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MECANISMOS DE TOLERÂNCIA AO AL^{3+} EM PLANTAS COMPARANDO ESPÉCIE DE CERRADO (*STYRAX CAMPORUM*), LIMOEIRO 'CRAVO' (*CITRUS LIMONIA* CV. CRAVO) E TRIGO (*TRITICUM AESTIVUM*)

Carolina de Marchi Santiago da Silva

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Ciências biológicas (Biologia Vegetal)

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TÍTULO DA TESE: Expressão gênica em mecanismos de tolerância ao Al3+ comparando espécies de cerrado (Styrax camporum) e limoeiro `Cravo' (Citrus limonia cv. Cravo)

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Sumário

Mecanismos de tolerância ao Al^{3+} em plantas comparando espécie de Cerrado (*Styrax camporum*), limoeiro 'cravo' (*Citrus limonia* cv. cravo) e trigo (*Triticum aestivum*)

Resumo

Presente em solos ácidos, o alumínio principalmente encontrado na sua forma tóxica (Al^{3+}) tem como principal efeito a diminuição do crescimento radicular. O Cerrado ocorre no centro-oeste do Brasil sob solos ácidos (pH <4) e com alto teor de alumínio (Al). *Citrus limonia* cv 'Cravo' Osbeck e *Styrax camporum* Pohl compartilham localização geográfica e características edáficas em que se estabelecem, no entanto exibem respectivamente sensibilidade e tolerância ao Al. O presente trabalho visa elucidar mecanismos envolvidos no estresse por Al^{3+} a nível de expressão gênica radicular. Para as duas espécies foi feita uma análise de transcriptoma para avaliar os efeitos do Al por uma perspectiva global, e a partir destes resultados alguns genes foram selecionados para avaliação ao longo de experimentos de hidroponia mantido por 60 dias. São discutidas estratégias de tolerância e sensibilidade ao Al nas duas espécies, juntamente com análises de biometria, quantificação hormonal e anatomia que corroboram o comportamento sensível de *Citrus* e tolerante de *Styrax* por diversas vias. Além disso, o último capítulo trata da dependência do pH em um importante mecanismo de tolerância ao Al em trigo envolvendo exsudação de ácido orgânico.

Palavras-chave: Alumínio, Citros, Transcriptoma, qRT-PCR, Plantas lenhosas do Cerrado

Al-tolerance mechanisms in plants comparing Cerrado species (*Styrax camporum*), 'Rangpur' lime (*Citrus limonia*) and wheat (*Triticum aestivum*)

Abstract

Present in acid soils, aluminum mainly found in its toxic form (Al^{3+}) has as main effect the decrease of root growth. Cerrado occurs in central-western Brazil under acid soils (pH <4) and high aluminum content (Al). *Citrus limonia* (Osbeck) and *Styrax camporum* (Pohl) share geographic location and edaphic characteristics in where they are settled, however exhibiting respectively Al-sensitivity and Al-tolerance. The present work aims to elucidate the mechanisms involved in Al^{3+} stress at root gene expression level. For both species, a transcriptome analysis was performed to evaluate the effects of Al in a global perspective, and from these results some genes were selected for evaluation in hydroponic experiments maintained for 60 days. Al tolerance and sensitivity strategies are discussed in both species, together with analyzes of biometrics, hormonal quantification and anatomy data that corroborate the Al-sensitive behavior of Citrus and Al-tolerance in Styrax by several routes. In addition, the last chapter deals with the dependence of pH on one important mechanism of Al tolerance in wheat involving organic acid exudation.

Key words: Aluminum, Citrus, Transcriptome, qRT-PCR, Cerrado woody plants

Introdução geral

Alternativas para aumentar a produção de alimentos têm sido estudadas em diversas áreas, já que em decorrência do crescimento acelerado da população mundial, a demanda torna-se maior. Atividades agropastoris teriam que aumentar a produção paralelamente ao crescimento populacional, mas áreas com as características de clima e solo necessárias para agricultura são escassas (Phalan et al. 2013).

Quarenta por cento dos solos agricultáveis são ácidos (pH < 5,0), sendo que na América do sul 60% dos solos têm pH abaixo de 4,0 (Von Uexküll and Mutert 1995). Além de características naturais do solo, chuvas ácidas e o uso de fertilizantes amoniacais contribuem para a acidez do solo (Kochian et al. 2004; Zheng 2010). Devido à larga ocorrência de solos ácidos em grandes proporções de áreas agricultáveis no Brasil (solos do Cerrado e Centro sul), esforços biotecnológicos para tornar possível a atividade agrária nestes solos têm sido feitos (Ryan et al. 2011).

A acidez do solo pode diminuir em até 60% a produtividade, dependendo da cultura (Blair et al. 2009). Para minimizar a acidez do solo são usadas técnicas como aplicação de calcário ($CaCO_3$ ou $MgCO_3$ - calagem) e alternância com cultura de leguminosas, técnicas essas com eficácia duvidosa, já que o investimento em calagem (± US$400/ha/ano – Ratter et al. 1997) não é refletido na neutralização do pH em todo o perfil do solo, mas apenas superficialmente (0-20 cm prof.) (Delhaize et al. 2009). Quando $pH_{solo} < 5$, aluminossilicatos são ionizados e o alumínio passa à forma ionizada (Al^{3+}), que é tóxica à maioria das plantas (Ulrich 1986). Outro problema de solos ácidos é a indisponibilidade de fósforo (P) para as raízes, porque o P fixa-se em compostos com ferro (Fe^{3+}) e/ou Al^{3+} (Kochian et al. 2004; Zheng 2010).

Altas concentrações de Al^{3+} no solo são percebidas (bioquimicamente) pelo ápice das raízes, que diminuem o crescimento radicular quase que instantaneamente (Delhaize et al. 2004; Sun et al. 2010). Esta diminuição do alongamento diminui a chance de absorção de água e nutrientes em sub-superfícies do solo. A presença de Al^{3+} no simplasto causa desordens fisiológicas celulares: aumento da concentração de cálcio (Ca^{2+}) no citosol, que culminam em desorganização do citoesqueleto e deposição de calose (Horst et al. 2010). Na parte aérea da planta, a toxicidade do Al^{3+} está relacionada ao processo fotossintético (Konrad et al. 2005; Jiang et al. 2008), que tem baixo rendimento devido principalmente ao dano causado às membranas dos tilacóides por espécies reativas de oxigênio (Horst et al. 2010; Ryan et al. 2011).

A diminuição no alongamento da raiz é o sintoma de toxicidade de Al^{3+} mais estudado. O crescimento e desenvolvimento da raiz dependem fortemente da concentração e transporte (distribuição simétrica) de auxinas (Perilli et al. 2012) e etileno no tecido radicular, hormônios vegetais que atuam em sintonia neste órgão (Kang et al. 1971; Swarup et al. 2007). Em solos ácidos, a elevada concentração de Al^{3+} induz maior síntese de etileno; este hormônio faz com que a distribuição polar de auxina fique alterada e com isso, o alongamento celular não ocorre corretamente, deixando as raízes mais curtas (De Cnodder et al. 2005; Ponce et al. 2005; Sun et al. 2010; Basu et al. 2011) e tortas (Kopittke et al. 2008). Estes últimos autores demonstraram que o Al^{3+} causa rigidez da rizoderme, enquanto que células das camadas internas do órgão continuam crescendo, causando rupturas e raízes tortas. Isso sugere que se a parede celular tem sua composição alterada (pela perturbação direta de Al^{3+} e/ou competição por Ca^{2+}), a extensibilidade celular é também alterada. De fato, o grau de metilação das pectinas, pela enzima pectina metil esterase e grupos carboxílicos da parede celular geram cargas negativas que normalmente são estabilizadas por Ca^{2+} (Carpita, N. C.; McCann 2000). O Al^{3+}, no entanto, se presente no apoplasto, pode competir por estas cargas negativas. O Al^{3+} ligado à parede celular impede sua expansão, deixando-a mais rígida (Kopittke et al. 2008) e limita a expansão por causar ainda comprometimentos enzimáticos na parede (Horst et al. 2010).

Frente a todos os tipos de dano que o Al^{3+} solúvel no solo pode causar às plantas, estas categorizam-se em três grupos: plantas que são sensíveis ao(s) (efeitos tóxicos do) Al^{3+}; as que são resistentes, com mecanismos para exclusão de Al^{3+}; e as que são tolerantes, com mecanismos para quelar e armazenar o Al^{3+} em formas menos tóxicas (Ryan et al. 2011). Os mecanismos de resistência e tolerância ao Al^{3+} podem ser determinados por um ou vários genes (Delhaize et al. 2012) dependendo da espécie, e pode ainda haver herança quantitativa, com fenótipos gradativos de resistência ou de tolerância (Kochian et al. 2004; Delhaize et al. 2012).

Por outro lado, o bioma Cerrado que originalmente ocorre (ou ocorria) no centro-oeste do Brasil (originalmente, 23% do território nacional), encontra(va-se) sobre solos ácidos ($pH < 4$), com alta concentração de Al^{3+} e baixa disponibilidade de nutrientes, principalmente P, Ca^{2+}, Mg^{2+} e Zn^{2+} (Haridasan 2008). Isso faz com que as espécies nativas desta vegetação sejam adaptadas a tais condições edáficas (Haridasan 2008). Assim, as espécies lenhosas do Cerrado são divididas em acumuladoras obrigatórias de Al^{3+} (não se desenvolvendo na ausência desse nutriente), acumuladoras facultativas e espécies não acumuladoras (Haridasan 1982; Haridasan et al. 1987).

Rawitscher (1948) já descrevera o sistema radicular profundo de espécies lenhosas do Cerrado. Franco (1998) e Hao et al. (2008) demonstraram indiretamente que estas espécies mostram sistema radicular profundo. Habermann and Bressan (2011) demonstraram por medidas diretas que espécies de savanas mostram raízes com 1m de comprimento após 100 dias da germinação. Assim, é possível que o Al^{3+} do solo do Cerrado esteja associado a alguma relação com a biossíntese, transporte e/ou ação de auxinas e/ou etileno, promovendo o crescimento em profundidade dessas raízes. Não que a presença de Al^{3+} seja essencial para esta característica. Mesmo porque para algumas plantas do Cerrado, o significativo crescimento de raízes não é plástico ao ambiente edáfico, mesmo se cultivadas em solos muito férteis e ausentes em Al^{3+} (Habermann and Bressan 2011). No entanto, espécies não-acumuladoras de Al^{3+} mostram crescimento de raízes significativamente maior quando cultivadas em 740 μM Al^{3+} do que se cultivadas na ausência desse nutriente (de Souza and Habermann 2012). Porém, até hoje, a ciência agronômica não mostrou interesse em entender o metabolismo de Al^{3+} nas espécies do Cerrado (de Souza and Habermann 2012).

Por outro lado, nas regiões norte, noroeste e central do estado de São Paulo e sul de Minas Gerais, que englobam áreas originalmente ocupadas pela borda sul da vegetação de Cerrado, a citricultura é desenvolvida com sucesso desde os anos 50 (de Souza and Habermann 2012). Porém os *Citrus* sp, ao contrário da vegetação nativa do Cerrado, podem exibir alta sensibilidade ao Al^{3+} solúvel em solos ácidos (Pereira et al. 2000; Jiang et al. 2008).

Para resistência a altas concentrações de Al^{3+} no solo o principal mecanismo já demonstrado consiste em secretar ácidos orgânicos pelas raízes (Kochian et al. 2004); o ácido liga-se ao Al^{3+} e impede que se ligue à parede celular ou seja absorvido. O tipo de ácido orgânico secretado depende da espécie, e também da especificidade do substrato da enzima extrusora (Ryan et al. 2011), podendo a secreção ser constituída de um ou mais tipos de ácido. A parte aérea também deve ter uma função na resistência ao Al^{3+} (Wu et al. 2013), mas são necessários mais estudos nesta área.

Os principais ácidos orgânicos na função de resistência à toxicidade do Al^{3+} são malato, citrato e oxalacetato (Kochian et al. 2004). A exsudação de citrato ocorre mais frequentemente nas plantas, talvez porque tenha maior poder para quelar o íon alumínio (Pereira et al. 2010). O citrato é também importante na distribuição de ferro nos organismos vegetais (Magalhaes 2010; Delhaize et al. 2012).

Segundo Ryan et al. (2011), o fator limitante para a exsudação de ácidos orgânicos não é a síntese do composto (que é sintetizado continuamente), e sim a

extrusão deste. O Al^{3+} do solo é responsável por ativar a função destas proteínas de transporte (Delhaize et al. 2009) sendo a transdução deste sinal mediada por quinases (Kumari et al. 2008).

Para a exsudação de malato na raiz existe a proteína ALMT (do inglês, aluminium activated malate transporter), exclusiva no reino *Plantae*. Já o citrato é secretado por um canal não específico (pode liberar outros compostos) que ocorre em todos os domínios vivos, pertencente à família MATE (do inglês, multidrug and toxic compound exudation Family) (Magalhaes 2010). O mecanismo de exsudação de oxalacetato ainda não é conhecido.

No mecanismo de tolerância, algumas espécies são capazes de acumular grandes quantidades de Al^{3+} (Mukhopadyay et al. 2012; Negishi et al. 2012). Em plantas acumuladoras, o Al^{3+} é absorvido pela raiz e complexado ao oxalacetato, depois o Al^{3+} troca de aceptor, ligando-se ao citrato para transporte no xilema, chegando ao mesofilo, onde o aceptor volta a ser o oxalacetato (Kochian et al. 2004). Segundo Horst et al. (2010), o mecanismo de armazenar o Al^{3+} no simplasto em complexos menos reativos evita o contato do cátion com a parede e por isso anula o efeito de restrição no alongamento celular.

O efeito do Al^{3+} na expressão gênica tem sido estudado analisando-se genes de síntese de malato e/ou citrato (Horst et al. 2010; Wang et al. 2013), transporte ALMT (Sasaki et al. 2004, 2006; Guo et al. 2007; Houde and Diallo 2008; Pereira et al. 2010), transporte MATE (Liu et al. 2009; Magalhaes 2010), síntese de etileno (Chandran et al. 2008; Sun et al. 2010), distribuição de auxina (Li et al. 2006; Goodwin and Sutter 2009; Sun et al. 2010), homeostase de cálcio (Chandran et al. 2008), genes relacionados à parede celular (Kumari et al. 2008; Maron et al. 2008; Horst et al. 2010), genes reguladores da expressão de outros genes (Delhaize et al. 2012), e genes envolvidos no próprio acúmulo de Al^{3+} (Delhaize et al. 2012). Embora a diversidade de genes estudados seja relativamente alta, as espécies nas quais são feitos estes estudos resumem-se basicamente a trigo, *Arabidopsis*, milho e cevada.

Diante do exposto, fica claro que há espaço para entendimento do metabolismo do Al^{3+} em espécies cítricas e, sobretudo, em espécies em que o Al^{3+} não é tóxico, mas benéfico, como as espécies nativas do Cerrado.

Objetivos

Ao submeter plantas de limoeiro 'Cravo' (*Citrus limonia* cv. Cravo) e de *Styrax camporum* (Styracaceae), a diferentes concentrações de Al^{3+} em solução nutritiva,

foram identificadas diferenças no transcriptoma em situações contrastantes e a partir disso quantificada a expressão de diferentes genes relacionados ao metabolismo de extrusão de Al^{3+} pela raiz (com destaque para o funcionamento do canal TaALMT1 em trigo), metilação em pectinas da parede celular, e relacionados à síntese de etileno (Et) e transporte de auxinas (IAA).

Foram testadas as seguintes hipóteses:

i) Por se tratar de um espécie sensível ao Al, o transcriptoma de *Citrus limonia* deve apresentar respostas de mecanismos de tolerância a este estresse;

ii) O balanço hormonal em *C. limonia*, considerando principalmente níveis de auxina e etileno, assim como genes associados à biossíntese e transporte destes hormônios devem sofrer alterações culminando em diminuição do crescimento radicular;

iii) De modo análogo, o transcriptoma de *Styrax camporum* deve apresentar expressão acentuada de genes envolvidos em mecanismos de tolerância à toxicidade do Al, em comparação à *C. limonia*;

iv) Em *S. camporum* canais de exsudação de ácidos orgânicos para malato e/ou citrato através dos canais ALMT e MATE devem ser induzidos em resposta ao Al durante todo o tempo do experimento levando ao crescimento normal das raízes. Além disso, diferentes respostas devem ocorrer nas raízes expostas a concentrações de Al contrastantes;

v) O canal TaALMT1 nas raízes de trigo deve exibir resposta específica à faixa ácida de pH, onde o Al assume sua forma tóxica Al^{3+};

Assim, apresenta-se a Tese de que o Al^{3+} beneficia o desenvolvimento radicular das espécies do Cerrado, em oposição às espécies agrícolas, cujo crescimento radicular, mediado por IAA e Et, é limitado pelo Al^{3+}.

CAPÍTULO I

Transcriptome analysis shows sensitiveness in *Citrus limonia* roots under aluminum stress

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Abstract

Under acidic conditions toxic aluminum (Al^{3+}) decreases root growth in sensitive species. A wide range of Al-tolerance/sensibility occurs in Citrus genus, but the most common rootstock in Brazilian citriculture ('Rangpur' lime) has been not widely studied yet. Root apexes from 'Rangpur' lime (*Citrus limonia*) plants grown in a nutrient solution containing different Al concentrations for 60 days were analyzed by RNA-seq and some differentially expressed genes were validated by qRT-PCR. We found 99 genes up-regulated in response to Al, highlighting citrate synthesis and its exudation by MATE (multidrug and toxic compound exudation) transporter. Genes related to secondary metabolism, pectin methylesterification, auxin response, defense to biotic and abiotic stresses, cell division, suberin deposition and nitrate uptake were also induced by Al^{3+} in root nutrient solutions. The overview of up-regulated genes in *C. limonia* roots infer that this species may be considered an Al-sensitive rootstock, despite some tolerance responses induced.

Key words: Al^{3+}; Gene expression; 'Rangpur' lime, MATE transporter

Introduction

In neutral soil conditions aluminum (Al) occurs mainly in Al_3SiO_4 form. In acidic soils (pH < 5) this complex is hydrolyzed to different Al species mainly Al^{3+}, which is toxic to most plant species (Kochian 1995). Plants sensitive to Al (barley for example) shows direct effects such as inhibition of root growth, leading to lower root reach and impacting crop productivity (Kochian 1995; Horst et al. 2010; Sun et al. 2010).

Root growth reduction induced by Al is documented in several species, i.e., wheat (Delhaize et al. 1993), maize (Ryan *et al.*, 1993), barley (Furukawa et al. 2007), *Arabidopsis* (Hoekenga et al. 2003) and woody species (Brunner and Sperisen 2013), including *Citrus* trees (Lin and Myhre 1991). Decrease in root growth may be caused mainly by physic obstacle (cell wall become stiffened due to Al binding) or hormonal effects (increase in ethylene synthesis leads to auxins imbalance) (Sun et al. 2010).

Al-tolerant species exhibits distinct strategies to cope with such disturbance. The main mechanism of resistance consists in organic acid (malate, citrate and oxaloacetate) efflux preventing Al absorption or binding (Kochian et al. 2015). Malate efflux is mediated by the aluminum activated malate transporters (ALMT) membrane channels (Sasaki et al. 2004; Sharma et al. 2016) and a non-specific channel that belongs to the multidrug and toxic compound exudation (MATE family), operates citrate efflux (Magalhaes 2010). While ALMTs occurs only in plants (Sharma et al. 2016), MATE genes are widely distributed in bacteria, fungi and mammals besides plants (Omote et al. 2006).

The cell wall composition also plays a role in Al-tolerance, where less negative charged carboxylic groups due to higher pectin methylation avoid Al binding (Horst et al. 2010). The action of enzymes like pectin methylesterase (PME) leads to free pectic carboxylic groups, allowing Al to bind to pectic nets in the cell wall (Horst et al. 2010). Once Al binds to the cell wall, it becomes rigid, making cell elongation more difficult. Therefore, any enzyme that interfere in PME activity could decrease Al binding in the cell wall and, consequently, enhance Al resistance (Schmohl et al. 2000).

Auxins play a central role in root growth (Cleland 2010) but its interaction with Al is still not clear. Disruption in polar auxin distribution was leading to arrest of root elongation in *Arabidopsis* Sun et al. (2010) and, on the other hand, Yang et al. (2014) found an Al-induced increased biosynthesis of indol-acetic acid (IAA) in this species

root apex. Nevertheless, in roots high concentration of auxin can cause strong inhibition of cell elongation (Rahman *et al.*, 2007).

The *Citrus* genus present a wide range of responses to Al, including transcriptional differences (Lin and Myhre 1991; Guo et al. 2017). *Citrus limonia* L. Osbeck ('Rangpur' lime) is widely used as rootstock in subtropical regions of the Americas, due its ability to cope with abiotic stresses, including drought (Ribeiro and Machado 2007). However, this species is considered sensitive to Al (Pereira et al. 2000; Santos et al. 2000). Although recently our group detected a CO_2 assimilation rate reduction in leaves from *C. limonia* plants during the exposition to 1480 µm Al (Banhos et al. 2016a) its impact on the molecular mechanisms in roots are still unknown. In this way, studies that describe *C. limonia* response to Al, at physiological or molecular level, could enable breeding programs.

High-throughput mRNA sequencing is one efficient approach to uncover the plant transcriptome in response to Al in species as *Fagopyrum tataricum* (Zhu et al. 2015), *Anthoxanthum odoratum* (Gould et al. 2015) and some *Citrus* species (Guo et al. 2017). This approach is essential to discover key genes that are regulated under Al toxicity. In this study, we analyzed the Rangpur lime root transcriptome in response to Al toxicity, having in mind the wide use of *C. limonia* as a rootstock in the Americas and the distinct molecular pathways in *Citrus* species to cope with Al stress, expecting to uncover candidate genes related to differential Al response within *Citrus* genus.

Material and methods

Plant materials and growth conditions

This experiment followed the same rationale our group did in a previous study (Banhos et al. 2016a). We used three-month-old (five leaves) 'Rangpur' lime (*Citrus limonia*) plants (7 ± 0.5 cm-high). Plants were kept in a hydroponic system and grown directly on an aerated nutrient solution inside opaque plastic boxes (50 cm in length x 30 cm in width x 15 cm in height; 20 L).

The nutrient solution, adapted from Clark (1975), consisted of the following macronutrients (in mM): N (NO_3^-) 0.93; N (NH_4^+) 0.40; P, 0.013; K, 0.90; Ca, 1.37; Mg, 0.33; S, 0.25; and micronutrients (in µM): Cl, 231.0; Fe (EDTA), 23.3; B, 8.33; Mn, 2.91; Zn, 0.76; Cu, 0.32; Mo, 0.31. This solution contained 0, and 1480 µM Al provided using $AlCl_3$ 6 H_2O. The pH of the solution was monitored daily and

maintained at 4.0 ± 0.1 to keep Al as soluble as possible, and the solution was totally replaced every 15 days.

Expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick), with eight holes (2.5 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with polyurethane foam strips that were placed around the plant collar. The boxes stayed on benches (80 cm from the ground) inside a greenhouse with semi-controlled conditions (air temperature 28.5 ± 0.7°C; relative humidity 63.3 ± 1.3%; 853.4 ± 175.1 μmol photons m^{-2} s^{-1}; approximately 14h of natural photoperiod).

After 60 days, a mixture of root tips (~ 0.5 cm) were sampled in 0 and 1480 μM Al conditions. The same experiment was repeated twice: the first experiment provided samples for RNA-seq and samples from the second experiment were used to validate gene expression in qRT-PCR.

Determination of Al accumulation

Roots were washed under deionized water and oven-dried at 60◦C to constant dry mass. These samples were ground and digested in a nitric:perchloric acids solution (v:v). The concentration of Al was quantified colorimetrically (Sarruge and Haag 1974).

RNA extraction and transcriptome analysis

Apexes were collected, frozen in liquid nitrogen and RNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Total RNA was precipitated with sodium acetate and ethanol. All samples had a A260/A280 nm ratio > 1.99. Samples were sent to transcriptome sequencing (RNA-seq) at BGI Tech Solutions (Hong Kong, China).

Two samples (bulk of root apexes at 0 and 1480 μM Al) were used for library construction, quality assessment (Agilent 2100 Bioanalyzer) and RNA sequencing in an Illumina HiSeq2000 equipment (100bp, pair-end sequencing). Raw reads from Illumina sequencing were submitted to quality control tests prior analysis. They were filtered using SOAPnuke (https://github.com/BGI-flexlab/SOAPnuke). Reads consisting only of adaptor sequences, reads which the percentage of unknown bases is greater than 10% and reads where the percentage of the low-quality bases (base with quality value ≤ 10) is greater than 50% in a read were discarded.

Clean reads were mapped to the sweet citrus genome (Xu et al. 2013) by using the *Short Oligonucleotide Analysis Package* (SOAPaligner/SOAP2) (Li et al. 2009), allowing up to five mismatches.

Gene expression levels were calculated using RPKM (*Reads per kilobase transcriptome per million mapped reads*) (Mortazavi et al. 2008). Digital expression profiles were obtained based on Audic and Claverie (1997) test. Genes with false discovery rate ≤ 0.05 and the absolute value of Log2Ratio ≥ 1 were considered differentially expressed genes (DEGs). The DEGs list was submitted to KEGG (Kyoto Encyclopedia of Genes and Genomes) database analysis (Kanehisa et al. 2007). They were also submitted to Gene Ontology (GO) enrichment analysis. All DEGs were assigned to GO terms in the database (http://www.geneontology.org/), calculating gene numbers for every term, then uses hypergeometric test to find significantly enriched GO terms in the input list of DEGs, based on 'GO:TermFinder (http://smd.stanford.edu/help/GO-TermFinder/GO_TermFinder_help.shtml). We opted to highlight only most proeminent differences between DEGs and all genes from the database.

qRT-PCR validation

For qRT-PCR analysis, five replicates were used to quantify gene expression. Each biological replicate consisted of one *C. limonia* plant under control (0 μM Al) or Al condition (1480 μM Al). Each biological sample was run in three technical replicates.

Total RNA was extracted from root samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Two micrograms of total RNA were treated with TURBO DNA-free™ (Ambion, Carlsbad, USA) and reverse transcribed using an oligo-dT primer and Super Script™ III (Life Technologies, Carlsbad, USA), according to the manufacturer's protocol. cDNAs were used to qRT-PCR analyses, where amplification conditions were the same of amplification efficiency tests.

We validated using quantitative PCR the transcriptional profile of seven genes (Wang et al. 2014): MATE (Cs7g01770), CS (Cs7g01170), PMEI (Cs2g01440), SAUR (orange11t04220), SLAH (Cs3g25590), Peroxidase (Cs2g28810) and Alcohol acyltransferase (Cs2g29820) since they are candidate genes related to Al tolerance and showed differential expression level in RNA-seq analysis. Based on the reference genome, we designed primers for qPCR (Table 1). As reference genes we used GAPC2 and EFα, as recommended by Mafra *et al.* (2012).

Five serial dilutions of genomic DNA from *Citrus limonia* were amplified using the internal primers in order to access the amplification efficiency during qPCR analysis. GoTaq® qPCR Master Mix with SYBR® fluorescence (Promega, Madison, USA) was used as the reagent in these tests, with the following cycle specifications: 95°C for 5 min (1 cycle), 95°C for 10 s, 58°C for 30 s, 72°C for 30 s (40 cycles) followed by a melting curve analysis (1% slope temperature; 60–95°C), performed in a 7500 Fast real time PCR system (Applied Biosystems, Foster City, USA). Amplification efficiencies are presented in Table 1.

qPCR data was analyzed based on the procedure of Pfaffl (2001). Expression was normalized using the geometric mean of the RQ values of GAPC2 and EFα.

A one-way analysis of variance (Anova) was performed between plants exposed to 0 and 1480 μM Al to test for differences in length, dry weight, [Al] and gene expression in roots after 60-days treatment. The Tukey test ($\alpha = 0.05$) was used to conduct post-hoc comparisons to estimate the least significant differences between mean results.

MATE phylogenetic tree

Phylogenetic analysis and tree visualization were developed in Geneious software version 11.0 (Kearse et al. 2012). ClustalW was used for the alignment of 30 MATE proteins from plants and one from the *Diplocarpon roseae* fungus as tree outgroup. The phylogentic tree was generated by Bayesian inference MCMC sampling in MrBayes (Ronquist and Huelsenbeck 2003) using the LG +I +G substitution model with four rate categories, chosen under the BIC in ProTest (Abascal et al. 2005). Four chains were run for 50 million of generations and trees were sampled every 5000 generations, with the first 5 00000 discarded as burn-in. GenBank accessions of the protein sequences used were included in the supplementary data.

Results

Transcriptome analysis of Citrus limonia *plants growth in Al conditions*

When compared with plants not exposed to Al, the aluminum content in this hydroponic experiment resulted in higher Al accumulation in roots. Moreover, root showed reduced length and dry weight (Figure 1).

We obtained 25,087,582 and 24,657,760 clean reads for 0 and 1480 μM Al libraries respectively, where most of them successfully mapped against *Citrus sinensis* genome (Table 2). Transcriptome analysis of root apexes from plants incubated with 0 and 1480 μM of Al presented 16,750 DEGs, being 99 genes up-regulated and 16,651 down-regulated by Al toxicity.

In order to identify transcriptional mechanisms triggered by Al, we compared differentially up-regulated genes in *Citrus limonia* to those found in *Citrus sinensis* and *Citrus grandis* under similar conditions (Guo et al. 2017). A total of 20 genes were upregulated in *Citrus limonia* and at least one *Citrus* species (*C. grandis* and/or *C. sinensis*) (Figure 2). Only two genes share upregulation in the three species: multidrug and toxic compound extrusion channel (MATE - Cs7g01770) and nuclear transport factor 2 (NTF2 - Cs6g09500) (Supplementary Table II).

DEGs annotation

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze DEGs, resulting in 209 categories. We selected a total of 11 categories (Table 3) that have a significant difference of genes present in DEGs.

Citrate cycle, *Oxidative phosphorylation* and *Calcium signaling* were the more represented pathways in DEGs categories responding to Al. On the other hand, genes belonging to *Cell cycle*, *RNA polymerase* and *Apoptosis* categories were more significant (higher percentage) in the genome than in DEGs, which means secondary importance to the imposed treatment (Figure 3).

A total of 13,587 genes from the genome and 8,538 DEGs were assigned to Gene Ontology (GO). Comparison of the 17 gene ontology categories enriched in DEGs with the whole *C. sinensis* genome (Figure 3), indicated that 10 categories had values twice higher in DEGs than in genome, including *post replication repair*, *dicarboxylic acid transport* and *amide transmembrane transporter activity*. Smaller differences can be found in categories as *small ribosomal subunit* and *intrinsic to organelle membrane*, which had only 25% increase in DEGs compared to the genome.

Gene ontology analysis using the 16,750 differentially expressed genes showed *Cellular metabolic processes* - biological process category as the most abundant in DEGs, followed by *Ion binding* - cell components category and *Nucleus* - molecular function category, responsible for 60.4, 34.1 and 32.2 % of genes respectively (Figure 4). In general, categories belonging to biological process were more frequent (higher percentage). Categories as *Organic anion transmembrane transporter activity*, *Ethylene*

binding and *Auxin binding* which are recognized in Al-tolerance (Sun et al. 2010; Ryan et al. 2011) and also occurred in *C. limonia* transcripts.

On *Ion binding* category, we could also identify genes responsible for *Zinc ion binding* (6.3% of genes), *Cobalt ion binding* (0.4% of genes) and *Copper ion binding* (1.6% of genes), showing that Al-response is common to other metal toxicities. On the same way, *Response to stress* category may include abiotic stresses as *Response to salt stress* (5.1% of genes), *Radiation response* (8.1% of genes), *Heat response* (2.1% of genes), *Response to osmotic stress* (5.4% of genes) and *Response to oxidative stress* (4.1% of genes).

In both databases (KEGG and GO) tricarboxylic acid cycle genes were shown markedly responsive to Al stress. Citrate synthase (CS) - a member of tricarboxylic acid cycle - was found in transcriptome analysis and validated by qRT-PCR as up-regulated by Al treatment (Figure 5).

Validation by qRT-PCR of DEGs directly related to Al response

Transcriptome results were validated considering a group of genes related to Al response including MATE transporter, pectin methylesterase inhibitor (PMEI), CS, small auxin up-regulated RNAs (SAUR), S-type anion channel (SLAH), peroxidase and Alcohol acyltransferase. In these qRT-PCR validation CS, MATE, peroxidase and SLAH gene expression were confirmed to be up-regulated while PMEI and SAUR were down-regulated by Al stress (Figure 6). Alcohol acyltransferase gene expression resulted in opposite behavior, showing up-regulation at transcriptome analysis and down-regulation at qRT-PCR validation.

After 60 days on hydroponic treatment the foldchange comparing 0 and 1480 μM Al resulted in high MATE up-regulation (215.7 foldchange) which was much more increased than SLAH (34.4 foldchange), CS (9.1 foldchange) and peroxidase (4.6 foldchange). PMEI and SAUR genes showed similar down-regulation rates (-18.9 and -20.6 foldchange respectively), that showed more pronounced reduction than alcohol acyltransferase (-1.4 foldchange) (Figure 6).

Phylogenetic analysis of Citrus limonia MATE

Using *C. sinensis* corresponding gene as reference (Wang et al. 2014 - Cs7g01770), we obtain the MATE gene of *Citrus limonia* (GenBank - MF680000). Due to MATE generalist function, a phylogenetic analysis based in Bayesian inference was developed including reported MATE proteins and putative proteins, as well as the new

C. limonia MATE putative protein. In the rooted tree resulted from the phylogenetic analysis two well sustain clades were observed clustering Al and non-Al responsive MATE (Figure 7). *C. limonia* protein clustered at MATE group responsible for Al-dependent citrate exudation in roots (Figure 7).

Discussion

The main plant response to Al stress is the decrease in root growth, which may occur in varying magnitudes. In our experiments, we found that *C. limonia* roots were 44% short in length and 50% lighter in dry weight when imposed to Al treatment (Figure 1B and 1C), which is related to Al content almost seven times higher than in control conditions (Figure 1A). *Citrus grandis* Al content difference was lower (almost three times higher under Al conditions) thus leading to a subtle decrease in root dry weight (33% smaller under Al conditions) while no statistical difference was observed in *C. sinensis* root dry weight (Guo et al. 2017). Although in different Al concentration and exposure time, these results suggest that *C. limonia* might be even more sensitive than *C. grandis* to Al stress.

After 60-days treatment most genes were inhibited, which indicated the inhibition of many physiological pathways. From the 99 up-regulated genes, 15 were only shared with *C. grandis* up-regulated genes, thus the higher similarity with *C. grandis* transcriptome could also be an indicative of sensitive profile in both species (Supplementary Table II). Three genes were only shared between *C. limonia* and *C. sinensis*, and two of them were shared among these three species – NTF2 and MATE (Figure 2).

Shared between *C. limonia* and *C. sinensis*, alcohol acyltransferase belongs to HXXXD-type acyltransferase group, which forms compounds on suberin and cutin polymers, conferring a barrier to the roots (Kosma et al. 2012). The up-regulation of this gene in *C. sinensis* found by Guo et al. (2017) is more evident than in *C. limonia*, once in the last, it appears up-regulated in RNA-seq but down-regulated in qRT-PCR analysis, thus conferring more Al-tolerance to the *C. sinensis*.

Transcriptional profiles of *C. limonia* and *C. grandis* were closely related. Among the 15 genes up-regulated by Al in both species we first highlight SLAH channels, involved in nitrate uptake (Wang et al. 2012) also allowing normal growth in acidic conditions (Zheng et al. 2015). Chalcone and stilbene synthase also deserve highlight due its involvement in secondary metabolism (Nopo-Olazabal et al. 2014).

Triggered by Al, fungus and bacteria defense-related genes were up-regulated in all the three Citrus species studied. In *C. sinensis* and *C. grandis* osmotin and thaumatin genes were respectively activated, while in *C. limonia* both genes were up-regulated exercising this function (Liu et al. 1994; Datta et al. 1999).

Ran, a small GTP-binding protein, is imported to nucleus when complexed to NTF2 triggering various signaling pathways, including cell proliferation by controlling mitotic cycle (Vernoud et al. 2003). When NTF2 was over-expressed in *Arabidopsis* this import was disrupted (Zhao et al. 2006). Thus, by NTF2 up-regulation in these three Citrus species, we may assume that Al lead to less mitotic division in roots even in *C. sinensis* that showed no diminution in root dry weight (Guo et al. 2017).

MATE channel was also up-regulated in these three Citrus species. Notwithstanding, here transcriptome data was validated by qPCR while *C. sinensis* and *C. grandis* studies validation were accomplished by quantification of organic acid (OA) secretion (Guo et al. 2017). In *C. limonia* we also found ABC transporters which were previously related to Al toxicity in roots, having function similar to MATE channel – detoxification of organic compounds (Yokosho et al. 2014; Chen et al. 2015).

OA exudation is a common mechanism in tolerant species which consists in release citrate, malate or oxaloacetate to chelate Al^{3+}. These compounds are intermediates in tricarboxylic acid cycle (TCA) therefore, these cycle constituents have markedly importance in Al tolerance. In fact, *Dicarboxylic acid transport* and *TCA* were among the categories more enriched in response to Al (Table 3 and Figure 4) when comparing DEGs set and genome gene ontology. Analysis of CS expression by qRT-PCR, as a transcript representative of TCA category, confirm its up-regulation under Al stress (Figure 5).

However, in OA efflux the channel responsible for exudation is considered more consistent with Al resistance than OA synthesis (Ryan et al. 2011), meaning that to cope with Al stress MATE channel expression should have higher impact than CS in citrate efflux. In fact, Al-increased MATE expression was more than 20 times higher than Al-increased CS expression in *C. limonia* roots (Supplementary Figure I). Also, CS was not up-regulated by Al in *C. sinensis* nor *C. grandis* roots even with OA secretion increased (Guo et al. 2017).

Citrus species show a wide range of Al-tolerance (Lin and Myhre 1991; Pereira et al. 2000). Due to higher ALMT gene expression and higher OA efflux *C. sinensis* was considered more tolerant to Al than *C. grandis* (Yang et al. 2012; Guo et al. 2017). Despite a markedly increase in MATE gene expression in *C. limonia* roots (which

indirectly means increasing citrate efflux – Figure 7), the inhibition on root growth make this species more Al-sensitive than other Citrus species previously studied.

A possible cause for root growth decreased relies on PMEI low gene expression. Cell wall pectin undergoes partial apoplastic demethylesterification through PME action, resulting in the exposure of free pectic carboxylic groups, which could serve as binding sites for Al in the wall (Zhu et al. 2014). Potato transformants that exhibited higher expression of PME accumulate more Al, produce more callose and their root growth are more inhibited when exposed to Al than wild types (Schmohl et al. 2000). Analogously, lower PMEI expression favors Al binding in cell wall sites. This assumption can be verified by the relation between PMEI down-regulation (Figure 5) and higher Al content in roots with 1480 μM Al in nutrient solution (Figure 1 B).

A second possible cause that results in reduced *C. limonia* root growth is related to SAUR gene expression. These genes are induced by auxins and act in apoplast acidification (Spartz et al. 2014), allowing cell wall expansion. Our data show SAUR down-regulation (more than 20 times fold change – Figure 5) under Al stress, which contributes to reduced root growth.

In short, gene expression analysis of *C. limonia* plants exposed to Al stress, reveals that high MATE and CS expression (i.e. high citrate efflux) is not enough to allow normal root growth in this species. Moreover, suggest that PMEI and SAUR genes down-regulation could be related with the root growth inhibition observed, by the modification of cell wall affinities and auxin responses respectively.

Acknowledgements

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Figures and tables

Table 1. Specific primers designed for qPCR validation using *C. limonia* root cDNA.

Gene	ID	Primer F	Primer R	Efficiency (%)	Amplicon size (bp)
MATE	Cs7g01770	CATTGACACAGCATTTATCGGC	CAAATGAGGTTGTAATATTAACTAATGGG	91.85	128
CS	Cs7g01170	CGCTAAGCCAGATGGAGAAC	ACAGTGGCACGATCTCTCAA	85.38	119
PMEI	Cs2g01440	CCACAAGAACGACAGCGATA	TTAGCGTAACTCGGCAAGGT	97.22	147
SAUR	orange11t04220	TGGGTTCACAACTCACAAGC	GAACAATACCAGGCAAACG	91.71	142
SLAH	Cs3g25590	TGGTCAGTGGTTCACGAAAG	TCTTTCCACCCCATGTTAGC	106.84	119
Peroxidase	Cs2g28810	TGCTGGTCTTGTGAGAATGC	GACTTCAAACCCTCGCAGAC	109.40	138
Alcohol acyltransferase	Cs2g29820	TATTTCCAATTTGGCAACGAC	CGATTTGTGAGCCATGTTG	96.45	124
EFα	Cs8g16990	TCAGGCAAGGAGCTTGAGAAG	GGCTTGGTGGGAATCATCTTAA	100.04	80
GAPC2	Cs2g24610	TCCTATGTTTGTTGTGGGTG	GGTCATCAAACCCTCAACAA	97.24	144

Table 2. Summary of clean reads and mapping against reference genome from control and Al-treated 'Rangpur' lime roots

Sample	Clean reads	Genome map rate (%)	Perfect match	Expressed genes
0 µM Al bulk	25087582	18145177 (72.33%)	8472000	22099
1480 µM Al bulk	24657760	21290207 (86.34%)	13654519	21199

Table 3. Genes classification according to KEGG database to metabolic pathways. DEGs pathways means differences in root gene expression after 60 days cultivated in nutritive solutions with 0 and 1480 μM Al.

KEGG pathway	DEGs (total mapped 8489)	Genome (total mapped 14955)	P value
Oxidative phosphorylation	72 (0.85%)	94 (0.63%)	4.66E+00
Citrate cycle (TCA cycle)	41 (0.48%)	52 (0.35%)	0.0007
Ubiquitin mediated proteolysis	161 (1.9%)	250 (1.67%)	0.0079
Calcium signaling pathway	40 (0.47%)	58 (0.39%)	0.0387
DNA replication	48 (0.57%)	72 (0.48%)	0.0556
Starch and sucrose metabolism	33 (0.39%)	51 (0.34%)	0.1573
ABC transporters	109 (1.28%)	181 (1.21%)	0.1926
Cell cycle	123 (1.45%)	277 (1.85%)	0.9999
RNA polymerase	100 (1.18%)	240 (1.6%)	0.9999
Apoptosis	194 (2.29%)	469 (3.14%)	1

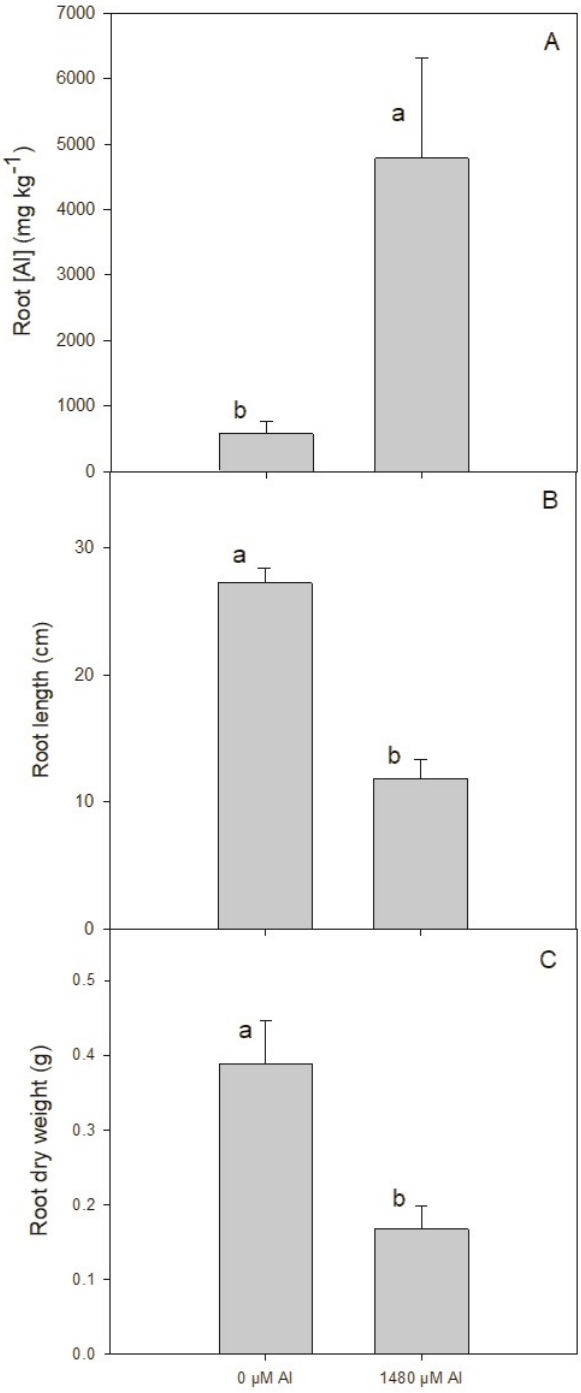

Figure 1. Al accumulation (A), length (B) and dry weight (C) in 'Rangpur' lime roots after 60 days cultivated in nutrient solution containing 0 and 1480 µM Al. Distinct letters indicates significant differences ($p < 0.05$) between treatments. Bars represent standard deviation.

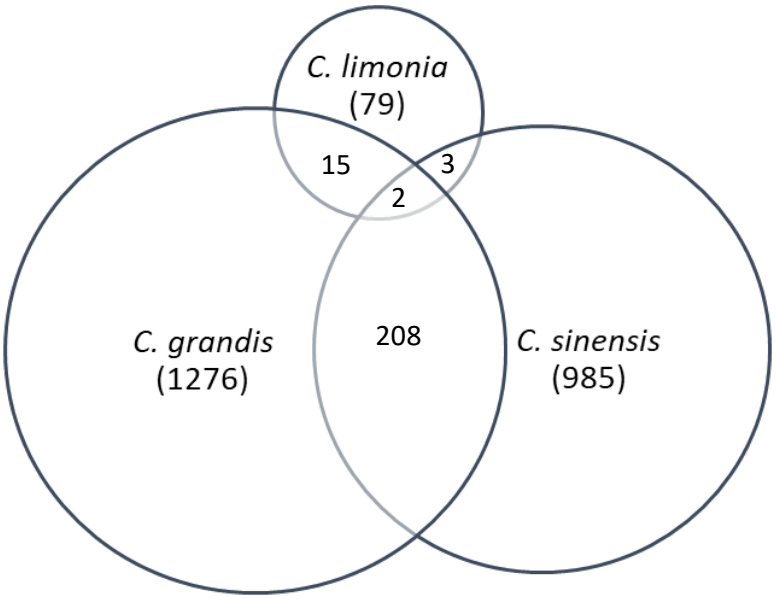

Figure 2. Common up-regulated genes in roots of three Citrus species under aluminum treatment.

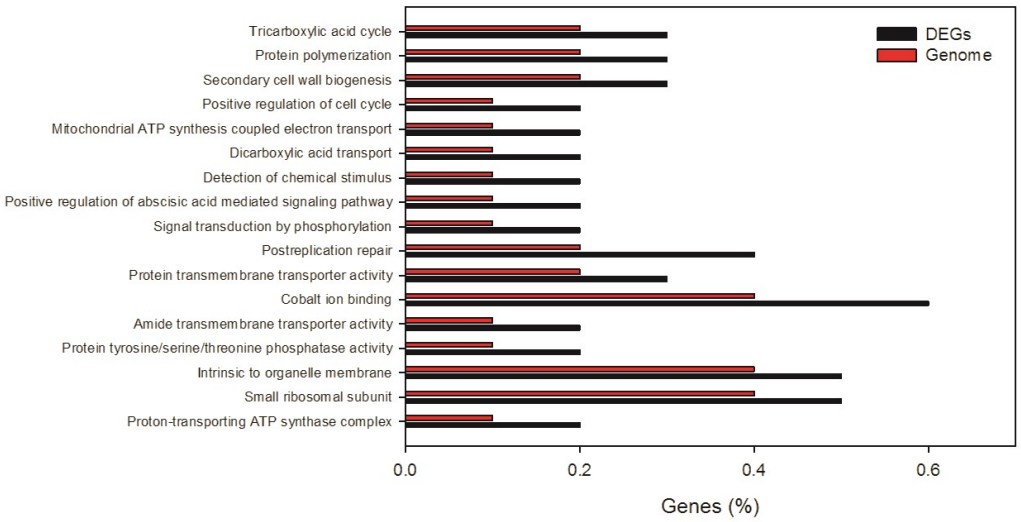

Figure 3. Gene ontology (GO) selected categories obtained for differential expressed genes (DEGs) and whole the genome. DEGs mean differences in root gene expression after 60 days cultivated in nutritive solutions with 0 and 1480 µM Al.

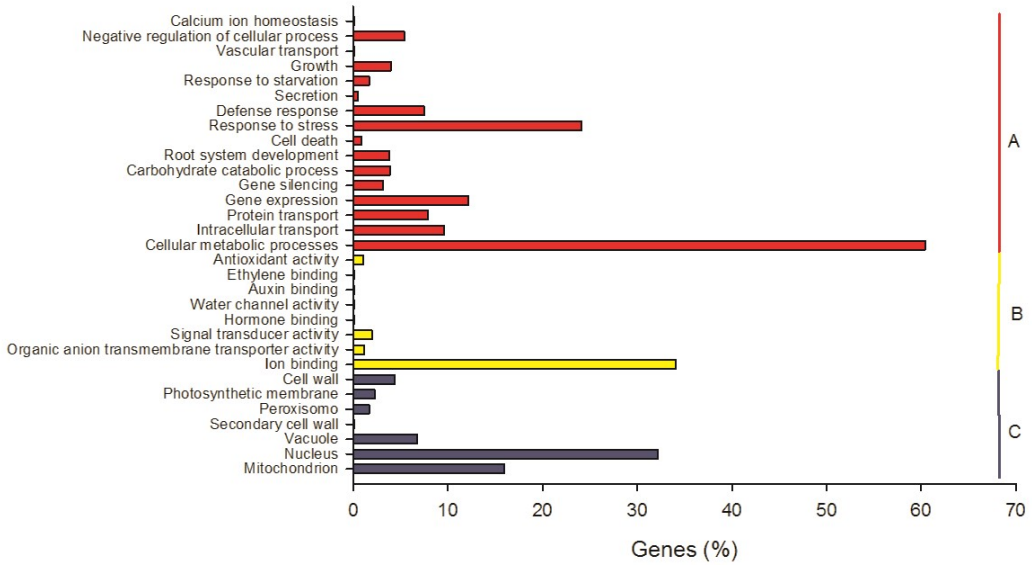

Figure 4. Gene ontology (GO) of selected differential expressed genes (different root gene expression after 60 days in nutritive solutions with 0 and 1480 µM Al). GO was divided in three groups: A – Biological process (8532 genes in total), B – Cell components (8109 genes in total) and C – Molecular function (8885 genes in total).

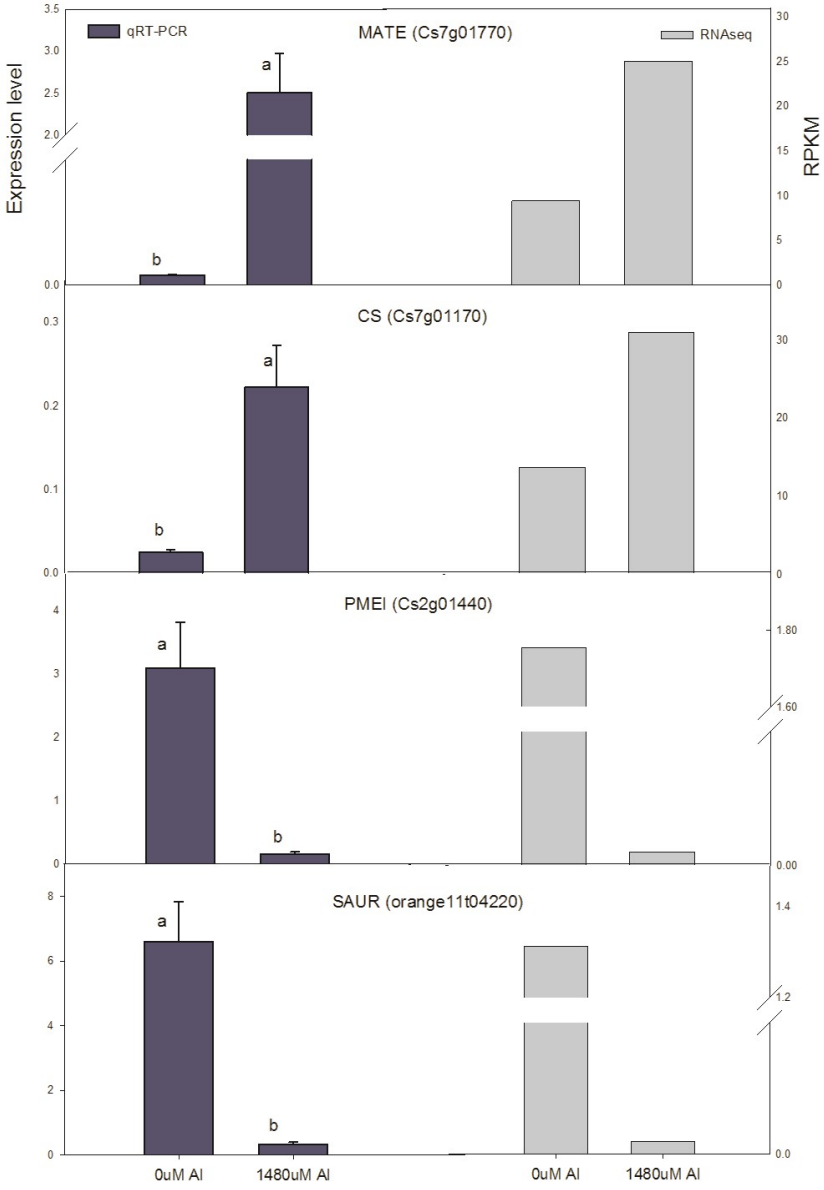

Figure 5. 'Rangpur' lime rootstock MATE, CS, PMEI and SAUR gene expression analysis by RNA-seq (in reads per kilobase per million mapped reads - RPKM) and real time PCR (qRT-PCR) after 60 days in nutritive solutions with 0 and 1480 µM Al. Distinct letters indicates significant differences ($p < 0.05$) between treatments. Bars represent S.D.

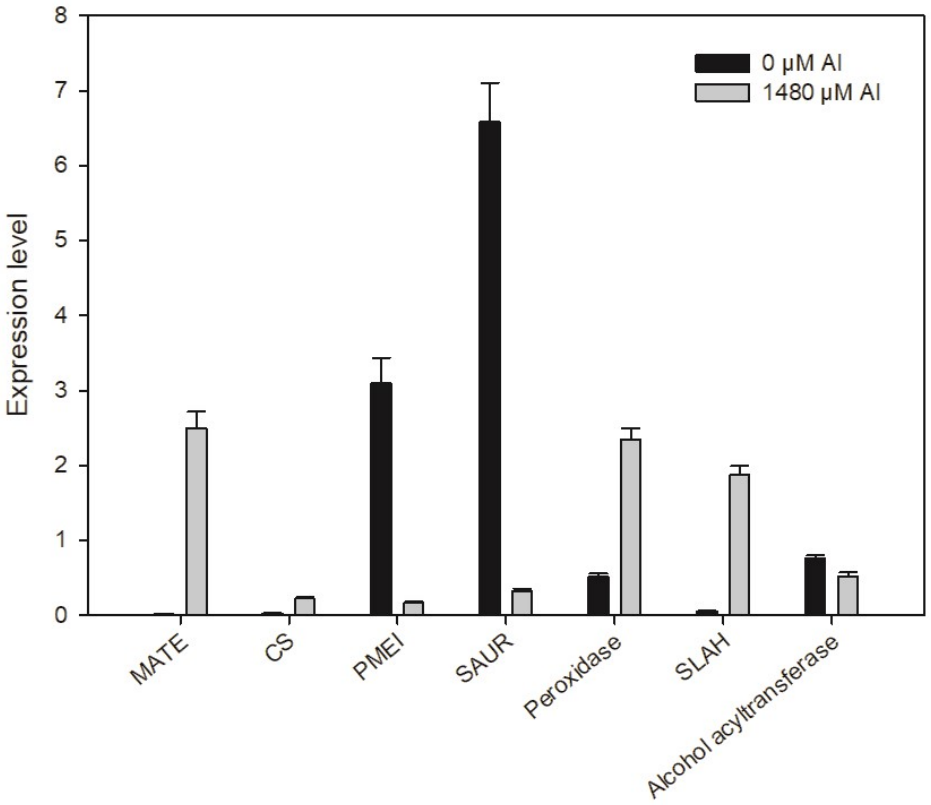

Figure 6 - Gene expression of multidrug and toxic compound exudation (MATE) transporter, citrate synthase (CS), pectin methyl esterase inhibitor (PMEI), small auxin up-regulated RNAs (SAUR), Peroxidase, S-type anion channel(SLAH) and Alcohol acyltransferase in root tips of 'Rangpur' lime plants grown in nutrient solutions containing 0 and 1480 µM Al at 60 days after planting. Bars represent standard difference.

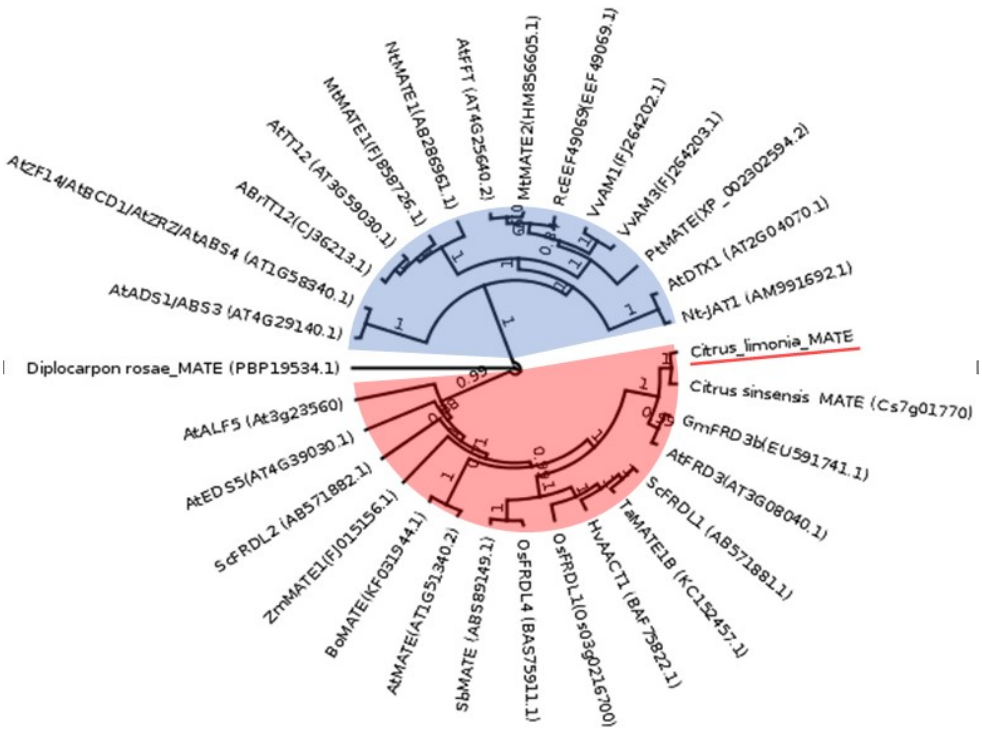

Figure 7. Maximum clade credibility tree of multidrug and toxic compound extrusion (MATE) proteins. Putative *Citrus limonia* MATE protein (Cl-MATE) is shown in red cluster with the plants Al-responsive MATE group highlighted in red and non Al-responsive MATE in blue highlight branch. A putative MATE from the ascomycete fungus *Diplocarpon roseae* was used as outgroup. Posterior probability values are indicated over each branch. Phylogenetic tree was generated by Bayesian inference of 31 MATE protein sequences using the MrBayes plugin in Geneious software. GenBank accessions of the protein sequences are in the Material and methods section.

Supplemetary Table I – Amino acid sequences used to construct the phylogenetic tree. Responsiveness to Al based on Liu et al.(2016), except for Citrus species.

Name	Accession code	Plant species	Cluster
SlMATE(MTP77)	BE354224	*Solanum lycopersicum*	Not responsive to Al
MtMATE2	HM856605	*Medicago truncatula*	
AtFFT	At4g25640	*Arabidopsis thaliana*	
RcEEF49069	EEF49069	*Ricinus communis*	
VvAM1	FJ264202	*Vitis vinífera*	
VvAM3	FJ264203	*Vitis vinífera*	
PtMATE	XP_002302594	*Populus trichocarpa*	
BrTT12	ACJ36213	*Brassica rapa*	
AtTT12	At3g59030	*Arabidopsis thaliana*	
MtMATE1	FJ858726	*Medicago truncatula*	
NtMATE1	AB286961	*Nicotiana tabacum*	
AtDTX1	At2g04070	*Arabidopsis thaliana*	
Nt-JAT1	AM991692	*Nicotiana tabacum*	
AtADS1	At4g29140	*Arabidopsis thaliana*	
AtZF14	At1g58340	*Arabidopsis thaliana*	
AtEDS5	At4g39030	*Arabidopsis thaliana*	Al responsive
CsMATE	Cs7g01770	*Citrus sinensis*	
ClMATE	MF680000	*Citrus limonia*	
EcMATE1	AB725912	*Eucalyptus camaldulensis*	
GmFRD3b	EU591741	*Glycine max*	
AtFRD3	At3g08040	*Arabidopsis thaliana*	
SbMATE	ABS89149	*Sorghum bicolor*	
OsFRDL4	Os01g0919100	*Oryza sativa*	
OsFRDL1	AB571881	*Oryza sativa*	
HvAACT1	BAF75822	*Hordeum vulgare*	
TaMATE1B	KC152457	*Triticum aestivum*	
ScFRDL1	AB571881	*Secale cereale*	
BoMATE	KF031944	*Brassica oleracea*	
AtMATE	At1g51340	*Arabidopsis thaliana*	
ZmMATE1	FJ015156.1	*Zea mays*	
ScFRDL2	AB571882	*Secale cereale*	

Supplementary Table II – List of up-regulated genes shared with one or two Citrus species.

		Up-regulation shared with *Citrus limonia*	
ID	**Description**	***Citrus sinensis***	***Citrus grandis***
Cs3g25590	S-type anion channel SLAH1		X
Cs2g29820	Alcohol acyltransferase	X	
Cs3g20680	Chalcone and stilbene synthase family protein		X
orange1.1t01309	RPOB [*Arabidopsis thaliana*]		X
orange1.1t00666	-		X
orange1.1t03318	AT2G01021 [*Arabidopsis thaliana*]		X
Cs4g16600	Cytochrome F		X
orange1.1t03320	-		X
Cs1g26280	Alpha/beta-Hydrolases superfamily protein [*Arabidopsis thaliana*]		X
orange1.1t01308	-		X
orange1.1t05667	CYP71A20 [*Arabidopsis thaliana*]		X
orange1.1t05688	CYP71B37 [*Arabidopsis thaliana*]		X
Cs3g09470	Hypothetical chloroplast RF21		X
orange1.1t03024	Pathogenesis-related thaumatin superfamily protein [*Arabidopsis thaliana*]		X
Cs7g01770	MATE efflux family protein [*Arabidopsis thaliana*]	X	X
Cs8g03430	Basic form of pathogenesis-related protein 1-like		X
Cs6g09500	Nuclear transport factor 2 (NTF2) family protein [*Arabidopsis thaliana*]	X	X
Cs8g07390	protein HYPER-SENSITIVITY-RELATED 4-like		X
Cs3g27360	Pentatricopeptide repeat-containing protein At2g01860	X	
Cs5g10550	Osmotin precursor [*Arabidopsis thaliana*]	X	

CAPÍTULO II

Aluminum-induced IAA biosynthesis may explain the Al susceptibility in *Citrus limonia*

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Abstract

In acidic soils (pH < 5.0), aluminum (Al) occurs as Al^{3+}, which is phytotoxic and reduces the root growth by hormonal imbalance and/or cell wall rigidity. However, the explanations for the decrease in root growth are not clear. A 60-day study was held with 'Rangpur' lime (*Citrus limonia*) plants grown in a nutrient solution containing different Al concentrations. We measured plant biometric data and used root apexes to analyze auxin (IAA) and ethylene quantification, expression in some Al-responsive genes and anatomic profiles. We found up-regulated expression of multidrug and toxic compound exudation (*Cl-MATE* channel), citrate synthase (*Cl-CS*) and pectin methylesterase inhibitor (*Cl-PMEI*) genes, but while *Cl-PMEI* was expressed at the beginning of the study, *Cl-CS* and *Cl-MATE* were up-regulated only after 60 days, suggesting a late and uncoordinated response to Al. Consequently, Al decreased the root growth and caused anatomical damage. In addition, genes related to IAA cell transport were not differentially expressed in the transcriptome analysis. High IAA, up-regulation of auxin-related small RNAs, as well as low ethylene production in the root tip suggest Al-induced IAA biosynthesis rather than Al-induced disruption in IAA distribution in root cells of this species.

Key words: Al^{3+}; Auxin; Efflux channels; Gene expression; 'Rangpur' lime; Root growth

Introduction

Aluminum is the third most abundant element in the Earth's crust and, in the soil, it naturally occurs as Al_3SiO_4 (Von Uexküll and Mutert 1995). Approximately 30-45% of soils from the world's ice-free land are acidic (pH < 5.0) (Von Uexküll and Mutert 1995) and, under this condition Al_3SiO_4 is hydrolyzed to Al^{3+}, which is toxic to most plants (Kochian 1995). In plants that are sensitive to aluminum (Al), it is covalently retained in the apoplast of root cells showing direct effects such as inhibition of root growth (Kochian 1995; Horst et al. 2010; Sun et al. 2010). It can also have indirect effects as reduced shoot growth (Jiang et al. 2008) and leaf gas exchange (Chen et al. 2005; Banhos et al. 2016a).

In plants not exposed to Al, ethylene upregulates auxin biosynthesis to inhibit root cell elongation (Swarup et al. 2007), the known crosstalk between ethylene and auxin for proper root growth. In plants exposed to Al, the toxicity perception was evidenced to occur at the root apex of maize (*Zea mays*) (Ryan et al. 1993), and the ethylene/auxin crosstalk seems to be affected. In fact, Al-induced ethylene biosynthesis could act as a signal to modify auxin *distribution* in roots by disrupting genes encoding proteins for polar transport of auxin, such as *At-AUX1* and *At-PIN2*, which eventually leads to arrest of root elongation (Sun et al. 2010). Al-induced increase in auxin biosynthesis could also lead to inhibition of cell elongation because, in roots, high concentration of auxin can cause inhibition of cell elongation (Ryan et al. 1993; Rahman et al. 2007). For instance, an Al-induced increased biosynthesis of indole-acetic acid (IAA) was found in the root apex of *Arabidopsis* (Yang et al. 2014).

Al toxicity has been demonstrated not only in *Arabidopsis* and maize, but also in woody species, like *Citrus* plants, where these mechanisms are still unclear. *Citrus limonia* or 'Rangpur' lime shows reduced root length after being exposed to 400 μM Al for 70 days (Pereira et al. 2003). This species shows significant drought resistance due to a vigorous root system, being important as rootstock in subtropical regions of the Americas (Ribeiro and Machado 2007). Thus, Al-induced decrease in root length has a tremendous impact on this species.

In a range of Al-tolerant species, including crop plants (Ryan et al. 2011) and woody species (Brunner and Sperisen 2013), one of the mechanisms to cope with Al involves the efflux of organic anions (malate, citrate, oxalate and succinate), which chelates the toxic Al form (Al^{3+}) preventing its absorption or binding to the cell wall.

Malate efflux is mediated by the ALMT channel - aluminum activated malate transporter (Sasaki et al. 2004), and a non-specific channel that belongs to the MATE family (multidrug and toxic compound exudation) operates citrate efflux (Magalhaes 2010). Considering organic acid exudation, two patterns are evident depending on Al response: no delayed response that corresponds to channel activation (pattern I) and response in a lag phase (pattern II), which involves gene expression activation (Ma 2000). In *Citrus grandis* and *C. sinensis*, the secretion of citrate and malate from excised roots exposed to Al was noted within hours, suggesting pattern I response in these species (Yang et al. 2011), but excised roots decrease their metabolism after a long time. In addition, to our knowledge, no gene expression of efflux channels has been evidenced in order to support such physiological data in *Citrus* plants.

Another mechanism to cope with Al disturbance relies on cell wall composition, which can interfere in Al affinity. In the absence of Al, the action of the pectin methylesterase (PME) leads to free pectic carboxylic groups, which in the presence of Al allows it to bind to pectic nets in the cell wall (Horst et al. 2010). Once Al covalently binds to the cell wall, it becomes rigid, limiting cell elongation. Therefore, the pectin methylesterase inhibitor (PMEI) enzyme impedes PME action and could decrease Al binding in the cell wall and, consequently, enhance Al resistance (Schmohl et al. 2000). The efflux of Al-organic acid complexes, enzymatic protection of pectic carboxylic groups in the cell wall, as well as IAA biosynthesis is well documented. However, the measurement of these parameters in whole plants with their roots exposed to Al for several days is lacking in the literature.

In a 60-day study, we grew young 'Rangpur' lime plants in a hydroponic system in the presence and absence of Al. We confirmed biometric parameters and also performed a transcriptome analysis, in which we expected to find differentially expressed genes related to the efflux of organic acid-Al complexes. We also evidenced organic acids biosynthesis and release from roots of intact whole plants. Although expecting to find MATE and ALMT gene families, as evidenced by citrate and malate effluxes in *Citrus grandis* and *C. sinensis* (Yang et al. 2011), we hypothesized that the expression of these genes is not enhanced due to the Al sensitiveness of 'Rangpur' lime plants (Pereira et al. 2003; Banhos et al. 2016a). We collected root tips and measured IAA and ethylene which, along with IAA polar transport genes possibly evidenced in the transcriptome and further gene expression analyses, made us predict that IAA distribution in root cells is also disrupted, as shown for *Arabidopsis* (Sun et al. 2010). In

addition, anatomical investigation of roots provided evidence to support the functional analyses.

Material and methods

Plant material and experimental conditions

We used three-month-old and 7 ± 0.5 cm-high 'Rangpur' lime (*Citrus limonia*) plants, showing approximately five leaves, for studying the effects of Al on root development within a 60-day period. The plants were kept in a hydroponic system and grown directly on an aerated nutrient solution inside opaque plastic boxes (50 cm in length x 30 cm in width x 15 cm in height; 20 L).

The nutrient solution was adapted from Banhos et al. (2016a) and shows a chemical composition based on the solution proposed by Clark (1975) (Clark 1975), having been used to test Al resistance in *Citrus* rootstocks (Santos et al. 2000; Banhos et al. 2016a). It consisted of the following macronutrients (in mM): N (NO_3^-) 0.93; N (NH_4^+) 0.40; P, 0.013; K, 0.90; Ca, 1.37; Mg, 0.33; S, 0.25; and micronutrients (in µM): Cl, 231.0; Fe (EDTA), 23.3; B, 8.33; Mn, 2.91; Zn, 0.76; Cu, 0.32; Mo, 0.31. In a previous study (Banhos et al. 2016a), we observed that 1480 µM Al causes Al-induced decrease in gas exchange rates in 'Rangpur' lime plants after 45 days. Therefore, besides macro and micronutrients, this solution contained 0, 370, 740, 1110 and 1480 µM Al provided through $AlCl_3$ 6 H_2O. The pH of the solution was monitored daily and maintained at 4.0 ± 0.1 to keep Al as soluble as possible, and the solution was totally replaced every 15 days. The nominal chemical composition of this solution was also tested on Geochem-EZ software (Shaff et al. 2010), resulting in more than 85% free Al^{3+} available. We also measured Al in the solutions using the colorimetric method (Sarruge and Haag 1974), and nominal 370, 740, 1110 and 1480 µM Al supply resulted in 214.4 ± 32.6, 400.2 ± 32.8, 907.7 ± 42.9 and 981.6 ± 67.9 µM Al.

Expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick), with eight holes (2.5 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with polyurethane foam strips that were placed around the plant collar. The boxes stayed on benches (80 cm from the ground) inside a greenhouse with semi-controlled conditions (air temperature 28.5 ± 0.7°C; relative humidity 63.3 ± 1.3%; 853.4 ± 175.1 µmol photons m^{-2} s^{-1}; approximately 14h of natural photoperiod).

Experimental design

After separating a group of plants for initial measurements (biometric data and biomass), we set up the plants in the hydroponic system, and measured the accumulated length of the main root every 7 days until 60 days after planting (DAP), when the number of leaves, leaf area per plant, shoot and main root lengths, as well as the biomass of organs, were assessed.

Using plants grown in the most contrasting treatments (0 and 1480 µM Al), we collected root tips to measure the concentration of indole-acetic acid (IAA) at 1, 3, 7, 15, 30 and 60 DAP, and ethylene production only at 60 DAP due to fine and insufficient roots before this date. After having conducted a transcriptome analysis using 'Rangpur' lime plants grown under 0 and 1480 µM Al, in the present study we collected root tips at 1, 7, 15, 30 and 60 DAP to assess the expression of some genes that had been differentially expressed and revealed by the transcriptome analysis. These were the *Cl-MATE* (Multidrug and toxic compound exudation), citrate synthase (*Cl-CS*), *Cl-SAUR 10* and *Cl-SAUR 15* (Small Auxin up-regulated RNAs) and pectin methyl esterase inhibitor (*Cl-PMEI*). The *MATE* gene family encodes membrane proteins that facilitate the efflux of organic anions such as citrate (Liu et al. 2009). The SAUR gene family is responsible for acidification of the apoplast during IAA action (Spartz et al. 2014). The *Cl-SAUR 10* showed close identity when compared to *Gm-SAUR X10A* and *Cl-SAUR15* had high identity when compared to *Gm-SAUR 15A*, being *Gm-SAUR X10A* and *Gm-SAUR 15A* the first characterized SAUR genes, both evidenced in *Glycine max* (McClure et al. 1989), which share similar functions in elongating tissues responding to auxin within few minutes (Jain et al. 2006). PMEI inhibits demethylesterification of pectic carboxylic groups in walls of root cells, which occurs through the action of pectin methylesterase (PME) (Giovane et al. 2004).

To check whether organic acids (OAs) were being synthesized and released by the roots, we cultivated a group of plants showing the same age and height as described above in the same nutrient solutions as previously described (Banhos et al. 2016a), containing 0 and 1480 µM Al. After seven days, we quantified the contents of oxalate, succinate, malate, and citrate in roots tips and also released in the solutions.

In addition, we collected root tips at 7, 15, 30 and 60 DAP to conduct an anatomical analysis of plants grown under 0 and 1480 µM Al in order to check anatomical disorders during the period of exposure to Al.

Biometric parameters

The lengths (cm) of roots (from the plant collar to the root tip) and stems (from the plant collar to the shoot apex) were measured with a ruler, and the number of leaves was counted. At 60 DAP (and also at 0 DAP for initial measurements) plants were separated into leaves, stems (plus petioles) and roots. The leaf area (cm^2) was measured with an area meter (LI-3100C, LI-COR, USA). The organs were dried at 60°C until constant mass to obtain the biomass (g) of organs and total plant biomass.

Indole-acetic acid analysis

Extraction and purification

Root apexes (0.5 cm in length) were washed in deionized water and stored at -80°C prior to analysis. Samples (420 µg fresh weight) were extracted using isopropanol:acetic acid (95:5; v:v) solution at 4°C. A solution containing 0.5µg of a labelled standard [$^{13}C_6$]-IAA (Cambridge Isotopes, Inc.) was added as internal standard (IS). After 2h, the solvent was separated by centrifugation at 14,000 *g* for 10 min at 4°C. The supernatant was concentrated to 50 µL, acidified to 2.5 pH, and IAA was extracted three times with ethyl acetate, according to Ludwig-Müller *et al.* (2008) (Ludwig-Müller et al. 2008). After purification, the solution was evaporated to dryness and re-suspended in 30 µL ethyl acetate for methylation with diazomethane.

GC-MS analysis

Quantification of free IAA was performed using gas chromatography coupled with mass spectrometry: a Hewlett Packard 6890 gas chromatograph (GC) coupled to an HP5973 mass selective detector. The GC column was HP-1701 (30 m, 0.25 mm I.D., 0.5 µm film thickness), with helium as the carrier gas at flow rate of 1 mL min^{-1}. The temperature program for analysis was 140°C for 1 min, followed by increase of 5°C min^{-1} up to 210°C, 1 min hold, increase to 280°C at 20°C min^{-1} then 1 min hold at 280°C. The ions were monitored at m/z 130 and 189, for the endogenous IAA, and m/z 136 and 195, for the labeled IS. The ratio 130:136 was used to calculate the endogenous amount of IAA.

Ethylene analysis

Root tips (~1 cm in length) of ~0.5 g were excised and placed into 5.0 mL glass vials containing 0.7% (m/v) agar (Sun et al. 2010). After 2h, 1 mL of the headspace was

taken from the vials using a syringe (Hamilton, Gastight, Nevada, USA), and injected into a gas chromatograph - GC (Trace 2000 GC, Thermo Fisher Scientific, USA). The GC was equipped with a 1.8 m Poropack N column at 120°C, flame ionization detector (FID) at 120°C, injector at 120°C and used nitrogen as the carrier gas (30 mL min^{-1}).

Gene expression analysis

We used quantitative PCR to analyze the expression of *Cl-MATE*, *Cl-CS*, *Cl-SAUR 10*, *Cl-SAUR 15* and *Cl-PMEI*, which had been differentially expressed in the presence of Al as revealed by the transcriptome analysis. Based on RNA-seq data, we designed primers to *Cl-MATE* (forward CATTGACACAGCATTTATCGGC and reverse CAAATGAGGTTGTAATATTAACTAATGGG), *Cl-CS* (forward CGCTAAGCCAGATGGAGAAC and reverse ACAGTGGCACGATCTCTCAA), *Cl-PMEI* (forward CCACAAGAACGACAGCGATA and reverse TTAGCGTAACTCGGCAAGGT), *Cl-SAUR 10* (forward TGGGTTCACAACTCACAAGC and reverse TGAACAATACCAGGCAAACG) and *Cl-SAUR 15* (forward AGGCGTGCTCTTATGGTTTC and reverse TTCTGAAAGGATGGGTGCTT). As reference genes we used *GAPC2* (forward TCCTATGTTTGTTGTGGGTG and reverse GGTCATCAAACCCTCAACAA) and *EFα* (forward TCAGGCAAGGAGCTTGAGAAG and reverse GGCTTGGTGGGAATCATCTTAA), which were proposed by Mafra et al. (2012).

Serial dilutions of genomic DNA from *Citrus limonia* were amplified using the internal primers in order to access the amplification efficiency during qPCR analysis. GoTaq qPCR Master Mix (Promega, Madison, USA) was used as the reagent in these tests, with the following procedural specifications: 95°C for 5 min (1 cycle), 95°C for 10 s, 58°C for 30 s, 72°C for 30 s (40 cycles) followed by a melting curve analysis (1% slope temperature; 60–95°C). Amplification efficiencies were 91.85% for *Cl-MATE*, 85.38% for *Cl-CS*, 97.22% for *Cl-PMEI*, 91.71% for *Cl-SAUR 10*, 91.55% for *Cl-SAUR 15*, 97.24% for *Cl-GAPC2* and 100.04% for *Cl-EFα*.

For quantitative analysis of gene expression, total RNA was extracted from root samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Total RNA (2 µg) was treated with RNase-free TURBO DNase (Ambion, Carlsbad, USA) and transcribed in reverse to cDNA using an oligo-dT primer and Super Script III, according to the manufacturer's protocol (Life Technologies, Carlsbad, USA). cDNAs were submitted to gene expression analysis using GAPC2 and the EFα genes in order to normalize the cycle threshold (Ct) values. The reagent and cycles used for the qRT-PCR analyses

were the same as those used for the amplification efficiency tests. In this protocol, every reaction was performed in triplicate.

Ct value of each sample, determined by the mean of the three technical replicates, was converted into relative quantities (RQ) using the function $RQ=E^{\Delta Ct}$. ΔCt is the difference between the lowest Ct value across all samples for the evaluated gene and the Ct value of the given sample. A normalization factor (NF) for each sample was calculated by the geometric mean of the RQ values of GAPC2 and EFα. Normalized-relative quantity (NRQ) of each sample was calculated as the ratio of the sample RQ and the appropriate NF. Individual fold change values were determined by dividing the sample NRQ by mean values of NRQ that were obtained from the calibrator, i.e., root samples of plants not exposed to Al. Following this, fold change in the control group always shows a mean value of 1.

Organic acids analysis

The plants were cultivated in Falcon® tubes containing the nutrient solutions (45 mL) with 0 and 1480 µM Al. Aeration was performed twice a day and pH was maintained at 4.0. The plants were maintained in laboratory conditions (25 ± 1 °C, photoperiod of 12h and photosynthetic photon flux density of 600 µmol m^{-2} s^{-1}, which returns saturating CO_2 assimilation rates). When necessary, the solutions were completed to 45 mL with deionized water.

Organic acids synthesized by the roots (internal OAs) were extracted by osmosis and alkaline gradient, immersing the root tips in 1.5 mL Na_2CO_3 40 mM for 24h. Then, we collected 1 mL of the extractor solution and dried at 80°C. Exudated OAs (released by the roots) were concentrated after drying both solutions (0 and 1480 µM Al) at 80°C.

Samples were esterified according to Fischer method (methylation) (Fischer and Speier 1895). To internal OAs samples, we added 400 µL of methanol (HPLC/Spectro) and 100 µL of sulfuric acid (7N) resulting in a 0.5 mL extracting solution. To exudated OAs samples, we added 700 µL of methanol (HPLC/Spectro) and 300 µL of sulfuric acid (7N) resulting in 1 mL extracting solution. After shaking, the samples were kept for 15 min at 70°C for catalyzing the reaction. After adding 1 mL of hexane (HPLC/Spectro) 100 µL of apolar phase were collected and analyzed using a gas chromatograph coupled to a mass spectrometer (GC-MS) system (GC-2010/GCMSQP2010 Plus, Shimadzu, Japan), with an automatic sample injector (AOC-20i).

In the GC-MS, we used a 30 m-length and 250 μm-diameter fused-silica microcolumn (RTX-5MS, Restek), and analytical ultra-pure helium (99.9999%, White Martins) was used as carrier gas. The injector temperature was 250°C (Splitless mode) and the injection volume was 1 μL. Column gas flow was maintained at 41 cm/s. The initial column temperature was 50°C with a 4 min step. After that, at a 10°C/min rate, it achieved 70°C. Then, it was increased to 250°C at a 25°C/min rate, and maintained for 0.8 min, completing 14 min running. Mass detector was a simple quadrupole type with 70eV electronic impact ionization. The GC-MS interface temperature was 250°C and 230°C to the ionizer. The detector potential was relative to tunning, with a 40 to 450 m/z detection range (scanner mode).

Anatomical analysis

Root tips (~ 0.5 cm in length and 1 mm in diameter) were collected and immediately fixed in Karnovsky solution (Karnovsky 1965). The samples were dehydrated in increasing ethanol series [30, 50, 70, 90 (one hour each), and 100% (three times, one hour each)], then infiltrated with resin (Historesin, Leica instruments, Germany) and ethanol 100%, at a ratio of 1:1, overnight. After 24h, samples were infiltrated with pure resin, reserved overnight and then polymerized in blocks. Longitudinal sections were obtained with a rotary microtome and mounted on permanent glass slides that were immersed (5 min) in a toluidine blue solution (pH 4.5) for staining (at room temperature) structures containing nucleic acids and lignin (O'brien et al. 1964). Then, the glass slides were washed under tap water to remove excess of dye and dried with a clean cotton cloth.

All sections were observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany) and the images were captured with a digital camera (DFC-290, Leica Microsystems, Germany) functionally attached to the DMLB.

The anatomical study was based on consecutively-sliced longitudinal sections of root tips containing the root cap, the quiescent center, and elongation, division and maturation zones.

Data analysis

Biometric data and the biomass of organs were measured using eight replications (plants). Concentration of IAA and ethylene production in the root tips was evaluated using three plants. For gene expression and OAs quantification in root tips and in the solution, five samples were used as replications.

A one-way analysis of variance (Anova) was performed between plants exposed to 0, 370, 740, 1110 and 1480 μM Al to test for differences in accumulated main root length at every evaluation date. The Tukey test ($\alpha = 0.05$) was used to conduct post-hoc comparisons to estimate the least significant differences between mean results. Biometric data and biomass of organs were subjected to student's t-test ($\propto = 0.05$) to verify differences between 0 and 60 DAP within each Al concentration. This same test was used to verify differences in IAA concentration, ethylene production, expression of genes and OAs synthesis/release between 0 and 1480 μM Al on distinct evaluation dates.

Results

Plants showed reduced plant growth, fewer leaves and decreased root development with the increase of Al concentration in the nutrient solution (Fig. 1).

Differences in root growth started after 14 DAP. At 21 and 28 DAP, plants exposed to Al showed similar values that were lower than those of plants not exposed to Al. From 35 DAP until 56 DAP plants exposed to 370 μM Al emerged as an intermediate treatment, showing values that were lower than those of plants not exposed to Al but higher than those of plants exposed to 740, 1110 and 1480 μM Al (Fig. 2).

When compared to initial values (0 DAP), the increment of leaf area (Fig. 3a), number of leaves (Fig. 3b), shoot length (Fig. 3c) and main root length (Fig. 3d) within 60 days reduced considerably with the increase of Al concentration in the nutrient solution. Plant biomass exhibited the same response pattern, showing reduced values of leaf (Fig. 4a), shoot (Fig. 4b), root (Fig. 4c), and total biomass (Fig. 4d) with the increase of Al concentration in the nutrient solution.

Plants exposed to 1480 μM Al showed higher IAA concentration in their root tips when compared to plants not exposed to Al, mainly at 3, 7 and 15 DAP (Fig. 5). At 60 DAP, ethylene produced in the root tips of plants exposed to 1480 μM Al was five times lower than that of plants not exposed to Al (Fig 6).

When exposed to 1480 μM Al, the gene responsible for *Cl-MATE* channel was 10.4 and 1.5 times more expressed at 1 and 7 DAP, respectively (Fig. 7a). However, at 60 DAP, *Cl-MATE* was 215 times more expressed in plants exposed to Al when compared to plants not exposed to Al (Fig. 7a). *Citrus limonia* citrate synthase gene expression was down-regulated at 15 DAP (1.6 times less expressed) and 30 DAP (5.3 times less expressed) in the presence of Al in relation to plants not exposed to Al.

However, *Cl-CS* was considerably up-regulated (9.1 times more transcripts) in the presence of Al at 60 DAP (Fig. 7b). *Cl-PMEI* gene expression, however, showed up-regulation at 7 DAP (1.8 times more transcripts), and then became down-regulated in response to Al, reducing to 3.4 (15 DAP), 10 (30 DAP) and 20 times less transcripts (60 DAP) in relation to plants not exposed to Al (Fig. 7c). The same up-regulation followed by down-regulation was observed for *Cl-SAUR 10*, which was 2.8 times more expressed at 7 DAP and reduced to 25 times less transcripts at 60 DAP (Fig. 8a). *Cl-SAUR 15* followed a similar response, showing down-regulation at 15 DAP (2 times less transcripts) and 60 DAP (33.3 times less transcripts) (Fig. 8b).

Roots tips OAs contents were found in all plants after seven DAP. However, in relation to plants not exposed to Al, only succinate was significantly reduced in roots of plants exposed to Al (Fig. 9a). After seven days, oxalate, succinate, and citrate were detected in the nutrient solutions and were higher in the solutions containing Al. Citrate was not found in the solution without Al, but approximately 2µmol of it was measured in the solution containing Al (Fig. 9b).

In plants not exposed to Al, cells with regular size and rectangular shape were noted in the cortex (Fig. 10b), while in plants exposed to 1480 µM Al thick a non-uniform round shape cells were found in the cortex (Fig. 10d), after 15 days of Al exposure. This anatomical pattern was reflected in the shape of the extreme root tip as plants exposed to Al showed a wider root tip (Fig. 10c, 10g and 10k) when compared to a sharp arrow-like root tip in plants not exposed to Al (Fig. 10a, 10e and 10i). When exposed to Al, more cellulosic deposition was observed on cell walls in the cortex and lignin in the vascular cylinder, as evidenced by stronger blue-green stains (Fig. 10d). The longer the exposure time to Al, the more apparent became this cellulosic cortical deposition as well as lignin deposition in the vascular cylinder (Fig. 10d, 10h, 10l). Anatomical ruptures could be observed on root epidermis of plants exposed to Al, mainly after 30 and 60 days of Al exposure (Fig. 10g and 10k), indicating an irregular pattern of epidermal cell division and elongation, which contrasted with plants not exposed to Al (Fig. 10e and 10f).

Discussion

'Rangpur' lime plants are sensitive to Al (Santos et al. 2000; Pereira et al. 2003; Banhos et al. 2016a) and here we show that, although they possess genes associated with Al resistance, as revealed by the transcriptome analysis (RNA-seq), their

expression is uncoordinated in this species. *Cl-MATE* and *Cl-CS* were conspicuously up-regulated when the roots were exposed to 1480 µM Al, but only at 60 DAP. We confirmed that 'Rangpur' lime plants had OAs inside their roots independently of the Al presence. This can be related to intense respiration activity (Krebs' cycle), as evidenced by the presence of mitochondria in root tips of soybean plants (Xu et al. 2010). After seven days, except for malate, OAs were significantly higher in the solution containing Al, indicating that OAs, including citrate, are secreted by this species in response to Al. Citrate efflux is mediated by *MATE* (Furukawa et al. 2007), and a cluster analysis of the *MATE* sequence found showed highest phylogenetic proximity to MATE genes related with this same function in different species (data not shown). CS and MATE can be both up-regulated by Al (Xu et al. 2010), but in transformant tobacco plants CS expression did not lead to the efflux of citrate by MATE channels (Delhaize et al. 2001), while in barley citrate exudation responds to Al (Zhao et al. 2003), indicating there is no consistent pattern in the expression of MATE and CS in response to Al. In the present study, *Cl-CS* and *Cl-MATE* were concomitantly up-regulated at 60 DAP, indicating a consistent pattern in the expression of these two genes in response to Al.

In soybean plants, only a 16% increase in citrate synthase *activity* occurred upon Al treatments after 6h (Xu et al. 2010). In *Vigna umbellata*, the citrate efflux mediated by MATE also occurred 6h following Al exposure (Liu et al. 2013). In the present study, *Cl-CS* was not significantly up-regulated at 7 DAP (Fig. 7b) corroborating the similar citrate concentration in root tips of both treatments (Fig. 9a). However, approximately 2 µmol citrate was found in the solution containing Al, at 7 DAP (Fig. 9b). These values may be comparable to the citrate secretion from *excised* roots of *Citrus grandis* and *C. sinensis* that reached a peak of 0.7 µmol citrate at 24h after exposure to 500 µM Al (Yang et al. 2011), which decreased after this period perhaps because excised roots reduce their metabolism after a long time. In the case of the whole root system of 'Rangpur' lime plants, the conspicuous up-regulation of *Cl-MATE* and *Cl-CS* only at 60 DAP may be a delayed response to mitigate the effects of Al, and investigation of OAs concentration in root tips and released in the solution at 60 DAP merits further investigation after refining the method of OAs detection presented here. Such delayed response to Al can be also supported by anatomical damage caused by Al and observed from 15 DAP until the end of the study (Fig. 10).

On the other hand, *Cl-PMEI* was up-regulated at 7 DAP. This indicates that pectic carboxylic groups in the cell wall are protected from demethylesterification processes through the action of PME, at least 7 days after exposure to Al. In fact, pectin

undergoes partial apoplastic demethylesterification through the action of PME, resulting in the exposure of free pectic carboxylic groups, which could serve as binding sites for Al in the wall (Zhu et al. 2014). Additionally, potato transformants that exhibited higher expression of PME accumulate more Al, produce more callose and their root growth is more inhibited when exposed to Al than wild types (Schmohl et al. 2000). Demethylesterification through the action of PME also occurs within hours (Franco et al. 2002). Therefore, up-regulation of a gene encoding inhibitors of this process at 7 DAP may not be considered a fast response, even in a 60-day experiment. In addition, this up-regulation was inverted, becoming down-regulated at 15, 30 and 60 DAP (Fig. 7c). Taken together, *Cl-PMEI* up-regulation at 7 DAP (but down-regulation from 15 to 60 DAP), while *Cl-MATE* and *Cl-CS* increased expression only at 60 DAP could indicate that these mechanisms, which are related to Al resistance in insensitive plants, are not only delayed (*Cl-CS* and *Cl-MATE*) but uncoordinated in 'Rangpur' lime plants, and this may explain the Al sensitivity in this species.

We confirmed that Al interferes with root elongation and root biomass production in 'Rangpur' lime plants. Plants not exposed to Al showed accumulated root length that was 10 times higher than that of plants exposed to 370 μM Al, and 16 times higher when compared to plants exposed to 740, 1110 and 1480 μM Al, at 56 DAP (Fig. 2). These expected symptoms, however, were associated with high concentration of IAA in root tips of plants exposed to Al, mainly at 3, 7 and 15 DAP (Fig. 5). In *Arabidopsis*, Al was also found to induce a localized enhancement of auxin biosynthesis in the root apex (Yang et al. 2014). Differently from stems, in cells (of the elongation zone) of root tips, high concentration of IAA can cause strong inhibition of cell elongation (Rahman et al. 2007; Cleland 2010). IAA concentration in root tips of plants exposed to Al was three- (7 DAP) and two-fold higher (15 DAP) than that of plants not exposed to Al (Fig. 5). Intriguingly, the expression of *Cl-SAUR 10* was also up-regulated at 7 DAP, when a peak of IAA concentration occurred in plants exposed to Al after which this gene became down-regulated until the end of the study, similar to what occurred with *Cl-SAUR 15* gene expression. This suggests that at 7 DAP auxin action was close to its maximum activity as SAUR genes are responsible for acidification of apoplast (Spartz et al. 2014). Alkalinization of the apoplast, on the other hand, is associated with inhibition of root cell elongation under elevated auxin concentration (Lüthen and Böttger 1993; Evans et al. 1994; Hager 2003). Thus, the up-regulation of *Cl-SAUR 10* occurring together with the peak of IAA concentration in roots of plants exposed to Al demonstrates that IAA and *Cl-SAUR 10* gene expression were *initially*

acting towards root cell elongation, but after that, *Cl-SAUR 10* and *Cl-SAUR 15* gene expressions were down-regulated while IAA concentration was kept high until 15 DAP (Fig. 5), which might have inhibited cell elongation. These responses of *Cl-SAUR 10* (up-regulation peaked at 7 DAP) and *Cl-SAUR 15* contrast with those of *Cl-CS* and *Cl-MATE* that peaked only at 60 DAP, reinforcing that such genes related with Al resistance are uncoordinated in 'Rangpur' lime plants. In addition, the transcriptome analysis of plants grown in the presence and absence of Al did not reveal any differential expression of genes responsible for membrane proteins associated with auxin polar transport, such as AUX1, AUX2 and PINs. Therefore, in the root tips of 'Rangpur' lime plants grown under 1480 μM Al, an increase in IAA concentration, and not an imbalance in IAA distribution, is likely to trigger the inhibition of root cell elongation and, consequently, root growth. This is evidenced by round shaped cells in the cortex of roots exposed to Al (Fig. 10d), indicating that these cells divide in a periclinal manner, contrasting with the rectangular cells in the cortex of roots not exposed to Al (Fig. 10b) that evidences an anticlinal cell division benefiting the normal growth in length.

Using *Arabidopsis*, Sun et al. (2010) argues that Al-induced ethylene biosynthesis is likely to act as a signal to change auxin *distribution* in roots by disrupting *At-AUX1* and *At-PIN2*-mediated auxin transport. Therefore, we infer that this disruption in auxin distribution leads to asymmetrical auxin distribution and, consequently, twisted root shape. Our anatomical analysis, however, indicated thick non-uniform round shape cells at *both* sides of the cortex of roots exposed to Al, resulting in a thick growth pattern at the extreme root tip of plants exposed to Al, where no twisted roots were observed on any evaluation date. This reinforces that Al-induced disruption in auxin distribution is unlikely to occur in 'Rangpur' lime plants when exposed to Al. In addition, although measured only at 60 DAP, ethylene production in plants exposed to Al was 1000 times lower than that in plants not exposed to Al. Thus, besides its role in disrupting IAA distribution that could not be evidenced in plants exposed to Al, such low ethylene production also corroborates the fact that leaves of these plants did not experience Al-induced abscission. This may be an evidence of a distinct mechanism for Al disruption of root cell elongation in this species, although measurement of ethylene production over time is needed in future studies with this species when disturbed by Al.

The ruptures in the epidermis of roots exposed to 1480 μM Al have also been observed in cowpea exposed to 600 μM Al for 48h (Kopittke et al. 2008; Blamey et al.

2011). These authors argue that these ruptures result from an increase in cell wall rigidity in the outer layers (epidermis and outer portions of the cortex) due to higher Al accumulation in these layers, as observed in barley (Ma et al. 2004) and maize (Stass et al. 2006). According to this, Al reduces elongation in outer layers while cells of the inner root layers continue to elongate, which possibly causes ruptures. We found irregular pattern of epidermal cell division and elongation in roots of plants exposed to Al, contrasting with those of plants not exposed to Al, which could corroborate the explanation given by Kopittke et al. (2008) and Blamey et al. (2011). However, we could not anatomically evidence that the inner root layers are suggestive of regular (anticlinal) cell division, as evidenced by non-rectangular round shaped cells in the whole cortex. Therefore, identifying distinct concentrations of Al in different portions of the root tip of 'Rangpur' lime plants exposed to Al is needed in future studies. For example, using Al-specific x-ray coupled to scanning electron microscope (SEM/EDS) could enable us to detect the presence of Al in different parts of leaves from Al-accumulating and non-accumulating species (Bressan et al. 2016).

In this study, we demonstrate that although possessing genes associated with Al resistance, 'Rangpur' lime plants are, in fact, sensitive to Al due to late expression of *Cl-MATE*, *Cl-CS* and *Cl-PMEI*, and also uncoordinated responses of these genes when the roots are exposed to Al in a 60-day study. In addition, we revealed that an increase in IAA concentration, rather than an imbalance in IAA distribution, as we expected, is associated with the inhibition of root cell elongation in this species.

Acknowledgements

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Figures and tables

Fig. 1 General view of the root system of 'Rangpur' lime plants grown for 60 days in nutrient solutions containing 0, 370, 740, 1110 and 1480 µM Al

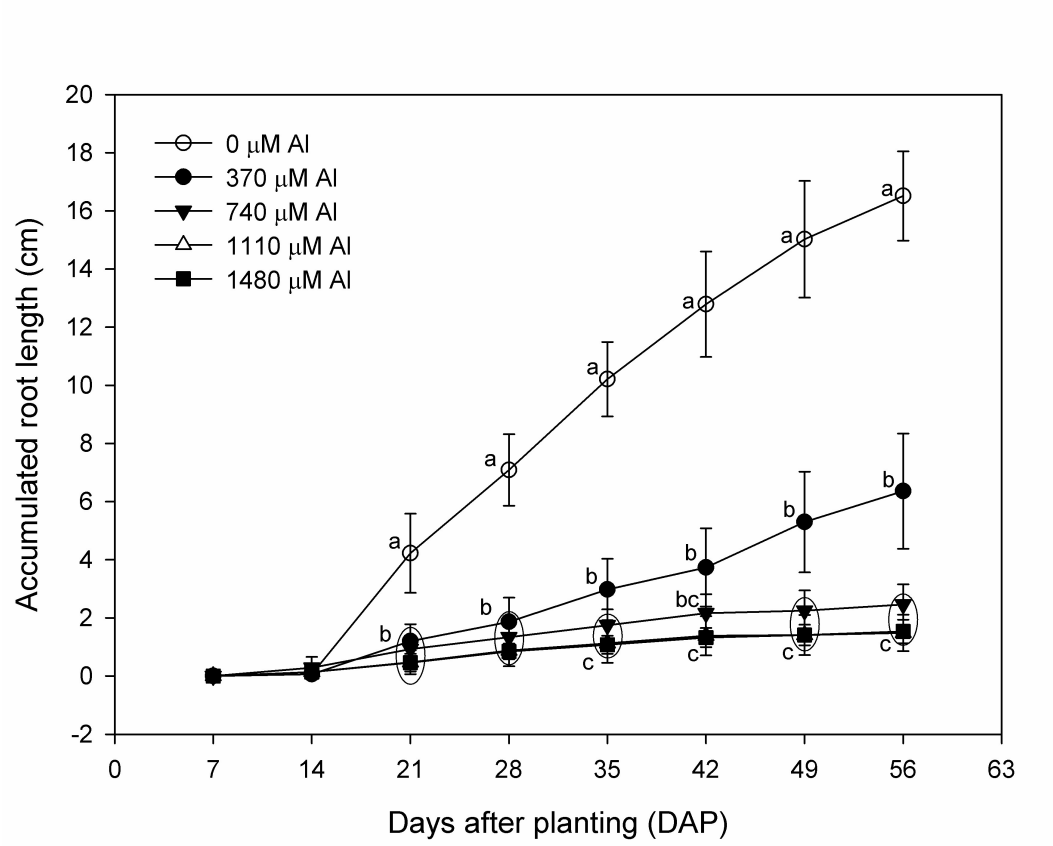

Fig. 2 Accumulated length of the main root of 'Rangpur' lime plants grown for 56 days in nutrient solutions containing 0, 370, 740, 1110 and 1480 μM Al. For each evaluation date, different letters represent significant differences ($P < 0.05$) between Al concentrations

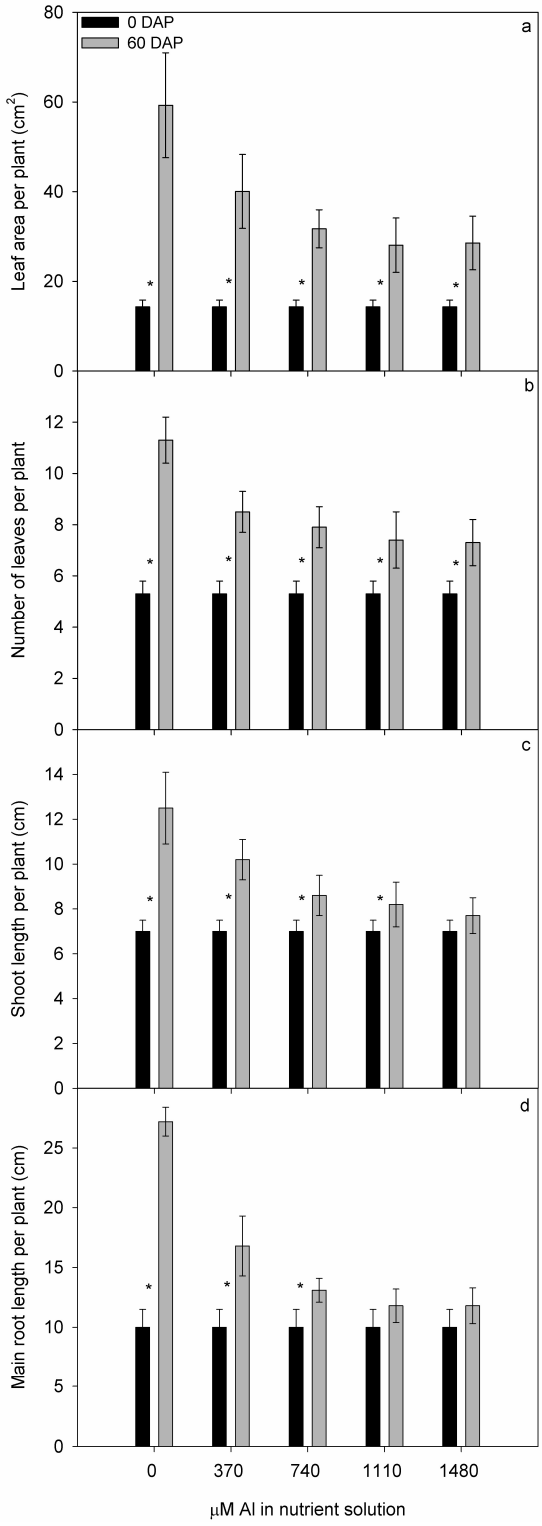

Fig. 3 Leaf area, number of leaves, shoot and root length of 'Rangpur' lime plants at 0 and 60 days after planting (DAP), grown in nutrient solutions containing 0, 370, 740, 1110 and 1480 µM Al. For each Al concentration, asterisks represent significant differences ($P < 0.05$) between 0 and 60 DAP

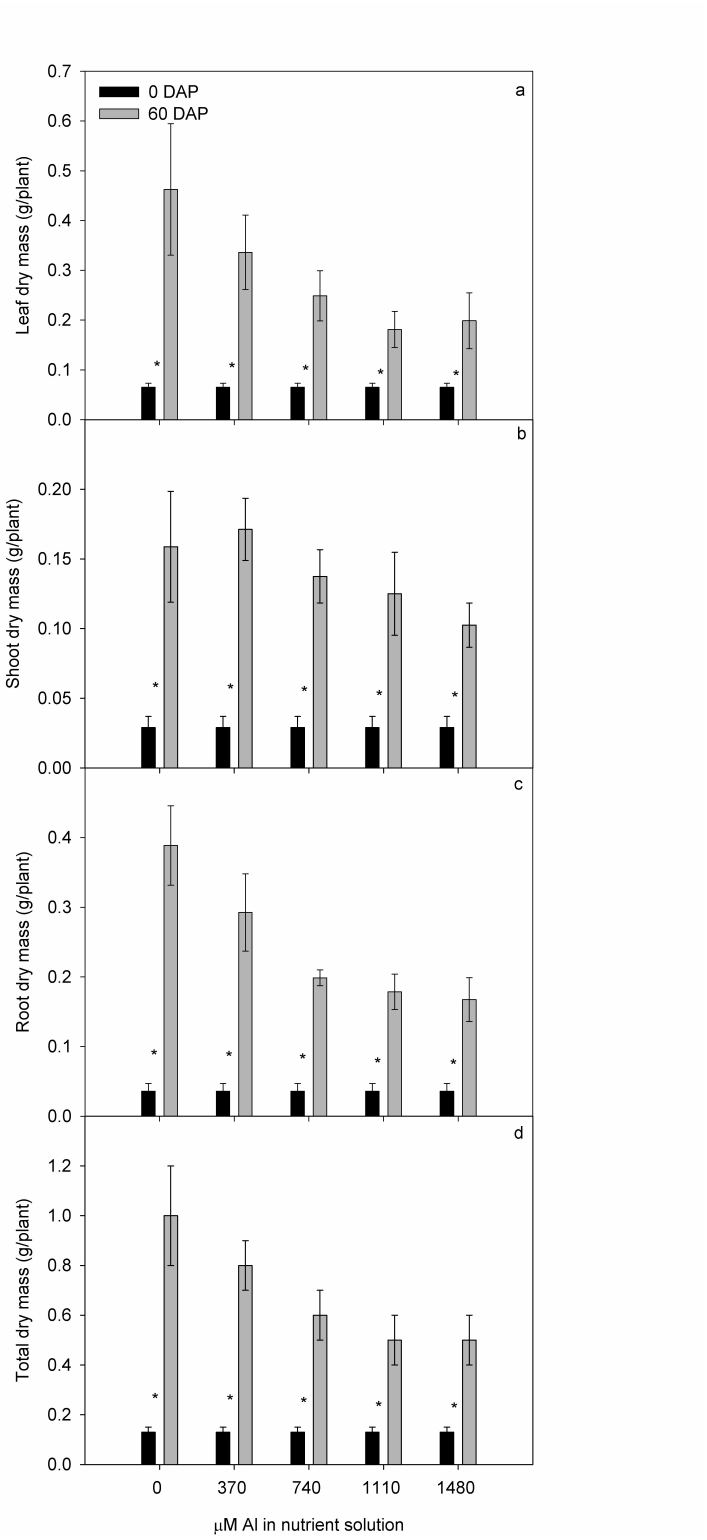

Fig. 4 Biomass of leaves, shoots, roots, and total biomass of 'Rangpur' lime plants at 0 and 60 days after planting (DAP), grown in nutrient solutions containing 0, 370, 740, 1110 and 1480 μM Al. For each Al concentration, absence of asterisks represents lack of significant differences ($P < 0.05$) between 0 and 60 DAP

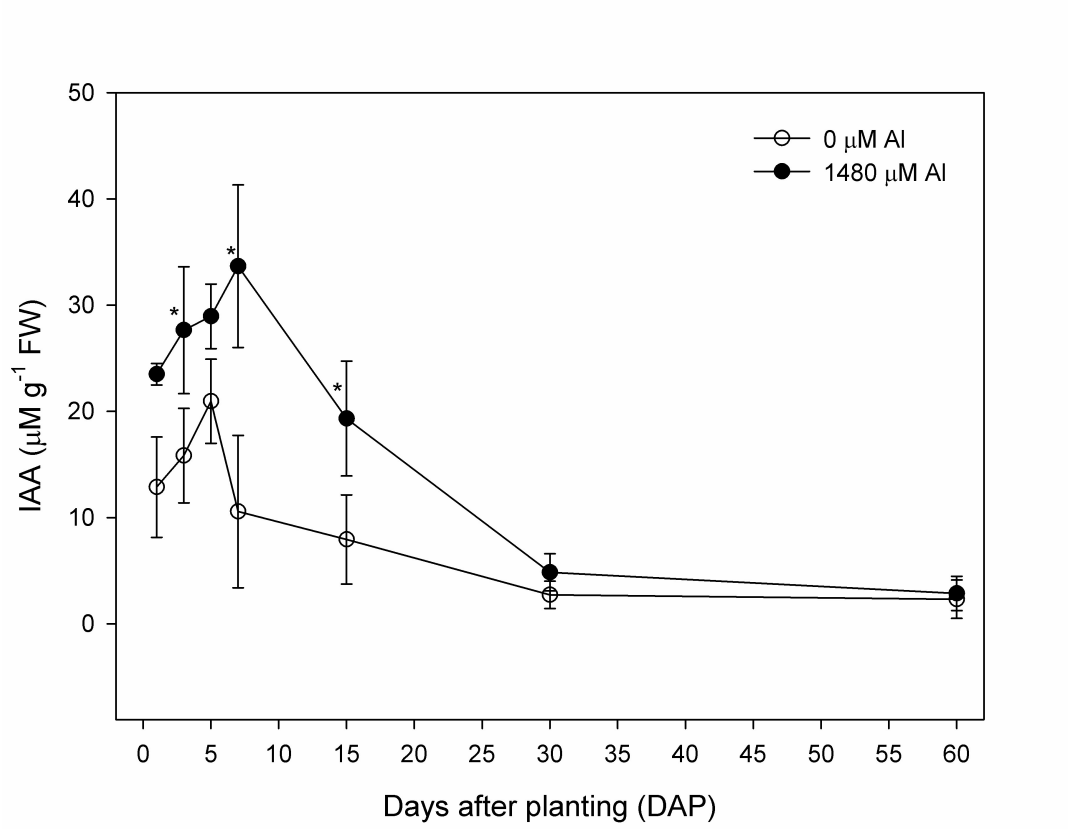

Fig. 5 Indol-acetic acid (IAA) concentration measured in root tips of 'Rangpur' lime plants grown in nutrient solutions containing 0 and 1480 μM Al at 1, 3, 7, 15, 30 and 60 days after planting (DAP). For each evaluation date, asterisks represent significant difference ($P < 0.05$) between 0 and 1480 μM Al

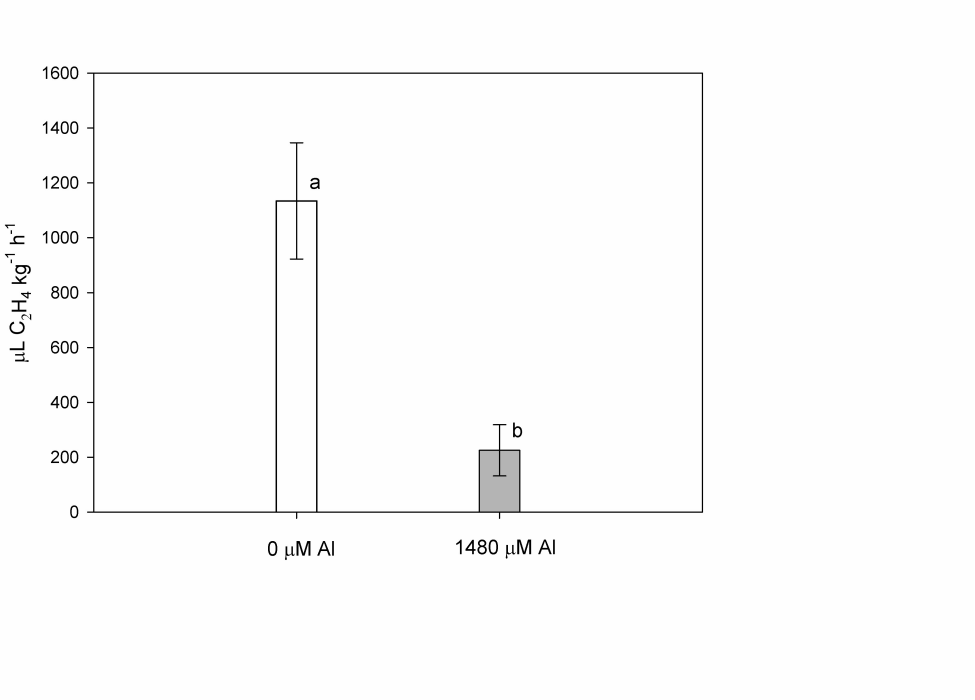

Fig. 6 Ethylene production in excised root tips of 'Rangpur' lime plants grown in nutrient solutions containing 0 and 1480 μM Al at 60 days after planting (DAP). Different letters represent significant differences ($P < 0.05$) between Al concentrations

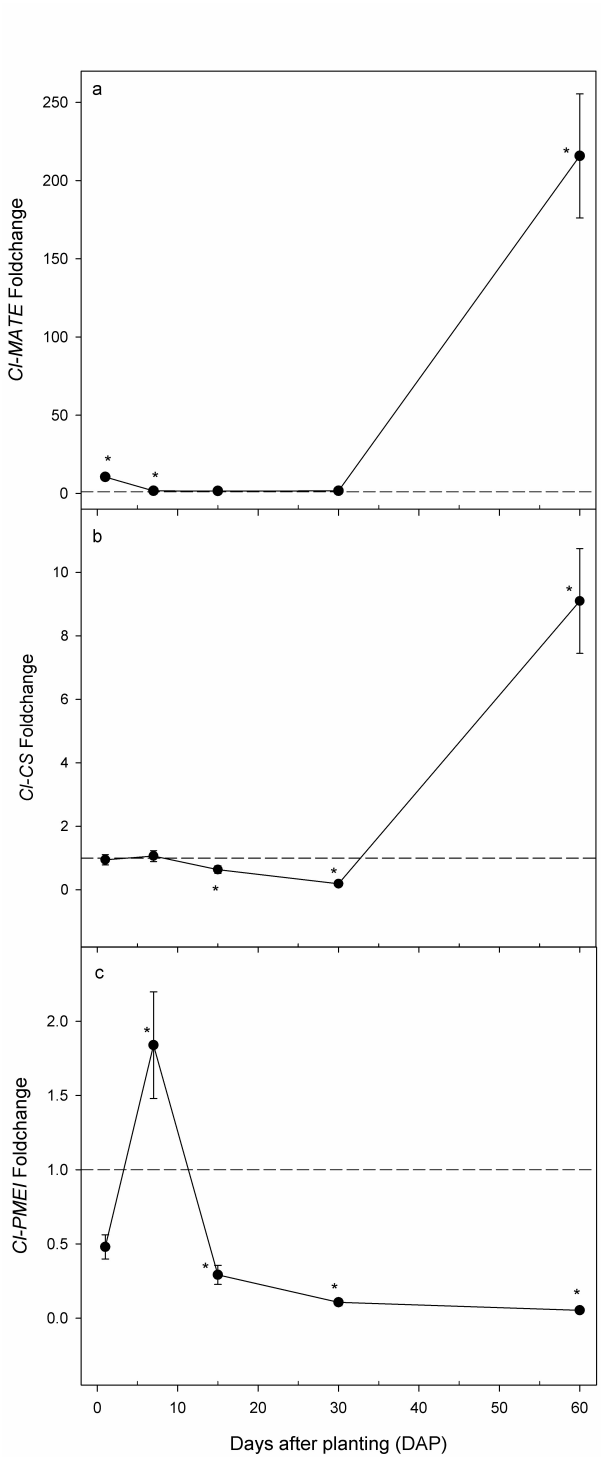

Fig. 7 Gene expression of MATE (multidrug and toxic compound exudation) channel, citrate synthase and pectin methyl esterase inhibitor in root tips of 'Rangpur' lime plants grown in nutrient solutions containing 0 and 1480 µM Al at 1, 3, 7, 15, 30 and 60 days after planting (DAP). For each evaluation date, asterisks represent significant difference ($P < 0.05$) between 0 and 1480 µM Al. Dashed line is a reference (one fold change in gene expression of plants not exposed to Al)

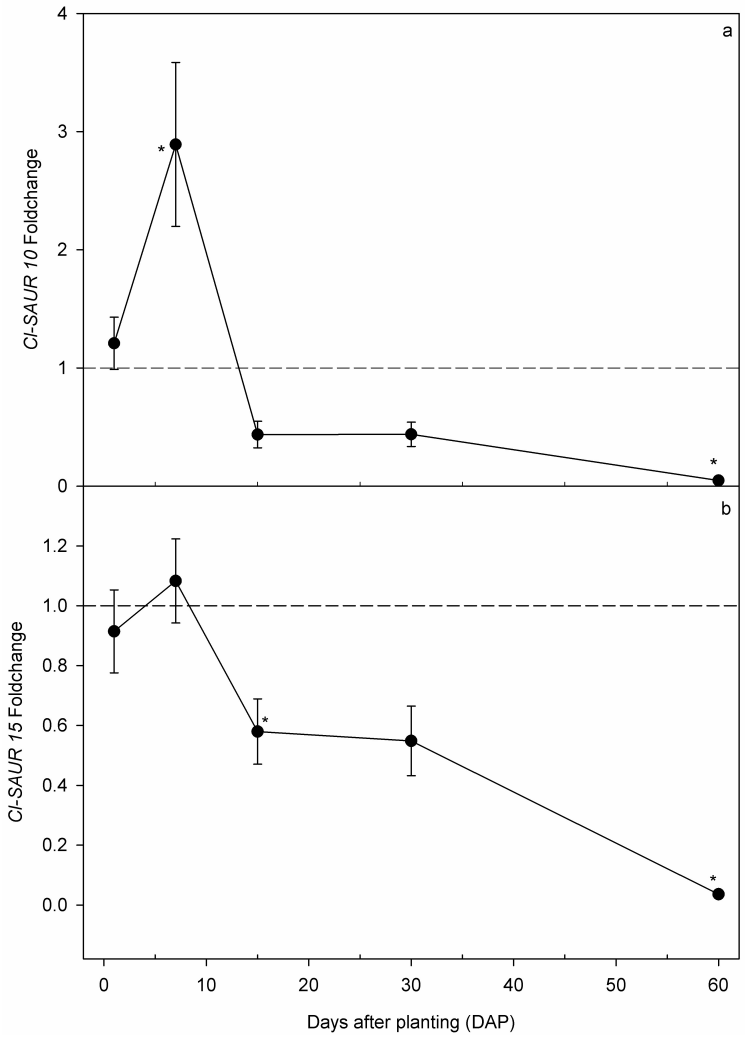

Fig. 8 Gene expression of SAUR (small auxin up-regulated RNAs) in root tips of 'Rangpur' lime plants grown in nutrient solutions containing 0 and 1480 µM Al at 1, 3, 7, 15, 30 and 60 days after planting (DAP). For each evaluation date, asterisks indicate significant difference ($P < 0.05$) between 0 and 1480 µM Al. Dashed line is a reference (one fold change in gene expression of plants not exposed to Al)

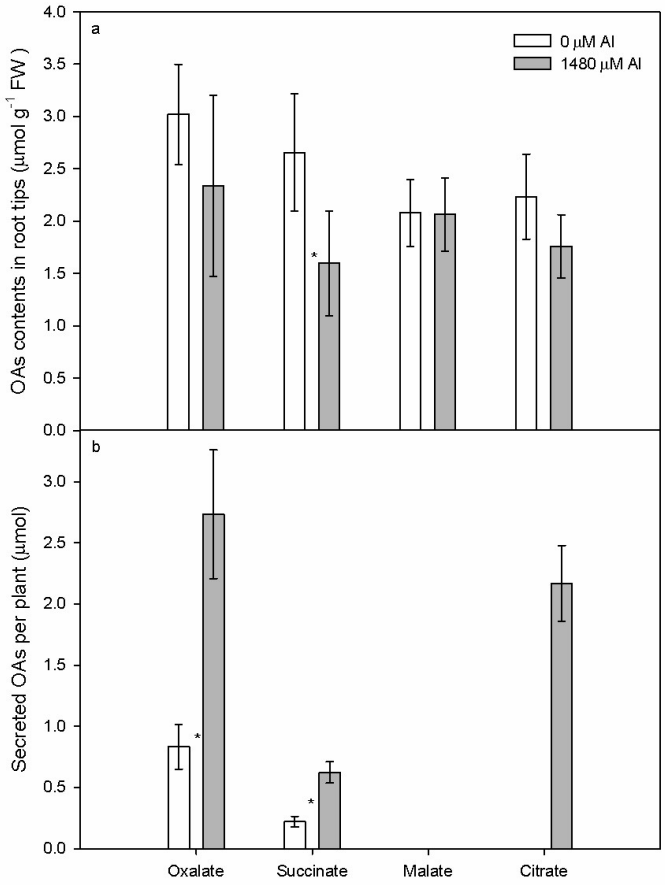

Fig. 9 Organic acids contents in roots tips (a) and released in the nutrient solution (b) by the roots of 'Rangpur' lime plants grown for seven days in nutrient solutions containing 0 and 1480 µM Al. For each OA, asterisks indicate significant difference ($P < 0.05$) between 0 and 1480 µM Al

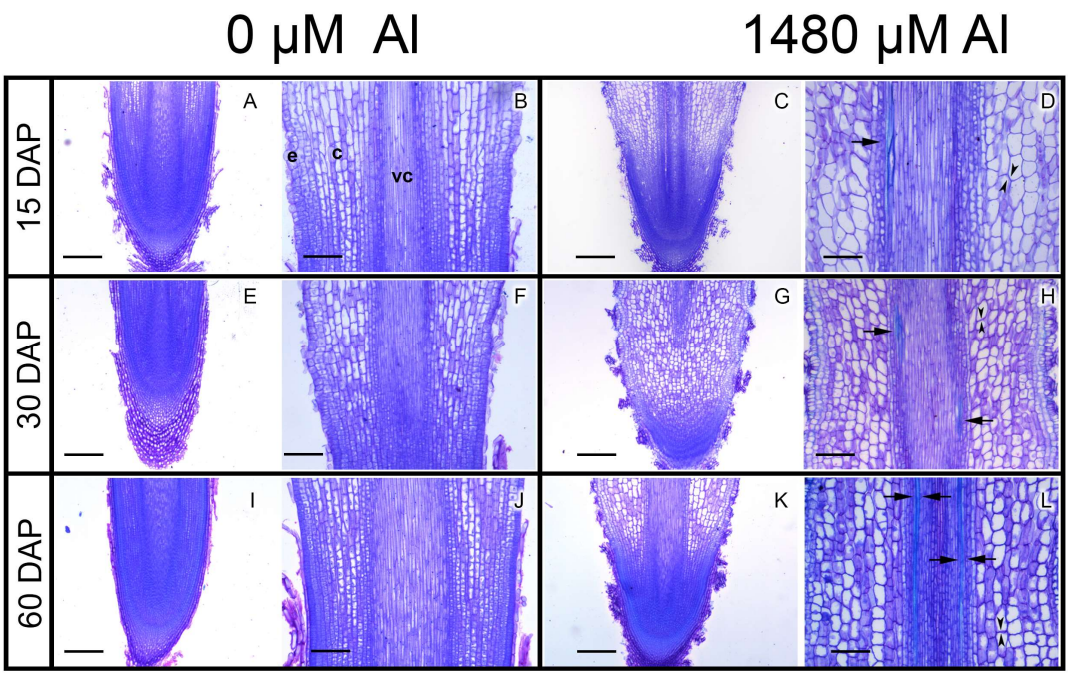

Fig. 10 Anatomical analyses of root tips of 'Rangpur' lime plants. A, B, E, F, I and J: grown in nutrient solution containing 0 µM Al; C, D, G, H, K and L: grown in nutrient solution containing 1480 µM Al; A, E, I, C, G and K: root apexes; B, F, J, D, H and L: root elongation zone. 15 DAP: A, B, C and D; 30 DAP: E, F, G and H; 60 DAP: I, J, K and L. c = cortex; e = epidermis; vc = vascular cylinder. Double-opposite head arrows: cellulosic deposition in the cortex. Single arrows: lignin deposition in the vascular cylinder. Double-opposite whole arrows: prominent lignin deposition in the vascular cylinder. Scale bars (µm): A, C, E, G, I and K = 100; B, D, F, H, J and L = 50

CAPÍTULO III

Transcriptome analysis shows aluminum-tolerance pathways in *Styrax camporum*, a moderate Al-accumulator from the Cerrado vegetation

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Abstract

Styrax camporum shrubs occurs in Cerrado (Brazilian savanna), presenting high Al-accumulation and deep roots. Once reports with non-crop species are rare, the mechanisms involved in *S. camporum* high Al-tolerance are unknown. In this study, we conducted a transcriptome analysis (RNA-seq) of Al-response in root apexes. The *de novo* assembly resulted in 30021 unigenes, being 1183 differentially expressed (994 were up-regulated). Differential expression annotation reveals transporter channels ALMT (aluminum activated malate transporter), MATE (multidrug and toxic compounds exudation) and other three ABC transporter up-regulated by Al as well as enzymes from tricarboxylic acid cycle, highlighting organic acid role in Al-tolerance. Were also up-regulated some hydrolases enzymes (pectin methylesterase), genes related to oxidative stress (peroxidases) and genes involved in hormonal pathways (auxin transporters). Compared to already studied species, the overview of transcript genes in *S. camporum* roots infer that the Al-tolerance mechanisms relies on similar pathways induction.

Key words: Al^{3+}; Gene expression; ALMT, MATE

Introduction

Brazilian savanna (Cerrado) occurs in acidic soils (pH < 4) with high Al content (Haridasan 2008) and even so Cerrado native wood species are characterized by higher root growth rate (Hoffmann and Franco 2003). Styracaceae is a widely distributed family at Cerrado biome, in which *Styrax camporum* accumulate high amounts of Al (more than 1.3 mg Al per g dry leaves) (Bressan et al. 2016). Despite these intriguing features, there is a lack of information about savanna woody species related to molecular mechanisms to cope with edaphic Al.

When pH < 5 the aluminum complexed with silicon is hydrolyzed to Al^{3+}, which is toxic to most plant species (Kochian 1995). Plants sensitive to Al shows direct effects such as inhibition of root growth, that might impact crop productivity (Ryan et al. 2011).

Root growth reduction induced by Al is documented in several species, i.e., wheat (Delhaize et al. 1993), maize (Ryan *et al.*, 1993), barley (Furukawa et al. 2007), *Arabidopsis* (Hoekenga et al. 2003) and woody species (Brunner and Sperisen 2013). Decrease in root growth may be caused mainly by physic obstacle (cell wall become stiffened due to Al binding) or hormonal effects (increase in ethylene synthesis leading to auxins imbalance) (Sun et al. 2010).

On the other hand, Al-tolerant species exhibits distinct strategies to cope with such disturbance. The main mechanism of resistance consists in organic acid (malate, citrate and oxaloacetate) efflux preventing Al absorption or binding (Kochian et al. 2015). Moreover, cell wall composition in tolerant species show less negative charged carboxylic groups due to higher pectin methylation, avoiding Al binding (Horst et al. 2010).

Malate efflux is mediated by the ALMT membrane channels - aluminum activated malate transporters (Sasaki et al. 2004; Sharma et al. 2016) and a non-specific channel, that belongs to the multidrug and toxic compound exudation MATE family, operates citrate efflux (Magalhaes 2010). While ALMTs occurs only in plants (Sharma et al. 2016) MATE genes are widely distributed in bacteria, fungi and mammals besides plants (Omote et al. 2006).

Another mechanism to cope with Al disturbance relies on cell wall composition, which can interfere in Al affinity. The action of enzymes like pectin methylesterase (PME) leads to free pectic carboxylic groups, allowing Al to bind to pectic nets in the cell wall (Horst et al. 2010). Once Al binds to the cell wall, it becomes rigid, making cell elongation more difficult. Therefore, any enzyme that interfers in PME activity

could decrease Al binding in the cell wall and, consequently, enhance Al resistance (Schmohl et al. 2000).

Auxins play a central role in root growth (Cleland 2010) but its interaction with Al is still not clear. Disruption in polar auxin distribution was leading to arrest of root elongation in *Arabidopsis* (Sun et al. 2010) and on the other hand (Yang et al. 2014) found an Al-induced increased biosynthesis of indol-acetic acid (IAA) in this species root apex. Nevertheless, in roots high concentration of auxin can cause strong inhibition of cell elongation (Rahman *et al.*, 2007).

High-throughput mRNA sequencing is one efficient approach to uncover the plant transcriptome in response to Al in non-model organisms, using *de novo* transcriptome assembly, such as *Fagopyrum esculentum* (Yokosho et al. 2014), *Hydrangea macrophylla* (Chen et al. 2015) and *Urochloa decumbens* (Salgado et al. 2017). This approach is essential to discover key genes that are regulated under Al toxicity. In this study, we analyzed *S. camporum* root transcriptome in response to Al toxicity, having in mind the peculiarities from Cerrado species coping with high Al contents, revealing as far as we know the first transcriptome from Cerrado woody species.

Material and methods

Plant materials and growth conditions

This experiment followed the same rationale our group did in a previous study (Banhos et al. 2016b). Mature fruits of *Styrax camporum* Pohl were collected from adult trees growing in a Cerrado area in Brazil (37 ha; 22°15'S and 47°00'W). After germination and growth in controlled conditions, eighteen-month-old *S. camporum* plants (26 ± 1 cm in height) were kept in a hydroponic system and grown directly on an aerated nutrient solution inside opaque plastic boxes (50 cm in length x 30 cm in width x 15 cm in height; 20 L).

The nutrient solution, adapted from Clark (1975), consisted of the following macronutrients (in mM): N (NO_3^-) 0.14; N (NH_4^+) 0.06; P, 0.002; K, 0.12; Ca, 0.20; Mg, 0.05; S, 0.03; and micronutrients (in μM): Cl, 30.6; Fe (EDTA), 3.3; B, 1.2; Mn, 0.41; Zn, 0.1; Cu, 0.04; Mo, 0.044. This solution contained 0 and 740 μM Al provided using $AlCl_3$ 6 H_2O. The pH of the solution was monitored daily and maintained at 4.0 ± 0.1 to keep Al as soluble as possible, and the solution was totally replaced every 15 days.

Expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick), with five holes (2.5 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with polyurethane foam strips that were placed around the plant collar. The boxes stayed on benches (80 cm from the ground) inside a greenhouse with semi-controlled conditions (air temperature 28.5 ± 0.7°C; relative humidity 63.3 ± 1.3%; 853.4 ± 175.1 μmol photons m^{-2} s^{-1}; approximately 14h of natural photoperiod).

After 60 days, a mixture of root tips (~ 0.5 cm) were sampled in 0 and 740 μM Al conditions. The same experiment was repeated twice: the first experiment provided samples for RNA-seq and samples from the second experiment were used to validate gene expression in qRT-PCR.

RNA extraction and transcriptome analysis

Apexes were collected, frozen in liquid nitrogen and RNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Total RNA was precipitated with sodium acetate and ethanol. All samples had a A260/A280 nm ratio > 1.99. Samples were sent to transcriptome sequencing (RNA-seq) at Omega bioservices (Norcross, GA, USA).

Two samples (bulk of root apexes at 0 and 740 μM Al) were used for library construction, quality assessment (Agilent 2100 Bioanalyzer) and RNA sequencing in an Illumina HiSeq2500 equipment (100bp, pair-end sequencing). Raw reads from Illumina sequencing were submitted to quality control tests using FASTQC (v. 0.11.5) (Andrews 2010) prior analysis. GC biased regions were removed using Fastx Trimmer (Fast Tool Kit package v. 0.0.14 - McKenna et al. 2010) and filtering by size and quality using Seqyclean (v. 1.9.12) (Zhbannikov et al. 2017). Base trimming was done from the 3' and 5' end of each read to remove bases with a quality below Q30. Reads shorter than 30 bp and the remaining orphan reads were removed before further analysis. Read abundance was normalized to 20X coverage using the *in silico* normalization tool in Trinity (v. 2.4.0) (Grabherr et al., 2011) to improve assembly time and minimize memory requirements. Filtering and normalization reduced the dataset to approximately 21 million normalized read pairs. The combined reads of the libraries were assembled *de novo* using Trinity with a minimum contig length of 300 bp.

Clean reads were re-mapped in contigs using Bowtie2 (v. 2.2.6) (Langmead and Salzberg 2012) using default parameters. Transcript abundance estimation of the *de novo*-assembled transcripts was done using RNA-seq by expectation maximization

(RSEM v. 1.3.0 - Li and Dewey 2011), which allows for an assessment of transcript abundances based on the mapping of RNA-seq reads to the assembled transcriptome. Differential expression analysis using TPM (Transcripts per million - Wagner et al. 2012) was performed using NOISeq package (v. 2.14.1) (Tarazona et al. 2011), a non-parametric approach for the identification of differentially expressed genes from count data with the ability to simulate replicates.

Firstly, we tested differences in libraries size (Supplementary Figure I) and then used to determinate differentially expressed genes between the roots with no Al and roots treated with 740 µM. The differential expression between roots of *S. camporum* transcriptomic sequences was assessed by the NOISeq-sim method (Tarazona et al. 2011), implemented in the statistical language R (http://www.R-project.org) and available at http://bioinfo.cipf.es/noiseq. NOISeq-sim computes differential expression between two experimental conditions for experiments without replications. The log ratio (M) and the absolute value of difference (D) were estimated to collect the information on fold change and also the absolute pseudo-counts difference. Read counts of each library mapped to the *S. camporum de novo* assembled contigs were used for the differential expression analysis. Lists of contigs that were differentially expressed at significant level ($q = 0.9$ and $prob > 0.9$) were used for further analysis.

Unigenes functional annotation in each library was assessed using Blast2GO Pro software (Conesa et al. 2005). A BLASTX search was performed with a cutoff E-value of 1e-06 against Viridiplantae sequences from theNCBI non redundant database, followed by attribution of Gene Ontology (GO) terms, Enzyme Commission (EC), InterProScan domains and mapping of annotations to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

qRT-PCR validation

For qRT-PCR analysis, five replicates were used to quantify gene expression. Each biological replicate consisted of one *S. camporum* plant under control (0 µM Al) or Al condition (740 µM Al). Each biological sample was run in three technical replicates.

Total RNA was extracted from root samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Two micrograms of total RNA were treated with TURBO DNA-free™ (Ambion, Carlsbad, USA) and reverse transcribed using an oligo-dT primer and Super Script™ IV (Life Technologies, Carlsbad, USA), according to the

manufacturer's protocol. cDNAs were used to qRT-PCR analyses, where amplification conditions were the same of amplification efficiency tests.

We validated using quantitative PCR the transcriptional profile of four genes (Supplementary data I) comprehending the main studied responses to Al toxicity: oxidative stress (evaluating GPX gene - contig TRINITY_DN6322_c0_g1_i1), organic acid exudation (evaluating MATE gene - contig TRINITY_DN19324_c0_g1_i1), cell wall affinity to Al (evaluating PME3 gene - contig TRINITY_DN18888_c0_g1_i1) and hormonal imbalance (evaluating PIN1 gene – contig TRINITY_DN19750_c4_g4_i2). Based on RNA-seq data we designed primers for qPCR (Table 1). As reference genes we used Actin and EFα, designed for Styrax by Silva et al. (2017).

Five serial dilutions of genomic DNA from *Styrax camporum* were amplified using the internal primers in order to access the amplification efficiency during qPCR analysis. GoTaq® qPCR Master Mix with SYBR® fluorescence (Promega, Madison, USA) was used as the reagent in these tests, with the following cycle specifications: 95°C for 5 min (1 cycle), 95°C for 10 s, annealing temperature for 30 s, 72°C for 30 s (40 cycles) followed by a melting curve analysis (1% slope temperature; 60–95°C), performed in a 7500 Fast real time PCR system (Applied Biosystems, Foster City, USA). Annealing temperatures and amplification efficiencies are also presented in Table 1.

qPCR data was analyzed based on the procedure of Pfaffl (2001). Expression was normalized using the geometric mean of the RQ values of Actin and EFα.

A one-way analysis of variance (Anova) was performed between plants exposed to 0 and 740 µM Al to test for differences in length, [Al] and gene expression in roots after 60-days treatment. The Tukey test ($\alpha = 0.05$) was used to conduct post-hoc comparisons to estimate the least significant differences between mean results.

Results

Root growth

After 60 days under 740 µM Al *Styrax camporum* plants were able to keep normal root growth, despite the Al content more than four times higher when compared to 0 µM Al treatment (Table 2), evidencing its Al-tolerance feature.

De novo *assembly: overall features*

RNA-seq *S. camporum* root apexes resulted in 50264 contigs => 300bp, with average length of 1176.6 bp (Figure 1), corresponding to 30021 unigenes. From *de novo* assembly data, *Styrax camporum* sequences share higher similarity degree with *Arabidopsis thaliana*, *Vitis vinifera*, *Juglans regia* and *Theobroma cacao* (Figure 2).

Differential expression

We identified 1183 differentially expressed genes, from which 994 genes were up-regulated and 189 genes down-regulated under Al treatment.

Root apexes from a second experiment were used to confirm RNA-seq analysis. We tested four unigenes (GPX, MATE, PME3 and PIN1) that shared the same transcriptional profile in both RNA-seq and qRT-PCR analysis (Figure 5).

DEGs Annotation

Among genes up- and down-regulated by Al in *S. camporum* roots, the more enriched terms when compared to entire assembled data set were *positive regulation of glycine hydroxymethyltransferase activity* and *negative regulation of photosynthesis, light reaction* at biological processes cluster; *chloroplast photosystem II* and *ribulose bisphosphate carboxylase complex* at cellular components cluster; and *glutamate synthase (ferredoxin) activity* and *oxidoreductase activity, acting on the CH-NH2 group of donors, iron-sulfur protein as acceptor* at molecular functions cluster (Figure 3).

Using KEGG database we found 171 differentially expressed enzymes occurred in six enzyme categories (following the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), belonging majoritary to hydrolases (64 sequences found), followed by oxidoreductases and transferases (both with 57 sequences found) (Figure 4), which acts respectively in hydrolysis of chemical bonds (Enzyme Code 3), electron transference (Enzyme Code 1) and transference of particular molecules (Enzyme Code 2). Enzymes from lyase, isomerase and ligase classes were less representative, acting in breaking chemical bonds in differente ways than hydrolysis (Enzyme Code 4), converting molecule in another isomer (Enzyme Code 5), and forming new chemical bonds among molecules (Enzyme Code 6), respectively.

Discussion

While almost all crops present strong reduction in root growth under Al^{3+} conditions some species are able to cope and even accumulate large amounts of Al in roots. Besides the high Al concentration in nutrient solution (740 μM Al instead of 39 μM Al used in maize for example - Maron et al. 2008) *Styrax camporum* had normal root growth (length and biomass), inferring different pathways to deal with Al than in crop Al-sensitive species.

Rather than preventing Al entrance in plant cell, some species absorb Al from soil solution sending its complex with organic acids to aerial parts, where Al can be accumulated in leaves, for example. This Al-accumulation is well studied in Hydrangea (Negishi et al. 2012) and tea plant (Matsumoto et al. 1976), but the mechanisms involved in such accumulation are still unclear. Plants from Cerrado also present high Al leaf content, reaching surprising values as 9.8 mg Al g^{-1} dry weight in the leaves in *Qualea parviflora* (Bressan et al. 2016). Although not being characterized as hyper Al-accumulator (Haridasan 1982), *Styrax camporum* shows Al leaf content as high as 1.3 mg Al g^{-1} dry weight (Bressan et al. 2016), which also deserves highlighting.

In *Styrax camporum* roots, the transcription factors STOP1/ART1 were not affected by Al treatment. These transcription factor STOP1/ART1 are documented as highly responsive do Al stress (Iuchi et al. 2007; Yamaji et al. 2009), leading to the main responses to cope with such disturbance: induction of ALMT (aluminum activated malate transporter), ALS and MATE (multidrug and toxic compound exudation) genes, responsible for malate exudation, anions storage in vacuole and citrate exudation, respectively. Up-regulation of STOP1/ART1 transcription factors were found in transcriptome analysis of *Fagopyrum esculentum* (Yokosho et al. 2014) under Al conditions, but was not differentially expressed in *Hydrangea macrophylla* (Chen et al. 2015) for example. Our analysis suggests that, in *S. camporum*, this transcription factor family appears not to be a major factor related to the activation of Al-tolerance mechanisms.

Among classical molecular responses to Al tolerance ALMT, MATE and other three ABC transporter genes were differentially expressed in *S. camporum* roots under 740 μM Al. After 60-days treatment, Al induced ALMT transcriptional profiles (2-fold change) and reprimed in the same magnitude MATE transcriptional profile in RNA-seq analysis (Figure 5). We can infer that the organic acid exudation mechanism in *S. camporum* relies on malate efflux by ALMT channel rather than citrate by MATE transporter. In other transcriptome studies analyzing Al effects ALMT gene was

induced in wheat roots (Houde and Diallo 2008) while in common bean and aspen MATE genes were up-regulated instead (Eticha et al. 2010; Grisel et al. 2010). Organic acid efflux occurs in many Al-tolerant species, allowing malate, citrate or oxaloacetate complexation with Al^{3+} and thus preventing Al binding at cell wall or its entrance in plant cell (Ryan et al. 2011; Kochian et al. 2015).

ALS1 gene belongs to a ABC transporters class, playing a role in deposit of anions in the vacuole (Larsen et al. 2007) thus conferring Al-detoxification. Even though ALS1 orthologous only occurred among not differentially expressed genes (contig TRINITY_DN16397_c0_g1_i4 – 75% identity), in *Styrax camporum* roots three genes encoding transmembrane domain ABC transporters were up-regulated, which may contribute to hyper Al-accumulator behavior in this species.

Although organic acid exudation relies more in transmembrane channels than in organic acid synthesis (Ryan et al. 2011), several transcriptome studies observed up-regulation in some enzymes from tricarboxylic acid cycle (TCA) such as malate dehydrogenase, citrate synthase, phosphoenolpyruvate carboxylase when plants were under Al conditions. Three enzymes from TCA cycle were up-regulated in *S. camporum* roots: isocitrate dehydrogenase, ACC oxidase and malate dehydrogenase, which corroborates findings in other species such as *Fagopyrum esculentum* (Yokosho et al. 2014) and *Citrus sinensis* (Guo et al. 2017) under Al conditions.

Due to its affinity to negative charged sites, Al^{3+} may bind to cell wall making it stiffened thus avoiding normal root growth (Horst et al. 2010). Al-tolerance/sensitivity interferes in cell wall enzymes such as β-glucosidase and pectin methylesterase (PME). The β-glucosidase enzyme belongs to hydrolases group, playing a role in cellulose degradation (cell wall loosening) thus permitting its expansion (Fry 2004). In *S. camporum,* hydrolases were the enzyme group more affected by Al (Figure 4) being three glucosidase genes up-regulated in these conditions, which was also observed in transcriptome analysis from Al-tolerant wheat (Guo et al. 2007). On the other hand, cell wall pectic matrix undergoes PME action exposing free carboxylic groups which have high affinity to Al (Franco et al. 2002). In fact, *S. camporum* roots showed down-regulation in PME3 gene expression (Figure 5), the opposite behavior obtained in maize Al-sensitive lines (Maron et al. 2008), thus also indicating a cause for *S. camporum* tolerance to Al.

The oxidative stress caused by Al is well documented in several species (Richards et al. 1998). In Al-sensitive species oxidative stress can be inferred by up-regulation of genes such as glutathione transferase, ascorbate peroxidase and superoxide

dismutase (Kumari et al. 2008; Maron et al. 2008; Guo et al. 2017). Besides its Al-tolerance mechanisms as organic acid exudation and cell wall composition, *S. camporum* roots also showed up-regulation in guaiacol peroxidase (Figure 5), and superoxide dismutase enzymes which does not affect root growth (Table 2).

Root growth is mediated by hormonal balance, with auxins playing a central role in this aspect. The crosstalk between hormones and Al disturbance was elucidated in *Arabidopsis* (Sun et al. 2010), where the authors discuss that Al induces ethylene production which disrupts auxin polar flux (evidenced by up-regulation of PIN proteins), leading to decrease in root growth. Here we found that *S. camporum* roots down-regulated PIN1 transcripts (Figure 5) in response to Al, thus the opposite behavior that showed by an Al-sensitive species.

In conclusion, our RNA-Seq data analysis revealed that there are several mechanisms for Al tolerance and accumulation in *Styrax camporum*, relying mainly on organic acid synthesis and exudation, cell wall composition, oxidative stress protection and hormonal pathways that culminate in normal root growth even under high Al concentrations. When compared to already studied species, the overview of transcript genes in *S. camporum* roots infer that the Al-tolerance mechanisms relies on similar pathways induction.

Figures and tables

Table 1. Specific primers designed (based on RNAseq) for qPCR validation using *Styrax camporum* roots cDNA.

Gene ID	Primer F	Primer R	Annealing temperature	Efficiency (%)	Product size (bp)
PIN	CGCTGTTTCTCTGTCCATCA	AGGTGGGTTGGAGATGTGAG	60 °C	94.55	141
PMEI	GCACGGCTTGAATCCTAGAG	CCCTGAGTTGGTTCATTTCC	64 °C	106.44	110
GPX	GGCATCAGCAAACCTACCTC	CCTATGGTGTGTGCTCCAGA	66 °C	92.95	117
Actin	AGGAGCTGGAGACTGCAAAG	GAAGAGGACTTCTGGGCAAC	60 °C	95.06	113
EFα	GCAACCACGCCAAAGTATTC	TGTTGTACACCTCAAAACCA	60 °C	89.36	131

Table 2. Biometric parameter (root length and weight) and Al root content in *Styrax camporum* plants cultivated for 60 days in nutrient solution containing 0 and 740 µM Al.

Measurements	**0 µM Al**	**740 µM Al**
Root length (cm)	29.7	29.2
Root dry weight (g)	1.94	2.18
Al content (mg g^{-1})	865.3	4848

Table 3. Summary of clean reads from control and Al-treated *Styrax camporum* root apexes after 60-days treatment.

***Styrax camporum* root apexes bulk**	**0 µM Al**	**740 µM Al**
Number of sequences (Q30 filter)	9523028	12242698
Reads	4761514	6121349
Overall alignment rate	87.43%	86.08%

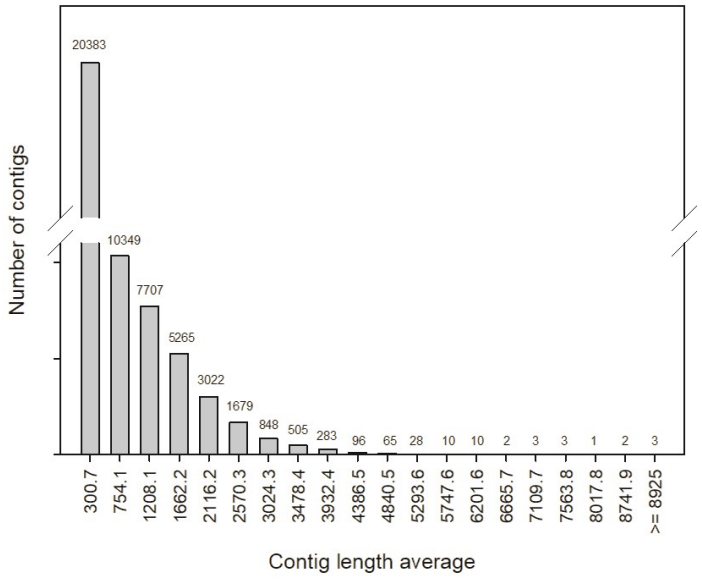


Figure 1. Distribution of contig lengths after merge two sequenced samples (control and Al treated) from *Styrax camporum* root apexes.

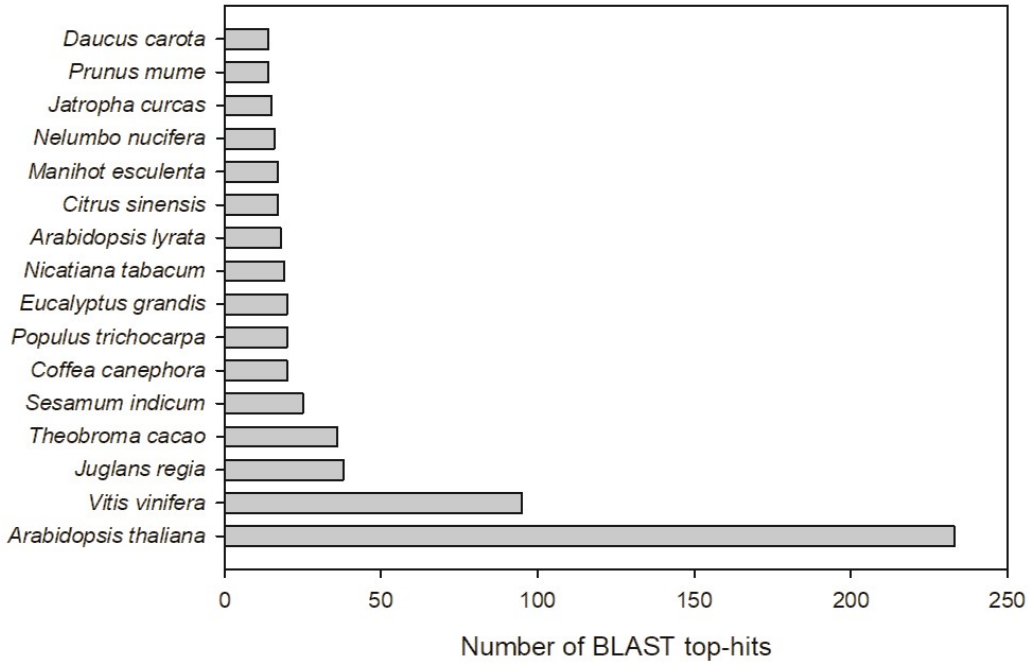


Figure 2. Best hits in species from Blast2GO software database when compared to *Styrax camporum* sequencing.

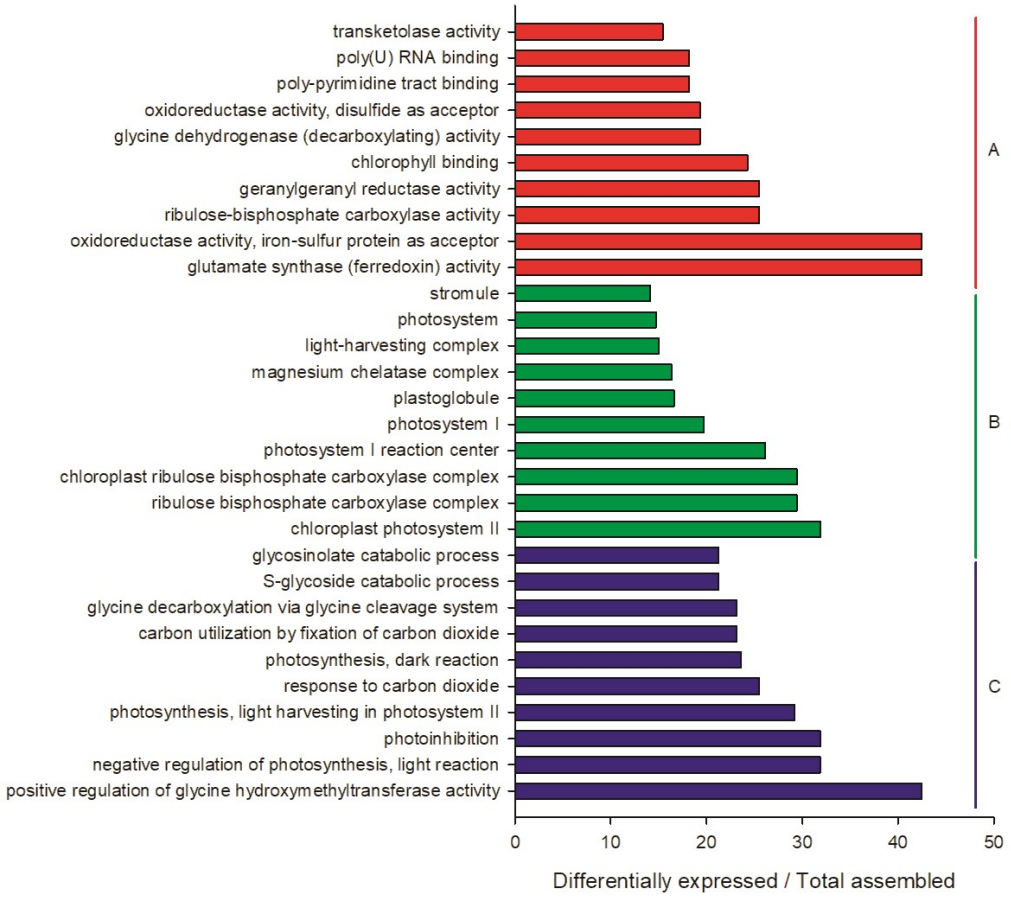


Figure 3. Top ten enriched categories in three groups: A- molecular functions, B- cell components and C- biological processes. Ratios between differential expression (DE) caused by Al and total assembled data, filtered by at least three genes at DE set.

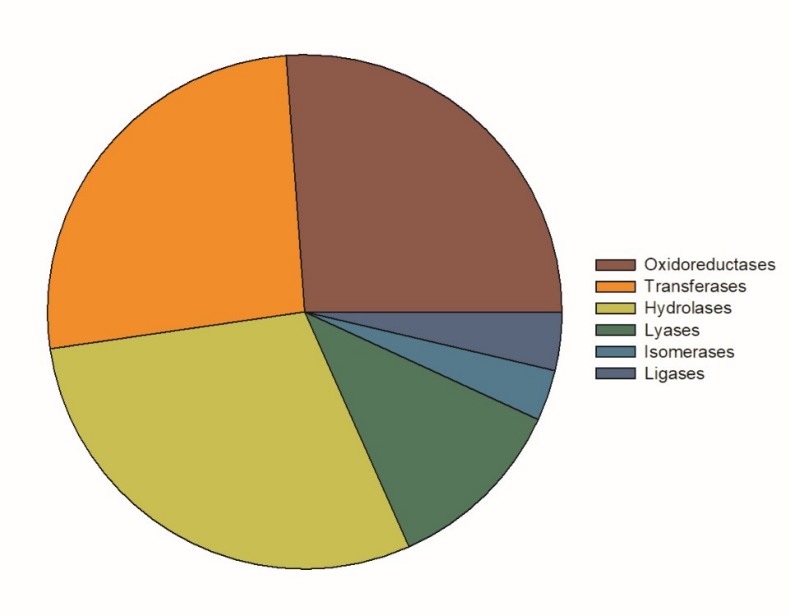


Figure 4. Enzyme code distribution in differentially expressed genes when comparing *Styrax camporum* roots at 0 and 740 µM Al.

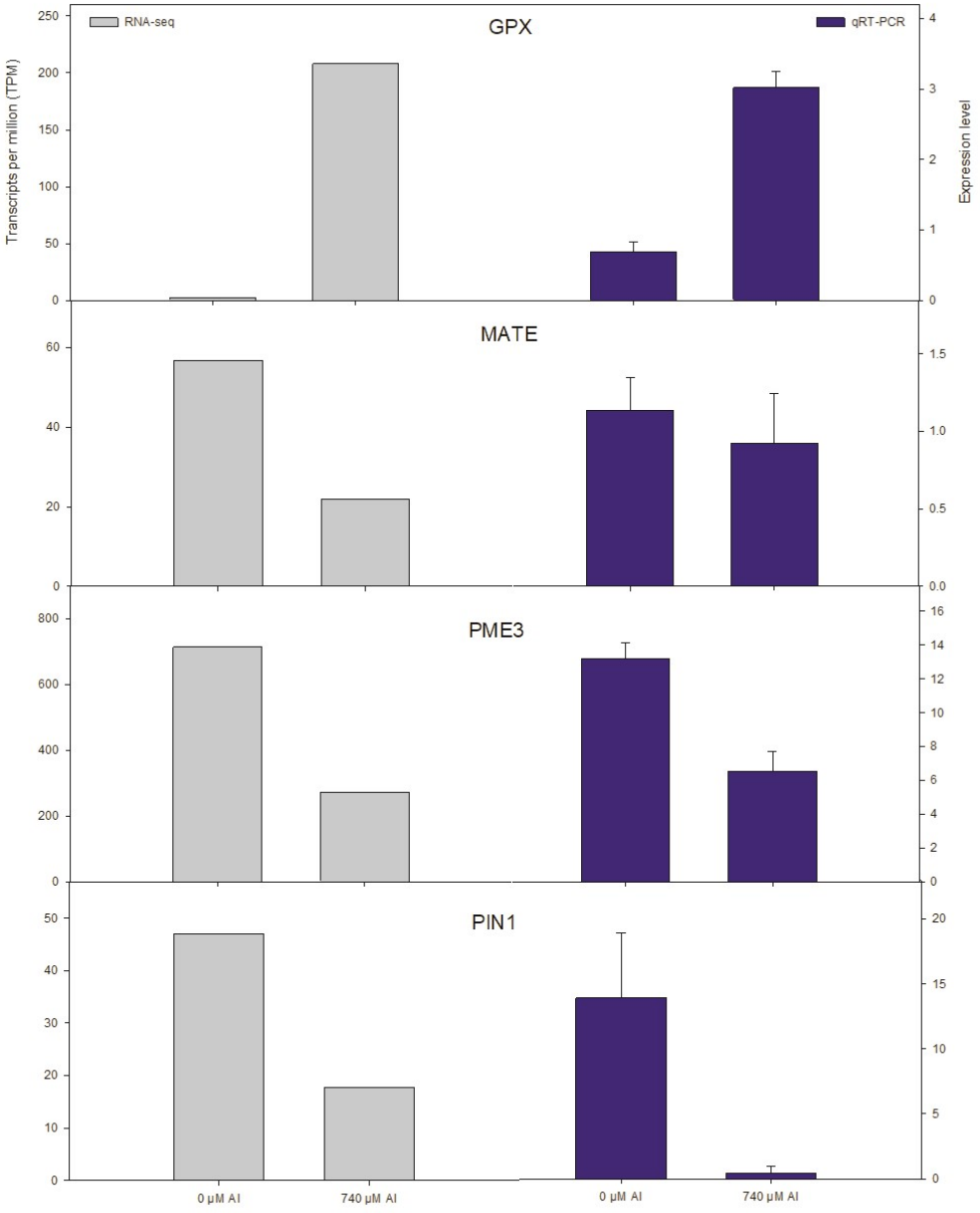
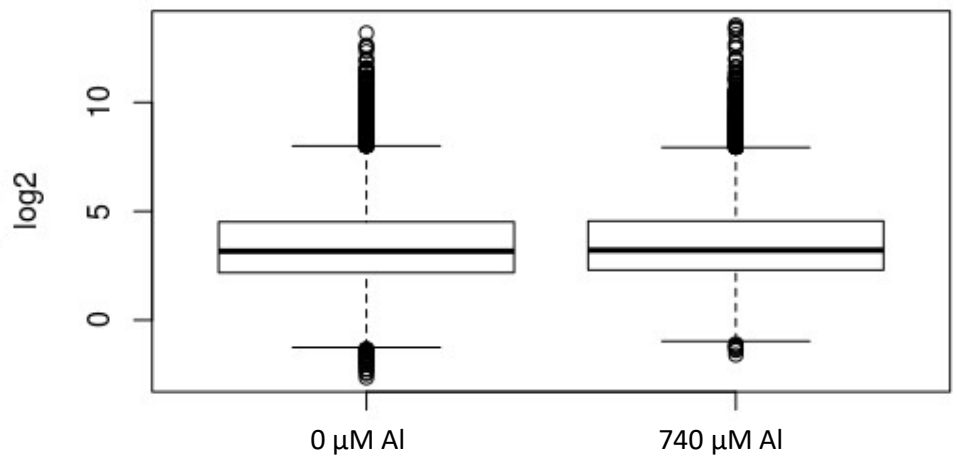


Figure 5. *Styrax camporum* GPX, MATE, PME3 and PIN1 gene expression analysis by RNA-seq (normalized by transcripts per million - TPM) and qRT-PCR after 60 days in nutritive solutions with 0 and 740 µM Al. Bars represent standard deviation after normalization by ΔΔCt method.



Supplementary Figure I. Library sizes (in log2 transcripts per million) in 0 µM Al bulk sample and 740 µM Al bulk sample, evidencing no normalization needed.

Supplementary data I. Sequences from referred contigs

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Multidrug and toxic compound exudation channel (MATE)
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Pectin methylesterase (PME3)
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CAPÍTULO IV

Styrax camporum, a moderate Al-accumulating plant, might rely on mechanisms associated with cell elongation and auxin metabolism to cope with Al

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Abstract

Occurring in acid soils with high Al^{3+} content, *Styrax camporum* accumulates aluminum (Al) in leaves with no detectable damage to its metabolism also keeping normal root growth. Despite well stablished mechanisms in Al-tolerance in crop species, there is still a lack of knowledge on Cerrado woody species reports. A 60-day study was held with *S. camporum* plants grown in a nutrient solution containing different Al concentrations. We measured plant biometric data and used root apexes to analyze expression in some Al-responsive genes and anatomic profiles. Higher Al concentration (1480 μM) triggered toxicity features leading to decrease in root growth, while at intermediate concentration (740 μM) biometric parameters were like control. Organic acid exudation by up-regulation in ALMT gene (aluminum activated malate transporter), was not enough to avoid cell wall Al binding, causing epidermis rupture at root apex. On the other hand, Al-tolerance in *Styrax camporum* roots seems to be linked with auxin-related genes as transport by PIN1 protein, inactivation metabolism by GH3 gene, and SAUR (small up-regulated RNAs) role in cell wall acidification.

Key words: Aluminum; Cerrado; Gene expression; Root growth

Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust (Von Uexküll and Mutert 1995) playing an important role in plant root decrease in acidic soils (pH < 5). Its free and toxic form (Al^{3+}) leads to considerable reduction in the root growth (Kochian et al. 2004). Studies about the mechanisms used by plants insensitive to Al (tolerant varieties and species) to cope with this metal have been widely published, but for native plants that naturally grow on soils that are acidic and rich in Al these studies are scarce.

Occurring in the Cerrado vegetation, broadly known as Brazilian savanna, *Styrax camporum* Pohl. (Styracaceae) is a tree (8-12 m in height) species widely distributed within the Cerrado physiognomies (Kissmann et al. 2012). This plant exhibits several adaptations in the aboveground organs (Habermann et al. 2011) and also belowground, such as long roots reaching deeper soil layers to survive seasonal drought (Habermann and Bressan 2011). This plant is a moderate Al-accumulating species, and it can store approximately 1500 mg Al per g dry leaves (Bressan et al. 2016). However, until now, the mechanisms by which these and other native plants from the Cerrado cope with the highly available Al in the soils of this vegetation (exchangeable Al availability ≈ 80%; see Haridasan 2008) remains unclear.

In plants that are sensitive to Al the first and conspicuous symptom of Al toxicity is the reduction in root growth (Eticha et al. 2010; Singh et al. 2017), and the Al binding to pectic nets in the root apoplast seems to be the first lesion of this metal (Kopittke et al. 2015). One of the most studied mechanisms of tolerant species to cope with Al relies on the exudation of organic acids (malate, citrate, oxalate and succinate), valid for herbaceous crop plants (Ryan et al. 2011) and woody species (Brunner and Sperisen 2013). Organic acid efflux is mediated by transmembrane channels: ALMT (aluminum activated malate transporter) responsible for malate exudation, first characterized in wheat (Sasaki et al. 2004), and MATE (multidrug and toxic compound exudation) an unspecific channel that exudes citrate responding to Al in barley (Furukawa et al. 2007).

Cell wall composition and hormonal interactions may also contribute to allow normal root growth (Brunner and Sperisen 2013). Pectin methylesterase (PME) enzymes remove methyl groups in the cell wall, presumably increasing the number of Al binding sites due to its negative charges (Horst et al. 2010; Zhu et al. 2014). This usually results in a more rigid cell wall due to the binding of Al, making root growth

more difficult. In fact, in plants sensitive to Al, the outer layers of the cortex and epidermis of root cells become more rigid due to the excess of Al in these cells causing ruptures on the epidermis (Koppittke et al., 2008; Blamey et al., 2011).

It has also been reported that Al induces ethylene biosynthesis, which in turn disturbs auxin polar transport leading to inhibition of root growth (Swarup et al. 2007; Sun et al. 2010). Therefore, the ethylene-auxin crosstalk for proper root elongation is expected to be disturbed in sensitive plants exposed to Al. Auxin polar transport by PIN proteins plays important roles in root growth (Blilou et al. 2005); GH3 gene is involved in auxin - amino acids complex, inactivating such hormone (Staswick et al. 2005); SAUR (small auxin up-regualted RNAs) allow cell wall acidification leading to cell expansion (Spartz et al. 2014). These gene can be influenced by different stress sources, thus interfering in auxin transport, availability and action.

In a previous study using one-year-old plants of *S. camporum*, we observed that 1480 μM Al in nutrient solution is toxic, causing a reduction in the shoot growth, leaf gas exchange and formation of lateral roots. Therefore, a concentration between 0 and 1480 μM Al could possibly show benefic or non-toxic effects of Al to this moderate Al-accumulating species, and this approach could be useful to study the molecular interactions of Al in the metabolism of this plant. Based on a transcriptome analysis performed in *S. camporum*, roots exhibited some differentially expressed genes that were selected to a time-course analysis (1, 15, 30 and 60 days). Then, we analyzed the transcription pattern of *ALMT*, *PIN*, *GH3*, *SAUR*, *ERF* and *PMEI* (pectin methylesterase inhibitor), as well as biometric parameters in plants submitted to 0, 740 and 1480 μM Al in nutrient solutions. We hypothesized that these genes, or at least *ALMT*, is more expressed in the presence of Al, as *S. camporum* is not a hyper-accumulator (> 1500 mg Al per kg dry leaves; Haridasan 2008). In addition, light and scanning electron microscopy analyses of roots were performed to provide evidence to support the functional analyses.

Material and methods

Plant material and experimental conditions

Ripe fruits of *Styrax camporum* were collected at a Cerradão remnant (22°15' S e 47°00' W) in the municipality of Corumbataí, São Paulo State, Brazil. After germination under controlled conditions, as suggested by (Kissmann and Habermann 2013), seedlings were transferred to greenhouse for eight months. The plants (26 ± 1 cm

in height) were then transferred to a hydroponic system and grown directly on an aerated nutrient solution inside opaque plastic boxes (50 cm in length x 30 cm in width x 15 cm in height; 20 L).

The nutrient solution was adapted from (Banhos et al. 2016b) and shows a chemical composition based on the solution proposed by Clark (1975). It consisted of the following macronutrients (in mM): NO_3^- 0.14; NH_4^+ 0.06; P 0.002; K 0.12; Ca 0.20; Mg 0.05; S 0.03; and micronutrients (in μM): Cl 30.6; Fe (EDTA) 3.3; B 1.2; Mn 0.41; Zn 0.1; Cu 0.04 and Mo 0.044. Besides macro and micronutrients, this solution contained 0, 740 and 1480 μM Al provided through $AlCl_3$ 6 H_2O. The pH of the solution was monitored daily and maintained at 4.0 ± 0.1 to keep Al as soluble as possible, and the solution was totally replaced every 15 days. The nominal chemical composition of this solution was also tested on Geochem-EZ software (Shaff et al. 2010), resulting in more than 85% free Al^{3+} available. We also measured Al in the solutions using the colorimetric method (Sarruge and Haag 1974), and nominal 740 and 1480 μM Al supply resulted in 400.2 ± 32.8 and 981.6 ± 67.9 μM Al.

Expanded polystyrene (Isopor®) 50 x 30 cm plates (2-cm thick), with eight holes (2.5 cm in diameter) each, were floated on the nutrient solution in the boxes, and the plants were fixed in these holes with polyurethane foam strips that were placed around the plant collar. The boxes stayed on benches (80 cm from the ground) inside a greenhouse with semi-controlled conditions (air temperature 28.5 ± 0.7°C; relative humidity 63.3 ± 1.3%; 853.4 ± 175.1 μmol photons m^{-2} s^{-1}; approximately 14h of natural photoperiod).

Experimental design

Using plants grown in the nutrient solutions containing 0, 740 and 1480 μM Al, we collected root tips at 1, 15, 30 and 60 days after planting (DAP) to assess the expression of some genes that had been differentially expressed and revealed by a previous transcriptome analysis performed using plants grown at 0 and 740 μM Al. These were the *ALMT* (aluminum activated malate transporter), genes related to the auxin metabolism – *PIN*, *GH3* and *SAUR*, genes related to the ethylene signaling (*ERF*), a gene related to the inhibition of the demethylesterification of pectic nets in the cell wall where Al covalently binds (Horst et al. 2010) – *PMEI*, and guaiacol peroxidase (*GPX*). *PIN1* is an auxin efflux carrier (Grieneisen et al. 2007), *GH3* promotes the auxin conjugation with amino acids, decreasing auxin availability in the metabolism (Staswick et al. 2005), and *SAUR* (small auxin up RNAs) is related to the acidification of the

apoplast (Spartz et al. 2014) for proper auxin action. *GPX* is an electron donor which may interfere in several pathways such as scavenging reactive oxygen species (ROS), lignin production and plant development (Amako et al. 1994).

We also collected root tips to perform a micromorphological analysis by SEM (scanning electron microscopy) at 1 and 60 DAP in order to observe possible damage to the epidermis of root tips of plants exposed to Al. At 60 DAP light microscopy anatomical analyses were conducted to observe the pattern of cells in root tips. At the end of the study we measured root biomass, the length of the main root and Al content in the root system.

Gene expression analysis

We used quantitative PCR to analyze the expression of seven Styrax camporum genes in roots (Table 1). As reference genes we used actin and elongation factor alpha (EFα), designed by Silva et al. (2017) for *Styrax* genus. Five replicates were used to quantify gene expression. Biological replicate consisted of one *S. camporum* individual under control (0 µM Al) or Al conditions (740 and 1480 µM Al). Each biological sample was run (qPCR) in three technical replicates.

Five serial dilutions of genomic DNA from *Styrax camporum* were amplified using the internal primers in order to access the amplification efficiency during qPCR analysis. GoTaq® qPCR Master Mix with SYBR® fluorescence (Promega, Madison, USA) was used as the reagent in these tests, with the following cycle specifications: 95°C for 5 min (1 cycle), 95°C for 10 s, annealing temperature for 30 s (Table 1), 72°C for 30 s (40 cycles) followed by a melting curve analysis (1% slope temperature; 60–95°C), performed in a 7500 Fast real time PCR system (Applied Biosystems, Foster City, USA). Amplification efficiencies are presented in Table 1.

Total RNA was extracted from root samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Two micrograms of total RNA were treated with TURBO DNA-free™ (Ambion, Carlsbad, USA) and reverse transcribed using an oligo-dT primer and Super Script™ IV (Life Technologies, Carlsbad, USA), according to the manufacturer's protocol. cDNAs were used to qRT-PCR analyses, where amplification conditions were the same of amplification efficiency tests.

qPCR data was analyzed based on the procedure of Pfaffl (2001). Expression was normalized using the geometric mean of the RQ values of actin and EFα.

Micromorphological analysis

For the scanning electron microscopy (SEM), the root segments were fixed in a 2.5% (v/v) Karnovsky (1965) solution (with 0.1 M phosphate buffer, at pH 7.3; overnight at 4 °C) and dehydrated in an increasing acetone series of 50, 70, 90, 95, and 2 × 100%, kept for 15 min in each step, before mounting these root segments on stubs. The images were obtained from a scanning electron microscope (TM 3000, Hitachi, Japan) operated at 15 kV.

Anatomical analysis

Root tips (~ 0.5 cm in length and 1 mm in diameter) were collected and immediately fixed in Karnovsky solution (Karnovsky 1965). The samples were dehydrated in increasing ethanol series [30, 50, 70, 90 (one hour each), and 100% (three times, one hour each)], then infiltrated with resin (Historesin, Leica instruments, Germany) and ethanol 100%, at a ratio of 1:1, overnight. After 24h, samples were infiltrated with pure resin, reserved overnight and then polymerized in blocks. Longitudinal sections were obtained with a rotary microtome and mounted on permanent glass slides that were immersed (5 min) in a toluidine blue solution (pH 4.5) for staining (at room temperature) structures containing nucleic acids and lignin (O'brien et al. 1964). Then, the glass slides were washed under tap water to remove excess of dye and dried with a clean cotton cloth.

All sections were observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany) and the images were captured with a digital camera (DFC-290, Leica Microsystems, Germany) functionally attached to the DMLB.

Biometric parameters

The lengths (cm) of the main root (from the plant collar to the root tip) were measured with a ruler. Then, the root sample was dried at 60°C until constant mass to obtain the root biomass (g).

Aluminum concentration in the root system

After drying and weighting the root samples, these were taken to the plant nutrition laboratory at the Campinas Agronomic Institute (IAC), Campinas, Brazil, for routine analysis of Al concentration. The samples were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, Al concentrations were determined by the atomic absorption spectrophotometer method (Sarruge and Haag 1974) and expressed as mg Al per g dry root.

Data analysis

Biometric and Al content in the roots were measured using five replications (plants). For gene expression five biological and three technical replications were used. A one-way analysis of variance (Anova) was performed to test for differences in length, dry weight, Al content and gene expression between roots of plants exposed to 0, 740 and 1480 μM Al. The Tukey test ($\alpha = 0.05$) was used to conduct post-hoc comparisons to estimate the least significant differences between mean results of the three treatments

Results

Plants exposed to 740 μM Al showed similar root length (Fig. 1A) and root biomass (Fig 1B) when compared to those not exposed to Al, and these parameters were significantly reduced in plants exposed to 1480 μM Al (Fig. 1A,B). As expected, Al contents in roots of plants exposed to both Al concentrations were approximately 10 times higher when compared to those not exposed to Al (Fig. 1C).

At 15 DAP, *ALMT* was 25 times more expressed in plants exposed to 740 μM Al when compared to those not exposed to Al, and this difference was only 10.6 times for plants exposed to 1480 μM Al (Fig. 2). On the other dates, *ALMT* was slightly down regulated, reaching its minimum response at 30 DAP in plants exposed to 1480 μM Al (4.8 times less transcription).

In relation to plants not exposed to Al, *PIN* was more expressed in plants exposed to 1480 μM Al, especially at 30 DAP (1.6 times more expressed when compared to 0 μM Al) (Fig. 3A). During the first 30 days of Al exposure, plants exposed to Al showed *GH3* expression that was not more than 1.8 times more expressed in relation to the control plants, but at 60 DAP plants exposed to 1480 μM Al showed four times more *GH3* transcripts while in plants exposed to 740 μM Al there was 16.8 times less expression of this gene (Fig. 3B). At 1 DAP, plants exposed to 740 μM Al showed 1.7 more *SAUR* transcripts in relation to plants not exposed to Al, although this response was considerably attenuated after 15 days (Fig. 3C). Plants exposed to 1480 μM Al showed down-regulation of SAUR at 1, 15 and 30 DAP (Fig. 3C).

Ethylene responsive factor (*ERF*) presented a different pattern of expression in each Al treatment. While plants exposed to 740 μM Al significantly decreased its up-regulation in relation to the control plants (8 times foldchange at 1 DAP to 2.7

foldchange down-regulation at 60 DAP), plants grown under 1480 μM Al increased its ERF expression, and reached up-regulation at 60 DAP with 4.3 times more transcripts than plants not exposed to Al (Fig. 4).

At 1 DAP, plants exposed to 740 μM Al showed pectin methyl esterase inhibitor (*PMEI*) almost 40 times more expressed in relation to the control plants, while in plants exposed to 1480 μM Al this up-regulation was only three times higher (Fig. 5). However, the high *PMEI* expression in plants exposed to 740 μM Al was considerably attenuated after 15 days, decreasing the transcription response pattern between plants exposed to both Al concentrations (Fig. 5).

Micromorphological evidence for Al damage were almost imperceptible at 1 DAP, but damage such as ruptures to the epidermis were more evident with the increasing of Al concentration in the nutrient solution as noted at 60 DAP (Fig. 6).

At 60 DAP, non-uniform flat cells with conspicuous nucleus were observed in the elongation zone of root tips of plants exposed to Al, mainly in those grown under 1480 μM Al (Fig. 7C). In these plants, the vascular cells (first vessel and sieve tube elements) could not be evidenced. The ruptures on the epidermis observed in SEM were also anatomically evidenced, with the increase of Al in the nutrient solution (Fig. 7B, C). These anatomical patterns were not observed in the control plants, and cells with regular size and rectangular shape were noted in the cortex as well as normal fusiform shape of the first vascular cells were present, showing no ruptures on the epidermis (Fig. 7A).

Discussion

A previous study conducted with adult plants from the Cerrado vegetation (Bressan et al. 2016) demonstrated that *Styrax camporum* accumulates approximately 1500 mg Al per kg dry leaves with no damage to its metabolism. However, these authors could not assess the Al content in their roots. When one-year-old plants of this species were grown in nutrient solution with 1480 μM Al, it was toxic to this species, reducing the flushing, leaf gas exchange, the functioning of the shoot apex, but the root system was less affected, even though it retained 2500 mg Al per kg root which corresponded to 70% of the plant's absorbed Al (Banhos et al. 2016b). In the present study, despite showing the same Al content in both Al treatments (Fig. 1C), when grown in 740 μM Al the root length and root biomass were not different from the control and the root was 35% longer (Fig. 1B) and had 60% more biomass (Fig. 1B) in

comparison to plants grown in 1480 μM Al (Fig. 1A). This confirms that 1480 μM Al could be toxic to this species, as pointed out by Banhos et al. (2016). In addition, there might be a specific Al availability in the soil, which is difficult to simulate in nutrient solutions, that allows this species to grow with no damage to the root system.

In the present study, we also aimed at understanding the factors that could possibly explain the increased tolerance of *S. camporum* to Al toxicity when looking at its root system. In species that are sensitive to Al, whose examples come from crop species (Ryan et al. 2011; Brunner and Sperisen 2013), the first and most conspicuous symptom is the reduction of the root growth (Horst et al. 2010; Singh et al. 2017), which can be detected within hours (Kopittke et al. 2008). In addition, it was recently evidenced that the primary lesion of Al is apoplastic, with Al being toxic by binding to the walls of outer cells, which directly inhibit their loosening in the elongation zone (Kopittke et al. 2015). It was demonstrated that 1480 μM Al impairs the formation of *lateral* roots of *S. camporum* (Banhos et al. 2016b), which occurs at the root maturation zone (Lavenus et al. 2013). Thus, the mechanism of resistance in this species could involve the protection against the binding of Al to the walls of root cells, in order to preserve the elongation zone. Notwithstanding, although *PMEI* was 40 times more expressed in plants exposed to 740 μM Al and only 4 times more expressed in those exposed to 1480 μM Al at the beginning of the study (1 DAP), the *PMEI* transcription was considerably reduced for both treatments after 15 days (Fig. 5). Moreover, the Al contents found in the roots of both treatments were similar and ten times higher than that of plants not exposed to Al (Fig. 1C). Thus, it is possible that *PMEI* transcription might have been activated at the beginning of the study in plants grown in 740 μM Al but for some reason it was not maintained and the role of this enzymatic protection against the Al binding in the apoplast of root cells merits further investigation in this species. These results corroborate the ruptures on the epidermis of plants exposed to Al, mainly in plants grown under 1480 μM Al (Fig. 6E,F, 7B,C). According to Kopittke et al. (2008) and Blamey et al. (2011), the ruptures on the epidermis of plants sensitive to Al is a result of an increase in cell wall rigidity on the epidermis and outer layers of the cortex due to more Al in these cells, as observed in barley (Ma et al. 2004) and maize (Stass et al. 2006). Therefore, the ruptures observed on the root epidermis of *S. camporum* could be related to these mechanisms proposed by Kopittke et al. (2008) and Blamey et al. (2011).

One of the most studied mechanisms to cope with Al involve the efflux of organic acids (malate, citrate, oxalate and succinate) by roots of plants that are tolerant

to this metal (Ryan et al. 2011; Singh et al. 2017). Malate efflux occurs through *ALMT* channels, whose activation can be triggered by Al within few minutes or a late efflux response due to increased *ALMT* expression. In the present study, up-regulation of *ALMT* occurred in both Al treatments, and only at 15 DAP. On the other dates, *ALMT* was slightly down regulated. This result contrasts with *ALMT* expression after 20 hours as observed in rye roots (Fontecha et al. 2007). Therefore, the putative mechanism to be present in woody plants from the Cerrado does not seem to be important in *S. camporum*. These results also agree with the fact that both Al treatments were not able to limit the Al accumulation in their roots (Fig. 1C).

Genes related to the auxin metabolism could also be involved among the mechanisms of Al resistance in *S. camporum*. Auxin distribution plays a central role in the root growth because *PIN* transporters allow symmetric distribution of auxin at the root tip (Grieneisen et al. 2007). PIN expression was up-regulated in the roots of plants exposed to 1480 µM Al mainly at 30 DAP (Fig. 3). This indicates that on this date auxin was being differentially distributed at the root tip, which corroborates impairment to root growth. In *Arabidopsis* under Al stress *PIN* expression was up-regulated, leading to disruption in IAA polar transport, what was associated with reduced root growth (Sun et al. 2010). These authors proposed that Al-induced ethylene biosynthesis is likely to act as a signal to alter auxin distribution in roots because ethylene is known to up-regulate auxin biosynthesis to enhance inhibition of root cell elongation in plants not disturbed by Al (Swarup et al. 2007). In the present study, *ERF*, a gene related to ethylene signaling (Hu et al. 2008), decreased its expression in plants exposed to 740 µM Al until 30 DAP, while in those exposed to 1480 µM Al *ERF* was up-regulated at 15 (1.4 times more expressed) and 60 DAP (4.3 times more expressed). Then, the overall up-regulation of *ERF* in plants exposed to 1480 µM could signal ethylene action more efficiently (Solano et al. 1998) and disrupt auxin distribution as evidenced by Sun et al. (2010). *GH3* was conspicuously up-regulated in the roots of plants exposed to 1480 µM Al at 60 DAP, while it was down-regulated in plants exposed to 740 µM Al (Fig. 3B). Down-regulation of *GH3* confers increased free auxin content (Staswick et al. 2005), possibly allowing the continuity of normal root growth for plants in 740 µM Al when compared to those in 1480 µM Al. In addition, plants exposed to 740 µM Al showed 2.7 more *SAUR* transcripts in relation to plants not exposed to Al, although this response was attenuated throughout the study, but plants grown in 1480 µM Al showed down-regulated SAUR expression throughout the entire study. Considering that auxin triggers transcriptional responses in few minutes (McClure et al. 1989) and that one of those

responses is mediated by *SAUR*, which acts in cell wall acidification allowing cell elongation (Hager 2003; Spartz et al. 2014), then, up-regulation of *SAUR* at the beginning of the study could contribute to the increased root length observed for plants grown in 740 μM Al when compared to those in 1480 μM Al. The above-mentioned discussion over genes related to auxin metabolism must be interpreted with care because the reasons used to corroborate the longer roots of plants grown in 740 μM Al are limited by timing, as *SAUR* interpretation of expression is in favor of these plants at the beginning of the study, *PIN* at 30 DAP and *GH3* at 60 DAP. Notwithstanding, these results are supported by the anatomical analyses. Plants exposed to 1480 μM Al showed non-uniform flat shape cells in the elongation zone evidencing abnormally elongated cells, while in plants exposed to 740 and 0 μM Al these effects were less evident (Fig. 7). Taken together, these results could suggest that the metabolism of auxin and its cellular responses (cytokinesis) is less affected by Al in *S. camporum*, as evidenced by extended damage with the increase of Al concentration in the nutrient solution.

This is the first investigation of molecular mechanisms that could explain the reduced sensitiveness to Al of *S. camporum*, a moderate Al-accumulating woody plant from the Cerrado vegetation. The results show that among possible genes involved in the Al resistance in this species, those associated with cell elongation and auxin metabolism should be better researched in future studies with Al-accumulating and non-accumulating species.

Figures and tables

Table 1. Specific primers designed for qPCR validation using *Styrax camporum* roots cDNA.

Gene	ID	Primer F	Primer R	Annealing temperature	Efficiency (%)	Product size (bp)
ALMT		AATTTCCACATCGCAACCTC	TGAGCAATGAAGCCACTGTC	60 °C	99.6	124
PIN		CGCTGTTTCTCTGTCCATCA	AGGTGGGTTGGAGATGTGAG	60 °C	94.55	141
GH3		GCCCCAACTACAACGTCAAT	TGCACCATACAAAACGAACC	60 °C	73.02	143
SAUR		CACTCCTACCTTGCGTCCTC	AACCCGAAAACAAGCAAGAA	62 °C	94.03	130
ERF		GGGGTGGTTCTGGGATTTAT	TTCATCCCTTCCCCTTTTCT	62 °C	83.75	110
PMEI		GCACGGCTTGAATCCTAGAG	CCCTGAGTTGGTTCATTTCC	64 °C	106.44	110
Actin		AGGAGCTGGAGACTGCAAAG	GAAGAGGACTTCTGGGCAAC	60 °C	95.06	113
EFα		GCAACCACGCCAAAGTATTC	TGTTGTACACCTCAAAACCA	60 °C	89.36	131

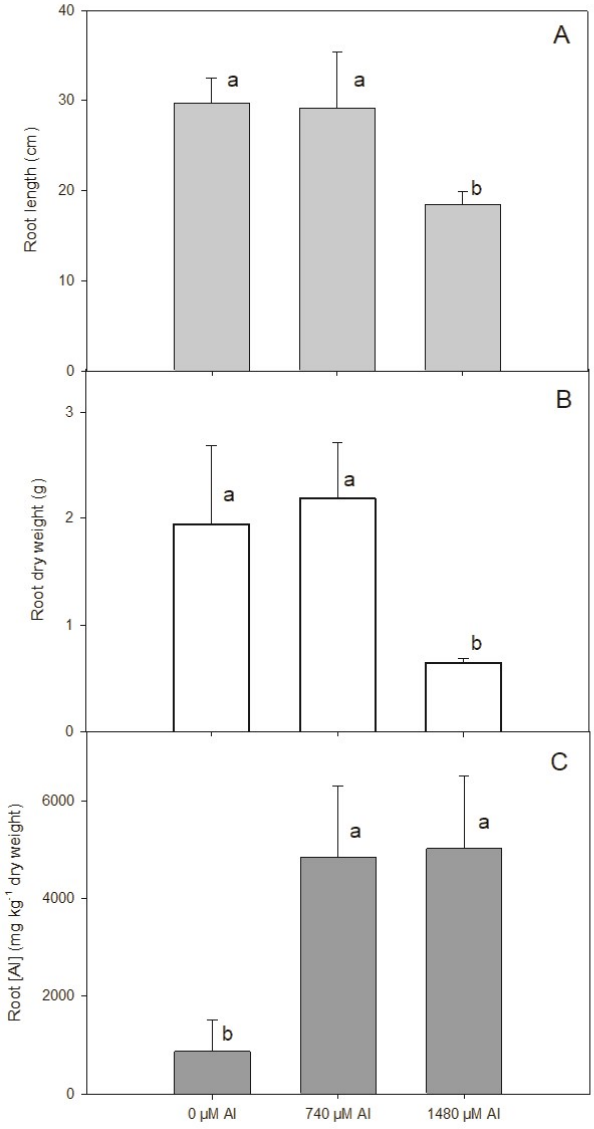

Figure 1. Length (A), dry mass (B) and Al content (C) in the roots of *Styrax camporum* after 60 days in nutrient solution containing 0, 740 and 1480 µM Al. Distinct letters indicate significant difference ($P < 0.05$) between treatments. Bars represent standard deviation.

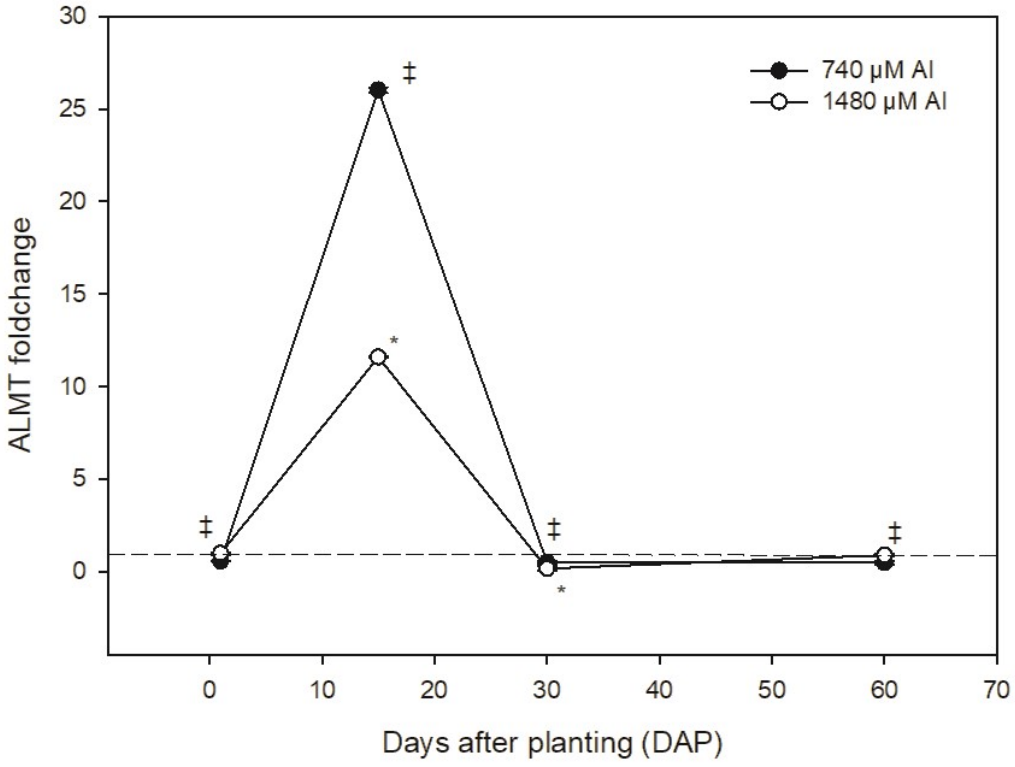

Figure 2. Gene expression of *ALMT* (aluminum activated malate transporter) in root tips of *Styrax camporum* grown in nutrient solutions containing 0, 740 and 1480 µM Al at 1, 15, 30 and 60 days after planting (DAP). For each evaluation date, double cross (‡) represent significant difference ($P < 0.05$) between 0 and 740 µM Al and asterisk, between 0 and 1480 µM Al. Dashed line is a reference (one fold change in gene expression of plants not exposed to Al)

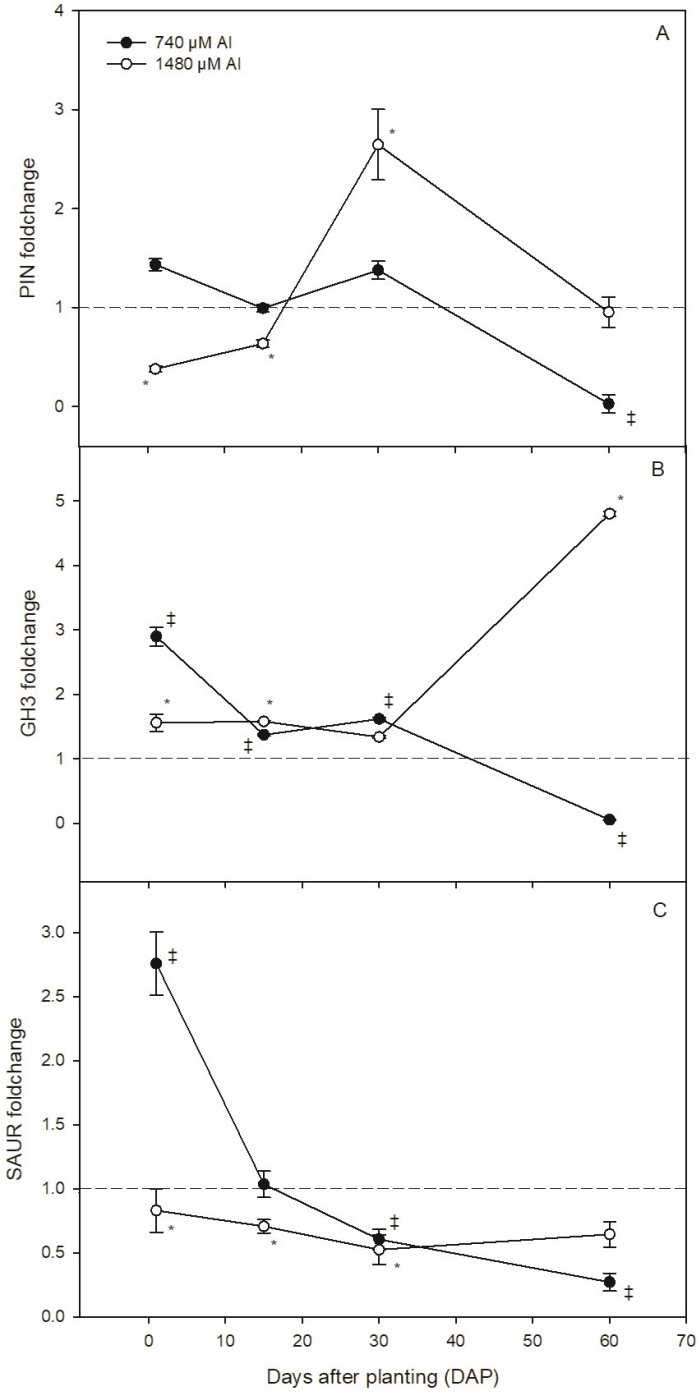

Figure 3. Gene expression related to auxins: *PIN1* - auxin efflux carrier (A), *GH3* (B) and *SAUR* – small auxin up RNAs (C) in root tips of *Styrax camporum* grown in nutrient solutions containing 0, 740 and 1480 µM Al at 1, 15, 30 and 60 days after planting (DAP). For each evaluation date, double cross (‡) represent significant difference ($P < 0.05$) between 0 and 740 µM Al and asterisk, between 0 and 1480 µM Al. Dashed line is a reference (one fold change in gene expression of plants not exposed to Al)

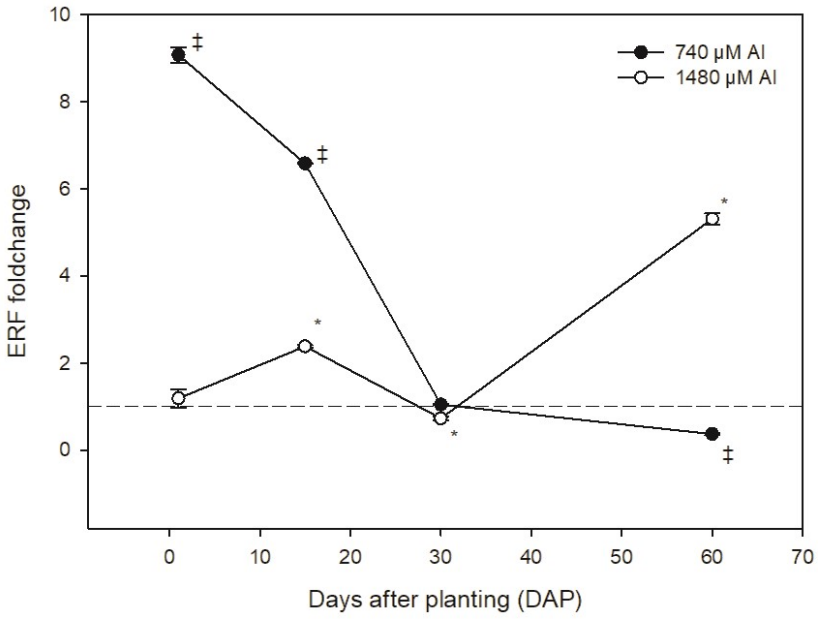

Figure 4. Gene expression of *ERF* (ethylene responsive factor) in root tips of *Styrax camporum* grown in nutrient solutions containing 0, 740 and 1480 µM Al at 1, 15, 30 and 60 days after planting (DAP). For each evaluation date, double cross (‡) represent significant difference ($P < 0.05$) between 0 and 740 µM Al and asterisk, between 0 and 1480 µM Al. Dashed line is a reference (one fold change in gene expression of plants not exposed to Al)

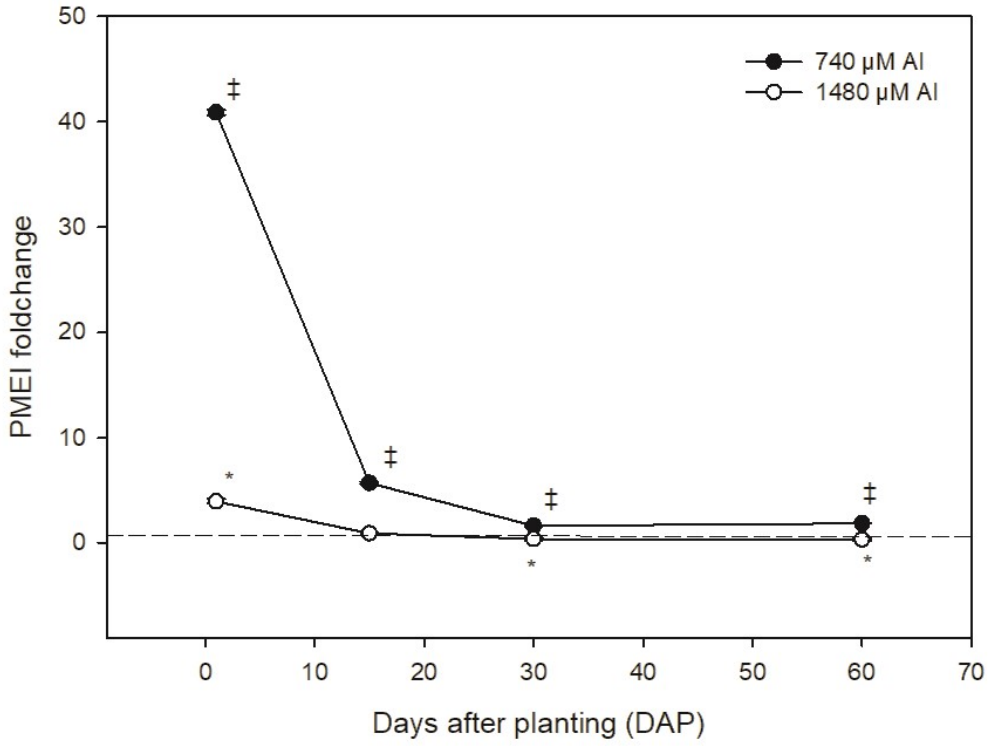

Figure 5. Gene expression of *PMEI* (pectin methyl esterase inhibitor) in root tips of *Styrax camporum* grown in nutrient solutions containing 0, 740 and 1480 µM Al at 1, 15, 30 and 60 days after planting (DAP). For each evaluation date, double cross (‡) represent significant difference ($P < 0.05$) between 0 and 740 µM Al and asterisk, between 0 and 1480 µM Al. Dashed line is a reference (one fold change in gene expression of plants not exposed to Al)

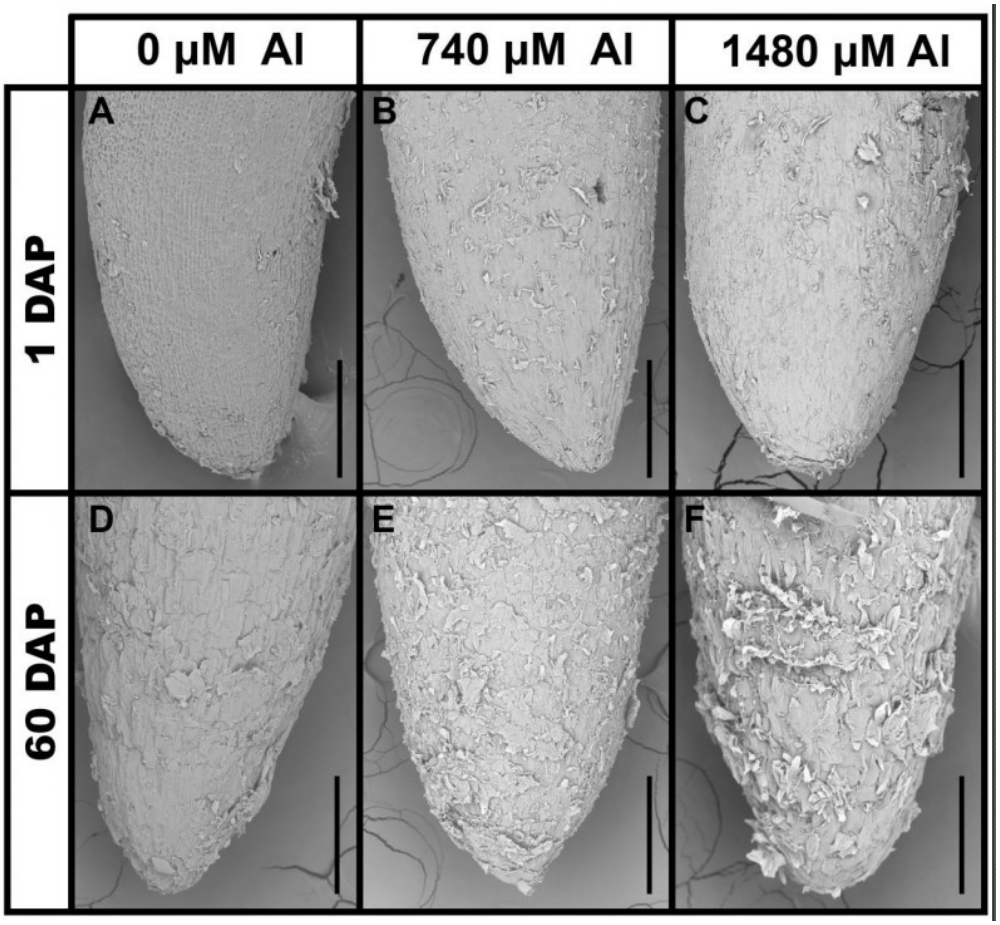

Figure 6. Micromorphological analysis by scanning electron microscopy in root tips of *Styrax camporum* grown in nutrient solutions containing 0, 740 and 1480 µM Al at 1 and 60 days after planting (DAP). Bar reference corresponds to 200 µM.

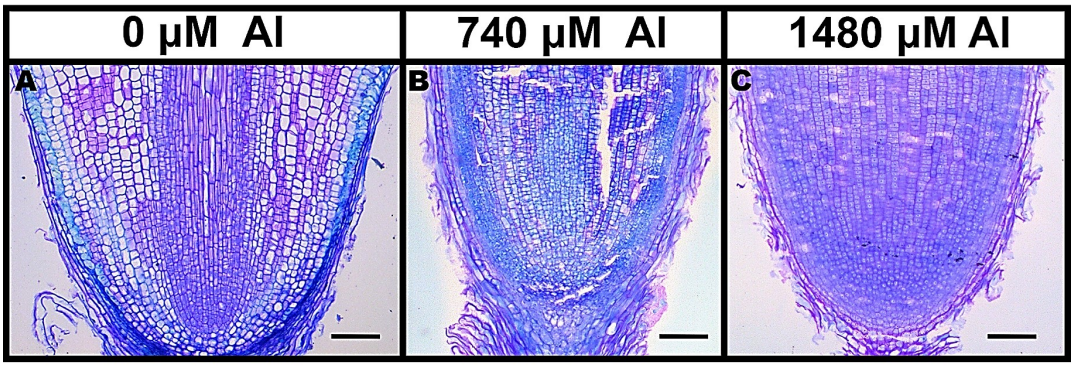

Figure 7. Anatomical analyses of root apexes of *Styrax camporum* plants. Grown for 60 days in nutrient solution containing 0 (A), 740 (B) and 1480 µM Al (C). Scale bars = 200 µm

CAPÍTULO V

Does *TaALMT1*, the major aluminium tolerance gene in wheat (*Triticum aestivum* L.), also confer tolerance to alkaline soils?

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Abstract

Both acidic and alkaline soils can constrain crop production. One of the major limitations in acid soils (pH<4.5) is the increased concentration of the toxic Al^{3+} cations. Wheat (*Triticum aestivum* L.) shows genotypic variation in Al^{3+} tolerance and the main mechanism controlling this variation is the Al^{3+}-activated efflux of malate anions from the root apices. This efflux occurs via an anion channel encoded by the gene *TaALMT1*. The higher *TaALMT1* expression in the roots apices of Al-tolerant cultivars of wheat is controlled by allelic variation in the promoter region of this gene. Recent studies have linked a tolerant-allele of *TaALMT1* with tolerance to high pH solutions and greater yields on alkaline soils than a sensitive allele. Furthermore, the TaALMT1 protein has been shown to facilitate malate efflux from wheat, tobacco (*Nicotiana tobacum* L.) suspension cells and *Xenopus* oocytes at higher pH and in the absence of Al3+ cations. The present study tested the hypothesis that an Al-tolerant allele of *TaALMT1* also confers tolerance to alkaline conditions by comparing growth of the near-isogenic wheat lines ET8 (Al^{3+}-tolerant) and ES8 (Al^{3+}-sensitive) that differ at a single genetic locus in high pH solution and in alkaline soils. We found no consistent differences in root growth between ES8 and ET8 in a range of hydroponic treatments with pH values that ranged from 4.3 to 9.1. These included specific treatments previously used to screen wheat cultivars for tolerance to high pH. Similarly, no differences in root growth were detected between the lines in four alkaline soils (pH 7.8 to 8.0) with contrasting mineralogy. Malate efflux was detected from ET8 in acidic solution with Al^{3+} but no substantial malate efflux was detected at pH 9.0 treatment with added Na_2SO_4. Results from these hydroponic experiments and soil trials are inconsistent with the hypothesis that the *TaALMT1* gene confers an advantage to wheat grown on alkaline soils.

Key words: wheat, malate, TaALMT1, aluminum, resistance, tolerance, alkaline, acidic, soil, boron

Introduction

Crop productivity can be limited by both low and high pH soils. While acid soils have pH<7.0 and alkaline soils have pH>7.0 yields crop production is generally not affected until pH is less than 5.0 or greater than 7.5. When pH is less than 4.5 aluminium is released from the aluminosilicates and other minerals such that trivalent aluminium cations (Al^{3+}) become the dominate monomeric Al species in solution (Ulrich 1986). Micromolar concentrations of Al^{3+} can rapidly inhibit root growth of wheat (Ryan et al. 1992) and the small root systems have reduced capacity for water and nutrient uptake (Foy 1984; Kochian 1995). Acid soils restrict nutrient uptake in other ways. Al^{3+} can directly inhibit the transporter absorbing K^+ and Ca^{2+} or form insoluble complexes with phosphorus which reduces its availability to plants (Piñeros and Tester 1993; Gassmann and Schroeder 1994; Zheng 2010).

Alkaline soils also limit plant growth through nutrient deficiencies and toxicities. Some micronutrients are less soluble at high pH (e.g. Fe and Mn) and the availability of other minerals is reduced through the formation of complexes. For instance, high calcium concentrations can reduce phosphate availability and HCO_3^- and clay minerals can reduced zinc availability (Adcock et al. 2007). Boron and sodium toxicities can be important constraints in some alkaline soils and HCO_3^-/ CO_3^{2-}-related stresses are also important (Millar et al. 2007).

Many plants have adapted to acidic soils by evolving mechanisms that either exclude Al^{3+} from the root cells or accumulate and sequester them in the vacuole (Ma et al. 2001; Kochian et al. 2015). A mechanism of Al exclusion now described in many species relies on the release or efflux of organic anions such as malate and citrate from the root apices (Ma et al. 2001; Delhaize et al. 2007; Ryan and Delhaize 2010; Kochian et al. 2015). These organic anions chelate Al^{3+} in the apoplast and rhizosphere and reduce its potential to damage the root tissues. Malate and citrate release occurs via membrane-bound transport proteins encoded by members of the *aluminium activated malate transporter* (*ALMT*) or the *multidrug and toxic compound exudation* (*MATE*) gene families, respectively (Sasaki et al. 2004; Delhaize et al. 2007, 2012; Furukawa et al. 2007; Magalhaes et al. 2007). In hexaploid wheat, (*Triticum aestivum* L.) Al resistance is strongly correlated with malate efflux from the root apices via an anion channel encoded by the *TaALMT1* gene on chromosome 4DL (Sasaki et al. 2004; Raman et al. 2005). *TaALMT1* expression is constitutively higher in the root apices of Al resistant cultivars compared to Al-sensitive cultivars and Al^{3+} cations are required to directly activate the TaALMT1 proteins to release malate from root apices. Al-resistant cultivars of wheat have greater *TaALMT1* expression

levels than sensitive cultivars due to the presence of repeated blocks of sequence in the *TaALMT1* promoter (Ryan et al. 2010). To date, the major variants or alleles of this region have been designated as Type I to Type VII but more are likely to exist. These alleles reflect the number and order of repeats most of which can be distinguished with genetic markers (Sasaki et al. 2006; Raman et al. 2008). In general, a greater number of repeats is associated with high *TaALMT1* expression, more malate efflux and enhanced Al resistance (Ryan et al. 2010). Most Al-sensitive cultivars of wheat have the Type I allele with no repeats in the promoter and low *TaALMT1* expression and low malate release. Many Al-resistant cultivars possess the Type V allele which has three large sequence repeats, higher *TaALMT1* expression and greater malate release than sensitive cultivars (Raman et al. 2008). ET8 and ES8 are near-isogenic wheat lines that vary in Al resistance at the *TaALMT1* locus. ES8 has the Type I allele for *TaALMT1* and ET8 has the Type V allele for *TaALMT1*.

McDonald et al. (2013) analyzed 233 trials over 12 years largely from South Australia and Western Australia to assess the contribution of various traits to the variation in grain yield. The study compiled the cultivars, average yields and rainfall events for those trials but did not include soil characteristics. They also measured aspects of root structure, nutrient efficiency and tolerance to certain abiotic stresses (salinity, high pH, Al and B) of the 52 cultivars studied and combined this information with their phenology. Among the root traits assessed, tolerance to salinity (as measured by Na^+ exclusion) was more frequently associated with high yields across all trials than any other trait. This was followed by tolerance to Al and then by tolerance to B toxicity. The authors found that cultivars tolerant to high pH were also more likely to be tolerant of B toxicity and, interestingly, cultivars that were more tolerant of B toxicity were also more likely to be resistant to Al toxicity (McDonald et al. 2013).

A similar retrospective study compared average yields from 208 wheat trials with the allelic variation in the major genes controlling phenology, plant height and Al resistance (Eagles et al. 2014). These trials were also conducted across southern Australia where top soil pH ranged from mildly acidic (pH 4.8 to 5.5) to mildly alkaline (pH 7.0 to 8.5). Over all sites and experiments no advantage to yield was detected for the Type V allele of *TaALMT1* (associated with Al resistance) compared to the Type I allele (associated with Al sensitivity) even on mildly acidic soils. This is consistent with previous studies that show that the benefit of *TaALMT1* is prominent in soil with pH<4.5 where Al^{3+} cations are prevalent (Tang et al. 2003). Unexpectedly, the Type V allele of *TaALMT1* was associated with better yields on mildly alkaline soils. Eagles et al. (2014) speculated that

the resistant allele of *TaALMT1* might either be protecting roots from the aluminate anions [$Al(OH)_4^-$] in alkaline subsoils (Brautigan et al. 2012) or enhancing nutrient availability. The latter idea assumes that TaALMT1 allows malate efflux in alkaline conditions just as it does in acidic soils and this somehow protects the roots from one or more of the stresses imposed by high pH soils.

At the time Eagles et al. (2014) published their results there was no evidence to support the idea that malate release via TaALMT1 could occur in alkaline conditions. The only function assigned to TaALMT1 was to facilitate the Al^{3+}-dependent release of malate in acidic conditions (Delhaize et al. 1993; Ryan et al. 1995; Sasaki et al. 2004). Yet soon after, further links between TaALMT1 and high-pH tolerance arose when (Ramesh et al. 2015) also concluded that ET8 wheat was more tolerant of alkaline conditions than ES8. After 20 h treatments in 1.8 mL solution (pH 9.0) ET8 seedlings grew roots and released more malate than ES8. Further experiments using *Xenopus laevis* oocytes expressing *TaALMT1* showed that malate release via TaALMT1 occurred at pH 7.5 in the absence of Al^{3+} if anions such as malate, citrate, SO_4^{2-}, Cl^-, NO_3^- or phosphate were present in the bathing media (Ramesh et al. 2015). The authors proposed that the malate efflux via TaALMT1 was balanced by proton release which decreased the apoplastic pH of ET8 roots and enhanced cell wall extension and/or nutrient acquisition. The efflux of organic anions from roots in alkaline soils can benefit plant growth under some conditions (Ström et al. 2005).

The results of Eagles et al. (2014) and Ramesh et al. (2015) are intriguing but uncertainties remain around these links between TaALMT1 function and high pH tolerance. Soil characterization in the Eagles et al. (2014) study was minimal and included only basic measurements of pH. The growth studies conducted by Ramesh et al. (2015) were short-term hydroponic experiments and none included soil. The present study used the near-isogenic wheat lines ET8 and ES8 to test the main tenets of this hypothesis in a series growth experiments in both hydroponic culture and in pot trials using a range of soils with contrasting properties.

Material and methods

Plant Material

ES8 (Al-sensitive) and ET8 (Al-tolerant) are near-isogenic lines that differ in Al tolerance at a single major locus at the *TaALMT1* gene on chromosome 4DL (Sasaki et al. 2004; Raman et al. 2005). These lines are further backcrosses derived of the ES3

and ET3 described previously (Delhaize et al. 1993). ES8 possesses the Type I allele of *TaALMT1* (*TaALMT1*-I) which is associated with low levels of *TaALMT1* expression in the root apices and low malate efflux. ET8 possesses the Type V allele of the *TaALMT1* (*TaALMT1-V*) which is associated with high levels of *TaALMT1* expression in root apices and greater malate efflux (Sasaki et al. 2006; Ryan et al. 2010).

Soil experiments

Growth was measured in six contrasting soils (Table 1). The Robertson soil (pH = 4.08; -34.596444, 150.605952) was acidic, Al-toxic and highly phosphorus P-fixing. An additional treatment with added KH_2PO_4 to raise the Colwell P of this soil from 70 to 239 mg kg^{-1} was included. This higher Colwell P was still P-limiting for wheat in longer-term growth studies (Liao et al. 2008). The Wallaroo soil was an acidic but non-Al toxic soil (pH = 5.39; -35.170848, 149.053645). The four alkaline soils were collected from the Eyre Peninsula (pH = 7.82; -33.1424, 134.6956), Streaky Bay (pH = 7.67; -32.847505, 134.386523), Mallala (pH = 7.95; -34.375834, 138.458392) and Redhill (pH = 8.02; -33.540905, 138.195753). Moisture content of the Robertson, Wallaroo, Eyre Peninsula and Streaky Bay soils were adjusted to 80% field capacity and placed in pots (20 cm high x 8.5 cm diameter). The growth experiments were conducted in a growth cabinet (day/night was 22ºC /18ºC for 16 h/8 h) or glasshouse (22ºC /18ºC) with natural light over 10 days. The Mallala and Redhill soils had high clay contents and prone to cracking. These soils were mixed with 20% coarse sand, adjusted to moisture contents of 50% and 60% field capacity, respectively, and placed in pots (19 cm high and 6.3 cm diameter). Experiments using these soils were run for seven days.

Hydroponic experiments

ET8 and ES8 grain were weighed (usually 45-55 mg) and imbibed on moist filter paper at 4 °C for two days and then germinated at room temperature for two days. The seedlings were planted in aerated hydroponic tanks or in various soils. Hydroponics experiments used aerated 20 L tanks (50 cm length x 30 cm width x 15 cm height) placed in growth chambers (16 h/8 h day/night at 22/18 ºC for) or in a glasshouse with natural light. Two basal hydroponic solutions were used that were modified by altering the pH and adding additional salts. Hydroponic solution 1 consisted of 500 µM $CaCl_2$, 150 µM $MgSO_4$, 500 µM KNO_3, 2 µM FeEDTA, 500 µM NH_4NO_3, 10 µM KH_2PO_4, 11 µM H_3BO_3, 2 µM $MnCl_2$, 0.35 µM $ZnSO_4$ and 0.2 µM $CuCl_2$. Various buffers

including 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or bis-Tris propane (BTP) were added to the basal solution along with a variety of salts including Na_2CO_3, $NaHCO_3$, Na_2SO_4 or Na_2SO_4. The pH of the solutions was adjusted as described in the text.

Hydroponic solution 2 was used previously to score wheat cultivars for tolerance to high pH (Millar et al. 2007; McDonald et al. 2013). The control solution comprised 0.5 mM $Ca(NO_3)_2$, 15 µM H_3BO_3 and 0.2 µM $ZnSO_4$ (pH 6). The high pH treatment also included 5 mM $NaHCO_3$, 1 mM Na_2CO_3 and 5 mM $CaCO_3$ (pH 9.1). The high pH solution was aerated with CO_2-free air by using a 10 M KOH trap and treatments were monitored daily for pH. Net root growth was compared in the control and high pH solutions after 10 days.

Measurements

In the hydroponic experiments the longest root, total root length or root biomass were measured. At the end of the experiment, roots and shoots were excised. Roots were scanned using WinRhizo™ to estimate total root length and then roots and shoots were dried at 70°C for two days and weighed. For harvesting soil-grown plants, the roots were washed out and scanned for total root length and then roots and shoots were dried and weighed. Relative root length (relative shoot biomass etc) were calculated as root length (shoot biomass) measured at the treatment pH relative to the control treatment.

Malate Efflux

Malate efflux was measured from excised root apices or from sterile seedlings grown in flasks as described previously (Ryan et al. 1995; Pereira et al. 2010). The high pH treatment included 10 mM Na_2SO_4 unbuffered at pH 9.0. Malate in the solutions was estimated with an enzymatic assay (Delhaize et al. 1993).

Boron tolerance

Root and shoot growth of ES8 and ET8 were measured after growth in 20 L hydroponic basal solution 1 with various H_3BO_3 concentrations. The first experiment measured growth after seven days in 0, 2.5 or 5 mM H_3BO_3 (pH 6.0) with 16 seedlings per treatment. The second experiment measured growth after 20 days in 0 or 4 mM H_3BO_3 (pH 6.0) using 10 to 13 replicate seedlings per treatment. Roots were either

scanned for total root length or dried with the shoots at 70^{o}C for two days and weighed to measure biomass.

Statistical analysis

Data from total root length, root and shoot dry weight were subjected to Student's t-test (P = 0.05). To test for statistical differences in relative root length (relative shoot biomass etc) between ES8 and ET8 we used an adapted statistical analysis described previously (Zhou et al. 2013).

Results

Soil experiments

Growth experiments were performed with soils collected from New South Wales and South Australia. Characteristics of these soils are summarized in Table 1. The first experiment compared root growth of ES8 and ET8 in soil collected from Robertson and Wallaroo. The Robertson soil was an acidic ferrosol (pH 4.1) with high levels of exchangeable Al while Wallaroo soil (pH 5.4) was low in exchangeable Al. Since Robertson soil also had a high phosphate buffering capacity (~1000 mg kg^{-1}) we included an extra treatment with added phosphorus. After 10 days growth in Robertson soil, with or without added P, total root length of ET8 was two to three-fold greater than ES8 which is consistent with their contrasting resistance to Al toxicity (Figure 1a). Root biomass did not differ between ET8 and ES8 in Robertson soil (Figure 1b) which is consistent with previous reports showing that while Al inhibits root elongation the roots become shorter and thicker. No differences in shoot biomass were detected between ET8 and ES8 (Figure 1c) which was also similar to previous reports. In Wallaroo soil (pH 5.4) total root growth, biomass and shoot biomass were the same for ET8 and ES8 (Figure 1a, b, c). These results demonstrate that root growth of ET8 was greater than ES8 in an acid soil containing toxic concentrations of Al. These differences were not observed in a pH 5.4 soil without Al toxicity.

A series of experiments then compared ES8 and ET8 growth in four contrasting alkaline soils from South Australia with pH values ranging from 7.7 to 8.0. These experiments measured final total root length, root biomass and shoot biomass. In all four soils no differences were detected between ES8 and ET8 for any parameter (Figure 2). Trials with the Mallala and Redhill soils were conducted over seven days while those with Streaky Bay or Eyre Peninsular were ten days long.

Hydroponic experiments

We first confirmed that the batches of ET8 and ES8 grain differed in Al resistance by growing seedlings for five days in hydroponics with and without 10 μM $AlCl_3$ (pH 4.3). ET8 and ES8 plants had similar net root growth in the control treatment but, in the presence of Al, root growth of ES8 was significantly inhibited whereas ET8 was unaffected (Figure 3). Relative root length (RRL) of ET8 was nine-fold greater than ES8.

Growth of ET8 and ES8 was then compared in a series of hydroponic treatments with various added salts and buffers to adjust pH. These treatments were designed to reflect published reports and mimic conditions in some alkaline soils. Some experiments measured net growth of the longest root while others measured total root length as mentioned in each case. Also included are calculations of relative root length or relative total root length to account for any variation in seed stocks. Relative values for each genotype are defined as the measurement in a high pH treatment compared to the value in a control, low pH treatment.

The first experiment measured root growth in hydroponic solution 1 amended with different anions and buffers. The control solution was pH 4.3 (no Al) while the high pH treatments used 5 mM $NaHCO_3$ to adjust pH to 8.1 or BTP adjusted to pH 9.5 (Figure 4 a,b). Root growth of ET8 tended to be greater than ES8 in both the pH 4.3 control solution and pH 8.1 solutions but the difference was significantly greater in the pH 8.1 treatment only. However relative root length did not differ between ET8 and ES8.

The second series of experiments used the hydroponic solution 1 (pH 6.0) as the control and a series of treatments with added Na_2CO_3, $NaHCO_3$ or Na_2SO_4 and buffers all at pH 8.1 (Figure 4 c,d; Figure 5). Individual treatments did show a significant difference in root growth between ET8 and ES8 for the control, unbuffered, and 2 mM Na_2SO_4 treatments but for relative root length the only statistically significant difference was for the 1 mM Na_2CO_3 treatment where ES8 was greater than ET8.

A third set of hydroponic experiments followed the method used previously to score wheat cultivars for tolerance to high pH (Millar et al. 2007; McDonald et al. 2013). The control treatment in this experiment was adjusted to pH 7.0 and the high pH treatment included a mixture of HCO_3^- and CO_3^{2-} (pH 9.1) aerated with CO_2-free air to minimize pH changes. This experiment was performed twice: one lasting for five days (Figure 6 a,b) and the other for ten days (Figure 6c-f). Measurements were made of the

longest root in the first experiment but the second experiment measured the longest root and total root growth. No differences in root length or total root length were detected between ET8 and ES8 for either experiment and, correspondingly, no differences in relative growth were detected in these measurements.

Malate efflux at high pH

Malate release was measured from excised root apices of ET8 and ES8 seedlings. Efflux occurred over one hour in a simple solution (0.2 mM $CaCl_2$, pH 4.5), with and without addition of 100 μM $AlCl_3$, or in 0.2 mM $CaCl_2$ and 10 mM Na_2SO_4 adjusted to pH 9.1 with NaOH. Malate efflux from ET8 was the same as ES8 for all treatments except for pH 4.5 with Al^{3+} where efflux from ET8 was four-fold greater than ES8. This is consistent with previous studies comparing Al-resistant and sensitive genotypes of wheat (Delhaize et al. 1993; Ryan et al. 1995) (Figure 7).

ET8 and ES8 responses to B toxicity

McDonald et al. (2013) reported that cultivars resistant to Al toxicity were also more likely to be tolerant to B toxicity. We tested whether the higher expression of *TaALMT1* in ET8 provided any protection from B toxicity by measuring root and shoot growth in various H_3BO_3 treatments. In the first experiment total root length and final shoot biomass were measured after 12 days in 0, 2.5 and 5.0 mM H_3BO_3 (Figure 8a-d). In a second experiment total root biomass and shoot biomass were measured after 21 days in 0 and 5 mM H_3BO_3 (Figure 8e-h). Growth was significantly inhibited at all B levels with the highest concentration reducing root growth to ~40% of controls. No significant differences in total root length, root biomass were detected between ET8 and ES8 in either experiment. Similarly no differences between ES8 and ET8 were detected in shoot biomass in either experiment. The results indicate that in short-term growth assays ET8 and ES8 do not differ in tolerance to B stress.

Discussion

In a study of 208 wheat trials largely covering southern Australia, Eagles et al. (2014) concluded that wheat with the Type V promoter allele of the *TaALMT1* gene (*TaALMT1-V*) tended to perform better on mildly alkaline soils than wheat with the Type I promoter allele (*TaALMT1-I*). In other words, cultivars that were tolerant of acid soils tended to yield better on mildly alkaline soils than cultivars that were sensitive to acid soils.

The inference was that *TaALMT1*, the major gene controlling tolerance to acid soils, was protecting plants from one or more of the stresses present in high pH soils. Until this study, the only function assigned to the TaALMT1 anion channel was to facilitate the Al^{3+}-dependent release of malate from roots. Therefore, the finding of Eagles et al. (2014) was unexpected since Al^{3+} cations occur in acid soils, not alkaline soils. For this idea to have any veracity TaALMT1 would have to function at high pH and in the absence of Al^{3+}.

Support for the conclusion of Eagles et al. (2014) was provided by Ramesh et al. (2015) who independently concluded that ET8 was more tolerant of alkaline conditions than ES8. Ramesh et al. (2015) compared net root growth in young ES8 and ET8 seedlings over 20 h in solutions containing 3 mM $CaCl_2$, 5 mM MES/BTP and 10 mM SO_4^{2-} (pH 9.0). Further experiments with seedlings as well as heterologous expression systems (tobacco suspension cells and *Xenopus* oocytes) demonstrated that malate efflux via TaALMT1 occurred at pH > 7.5 if 10 mM concentrations of organic or inorganic anions were added to the external media (Ramesh et al. 2015). The model they proposed suggested that protons released with the malate lowered the apoplastic pH of the root cells which facilitated cell wall loosening and cell elongation.

However Eagles et al. (2014) compared wheat varieties and did not use near-isogenic lines while Ramesh et al. (2015) did not perform soil trials. The present study tested the main tenets of this hypothesis using the near-isogenic wheat lines ET8 and ES8. These require that the Al-resistant ET8 grows better than ES8 in high pH solution and alkaline soil and that ET8 releases malate at high pH in the absence of Al^{3+}.

We confirmed that our stocks of ET8 and ES8 differed in Al^{3+} resistance both in acid soil and hydroponic experiments. The only difference in root growth between ET8 and ES8 in the soil experiments occurred in an acid ferrosol that contained high levels of exchangeable Al. No differences in root growth were detected in four other alkaline soils of contrasting mineralogy. In hydroponic experiments, large differences in root growth between ES8 and ET8 occurred in an acidic solution with toxic levels of Al^{3+}. No consistent differences in root growth were detected in any of the hydroponic treatments ranging from pH 8.0 to 9.1. This included specific solutions previously used to screen wheat cultivars for tolerance to high pH. Malate efflux was measured from ET8 in acidic solution with Al^{3+} but no substantial malate efflux was detected in another treatment at pH 9.1.

From their survey of wheat yields over southern Australia, McDonald et al. (2013) concluded that cultivars tolerant of Al toxicity were no more likely to be tolerant of high pH. This not support the model presented by Eagles et al. (2014) but it is consistent with

other information. For example Krichauff, one of the most successful wheat cultivars in the highly-alkaline soils of South Australia, has the Type I promoter allele for *TaALMT1* and is sensitive to Al toxicity (Millar et al. 2007; Raman et al. 2008). This suggests that TaALMT1 is unlikely to be a major contributor to high pH tolerance or, at least, is not an important source for breeding purposes.

Eagles et al. (2014) noted that in their analysis the soils in their survey could have been characterized more thoroughly. Of particular importance here is pH because only general categories were provided for pH in the topsoil (mildly acidic, neutral, mildly alkaline). Eagles et al. (2014) argued that many soils in the "mildly alkaline" category could have been much more alkaline at depth. They proposed that the *TaALMT1-V* could have been protecting plants from the aluminate anion, $Al(OH)_4^-$, which is more prevalent at high pH and potentially toxic to plants (Brautigan et al. 2012). However no mechanism was provided as to how malate efflux via TaALMT1 could afford protection from aluminate. It could be equally argued that a mildly alkaline topsoil could overlay a more acidic subsoil. The application of lime to correct sub-soil acidity is now becoming routine and since lime can take years to move down the profile, especially in minimal till production systems, it is common for soils to have a much higher pH in the topsoil than at depth (Helyar 1991; Dolling et al. 2001). Therefore it is possible that, for a subset of the mildly alkaline soils, *TaALMT1–V* was protecting plants from subsoil acidity. Admittedly, if this was occurring we would have also expected *TaALMT1-V* to provide a yield advantage in mildly acidic soils for the same reasons, but this was not observed (Eagles et al. 2014).

McDonald et al. (2013) concluded that the genotypic variation in high pH tolerance was relatively small among the cultivars they tested and that tolerance to high pH *per se* was less important than the other sub-soil limitations that occur on alkaline soils such as salinity and B toxicity. Approximately 30% of cropping soils in South Australia have B concentrations above the recommended levels of 15 mg/kg soil (Government of South Australia, Department of Environment and Natural Resources 2004). Indeed, McDonald et al. (2013) found that cultivars tolerant of B toxicity were more likely to be tolerant of high pH and more resistant to Al toxicity. Whether this association is related by a common physiological function, genetically linked traits or just co-incidental, is unclear.

Tolerance to B toxicity in plants generally depends on excluding B from the roots and shoots. An important mechanism of B tolerance in barley relies on the efflux of borate anions from root cells via an anion channel encoded by *HvBOT1* on chromosome 4H (Reid 2007; Sutton et al. 2007). Several QTL for B tolerance in wheat are associated with superior yields in the high-B soils of southern Australia (Paull et al.

1991; Nable et al. 1997; Jefferies et al. 2000). The two major QTL on chromosome 7BL (*Bo1*) and on chromosome 4AL (*Bo4*) contain genes that encode anion channels similar to *HvBOT1* and presumably function in the same way - even though they are not direct orthologues of the barley gene (Pallotta et al. 2014).

Interestingly, a link between ALMT transporters and B tolerance was reported recently in rice. Over-expression of the rice *OsALMT4* gene enhanced the B tolerance of the transgenic lines (Liu et al. 2017) and the authors proposed that OsALMT4 might be facilitating the efflux of borate anions in a similar manner as the BOT1 proteins in barley and wheat. Those findings and the study of McDonald et al. (2013) prompted us to directly compare the B tolerance of ET8 and ES8 to determine whether different alleles of *TaALMT1* could explain the observation of Eagles et al. (2014). However we found no significant differences in root or shoot biomass between ET8 and ES8 grow in toxic concentrations of B. This indicates that TaALMT1 does not substantially contribute to B tolerance, at least in these short-term growth assays.

Conclusions

We performed a series of short-term growth trials to test the conclusions of Eagles et al. (2014) and Ramesh et al. (2015) that the Al resistance gene *TaALMT1* also improves growth and performance at high pH. We found no consistent evidence that ET8 or ES8 differed in their tolerance to alkaline soils, high pH solutions or to B toxicity. We could not corroborate previous reports that malate efflux occurs via TaALMT1 under alkaline conditions. Future work should test these findings with field trials on a range of alkaline soils.

Figures and tables

Table 1. Chemical analysis and fertility parameters of the soil types used in the experiments.

	Soil types						
	Robertson (NSW)	**Robertson +P (NSW)**	**Wallaroo (NSW)**	**Streaky Bay (SA)**	**Eyre Pen. (SA)**	**Mallala (SA)**	**Redhill (SA)**
pH ($CaCl_2$)	4.1	4.2	5.4	7.7	7.8	8.0	8.0
C (%)	5.23	5.58	1.17	4.44	4.16	1.48	0.770
N (%)	0.365	0.394	0.100	0.205	0.174	0.0625	0.0495
Extr S (mg/kg)	27.2	20.9	16.3	10.0	17.3	43.9	67.0
B (mg/kg)	0.921	1.030	0.244	2.98	5.71	4.24	18.4
Exch Al (cmol+/kg)	3.85	3.09	0.018	0.014	0.013	0.014	0.009
Cu (mg/kg)	0.459	0.682	0.508	0.102	0.114	3.09	3.10
Fe (mg/kg)	32.8	41.5	61.8	4.27	4.45	6.83	10.0
Mn (mg/kg)	3.82	31.0	21.4	5.57	4.95	13.4	17.8
Zn (mg/kg)	0.18	0.49	0.93	0.56	1.05	0.83	1.29
Exch Ca (cmol+/kg)	0.73	1.26	3.70	24.0	27.4	26.4	18.9
Exch K (cmol+/kg)	0.655	1.90	0.706	1.14	2.86	1.17	1.06
Exch Mg (cmol+/kg)	0.230	0.489	0.540	2.19	3.95	8.35	5.44
Exch Na (cmol+/kg)	0.087	0.121	0.085	0.164	0.945	3.081	3.298
Extr K (mg/kg)	673	357	489	213	376	362	856
Cl (mg/kg)	6	8	10	11	42	197	156
Amm-N (mg/kg)	9.77	4.14	39.7	1.97	6.94	4.56	3.88
Nitrate N (mg/kg)	9.09	158	4.6	99.2	44.2	9.91	13.1
Colwell P (mg/kg)	70.6	239	45.0	100	59.3	12.5	23.1
Olsen P (mg/kg)	13	50	18	22	12	2	7
PBI unadj (mg/kg)	1067	881	41.2	182	210	160	63
PBIcol (mg/kg)	1143	1094	48.9	208	226	163	67

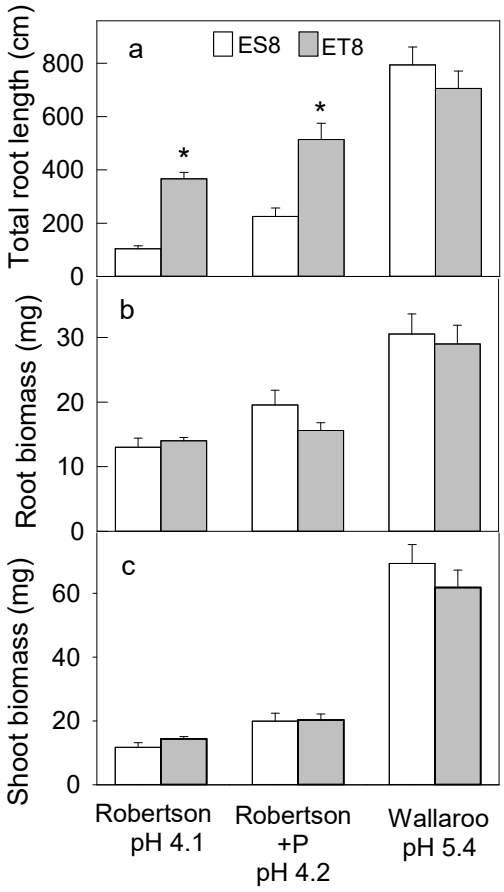

Figure 1 Root and shoot growth of ET8 and ES8 in acidic soils. Final root length (a), root biomass (b) and shoot biomass (c) of ET8 and ES8 plants was measured after 10 days growth in the Robertson and Wallaroo soils. Data show the mean and SE (n=6-8). An asterisk indicates a significant difference between ES8 and ET8 for that soil ($P<0.05$).

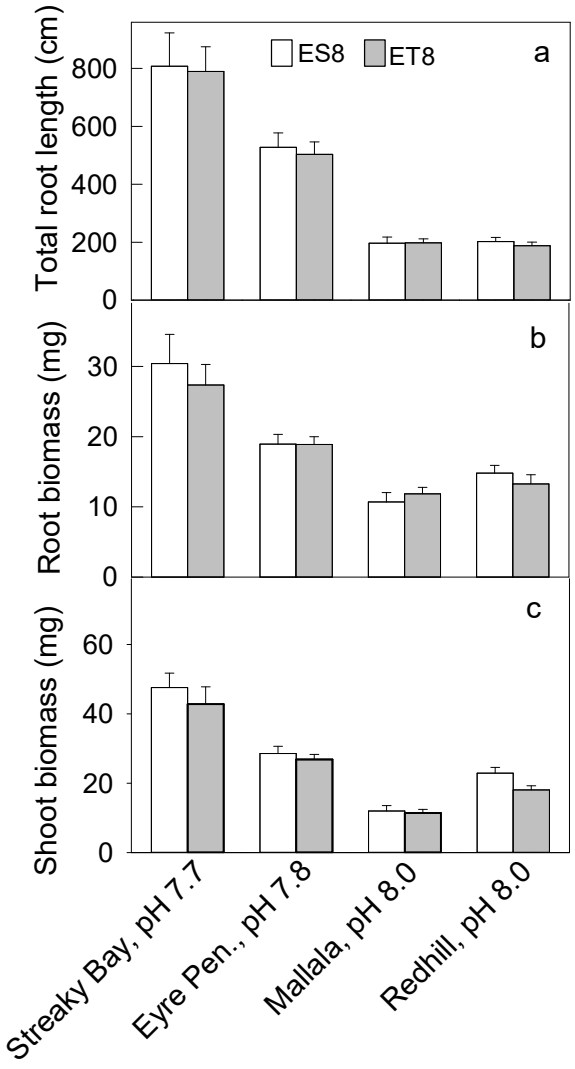

Figure 2 Root and shoot growth of ET8 and ES8 in alkaline soils. Total root length (a), root biomass (b) and shoot biomass (c) of ET8 and ES8 plants was measured after 10 days growth in the Streaky Bay and Eyre Peninsula soils and after seven days in the Mallala and Redhill soils. Data show the mean and SE (n=6-8). Absence of an asterisk indicates no significant differences between ES8 and ET8 for each soil ($P>0.05$).

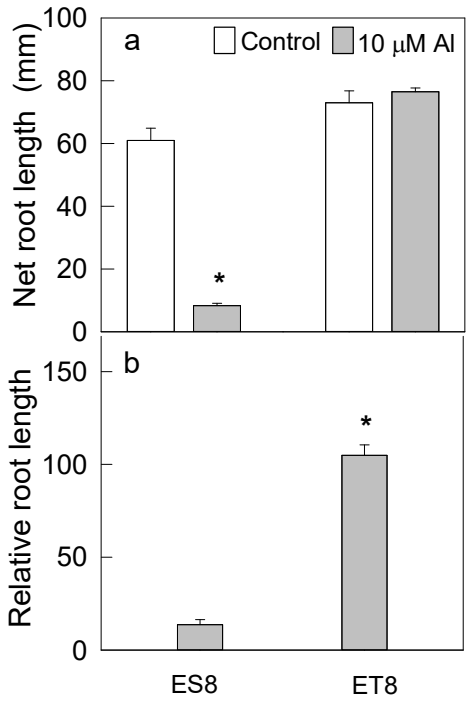

Figure 3. Comparing the Al^{3+} tolerance of ET8 and ES8. (a) Net root length (net growth of longest root) and (b) relative root length of ES8 and ET8 after five days growth in hydroponic solution 1 (unbuffered) with and without 10 μM $AlCl_3$ (pH 4.3). Data show mean and SE (n=4). Error bars on the data in b are corrected SE. An asterisk in (a) indicates a significant difference between treatments for each line, whereas an asterisk in (b) indicates a significant difference between ES8 and ET8 using a Students t test ($P<0.05$).

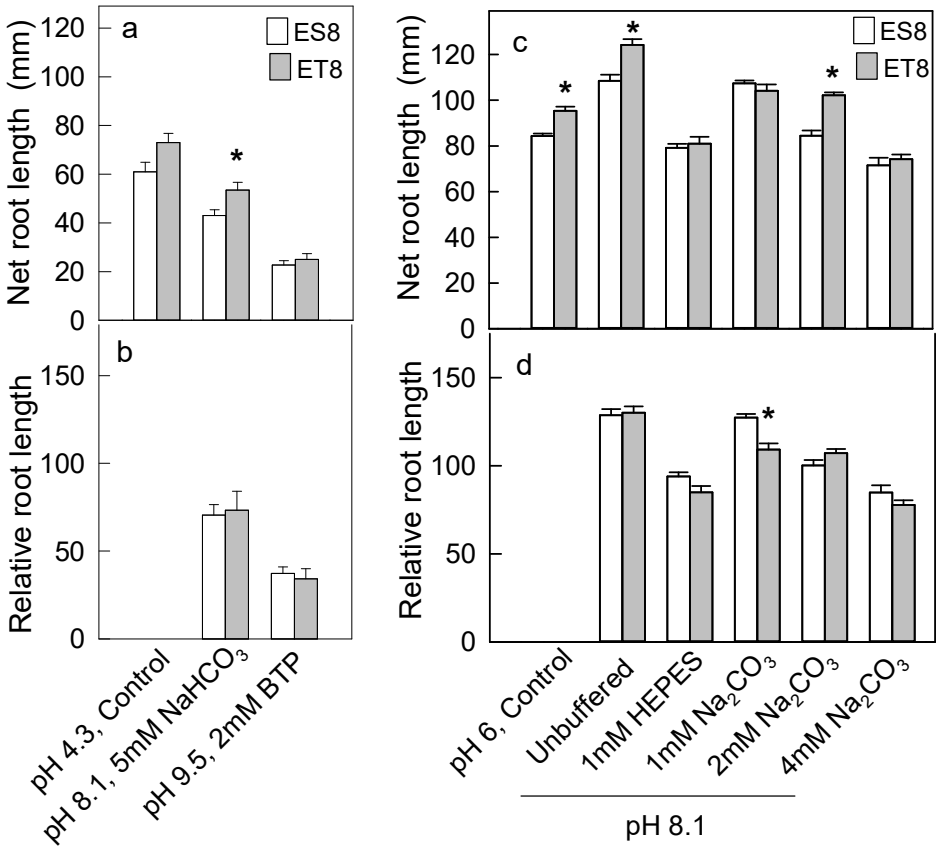

Figure 4. Comparing root growth of ET8 and ES8 in various treatments. Experiment 1 shows net root length (net growth of longest root) in three treatments (a) and relative root length (b) compared to growth in the control (pH 4.3). Experiment 2 shows net root length in six treatments (c) and relative root length (d) compared to the control (pH 6.0). Measurements were collected after five days in hydroponic solution 1 amended as shown. Data show mean and SE (n= 4 to 6). Error bars on the data in b and d are corrected SE. An asterisk indicates a significant difference between ES8 and ET8 using a Students t test ($P<0.05$).

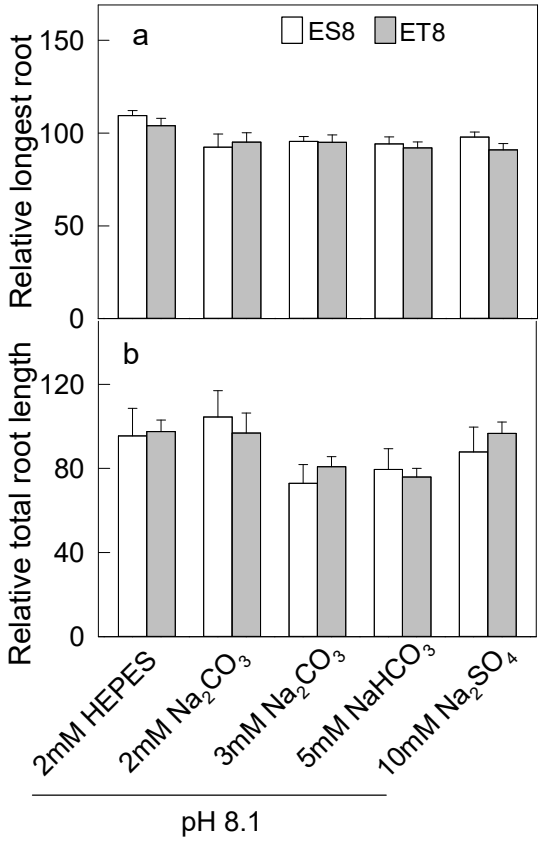

Figure 5. Root growth of ET8 and ES8 at high pH. Relative longest root and relative total root length of ET8 and ES8 lines after five days growth in hydroponic solution 1 with added treatments as shown. The control treatment was adjusted to pH 6.0. Data show mean with corrected SE (n= 5 to 6).

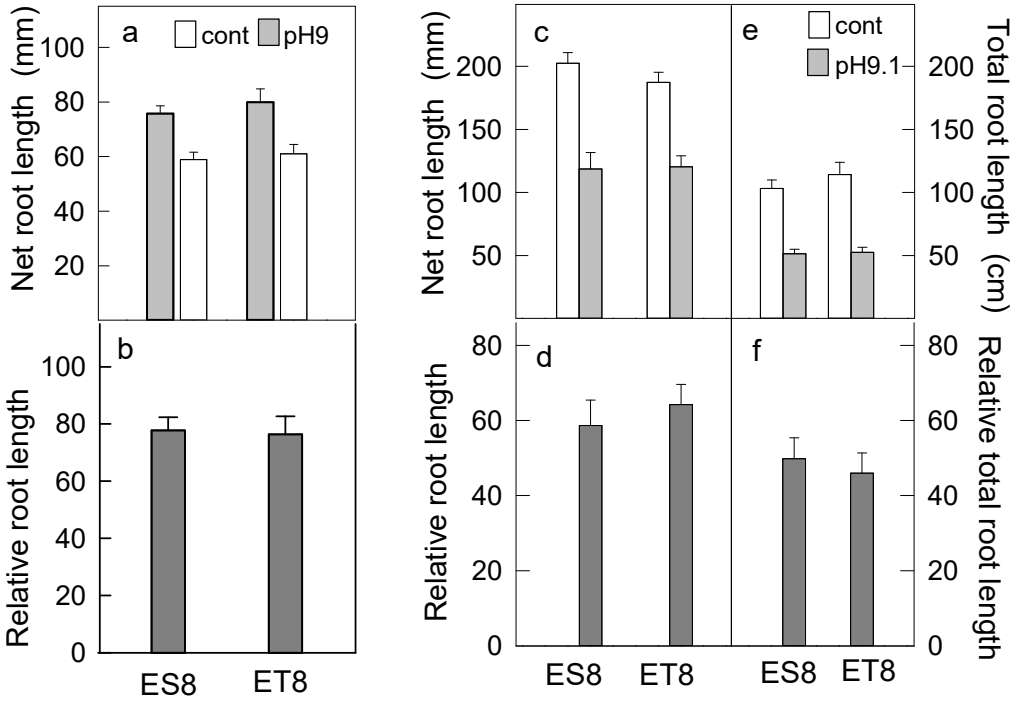

Figure 6 Root growth of ET8 and ES8 using hydroponic solution 2. Root growth of ET8 and ES8 were compared in hydroponic solution 2 as described by McDonald *et al*. (2013). Experiment 1 (a, b) compared root length (net growth of longest root) and relative root length after five days. Experiment 2 (c-f) compared root length (c) and total root length (e), as well as relative root length (d) and relative total root growth (f) after 10 days. The control solution (cont) was adjusted to pH 7 and the high pH solution was ~pH 9.1. Mean and SE (n=9 -10) and the absence of asterisks means no significant difference between lines ES8 and ET8 (P 0.05). Error bars on the data in b, d and f are corrected SE.

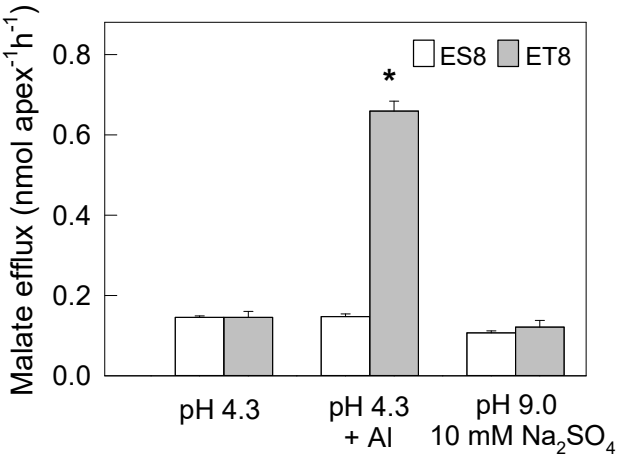

Figure 7 Malate efflux from the roots of ET8 and ES8. Malate efflux from the excised root apices of ES8 and ET8 seedlings treated with 0.2 mM $CaCl_2$ with and without 100 µM $AlCl_3$ (pH 4.3) or 0.2 mM $CaCl_2$ with 10 mM Na_2SO_4 (pH 9.0, unbuffered). Data show the mean and SE (n=5). An asterisk indicates a significant difference between ES8 and ET8 (P>0.05).

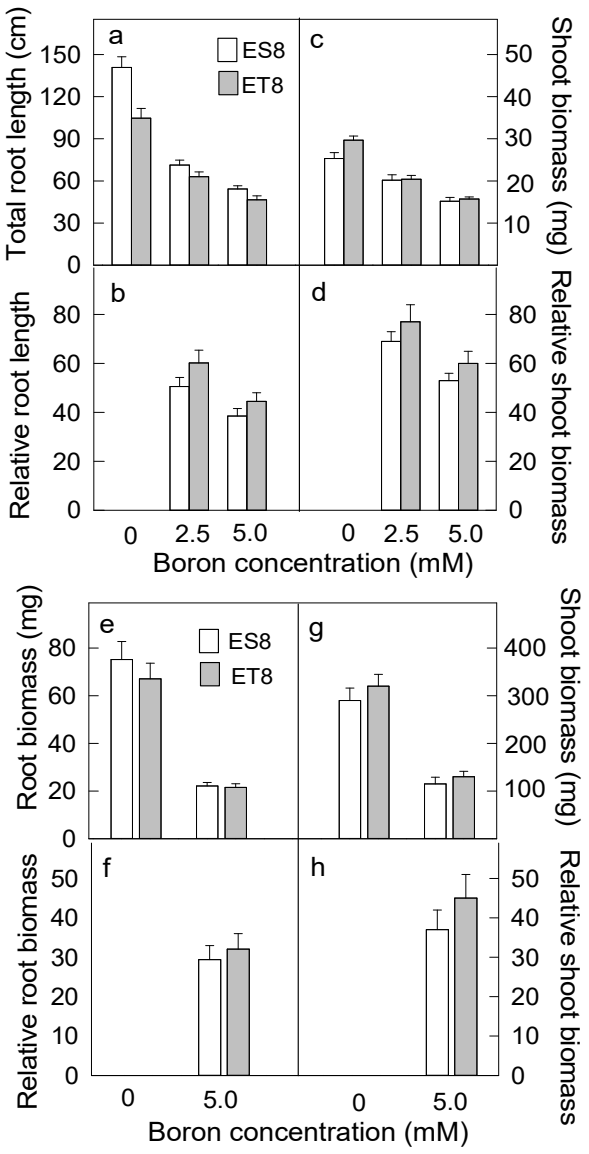

Figure 8 Comparing the B tolerance of ET8 and ES8. Results from two experiments are shown. Experiment 1 (a-d) shows total root length and shoot biomass after seven days growth in hydroponic solution 1 with 0, 2.5 or 5 mM H_3BO_3 (pH 6.0) Also shown are relative total root growth and relative shoot biomass. Data show the mean and SE (n=16). Experiment 2 (e-h) shows root and shoot biomass after 20 days growth in hydroponic solution 1 with 0 or 4 mM H_3BO_3. Also shown are relative root and shoot biomass. Data show the mean and SE (n=10 to 13). Error bars on the data in b, d, f and h are corrected SE. Absence of an asterisk indicates no significant differences between ES8 and ET8 for each hydroponic solution ($P>0.05$).

Conclusões

Diante das discussões baseadas em experimentos realizados com *Citrus limonia* e *Styrax camporum* frente à toxicidade do Al, bem como uma análise de funcionamento de um dos canais mais importante da tolerância ao Al (*TaALMT)* conclui-se que:

i) Apesar de considerada uma espécie sensível, o transcriptoma de *C. limonia* exibe genes de tolerância ao Al como a indução de expressão no canal MATE (responsável pela exsudação de citrato);
ii) Mecanismos de tolerância ao Al ocorrem de forma desorganizada com o passar dos dias de experimento. Além disso, a diminuição na concentração de etileno e o aumento nos níveis de auxina contribuem significativamente para redução no crescimento radicular de *C. limonia* a 1480 µM Al;
iii) O transcriptoma de *Styrax camporum* revelou indução de genes de tolerância ao Al em diversas vias, como por exemplo para exsudação de ácido orgânico (ALMT, efluxo de malato);
iv) De acordo com o tempo de tratamento a resposta de tolerância ao Al dá-se preferencialmente em uma via. Além disso, de modo geral em *S. camporum* mostra-se tolerante ao Al no nível de 740 µM Al enquanto que a 1480 µM Al as respostas indicam suscetibilidade;
v) O canal *TaALMT1* atua na exsudação de malato unicamente em condições ácidas e na presença de Al, amenizando o estresse causado pelo Al^{3+};

Assim, em determinadas concentrações o Al^{3+} pode beneficiar o desenvolvimento radicular das espécies do Cerrado, mas quando o teor de Al é mais elevado (1480 µM) o crescimento radicular é reduzido tanto em *C. limonia* quanto em *S. camporum*.

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