Reduction of Al-induced oxidative damage in wheat

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Abstract

Aluminum stress inhibited root growth and caused serious oxidative damage in wheat (Triticum aestivum L.) seedlings. Under Al stress, added Ca significantly improved root growth, decreased Al, H2O2, and TBARS content and increased O2 generation, Ca content, improved CAT activity, and decreased OXO and FA-POX activity. Interestingly, Ca levels did not have any significant effect on SOD activity under Al stressed condition. Histochemical data also showed that added Ca decreased the accumulation of phenolic compounds synchronously with the reduction of Al and H2O2 content in the Al-stressed roots. Both biochemical and histochemical data revealed that added Ca reduced oxidative coupling reactions (i.e. cross-link formation) of phenolic compounds by decreasing FA-POX and thus improving the root growth of wheat seedlings under Al stress. The results suggest that the exogenously supplied Ca may be involved in alleviating Al-induced oxidative damage by regulating ROS level via antioxidant enzyme activities and improving root growth by displacing Al from the root surface.

Keywords: Aluminum, oxidative damage, H2O2, wheat, Triticum aestivum.

Abbreviations: CAT- catalase; FA POX- Ferulic acid peroxidase; SOD- superoxide dismutase, OXO- oxalate oxidase, ROS- reactive oxygen species.

Introduction

Aluminum toxicity is an environmental challenge (Delhaize and Ryan, 1995; Kochain, 1995; Ma, 2000) affecting a number of metabolic functions in plants through the generation of reactive oxygen species [ROS] (Darko et al., 2004; Kawano et al., 2004). The excessive generation of ROS including superoxide radical (O2−•), hydroxyl radical (•OH), and hydrogen peroxide (H2O2) is an unfortunate consequence of Al stress in plants (Boscolo et al., 2003; Cakmak and Horst, 1991). Impaired electron transport processes in mitochondria cause excess generation of ROS during Al stress (Yamamoto et al., 2002; Kobayashi et al., 2004). The activities of oxalate oxidase (Delisle et al., 2001) and NADH-dependent cell-wall peroxidase (Tamas et al., 2004) in the apoplast are also responsible for ROS generation. However, the generated ROS have the potential to interact non-specifically with many cellular components, triggering peroxidative reactions and causing significant damage to membranes and other essential macro-molecules such as lipids, protein and nucleic acids (Dat et al., 2000; Lin and Kao, 2000; Foyer et al., 1994). Therefore, ROS levels must be carefully monitored and controlled in cells. Plants possess antioxidant defense systems with enzymatic and non-enzymatic components, which normally maintain ROS balance within the cell. For instance, plants use a diverse array of enzymes like superoxide dismutases (SOD), oxalate oxidase (OXO), catalases (CAT) and peroxidases as well as low molecular mass antioxidants like ascorbate and reduced glutathione (GSH) to scavenge different types of ROS (Mittler, 2002; Pourtaghi et al., 2011). Generally SODs catalyze the dismutation of O2−• to H2O2 in different cellular compartments. But the generated H2O2 is also toxic to cells and has to be further detoxified by CAT and/or peroxidases to H2O and O2. Many investigators have reported that exogenously added Ca reduced Al toxicity (Kinraide and Parker, 1987; Kinraide et al., 1994; Taylor et al., 1988; Hossain et al., 2004) and supported the “Ca-displacement hypothesis” (Ryan et al., 1994). However, this hypothesis conflicts with the report of Schofield et al., (1998), who stated that Al induced root growth inhibition is signal-mediated rather than by Ca-displacement. Hence, causes of Al toxicity and its reduction by exogenous Ca supply is still being debated. However, no information is so far available on the generation of ROS as well as antioxidant enzyme activities during Al*−Ca2+ interactions in plants. This study was therefore designed to investigate influence of exogenous Ca supply on ROS generation, antioxidant enzyme activities and lipid peroxidation under Al stress and to elucidate the physiological mechanism of Al stress mitigated by Ca in wheat seedlings.

Results

Growth response of wheat seedlings

Root elongation was inhibited by aluminum (Table 1). After 24 h treatment with 50 µM Al, the root length at 250 µM Ca was 27% of its control. In contrast, the root length was 73% of the control when Ca supply was increased from 250 to 2500 µM. The root elongation inhibition was significantly mitigated by additional Ca supply during Al stress. 

Al and Ca content in the roots

Aluminum content in the roots decreased significantly when Ca supply was increased from 250 µM to 2500 µM (Table 1). Al content was very low in control roots without Al treatment.
In the absence of Al, Ca content in the roots increased with the increasing supply of Ca in culture solution. Under Al stress, Ca content in the roots decreased drastically and it was greater in the presence of 250 µM Ca than in 2500 µM Ca. The results indicated that increased Ca supply improved Ca content in root tissues.

**Effects of Ca on accumulation of Al and phenolic compounds in roots**

The accumulation of Al in the roots was observed using hematoxyline stain (Fig.1A). Aluminum was not accumulated in the control roots (data not shown). Staining with hematoxylin revealed that more Al accumulated in roots (detected by stain area and intensity) treated with 50 µM Al in the presence of 250 µM Ca. Both stain intensity and stain area were decreased significantly when Ca supply was increased from 250 to 2500 µM. The cross-section also showed that Al accumulated mainly in the epidermal and cortical cells in the root tissues. In the absence of Al, there was no difference in phenolic compounds accumulation between 250 to 2500 µM Ca treatments (Fig. 1B). Significant differences in phenolic compounds accumulation were observed between 250 and 2500 µM Ca treatments under Al stress. The results indicated that (i) Al induced phenolic compounds and (ii) additional Ca reduced Al-inducible phenolic compounds accumulation in the roots.

**In situ H$_2$O$_2$ detection**

*In situ* generation of H$_2$O$_2$ in Al-stressed and non-stressed wheat roots was detected using histochemical procedure (Fig. 2). The DAB detectable H$_2$O$_2$ was observed only in the Al treated roots and not in the control roots. The magnitude of H$_2$O$_2$ generation depended on the Ca concentration in the culture solution containing 50 µM Al. Reduced H$_2$O$_2$ generation was observed at the increased level (2500 µM) of Ca. According to Thordal-Christensen et al. (1997), DAB polymerizes as soon as it comes into contact with H$_2$O$_2$. In our study, the highest DAB polymerization was observed in roots at low level (250 µM) of Ca with 50 µM Al. The DAB reaction was reduced, when Ca supply was increased from 250 to 2500 µM.

**Oxidative damage**

Thiobarbituric acid reactive substances (TBARS) content is an established indicator of lipid peroxidation. Aluminum stress significantly increased lipid peroxidation as compared with the controls (Fig. 3). Again, when exogenous Ca supply was increased from 250 to 2500 µM under Al stress, inhibition of membrane lipid peroxidation was improved significantly.

**Enzymes activity in antioxidant systems**

Aluminum stress significantly increased SOD activity as compared to the controls (100%) (Fig. 4). In the absence of Al, SOD activity was slightly higher at 2500 µM Ca than at 250 µM Ca (data not shown). The results also showed that exogenously added Ca had no effect on SOD activity under Al stress. Oxalate oxidase is an important H$_2$O$_2$ generating enzyme in the extracellular compartments. Aluminum stress increased the OXO activity and addition of Ca decreased the activity. Catalase is an important H$_2$O$_2$ detoxifying enzyme in cellular environments. Its activity in the Al stressed roots decreased significantly in comparison to the controls. Exogenously added Ca improved CAT activity during Al stress. Aluminum stress considerably enhanced the enzyme activity of FA-POX and additional Ca decreased the activity.

**Discussion**

Root elongation was inhibited by Al and exogenously supplied Ca efficiently improved root growth in Al-stressed wheat seedlings (Rengel, 1992; Hossain et al., 2004). In the present study, the root elongation inhibition of Al-stressed wheat seedlings was significantly improved by the added Ca (Table 1). In addition, the exogenously supplied Ca alleviated Al-induced oxidative damage (Fig. 3) in the roots of wheat seedlings. Generally, oxidative damage occurred due to serious imbalance between ROS generation and their detoxification in the cellular environment (Mittler, 2002). Therefore, Al-induced oxidative damage is obviously related to Al-induced ROS imbalance, which might be attributed to excess ROS generation and/or low detoxification or by the action of both. In the present study, DAB-detectable H$_2$O$_2$ was visualized only in the Al treated roots (Fig. 2). Exogenously added Ca reduced the level of H$_2$O$_2$ and also reduced Al-induced oxidative damage (Fig. 3). The results suggest that reduced H$_2$O$_2$ yield may be one possible cause of alleviation of Al-induced oxidative damage by high exogenous Ca supply during Al stress, since Al stress-induced oxidative damage is associated with increased H$_2$O$_2$ generation (Ezaki et al., 2000; Hossain et al., 2005; Vranova et al., 2002). Similarly, Al-induced oxidative damage was observed in maize (Boscolo et al., 2003), pea (Kobayashi et al., 2004), soybean (Cakmak and Horst, 1991) and in tobacco cultured cells (Yamamoto et al., 1997). Al stress increased SOD activity (Fig. 4), which rapidly dismutated O$_2^-$ into H$_2$O$_2$ (Cakmak and Horst, 1991). The increased H$_2$O$_2$ generation during Al stress may therefore be the result of high SOD activity. However, the difference in H$_2$O$_2$ yields between 250 and 2500 µM Ca under Al stress could not be explained by the activity of SOD alone, because the difference in SOD activity (% of controls) between 250 and 2500 µM Ca was not significant under Al stress (Fig. 4).

**Table 1. Effect of exogenous Ca supply on root length and content of Al and Ca in seedling roots treated with 50 µM Al for 24 h.**

<table>
<thead>
<tr>
<th>Treatments (µM)</th>
<th>Root length (mm)</th>
<th>Al content (µg g$^{-1}$DW)</th>
<th>Ca content (µg g$^{-1}$DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al 0 + Ca 250</td>
<td>14.2 ± 0.7</td>
<td>45 ± 12</td>
<td>1094 ±112</td>
</tr>
<tr>
<td>Al 50 + Ca 250</td>
<td>3.8 ± 0.5</td>
<td>2135 ± 115</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>Al 0 + Ca 2500</td>
<td>15.3 ± 0.6</td>
<td>33 ± 10</td>
<td>2149 ± 92</td>
</tr>
<tr>
<td>Al 50 + Ca 2500</td>
<td>11.5 ± 1.0</td>
<td>1277 ± 125</td>
<td>205 ± 17</td>
</tr>
</tbody>
</table>

Values are means ± SE (n=6)
Fig 1. Light micrographs of (A) hematoxylin stained root tissues for detection of Al accumulation, and (B) toluidine blue stained root tissues for detection of phenolic compounds. (Note: A-Left shows Al accumulation in epidermal and cortical layer of roots; A-Right shows Ca has effectively displaced Al from the root tissues; B-dark blue indicates high phenolic compounds, aluminum treatments were performed in solutions containing only 250 and 2500 µM Ca)

This indicated that Ca might have some effect on other H$_2$O$_2$ metabolizing enzyme activities in addition to SOD activity. In this regard, OXO activity with increased Ca supply under Al stress was investigated, since Al enhanced OXO activity and increased H$_2$O$_2$ generation (Delisle et al., 2001). Significant decrease in OXO activity was observed at 2500 µM Ca in comparison with 250 µM Ca under Al stress (Fig. 4). Generally, OXO catalyzes oxidation of oxalic acid (stored as Ca salt) by molecular oxygen, yielding CO$_2$, Ca$^{2+}$ and H$_2$O$_2$ (Lane et al. 1993). Therefore, the increased Ca supply improved Ca-influx under Al stress (Table 1) and thus suppressed OXO activity (Fig. 4). CAT activity decreased under Al stress, but exogenous Ca supply elevated CAT activity significantly (Fig. 4). Increased Ca supply improves Ca-influx and the Ca$^{2+}$ ions may form complex with calmodulin (CAM) in the apoplasm as well as in the cytoplasm (Yang and Poovaiah, 2002). According to Yang and Poovaiah (2002), this Ca$^{2+}$/CAM complex binds to plant catalase and enhances its activity. Therefore, H$_2$O$_2$ level is decreased in Al-stressed seedlings by the increased supply of Ca. The reduced generation of H$_2$O$_2$ (Fig. 2) in the presence of 2500 µM Ca was in agreement with the above statement. High exogenous Ca supply during Al stress reduced the amounts of H$_2$O$_2$ in our present study (Fig. 2) and ferulic acid in our previous study (Hossain et al., 2006) and thus decreased FA-POX activity (Fig. 4) because they are substrates of FA-POX. The high Ca supply also reduced accumulation of phenolic compounds (Fig. 1B) and alleviated Al-induced inhibition of cell wall extension (Table 1). The results are in agreement with the findings of Tabuchi and Matsumoto (2001), who reported that Al stress increased phenolic compounds such as ferulic acid as well as its peroxidative product, diferulic acid in wheat roots. According to “Ca-displacement” hypothesis, Al displaces Ca from the common negative binding sites of root surface, particularly from the cell wall and plasma membrane and caused Al toxicity. Increased Ca supply decreased Al content and improved Ca-influx that was reflected by Ca content (Table 1) in the roots, due to ameliorant-toxicant competition (Kinraide, 1998; Hossain et al., 2004). In agreement with the statement, reduced Al accumulation (Fig. 1A) was also observed in the case of 2500 µM Ca supply. In considering the overall mechanism of action, it may be concluded that increased Ca supply not only alleviates Al-induced root growth inhibition but also protects membranes from oxidative damage by regulating ROS via antioxidant enzyme activities and/or by displacing Al from the root surface.

Materials and methods

Plant material

Seeds of wheat cultivar, Kalyansona, were surface sterilized with 1% NaOCl for 5 minutes, rinsed thoroughly with distilled water, and germinated on moist filter paper at 25 °C. Germinated seeds with uniform roots were transferred to plastic containers with 250 µM CaCl$_2$ plus other nutrients as described by Hossain et al., (2004). The seedlings were
**Fig 3.** Effect of Ca supply on lipid peroxidation in roots of Al treated wheat seedlings. (Note: Ca was supplied at 250 or 2500 μM during 24 h treatment with 0 or 50 μM Al; seedlings were grown in full nutrient solutions, except 250 μM Ca, at pH 5 for 48 h from germination; aluminum treatments were performed in solutions containing only 250 or 2500 μM Ca; values are means of three replicates)

**Fig 4.** Effects Ca supply on activity of antioxidant enzymes in wheat roots. (Note: Ca supply was 250 or 2500 μM; antioxidant enzyme activities were SOD, OXO, CAT and FA-POX under 0 or 50 μM Al stressed condition for 24 h; seedlings were pre-cultured for 48 h without Al; relative activity was calculated on the basis of the corresponding controls considered as 100; values are means of three replicates; error bars represent SE, with n=6)
cultured for 48 h at pH 5 in the growth chamber under a control environment as described by Koyama et al. (2001). The culture solution was renewed every 24 h. Each container had 4 x 6 plants and the pH of the nutrient solution was adjusted to 5.0 daily using 0.1M HCl.

**Treatments**

After 48 h, the seedlings were transferred to experimental containers with treatment solutions containing AlCl₃ and CaCl₂. In addition to the two elements, the pH of the nutrient solution was adjusted to 4.5 using HCl before transplanting. The experimental treatments consisted of two levels each of Al (0, 0.05 mM Al) and Ca (0.25 and 2.5 mM Ca). The experimental containers were arranged in a randomized complete block design in three replicates, with a total of 12 containers. After 24 h treatment, root lengths were measured, and 10-mm roots from the apical part were sampled to determine TBARS level and activities of antioxidant enzymes.

**Al and Ca contents**

After completion of root growth measurement, 10-mm root tips were cut and dried in an oven at 70-80 °C for 24 h and dry weights were determined. The Al content in roots was determined as described by Hossain et al. (2004). Briefly, 20 mg dry root samples were digested using 10 N H₂SO₄ and 30% H₂O₂, and Al in the samples was determined by pyrocatechol violet (PCV) method using a spectrometer (Kerven et al., 1988). Calcium was determined by atomic absorption spectrophotometry.

**Histochemical analyses**

The cellular H₂O₂ yield in intact roots was detected using DAB (3, 3'-diaminobenzidine tetrachloride) solution, as described by Thordal-Christensen et al. (1997) with slight modification (0.01% DAB). H₂O₂ was visualized as brown color at the site of DAB polymerization. Aluminum accumulation was detected by hematoxylin stain following the method of Hara et al., (1999). Transverse root sections of 40 µm thickness were obtained from a region 440-480 µm from the tip. The stained sections were mounted on glass slides and visualized under light microscope. Phenolic compounds in the treated roots were observed following the method of Mellersh et al. (2002). Briefly, roots were washed after 24 h treatment and stained briefly in a solution of 0.05% toluidine blue in 50 mM citrate buffer (pH 3.5). After staining, roots were observed under a light microscope.

**Lipid peroxidation**

Thiobarbituric acid reactive substances (TBARS) were estimated according to Hodges et al (1999). The reaction mixture in a total volume of 4 ml containing 1 ml of extracts and 3 ml of 2% (w/v) TBA (2-thiobarbituric acid) made in 20% trichloroacetic acid (TCA), was heated at 95 °C for 30 min and quickly cooled on ice. After centrifugation at 10,000×g for 10 min, the absorbance of the supernatant was measured at 532 nm. A correction for non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The lipid peroxides were expressed as nmol TBARS g⁻¹ FW using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

**Preparation of extracts for enzymes assays**

Fresh roots samples (10-mm) were used for enzyme assays. Root samples (g/10 ml) were homogenized in a chilled pestle and mortar with extracting buffer including 1 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 15,000×g for 20 min at 4 °C. The supernatant was used for enzyme activity and protein content assays. An aliquot of the extract was used to determine its protein content by the method of Bradford (1976) utilizing bovine serum albumin as the standard. All spectrophotometric analyses were conducted on a Hitachi (U-2000) spectrophotometer.

**Superoxide dismutase (SOD) activity**

Total SOD activity was measured according to the method of Beyer and Fridovich (1987).

**Oxalate oxidase (OXO) activity**

OXO activity was performed according to the method described by Hu et al. (2003) with slight modification. Briefly, the assay mixture (2 ml) containing 50 mM Na succinate (pH 3.5) and 50-100 µg protein was incubated at 37 °C. The reaction was started by adding 10 mM oxalic acid. The absorbance of the solution was measured at 550 nm.

**Catalase (CAT) activity**

CAT activity was measured according to Chance and Herbert (1950).

**Ferulic acid peroxidase (FA-POX) activity**

Ferulic acid peroxidase (FA-POX) activity was measured using the procedure of Sanchez et al. (1996) with slight modification. The 2 ml reaction mixture contained 90 mM sodium phosphate buffer (pH 4.0-5.5), 0.5 mM H₂O₂, 40 µM ferulic acid and 10 µg enzyme extract. The increase of absorbance due to oxidation of ferulic acid was measured at 310 nm. In each case, relative enzyme activity was calculated on the basis of corresponding control values, considered as 100.

**References**


Cakmak I, Horst WJ (1991) Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean (Glycine max) Physiol Plant 83: 463-468
