacionado com a tolerância ao Al em milho.

O QTL denominado *Alm1*, identificado por Sibov et al (1999) também possibilitou a localização de alguns genes identificados no presente estudo de microarray (Figura 7). Entre eles está o fator de replicação C (subunidade 2). Esse tipo de fator é muito importante para a replicação e reparo do DNA (Furukawa et al., 2003). Dois estudos recentes demonstraram que mutantes de Arabidopsis para um gene dessa família (RFC3) apresentavam defeitos na proliferação celular em folhas e raízes. Além disso, essas plantas tornavam-se mais susceptíveis a infecção por patógenos (Xia et al., 2009; Xia et al., 2010). Portanto, assim como as ciclinas descritas anteriormente, a expressão de genes relacionados com a manutenção do DNA, parece ser um mecanismo importante para a sustentação do crescimento radicular perante o estresse proporcionado pelo solo ácido.

O gene Zm.5637.1.A1_at (*xyloglucan endotransglycosylase protein 8 precursor*), constitutivo da Cat100-6, também foi mapeado dentro do QTL *Alm1*. Esse gene integra novos xiloglucanos recém sintetizados na nova parede contribuindo para o crescimento radicular (Nishitani, 1997; Thompson e Fry, 2001). É importante ressaltar que os últimos genes aqui discutidos de certa forma trabalham juntos para a manutenção da divisão e do crescimento radicular.

O gene Zm.5268.1.S1_at (*phosphoglycerate mutase-like*) catalisa uma etapa reversível da glicólise, é um gene constitutivo da Cat100-6. Mazarei e colaboradores (2003) verificaram que o ortólogo desse gene em Arabidopsis é expresso nos meristemas apicais do caule e raiz em condições normais de crescimento e esse gene pode ser ativado pela auxina e pela infecção de

patógenos. Esse gene também foi induzido em raízes de Arabidopsis em resposta ao nitrato (Wang et al., 2003). Portanto esse gene é induzível por estresses bióticos e abióticos, mas o fato da Cat100-6 apresentar a expressão desse gene de forma constitutiva pode acarretar em uma maior flexibilidade metabólica perante o estresse no crescimento em solo ácido.

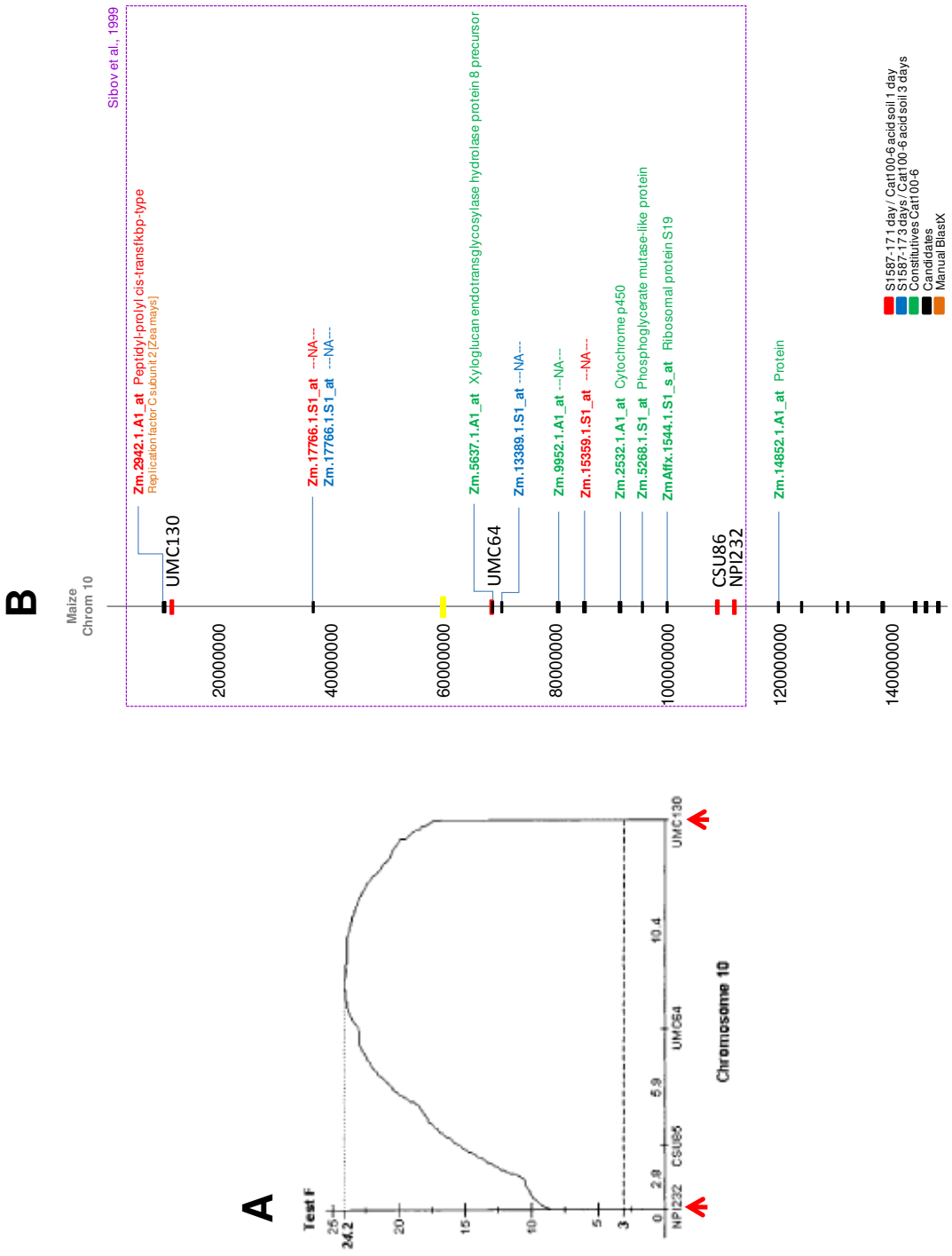


Figura 6. Representação da posição dos marcadores do *Alm1*. A: Gráfico retirado do trabalho de Sibov et al. (1999). Os marcadores identificados por setas vermelhas indicam a região cromossômica com maior contribuição para a tolerância ao Al no cromossomo 10. B: Representação esquemática do cromossomo 10 de milho. À esquerda está a posição em pares de base. O traço amarelo representa a posição do centrômero; os traços vermelhos representam as posições dos marcadores identificados e em preto as posições dos genes identificados por microarray. As cores de cada gene identificado representa a lista da qual esse gene foi obtido, caso haja outra anotação em laranja, significa uma anotação adicional obtida por BlastX manual.

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CAPÍTULO III

Transcriptional profile of maize leaves under acid soil growth

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Transcriptional profile of maize leaves under acid soil growth

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Key words: acid soil, abiotic stress, leaves, microarray, *Zea mays*

Running title: Transcriptional profile of maize leaves under acid soil growth

Accession numbers: The gene expression data was deposited at The Gene Expression Omnibus (GEO) Database under access number GSE22479

Supplementary Materials: All the supplementary data are available in the annexed CD.

Abstract

Aluminum toxicity limits crops yield worldwide. The primary symptom of Al toxicity is the inhibition of root growth, and few works have addressed the events that take place in the aerial part of the plant. Moreover, Al tolerance has been extensively studied using hydroponics despite the fact that this method does not maintain all root apex components necessary for the proper root growth such as allow soil as a substrate. We have compared the transcriptome of leaves from maize seedlings grown in a control soil without free Al and in an acid soil containing toxic levels of Al. Our data demonstrated that acid soil triggered several changes in the transcriptome of an Al-tolerant genotype (Cat100-6), while S1587-17, an Al-sensitive genotype, had no differentially expressed genes. Several genes related to the metabolism of ROS were identified. Additionally, genes associated to photosynthesis and photorespiration were down-regulated. Interestingly, genes encoding for enzymes from TCA cycle were up-regulated, suggesting a role for the organic acids produced in the leaves as part of the plant responses to Al in the roots. This study provided for the first time insights about the effects of acid soil with toxic levels of Al on the transcriptome of maize leaves.

Key words: acid soil, abiotic stress, leaves, microarray, *Zea mays*

Introduction

Acid soils causes low yield in many crops due the presence of stresses such as low pH, toxicity of aluminum (Al), manganese (Mn), and iron (Fe), and deficiencies of nitrogen (N), phosphorus (P), calcium (Ca), and magnesium (Mg). About 30% of the world's soils are acidic and 60% are located in to tropical and subtropical areas [1]. One of the major problems on acid soils is Al phytotoxicity. In soils with pH above 5, Al is precipitated which has no phytotoxic effect. At lower pH this compound is solubilized and Al is released interfering on plant growth.

The most evident and studied consequence of Al exposure is the root growth inhibition [2] which lead the focus on the root tip, which it is the most sensitive tissue from roots when challenged with Al [3-4] and little attention has been paid to the aerial portion of the plant. Al can be transported to the shoot through the phloem [5] and directly affect the photosynthetic activity [6] or provoke the stomata closure [7], lipid peroxidation and activate antioxidant enzymes [8], enhances the activities of enzymes related to carbon assimilation in citrus [9], and reduce chlorophyll content [10]. In maize, Al effects in leaves includes the variation of the activity of Calvin cycle enzymes [11], disorganization of tylacoid structure, inhibition of photosystem II [12], enhanced of ethylene production, lipids peroxidation and activities of antioxidants enzymes [13].

Results achieved so far regarding the discovery of genes involved in Al tolerance were obtained using hydroponic culture to assess transcriptional profiles of roots from wheat [14], Arabidopsis [15], maize [16-17] and common bean [18]. To our knowledge no studies concerning transcriptional changes in leaves due Al stress have been published so far. Hydroponic culture does not create the best condition for the rizosphere development [19].

In this study we present the result of a large scale analysis of transcriptional profiling using Affymetrix microarray of maize leaves grown on acid soil containing toxic levels of Al. We used two varieties contrasting for Al tolerance and we identified almost 3,000 genes

differentially expressed due to acid soil treatment in the Al-tolerant genotype. Surprisingly, the Al-sensitive genotype showed no changes in the expression profile. Our data highlight several metabolic modifications in the Al-tolerant genotype in response to the growth in acid soil in the Al-tolerant genotype such as photosynthesis, photorespiration and TCA cycle, as well as the production and scavenging of reactive oxygen species.

Materials and Methods

Soil

The soil used is a Dark Red Latossol collected at Fazenda Santa Elisa, Campinas, SP, Brazil, at the location 22°59'09"S 47°05'25"W. It was collected at 20-40 cm depth, sieved through a 4 mm mesh and kept inside plastic boxes protected from light. Soil analysis performed by the Agonomic Institute of Campinas (IAC) demonstrated that the original soil presented pH 4.1 and Al content of 10 $mmol_c/dm^3$. To prepare the acid soil treatment the original soil received fertilization to avoid nutritional stress. The control soil was incubated with 0.8g of $Ca(OH)_2$ per Kg of soil for one week prior the fertilization with the same amount of nutrient provided to the acid soil. The incubation with $Ca(OH)_2$ increased the pH to pH 5.5 and the presence of Al free Al could not be dedected (data not shown). The fertilization was comprised by the following nutrients (mg/Kg of soil): 56 of N; 38.75 of P; 78 of K; 32 of S; 60 of Mg; 0.5 of B; 0.5 of Cu; 0.01 of Mo; 1.0 of Zn. The acid soil also received the correction of Ca through the addition of a $CaCl_2$ solution to compensate the $Ca(OH)_2$ added to the control soil. The soil was mixed several times to diminish the natural variability of the physical and chemical properties and insure the homogeneity of the nutrient added.

Plant material and growth conditions

Seeds from the tropical maize (*Zea mays* L.) inbreed lines Cat100-6 and S1587-17 were obtained at University of Campinas. Seed were geminated for 2 days in moist filter paper. Only seedlings with similar initial root length were transferred to the soil plastic bags (500 mL each) filled with 1 kg of each soil (with 15% water – mL/Kg). Each bag received 20 seeds which were grown in a growth chamber at 26°C (light: dark, 16:8h). The bags weighted and then twice a day the weight was completed with distillated water to keep the humidity at 15%.

Dry Weight (DW)

After soil cultivation the germination bags were cut with a scalp and the soil was gently removed to expose the plants. Each plant was washed in running water to remove the excess of soil and then gently dried with absorbing paper to remove the excess of water and the whole plant liofilized for 24h. The DW was accessed in an analytical balance.

Carbon isotopic composition

Young maize leaves were collected and lyophilized. Carbon isotopic composition was determined at the Stable Isotope Laboratory (CENA-USP, Piracicaba, Brazil) using PDB as standard. Carbon isotopic discrimination was calculated according to [20]: $\Delta = (\delta a - \delta p)\, \delta a/(1+\delta p)$, where δa is the isotopic composition of the dry leaf and δp is the isotopic composition of the air.

RNA extraction

The RNA was extracted with RNeasy Plant Mini Kit (Quiagen, EUA). The RNA was evaluated in agarose/formaldehyde gel, quantified in spectrophotometer and stocked at -80°C.

Microarray hybridization and analysis

For the microarray experiment, three independent replicates were used totalizing 24 samples. Two µg of each RNA sample were processed and hybridized to the Affymetrix GeneChip® Maize Genome Array as described by the manufacture's protocol. The hybridization, staining, washing and scanning were performed at Laboratório Nacional de Luz Sincrontron (LNLS), Campinas, SP, Brazil, with the use of the Command Console Software (Affymetrix, USA). The data was normalized by the RMA method, $\log_2$ transformed and loaded into SAS (SAS Institute, USA) to perform the contrasts. The One-way Analysis of Variance (ANOVA) was the statistical method used and allowed us to separate the contribution of each effect on the expression level of a given gene. The model used was: $y_{ikl} = \mu + G_i + Ta_k + (G \times Ta)_{ik} + (G \times Ta)_{ik} + \xi_{ik}$ where μ is the sample mean, G_i represents the Genotype Effect for the i^{th} Gentotype (e.g. Cat100-6 or S1587-17) (df = 1), Ta_k is the Effect of the k^{th} Treatment (e.g. acid soil or control soil) (df = 1), $(G \times Ta)_{ik}$ is the Effect of interaction between Genotype and Treatment (df = 1), and ξ_{ik} is the residual error. Least-square means for each gene in each sample was generated and the Differential Estimates (DE) in expression were calculated as the difference between least-square means for each of the terms in the model. DE values were calculated between acid and control soil treatments and also between genotypes. False discovery rate (FDR) was set to 10% to control Type I errors [21]. Qvalues were calculated from Pvalues using the software qvalue from the R platform [22]. Only the genes with DE above 1 were further analyzed. The generated list of differentially expressed genes were annotated with the use of Blast2GO software www.blast2go.org [23-24] using all default settings.

Real Time qPCR

RNA from other two independent replicates was treated with DNase I Aplification Grade (Invitrogen, USA) and the cDNA were synthesized from 2 µg of RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA). The Real Time qPCR were performed with a ABI 7500 (Applied Biosystems, USA) using Sybr Green I PCR Master Mix (Applied Biosystems, USA). Primers were designed using Primer Express 2.0 software (Supplemental Material - Table S2). The efficiency of each pair was tested with a relative standard curve experiment. The maize tubulin gene (Zm.6045.1.A1_s_at) was used as an endogenous control. As all primers efficiency was near to 100% the relative expression was calculated by the ΔΔCt method. For microarray validation, the ΔCt values were calculated for each gene in each sample, $\log_2$ transformed and plotted against its corresponding least-square means data from de microarray.

Results and Discussion

Physiological analysis of maize leaves from plants grown in acid soil

The works aiming to elucidate plants transcriptional responses or proteome changes in response to Al exposure have used hydroponic culture [15-18,25]. Because this method does not allow the maintenance of all root apex components and the root architecture we decided to use a soil substrate, closer to the field conditions. These studies also look up for changes in the roots, and no large-scale study was ever made in leaves to elucidate transcriptional responses.

The use of soil as a growth substrate has been applied only to evaluate phenotypes [26-28]. Few works have been done to elucidate tolerance responses to Al with other methods rather than hydroponic and, additionally, the changes in expression profile in leaves has never been evaluated. The soil used in the present study is a Dark Red Latossol with pH 4.1 and Al content

of 10 $mmol_c/dm^3$. As a control we used the same soil with pH corrected to 5.5 (See Materials and Methods). The S1587-17 (Al-sensitive) is a somaclonal variation regenerated from callus culture of the Cat100-6 (Al-tolerant) line [29] and these lines have been used in several studies on Al tolerance, always using hydropony [16-17,30-33]. As shown elsewhere the evaluation the relative root growth (RRG), Al content in root tips and callose deposition due acid soil treatment after 1 day and 3 days was able to discriminate both genotypes (Mattiello et al., Submitted). In this study, Al-sensitive plants were severely affected by Al in both time points, while Al-tolerant plants were affected only after 3 days, and at a lower extension than Al-sensitive plants.

S1587-17 presented a more vigorous growth than Cat100-6 in this short-term experiment either in control or acid soil (Figure 1), which is reflected in the dry weight measurements (Figure 2). However, while the dry weight of Cat100-6 is practically unaltered during acid or control soil growth, the Al-sensitive genotype has its mass reduced to 88% after growing in the acid soil. A similar result can be observed comparing the roots from these two maize lines grown in nutrient solution without Al. After a longer (7 days) exposure to Al, S1587-17 roots were severely affected [29], suggesting that longer experiments in soil would keep the same tendency and allow a better discrimination between the leaf growth in these two genotypes.

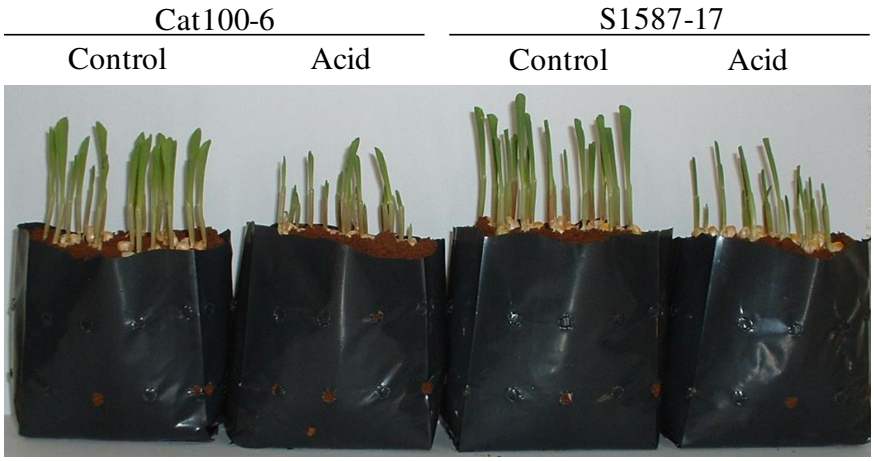

Figure 1. Phenotype of maize plants grown on acid or control soil. Plants were grown on acid (pH 4.2) and control (pH 5.5) soil for three days.

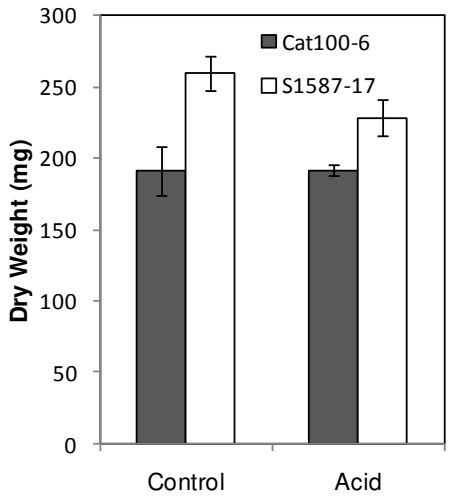

Figure 2. Effect of acid soil saturated with Al^{3+} on maize whole plant dry weight. Plants were grown on acid (pH 4.2) and control (pH 5.5) soil for three days. The vertical error bars represent mean ± SD (n=5).

The two maize genotypes had different carbon isotopic composition ($\delta^{13}C$) in the leaves (-10.4 $^{o}/_{oo}$ in Cat100-6 and -11 $^{o}/_{oo}$ in S1587-17) when growing in control soil. These values were not affected by the growth in acid soil (data not shown), indicating that ^{13}C discrimination was not affected by the Al stress. This may be explained by the short term of the experiment and by the fact that the young seedlings still relay at least in part from the carbon resources mobilized from the seed. These differences may be due to metabolic differences between genotypes rather than growth under stressful conditions.

Gene Expression Profiling

The transcriptional profile of leaves from the two maize genotypes was evaluated with the Affymetrix GeneChip® Maize Genome Array. Volcano plots allowed the integration of biological relevance (DE – Differential Estimate, see Materials and Methods section) with statistical significance (FDR or Qvalue) of large-scale analysis. The contribution of each effect in the expression of any given gene was done using an ANOVA analysis. The Genotype Effect (G_i)

is shown in Figure 3A and it is clear the wide range of the distribution of DE with high statistical relevance. The genes with positive DE were more expressed in the Al-tolerant variety while negative DE represent genes more expressed in the Al-sensitive variety. These data indicates that the two genotypes present different expression patterns despite the stressor.

The Treatment Effect (Ta_k – Figure 3B) presented lower number of DE with high statistical relevance, despite the fact that the amplitude was narrower when compared to the Genotype Effect. When this effect is desiccated in two other plots, one for each maize line, a higher number of genes is elucidated. This analysis is also known as the Effect of interaction between Genotype and Treatment ($(G \times Ta)_{ik}$). For Cat100-6 (Figure 3C) 1805 genes were up regulated (positive DE) while 1165 was repressed (negative DE) (Table S1 – Supplemental Material). On the other hand, S1587-17 (Figure 3D) presented no differentially expressed genes. This indicates striking differences between from the two maize varieties in their leaf responses to acid soil treatment. At this stage there is no supporting data to explain the absence of changes in the transcriptional profile from the Al-sensitive maize genotype. The high level of transcripts with differential expression in S1587-17 roots indicates this line is able to perceive the signals derived from the toxic conditions found in the acid soil. We can speculate that S1587-17 leaves do not receive any signals from the stressed roots, which are received in the case of Cat100-6 leaves.

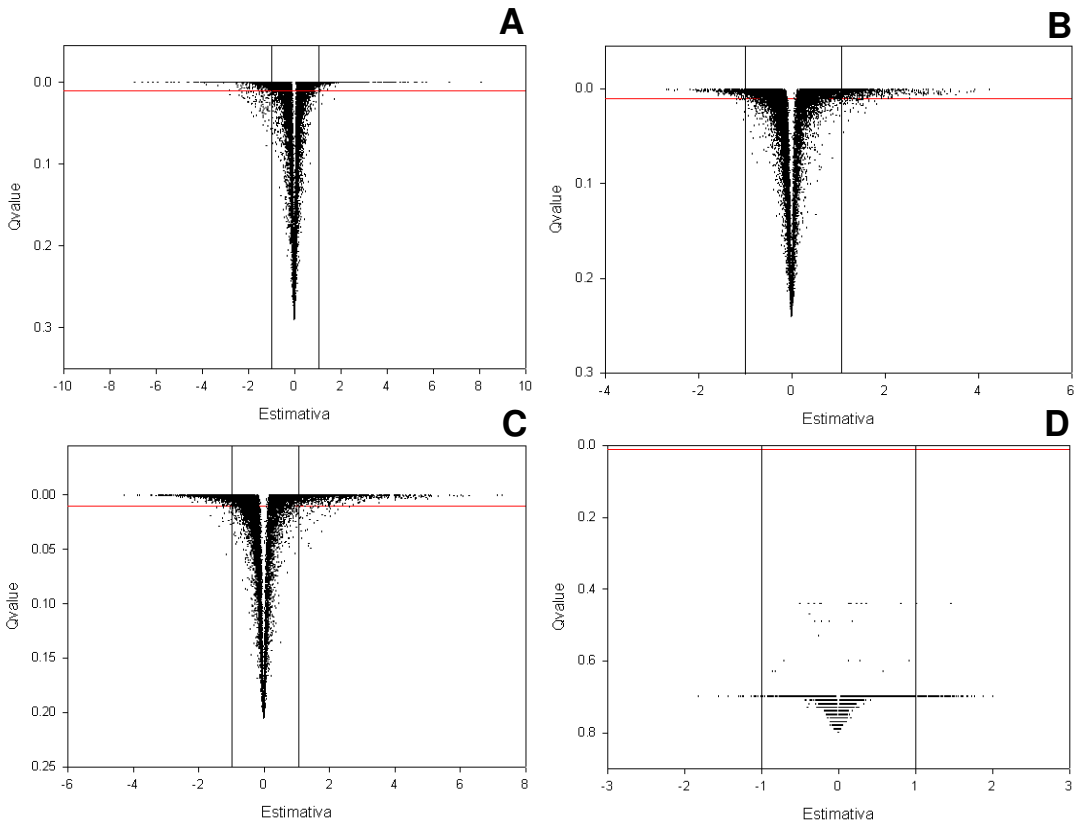

Figure 3. Volcano plots representing each Effect or Interaction of Effects. Estimates were calculated as the difference between the least-square means for each contrast (x-axis). Estimates equal to zero represents no expression change and estimates different from zero represents genes expression modification. A: Genotype Effect; B: Treatment Effect; C: Interaction Effect between Genotype and Treatment for Cat100-6; D: Interaction Effect between Genotype and Treatment for S1587-17. The red line represents a FDR of 1%, and data points above this line represent significant observation (y-axis represent Qvalues). Note that the Estimate axis is different for each plot.

Due to the large number of genes identified in Cat100-6, a functional analysis was performed with the identification of the Gene Onthologies to facilitate the recognition of the pathways affected by Al toxicity. In the first day most of up-regulated genes were involved with biosynthetic processes, cellular macromolecular, protein and geral metabolic processes (Figure 4A). On the other hand, most genes down-regulated encoded proteins involved to cellular response to oxidative stress, carbohydrate metabolism and lipid metabolic process (Figure 4B).

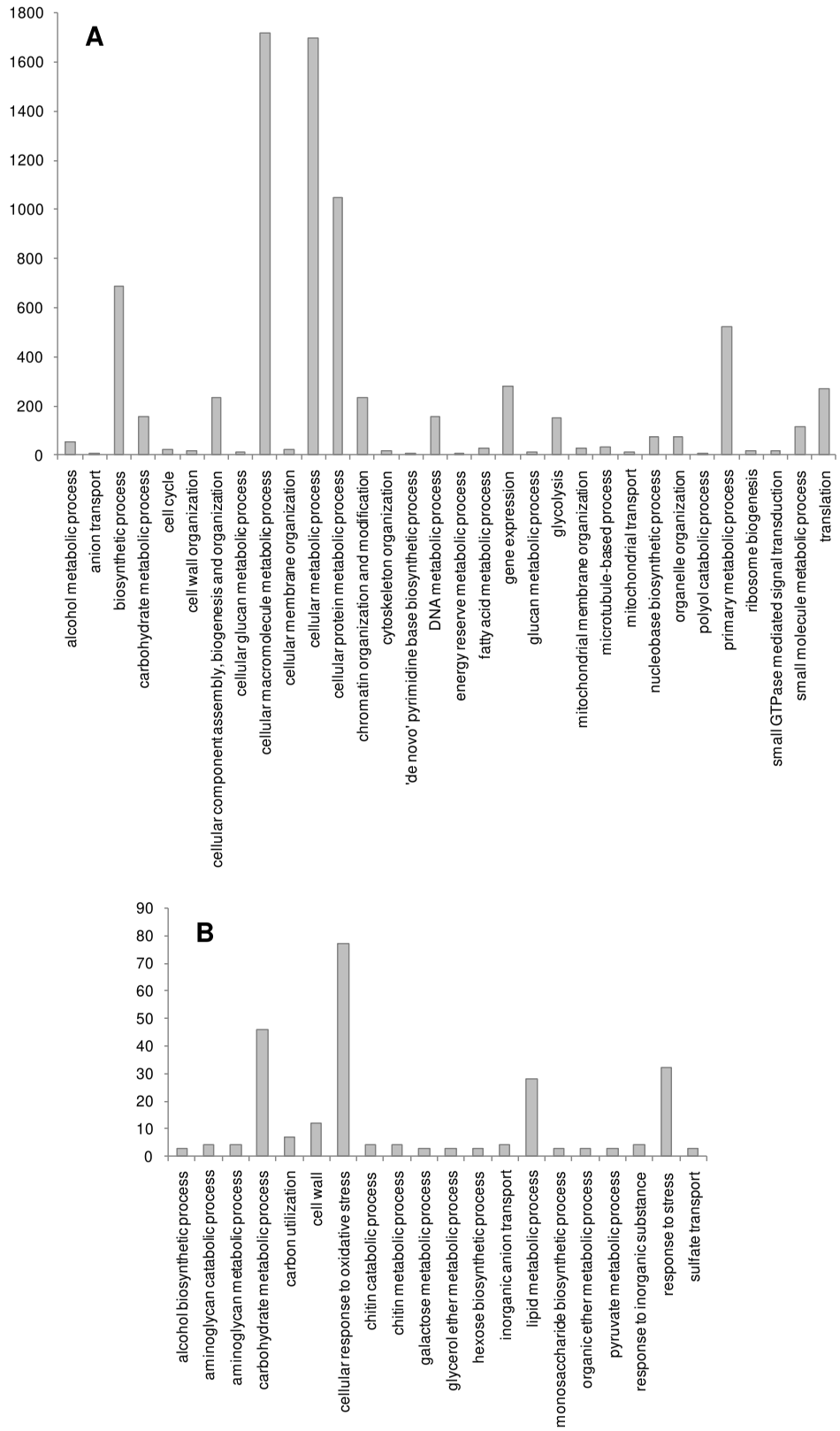

Figure 4. Function analysis of genes differentially expressed in Cat100-6 after 3 days of treatment in acid soil. A: Up-regulated; B: Down-regulated. All the genes that did not present Gene Onthologies were removed from the analysis. The y-axis represents the number of genes belonging to the respective biological function.

As up-regulated genes we can mention a dirigent protein (DE: 5.03), which is related to the production of lignans and lignins [34] and citrate synthase (DE: 4.27), a key enzyme for the TCA cycle. Among down-regulated genes is sucrose phosphate synthase (DE: -4.25), which may indicate the down-regulation of sucrose biosynthesis.

Effect on photosystems

Our microarray analysis indicated that all genes encoding components of the photosystems were down-regulated in the Al-tolerant genotype as shown in Figure 5 and Table 1. Several studies demonstrated that CO_2 assimilation was reduced upon Al exposure, as observed in *Citrus ssp*. [9,35-36], tomato [37], *Guercus glauca* Thumb [38] and maize [11,39]. Akaya and Takenaka [38] demonstrated that Al induces stomata closure, but this may not be the main cause of the photosynthesis reduction observed in Cat100-6, since the stomatal conductance and internal carbon concentration were not significantly affected due to acid soil treatment (data not shown). These data from Cat100-6 is in agreement with a study with tomato also showing no alteration in stomatal conductance and internal carbon concentration after Al exposure [37].

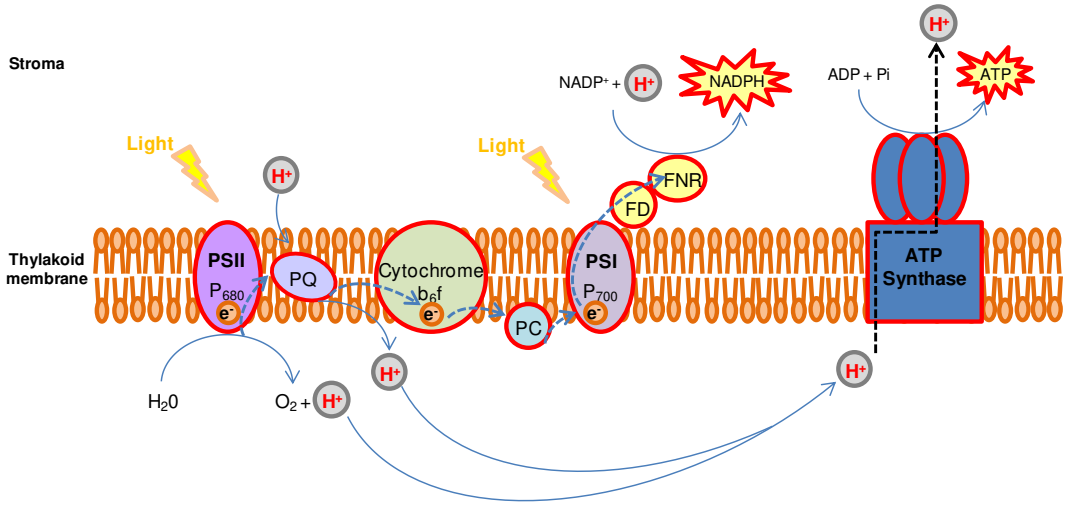

Figure 5. Representation of photosynthesis light-dependent reactions that takes plance inside chloroplasts. All the components with red outline represent genes down-regulated in Cat100-6 after acid soil growth.

Table 1. Genes related to photosynthesis identified as differentially expressed in Cat100-6 after acid soil grown (FDR 1%; DE ≥ 1).

GeneID	Estimate	Qvalue	Annotation
ZmAffx.1462.1.S1_at	-1,75	0,0075	ATP synthase beta subunit
ZmAffx.1432.1.S1_at	-1,38	0,0033	ATP synthase cf0 subunit iii
ZmAffx.1431.1.S1_at	-1,43	0,0052	ATP synthase cf0 subunit iv
Zm.5003.1.A1_at	-1,32	0,0022	cytochrome b561
Zm.5003.2.A1_a_at	-1,36	0,0014	cytochrome b561
Zm.5003.2.A1_x_at	-1,36	0,0011	cytochrome b561
ZmAffx.1507.1.S1_at	-1,40	0,0036	cytochrome b6
ZmAffx.1509.1.S1_at	-1,71	0,0018	cytochrome b6 f complex subunit iv
Zm.15923.1.S1_x_at	1,07	0,0010	cytochrome c
ZmAffx.1336.1.S1_at	-1,64	0,0020	cytochrome c heme attachment protein
ZmAffx.1467.1.S1_at	-1,07	0,0055	cytochrome f
Zm.1630.1.S1_at	-1,14	0,0037	ferredoxin-1
Zm.6.1.A1_at	-1,80	0,0010	ferredoxin-dependent glutamate synthase
Zm.3410.1.A1_at	-1,07	0,0026	ferredoxin-$NADP^+$ reductase
Zm.272.1.A1_at	-1,49	0,0016	ferredoxin-$NADP^+$ reductase
Zm.18989.1.S1_at	-1,71	0,0005	ferric reductase-like transmembrane component
ZmAffx.1465.1.S1_at	-1,26	0,0038	photosystem I assembly protein ycf4
ZmAffx.1455.1.S1_at	-2,00	0,0064	photosystem I p700 apoprotein a1
ZmAffx.1454.1.S1_at	-1,47	0,0061	photosystem I p700 apoprotein a2
ZmAffx.1156.1.S1_s_at	-2,25	0,0039	photosystem I subunit b
ZmAffx.1338.1.S1_at	-1,35	0,0077	photosystem I subunit VII
Zm.14566.1.S1_a_at	-1,12	0,0017	photosystem II 11 kd protein
ZmAffx.1489.1.S1_at	-1,26	0,0047	photosystem II 47 kda protein
ZmAffx.1492.1.S1_at	-1,45	0,0052	photosystem II phosphoprotein
ZmAffx.1435.1.S1_at	-1,24	0,0033	photosystem II protein d1
ZmAffx.1441.1.S1_at	-1,08	0,0065	photosystem II protein d2
ZmAffx.1439.1.S1_at	-1,35	0,0039	photosystem II protein i
ZmAffx.1491.1.S1_at	-1,50	0,0061	photosystem II protein n
ZmAffx.1445.1.S1_at	-1,08	0,0021	photosystem II protein z
Zm.3414.1.A1_at	-1,23	0,0020	photosystem II reaction center w protein
Zm.4960.1.S1_at	-1,35	0,0007	plasma membrane H^+ ATPase
Zm.13934.1.S1_at	-1,91	0,0034	plasma membrane H^+ ATPase
Zm.13376.1.S1_s_at	-1,29	0,0035	plasma membrane H^+ ATPase
Zm.7003.1.A1_at	-1,04	0,0021	plastoquinol-plastocyanin reductase

ROS scavenging and production genes

Plant cells produce ROS (Reactive Oxygen Species) normally due to respiration and other biochemical processes that provoke the reduction of oxygen molecules. There are several enzymes that produce ROS and others responsible for ROS scavenging, but when there is an unbalance, such as caused by abiotic stresses, the production of ROS is higher that the power of cell proteins to overcome them. It has been demonstrated that plants treated with Al uses only a portion of the light received which can result in the overwhelming production of ROS causing damage to the photosynthetic apparatus and cell structure [40-41]. Additionally, the photosystem repair is highly inhibited by oxidative stress [42-43]. Peroxidases are important ROS generating enzymes and several genes encoding these enzymes were found in our study (Table 2). This expression profile is in agreement with a study in maize showing increased activity of anti-oxidative enzymes in shoots when roots were exposed to Al [13]. We were able to identify a few ROS scavenging enzymes, and they presented different expression profiles, with some being up-regulated and others down-regulated, indicating a complex response (Table 2). Rice leaves treated with an Al solution had reduced activity of superoxide dismutase , while an increase was observed in the activity of catalase, gluthatione reductase, peroxidase and ascorbate peroxidase, indicating the involvement of ROS scavenging enzymes to suppress harmful effects of Al on biochemical processes taken place in leaf cells [8].

Table 2. ROS producing and ROS scavenging genes identified as differentially expressed in Ca100-6 after acid soil grown (FDR 1%; DE ≥ 1).

GeneID	Estimate	Qvalue	Annotation
Zm.18111.1.S1_at	1,73	0,0005	peroxidase
Zm.2136.1.S1_at	-1,77	0,0004	peroxidase
Zm.2766.1.A1_at	-2,73	0,0004	peroxidase
Zm.2707.1.S1_at	-2,99	0,0004	peroxidase
Zm.4541.1.S1_at	1,91	0,0006	peroxidase 16 precursor
Zm.4894.1.A1_at	-1,99	0,0018	peroxidase 52
Zm.4338.1.A1_at	1,14	0,0030	peroxidase 72
Zm.16830.2.S1_at	1,18	0,0007	peroxidase atp6a
Zm.11864.1.S1_at	1,61	0,0004	peroxidase family expressed
Zm.2505.1.S1_at	2,62	0,0004	peroxidase like protein
Zm.405.1.A1_at	-1,86	0,0004	peroxidase p7x
Zm.3093.1.S1_at	-2,08	0,0020	peroxidase precursor
Zm.3497.1.A1_at	-1,10	0,0016	glutathione peroxidase
Zm.550.1.A1_at	1,68	0,0004	glutathione s-transferase
Zm.628.1.A1_at	1,59	0,0007	glutathione s-transferase
Zm.883.1.A1_at	1,54	0,0005	glutathione s-transferase
Zm.553.1.S1_at	-1,69	0,0009	glutathione s-transferase
Zm.546.1.A1_at	1,68	0,0019	glutathione s-transferase 6
Zm.545.1.S1_at	-1,04	0,0053	glutathione s-transferase gstf2
Zm.564.1.S1_a_at	-1,57	0,0007	glutathione s-transferase gstu6
Zm.558.1.S1_s_at	-2,21	0,0008	glutathione s-transferase gstu6
Zm.16447.1.S1_at	-1,14	0,0047	ABC transporter
Zm.5635.1.A1_at	1,04	0,0004	ABC transporter family protein
Zm.14399.1.S1_at	-1,47	0,0014	ABC transporter family protein
Zm.5809.1.A1_at	-1,66	0,0012	ABC transporter-like
Zm.5195.1.A1_at	1,95	0,0028	ATP binding protein
Zm.2928.1.S1_at	1,25	0,0005	ATP binding protein
Zm.3548.1.A1_at	-1,43	0,0010	ATP binding protein
Zm.915.1.S1_at	1,94	0,0004	multidrug pheromone mdr abc transporter family

Carbon fixation

The Calvin cycle is conducted by a group of enzymes in the chloroplast stroma that participates in the carbon fixation. The initial compound is the ribulose-1,5-bisphosphate which must be recovered at the end of the cycle. The key enzyme is the ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) and it posses and unspecific reaction site that can bind to both

oxygen (leading to photorespiration) or carbon dioxide (entering the Calvin cycle). Plant with C_4 metabolism concentrate CO_2 in carboxylate forms prior to the Calvin Cycle and genes related to this step, such as pyruvate phosphate dikinase (Zm.4471.2.S1_at), NAD (P) malic enzyme (Zm.15.1.A1_a_at) and Rubisco itself (Zm.514.1.A1_at) were down-regulated in Cat100-6 plants. These data suggest that growing in acid soil interferes in the expression of genes responsible for the first steps of carbon assimilation. These results are in disagreement with a previous report in other maize genotypes that indicated enhanced activity of the NADP-malic enzyme and pyruvate phosphate dikinase in response to Al [11]. However, several studies indicated that the treatment of plants with toxic metals affect negatively the activity of enzymes from the Calvin cycle in species such wheat, *Phaseolus vulgaris*, rye, pea, barley and maize [44-47]. On the other hand, in tangerine, Al reduced the CO_2 assimilation, stomatal conductance and internal CO_2 concentration, but the activity of the Calvin cycle enzymes such as NADP-gyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase e fructose-1,6-bisphosphate and Rubisco were increased [9]. Taken all together, these data indicate that the Al response can vary between species and even between genotypes of the same species.

Under stressful conditions stomata may close, reducing the CO_2 concentration and resulting in increased photorespiration, since Rubisco can bind to both CO_2 and O_2. As mentioned before, our physiological data showed that the putative reduction on photosynthesis rate was not due stomata closure and subsequent reduction in the internal carbon concentration, which would deviate Rubisco activity to photorespiration. In fact, genes related to this pathway were down-regulated (Rubisco - Zm.514.1.A1_at and ferredoxin-dependent glutamate synthase – Zm.6.1.A1_at). These data suggest that a decrease in photosynthesis rate would not correlated with increased expression of genes related to photorespiration, corroborating with the unmodified internal CO_2 concentration due to acid soil exposure.

Organic acids production

Organic acid exudation is considered the main Al-resistance mechanism [2]. Several genes from the TCA cycle were up-regulated in maize leaves such as those encoding pyruvate dehydrogenase (Zm.434.1.A1_at), citrate synthase (Zm.15069.1.A1_at), malate dehydrogenase (Zm.17850.1.S1_at, Zm.3845.2.A1_at, Zm.5897.1.A1_at and Zm.2061.1.A1_at) and isocitrate dehydrogenase (Zm.5861.1.A1_at). Interestingly, such changes did not take place in maize roots from plants grown in the same conditions (Mattiello et al., Submitted) or in hydroponics [16-17]. Increased production of malate and citrate was already reported in the leaves of *Citrus grandis* in response to Al [48]. Additionally, two works have already demonstrated that photosynthesis and organic acid accumulation in leaves have an important role in citrate exudation when soya seedlings are challenged with Al [49-50]. Interestingly, the differential expression of organic acid transporters was observed in maize plants grown in hidroponics [17], but this was not the case in the roots of soil grown maize plants (Mattiello et al., Submitted). Taken all together, these results let us to suggest that organic acids production was stimulated in the leaves and then transported through the vascular system down to the roots and consequently exudated, without the need of the increased of expression of transporters genes when plants are grown in the soil.

This study reports for the first time the transcriptional changes in maize leaves from plans grown on acid soil with phytotoxic levels of Al. Our data demonstrated that the transcriptome of Cat100-6 presented major changes while the Al-sensitive genotype (S1587-17) revealed no differentially expressed genes.

Several genes encoding for ROS generating or ROS scavenging gene were identified, revealing the role of oxygen reactive species in the leaves. Additionally, genes related to photosynthesis and photorespiration were down-regulated. Interestingly, genes encoding for enzymes that belong to the TCA cycle were up-regulated in leaves but not in roots (Mattiello et

al., Submitted), leading us to speculate on a role of the organic acids produced in the aerial part of the plant in the root defense against the Al in soil.

Validation of gene expression profiles using qPCR

Five genes with different expression patterns were selected and Real Time qPCR was performed to validate the microarray results. This validation was done with two other biological replicates (different from the replicates used for microarray experiment). A significant correlation between the two data sets was observed ($R^2 = 0.7592$), as indicated in Figure 6.

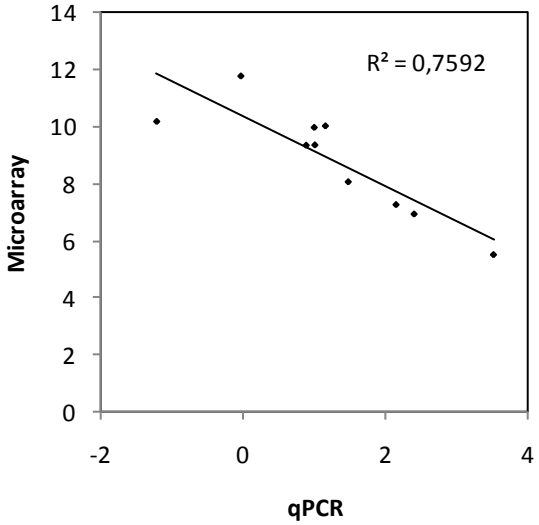

Figure 6. Real Time qPCR validation of microarray results. qPCR data were log2 transformed and plotted against microarray data (least-square means). The correlation is negative because in qPCR data, the more the gene is expressed the lower is its Ct value.

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Description of Additional Files

Table S1: Annotation of genes differentially expressed in Cat100-6 after acid soil growth for three days (FDR = 1% and DE ≥ 1).

Table S2: Affymetrix sequences and primers used for Real Time qPCR validation of microarray experiment.

DISCUSSÃO GERAL

A toxidez de alumínio (Al) é o principal fator limitante da produtividade agrícola em grandes áreas do Brasil e do mundo. A resposta desenvolvida pelas plantas contra esse íon indica uma ampla diversidade de respostas e sua compreensão pode ser ampliada com a identificação de genes responsivos à exposição ao Al. Este projeto teve como objetivo ampliar o conhecimento sobre a fisiologia e a regulação gênica de raízes e folhas de genótipos contrastantes de milho (Cat100-6 (tolerante) e S1587-17 (sensível) cultivadas em solo ácido com concentrações fitotóxicas de Al.

A avaliação da variabilidade genética em relação à tolerância ao Al geralmente é executada em condições hidropônicas (Sephar, 1994; Delhaize et al., 2003; Delhaize et al. 2004; Wenzel et al., 2003; Anoop et al., 2003; Sasaki et al., 2004). Enquanto que alguns estudos demonstram que não há correlação entre a avaliação em hidroponia ou cultura em solo (Sartain e Kamprath, 1978; Noble et al., 1987), outros demonstram que existe alguns exemplos de correlação positiva entre os dois métodos (Baier et al., 1995; Urrea-Gómez et al., 1995). O uso de solo ácido e corrigido também possibilitou a distinção fenotípica de triticale, Arabidopsis e cevada (Ma et al., 2001; Kobayashi et al., 2005; Delhaize et al., 2009).

O solo utilizado no presente estudo contém 10 $mmol_c/dm^3$ de Al (equivalente a 90 ppm) e pH de 4.2, condições capazes de elicitar a resposta fitotóxica do Al (Marschener, 1991; Wersuhn et al., 1994; Kirkpatrick et al., 1995; Kinraide, 2003). As linhagens de milho Cat100-6 e S1587-17 foram cultivadas por um ou três dias em solo não corrigido (pH 4,1) ou corrigido com $Ca(OH)_2$ (pH 5,5). O genótipo S1587-17 apresentou uma maior inibição do crescimento radicular nos períodos amostrados algo que está altamente correlacionada com a acumulação de Al nos ápices

radiculares, já que o genótipo sensível, S1587-17, acumulou quantidades significativamente maiores de Al que o genótipo tolerante, Cat100-6. A quantidade de Al adsorvido pelo ápice radicular é um indicativo da resistência e sensibilidade de diferentes espécies perante o estresse (Larsen et al., 1998; Wenzl et al., 2001; Yamamoto et al., 2001). Nossos resultados corroboram outro estudo de nosso grupo que utilizou as mesmas variedades cultivadas em hidroponia na presença e na ausência de diferentes concentrações de Al durante a avaliação de exsudados (Jorge e Menossi, 2005).

Vários estudos demonstraram a relação entre o alumínio fitotóxico e a deposição de calose, sendo que este um parâmetro utilizado para avaliar a tolerância de diferentes espécies frente ao estresse (Wissemeier e Horst, 1995; Llugany et al., 1994; Zhang et al., 1994; Wissemeier et al., 1998). A calose é composta de 1,3-β-glucans em um grau de polimerização suficiente para torná-lo insolúvel (Kauss, 1992). Sua função é fechar as conexões entre protoplastos de células contíguas e a deposição pode ser bem rápida, na faixa de minutos (Kauss, 1987). Nossos dados indicam maior produção de calose no genótipo sensível em comparação com o genótipo tolerante. Estudos também já demonstraram que a calose pode ser detectada antes mesmo da inibição do crescimento radicular (Wissemeier e Horst, 1995; Zhang et al., 1994). Estes dados confirmam as diferenças entre as duas linhagens em condições de solo, abrindo perspectivas para entender pela primeira vez a base molecular das alterações das plantas em condições próximas à realidade de campo.

A partir das hibridizações com os chips da Affymetrix do RNA extraído de raízes foram identificados possíveis candidatos a tolerância ao Al dentre eles: genes codificantes para uma lipase hidrolase com motivo GDSL, uma retinol desidrogenase, um fator de transcrição da família WRKY, uma proteína rica em glicina e duas proteínas desconhecidas. Adicionalmente, com um experimento de hidroponia separamos as variáveis pH e presença de Al, ambas

condições diferenciais em nosso tratamento com solo. Entre os candidatos observados nos ensaios em solo identificamos genes que respondem à presença do Al e não à acidez, delimitando assim os genes com possíveis papéis na tolerância ao Al presente no solo ácido a apenas três: retinol desidrogenase, uma proteína desconhecida e um fator de transcrição WRKY.

O mRNA do homólogo da retinol desidrogenase em uva foi descrito por Coupe e colaboradores (1994). Nessa enzima foi possível identificar um sítio de ligação NAD/FAD desidrogenase e uma região conservada da família ribitol desidrogenase. Um membro dessa família de desidrogenases de Arabidopsis (SDR1) funciona como uma molécula que une a sinalização de nutrientes com a via de biossintese hormonal já que gene faz parte da via de produção de ABA, algo crítico para o crescimento e desenvolvimento da planta, resposta a estresses e sinalização por glicose (Cheng et al., 2002). Dessa forma, o gene Zm.19227.1.S1_at pode ser um possível candidato a participar da via de tolerância proporcionando maior produção hormonal.

A proteína codificada pelo gene Zm.1871.1.A1_at não foi identificada, mas este apresenta um domínio conservado típico de metiltransferases que são responsáveis pela metilação de diversos componentes celulares como DNA, RNA, proteínas e pequenas moléculas (Klimasauskas e Weinhold, 2008). Proteínas com esse domínio também apresentam papéis importantes na resistência a doenças, crescimento e desenvolvimento (Joshi et al., 1998). Estudos prévios em arroz (Fukuda et al., 2007), Arabidopsis (Zhao et al., 2009) e tomate (Zhou et al., 2009) também identificaram membros dessa família como ativados mediante o estresse por Al. Esse é o primeiro relato de uma putativa função de um gene com esse domínio na tolerância ao Al em milho.

A família de fatores de transcrição WRKY apresenta um número elevado de membros (Rushton e Somssich, 1998) e já foi demonstrada a indução de alguns deles após ferimentos,

infecção por patógenos e estresses abióticos (Eulgem et al., 2000; Marè et al., 2004; Fowler e Thomashow, 2002; Seki et al., 2002). Além disso, membros dessa família são identificados após tratamento com Al (Maron et al., 2008; Kumari et al., 2009; Goodwin e Sutter, 2009; Zhao et al., 2009).

Diversos processos metabólicos da linhagem sensível são afetados pelo tratamento em solo ácido e podem estar envolvidas na inibição radicular, como a produção de ROS e a modificação da parede celular, através da produção de lignina, celulose e calose, bem como os processos relacionados com a produção, transporte ou reposta à auxina e etileno.

O mapeamento cromossômico dos genes das listas que consideramos possíveis responsáveis pela tolerância ao Al na Cat100-6 permitiu a identificação de genes localizados dentro de QTLs de milho previamente descritos na literatura como responsáveis pelo fenótipo tolerante (Sibov et al., 1999; Ninamango-Cárdenas et al., 2003). No QTL_1, foi mapeada uma proteína ligadora de RNA de dupla fita o que pode indicar que mecanismos de silenciamento gênico por miRNAs podem estar atuantes em genes alvo que contribuem para a tolerância ao Al. Dentro do mesmo QTL foi mapeada uma a*cyl-Coa thioesterases* podem estar relacionadas com a produção de ácidos graxos ou na β-oxidação de lipídeos (Tilton et al., 2000).

Nos QTLs *Alm2* e QTL_2 encontramos o gene *SMP1* (*Swellmap1*) que constitui uma proteína ligadora de ácidos nucléicos como RNA em fita simples, que pode favorecer a permanência da divisão celular mesmo quando exposto a solo ácido com concentrações fitotóxicas de Al. Outro gene identificado no QTL_2 e *Alm2* foi um inibidor de proteinase do tipo *subtilisin-chymotrypsin*. Inibidores de proteases podem ser induzidos por ferimentos ou tratamento com metil-jasmonato (Botella et al., 1996; Choi et al., 2000), estresse salino (Lopez et al., 1994; Huang et al., 2007), seca (Gosti et al., 1995; Huang et al., 2007) e patógenos (Choi et al., 2000). Adicionalmente, a superexpressão do gene *OCPII* em arroz aumentou significativamente a tolerância a seca e

estresse salino (Huang et al., 2007). Esses estudos demonstram que o gene constitutivo na variedade Cat100-6 pode fazer parte do sistema de tolerância a solo ácido por controlar a atividade de proteases.

No QTL_4 foram mapeados genes como de uma proteína *pirin-like* e ciclinas que estão relacionadas com alterações celulares durante o processo de apoptose (Foo e Nolan, 1999) e manutenção da divisão celular das células de ápice radicular (Doerner et al., 1996a; Doerner et al., 1996b), respectivamente. Boscolo et al. (2003) verificaram que a variedade tolerante não apresenta morte celular detectável quando exposta ao Al, com o qual podemos especular a contribuição do gene *pirin-like* para tal resultado. Como nosso próprios dados demonstram, a linhagem Cat100-6 não sofre inibição do crescimento radicular de forma severa, o que pode ser uma contribuição da expressão de ciclinas.

No QTL *Alm1* mapeamos o fator de replicação C (subunidade 2), muito importante para a replicação e reparo do DNA (Furukawa et al., 2003). Portanto, assim como as ciclinas descritas anteriormente, a expressão de genes relacionados com a proliferação celular e manutenção do DNA parece ser um mecanismo importante para a manutenção do crescimento radicular perante o estresse proporcionado pelo solo ácido. Além disso, mapeamos o gene *xyloglucan endotransglycosylase protein 8 precursor* responsável pela integração novos xiloglucanos recém sintetizados na nova parede, contribuindo para o crescimento radicular (Nishitani, 1997; Thompson e Fry, 2001). Neste mesmo QTL encontra-se o gene da *phosphoglycerate mutase-like* que catalisa uma etapa reversível da glicólise. Esse gene é induzido por auxina, infecção por parasitas (Mazarei et al., 2003) e nitrato (Wang et al., 2003). Portanto, esse gene é induzível por estresses bióticos e abióticos, o que demonstra a modificação do metabolismo da planta frente a estresses.

Para aprofundarmos os estudos de tolerância a solo ácido das variedades contrastantes de milho, efetuamos, pela primeira vez na literatura, o transcriptoma de folhas coletadas após três dias de cultivo em solo ácido ou solo corrigido com microarrays da Affymetrix. O transcriptoma das folhas indica profundas alterações na linhagem tolerante, em contraposição à ausência de alteração significativa nas folhas da linhagem sensível. Todos os genes referentes à fotossíntese e a fotorrespiração foram regulados negativamente pelo tratamento em solo ácido na linhagem tolerante o que pode ter contribuído, em parte, para a diminuiçãoda taxa fotossintética na Cat100-6. Já foi demonstrado anteriormente que o tratamento com Al diminui consideravelmente a assimilação de CO_2 em *Citrus ssp*. (Chen et al., 2005 a e b; Jiang et al., 2008), tomate (Simon et al., 1994 a e b) e milho (Lindon et al., 1999). Já foi postulado que em plantas tratadas com Al apenas uma fração da energia luminosa recebida é aproveitada e o excesso de energia absorvida pode resultar na produção de espécies reativas de oxigênio, causando dano no aparato fotossintético e estrutura celular (Chen e Cheng, 2003; Chen et al., 2005b). Além disso, o reparo dos sistemas fotossintéticos é altamente inibido por estresse oxidativo (Nishiyama et al., 2001; Nishiyama et al., 2004), estresse salino (Allakhverdiev et al., 2002; Allakhverdiev e Murata, 2004), estresse por frio (Allakhverdiev e Murata, 2004) e estresse por calor (Allakhverdiev et al., 2008). Contudo, o ciclo do ácido cítrico está ativado indicando uma putativa participação dos ácidos orgânicos produzidos na porção aérea da planta na resposta ao Al possivelmente através da sua translocação para as raízes através do sistema vascular.

Em suma, nossos dados mostram que o transcritoma de raízes e folhas de milho são alterados em resposta ao crescimento em solo contendo níveis altos de Al. Essas informações permitiram a identificação de diversos processos metabólicos afetados, bem como alguns pontos que podem ser relevantes para explicar a tolerância ao Al.

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CONCLUSÕES

- O uso de solo é uma estratégia eficiente para avaliar genótipos contrastantes de milho para a tolerância ao Al, mais próxima da situação natural das plantas;
- A análise de expressão gênica em larga escala permitiu a identificação de diversas vias metabólicas da linhagem sensível que são afetadas pelo tratamento em solo ácido. Dentre elas podemos destacar o acúmulo de precursores para a produção de lignina, celulose, síntese de etileno e auxina, que estão associados à inibição radicular;
- Dentre os genes candidatos a um papel mais relevante na tolerância ao alumínio observada na linhagem Cat100-6 estão os que codificam uma lipase hidrolase com motivo GDSL, uma retinol desidrogenase, um fator de transcrição WRKY, uma proteína rica em glicina e duas proteínas sem identificação;
- O estudo de expressão gênica em hidroponia permitiu diferenciar a expressão de genes em relação à modificação de pH e à presença de Al, delimitando assim os genes candidatos a tolerância ao Al presente no solo ácido a apenas três: retinol desidrogenase, um fator de transcrição WRKY e uma proteína desconhecida;
- O mapeamento dos genes obtidos no experimento de microarray de raízes submetidas a tratamento com solo ácido permitiu a identificação daqueles que se encontram dentro de QTLs previamente descritos associados à tolerância ao Al em milho. Dentre eles podemos destacar aqueles relacionados com a possível participação de silenciamento gênico por miRNAs e genes envolvidos na manutenção da divisão celular;

- O perfil transcricional observado em folhas indica grandes alterações na linhagem tolerante, em contraposição à ausência de alteração significativa nas folhas da linhagem sensível;
- Genes relacionados com a fotossíntese e a fotorrespiração foram regulados negativamente pelo tratamento em solo ácido na linhagem tolerante. Contudo, o ciclo do ácido cítrico foi ativado indicando que a participação da produção de ácidos orgânicos na parte aérea da planta pode auxiliar na resposta ao Al.