

## Crosstalk of arabinogalactan protein, auxin, gibberellin, and callose in aluminum-treated Tea seedlings

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### Abstract

Arabinogalactan proteins (AGP) are a class of cell surface plant peptidoglycans which have been implicated in root elongation and signal transduction pathways. Aluminum promotes the elongation of tea (*Camellia sinensis* L.) roots. In this study the possible involvement of AGP in regulation of auxin, gibberellin, and callose contents after exposure of tea seedlings to 400  $\mu$ M Al was evaluated. The results show that the Al-induced elongation of tea roots was accompanied by significant increase of the AGP. Maximum contents of endogenous GA<sub>3</sub>, 270% of the control group were detected after 6 hrs. of the Al treatment. The content of IAA, rapidly (6 hrs. of the treatment) and remarkably declined due to Al to 50% of the control. In addition Al exposure for 6 and 24h periods decreased the callose content of tea seedlings by 20% and 50% of that of the control cells, respectively. However, Al treatment for 6 hrs. increased the gene expression (167% of the control), and the activity of  $\beta$ -1, 3-glucanase (150% of the control). The findings suggest a cross-talk between AGP, callose, and the two main growth hormones auxin and gibberellin in tea seedling which was started by Al exposure and resulted in elongation of the roots.

**Keywords:** Aluminum, Arabinogalactan proteins, Auxin; Callose, Gibberellin, Tea seedling.

### Introduction

Arabinogalactan proteins (AGP) are a class of cell surface plant peptidoglycans anchored to the cell membrane through a C terminal glycosylated phosphatidylinositol (GPI). Contribution of AGP in plant cell signaling pathways is evidenced by the fact that AGP is cleaved from its anchor by phospholipase C and releases into the periplasm as freely soluble form. Purified AGP from embryogenic cells can stimulate embryogenesis in non-embryogenic ones (Mallon *et al.*, 2013).

More studies, however, suggest that AGP plays an important role in cell wall loosening and plant cell growth. It has been suggested that in expanding cell walls, AGP can plasticize pectin and also may function as lubricating agents among cellulose microfibrils (Lampert and Varnai, 2012). Application of  $\beta$ -D-glycosyl Yariv reagent, which specifically crosslinks 1,3- $\beta$ -linked AGP, inhibited the cell elongation in carrot suspension cultures and Arabidopsis seedling roots (Ding and Zhu, 1997 ;Thompson and Knox, 1998).

The involvement of AGP in the functions of phytohormones has been suggested by the observation inducing that Yariv reagent inhibited GA-promoted induction of  $\alpha$ -amylase in barley (*Hordeum vulgare*) aleurone protoplasts (Suzuki *et al.*, 2002). The transcription level of AGP in *Cucumis sativus* hypocotyls increased not only in response to GA but

also to IAA. Application of Yariv reagent inhibited IAA/GA induced cell elongation in hypocotyls of *Cucumis sativus* and seedling roots of *Arabidopsis thaliana* (Park *et al.*, 2003; McCartney *et al.*, 2003).

Ionic aluminum (mainly Al<sup>3+</sup>) is highly toxic to plants particularly in acid soils. Low concentrations of Al<sup>3+</sup> rapidly inhibit root elongation, and restrict water and nutrient uptakes (Sun *et al.*, 2010). It has been accepted that Al affects microtubules and actin microfilaments in elongating cells of root apices (Sivaguru *et al.*, 2000). The callose deposition has been shown to be an early sign of Al toxicity in plants and is considered a reliable indicator of Al sensitivity (Smith *et al.*, 2011). Al induced callose formation has often been associated with inhibition of root growth. With respect to the critical role of auxin and gibberelic acid in plant cell growth, it is plausible that Al toxicity is mediated by its direct or indirect interactions with these hormones. Less inhibition of root elongation by Al<sup>3+</sup> was observed in Arabidopsis mutants of auxin polar transport; *pin2* and *aux1* than wild-type plants. Moreover, application of the auxin polar transport inhibitor, naphthylphthalamic acid, substantially alleviated the Al<sup>3+</sup>-induced inhibition of root elongation. (Sun *et al.*, 2010). Inhibition of root growth of Arabidopsis was attributed to the accumulation of auxin in the transition zone of the apex (Yang *et al.*, 2014). Disruption of polar auxin transport or signaling

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diminishes the effects of GA on root elongation (He *et al.*, 2012). Auxin promotes the GA-induced destabilization of some of the DELLA proteins, which plays a key integrative role in the phytohormone signal response network (Band *et al.*, 2011).

Arabinogalactan proteins are signaling molecules whose role in wall loosening is well documented. The mediators transmitting signals from plant hormones for cell elongation are well known as well. Tea (*Camellia sinensis* L.) is a major commercial crop whose root elongation is promoted by Al (Ghanati *et al.*, 2005). However the mechanisms and putative molecules mediating stimulatory effects of Al on this plant is not yet well understood. Therefore, the present study was undertaken in order to clarify possible involvement and cross-talk of AGP, IAA, and GA<sub>3</sub> in Al-induced elongation of tea seedling roots.

## Materials and methods

**Plant materials, analysis of cell lengths and Al content:** Tea seeds (*Camellia sinensis* L. cv Yabukita) were surface sterilized through subsequent washing with detergent, sodium hypochlorite (containing 5% active chlorine), EtOH 70%, and rinsing with distilled water. Then they were allowed to germinate in moistened vermiculite at 25 °C in darkness. After 20 days, the two-leaf seedlings were washed thoroughly and then transferred to continuously aerated one-fourth-strength Hoagland solution (pH 4.5), and the medium was renewed every 3 days. Since it has been shown that the response of the plant to Al may be related to the immobilization and detoxification of Al by phosphorus (Zheng *et al.*, 2005), P was omitted from the nutrient solution throughout this experiment. After 1 week the plants were treated with or without 400 μM AlCl<sub>3</sub> and harvested at 6, 24, 96, and 192h intervals. Previous reports have stated that 400 μM Al is the highest concentration at which the growth of the plant was not only continued but also was stimulated (Ghanati *et al.*, 2005). Free hand longitudinal sections of elongation zone (3 mm from tip) were obtained from 5 seedlings of each treatment and were studied under a light microscope equipped with a digital camera (BH2, Olympus, Tokyo, Japan). The length of at least 20 cells in each section was measured using Image J analysis software (1.4.3.67).

For determination of Al content of root apices, seeds and seed coats, the samples were ashed at 550 °C, dissolved in dilute HCl, and their Al content was determined by Eriochrom cyanide R at 584 nm by a spectrophotometer (Cintra 6, GBC, Melbourne, Australia) (Khanhuathon *et al.*, 2005).

**Biochemical analysis:** The segments of 30 mm from the root tips of the seedlings (30 tips for each treatment) were extracted in 3 mL of absolute MeOH on an ice bath overnight. Then the extract was concentrated via evaporation, and filtered by sterile syringe filter (0.22 μm). Aliquots (20 μL) of the filtrates were applied to HPLC (Knauer, Germany). The system was equipped

with a HPLC pump K1001 and a C18 column (Perfectsil Target ODS3, 5 μm, 250 × 4.6 mm, MZ-Analysentechnik, Mainz, Germany). Phytohormones were eluted at a flow rate of 1 mL min<sup>-1</sup> with a gradient of MeOH: acidic water (deionized water containing 0.67% acetic acid, pH 3.0). The gradient was as follows: 60:40 to 55:45 (10 min), to 80:20 (20 min) and to 60:40 (25 min). Gibberellic acid (GA<sub>3</sub>) and IAA were detected at 220 nm using an ultraviolet detector (PDA, Berlin, Germany) and were quantified by comparison of their retention times and peak area with genuine standards (Sigma) (Razifah *et al.*, 2014).

AGP were quantified by a colorimetric assay with Yariv reagent (Lampert, 2013). Ten root tips of each treatment were fine powdered by liquid nitrogen using a mortar and pestle and then were incubated in 2% w/v CaCl<sub>2</sub> for 2-3 hrs. at room temperature on a magnetic stirrer. Afterward, 200 μL of β-D-Glucosyl-Yariv reagent (1 mg/mL in 2% CaCl<sub>2</sub>) was added to tubes, mixed well, and incubated for 30 mins at room temperature. After centrifugation, the supernatant was discarded and the remaining cell wall pellet was washed twice with the 1 mL 2% CaCl<sub>2</sub>. One milliliter of NaOH (20 mM) was added to the salt-washed Yariv reagent-AGP complex and the absorbance was measured at 457 nm using Gum Arabic as a standard.

Callose content of root samples was quantified fluorometrically with excitation at 393 nm and emission at 484 nm, as described by Dahajipour *et al.* (2011). For the assay of β-1, 3-glucanase activity, the method of Miller (1959) was followed with some modifications. In brief, the samples were homogenized in sodium acetate buffer (50 mM, pH 5.5), followed by centrifugation at 15,000g for 30 mins at 4 °C. The supernatant was used for the enzyme assay. The reaction mixture contained 100 μL of 0.1% curdlan as a substrate and 100 μL of enzyme solution. The mixture was incubated at 35 °C for 2 hrs. The reaction was terminated by adding 2 mL of 3,5-dinitrosalicylic acid reagent. The absorbance was measured at 540 nm. One unit of β-1,3-glucanase activity was defined as the quantity required to release 1 μM of glucose per minute.

**Molecular analysis:** Total RNA was isolated with the Ribospin™ total RNA purification kit (GeneAll Biotechnology, Seoul, South Korea) and 2 μg of total RNA was used to generate a cDNA library using a first-strand cDNA synthesis kit (Fermentas; Canada) according to the manufacturer instructions. Quantitative real-time PCR (Applied Biosystem/MDS SCIEX, Foster City, USA) was used to analyze the expression of β-1,3-glucanase gene with iQ™SYBR® Green Supermix master mix. The primer was designed from the published sequence using the Oligo 5.0 software (Table 2). The following primer pairs were used to amplify the genes of interest: β-1, 3-glucanase 5'-TGCTGCAAGCTGCTTTAGAT - 3' & 5'-CCACGGTGTCTGGTTCATAG -3' and housekeeping gene GAPDH 5'-GGT GCC AAG AAG GTT GTC AT -3' & 5'-TAA CCT TAG CCA AGG GAG CA -3'. The

expression level of  $\beta$ -1, 3-glucanase was determined relative to the expression levels of GAPDH.

**Statistical analysis:** All experiments and observations were repeated three times with at least 30 segments of 30-mm root tips each. The experiment was structured following a Completely Randomized Design (CRD) with three replications. For all variables, analysis of variance (ANOVA) was performed. The significance of differences among treatment means were compared by Statistical analyses was done using SPSS software (version 16, Chicago, IL, USA). One-way ANOVA was used and a least significant difference (LSD) test was applied for multiple means' comparisons at a significance level of  $P \leq 0.05$ .

### Results

Regarding the high availability of Al in humus soils of tea fields, the presence of Al in tea seeds was plausible. As shown in Table 1, considerable amount of Al was found in tea seeds before any treatment ( $321.4 \text{ mg Kg}^{-1} \text{ DW}$ ). About 85% of seed Al content was accumulated in seed kernels, 55% of which was transferred to the emerging seedling. It was interesting that 79% of Al content of seedlings was found in their root tips (Table 1).

Aluminum content of root tips increased from  $115.8$  to  $177.6 \text{ mg Kg}^{-1} \text{ DW}$ , after 6 hrs. exposure of tea seedlings to  $400 \mu\text{M}$  Al. However exposure to Al up to 24 hrs. did not significantly increase the Al content of tea root tips, compared to 6 hrs. (Table 1).

Callose formation, activity and gene expression of  $\beta$ -1, 3-glucanase Microscopic observations of root tips following Image J analysis showed an average increase of  $20 \mu\text{m}$  in the longitudinal dimension of cells in the elongation zone of tea seedlings after 6 hrs. exposure to Al (Fig.1).

As shown in Fig. 2, exposure to Al reduced callose content of tea root tips, compared to the control group. Variations of the activity of callose degrading  $\beta$ -1, 3-glucanase enzyme and expression of its gene are depicted in Fig. 3. As shown, the activity and gene expression of  $\beta$ -1, 3-glucanase after 6 and 24 hrs. of Al treatment were significantly higher than the control groups.

Determination of AGP content of seedling root tips by Yariv reagent showed a significant increase in its amount after 6 hrs. Al treatment, as compared with the control group (Fig.4). However, the content of AGP in tea root tips decreased to 70% of the control after 24 hrs. treatment with Al (Fig.4).

Endogenous content of IAA drastically reduced to 50 % of the control, after 6 hrs. exposure to Al (Fig.5a). Although IAA content of Al-treated seedlings gradually increased after 6 hrs., it was still lower than the control (Fig.5a). Aluminum increased the GA3 content of tea seedling root tips to 200% of the control cells after 6 hrs. (Fig.). The content of GA3 in 24 hrs. Al-treated seedlings however, was identical to the control group (Fig.5b).

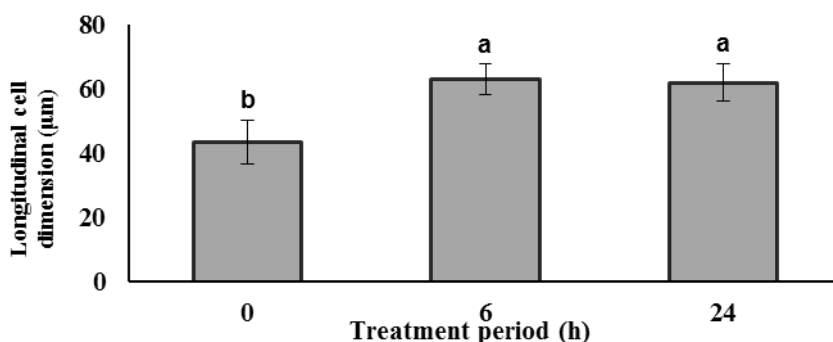
### Discussion

Growth of tea plants was clearly stimulated by Al. It is well known that Al alters a number of processes involved in regulatory pathways of the plant cell (Liu *et al.*, 2014). Treatment of tea seedlings with Al in the present study significantly increased the length of elongation zone cells, 6 hrs. of the treatment, while the tendency of cell elongation afterward was not remarkable. Signal transduction of developmental and environmental cues are perceived through a route of cell wall, plasma membrane, and cytoskeleton. The connections between plasma membrane and cell wall can be mediated by a number of proteins such as AGP, wall-associated kinase, endo-1-4- $\beta$ -D-glucanase, and cellulose synthase (Sardar *et al.*, 2006). Rapid response of elongation zone cells of tea seedlings to Al was accompanied by a significant rise of AGP at 6 hrs. and its decline at 24 hrs. Like other GPI anchored proteins, AGP contributes in signaling via different mechanisms among which cleavage of the anchor and releases of soluble AGP is the most important in transduction of the growth signal to the other cells (Showalter 2001). Other proposed mechanisms by which AGP contributes in growth include the direct transduction of a signal via its GPI anchor to downstream proteins and indirect interaction with them by triggering intracellular second messengers such as phosphatidylinositol and diacylglycerol by phospholipase cleavage of GPI anchors (Ruelland *et al.*, 2015; Saha *et al.*, 2016). Whether direct or indirect, increase of AGP may trigger the expression/regulation of the proteins involved in hormone signaling. It has been proposed that common hormone-mediated pathways are involved in modulating root growth triggered by Al in plants (Sun *et al.*, 2016), although the exact mechanism is yet to be clarified. In the present work the GA3 content was increased after 6 hrs. of the treatment of tea seedlings with Al while the auxin content declined. Gibberellin biosynthesis is transcriptionally controlled through the activity of the DELLA proteins, which act as negative regulators in the signaling pathway. Interestingly, the expression of two feedback-regulated genes i.e., GA20ox2 and GA3ox1, which conduct the terminal steps of GA3 biosynthesis, shows the largest changes in response to alterations in DELLA levels (Hedden and Thomas, 2012). From the results presented here it seems that binding of GA3 to its receptor, inhibited DELLA proteins and resulted in increase of GA3 contents of Al-treated plants. Another critical DELLA target gene is the gene encoding an Aux/IAA protein. The latter acts as a negative element in the auxin signaling pathway (Gallego-Bartolome *et al.*, 2011c). Inhibition of DELLA upon increase of GA3 in Al-treated seedlings resulted in increase of Aux/IAA protein and subsequent decrease of IAA.

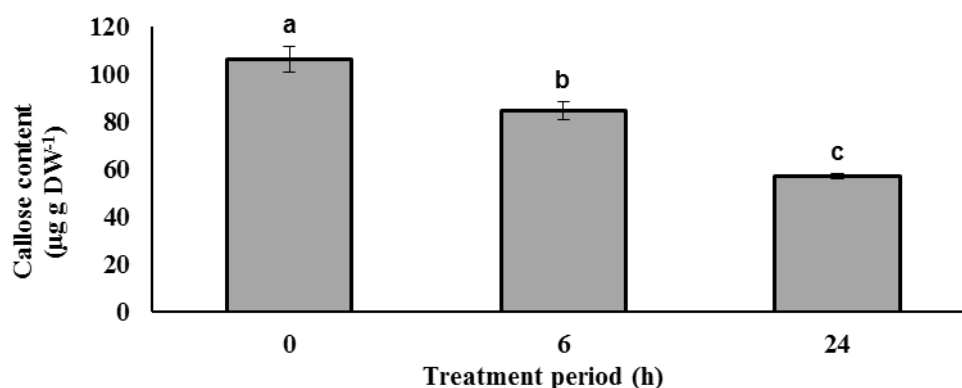
Previous studies have shown the involvement of AGP in cell wall assembly (Torode *et al.*, 2016). Direct evidence was provided by Vissenberg *et al.* (2001) where Yariv reagent inhibited cellulose deposition on the protoplasts of cultured tobacco cells and inhibited

**Table 1** Distribution of Al in the different parts of Tea seeds and seedlings before and after treatment with 400 Mm Al. Data present mean  $\pm$  SD, n = 6. Means with different letters are significantly different at  $P \leq 0.05$  by LSD test.

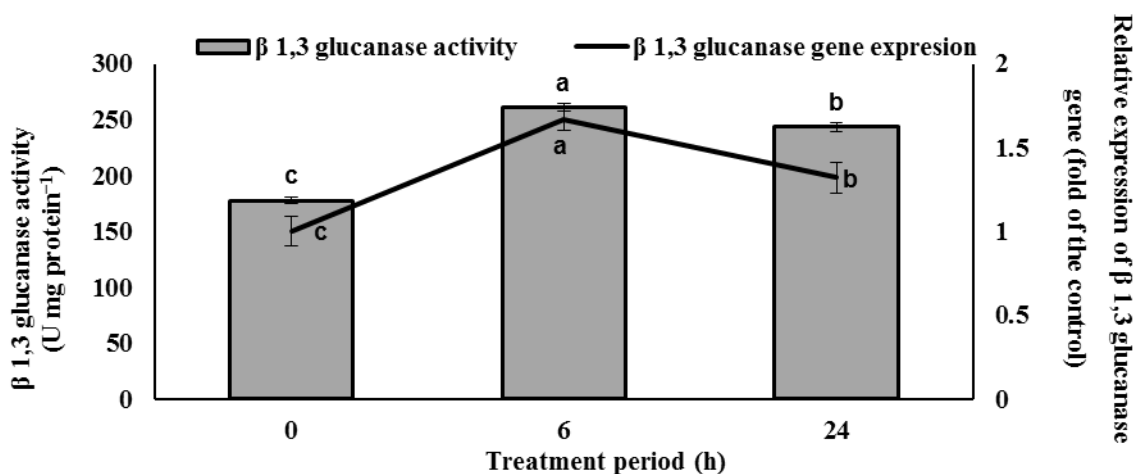
	Al content (mg/kg DW)
Seed	321.4 $\pm$ 25.5 <sup>a</sup>
Seed coat	48.7 $\pm$ 0.5 <sup>f</sup>
Seed kernel	272.6 $\pm$ 10.3 <sup>b</sup>
Seedling	148.9 $\pm$ 8.8 <sup>d</sup>
Root tip (0 Al/0h)	115.8 $\pm$ 9.4 <sup>e</sup>
Root tip (400 $\mu$ M Al/6h)	177.6 $\pm$ 7.9 <sup>c</sup>
Root tip (400 $\mu$ M Al/24)	183.2 $\pm$ 10.0 <sup>c</sup>



**Fig.1** The longitudinal dimension of cells in the elongation zone of tea seedlings before and after exposure to 400  $\mu$ M Al. Data present mean  $\pm$  SD, n = 300. Means with different letters are significantly different at  $P \leq 0.05$  by LSD test.



**Fig.2** Callose content of tea seedling root tips before and after exposure to 400  $\mu$ M Al. Data present mean  $\pm$  SD, n = 30. Means with different letters are significantly different at  $P \leq 0.05$  by LSD test.



**Fig.3** Effect of Al on the activity and gene expressions of  $\beta$ -1,3-glucanase in tea seedling root tips before and after exposure to 400  $\mu$ M Al. The expression of  $\beta$ -1,3-glucanase was analyzed by real-time qPCR, using GAPDH as an internal control. Data present mean  $\pm$  SD, n = 30. Means with different letters are significantly different at  $P \leq 0.05$  by LSD test.

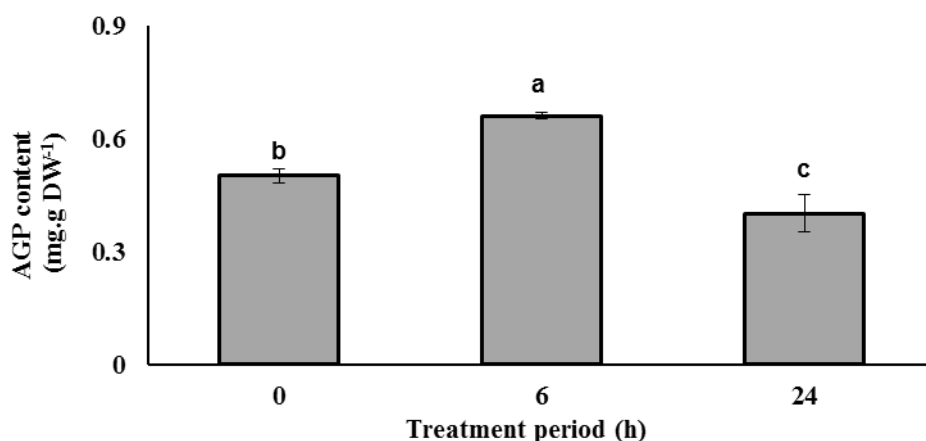


Fig.4 Arabinogalactan content of tea seedling root tips before and after exposure to 400  $\mu$ M Al. Data present mean  $\pm$  SD, n = 30. Means with different letters are significantly different at  $P \leq 0.05$  by LSD test.

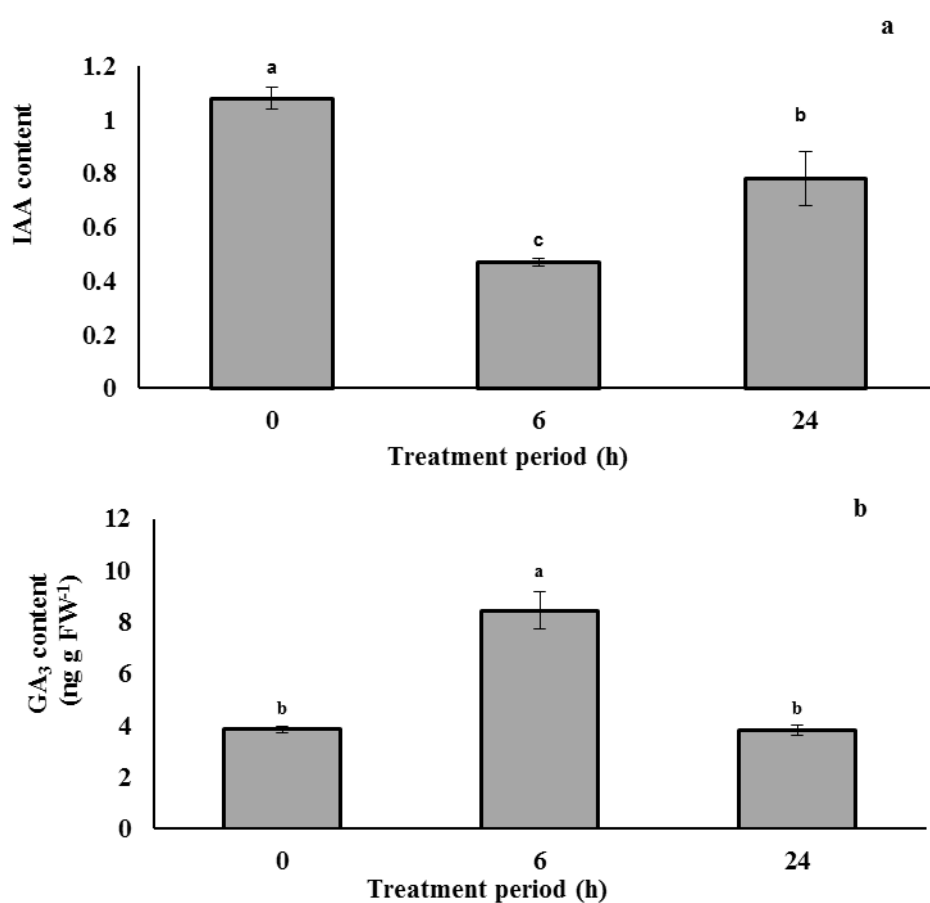


Fig.5 Endogenous contents of IAA (a) and GA<sub>3</sub> (b) of tea seedling root tips before and after exposure to 400  $\mu$ M Al. Data present mean  $\pm$  SD, n = 30. Means with different letters are significantly different at  $P \leq 0.05$  by LSD test.

the elongation of these cells. Applying microarray analysis in *Arabidopsis* cell cultures, Guan and Nothnagel (2004) found a high similarity between gene expression profile induced by Yariv treatment with those induced by wounding where the expression of both callose synthase and  $\beta$ -1, 3-glucanase genes were altered. Callose is also synthesized in response of Al by sensitive plants, and its content is closely and positively correlated with the degree of the growth inhibition

(Zhang *et al.* 1994; Horst *et al.*, 1997). Interestingly, in the present study, Al treatment reduced the content of callose in tea seedlings and it was accompanied by a remarkable induction of activity of  $\beta$ -1, 3-glucanase and the expression of its gene.

Altogether, the data presented here suggests that rapid elongation of tea seedlings in response to Al was mediated by AGP and its interactions with IAA, GA<sub>3</sub>, and callose.



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