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Changes in antioxidant gene expression and induction of oxidative stress in pea (*Pisum sativum* L.) under Al stress

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Abstract Aluminium toxicity has been recognized as a primary growth-limiting factor in acid soil, resulting in a decrease in plant growth and production. In this experiment we have studied the induction of oxidative stress and changes in antioxidant gene expression in pea (var. ALASKA) under aluminium (Al) stress. We have found that Al treatment affected the growth of pea plant and induced oxidative stress with a change in antioxidant gene expression profile. While the expression of glutathione-s-transferase (*GST*) and catalase (*CAT*) was more in root, cytosolic Ascorbate peroxidase (*cAPX*) expression increased in shoot under aluminium stress. Copper- Zinc Superoxide dismutase (Cu-Zn SOD) gene expression was higher after 24 h but decreased after 48 h along with elevated expression of manganese superoxide dismutase (*MnSOD*) and iron-superoxide dismutase (*FeSOD*) at higher aluminium contentrations after 24 and 48 h. Aluminium stress elevated hydrogen peroxide (H_2O_2) level and affected the growth. The proline content did not change significantly, whereas glutathione content increased with a decreased ascorbate content under Al stress. The present study indicates that aluminium treatment affected the antioxidant gene expression and induced oxidative stress in pea plant.

Keywords Aluminium · Oxidative stress · Antioxidants · Pea · Gene expression

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Introduction

Aluminium (Al) being the third most abundant metal in the earth's crust poses a serious threat to crop productivity in acid soils, which comprise almost half of the arable land (Panda and Matsumoto 2007; Panda et al. 2009). Aluminium is a light metal that makes up 7% of the earth's crust, occurring as harmless oxides and aluminosilicates. Nevertheless, if the soil pH becomes acidic, as is now the case on 40% of the arable lands in the world, Al is solubilised into toxic forms, such as $[Al(H_2O)_6]^{3+}$, generally referred to Al^{3+}. Aluminium toxicity has been recognized as a primary growth-limiting factor in acid soil, resulting in a decrease in plant growth and production (Kochian 1995). Due to the agronomic importance of Al^{3+} toxicity, it is necessary to improve the Al tolerance of crops, which is possible using conventional plant

breeding technology (Pinto-Carnide and Guedes-Pinto 2000; de Camargo and Filho 2001; Poschenrieder et al. 2008) or biotechnological methods, including in vitro cell and tissue cultures (Arihara et al. 1991), cytogenetic approaches (Miller et al. 1997) and the creation of transgenic plants, for example by the overexpression of the citrate synthase gene (CSb) (de la Fuente et al. 1997).

The toxic effects of aluminium begin in plant roots within minutes of exposure and include root growth inhibition, callose accumulation, cytoskeletal damage, plasma membrane rigidification, alterations of the membrane surface charge, membrane lipid peroxidation, imbalance in Ca^{2+} homeostasis, induction of oxidative stress in plant mitochondria, opening of mitochondrial membrane permeability transition pores causing high-amplitude swelling of mitochondria, collapse of inner membrane potential, and several other bioenergetic alterations resulting in cell death (Panda and Matsumoto 2007; Poschenrieder et al. 2008; Panda et al 2009).

In plants, ROS are produced continuously as by product of various physiological and metabolic pathways, such as photosynthesis, photorespiration and CO_2 assimilation (Foyer and Noctor 2000). Furthermore, ROS production is increased by several environmental stress, such as exposition to high levels of light, drought, heavy metals, salt, temperature extremes, air pollution, UV radiation, herbicides and pathogen attacks (Shao et al. 2008). Whether ROS will act as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time. To test the induction of oxidative stress in an Al-tolerant pea plant and simultaneous changes in antioxidative gene expression pattern, the present investigation was undertaken.

Materials and methods

The variety of pea which was selected for the experiment was Al-tolerant variety—Alaska. Pea seeds were surface sterilized in 0.1% mercuric chloride for 5 min and washed in sterile distilled water and plated in petriplates moistened with distilled water. Pea (*Pisum sativum* L. cv Alaska) seedlings (4 d after germination) were treated with $AlCl_3$ (0, 10, 50 μM) in an aerated 100 μM $CaCl_2$ solution, pH 4.75, for up to 24 h at 25°C under a 12-h photoperiod. Root elongation during Al treatment was determined as described (Yamamoto et al. 2001).

Quantitation of Al

Plant tissue was digested with acids, and the concentration of Al was determined using an atomic absorption spectrophotometer with graphite furnace atomizer (model Z-9000; Hitachi, Tokyo) as described previously (Yamamoto et al. 2001).

Determination of hydrogen peroxide content

Hydrogen peroxide (H_2O_2) content was measured according to Sagisaka (1976). One gram of root tissue was homogenized in 5% trichloroacetic acid (TCA) and the homogenate was centrifuged at 17,000*g* at 0°C for 10 min. The reaction mixture contained 1.6 ml of supernatant root extract, 0.4 ml TCA (50%), 0.4 ml ferrous ammonium sulfate and 0.2 ml of potassium thiocyanate. The absorbance was recorded at 480 nm.

Determination of Proline content

Proline concentration in pea was determined following the method of Bates et al. (1973). Leaf sample (0.5 g) was homogenized with 5 ml of sulfosalicylic acid (3%) using mortar and pestle and filtered through Whatman No. 1 filter paper. The volume of filtrate was made up to 10 ml with sulfosalicylic acid and 2.0 ml of filtrate was incubated with 2.0 ml glacial acetic acid and 2.0 ml ninhydrin reagent and boiled in a water bath at 100°C for 30 min. After cooling the reaction mixture, 6.0 ml of toulene was added and after cyclomixing it, absorbance was read at 570 nm.

Measurement of antioxidant enzyme activity

Plant tissue was homogenized with phosphate buffer, pH 6.8 (0.1 M) in pre chilled mortar and pestle. The extract was centrifuged at 4°C for 15 min at 12 000 *g* in a cooling centrifuge. The supernatant was used for the assay of guaiacol peroxidase (GPx, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1) and glutathione reductase (GR, EC 1.6.4.2). The GPx activity was assayed as per the method of Chance and Maehly (1955). The 5.0 ml mixture comprised of 3.0 ml

phosphate buffer (pH 6.8), 1 mL (30 mM) H_2O_2 and 1 ml enzyme extract. The 3.0 ml reaction mixture comprise of 0.1 M phosphate buffer (pH 6.8), guaiacol (30 mM), H_2O_2 (30 mM) and 0.3 ml enzyme extract. The rate of change in absorbance at 420 nm was measured. The GPX activity was expressed as μmol (H_2O_2 destroyed) g^{-1}(f. m.) min^{-1}. The assay of SOD was done as per the method of Giannopolitis and Ries (1977). A total of 3 ml assay mixture for SOD contained 79.2 mM Tris-HCl buffer (pH 6.8), containing 0.12 mM EDTA and 10.8 mM tetraethylene diamine, bovine serum albumin (0.0033%), 6 mM nitroblue tetrazolium (NBT), 600 μM riboflavin in 5 mM KOH and 0.2 mL enzyme extract. Reaction was initiated by placing the glass test tubes in between two fluorescent tubes (*Philips 20 W*). By switching the light on and off, the reaction was started and terminated, respectively. The increase in absorbance due to formazan formation was read at 560 nm. Under the above condition, the increase in absorbance in the absence of enzyme was 100% and 50% initial was taken an equivalent to 1 unit of SOD activity. Glutathione reductase (GR) was assayed by the method of Smith et al. (1988). The reaction mixture contained 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM 5,5′-ditiobis-2 nitrobenzoic acid (DTNB) in 0.01 M potassium phosphate buffer (pH 7.5), 2 mM NADPH, 1 ml enzyme extract and distilled water to make up a volume of 2.9 ml. Reaction was initiated by adding 2 mM oxidized glutathione or glutathione disulphide (GSSG). The increase in absorbance at 412 nm was recorded at 25°C over a period of 5 min. The activity is expressed as ΔA412 g^{-1} (f. m.) s^{-1}.

Metabolite determination

The extraction and estimation of glutathione was done according to Griffith (1980) method. The root tissue was homogenized in 5 % (m/v) sulfosalicylic acid and the homogenate was centrifuged at 10,000*g* for 10 min. A total of 1 ml supernatant was neutralized with 0.5 ml of potassium phosphate buffer (pH 7.5). Total glutathione content was measured by adding 1 ml neutralized supernatant to a standard solution mixture consisting of 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing 1 ml EDTA, 0.2 ml of 6 mM 5,5′dithiobis (2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH and 0.1 ml of 1 U yeast GR Type-III (Sigma Chemical, St. Louis, USA). The change in absorbance was measured at 412 nm and followed at 25 ± 2°C until the absorbance reached 0.5 units. The ascorbate extraction and estimation was done by the method of Oser (1979). The reaction mixture contained 2 ml 2 % Na-molybdate, 2 ml 0.15 M H_2SO_4, 1 ml 1.5 mM Na_2HPO_4 and 1 ml root tissue extract. It was mixed and incubated at 60°C in water bath for 40 min, then cooled, centrifuged at 3,000*g* for 10 min and absorbance was measured at 660 nm.

Antioxidant gene expression

Frozen root and leaf tissue (approximately 100 mg) was ground thoroughly in liquid nitrogen using a mortar and pestle. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). The concentration of the RNA was determined spectrophotometrically at 260 nm (NanoDrop). The RNA purity was also checked spectrophotometrically by means of the 260/280 ratio and also electrophoretically. First strand cDNA synthesis was primed with an oligo(dT)16 primer according to the manufacturer's instructions using Superscript reverse transcriptase reagents (Invitrogen), and equal amounts of starting material (RNA) were used (1 μg). Quantitative PCR was performed with the ABI Prism 9600 (Applied Biosystems), thermal cycler. The following genes were amplified: *CAT,* cAPX, MnSOD, FeSOD, CuZn-SOD and GR. As a reference gene *18S rRNA* was used. PCR amplifications were performed in a total volume of 50 μl containing 1 μl cDNA sample, 5 μl 10× buffer, 4 μl dNTPs, 1 μl Forward and Reverse primers, 01 μl ex Taq DNA Polymerase and rest RNase free water. (Table 1) All samples were tested in duplicate for the housekeeping gene as well as for the genes of interest. For Real time PCR analysis appropriate primers (see table) were used to score 500 bp partial fragments. Quantitative PCR (Q-PCR) was performed on the Light Cycler instrument with the Light Cycler Fast Start DNA Master SYBR Green 1 kit (Roche) for the amplification of *CAT, cAPX, MnSOD, FeSOD, CuZnSOD and GR* or *18 S r RNA* according to the manufacturer's protocol. Gene expression data were calculated relative to the reference gene, i.e. 18srRNA (Livak and Schmittgen 2001).

Table 1 Primer sequences for amplification of genes in RT-PCR and Real Time PCR of Pea

Gene	Genebank accession no.	Forward (5′–3′)	Reverse (3′–5′)
CAT	AB087838.1	CTATTGGAAGATTATCATCT	AGAATTCTTGATTTCTTCTA
cAPX	X62077	TGGCACTCTGGGTACTTT	GATTTGAGGGACCATGGACT
MnSOD	U30841	ACGCTCCCGGTCTCGCTTA	AGAGGCTTGTGGTTGAAACC
Cu,Zn-SOD	AB189165	AGTCAGGAGGGAAATGGTCC	AAACTACTGGAAATGCTGGT
FeSOD	AJ496175	CTGATCTAGAGGGAAGTCA	TGTATGGGAGCATGCTTACT
GST	AB087837	ATCACTAGAAATTACAAGGG	TTCCGTTCGTTGAAAGATTC
18srRNA	AY143480	AGTTTGAACACATGCGGTGG	TTTCGTCTCATGGTTGGTTG

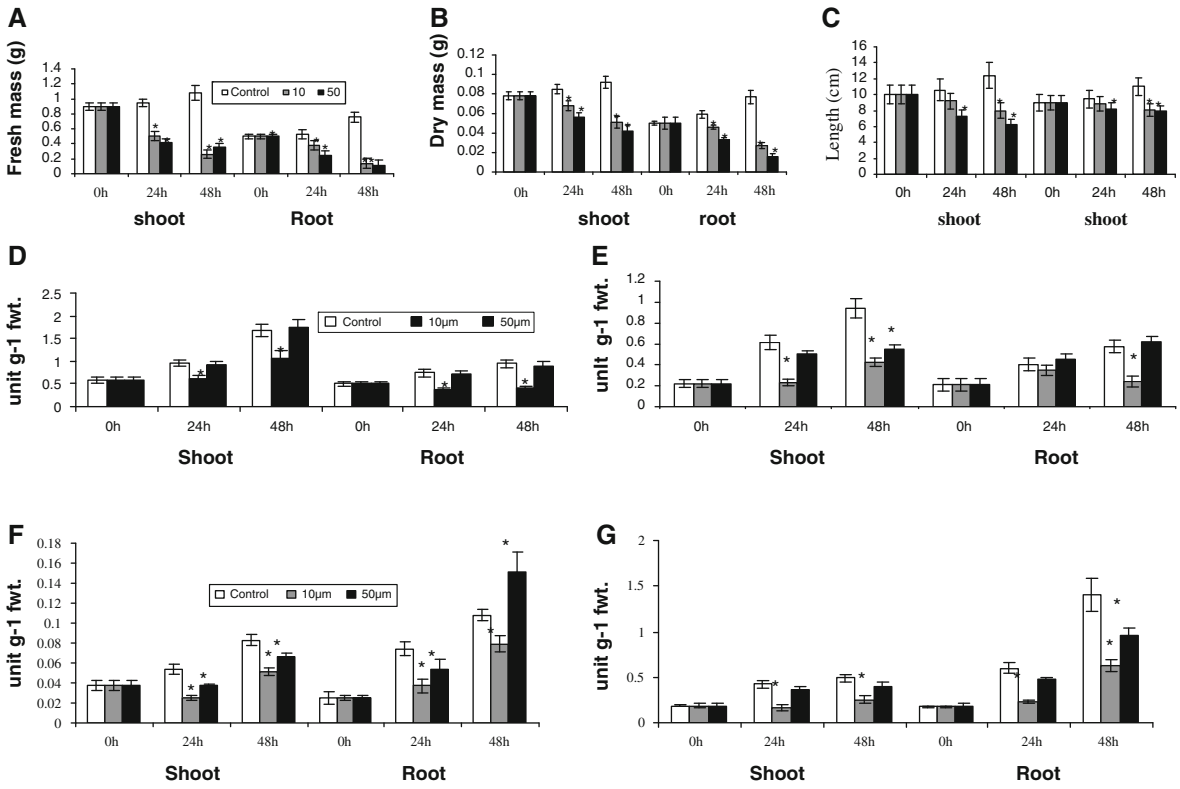

Fig. 1 Changes in fresh (**a**) and dry mass (**b**) and length (**c**) in root and shoot of *Pisum sativum* L. seedling after 0, 24 and 48 h of Al treatment[control(0), 10 and 50 μM]. Changes in activities of APX (**d**), CAT (**e**), GR (**f**) and SOD (**g**) in root and shoot of *Pisum sativum* L. seedling after 0, 24 and 48 h of Al treatment [control (0), 10 and 50 μM]. Data presented are mean ± SE. * Indicates significant difference from control at $P < 0.05$ by Tukey test

Statistical analysis

Each experiment was repeated thrice and data presented are mean ± standard error (SE). The results were subjected to ANOVA using GLM factorial model on all the parameters. Tukey test was used for comparison between pairs of treatments. The data analysis was carried out using statistical package, SPSS. 10.

Results and discussion

Growth changes under Al stress

Aluminium toxicity affected growth (Fig. 1a–c) which has been established in terms of root and shoot length of growing pea seedling as reported in a variety of other plants such as rice, wheat (Ryan et al. 1992), soybean (Hai et al. 1989) etc. We also

recorded a decrease of fresh and dry mass of root and shoot under Al treatment besides root growth inhibition which was correlative to the Al-uptake data and which is the characteristic of Al toxicity (Panda et al. 2009).

Changes in gene expression profile of major antioxidant genes under Al stress in pea

Genetic studies in seedlings of *Arabidopsis thaliana* and wheat (*Triticum aestivum*) and in cultured tobacco cells also suggest a link between Al stress and oxidative stress, since Al induces the expression of several genes that are also induced by oxidative stress. Some of the genes encode antioxidant enzymes (eg.:- GST, GPX, CAT, SOD) commonly induced by Al treatment and oxidative stress (Ezaki et al. 2000; Rodriguez-Milla et al. 2002). In the present study *GST* expression was seen more in root than in shoot (Figs. 3a, 4). In root under 10 μM Al, *GST* was almost same as that of control but in 50 μM Al *GST* increased five fold after 24-h suggesting the effect of Al-stress, though the increase fell by two fold after 48 h. The root showed two and three fold increase in *CAT* expression after 24 h and 48 h respectively in 50 μM-Al which was negligible in shoot (Figs. 3b, 4a) after 48 h of 50 μM-Al treatment, though after 24 h it showed two fold increase indicating the involvement of *CAT* in controlling damage caused by H_2O_2. In shoot 10 μM-Al showed considerable increase in expression specially after 24 h compared with 50 μM Al. But in root there was no significant change observed except in 50 μM after 48 h, which showed almost three fold increase in its expression. *cAPX* expression was increased in both the Al concentrations in shoot (Figs. 3c, 4). Based on metal cofactor, *SOD*s are classified as *Cu-Zn SOD*, *Mn SOD* and *Fe SOD* (Alscher et al. 2002). In root after 48 h the *Cu-Zn SOD* (Figs. 3b, 4) showed decreased expression, whereas after 24 h 50 μM Al showed much high expression. *Fe-SOD* (Figs. 3c, 4) showed gradual increase in its expression after 24 and 48 h of treatment. In case of *MnSOD* (Figs. 3b, 4) only 50 μM treatment showed elevated expression of the gene. After 24 h, at 10 and 50 μM Al, *Cu-ZnSOD*, *MnSOD* expression increased whereas at only 10 μM *FeSOD* showed increased expression in shoot (Fig.3c,4). But after 48 h except 10 μM of Al all the other treatments showed low *FeSOD* expression. The increase in these different types of SODs suggests their involvement in defense mechanism under oxidative stress generated in the cell under Al stress characterizing the Al-tolerant Pea variety. (Richards et al. 1998; Ushimaru et al. 1999). The changes in expression of *SOD*s are also studied in other plants like *Arabidopsis thaliana* (Attia et al. 2008). Basu et al. (2001) overexpressed *MnSOD* in *Brassica napus* and found a modest Al-resistance. The gene expression pattern of oxidative stress related enzymes were also studied in *Citrus sinensis* (Peroni et al. 2007). These genes encode peroxidase (*POX*), glutathione- Stransferase (*GST*), blue copper-binding protein (*BCB*) and Bowman-Birk protease inhibitor (Richards et al. 1998), and some of them (*POX, GST, BCB*) have antioxidant functions. Furthermore, the transformation and overexpression of these antioxidant genes in *A. thaliana* improves root growth under Al exposure (Ezaki et al. 2000).

Changes in activities of major antioxidant enzymes under Al stress

Changes in activities of major antioxidant enzymes in growing pea seedlings under Al stress were significant. Glutathione reductase activities increased in root cells whereas the same was decreased in shoot of pea plant under Al stress (Fig. 1f). Al stress caused decline SOD activities in root and shoot of growing pea seedling relative to control as depicted in Fig. 1g. Comparing with control plants APX activities showed significant decline at 10 μM in roots and shoot after 24 and 48 h of stress imposition (Fig. 1d) but at 50 μM Al stress APX activities did not show any significant changes. As shown in Fig. 1e, Al stress caused decrease in CAT activities in shoot whereas it did not change significantly after 48 h of Al treatment. Peroxidase (POX) activities increased in both root and shoot of Al stressed pea seedling (Fig. 2a). The activity of CAT (Fig. 1e) here decreased due to Al-treatment, is a signal of stress imposed by ROS formation i,e, excess of H_2O_2 accumulation might have created this situation. Only in case of 50 μM Al treatment root tissue showed increased activity. Darko et al. (2004) showed that increased activity of CAT in Al-tolerant wheat plants may contribute to the intensive detoxification of Al-induced ROS. However, in rice seedling decline in CAT activity was noticed with 80 μM-Al (Sharma

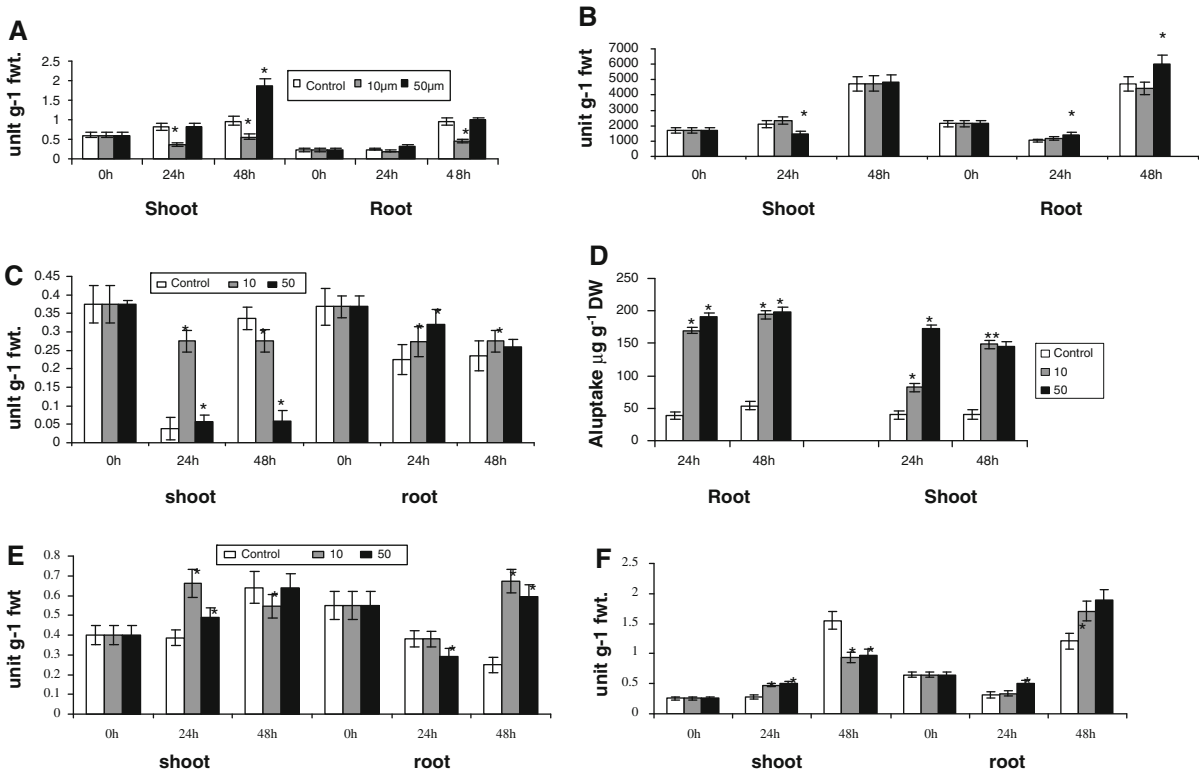

Fig. 2 Changes in activities of GPX (**a**), glutathione content (**b**), H_2O_2 content (**c**), Al uptake (**d**), total ascorbate (**e**) and proline content (**f**) in root and shoot of *Pisum sativum* L. seedling after 0, 24 and 48 h of Al treatment [control (0), 10 and 50 mM]. Data presented are mean ± SE. * Indicates significant difference from control at $P < 0.05$ by Tukey test

and Dubey 2007), might be due to inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits under such condition (Ushimaru et al. 1999). APX has higher affinity for H_2O_2 than any other H_2O_2 scavenging enzymes (Sharma and Dubey 2007). It is also known as one of the important enzyme of Ascorbate-Glutathione cycle (AGC). In the present study, APX activity decreased (Fig. 1d) in both the Al concentrations for root and shoot which indicates accumulation of ROS in cells (Peroni et al. 2007). On the other hand POX is also known to protect cells from oxidative injury by detoxifying H_2O_2 (Peroni et al. 2007; Smeets et al. 2008). In our experiment we found that POX (Fig. 2a) activity increased only in 24 and 48 h of 50 µM Al treatment in root and shoot respectively suggesting that POX helps in tolerance and scavenging of H_2O_2. According to Meriga et al. (2004), decreasing tendency of POX and SOD with exposure to Al starts where the amount of free radicals exceeds cell's capacity i.e., enzymatic activities and cell's metabolic functions start decreasing and if unchecked ultimately lead to DNA damage. In our study, we found that the activity of SOD (Fig. 1g) also declined indicating the induction of oxidative stress under such condition.

Changes in metabolites and antioxidant function inside cells under Al stress

Biochemical studies indicate that Al ions have a strong affinity to biomembranes (Akeson et al. 1989; Jones and Kochian 1997) and cause the rigidification of the membranes (Deleers et al. 1986), which seems to facilitate the radical chain reactions mediated by iron (Fe) ions and to enhance the peroxidation of lipids. The peroxidation of lipids is the most prominent symptom of oxidative damage. H_2O_2, the reactive oxygen species (ROS) (Shao et al. 2008), was found in elevated levels (Fig. 2c) under 10 and 50 µM Al-treatment which suggests induction of

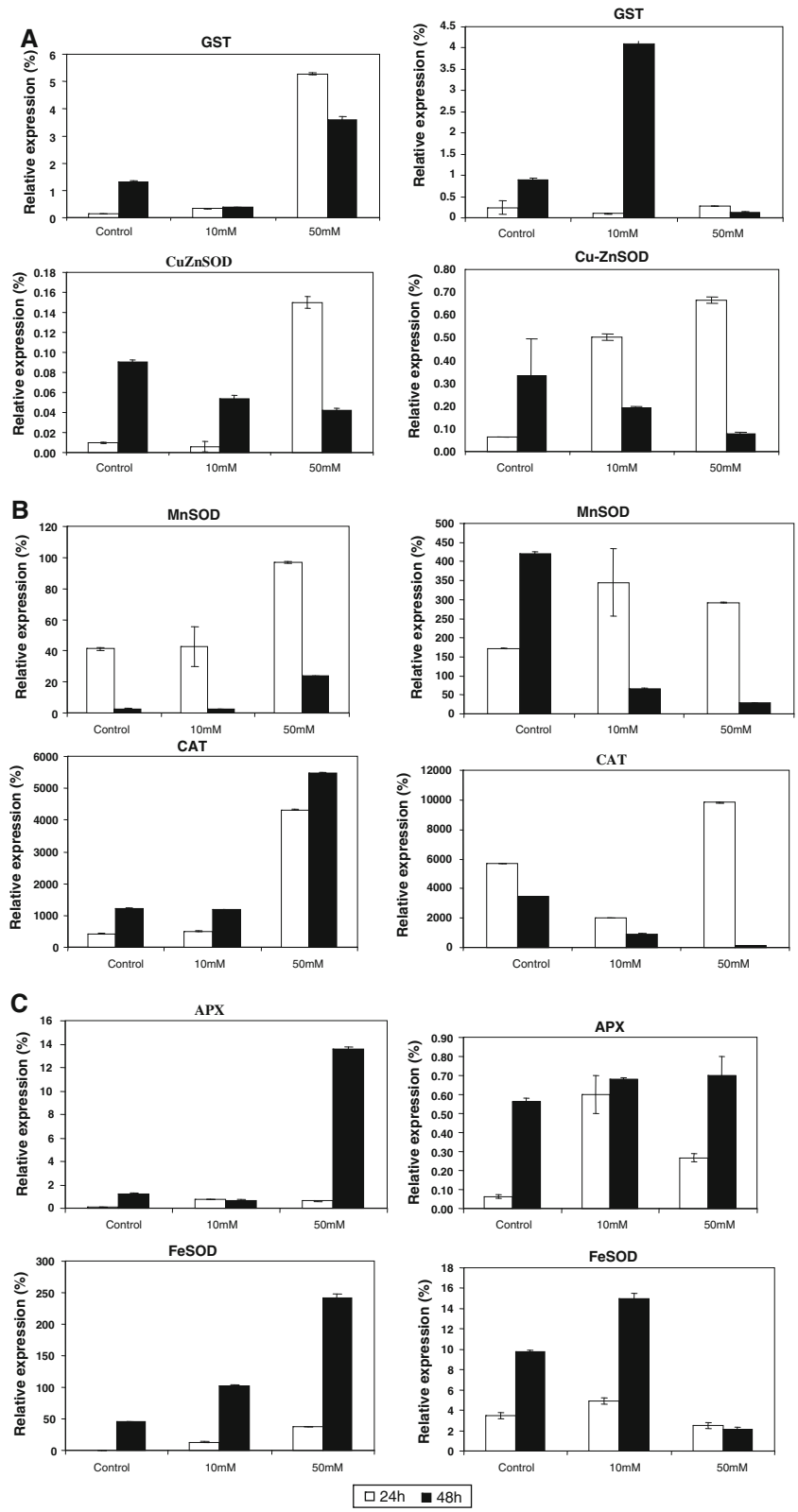

Fig. 3 Semi-quantitative (Q)–Polymerase chain reaction showing changes in mRNA levels of antioxidants genes in the root and shoot of *Pisum sativum* under 24 and 48 h of Al treatment. 18s rRNA was used as internal control. Data presented are means of three independent experiments ± standard errors

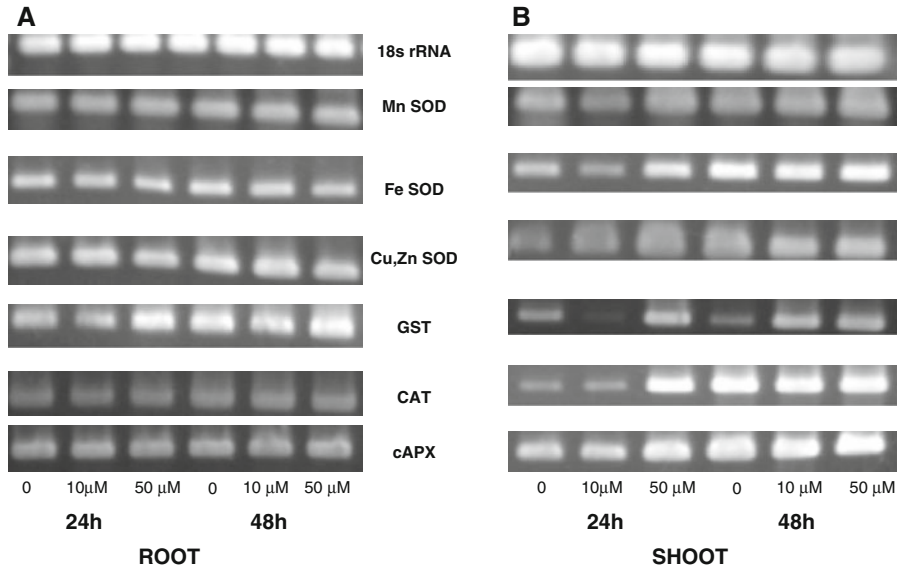

Fig. 4 Reverse Transcriptase (RT)-PCR showing changes of antioxidant genes expression in the root (**a**) and shoot (**b**) of *Pisum sativum* under 24 and 48 h Al treatment. The bands represent PCR optimised at 28 cycles to show changes in expression with reference to internal control (18s rRNA)

oxidative stress. Figure 2d also indicates increase in Al content in roots and shoots of growing pea seedling after 24 and 48 h of Al treatment. The ROS elevation also indicates failure of defense system to combat the oxidative damage. Thus it is likely that the Al-induced ROS production is one of the decisive factors for Al-induced inhibition of root elongation (Yamamoto et al. 2001; Panda et al. 2009). Similar case has been observed by Sharma and Dubey (2007) in 160 μM-Al treated rice seedling. H_2O_2 in cells is eliminated by the activation of AGC (Ascorbate-Glutathione Cycle). The cycle includes participation of ascorbate, glutathione, APX, GR etc. (Noctor and Foyer 1998). The elimination of H_2O_2 involves the conversion of H_2O_2 to water molecule (Peroni et al. 2007). Glutathione plays an important role in protecting plants from oxidative stress induced by some heavy metals (Xiang and Oliver 1998). In root (Fig. 2b) though glutathione content showed an increase indicating its antioxidative role but in shoot an opposite condition was observed except at 10 μM (after 24 h) Al treatment. A short term lack of reduced glutathione may favour the accumulation of ROS and disturb developmental processes (Schutzendubel and Polle 2001). GR, an enzyme of AGC also decreased (Fig. 1f) under the treatment of Al in pea but in rice reverse case was observed by Sharma and Dubey (2007) which may be due to species and Al dose variation. Loss in GR due to heavy metal exposure was also studied in pea by Zn, Cu and Fe (Bielawski and Joy 1986). The maintenance of ascorbate in its reduced form is achieved by the operation of AGC (Miyake and Asada 1994). In the roots of pea after 24 h of treatment (Fig. 2e), ascorbate concentration decreased but after 48 h it increased again. However, in shoot except 10 μM (24 h), rest of the treated types showed decrease in activity and even no change (50 μM after 48 h). The proline content did not show an increase in shoot under Al treatment whereas in roots it increased with the increase in Al concentration (Fig. 2f). Proline accumulation in root may be attributed to a protection from ROS under Al stress. Though there were enhancements in major antioxidant genes expression under Al stress in pea root and shoot tissues with changes in the activity of components of antioxidant machinery in pea tissue, the growth inhibition may be attributed to an imbalance in antioxidant protection and ROS production under Al stress.

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