

Aluminum Affects the Antioxidant Activity of Tobacco Cells and the Expression of Salicylate-Induced Protein Kinase (SIPK)

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Abstract

It has been shown that Aluminum (Al) toxicity results in over-production of reactive oxygen species (ROS). Despite the fact that ROS can induce MAPK activation, no direct genetic evidence has linked ROS-induced MAPK activation with Al toxicity. The effect of Al on the activation of antioxidant systems in connection with the expression of salicylate-induced protein kinase (SIPK), was examined in tobacco cells. Suspension-cultured tobacco cells (*Nicotiana tabacum* L. cv. 'Barley 21') were treated with or without 80 μ M Al. Certain parameters related to antioxidant activity were measured. A MAPKK inhibitor (PD98059) was also applied together with or without Al treatments and semi quantitative RT-PCR was applied to show the expression level of the SIPK gene. Treatment with Al rapidly increased the radical scavenging capacity of cells, catalase and superoxide dismutase activities, and expression of the SIPK gene, as compared to the control cells. In the presence of Al + PD98059, a decrease in catalase and superoxide dismutase activities was observed, compared to those cells which were treated only with Al. These results suggested that a short treatment with Al induced the activity of certain antioxidant enzymes in tobacco cells and that this response was mediated by a MAPK signal transduction pathway.

Keywords: aluminum, antioxidant system, MAPK cascade, Reactive Oxygen Species (ROS), Salicylate-Induced Protein Kinase (SIPK), tobacco cells

Introduction

Aluminum (Al) toxicity is the most widespread form of metal toxicity experienced by plants grown in acidic soils (Zhang *et al.*, 2010). The steps that initiate the signaling response of plants to Al are not known. Although it is assumed that Al exerts its toxic effect in the apoplast through interaction with the negative binding sites of the cell walls (Schmohl and Horst, 2000), at the cellular level plasma membrane is considered as the primary target of Al. Strong affinity of Al for bio-membranes and its interference with membrane lipids or membrane proteins can increase the membranes rigidity, which seems to facilitate the radical chain reactions mediated by Fe ions enhancing the production of reactive oxygen species (ROS), and peroxidation of membrane lipids (Cakmak and Horst, 1991; Yamamoto *et al.*, 2003).

It has been shown that Al can act upon the signal transduction pathway specifically reducing the activity of phospholipase C (PLC), consequently followed by inhibition of IP₃, regulation of calcium release and activation of protein kinases, e.g., MAPK (Jones *et al.*, 1995). It is now clear that heavy metals activate MAPKs in several plant systems. For example exposure of alfalfa (*Medicago sativa*) seedlings to excess Cu or Cd ions activated four distinct mitogen-activated protein kinases (Jonak *et al.*, 2004). Despite the

fact that ROS can induce MAPK activation, no direct genetic evidence has linked ROS-induced MAPK activation with Al toxicity. MAPK cascades minimally consist of a MAPKKK-MAPKK-MAPK module that is linked in various ways to upstream receptors and downstream targets. In tobacco the major ROS-induced MAPK is salicylate-induced protein kinase (SIPK) (Samuel and Ellis, 2002). In the present study rapid effects of Al on lipid peroxidation (LPO), radical scavenging capacity and activation of superoxide dismutase (SOD) and catalase (CAT), as well as the expression of SIPK in suspension-cultured tobacco cells, were investigated.

Materials and methods

Suspension-cultured tobacco (*Nicotiana tabacum* L. cv. 'Barley 21') cells were grown in a modified MS medium without glycine, and containing 3% (w/v) sucrose. The cells in their logarithmic growth phase were treated with or without 80 μ M aluminum in the form of AlCl₃, for 1 h. Potentially useful tools in the analysis of MAPK pathways are commercially available, specific inhibitors of certain steps in the pathway. Therefore, a group of cells were also treated with 80 μ M Al in the presence of 40 μ M PD98059, as a non-competitive inhibitor of activation of MAPKK. Al and PD98059 were added to the cell suspensions after

filter sterilization. After the desired periods of treatment, the cells were harvested, washed thoroughly with medium, frozen in liquid N₂ and kept at -80°C for further analysis.

Determination of the level of lipid peroxidation

The level of damage of membranes was determined by measuring malonyldialdehyde (MDA) as the end product of peroxidation of membrane lipids. The traditional method for MDA determination has been the thiobarbituric acid (TBA) assay owing to its simplicity. In this method, MDA reacts with TBA via an acid-catalyzed nucleophilic-addition reaction, yielding a pinkish-red chromophore with a maximum absorbance at 532 nm. However, TBA is not specific for MDA, and many other compounds e.g., simple carbohydrates and pigments which are present in many plant extracts, may interfere with the TBA assay. To avoid these interferences, improved methods have been developed (Du and Bramlage, 1992; Hodges *et al.*, 1999). A 0.5 g aliquot of frozen powder was added to 5.0 ml 0.1% (w/v) trichloroacetic acid and centrifuged at 10,000 ×g for 5 min. A 1 mL aliquot of supernatant was added to 1 mL 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid. The mixture was heated at 100°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000 ×g for 10 min, the A₅₃₂, A₆₀₀ and A₄₄₀ values of the supernatant were recorded. The value for nonspecific absorption at 600 nm was subtracted and a standard curve of sucrose (from 2.5 to 10 mmol mL⁻¹) was used to correct the results from the interference of soluble sugars in samples, reading A₅₃₂ and A₄₄₀. Malonaldehyde content was calculated using its absorption coefficient of 157 mmol⁻¹ cm⁻¹ and expressed as nmol g⁻¹FW.

Extraction and assay of the enzymes involved in ROS scavenging

Frozen samples (200 mg fresh weight) were homogenized in 3 mL HEPES-KOH buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 12,000 ×g for 20 min. All operations were performed at 4°C. In the supernatant, SOD was assayed by a photochemical method (Cakmak and Horst, 1991). The reaction mixture (3 mL) consisted of 50 mM HEPES-KOH buffer (pH 7.8), 0.1 mM EDTA, 50 mM Na₂CO₃ (pH 10.2), 12 mM L-methionine, 75 μM NBT, 300 μL enzyme extract and 1 μM riboflavin. One unit SOD activity was defined as the amount of enzyme required to result in a 50% inhibition of NBT reduction measured at 560 nm.

Activity of CAT was measured in a reaction mixture consisting of 25 mM Na-phosphate buffer (pH 6.8), 10 mM H₂O₂ and adequate amount of enzyme extract. The decomposition of H₂O₂ was followed by the decline in absorbance at 240 nm (Cakmak and Horst, 1991; Ghannati *et al.*, 2005). Protein contents were determined by the method of Bradford (1976), using BSA as standard.

Radical scavenging capacity

Evaluation of the total radical scavenging capacity of the cell extracts was conducted by using the stable 2,2'-diphenylpicrylhydrazyl radicals (DPPH). In this method, the hydrogen atom level (or electron-donating ability) is measured from the bleaching of the purple-colored ethanol solution of DPPH. One-half mL of various ethanol extracts were diluted 1/10 and were added to 2.5 mL of a 1 mM ethanol solution of DPPH. After 40 min of incubation at room temperature, the absorbance was read against a blank at 517 nm. The total capacity of scavenging of DPPH in percent was calculated as follows (Burits and Bucar, 2000; Stef *et al.*, 2009):

Total radical scavenging capacity (%) = (A blank – A sample)/A blank × 100.

RNA extraction and RT-PCR analysis

Total RNA was extracted according to the procedure previously described by Seyedi *et al.* (1999). Samples (ca. 2 g) were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was extracted in 4 mL of a buffer containing 50 mM Tris-HCl, pH 8.0, 4.0% (w/v) p-aminosalicylate, 1.0% (w/v) tri-isopropyl naphthalene sulfonate (sodium salt), and 2.0% (v/v) mercaptoethanol. The aqueous phase was blended with an Ultra-Turrax in an equal volume of phenol [containing 0.1% (w/v) hydroxyquinoline]: chloroform (1:1, v/v). The homogenate was partitioned by centrifugation at 5,800 ×g for 20 min. The total nucleic acids within the aqueous phase were precipitated by addition of 0.1 volume of 3 M sodium acetate and 2.2 volumes of ethanol. After 4 h at -20°C, the nucleic acids were centrifuged at 20,000 ×g for 30 min. The pellet was re-suspended in 500 μL of H₂O and LiCl was added to a final concentration of 2.7 M. The RNA was selectively precipitated at 4°C overnight and centrifuged in a microfuge for 10 min to collect the pellet. The RNA was washed sequentially in 3 M sodium acetate and 70% ethanol before drying and re-suspending in water. The RNA absorbance was scanned between 320 and 220 nm and quantified at 260 nm before storage at -70°C.

Semi-quantitative RT-PCR was performed with Accupower RT premix (Bioneer, Daejeon, Korea) following the manufacturer's protocol using gene-specific primers of *Nicotiana tabacum* SIPK (Seo *et al.*, 2007) (forward: 5'-GGA AGG CTG AGG CAA TAA CA-3', reverse: 5'-GTA CAA AGG GCA GGG ACG TA-3') and 18S rRNA from *Crocus sativus* (forward, 5'-TCC ACC ACC ACA GAG AGA GG-3', reverse 5'-GAC AGT GCT CCT CAG ATA AA-3') as a housekeeping gene.

Quantification of RT-PCR analysis was performed using UV tech software (version 12.5.0.0). Sequence analysis of the PCR products conducted using BLAST confirmed 100% similarity between PCR products and expected sequence of tobacco SIPK.

Statistical analysis

All of the experiments were carried out with at least three independent repetitions. All values are shown as the mean \pm SD. Statistical analysis was performed using Student's T-test and the differences between treatments were expressed as significant at level of $p \leq 0.05$.

Results and discussion

In comparison with the control cells, Al treatment rapidly and significantly lowered the level of LPO of tobacco cells (Fig. 1).

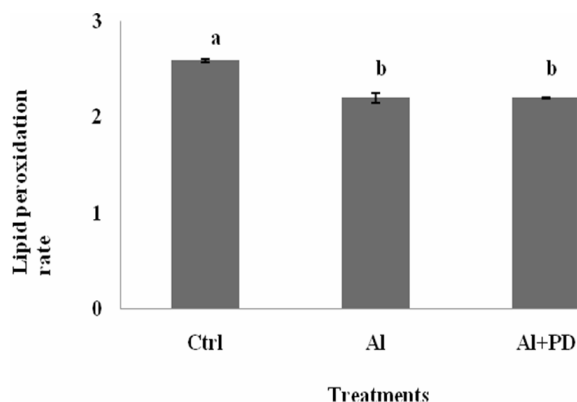


Fig. 1. Effect of Al on the rate of lipid peroxidation in suspension cultured-tobacco cells. Data are presented as the means \pm SD with $n = 3$. Bars with different letters are significantly different at $p \leq 0.05$, according to the Student's T-test

Treatment with Al for 1 h, also caused significant increase of SOD and CAT activity, which was accompanied by significant increase of total scavenging capacity of tobacco cells (Fig. 2).

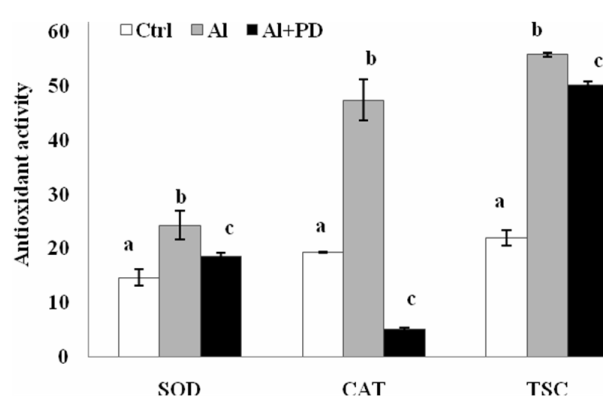


Fig. 2. Effect of Al on the antioxidant activity of tobacco cells. SOD was calculated as Unit/ mg protein, CAT as ΔA_{240} /mg protein, and TSC (total scavenging capacity) as % of scavenging DPPH. Data are presented as the means \pm SD with $n = 3$. Bars with different letters are significantly different at $p \leq 0.05$, according to the Student's T-test

Treatment of tobacco cells with Al, also increased the expression of the SIPK gene, compared to the control cells (Fig. 3).

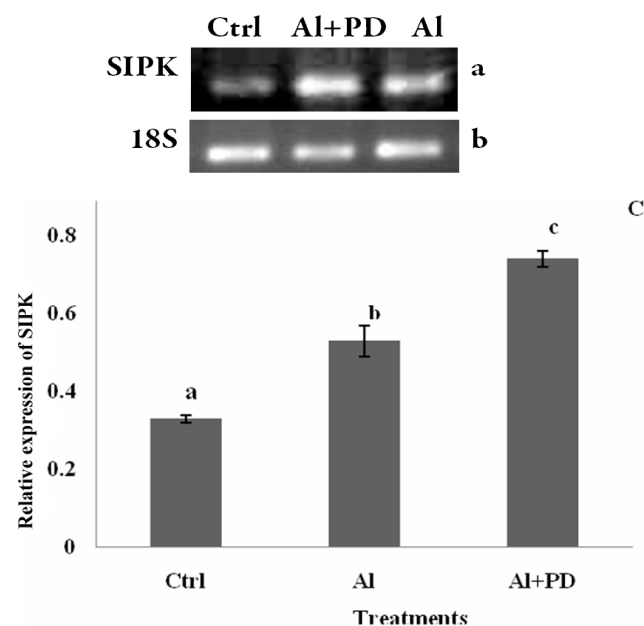


Fig. 3. RT-PCR analysis of the expression of the SIPK gene (a), in comparison with the 18S rRNA gene (b), in suspension cultured-tobacco cells, and quantification of the expression level using UV tech software (c). Data are presented as the means \pm SD with $n = 3$. Bars with different letters are significantly different at $p \leq 0.05$, according to the Student's T-test

The rate of membrane lipid peroxidation of the cells lowered by Al together with the MAPKK inhibitor PD98059 (PD), compared to that of the control cells, but there was no significant difference between the level of LPO in Al and Al+PD treatments (Fig. 1). Treatment of tobacco cells with Al+PD significantly decreased radical scavenging capacity, and the activities of SOD and CAT, which were increased by the treatment with Al alone (Fig. 2).

The relative expression of the SIPK gene in Al+PD treated tobacco cells was significantly higher than those of either control or Al-treated cells (Fig. 3).

Aluminum itself is not a transition metal and does not catalyze redox reactions; nonetheless, the involvement of oxidative stress in Al toxicity has been suggested (Ghanati *et al.*, 2005; Yamamoto *et al.*, 2003). Rapid and temporary induction of a 58 KDa protein kinase in response to a toxic concentration of Al has been monitored immunologically in suspension cultured-coffee cells (Arroyo-Serlalta *et al.*, 2005). Despite the fact that ROS can induce MAPK activation, no direct genetic evidence has linked ROS-induced MAPK activation with antioxidant enzymes activation due to Al stress. It is striking that so many stresses that elicit ROS accumulation in plant cells, consis-

tently appear to activate MAPK modules as one of their earliest effects. The MAPK signal transduction cascade is the route through which eukaryotic cells deliver extracellular messages to the cytosol and nucleus (Morris, 2001). A variety of stress responses have been found to involve the rapid activation of a specific subset of plant MAPKs, e.g. Arabidopsis MPK6, wound-induced protein kinase (WIPK) and SIPK in tobacco, and salt stress-induced MAPK (SIMK) in alfalfa (Cardinale *et al.*, 2000; Yuasa *et al.*, 2001; Zhang and Liu, 2001).

Reactive oxygen species production is closely related to the response of plants to heavy metals (Nagajyoti *et al.*, 2010). It is also clear that heavy metals activate MAPKs in several plant systems (Pitzschke and Hirt, 2006). Depending on heavy metal identity, four alfalfa MAPKs become activated in a complex pattern (Jonak *et al.*, 2004). In tobacco, the major ROS-induced MAPK is SIPK because one of the central functions of SIPK in the cell is to help monitor and regulate the cellular redox state. Activation of SIPK might play an important role in determining the response and ultimate fate of the stressed cells (Samuel and Ellis, 2002; Samuel *et al.*, 2005).

Al-induced oxidative stress has been most commonly attributed to alterations in membrane structure, which then favor radical chain reactions mediated by Fe, resulting in the formation of lipid peroxides (Yamamoto *et al.*, 2003). The rapid and significant increase of SOD activity in Al-treated tobacco cells was probably triggered to scavenge membrane-damaging superoxide radicals, which in turn resulted in the production of more hydrogen peroxide. Thus, the activation of CAT to detoxify excessively produced H_2O_2 is reasonable. Superoxide dismutase and CAT are supposed to be the key enzymes in detoxification of ROS; however, the involvement of other enzymes (e.g., glutathione reductase, ascorbate peroxidase), as well as nonenzymatic antioxidants (e.g. ascorbic acid, polyphenols, etc.) should not be overlooked. Although such components were not measured in the present experiments, the significant increase of total scavenging capacity of Al-treated tobacco cells implies that Al has triggered many nonenzymatic and enzymatic routes, resulting in lowering peroxidation of membrane lipids.

Increase of SIPK expression in Al-treated tobacco cells in comparison with the untreated (control) cells suggests that SIPK (and probably other members of MAPK cascade) are involved in transmitting the Al stress signal. Activation of SIPK both by harpin and direct oxidants was interrupted by pre-treatment with the MAPKK inhibitor, PD98059 (Samuel *et al.*, 2005). Use of this inhibitor with Al was appropriate to show the relationship between Al-induced SIPK expression and activation of antioxidant systems. The result presented here demonstrated that challenge with Al+PD98059 rapidly enhanced the expression of SIPK. The exact mechanisms by which inhibition of kinase activity of MAPKK influenced the expression of the SIPK gene, remains to be clarified, but it is likely that to-

bacco cells try to compensate the inhibition of the activity of an upstream protein in the MAPK cascade by increasing transcripts levels of a downstream module, SIPK.

Inhibition of the kinase activity of MAPKK in those cells treated with Al+PD98059 suppressed the activity of both SOD and CAT, an effect more pronounced regarding catalase activity. Time course monitoring of expression of the CAT gene in the presence of MAPK inhibitors, with or without Al, may help to clarify whether MAPK regulates ROS scavenging proteins at the transcriptional or postranscriptional level.

Most previous researches have shown adverse effects of Al on plant growth and enhancement of ROS. They attributed this to the existence of Al in acidic conditions as polyvalent cations which bind strongly to the negative charges in the cell (Poschenrieder *et al.*, 2008; Rbia *et al.*, 2011; Rengel and Reid, 1997; Zhang *et al.*, 2010). Previous study showed, however, that most of the supplied Al was absorbed by tobacco cells in MS medium, pH 5.5 (Shokuhi and Ghanati, 2007). It is generally assumed that under conditions with pH higher than 5.0, Al absorption would be limited, because Al forms insoluble species such as aluminum hydroxide. However, working on the effect of Al uptake on disease resistance of *Nicotiana rustica*, Zhang *et al.* (2010) showed that aluminum hydroxide also induced H_2O_2 accumulation through non-enzymatic and enzymatic regulation, ultimately resulting in resistance to tobacco wilt disease. Hydrogen peroxide is phytotoxic at higher concentrations but may function as a secondary messenger at low concentrations. Therefore, it may play two different roles in plant adaptation to both biotic and abiotic stresses: exacerbating damage or signaling the activation of defense responses (Dat *et al.*, 2000).

Conclusions

The results presented here showed that Al rapidly promoted the activity of ROS scavenging systems in tobacco cells, probably by production of low amounts of hydrogen peroxide, and that this effect was somehow mediated by a MAPK route.

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