Gene expression profiling in maize roots under aluminum stress

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Abstract

To investigate the molecular mechanisms of Al toxicity, cross-species cDNA array approach was employed to identify expressed sequence tags (ESTs) regulated by Al stress in root tips of Al-tolerant maize (*Zea mays*) genotype Cat100-6 and Al-sensitive genotype S1587-17. Due to the high degree of conservation observed between sugarcane and maize, we have analyzed the expression profiling of maize genes using 2 304 sugarcane (ESTs) obtained from different libraries. We have identified 85 ESTs in Al stressed maize root tips with significantly altered expression. Among the up-regulated ESTs, we have found genes encoding previously identified proteins induced by Al stress, such as phenyl ammonia-lyase, chitinase, Bowman-Birk proteinase inhibitor, and wali7. In addition, several novel genes up- and down-regulated by Al stress were identified in both genotypes.

Additional key words: abiotic-stress, Al-sensitive, Al-tolerant, heterologous-hybridization, *Saccharum* sp., *Zea mays.*

Introduction

Aluminum (Al) toxicity is one of the major limiting factors to plant growth in acid soils. The most dramatic symptom of Al toxicity is the inhibition of root growth, which has become a widely accepted measure of Al stress in maize (Magnavaca *et al*. 1987, Cançado *et al*. 1999). Although Al toxicity primarily restricts root growth, a myriad of different symptoms appear in both roots and shoots that are often mistaken with soil nutrient deficiencies (Foy *et al.* 1978). At the cellular level, Al has been shown to affect lipid peroxidation (Yamamoto *et al.* 2001), inositol 1,4,5-triphosphate signaling transduction (Jones and Kochian 1995), cytoplasmic calcium homeostasis (Zhang and Rengel 1999), microtubules and actin organization in cell elongation (Blancaflor *et al.* 1998), and callose formation and deposition (Horst *et al.* 1997).

Although Al is responsible for promoting serious metabolic dysfunctions, some plants have evolved Al tolerance mechanisms that enable them to grow in acid soils with toxic concentrations of Al ions (for a review see, Ma *et al.* 2001, Kochian *et al.* 2004). Therefore, a comprehensive understanding of the molecular mechanisms underlying Al toxicity and tolerance in plants could provide important insights into the development of new cultivars with improved Al stress tolerance.

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Abbreviations: ABA - abscisic acid, *Amp*^{r} - ampicilin resistance gene, BLAST - basic local alignment search tool, CAD - cinnamyl alcohol dehydrogenase, CV - coeficient variation, DAHP - 3-deoxy-D-arabino-heptulosonate 7-phosphate, dATP - 2'-deoxy-adenosine 5'-triphosphate, dCTP - 2'-deoxycytidine 5'-triphosphate, DEPC - diethylpyrocarbonate, dGTP - 2'-deoxiguanosina 5'-tri-phosphate, dTTP - 2'-deoxitimine 5'-triphosphate, EDTA - ethylenediaminetetracetic acid, E-value - expectation value, maize GDB - maize genetics and genomics database, MDRH - monodehydroascorbate reductase, PAL - phenylalanine ammonia-lyase, PAM - percent accepted mutation, TCA - tricarboxylic acid, TMB - thiol-monophosphate biosynthesis.
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Al toxicity can promote profound changes in gene expression, altering the control of normal physiological processes (Kochian *et al*. 2004). It has been repeatedly observed that Al affects the expression of several genes, including those encoding pathogen-, wounding-, and oxidative stress-induced proteins (Cruz-Ortega and Ownby 1993, Snowden and Gardner 1993, Snowden *et al.* 1995, Cruz-Ortega *et al.* 1997, Hamel *et al.* 1998, Richards *et al*. 1998, Ezaki *et al.* 2000, Watt 2003, Xiao *et al*. 2005). These studies have been restricted to wheat and *Arabidopsis*. In maize, a crop with a wide range of Al tolerant germplasms, little is known about the Al stress-regulated gene expression, except for a gene encoding a glutathione S-transferase recently identified (Cançado *et al*. 2005).

The chelation of Al anions by organic acid molecules, the most accepted Al tolerance mechanism in plants, is still controversial in maize. Pellet *et al*. (1995) and Jorge and Arruda (1997) working with a reduced number of maize genotypes, showed that the amount of citrate released by roots exposed to same Al concentration are higher in Al-tolerant genotypes than in Al-sensitive genotypes. However, citrate released by roots exposed to Al was observed either in Al-tolerant or in Al-sensitive genotypes (Piñeros *et al*. 2005). Possibly there is more than one mechanism involved with Al tolerance in maize. Recently, Tamás *et al*. (2006) observed that small increases in pH promoted by barley seedlings cultivated on filter paper were able to decrease the Al toxicity.

Colinearity and synteny between related species have been largely studied through comparative mapping, specifically within the *Poaceae* family that contain many of the more important cereal crops. Comparative maps among different grass species demonstrate that the information from one species can be extrapolated to other ones, for breeding, ecology, evolution and molecular biology purposes (Guimarães *et al*. 1997). Several authors showed that the degree of cross-hybridization between maize and sugarcane oscillated from 68 to 97 % (D'Hont *et al*. 1994, Da Silva *et al*. 1993, Grivet *et al*. 1996, Asnaghi *et al*. 2000), indicating that sugarcane and maize could benefit from comparative analyses. Thus, the close genetic relationship between maize and sugarcane (Bennetzen and Freeling 1997, Draye *et al*. 2001, Kellogg 2001) prompted us to use cDNA arrays filters containing sugarcane ESTs to investigate gene expression in roots of two maize inbreed lines (Cat100-6 and S1587-17) cultivated in nutrient solution with different levels of Al stress.

Materials and methods

Plants and treatments: Two non-isogenic maize (*Zea mays* L.) lines, Cat100-6 and S1587-17, rated as Al-tolerant and Al-sensitive due its root growth in nutrient solution with Al (Moon *et al.* 1997), were obtained from the germplasm collection of the Universidade Estadual de Campinas, Campinas, Brazil. Sugarcane (*Saccharum* sp.) genotypes, growth conditions, and the SUCEST (Sugarcane EST project, http://sucestfun.cbmeg.unicamp.br/sucestfun/) EST libraries are described in Vettore *et al*. (2001, 2003).

Maize seeds were surface-sterilized with 70 % (v/v) ethanol for 1 min, 0.5 % (v/v) sodium hypochloride for 20 min, rinsed four times with sterile water and germinated at 28 ^{o}C between two layers of moist filter paper for 3 d. Seedlings were grown in nutrient solution at pH 4.2 continuously aerated as described in Jorge *et al.* (2001) and under 16-h photoperiod at irradiance of 80 to 100 μmol m^{-2} s^{-1} and temperatures of 26 ^{o}C during the day and 20 ^{o}C at night. The relative growth rate (RGR) was calculated as the percentage of the root length increase of Al treated seedlings relative to the root length increase of Al untreated seedlings (during 24 h) as described in Moon *et al.* (1997).

In cDNA array experiments, 3-d-old seedlings of Cat100-6 and S1587-17 were exposed for 24 h to 0 (control), 75 and 283 μM of $AlK_3(SO_4)_3$, corresponding to 15 and 50 μM of Al^{3+} activity calculated using *GEOCHEM-PC 2.0* (Parker *et al*. 1995). The same concentrations of Al were used for the RNA-gel blot analyses, except for the sugarcane EST-probed RNA-gel blots. These blots also included two additional Al doses: 15 and 520 μM of $AlK_3(SO_4)_3$ corresponding to 5 and 75 μM of Al^{3+}. Three distinct biological replicates were used for cDNA array analysis and two extra biological replicate were used for RNA-gel blot analyses: one for maize cDNA-probed RNA-gel blots and another for sugarcane EST-probed RNA-gel blots. Each experimental unit was constituted for 30 maize seedlings.

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

RNA isolation, cDNA synthesis and probe preparation: Total RNA was isolated from roots according to Logemann *et al.* (1987) with minor modifications. Thirty root tips about 5 mm long from each treatment were excised, frozen in liquid nitrogen and ground in extraction buffer [8 M Guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 2 % (v/v)

2-mercaptoethanol]. After extraction with 1 volume phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), the suspension was separated by centrifugation (5 000 *g*, 15 min), the aqueous phase was recovered by ethanol precipitation and the pellet was resuspended in DEPC-treated water.

The cDNA probes were produced as described by Schummer *et al.* (1999) with minor modifications. About 30 μg of total RNA were reverse transcribed with *Superscript II* (*Invitrogen*, Carlsbad, USA) using an oligo-dT18V (3 μM) primer, with 111 TBq $mmol^{-1}$ [α-^{33}P]dCTP and unlabeled dATP, dGTP, and dTTP (1 mM each) for 20 min at 42 °C. Unlabeled dCTP was then added to a final concentration of 1 mM and the reaction continued for another 40 min. The cDNA probes were purified by using ProbeQuant G-50 microcolumns according to the manufacturer's instructions (*Amersham Biosciences*, Piscataway, USA) and the radiolabeled probe intensity was normalized with the aid of a *1217 Rack Beta* liquid scintillation counter (*LKB Wallac*, Turku, Finland).

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

cDNA array analysis: The median value of all spot intensities obtained with the *Amp^r* probe was determined. The CV of these median values was used to assess fluctuations in the DNA amount between replicate filters. Only filters with CV values lower than 10 % were used for subsequent analysis (Nogueira *et al.* 2003). The *Amp^r* probe was removed by boiling and the filters were re-hybridized with cDNA probes synthesized from RNA samples (Schummer *et al*. 1997). Subsequently, the filters were sealed in plastic film, immediately exposed to imaging plates for 96 h and scanned in a phosphorimager *FLA3000-G* (*Fujifilm*, Tokyo, Japan). Each set of filter was hybridized three times, each time with cDNA probes synthesized from distinct RNA biological replicates. Signal was quantified using *Array Vision* software (*Imaging Research*, St. Catherines, Canada). Grids were predefined and manually adjusted to obtain optimal spot recognition, and spots were then quantified individually.

Filtered and normalized cDNA array data were analyzed using the significance analysis of microarrays (*SAM*) software (Tusher *et al*. 2001) with parameters chosen in order to lead to conservative selections of differentially expressed genes. Treatment (75 μM and 283 μM of Al) comparisons against their control (absence of Al) were performed within each maize genotype, and genotype comparisons were performed within each Al concentration. For all comparisons, *SAM* parameters were set as follows: minimum fold-change was set to 1.5 at least for one comparison and the Δ-value was chosen as the minimum value that leads to an estimated false discovery rate threshold of 1 % or less.

RNA gel blot analysis: Samples of total RNA (20 μg) extracted from 30 maize root tips were separated in 1 % (m/v) formaldehyde-agarose gels. RNA blotting and filter hybridization were performed in hybridization solution containing 50 % formamide and incubated at 42 °C for 18 h, according to Sambrook *et al.* (1989). After hybridization, RNA filters were washed according to Sambrook *et al.* (1989). RNA filters were exposed to imaging plates for 18 h and then scanned in a phosphor-imager. Further, the RNA filters were hybridized one additional time with a *26S rRNA* probe to confirm equal RNA loading. The expression intensity was quantified using the *Image Gauge V.4.0* software (*Fujifilm*).

Bioinformatics analysis: Comparative sequence analysis was performed with *BLASTx* and *BLASTn* algorithms (Altschul *et al.* 1997) against GenBank database (http://www.ncbi.nlm.nih.gov). Matches were considered significant when E-values were below 10^{-5} and PAM120 similarity scores were above 80 (Newman *et al.* 1994).

Results

Effect of Al on root growth of Cat100-6 and S1587-17: Although Cat100-6 is tolerant to Al, both genotypes showed inhibition of the root growth under high Al concentrations after 24 h. The most evident effect of Al was observed with the two highest doses. Cat100-6 showed mild symptoms of Al stress (root growth reduction around 20 and 25 % in 283 and 520 μM Al, respectively), whereas S1587-17 displayed a much more prominent root growth reduction (higher than 40 and 55 %, respectively; Fig. 1). In addition, root tips damage was observed only in S1587-17 (Fig. 2*E*,*F*).

Identification of Al-stress responsive ESTs using cross-species cDNA arrays: Before cDNA probe hybridization, the arrays filters were hybridized with a probe corresponding to the *Amp^r* sequence of the plasmid vector (see Materials and methods) and the signal intensity was measured. Around 98 % of ESTs spotted on the filters had ratios ranging from 0.5 to 1.5, indicating that the DNA variation between replicate spots was less than 1.5-fold for most ESTs (data not shown). Thus, only those sugarcane ESTs displaying signal intensities at least 1.5-fold above or below the signal intensity of the control

treatment (0 μM Al) for at least one Al treatment (75 or 283 μM Al) in all cDNA array replicates were considered for further data analysis.

The array filters containing sugarcane ESTs were tested with different sets of cDNA probes synthesized from total RNA of three independent experiments to verify reproducibility. The normalized signal intensity of each spot was determined after subtracting the local background intensity. During the quantification analysis, the software automatically discarded spots whose signal intensity was similar or under the local background intensity. Finally, 12 spots representing the empty plasmid vector were used in each filter as a negative control to assess nonspecific hybridization. These spots have not consistently produced a hybridization signal above background (data not shown) indicating absence of nonspecific hybridization.

As a result, 85 sugarcane ESTs showing at least a 1.5-fold induction or repression were effectively selected (Table 1). The significance of this approach for screening Al-stress responsive ESTs was demonstrated by the identification of genes encoding proteins that had already been reported in other plant species as Al-stress induced, such as phenyl ammonia-lyase (Snowden and Gardner 1993), chitinase (Nagy *et al*. 2004), Bowman-Birk proteinase inhibitor (Snowden and Gardner 1993, Richards *et al*. 1994), and wali7 (wheat aluminum induced Richards *et al*. 1994).

Among the 85 Al-stress responsive ESTs detected

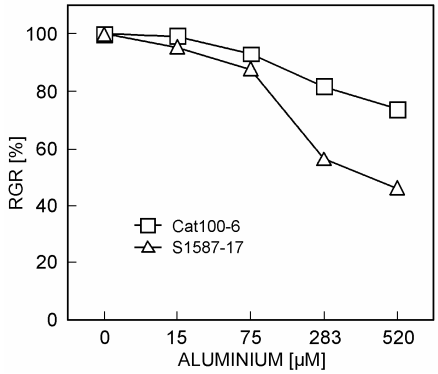

Fig. 1. Dose-response curves for relative growth rate (RGR) of roots of Cat100-6 (*squares*) and S1587-17 (*triangles*) after 24-h exposure to nutrient solution containing 0, 25, 75, 283, and 520 μM of $AlK_3(SO_4)_3$ (corresponding to 0, 5, 15, 50, and 83 μM of Al^{3+}).

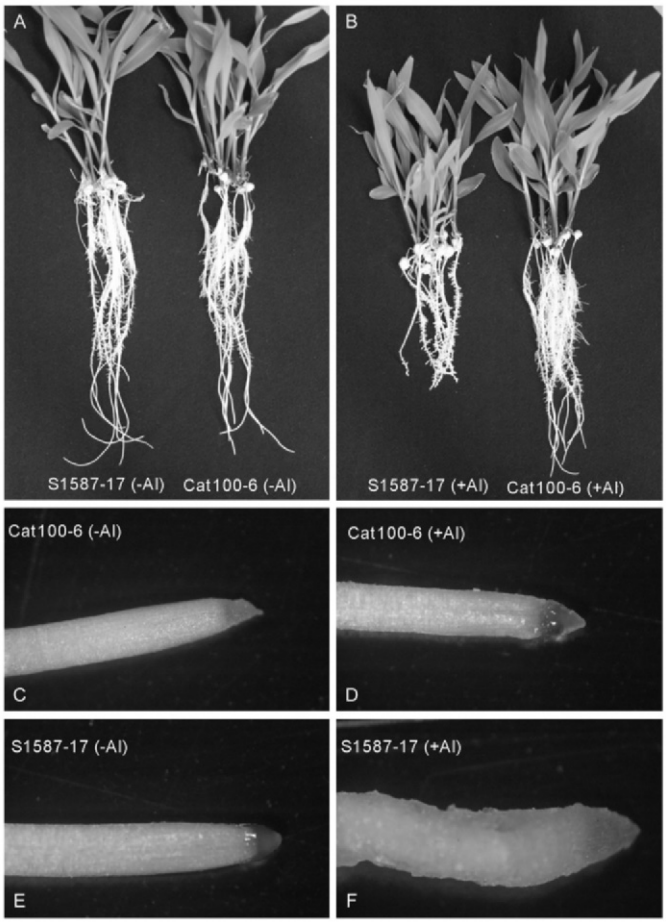

Fig. 2. Cat100-6 and S1587-17 roots after 7 d (*A* and *B*) and 24 h (*C*, *D*, *E*, and *F*) in nutrient solution with 0 (-Al) or 520 (+Al) μM $AlK_3(SO_4)_3$.

Table 1. Expression ratios of Al-altered sugarcane ESTs: [a]Expression ratios of treated intervals (75 and 283 μM of Al) in relation to its control (0 μM of Al): C75 = Cat100-6 under 75 μM of Al; C283 = Cat100-6 under 283 μM of Al; S75 = S1587-17 under 75 μM of Al; S283 = S1587-17 under 283 μM of Al. [b]Expression ratios of Cat100-6 in relation to S1587-17: C/S 75 = Cat100-6/S1587-17 under 75 μM of Al; C/S 283 = Cat100-6/S1587-17 under 283 μM of Al. [c]Classes dividing the genes in six general categories: I) Gene regulation; II) Sugar metabolism; III) Plant stress response; IV) Signal transduction; V) Other functions; and VI) Unknown and no hit proteins. [d]Description indicates the putative functions of the gene products expected from similarity sequences. The putative sequences were assigned using BLASTx and BLASTn algorithms.

Accession	Class[c]	BLAST (E-value)	Description[d]	Ratios[a]				Ratios[b]	
				C75	C283	S75	S283	C/S 75	C/S 283
Induced in both Cat100-6 and S1587-17									
CA095811	III	JC5843 (2E-46)	chitinase III	1.3	4.3	1.3	1.7	2.6	7.8
CA101236	VI	NP_922793 (2E-66)	unknown protein	0.4	1.9	1.5	2.3	0.3	3.0
CA102633	VI	XP_469468 (E-129)	unknown protein	0.7	1.9	3.4	2.5	0.9	3.0
CA098848	III	BAB63915 (0)	ERD protein	3.0	1.7	0.2	2.1	4.4	1.2
CA095754	III	1OM0A (E-119)	xylanase inhibitor protein	1.5	1.7	2.6	1.2	1.9	4.5
CA102690	II	CAA27681 (0)	alcohol dehydrogenase 1	0.7	2.3	1.5	1.3	0.3	1.0
CA096776	I	AAC69625 (E-115)	WD-40 protein	1.8	1.3	1.5	1.4	1.6	1.2
CA102731	II	AAF85966 (E-104)	sucrose synthase	3.1	3.2	1.9	1.8	1.1	0.9
CA064600	III	BAA97804 (4E-46)	β-glucuronidase precursor	1.0	1.9	0.7	1.7	1.7	1.6
CA064719	III	BAB19963	ASR protein	4.9	6.6	1.7	2.3	1.4	2.2
CA095678	III	P81713 (2E-07)	Bowman-birk proteinase inhibitor	1.5	1.3	18.7	46.6	1.4	1.1
CA095602	VI	-	no hit	1.4	2.2	1.3	1.5	1.2	2.1
CA097100	VI	-	no hit	3.2	1.1	2.4	65.6	2.5	0.7
CA095919	I	P49625 (E-116)	60S ribosomal protein L5	1.9	1.2	2.2	2.0	1.4	1.0
CA097212	II	2008300A (0)	sucrose synthase	1.5	1.3	2.1	2.3	1.7	1.1
Induced only in Cat100-6									
CA102689	VI	-	No hit	0.7	1.7	0.9	0.5	0.3	1.3
CA120042	III	BAD14927 (0.0)	DAHP synthetase	2.9	4.9	1.1	1.1	0.3	1.0
CA064763	III	AAL40137 (0)	PAL	1.3	3.7	1.1	1.1	1.0	3.0
CA064602	III	CAA13177 (7E-62)	CAD	3.0	1.6	0.7	0.8	3.4	1.9
CA097041	VI	-	no hit	1.9	1.1	0.5	0.6	3.3	2.0
CA064608	II	CAB87248 (9E-33)	glycerol 3-phosphate permease	2.3	2.7	0.8	1.4	5.9	3.8
CA064780	VI	NP_683323 (E-14)	unknown protein	1.0	1.5	0.4	0.5	2.3	1.8
CA064605	IV	AAB97114 (2E-89)	small GTP-binding protein	2.2	1.8	0.9	0.2	1.2	3.0
CA064810	VI	-	no hit	1.7	1.5	0.8	0.8	1.4	1.3
CA096090	III	AAC49972 (2E-28)	hypersensitive response protein	1.8	2.0	1.4	1.0	1.0	1.6
CA095938	VI	-	no hit	1.6	1.2	0.7	0.6	1.9	1.5
CA096097	III	NP_077728 (9E-67)	putative ferredoxin reductase	1.8	1.7	1.0	0.8	1.3	1.5
CA064787	III	AAK71314 (E-102)	papain-like cysteine peptidase	1.6	1.3	1.4	0.6	1.5	3.6
CA095663	I	P05621 (E-38)	histone H2B.2	1.5	1.1	1.2	1.3	0.7	0.5
CA096797	VI	XP_467727 (7E-48)	unknown protein	0.9	2.3	0.8	0.4	0.7	4.1
CA101237	III	Q40977 (4E-26)	MDRH	1.0	2.3	1.5	0.8	0.5	2.0
CA097462	I	NP_958815 (2E-14)	LUC7-like protein	0.7	1.8	0.2	0.2	1.7	5.2
CA097457	V	S72526 (0)	vacuolar H^+-pyrophosphatase	1.9	1.5	1.2	1.4	1.3	0.8
CA064742	III	AAM75139 (5E-39)	alkaline α-galactosidase	1.5	0.9	0.8	1.3	6.0	2.9
CA097200	VI	-	no hit	1.6	1.3	0.5	0.8	2.3	2.0
CA102716	III	AAC37416 (7E-50)	*Wali7*	1.1	1.6	1.2	1.0	1.1	2.1
CA096094	I	AAF76167 (E-174)	nuclear cap-binding protein	1.2	1.9	1.0	0.7	0.9	2.0
CA097210	V	S52030 (E-24)	oleosin 17	0.8	1.5	0.6	0.7	1.2	1.7
CA095623	III	CAD27730 (5E-41)	xylanase inhibitor	0.9	1.8	1.0	1.3	0.7	1.4
CA096135	I	AAG60059 (E-173)	acetyltransferase-related protein	0.9	1.8	1.1	0.9	0.7	1.6
CA095852	I	CAA58669 (2E-28)	ribosomal protein S27	1.4	1.7	0.9	1.0	0.9	1.1
Induced only in S1587-17									
CA064736	V	AAB72111 (6E-57)	BP-80 vacuolar sorting receptor	0.9	0.9	3.4	0.8	0.1	0.7

cont.

CA102674	I	AAN15557 (4E-52)	ABI3-interacting protein 2	0.8	1.1	2.4	0.6	0.3	1.4
CA102687	VI	XP_475538 (8E-38)	unknown protein	0.6	1.0	3.4	0.7	0.3	1.9
CA120045	I	NP_919951 (E-115)	putative retroelement	0.7	0.8	12.9	1.2	0.0	0.4
CA102772	II	CAA54609 (7E-75)	UTP-glucose glucosyltransferase	1.0	0.9	4.2	0.8	0.3	1.1
CA101178	VI	AAF01557 (2E-28)	unknown protein	1.2	1.1	1.9	1.0	0.3	0.6
CA097428	III	AAM64219 (2E-07)	cadmium induced protein CdI19	0.9	0.5	2.1	1.0	0.7	0.8
CA097438	III	CAA31077 (8E-36)	ABA-inducible protein	0.9	0.4	1.7	1.2	0.5	0.4
CA101174	VI	-	no hit	0.6	0.5	2.9	1.6	0.6	1.0
CA095885	I	T52344 (2E-87)	OsNAC5 protein	0.3	0.6	1.3	2.8	0.7	0.7
CA097119	VI	CAE03862 (3E-91)	unknown protein	1.0	1.1	1.8	1.3	0.6	0.9
CA064657	V	CAA45024 (5E-94)	aspartate aminotransferase	0.8	0.8	2.9	2.2	1.3	1.5
CA097111	III	JC5845 (2E-84)	chitinase III	1.1	0.9	0.9	0.9	5.5	5.4
CA064710	II	AAF70821 (0)	β-galactosidase	0.3	0.3	3.8	1.4	0.7	0.9
CA096803	IV	XP_469542 (3E-99)	putative ATP-binding protein	0.7	0.6	0.9	2.5	1.4	1.0
CA097066	VI	CAE05958 (E-129)	unknown protein	1.2	1.1	0.9	2.1	2.2	1.1
CA097909	VI	XP_476424 (E-22)	unknown protein	0.2	0.4	40.2	15.0	0.2	0.8
CA101149	I	T01996 (6E-45)	nucleoid DNA-binding protein	0.8	0.4	2.2	4.1	2.0	0.5
CA064656	V	P23687 (E-156)	prolyl oligopeptidase	1.2	1.2	1.8	2.3	1.3	0.8
CA096567	II	CAA39454 (0)	enolase	1.2	0.6	1.6	1.9	1.4	0.5
CA096578	I	O15818 (E-105)	putative eIF-3	0.9	0.6	1.3	1.6	1.0	0.6
CA102758	V	O13656 (6E-24)	mitochondrial import receptor	1.0	0.8	1.2	2.6	1.1	0.4
CA119990	III	JC2510 (0)	β-tubulin	1.1	0.9	1.6	2.1	1.3	0.9
CA064621	III	CAA55893 (E-140)	putative imbibition protein	1.0	1.3	1.1	1.9	1.8	1.3
CA064717	VI	-	no hit	1.2	1.0	1.1	2.1	2.9	1.5
CA097411	I	AAC25599 (3E-10)	CRP1 protein	1.2	0.9	0.9	2.3	2.2	0.8
CA101244	III	NP_005856 (E-43)	putative serine peptidase	1.0	0.6	0.8	1.5	3.9	1.3
CA119993	IV	CAA10660 (E-151)	Ca^{2+}-ATPase	1.1	1.1	1.8	2.2	1.6	1.5
Repressed in both Cat100-6 and S1587-17									
CA095925	II	AAG28503 (E-151)	hexokinase	0.6	0.2	0.3	0.4	1.2	0.4
Repressed only in Cat100-6									
CA095622	VI	-	no hit	0.4	0.5	0.7	0.8	0.8	0.9
CA095948	VI	AAN41388 (2E-17)	unknown protein	0.6	0.4	0.9	1.1	1.7	0.7
CA097085	VI	-	no hit	0.7	0.4	0.7	0.6	1.4	0.8
CA096779	I	P12629 (7E-60)	50S ribosomal protein L13	0.3	0.5	1.2	0.8	0.2	0.5
CA099555	I	CAB75508 (3E-14)	ABI3-interacting protein	0.5	0.6	1.5	1.1	0.7	1.2
CA101148	I	T01996 (6E-45)	nucleoid DNA-binding protein	0.8	0.4	1.3	1.5	1.6	0.8
CA095886	I	CAA63194 (E-155)	ribonucleotide reductase R2	0.5	0.8	0.9	1.3	0.5	0.6
Repressed only in S1587-17									
CA101271	III	AAM23263 (E-30)	DnaJ-like protein	0.9	0.7	1.1	0.4	0.4	0.9
CA096690	VI	BAB11002 (E-138)	unknown protein	0.9	0.7	0.3	0.3	4.2	3.6
CA095710	I	BAA95894 (2E-72)	putative reverse transcriptase	1.0	0.7	0.5	0.2	4.0	6.5
CA095910	V	AAF74980 (E-121)	cystathionine b-lyase	1.1	0.9	0.3	0.7	2.6	1.0
CA097854	I	BAA31739 (2E-05)	COP1-Interacting protein 7	0.9	0.7	0.4	0.8	2.8	1.1
CA098865	I	AAT40500 (E-50)	putative reverse transcriptase	0.9	1.1	0.5	0.7	1.7	1.3
CA064644	V	AAM60860 (E-136)	TMB	0.6	1.1	0.4	1.0	3.1	1.9
CA064616	I	AAD45720 (2E-31)	zinc finger protein	1.2	1.0	1.9	0.9	10.6	16.3

in our array data, 41 (48.2 %) and 43 (50.5 %) were up-regulated in Cat100-6 and S1587-17, respectively, while 15 (17.6 %) were up-regulated in both genotypes (Table 2). The number of ESTs down-regulated in Cat100-6 and S1587-17 were 8 (9.4 %) and 9 (10.6 %), respectively, totalizing 18.8 % of all ESTs significantly altered by Al (Table 2).

The up-regulated sugarcane ESTs in Cat100-6 and S1587-17 were distributed in three main groups (Table 2). The putative functions of each EST were assigned (Table 1) based on *BLASTx* and *BLASTn* sequence similarities and best E-value of sequences whose function was previously confirmed and characterized in the literature (NCBI, http://www.ncbi.nlm.nih.gov/blast). The first group contained ESTs encoding proteins previously described as Al-stress responsive, such as phenylalanine ammonia-lyase, *wali7* and Bowman-Birk proteinase inhibitor. The second group

contained ESTs encoding proteins responsive to other biotic and abiotic stresses, but not reported as Al-stress responsive until now. This group included xylanase inhibitor protein, OsNAC5 (*Oryza sativa* No Apical-Meristem Cup-shaped-cotyledon-2) protein, cadmium induced protein CdI19, and a ribosomal protein S27. The third group contained ESTs encoding unknown proteins, proteins with no hits in public databases, and proteins not reported as stress-responsive, such as nucleoid DNA-binding protein cnd41 (chloroplast nucleoids domain), mitochondrial import receptor tom40, BP-80 vacuolar sorting receptor and histone H2B.2.

To estimate the relative contribution of ESTs altered by Al stress from each *SUCEST* library used in the array experiments, we calculated the number of ESTs identified as altered by Al from each cDNA library (Fig. 3). Interestingly, the library CL6 accounted for most of the Al-stress up regulated ESTs (44 sequences of 69) and almost all of the Al-stress repressed ESTs identified in Cat100-6 and S1587-17 (13 sequences of 16), showing this library was the principal source of Al-altered ESTs.

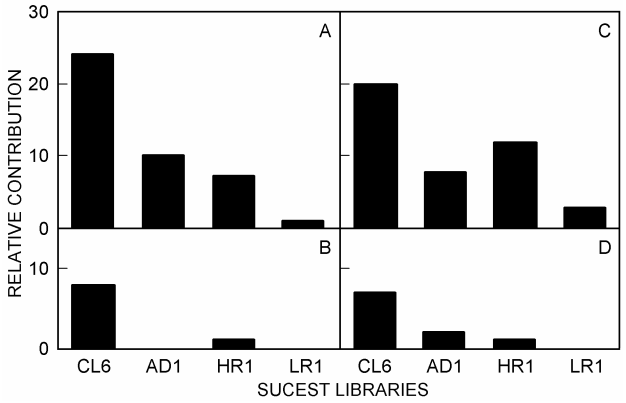

Fig. 3. Relative contribution of the SUCEST libraries used in the cDNA array experiments during the identification of ESTs altered by Al stress. The values represent the number of ESTs altered by Al, identified from each cDNA library (see "Results"). *A* - Cat 100-6 induced ESTs, *B* - Cat100-6 repressed ESTs, *C* - S1587-17 induced ESTs, *D* - S1587-17 repressed ESTs; CL6 - heat and cold-treated and untreated *callus*, AD1 - sugarcane plantlets infected with *Acetobacter diazotroficans,* HR1 - plantlets infected with *Herbasperillum rubrisubalbicans,* and LR1 - leaf roll tissue.

Table 2. Aluminum regulated ESTs identified by DNA array expression profile. [a]ESTs homologous to previously described as Al-induced genes; [b]ESTs homologous to previously described general-stress responsive genes; and [c]ESTs encoding proteins that have not been described previously as Al-induced or other stress-induced.

	Cat100-6 ESTs number	[%]	S1587-17 ESTs number	[%]
Up-regulated ESTs	41	48.2	43	50.6
Known Al-responsive[a]	5	5.9	6	7.0
Other stress-responsive[b]	17	20.0	14	16.5
Novel Al-responsive[c]	19	22.3	23	27.1
Down-regulated ESTs	8	9.4	9	10.6
Unknown ESTs Al up-regul.	4	4.6	7	8.1
Unknown ESTs Al down-regul.	1	1.2	1	1.2
No hit ESTs Al up-regul.	7	8.1	4	4.6
No hit ESTs Al down-regul.	2	2.4	0	0

RNA gel blotting analyses: RNA-gel blotting analyses using total RNA were performed for specific genes in order to validate the cDNA array data. The total RNA used as sample to make the maize cDNA-probed RNA-gel blots and the sugarcane EST-probed RNA-gel blots were extracted from independent biological samples. The Fig. 4 shows the expression profile of four maize genes with high similarity to: a PAL (Genbank Accession CO460668); an ABA and ripening-inducible like protein (CO445516); a sucrose synthase (CO465013); and a DAHP synthetase (CO458940). The expression profiles obtained from RNA gel-blots and cDNA arrays were similar, indicating consistency between the two data sets.

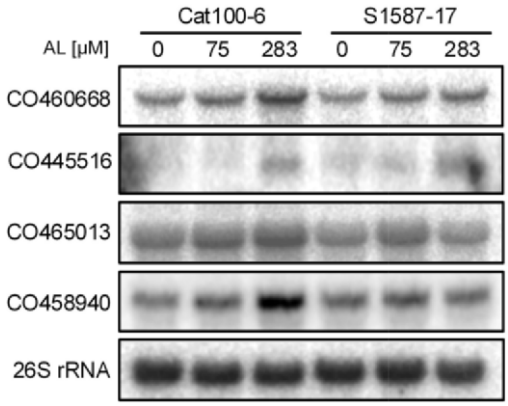

Fig. 4. Maize cDNA-probed RNA-blots of genes altered by Al. In the RNA gel blots, each lane was loaded with 10 μg of total RNA isolated from maize root tips exposed to increasing Al concentrations (0, 75 and 283 μM). The RNA loading was monitored using a *26S* rRNA gene as probe.

To investigate the cross-hybridization between sugarcane and maize in the RNA-blots, sugarcane probes were prepared using selected ESTs spotted onto the array filters. The Fig. 5 shows the expression profile of six sugarcane ESTs representing a PAL (CA064763), an ABA- and ripening-inducible like protein (CA064719), a protein homologous to B12D domain (CA097100), a chitinase class III (CA095811), a β-glucuronidase (sGUS) precursor (CA064600), and a hypothetical protein with no conserved domain (CA101236). The sugarcane ESTs showed 85.8 % (PAL - CO520609), 81.2 % (abscisic acid- and stress-inducible protein - BM499107), 41.6 % (chitinase class III - BE510590), and 52 % (β-glucuronidase (sGUS) precursor - CF024252) identity with maize EST sequences from the maize GDB (http://www.maizegdb.org/blast.php). The ESTs CA097100 and CA101236 produced no significant hits against maize GDB. Sugarcane ESTs displayed comparable expression

patterns to their homologs in maize (Figs. 4,5), pointing out that cross-species cDNA arrays are useful to examine gene expression in close-related plant species.

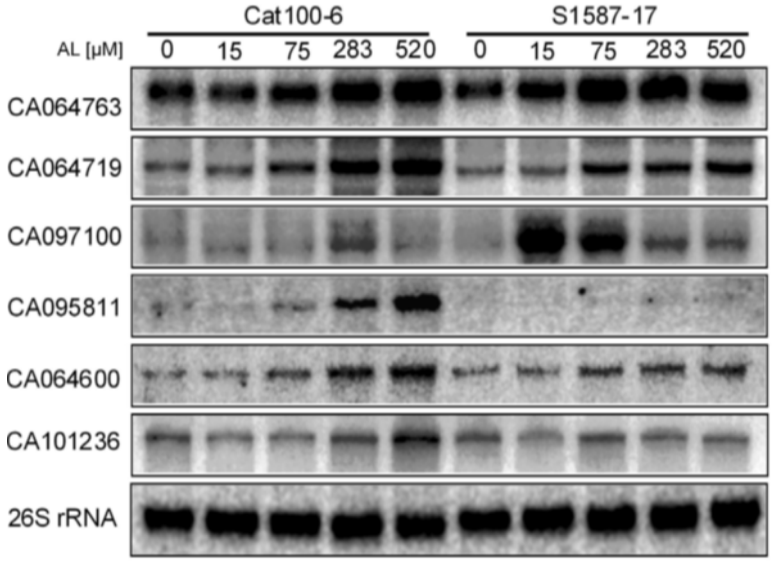

Fig. 5. Sugarcane EST-probed RNA-blot of genes altered by Al. In the RNA gel blots, each lane was loaded with 10 μg of total RNA isolated from maize root tips exposed to increasing Al concentrations (0, 15, 75, 283 and 520 μM). The RNA loading was monitored using a *26S* rRNA gene as probe.

Discussion

Using cross-species cDNA array transcript profiling, in which the tester was the cDNA obtained from maize root tips and the target was the sugarcane ESTs spotted onto nylon filters, we have identified 85 ESTs with expression significantly altered by Al stress (Table 1). Importantly, some of these ESTs are known as Al-stress responsive genes in other plants species (Table 2), demonstrating the suitability of our approach. Additionally, RNA-gel blot analyses showed similar expression profiles among maize and sugarcane homolog Al-stress responsive genes (Figs. 4,5). These data indicate that cross-species cDNA filter arrays can successfully be used to address important biological questions; maybe even in species whose do not have available cDNA array platforms.

Complex traits, such as responses to biotic and/or abiotic stresses, require the coordinated action of several genes. Thus, genomics approaches can be extremely useful to study such complex network among functional genetic modules (Aubin-Horth *et al.* 2005). Since many genes might be involved in more than one biological process, their functional categorization is not a simple task. We have attempted to group the ESTs identified in this work into six main class based on their putative function (Table 1). The first class comprises ESTs involved in transcriptional and post-transcriptional regulation. ESTs encoding proteins and enzymes involved in sugar metabolism and other sugar-dependent processes constitute the second class. The third class contains ESTs previously reported to be stress-responsive, including genes characterized as regulated by Al-stress. The fourth class includes ESTs involved in signal transduction pathways, while the fifth class is composed of EST encoding proteins whose known function does not match with any of the classes described above. The last class encompasses ESTs encoding unknown proteins and ESTs with no significant hit in public databases. This variety of functions suggests that several metabolic processes were altered in both Al-tolerant and Al-sensitive maize lines growing under Al stress.

The CL6 library was the main source of EST selected in the DNA array experiment. This library was constructed from a mixture of heat- and cold- treated and untreated sugarcane *callus* tissues (Vettore *et al.* 2001). Vettore *et al.* (2003) have hypothesized this library is a good source of biotic and abiotic stress-related ESTs, indicating that possibly many of the ESTs selected in this work might be involved with general stress response instead of Al-stress specific response.

Several genes involved with gene expression control were up- or down-regulated by Al stress in both maize lines, although some up-regulated and few down-regulated transcripts were genotype-specific (Table 1). For example, an EST encoding the ribosomal protein S27 (CA095852) was up-regulated only in Cat100-6 plants growing in presence of Al. Another gene involved in regulation of gene expression, which encodes a H2B histone (CA095663), was up-regulated in Cat100-6. ESTs encoding the CBP80 (Cap-Binding Protein 80 kDa, CA096094) and LUC7 (lethal unless CBC, CA097462)

were also up-regulated in Cat100-6. In S1587-17 genotype, the expression of a gene encoding a plant-specific NAC domain-containing transcription factor (CA095885) was induced when roots were submitted to Al stress (Table 1). The EST encoding to an ASR protein (ABA [abscisic acid]-, stress-, and ripening-induced, CA064719) involved in transduction pathway of sugar and ABA signaling (Çakir *et al.*, 2003) was up-regulated in both Cat100-6 and S1587-17 genotypes. Taken together, these findings suggest that primary and/or secondary effects trigged by Al stress might affect the gene expression-controlling pathways in maize toot tips.

The transcript level of a gene encoding the enzyme DAHP synthase (CA120042) was increased up to four times in Cat100-6, while remaining constant in S1587-17 (Table 1). DAHP synthase are involved with synthesis of plant secondary metabolites, including lignin, anthocyanic pigments, auxin, and antimicrobial phytoalexins (Keith *et al.* 1991). MDRH (CA101237) was another enzyme whose gene was up-regulated in roots of Cat100-6 growing under Al stress. This enzyme is involved with alleviation of oxidative stress (Murthy and Zilinskas 1994).

A remarkable feature in plant development is the ability of exhibiting a number of adaptative and protective responses to environmental stresses. Two ESTs encoding to PAL (CA064763) and CAD (CA064602), which are important enzymes involved with lignification processes, were strongly up-regulated in the presence of Al (Table 1). The activation of the phenylpropanoid metabolism has been reported during various biotic or abiotic stresses, such as wounding, pathogen attack, UV irradiation, heavy metals, and drought (Dixon and Paiva 1995). PAL has often been suggested as a constituent of the Al-stress alleviation mechanism in plants (Snowden and Gardner 1993, Snowden *et al.* 1995, Hamel *et al.* 1998). One reasonable explanation for the augment in PAL and CAD transcripts will be the cellular response to secondary effects trigged by the Al-stress.

The transcript level of an EST encoding to a chitinase class III was increased several times in root tips of Cat100-6 exposed to Al (Table 1, Fig. 5). The role of chitinases in plants is considered to be part of their defense mechanism against fungal pathogens (Watanabe *et al.* 1992). The increase in the transcription of chitinase genes or in chitinase activity may be induced by other external stimuli such as wounding, drought, cold, ozone, heavy metals, salinity and UV light (revised by Kasprzewska 2003). This enzyme was also recently appointed as involved in Al-stress alleviation in plants of Norway spruce (*Picea abies*) (Nagy *et al*. 2004).

The secretion of organic acid from roots has been hypothesized as an efficient mechanism of Al tolerance in plants, including maize (Pellet *et al*. 1995, Jorge and Arruda 1997). However, Piñeros *et al*. (2005) have showed increase in citrate level exuded by roots of Al-stress sensitive maize, indicating that citrate release will not be the major responsible for Al-tolerance acquisition in this specie. We have not identified Al-stress responsive ESTs encoding enzymes involved with biosynthesis of organic acids, even though ESTs corresponding to genes encoding four enzymes of the TCA cycle (aconitase, isocitrate dehydrogenase, fumarase and malate dehydro-genase) were present in our arrays (data not shown). This result agrees with Xiao *et al.* (2005) result, who were not able to find Al-stress induced genes associated with biosynthesis of organic acids in wheat cDNA arrays. In fact, internal root organic acid concentration was reported as unrelated with Al tolerance or root organic acids release in maize (Mariano *et al*. 2005). Furthermore, it has been recently showed in wheat and *Arabidopsis* that transport rather than synthesis might be the "bottle neck" of Al-stress tolerance induced by organic acid exudation (Hoekenga *et al*. 2006, Sasaki *et al*. 2004). However, a novel family of Al-activated organic acid transporters recently identified in wheat (Sasaki *et al*. 2004) and *Arabidopsis* (Hoekenga *et al*. 2006) does not seem to be related to Al tolerance acquisition in maize (Piñeros *et al*. 2008).

In this work we have increased the understanding of genetic mechanisms triggered by Al stress or by secondary effects of Al stress in maize roots tips, unravelling genes involved with several biological processes in both Al-tolerant and Al-sensitive genotypes. However, additional experiments are required to confirm whether alteration in transcript levels indeed reflects acquisition of Al tolerance. The use of maize transgenic plants overexpressing or silencing Al-stress responsive genes in the root tips may clarify their roles in the Al tolerance.

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