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ABSTRACT

Oxidizing agents are a normal product of aerobic metabolism and may also be encountered from environmental sources. Aluminum is one proposed source of environmental oxidative stress in plants, and is a major component of soils and an inhibitor of plant growth. Plant cells have evolved universal mechanisms to mediate oxidative stress including the enzyme glutaredoxin, which utilizes a disulfide oxidation-reduction mechanism to detoxify free radicals. A preliminary study suggested that the soybean glutaredoxin gene is expressed preferentially in roots and stems of soybean seedlings in response to toxic levels of Al in soils. To expand our understanding of our initial results, we examined the roots, stems, and leaves of soybean seedlings grown in soil treated with 0, 20, and 50 mM $AlCl_3$ and for periods of 10, 20, or 30 days. We also attempted to more precisely quantitate the differential soybean glutaredoxin response within the plant tissues by adding an 18s rRNA internal control to the RT-PCR amplification reaction. The preliminary results proved inconclusive as 18s gene transcript amplification was inconsistent and glutaredoxin gene amplification was seen only in roots and leaves at 20 days. In the future the RT-PCR reaction needs to be optimized and the experiments repeated, as the results of this preliminary study did not negate the validity of our assumptions.

Introduction

Oxidizing agents are a normal product of aerobic metabolism and may also be encountered from environmental sources. Oxidizing agents within living cells can add or remove electrons from macromolecules and may result in conditions such as growth inhibition, cancer, neural degenerative diseases, or other abnormalities (Grant, 2001; Lozano et al., 1994; Halliwell, 1999). One proposed source of environmental oxidative stress in plants, aluminum, is a major component of soils and an inhibitor of plant growth. While Al is insoluble in a neutral or weakly acidic pH, Al ions become increasingly available and potentially phytotoxic as soil becomes acidic. Al initially confers its toxic effects by binding phospholipids and/or retarding the movement of cations and increasing the movement of anions to the membrane (Kochian, 1995). Once incorporated into cells, positively charged Al ions are attracted to the negatively charged phosphates of DNA in the nucleus. Al subsequently binds the DNA, inhibiting cell division and root elongation (Silva et al., 2001). Recent work suggests that Al may also function as a source of oxidative stress to organisms. Richards et al. (1998) confirmed that some genes in *Arabidopsis thaliana* respond similarly to both oxidative and Al stress. Ezaki et al. (2000) proposed that common mechanisms mediate both Al toxicity and oxidative stress.

Organisms have evolved diverse mechanisms to respond to toxic levels of Al. Exclusion mechanisms are one common way that plants avoid Al toxicity. Plants exude organic acids such citrate, oxalate, or malate in order to keep Al from entering the roots (Kochian, 1995). Plants that do accumulate large concentrations of Al without detrimental effects may use oxalic acid to complex the Al ions (Ma et al., 1997). Plant cells additionally use

universal disulfide oxidation-reduction mechanisms to mediate oxidative stress. In these systems, electrons are transferred from reduced cysteines to toxic oxidants, rendering them incapable of participating in damaging reactions (Lozano et al., 1994). Glutaredoxin is one such protective protein reducing agent that actively scavenges oxygen free radicals in animals, plants, and bacteria (Minakuchi et al., 1994; Halliwell, 1999; Grant, 2001).

Glutaredoxins have now been described from a variety of plants and plant tissues: in seed aleurone (Minakuchi, et al., 1994) and genomic DNA (Sha et al., 1997) from rice; in sieve-tube exudates from castor bean (Szederkenyi et al., 1997; in chloroplasts and mitochondria from spinach leaves (Morell et al., 1995); in *Arabidopsis thaliana* (Tresmousaygue et al., 1997) and *Aleurites fordii* (tung) (Tang et al., 1998); and in fruit of tomato (Chevalier et al., 1999). More recently, Rouhier et al. (2002) described the isolation of glutaredoxin from poplar sieve tube exudates. In 2000, we isolated and initially characterized a novel glutaredoxin cDNA from soybean (Thomas-Bostic and Stephenson, 2001). The soybean glutaredoxin cDNA sequence shares highly conserved characteristics with glutaredoxin genes in plants and other species. Indeed, the soybean glutaredoxin sequence contains the identical Cys-Pro-Phe-Cys active site found in tomato and *Arabidopsis* (Chevalier et al., 1999; Tresmousaygue et al., 1997). From a socioeconomic perspective identification of the glutaredoxin gene in soybean is particularly significant because soybean is a major crop worldwide and soybean consumption has been steadily rising due to the health benefits of soy-based foods (Henkel, 2000; Harvilicz,1999; Scheraga, 1997).

Because Al is a major source of soil toxicity in agricultural soils worldwide, we investigated growth responses of soybean seedlings to Al in the soil and response of the soybean glutaredoxin gene to Al (Thomas-Bostic and Stephenson, 2001). Initial indications from research in yeast suggested that glutaredoxin is expressed differentially in response to protein oxidative stress (Grant, 2001). We speculated that soybean might have systems to detoxify Al ions that had bypassed the plant's initial avoidance mechanisms and hypothesized that the soybean glutaredoxin gene would be expressed differentially in roots and stems when soybean was grown in soils containing toxic levels of Al, with transcript levels increasing in direct proportion to the levels of Al in the soil. To test this hypothesis we examined the roots, stems, and leaves of soybean seedlings grown in soil treated with 0, 20, and 50 mM $AlCl_3$. We did observe a differential gene response, particularly in the roots and stems (Thomas-Bostic and Stephenson, 2001). Glutaredoxin gene expression in these initial experiments increased within the roots and stems when compared with the control, which is consistent with what is known about Al toxicity. The results are consistent with our hypothesis and suggest that glutaredoxin may be involved in neutralizing toxic Al compounds within the vascular tissue of soybean plants, thus limiting the potential for damage that could be done to the whole plant by toxic compounds that escape controls at the root level (Szederkenyi et al., 1997). We also observed that glutaredoxin gene expression in soybean leaves was constitutively high regardless of level of soil Al, suggesting a multi-functional role for glutaredoxin within the soybean plant (Thomas-Bostic and Stephenson, 2001).

Based on these preliminary results that indicated that soybean glutaredoxin is expressed preferentially in roots and stems of soybean seedlings in response to toxic levels of Al in soils (Thomas-Bostic and Stephenson, 2001), we expanded on our original experiment to include mature soybean plants as well as seedlings. In addition, we attempted to more precisely quantitate the differential soybean glutaredoxin response within the roots, leaves, and stems by adding an internal control to the RT-PCR amplification reaction.

Materials and Methods

Plant materials and growth conditions

Experimental plants were *Glycine max* [L] Merr. Cv Wye, a non-nodulating cultivar of soybean. Protocol for soybean growth was as established in our original study for 10-day-old seedlings (Thomas-Bostic and Stephenson, 2001). Al was added once to the soil as the chloride salt ($AlCl_3$), prepared at 0, 20, and 50 mM concentrations. 200 mL of $AlCl_3$ solutions were added to 4-inch pots intended for collection at 10 days. 500 mL of $AlCl_3$ solutions were added to 1-gallon pots intended for plant collection at 20 and 30 days. The Al solutions were added to each pot at planting and allowed to fully absorb into the soil. Plants were grown for 10, 20, or 30 days and the roots, stems, and primary leaves were collected separately. Height was measured from soil to primary leaf node, and primary leaf mass (fresh weight) was measured. Tissues were excised with a razor, then immediately flash–frozen in liquid nitrogen and stored at -70°C.

Extraction of Total RNA and Reverse Transcription of poly A RNA

Total RNA was extracted from frozen tissue using GITC (guanidine isothiocyanate) to denature the RNA and inactivate RNases. The components for the extraction were from the RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA), and the detailed protocol followed the manufacturer's instructions. Modifications to the detailed protocol were as described in our initial

characterization of soybean glutaredoxin (Thomas-Bostic and Stephenson, 2001). Poly A RNA was reverse transcribed as described in our original protocol (Thomas-Bostic and Stephenson, 2001). Ambion (Austin, TX) supplied the Random Decamer primers used at a final concentration of 1 µM.

Polymerase Chain Reaction (PCR)
3 µL of the reverse transcription reaction provided the template for PCR. For the soybean 18s rRNA template we used the sequence identified by Grabeau (1985) available from GenBank as Accession #M16859. The 18s rRNA sequence was co-amplified in the PCR rxn as an internal control (Ambion, Inc., no date). 18s primers were synthesized by Bio-Synthesis Inc. (Lewisville, TX). Oligonucleotide primer design was facilitated by PCR PRIMER3 (Whitehead Institute, MIT) design software available on Biology Workbench (www.workbench.sdsc.edu). The sequence of the 18s forward primer was 5' (TATCTTGCAGGTGACGACG) 3' and the reverse primer 5' (CGATTCCAACTTCATGTTCC) 3'. The soybean glutaredoxin cDNA primers were as described in our original protocol (Thomas-Bostic and Stephenson, 2001). The cDNA templates derived from reverse transcription were amplified using HotStarTaq DNA Polymerase (Qiagen) according to manufacturer's instructions. Each 50 µL reaction contained 3 µL of the RT reaction, 0.2 pmol of each soybean 18s primer and 0.7 pmol of each soybean glutaredoxin primer. Sigma (St. Louis, MO) supplied the dNTPs. Amplification was carried out per our original protocol (Thomas-Bostic and Stephenson, 2001).

Gel Analysis of PCR-amplified DNA
Visualization of the PCR-amplified DNA was accomplished using protocols described in our original experiment (Thomas-Bostic and Stephenson, 2001). Ethidium bromide-stained gels were photographed with a Kodak DC290 digital camera and the images scanned to a computer and manipulated using Kodak 1D LE 3.5 software.

Results
Macroscopic growth of soybean plants grown in varying levels of soil Al
Plants respond to toxic levels of metals in soils at both the macroscopic and molecular levels (Kochian, 1995). To examine the affect of Al in soil on the vegetative growth of soybean we grew soybean seedlings for 10, 20, or 30 days in 0, 20, and 50 mM $AlCl_3$ (Fig. 1a). Even at the highest concentration of $AlCl_3$ soybean plants exhibited a 96% germination rate (data not shown). In soybean plants grown at varying concentrations of soil Al plant height (Fig 1a; Table 1a), leaf mass (Fig 2; Table 1b) and root length (data not shown) showed an inverse correlation with the level of applied $AlCl_3$. Control plants growing in 0 mM $AlCl_3$ reached the greatest heights (Fig 1a; Table 1a), had broader leaves (Fig 2; Table 1b), and longer roots (data not shown) than plants grown in 20 or 50 mM $AlCl_3$ in both seedlings and mature soybean.

Glutaredoxin and 18s rRNA gene expression in soybean seedlings grown in varying levels of soil Al
Examination of soybean plants grown in soils with varying levels of Al demonstrated that soybean could grow in high levels of Al (Fig. 1-2), although growth was reduced as Al concentration increased (Fig 1-2; Table 1). We hypothesized that the glutaredoxin oxidation-reduction system could be involved in mediation of soybean tolerance to Al. In order to examine this question, we measured the accumulation of the glutaredoxin transcript in the roots, stems, and leaves of seedlings growing in soils containing 0, 20, 50 mM $AlCl_3$ and compared them to the accumulated transcript levels of our internal 18s rRNA control. After 10, 20, and 30 days, we harvested the tissues, isolated total RNA, reverse transcribed the mRNA, and co-amplified the resulting glutaredoxin cDNA sequence using PCR together with the 18s rRNA control sequence. Gel analysis indicated that glutaredoxin cDNA was amplified only in 20-day old root and leaf tissues of plants grown at the highest level of soil Al (Fig. 3b); no 18s rRNA was amplified in specific samples at 10d (Fig. 3a) and 20 d (Fig. 3b).

Discussion
Glutaredoxin, the principle component of the glutaredoxin-glutathione disulfide oxidation-reduction system, is one mechanism living cells use to mediate oxidative stress and is an enzyme of particular interest. Glutaredoxin may regulate enzymatic activities, integrate oxidation-reduction reactions, and inactivate toxic oxidant molecules within cells (Minakuchi et al., 1994; Halliwell, 1999). Al is one element that can be toxic to plants growing in acidic soil and is extremely common in soils worldwide (Matsumoto, 2000; Kochian, 1995). Studies have suggested that some of this toxicity may be a function of oxidative stress (Richards et al., 1998; Ezaki et al., 2000). In earlier experiments we isolated a novel glutaredoxin cDNA from soybean and investigated how soybean seedlings respond to differing levels of Al in the soil. We observed that our soybean glutaredoxin cDNA exhibits characteristics that are consistent with previously described plant glutaredoxins, such as the glutaredoxin found in seed aleurone of rice, vascular tissue of the castor bean, spinach leaves, and young tomato fruit (Thomas-Bostic and Stephenson, 2001). Based on a literature review and our initial experimental results, we hypothesized that the soybean glutaredoxin gene would be expressed differentially in the organs of

the soybean when compared with an internal control.

We examined the roots, stems, and leaves of soybean seedlings grown in soil treated with 0, 20, and 50 mM $AlCl_3$ and for periods of 10, 20, or 30 days. We attempted to determine whether our original results (Thomas-Bostic and Stephenson, 2001) were truly a differential gene response by co-amplifying the soybean glutaredoxin cDNA with an 18s rRNA control. Use of an internal standard offers a frame of reference and potential for more accurate quantitation of RNA levels (Ambion Inc., no date). The nucleotide sequence of the soybean mitochondrial 18s ribosomal RNA gene shows evidence for a slow rate of divergence in the plant mitochondrial genome (Grabeau, 1985) and is the source for the 18s sequence amplified. The 18s gene transcript expression was consistent in roots and leaves at 10 d roots and variable in roots and leaves at 20 d. Glutaredoxin gene expression was limited in roots and leaves at 20 d and nonexistent in other plant tissues and time points. Results of the glutaredoxin expression analysis are thus inconclusive.

A better understanding of soybean glutaredoxin will emerge as research explores the boundaries of this gene and its protein products. The immediate needs for future work are optimization of the RT-PCR reaction and repetition of the experiments, as the results during this trial did not negate the validity of our assumptions, and this investigation should eventually prove fruitful. During the preliminary trials described here, the abundant 18s template may have utilized available nucleotides, leaving few available for glutaredoxin sequence amplification. Therefore, in the future nucleotides available for DNA synthesis should be increased and/or the concentration of the 18s template should be reduced. RNA gel analysis will be a useful tool to determine whether RNA is available for reverse transcription and total RNA extractions should be repeated from tissues that did not yield RNA during this preliminary experiment. Ultimately, the parameters of soybean glutaredoxin expression should be expanded to include other forms of Al and other potential sources of oxidative stress. Furthermore, future researchers will need to verify that expression of soybean glutaredoxin gene leads to an active glutaredoxin enzyme.

It is conceivable that an enhanced plant glutaredoxin gene working in conjugation with other genes activated by oxidative stress may be engineered into soybean or other plants, and developed into viable mechanisms for phytoremediation, where plants are used to stabilize, collect, or chemically change contaminants to non-hazardous forms (Weatherford, et al., 1997; Rouhier, et al., 2001). Eventually, glutaredoxin gene expression could be enhanced to enable plants to bioaccumulate heavy metals, leading to the possibility that toxic lands could be reclaimed, especially lands that had been ruined by pollutants (Weatherford et al., 1997).

Table 1. Response of vegetative growth in soybean to varying levels of soil A1. *Plants were grown soils containing 0, 20, and 50 mM $AlCl_3$ for 10, 20, or 30 days. After the allotted time period plants were harvested and we measured plant height at the primary leaf node and primary leaf mass. We observed an inverse relationship between vegetative plant growth and Al concentration. Control plants showed the most robust growth with greatest plant height (a) and the broadest leaves (b). Plants grown in 50 mM $AlCl_3$ experienced the most growth repression.*

a.

	Plant Height at Primary Leaf Node		
Age	**A1 Concentration**	**Plant Height (cm)**	**SD of Height (± cm)**
10 D	0 mM $AlCL_3$	21.3	3.2
	20 mM $AlCL_3$	18.2	2.4
	50 mM $AlCL_3$	16.0	1.7
20 D	0 mM $AlCL_3$	37.8	5.9
	20 mM $AlCL_3$	32.8	3.7
	50 mM $AlCL_3$	29.8	4.8
30 D	0 mM $AlCL_3$	54.3	7.1
	20 mM $AlCL_3$	46.5	5.3
	50 mM $AlCL_3$	39.6	6.1

b.

	Primary Leaf Mass		
Age	**A1 Concentration**	**Leaf mass (g)**	**SD of Mass (± g)**
10 D	0 mM $AlCL_3$	0.126	0.012
	20 mM $AlCL_3$	0.120	0.018
	50 mM $AlCL_3$	0.096	0.004
20 D	0 mM $AlCL_3$	198	18
	20 mM $AlCL_3$	191	15
	50 mM $AlCL_3$	171	12
30 D	0 mM $AlCL_3$	258	28
	20 mM $AlCL_3$	249	23
	50 mM $AlCL_3$	234	21

Figure 1. Growth response of soybean plants grown in differing levels of soil aluminum. *Experimental plants were Glycine max [L] Merr. Cv Wye, a non-nodulating cultivar of soybean (Fig 1a). Soybean plants were grown for 10, 20, or 30 days in soil containing 0, 20, or 50 mM AlCl3. Soybean responded differentially to varying levels to Al in soil. Seedlings had 96% germination rate regardless of the Al concentration in the soil. Height was measured from soil to the primary leaf node. Control plants were the tallest while plants grown in soils containing 50 mM AlCl3 showed the smallest mean heights (Fig 1b).*

a.

b.

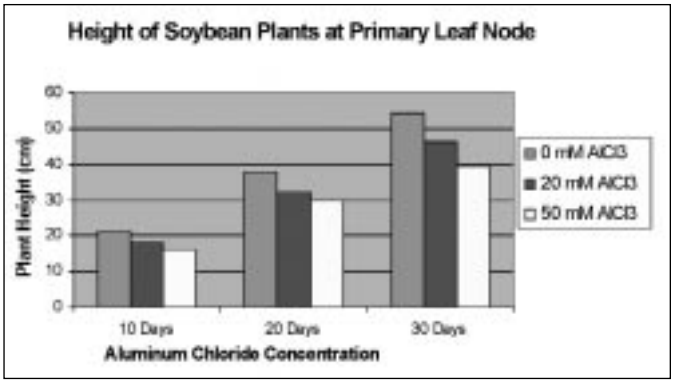
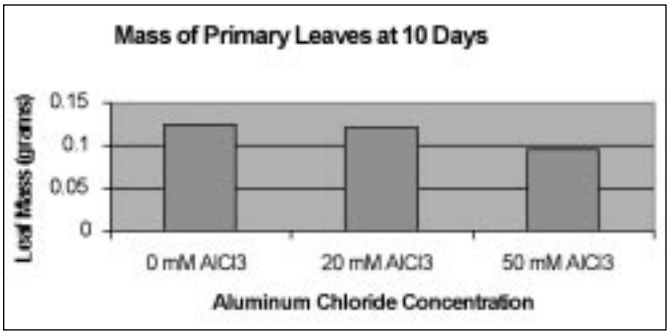
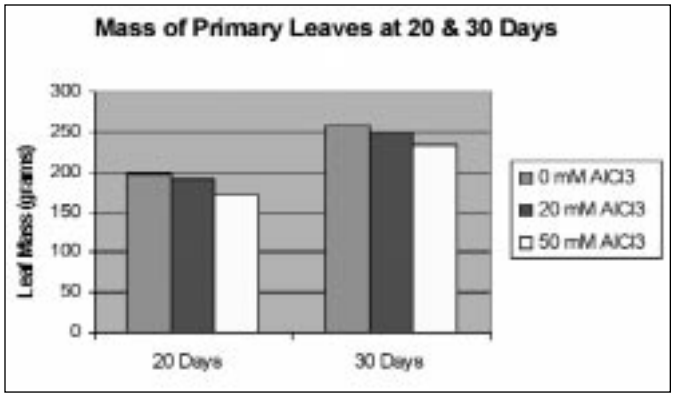


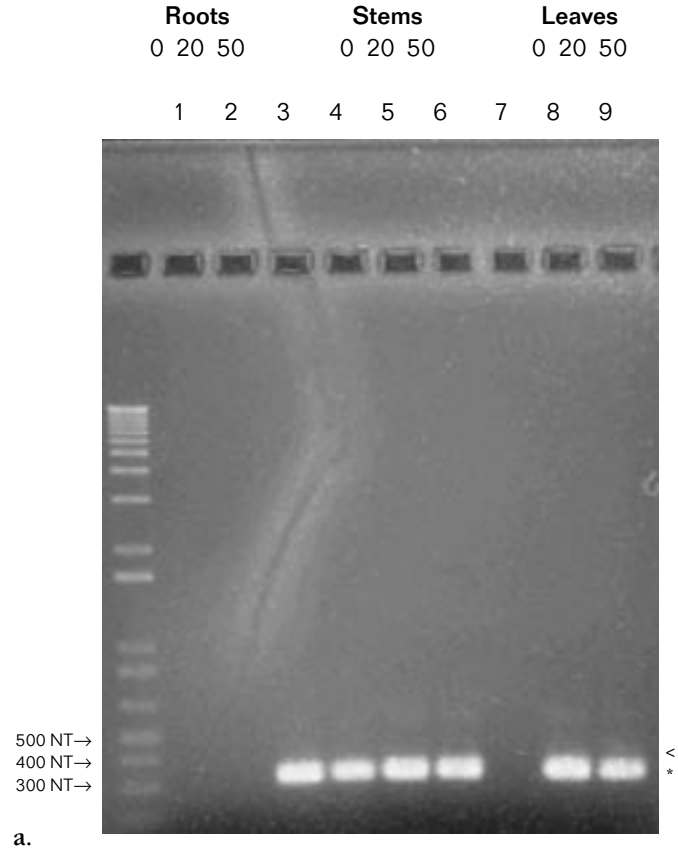
Figure 2. Mass of soybean primary leaves grown at differing levels of soil aluminum. *Soybean plants were grown for 10 (Fig 2a), or for 20 or 30 (Fig 2b) days in soil containing 0, 20, or 50 mM $AlCl_3$. Fresh weight mass was recorded for primary leaves. We observed an inverse relationship between primary leaf mass and Al level. The primary leaves of the control plants showed the highest mean mass while those from plants grown in 50 mM $AlCl_3$ showed the lowest mean mass.*

a.



b.





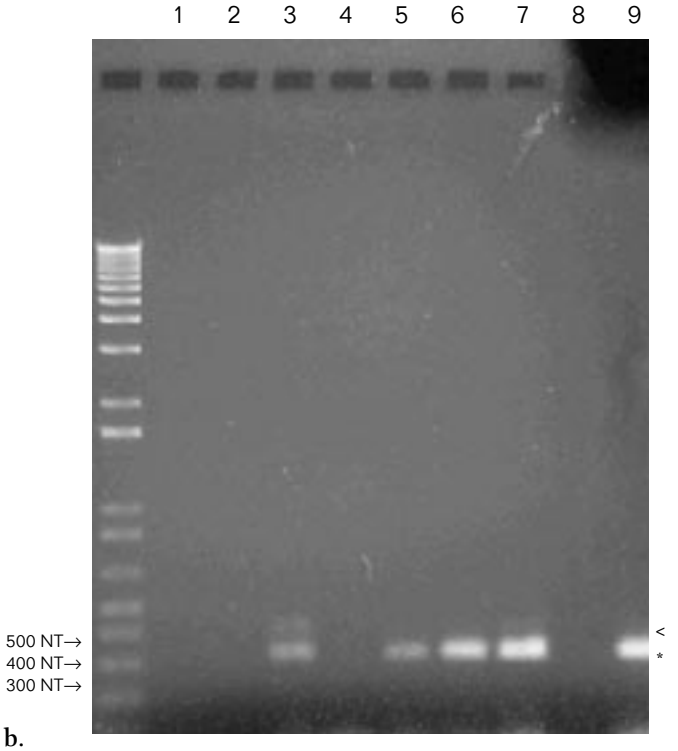


Figure 3. Soybean glutaredoxin gene response to varying levels of aluminum toxicity in soil. *Soybean plants were grown for 10 (Fig 3a) or 20 (Fig 3b) days in soils containing 0, 20, or 50 mM Al. Roots, stems, and leaves were harvested and total RNA extracted. Soybean glutaredoxin transcript and 18s rRNA were co-amplified using RT-PCR. The PCR products were separated by gel electrophoresis, stained with ethidium bromide and photographed. The first lane shows a size ladder in nucleotides (nt). Lanes 1, 4, and 7 indicate plants grown in 0 mM Al; lanes 2, 5, and 8 indicate plants grown in 20 mM Al; lanes 3, 6, and 9 indicate plants grown in 50mM Al. The * indicates the predicted mobility of the amplified 18s rRNA (338 nt). The < indicates the predicted mobility of the amplified glutaredoxin cDNA (425 nt).*

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