

Differential Effect of Aluminum on DNA Synthesis and CDKA Activity in Two *Coffea arabica* Cell Lines

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ABSTRACT

Aluminum (Al) is widely known to inhibit root growth: however, the exact mechanism of root growth inhibition is uncertain. To address the effect of Al on some components of the culture cycle, we used two *Coffea arabica* cell lines, L2 (Al-sensitive) and LAMt (Al-tolerant), and evaluated [³H]-thymidine incorporation into DNA and cyclin dependent kinase type A (CDKA) activity. We used cell cultures of the 7th day that were incubated with AlCl₃ (100 μM) during different periods of time and observed a decrease of 47% in the rate of [³H]-thymidine incorporation into DNA when the cells were incubated in the presence of Al in the L2 cell line as compared with the LAMt cell line, which showed an increase of 150% over the control.

A 1,132 bp cDNA for CDKA was amplified from *C. arabica* cells using real-time polymerase chain reaction (RT-PCR) and 3'-RACE, which encoded a 294 amino acid polypeptide. Incubation of the cells with AlCl₃ (100 μM) causes inhibition of CDKA activity up to 72% in the L2 cell line but it was stimulated up to 70% in the LAMt cell line. The Al effect on expression of CDKA transcripts estimated by RT-PCR showed no effect upon AlCl₃ incubation between the two cell lines. Our results suggest that Al affects cell cycle elements differentially in the two tested cell lines.

Key words: *Coffea Arabica*; Aluminum; DNA synthesis; Cyclin-dependent kinase

INTRODUCTION

Aluminium toxicity is one of the major factors that limit plant growth in acids soils. The most important effect of Al toxicity is a dramatic reduction in root

growth. Al exposure inhibits root elongation within 1 h. This rapid inhibition is primarily due to a block in cell elongation rather than in cell division, whereas over longer periods (> 24 h) both cell division and elongation are inhibited (Jones and others 1995).

Al has been shown to affect a large number of cellular processes, especially the uptake of K⁺ and

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Ca²⁺ (Huang and others 1992; Liu and Luan 2001), cytoskeletal dynamics (Sivaguru and others 1999), callose synthesis (Zhang and others 1994), lipid peroxidation (Yamamoto and others 2001), and signal transduction mechanisms (Jones and Kochian 1995; Piña-Chable and Hernández Sotomayor 2001; Martínez-Estévez and others 2003a, 2003b). It has been reported that Al interferes with cell division in plant roots and is associated with DNA (Morimura and Matsumoto 1978). Minocha and others (1992) observed a decrease up to 50% in the DNA synthesis in *Catharanthus roseus* cells in response to Al toxicity, which suggests that Al might be affecting the expression of genes involved in the regulation of the cell cycle.

The major checkpoints of the eukaryotic cell cycle are situated at the G1-S and G2-M transitions. Progression through these boundaries is catalyzed by cyclin-dependent kinases (CDKs). The catalytic activity of these protein kinases is regulated by the association with their regulatory subunits, cyclins, which also determines the substrate specificity and the subcellular localization of the CDKs complex (Colasanti and others 1993; Bögre and others 1997). The activity of the complexes is further controlled by a number of mechanisms including phosphorylation/dephosphorylation, interaction with inhibitory proteins, proteolysis, and intracellular trafficking (Dewitte and Murray 2003). During the past decade, many key cell cycle regulators have been characterized in plants, including CDKs, cyclins, CDK inhibitors, and a suppressor of the cell cycle block/CKS protein (Mironov and others 1999; Dewitte and Murray 2003).

Plants, like animals, possess several CDKs, but their functions have been poorly defined (Mironov 1999). Based on sequence similarity, plant CDKs can be subdivided into a few distinct groups (Dewitte and Murray 2003). The best characterized group (A-type) comprises plant CDKs that are most closely related to the mammalian CDC2 and CDK2 and contain the same PSTAIRE motif. A-type CDKs can partially complement yeast *cdc2/CDC28* mutations and are therefore supposed to be functional homologs of the yeast CDKs (Colasanti and others 1991; Ferreira and others 1991; Hata 1991; Hashimoto and others 1992; Imajuku and others 1992; Setiady and others 1996).

To understand the mechanism by which aluminum inhibits cell growth we used suspension cells of *C. arabica* L. as a model (Martínez-Estévez and others 2001b) and obtained an Al tolerant (LAMt) cell line (Martínez-Estévez and others 2003b). In the present work, we evaluated the effect of Al on CDKA activity and DNA synthesis in two

C. arabica cell lines, the Al sensitive cell line L2 and the Al tolerant cell line LAMt. Our results suggest that Al affects CDKA differentially in the two cell lines.

MATERIALS AND METHODS

Cell culture

An embryogenic suspension culture of *C. Arabica* was obtained by desegregation of callus by Martínez-Estévez and others (2001b) and maintained in MS medium (Murashige and Skoog 1962) at pH 5.8 supplemented with 100 mg/l myo-inositol, 10 mg/l thiamine, 25 mg/l cysteine, 3% sucrose, 3 mg/l 2,4-dichlorophenoxyacetic acid, and 1 mg/l 6-benzylamine purine. The embryogenic suspension culture was subcultured every 14 days. To obtain aluminum toxicity both cell lines (L2 and LAMt) were maintained at pH 4.3 and half ionic strength as described elsewhere (Martínez-Estévez and others 2001b, 2003b). Fresh weight was measured by filtering the cells. The cells were separated from the culture medium by filtration (using a filter paper with medium pore), then the cells were transferred from the filter paper to a new and clean paper on the balance. The final result was expressed in grams.

Incorporation of ³H-thymidine into DNA

DNA synthesis was monitored by pulse labeling as described by Minocha and others (1992) with some modifications: 370 kBq of [³H]-thymidine were added into 1 ml of cell suspension and were incubated at 28°C for 30 min. Then 3 ml of ice-cold 4% perchloric acid (PCA) was added to each tube and the tubes were incubated on ice for 15 min, after which the cells were filtered through GF/A glass microfiber filters (24 mm, Whatman International Ltd., Maidstone, England) premoistened with 4% PCA using a multifilter manifold under vacuum. The filters were washed successively with 4% PCA, 70% ethanol, and 100% ethanol. The filters were dried at 50°C for 1 h and counted for radioactivity in a scintillation counter (Beckman LS-6500, México).

CDKA kinase activity and immunoblot analysis

Protein extraction, p13^{suc1}-Sepharose affinity binding and histone H1 kinase assays were carried out as previously described (Magyar and others 1993) with

some modifications: cells were homogenized with a politron in extraction buffer (25 mM Tris-HCl, pH 7.5; 15 mM MgCl₂; 15 mM EGTA; 15 mM p-nitrophenylphosphate; 100 mM NaCl; 1 mM DTT; 0.1% Tween 20; 1 mM sodium orthovanadate; 10 µg/ml leupeptin; 1 mM phenyl-methyl-sulphonyl-fluoride; 20 µg/ml aprotinin) and centrifuged at 25 000 × g. The protein content was determined by using the bicinchoninic acid protein assay kit (Smith and others 1985).

Two hundred micrograms of total protein in extraction buffer was incubated overnight at 4°C with 10 µl 25% (v/v) p13^{suc1}-Sepharose beads. The protein-protein precipitates were washed three times with washing buffer (50 mM Tris-HCl pH 7.5; 250 mM NaCl; 5 mM EGTA; 5 mM EDTA; 0.1% Tween 20; 2 µg/ml aprotinin) and once with kinase buffer (50 mM Tris-HCl, pH 7.5; 15 mM MgCl₂; 5 mM EGTA; 1 mM DTT).

The kinase reaction mixture consisted of 50 mM Tris-HCl, pH 7.5; 15 mM MgCl₂; 5 mM EGTA; 1 mM DTT; 100 µg histone H1 and 370 kBq of [γ -³²P]ATP in 30 µl of total volume. Kinase reactions were started by the addition of 30 µl reaction mixture to the washed p13^{suc1}-Sepharose beads and stopped after 15 min at 28°C with the addition of Laemmli buffer (Laemmli 1970). The samples were analyzed by 12% SDS-PAGE and autoradiography. For immunoblot, proteins were separated in 12% SDS-PAGE and electroblotted on to Nitroplus transfer membrane for 1 h at 50 V. Filters were probed with polyclonal antibodies against cdc2a (1:3000) from Santa Cruz Biotechnology (USA), and visualized by ECL chemiluminescence.

Gene isolation

RNA was isolated from cell suspension cultures using Trizol (Invitrogen Life Technologies, USA) according to the manufacturer instructions. Total RNA (5 µg), 10 µM oligo dT primer and nuclease-free water were incubated at 70°C for 5 min followed by 1 min on ice. Reverse transcription was performed at 37°C for 1 h with 200 U M-MLV reverse transcriptase (Invitrogen Life Technologies, USA), 0.1 mM DTT, 40 U RNasin (Promega, USA), and 0.5 mM dNTP's. The amplification of the 3' cDNA end was performed with the Gene Racer kit (Invitrogen Life Technologies, USA) according to the manufacturer instructions. The primers used (0.5 µM) were gene race 3' (Invitrogen Life Technologies, USA) and the CDKA specific forward primer (5'-TGGATCAGTATGAGAAGGTGGAGAAGAT-3', 2-29 position from the translation initiation site), 3.75 U Expand Long Template System (Roche,

USA), 1.75 mM MgCl₂, and 350 µM dNTPs. The cycling conditions were 94°C 2 min, 1 cycle; 94°C 20 s, 55°C 1 min, and 68°C 10 min, for 35 cycles. The PCR products were fractionated on 1% agarose gel and the corresponding 1.1 kb fragment was cut. The purified PCR product was ligated into the Zero Blunt TOPO PCR (Invitrogen Life Technologies, USA) vector and sequenced on both directions. DNA sequences were compared to sequence databases using the BLASTN algorithms (Altschul and others 1997). Deduced amino acid sequences were aligned with known sequences using the Mult Alin program (Corpet 1988).

Southern-blot analysis

Genomic DNA was isolated from cell suspension cultures according to the method of Dellaporta and others (1983). DNA (50 µg) was digested with Bam H I, Xba I or both restriction enzymes, separated on a 0.7% agarose gel and blotted onto charged nylon membranes (Hybond N⁺, Amersham Pharmacia Biotech, England). For the probe labeling and hybridization we used the Gene Images Alkphos direct labeling and detection system (Amersham Biosciences, USA) kit according to the manufacturer instructions. The hybridization was carried on at 65°C in 2 M urea, 0.1% SDS, and 150 mM NaCl. A 631 pb probe was used that contains the cyclin binding domain of the CDKA. The probe was amplified by PCR with the following set of primers 5'-TGGATCAGTATGAGAAGGTGGAGAAGAT-3' position 2-29 from the gene cdc2a and 5'-TCGATCTCAGAATCTACAG GAAACAGTG-3', complementary to position 604-632.

RT-PCR

Total RNA was extracted using the RNeasy plant mini kit (Qiagen, USA). Reverse transcription reactions were performed using 2 µg of RNA, 200 U of M-MLV reverse transcriptase and 2.5 µM oligo dT from Invitrogen Life Technologies (Gene Racer Kit) primer as described previously in gene isolation. The PCR reactions were performed with 0.4 µM each of CDKA forward 5'-GGAACGTATGGAGTGGTGT-ACAA-3' (from position 37 to 59 of the CDKA gene) and reverse 5'-TCTATCTCAGAATCCCCAG GGAA-CAAAG-3' (located between base 604 and 632) primers, 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), 5 mM MgCl₂, and 200 µM dNTP Mix. Cycling conditions were 95°C 10 min 1 cycle, 94°C 20 s, 65°C 20 s, and 72°C 30 s, for 24 cycles. Tubulin PCR reactions were performed to normalize the results. The PCR reactions were per-

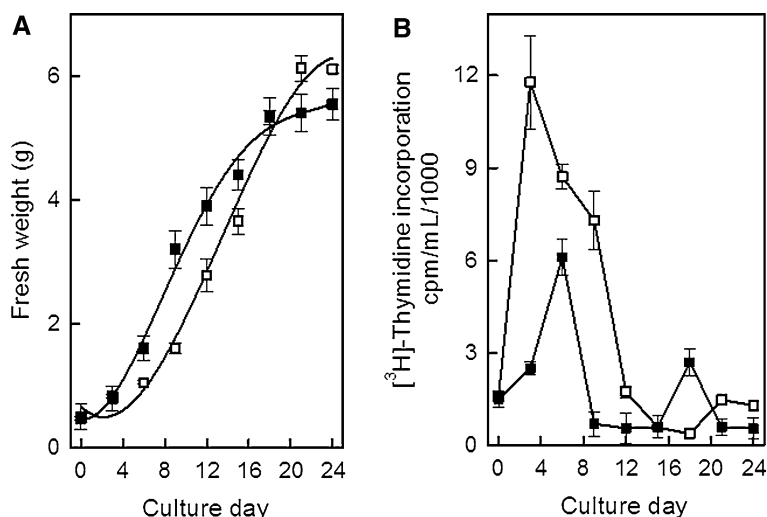


Figure 1. Cells from *Coffea arabica* suspension cell line L2 (closed symbols) or LAMt (open symbols) were evaluated as fresh weight (A) or [³H]-thymidine incorporation into DNA (B) for 24 days. Each data point represents mean \pm SE of three independent experiments.

formed with 0.4 μ M each of tubulin forward 5'-ATCCAGTTTGTCTCGACTGG-3' and reverse 5'-CATA-TGTAAGGAACCAAGGTAG-3', which amplify a 453 bp fragment of the tubulin gene. The reaction conditions were similar to those from CDKA gene amplification using the same cDNA from each condition.

RESULTS

Thymidine incorporation into DNA in response to Al treatment

To determine the effect of Al on DNA synthesis, we first characterized [³H]-thymidine incorporation into DNA and cell growth during the culture cycle in the L2 and LAMt *C. arabica* cell lines. Fresh weight and thymidine incorporation were evaluated every third day, from the day of subculture (day 0) to 24 days of culture. These *C. arabica* cell lines, previously characterized (Martínez-Estévez and others 2001b; 2003b), present a typical growth curve where the lag phase continues to the sixth day and the logarithmic growth phase finishes on day 12, after which there is a linear phase from day 14 to day 20 and a stationary phase that starts around day 22. In these reports we have measured the growth curves of the cells under different conditions and found that it takes from 6 to 7 days to start growth depending on the conditions tested. Because the experiments we carried out in this study take 6 h or less, it would be unlikely that the mitotic index would be affected, as the number of cells entering mitosis versus the total number of cells may change but only after days of the experiment. Figure 1A shows that both cell lines

presented a very similar pattern of growth referred to as fresh weight. On DNA synthesis measured as the rate of [³H]-thymidine incorporation, we observed that the higher thymidine incorporation in the L2 cell line is on day 6 of the lag phase, and in the LAMt cell line it was on the third day and it remained high until the ninth day of the logarithmic growth phase.

Cells from the 7th day of culture were incubated for different periods of time, from 0.5 h up to 9 h, in the presence or absence of 100 μ M AlCl₃ and [³H]-thymidine incorporation into DNA was evaluated as stated in *Materials and Methods*. Although incubation of the sensitive line with AlCl₃ decreases [³H]-thymidine incorporation from the first 30 min (more than 50%), the tolerant line did not behave in the same way. First there was an increase after 1 h of incubation with AlCl₃ up to 150% and then the levels of [³H]-thymidine incorporation return to base levels (Figure 2).

Isolation of *C. arabica* CDKA gene

With the results obtained in the [³H]-thymidine incorporation on the L2 and LAMt *C. arabica* cell lines, we aimed to correlate these results with the expression of the key cell cycle regulator CDKA at the transcriptional and translational levels.

For cloning the cDNA, we took advantage of the highly conserved 5' regions of the different CDKAs to generate a primer from the second to the twenty-ninth position of the known cDNAs and used an oligo dT primer. RT-PCR was performed using RNA isolated from day 7 of culture. The products from 3'-RACE were purified and cloned. After PCR and enzyme digestion confirmation, the products were sequenced. By comparing and aligning the

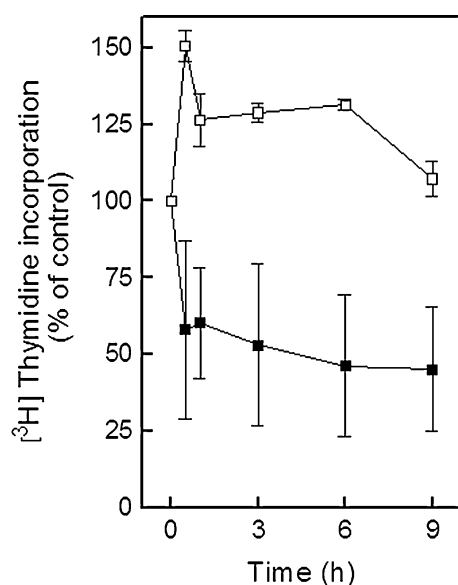


Figure 2. Effect of aluminum on [^3H]-thymidine incorporation into DNA. Cells from the 7th day of culture were incubated in the presence of 100 μM AlCl_3 for different periods of time and [^3H]-thymidine incorporation was determined as in Materials and Methods. Results represent the mean of three to six independent experiments expressed as percentage of the [^3H]-thymidine incorporated in the absence of AlCl_3 , which was considered as 100%. L2 and LAMt (closed and open symbols, respectively).

sequences a 1,132 bp with an open reading frame of 882 nucleotides encoding a 294 amino acids product was obtained (accession number AJ496622). The comparison of this sequence at the amino acid level with five plant CDKA sequences reported in GenBank (Figure 3), revealed a high degree of identity (86%–95%). The predicted protein contains all of the functionally important regions of CDKA such as the cyclin binding domain (residues 39–63, the EGVSTAIRESILLKE hallmark), the T-loop area (residues 147–172), and the amino acid residues known to be important for the regulation of CDKA activity via phosphorylation and dephosphorylation (residues Thr-14, Tyr 15, and Thr-161) (Ferreira and others 1991; Fobert and others 1996; Joubès and others 1999, 2000). This extensive structural similarity to plant CDKAs proteins supports the identification of the CDKA from *C. arabica*.

Genomic Southern blot analysis

Genomic DNA was extracted from tissue culture cells to evaluate if one or more genes of CDKA are present in *C. arabica*. The Southern blot was carried out with 50 μg of digested genomic DNA with the restriction enzymes *Bam* HI and/or *Xba* I. We used a

probe that contains the first 631 bp of *C. arabica* CDKA, which includes the conserved 5' region of the gene enclosing the cyclin binding domain common to all CDKA known sequences. The Southern hybridization was carried out under stringent conditions and showed one primary hybridizing band in all cases suggesting that there may be only a single copy of the CDKA gene on the *C. arabica* genome (Figure 4).

CDKA activity and gene expression in response to Al treatment

The effect of Al on CDKA activity on both cell lines was determined after identifying the sequence of the CDKA cDNA from *C. arabica*. Cells from day 7 after subculture were incubated with 100 μM AlCl_3 for different periods of time from 0.5 h to 9 h and CDKA activity was measured as described in *Materials and Methods*. We observed in the L2 cell line a decrease in the CDKA specific activity up to 75% when the cells were incubated with AlCl_3 ; this effect was time-dependent (Figure 5D). In the LAMt cell line there was an increase in the specific activity up to 70% that was also time-dependent.

Because of the results on CDKA activity, we decided to check if the effect of Al-treatment of the cells was due to different expression levels of the CDKA gene. The expression of CDKA transcripts on the L2 and LAMt *C. arabica* cell lines in response to 100 μM of AlCl_3 treatment during 0, 0.5, and 6 h was evaluated by semi-quantitative real-time polymerase chain reaction (RT-PCR) amplification of the cDNA transcript. Figure 5A shows the quantitative amplification profile of different RT-PCR cycles from which 24 cycles were selected as the linear range for amplification. A similar curve was carried out for the amplification of tubulin as a control gene. To our surprise, the results presented in Figures 5A and 5B showed a lack of effect on the CDKA expression levels during Al treatment in both cell lines. A ratio from the quantification of the expression of CDKA/tubulin RT-PCR showed only minor changes in the amount of CDKA expressed under the tested conditions as seen in Figure 5B. Furthermore, the increase in CDKA activity is independent of de novo protein synthesis, because the results of an experiment in the presence of cycloheximide were the same as results we obtained previously (data not shown). With antibodies against cdc2a, no difference in the amount of this protein was observed with the different treatments of the cells (L2 and LAMt) with AlCl_3 (Figure 5D). Even more, RT-PCR showed no significant differ-

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AJ496622     MDQYKVEKIGEGTGVVYKARDKSTNETIALKKIRLEQEDEGVPSTAIREISLLKEHQH
60D50738     MDQYKVEKIGEGTGVVYKARDRVNNTETIALKKIRLEQEDEGVPSTAIREISLLKEHQH
60AF194820   MDQYKVEKIGEGTGVVYKARDRVNNTETIALKKIRLEQEDEGVPSTAIREISLLKEHQH
60X77680     HEQYKVEKIGEGTGVVYKARDRLTNETIALKKIRLEQEDEGVPSTAIREISLLKEHQH
60M60526     HEQYKVEKIGEGTGVVYKALDKATNETIALKKIRLEQEDEGVPSTAIREISLLKEHQH
60D10850     MDQYKVEKIGEGTGVVYKARDKVNTETIALKKIRLEQEDEGVPSTAIREISLLKEHQH 60
*:***** *:*****

AJ496622     GNIVRLQDVVHSEKRLYLVEFYLDLCLKKHMDSPEFSKDPRLVKMFLYQILRGIA YCHS
120D50738    ANIVRLQDVVHSEKRLYLVEFYLDLCLKKHMDSPEFSKDPRLVKMFLYQILRGIA YCHS
120AF194820  GNIVRLQDVVHSEKRLYLVEFYLDLCLKKHMDSPEFAKDPRLVKTFLYQILRGIA YCHS
120X77680    GNIVRLQDVVHSEKRLYLVEFYLDLCLKKHMDSPELAKDPRLIKTFLYQILRGIA YCHS
120M60526    GNIVRLQDVVHSEKRIYLVFEFYLDLCLKKFMDSCPEFAKNPTLIKSYLYQILHGVA YCHS
120D10850    SNIVRLQDVVHSEKRLYLVEFYLDLCLKKHMDSPPDFSKDLHMIKTYLYQILRGIA YCHS 120
.***:*.*****:*****.*** *:***: :*:*****:*****

AJ496622     HRVLHRDLKPNQLLIDRRTNALKLADFGLARAFGIPVRTFTHEVVTLWYRAPEILLGSRH
180D50738    HRVLHRDLKPNQLLIDRRTNALKLADFGLARAFGIPVRTFTHEVVTLWYRAPEILLGSRH
180AF194820  HRVLHRDLKPNQLLIDRRTNALKLADFGLARAFGIPVRTFTHEVVTLWYRAPEILLGSRH
180X77680    HRVLHRDLKPNQLLIDRKTNAKLADFGLARAFGIPVRTFTHEVVTLWYRAPEILLGSRH
180M60526    HRVLHRDLKPNQLLIDRRTNALKLADFGLARAFGIPVRTFTHEVVTLWYRAPEILLGARQ
180D10850    HRVLHRDLKPNQLLIDRRTNALKLADFGLARAFGIPVRTFTHEVVTLWYRAPEILLGSHH 180
*****:*****:*.*****:*****:*** *:***: :*:*****:***

AJ496622     YSTPVDVWSVGCIFAEMVNQRPLFPDSEIDELFKIFRVMGTPNEDTWPGVTSLPDFKSA
240D50738    YSTPVDVWSVGCIFAEMVTQRPLFPDSEIDELFKIFRVMGTPNEDTWPGVTTLPDFKSA
240AF194820  YSTPVDVWSVGCIFAEMVNQKPLFPDSEIDELFKIFRILGTPNEDTWPGVTSLPDFKSA
240X77680    YSTPVDVWSVGCIFAEMVNQRPLFPDSEIDELFKIFRVLGTPNETTWPGVTSLPDFKSA
240M60526    YSTPVDVWSVGCIFAEMVNQKPLFPDSEIDELFKIFRILGTPNEQSWPGVSLPDFKTA
240D10850    YSTPVDIWSVGCIFAEMISQKPLFPDSEIDQLFKIFRIMGTPYEDTWPGVTSLPDYKSA 240
*****:*****:*.*****:*****:*** *:***: :*:*****:***

AJ496622     FPRWLSQDLATVVPNLDAAGLDLLRKMCLDPSKRITARNALAEHEYFKDIGFVP
294D50738    FPKWPSKDLATVVPNLDGAGLDDLKMLRLDPSKRITARNALAEHEYFKDIGYV-
293AF194820  FPKWPSKDLATVVPPTLEKAGVDLLSKMLFLDPTKRITARSALAEHEYFKDIGFVP
294X77680    FPKWPAKDLATVVPGLEPAGIDLLSKMLCLEPSKRITARSALAEHEYFKDLGFVP
294M60526    FPRWQAQDLATVVPNLDPAAGLDDLKMLRYEPSKRITARQALEHEYFKDLEVVQ
294D10850    FPKWKPDTLETVFPNLDPDGVDLLSKMLLMDPTKRINARAALAEHEYFKDLGGMP 294
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Figure 3. Comparison of the amino acid sequence deduced from the nucleotide sequences of *C. arabica* CDKA (AJ496622), *Nicotiana tabacum* (D50738), *Populus tremula* (AF194820), *Picea abis* (X77680), *Arabidopsis thaliana* (D10850), and *Zea mays* (M60526). The cyclin binding domain is boxed, as are the threonine 14 and tyrosine 15 residues required for the activity of the enzyme.

ence in the level of RNA expression of CDKA (data not shown), suggesting that the changes in the enzymatic activity were at the posttranscriptional level.

DISCUSSION

Aluminum is the most abundant metal in the Earth's crust. When the pH of the soil is below 5, soluble forms of Al accumulate producing negative effects on several plant processes. Several theories have traced the mechanisms of aluminum toxicity to its effects on the signal transduction pathways, and its inhibition of root growth and cell division (Liu and Jiang 2001).

To access aluminum toxicity on the cell cycle we decided to analyze one of the key enzymes for the process, such as CDKA, utilizing a previously well characterized suspension cell model with two existing cell lines, one sensitive and one tolerant (Martínez-Estévez and others 2003b).

No difference was evident in the cell growth pattern in either line, whereas a difference on the

pattern of [³H]-thymidine incorporation into DNA was observed. The LAMt cell line constantly incorporated up to 100% more thymidine during the first days of cell culture as seen in Figure 1. When the amount of [³H]-thymidine incorporation was compared between the two cell lines on day 7, the tolerant cell line behaved in a non-linear fashion showing two reproducible peaks of incorporation in the presence of Al (Figure 2). This may represent two different mechanisms for this cell line to tolerate aluminum. Few studies have dealt with the effect of Al on DNA synthesis in suspension cells. DNA synthesis has been studied in *Catharantus roseus* cells grown in cultures for 3 days and showed a 20%–30% increase in the rate of incorporation of [³H]-thymidine within 4 h of addition of Al, although after 16 h there was a strong inhibition of DNA synthesis activity. The concentrations of Al used in that study were extremely high, ranging from 0.2–1 mM (Minocha and others 1992; Zhou and others 1995). In the present work we used 0.1 mM of AlCl₃, which is a more physiological concentration for Al toxicity found in contaminated soils (Martínez-Estévez and others 2003b).

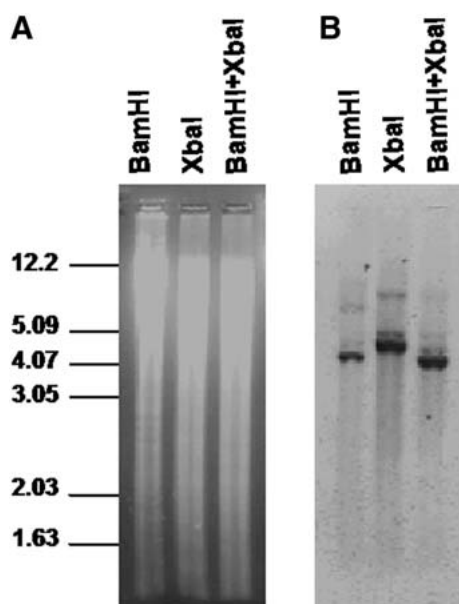


Figure 4. Southern blot analysis of *C. arabica* genomic DNA. Genomic DNA (50 µg) was digested with the restriction enzymes *Bam* H I and/or *Xba* I as indicated at the top of each lane. Digested DNA was blotted and hybridized with a 631 bp fragment of the *C. arabica* CDKA gene including the cyclin binding domain. The sizes of molecular weight markers in kilobase pairs are indicated on the left.

The *C. arabica* suspension cells respond to Al treatment in a different way, in that the tolerant line was less sensitive to DNA synthesis inhibition. It is unknown at this time whether the tolerant line produces more organic acids that would interact with the Al or whether it is capable of removing some of the Al via compartmentalization into vacuoles. These two mechanisms have been previously established for Al-tolerant plants (Feng and others 2001; Kochian and others 2004). Regardless of the mechanism of tolerance, the lack of DNA synthesis inhibition points to changes in the cell cycle machinery. To test if the cell cycle had been affected, we analyzed CDKA, a sensitive enzyme that is a key part of the cell cycle progression and that has been involved as a regulator of stress responses (West and others 2004). Sequence comparison of CDKA from several plants shows a high degree of conservation with the CDKA amino acid sequence of *C. arabica* we cloned, as shown in Figure 3. Southern blot analysis points to a single copy of this gene. The information from the gene bank shows a variation in the number of CDKA genes from plants like *Arabidopsis thaliana* that carry only one copy of the gene in their genome to plants like *Oryza sativa*

or *Zea mays* that can carry several copies of the gene. Our results indicate that *C. arabica* contains one copy of the gene, and its expression is insensitive to Al under tested conditions.

CDKA has been shown to be involved in the sensing of oxidative stress in *Lycopersicon esculentum* and salt stress in *Arabidopsis thaliana* (West and others 2004). CDKA activity in the LAMt cell line showed an increase during the first 6 h of incubation with Al as compared to the sensitive cell line L2, which showed the opposite trend. Although to our knowledge this is the first report on the effect of Al toxicity on CDKA activity, this enzyme appears to play a key role in the response to Al in the tolerant cell line. Our results show that CDKA is regulated at the post-transcriptional stage, because no significant increase or decrease was observed in the RNA levels of CDKA, as shown in Figure 5. A possible phosphorylation/dephosphorylation regulatory step may be involved in the signal transduction mechanism to regulate the CDKA activity during Al stress, or an increased pool of cyclins or decreased number of CDKA inhibitors could be involved in regulating this enzyme (Ferreira and Hemerly 1991).

Recognition of the pathways involved in the perception of aluminum is a key element in understanding the way in which some plants tolerate its toxicity. We have previously shown that Al specifically inhibits phospholipase C enzyme followed by a distinctive increment in some phospholipid kinases (Martínez-Estévez and others 2003a). Also, addition of aluminum to the cells induced a rapid and transient activation of a protein kinase that has characteristics of a MAP kinase (Arroyo-Serralta and others 2005). It is not surprising that a sensitive enzyme that is regulated by phosphorylation such as CDKA is also regulated in response to Al toxicity and that this may play a key role in the tolerance of the LAMt line.

Together our results show that the Al-tolerant cell line LAMt has a different response in the presence of Al. Furthermore, the increase in CDKA activity together with the [³H]-thymidine incorporation into DNA show that the LAMt cell line utilizes a different mechanism to cope with Al toxicity at the cell cycle level.

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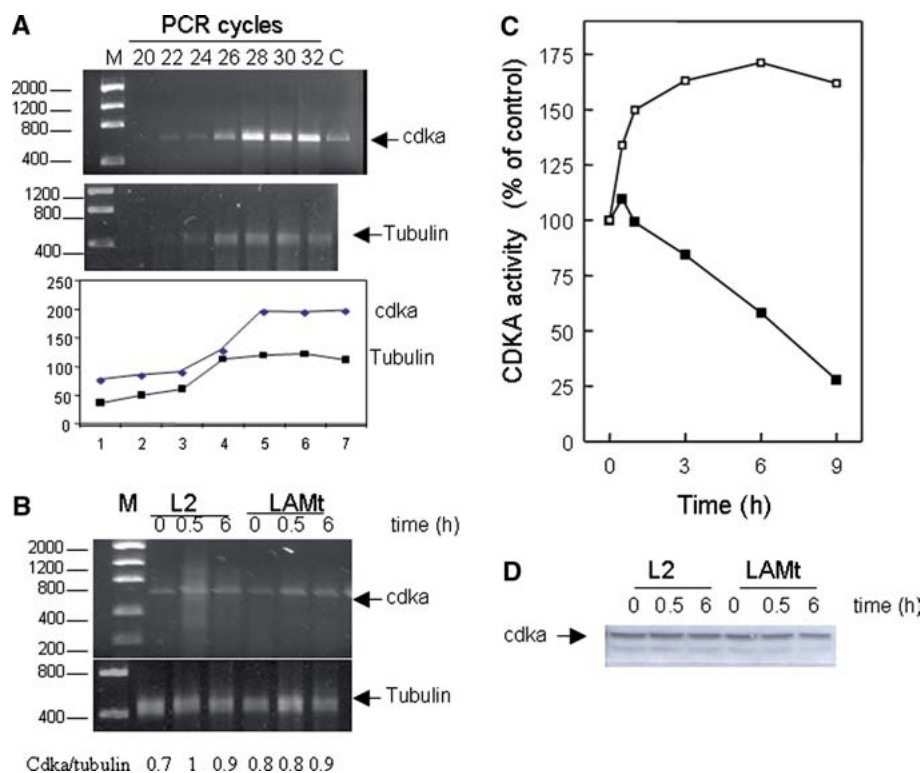


Figure 5. Effect of Al on *C. arabica* CDKA gene expression and CDKA activity. Cells from the 7th day of culture were incubated in the presence of 100 μ M AlCl₃ for different periods of time after which RT-PCR was performed according to *Materials and Methods*. **A.** Semi-quantitative RT-PCR for the CDKA and tubulin gene in the L2 cell line was run at different numbers of cycles. The graph shows the quantification of the bands that was carried out with NIH ImageQuant; **B.** 24 cycles of CDKA RT-PCR and 28 cycles for tubulin RT-PCR from the L2 and LAMt cell lines at different times of incubation with Al. The PCR of a plasmid containing the sequence of CDKA was used as a positive control C, and indicated with an arrow. **C.** CDKA activity was performed according to the technique outlined in *Materials and Methods*. Results represent the mean of three independent experiments expressed as percentage of CDKA activity in the absence of AlCl₃, which was considered as 100%. L2 and LAMt lines (closed and open symbols respectively). **D.** Western blot analysis of the CDKA protein as described in *Materials and Methods*.

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