

**Improvement of aluminum tolerance through scavenging reactive  
oxygen species and lipid peroxide-derived aldehydes**

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活性酸素と過酸化脂質由来アルデヒド消去による  
アルミニウム耐性の改善

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## Summary

Aluminum (Al) toxicity is a major factor limiting plant growth and productivity in acid soils. Al ions inhibit plant growth partly by causing oxidative damage that is promoted by reactive oxygen species (ROS) and can be prevented by improving antioxidant capacity. Ascorbic acid (AsA) and glutathione (GSH) are the major antioxidants in plants, which are regenerated by the action of monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). However, the functions of DHAR, MDAR and GR in Al tolerance have not been characterized. In the present study, I investigated the role of MDAR, DHAR and GR in AsA and GSH regeneration during Al stress using transgenic tobacco (*Nicotiana tabacum*) plants overexpressing AtMDAR (MDAR-OX) or AtDHAR (DHAR-OX), transgenic Arabidopsis (*Arabidopsis thaliana*) overexpressing AtGR (GR-OX), and the control plants wild-type tobacco SR-1 and Arabidopsis Columbia (Col).

DHAR-OX plants showed rapid root growth than wild-type (SR-1) plants after exposure to  $\text{AlCl}_3$  for 14 d on agar plate, but MDAR-OX plants did not. There was no difference in Al distribution and accumulation in the root tips among SR-1, DHAR-OX and MDAR-OX plants after treatment with 500  $\mu\text{M}$   $\text{AlCl}_3$  for 24 h in hydroponic medium. However, DHAR-OX plants showed lower hydrogen peroxide content, less lipid peroxidation and lower level of oxidative DNA damage than wild-type SR-1 plants, whereas MDAR-OX plants showed the same extent of damage as SR-1 plants. Compared with SR-1 plants, DHAR-OX plants consistently maintained a higher AsA level both with and without Al exposure, while MDAR-OX plants maintained a higher AsA level only

without Al exposure. Also, DHAR-OX plants maintained higher ascorbate peroxidase (APX) activity under Al stress. The higher AsA level and APX activity in DHAR-OX plants contributed to their higher antioxidant capacity and higher tolerance to Al stress. These findings show that the overexpression of DHAR, but not of MDAR, confers Al tolerance, and that maintenance of a high AsA level is important to Al tolerance.

Arabidopsis plants overexpression GR also showed Al tolerance as compared with wild-type Col plants. Under Al stress, GR transgenic plants exhibited better root elongation, lower hydrogen peroxide content and less lipid peroxidation compared to wild-type plants. Although no difference in Al accumulation and the activities of superoxide dismutase (SOD), catalase (CAT) and dehydroascorbate reductase (DHAR) were observed in roots of transgenic and wild-type plants after 24-h Al treatment, GR transgenic plants showed higher activities of GR and ascorbate peroxidase (APX), and higher levels of GSH and AsA than wild-type plants. Thus, overexpression of GR did not affect Al accumulation and the activities of other antioxidant enzymes. These results demonstrate that overexpression of GR improves the antioxidant capacity of Arabidopsis through increasing GSH and AsA level in the cell, leads to suppression of H<sub>2</sub>O<sub>2</sub> generation and lipid peroxidation, and results in enhanced tolerance to Al stress.

Lipid peroxidation, in the downstream of ROS, is a common symptom of Al toxicity, and it increases with increasing Al concentration. From animal cell studies, it is now recognized that the toxicity of lipid peroxide (LOOH) is largely ascribed to LOOH-derived aldehydes. In plants, a close correlation between the level of LOOH-derived aldehydes (determined as thiobarbituric acid-reactive substances (TBARS)) and cellular damage has been shown in environmental stresses caused by heat, chilling, UV-B radiation, salinity,



heavy metals and Al. Thus, it is possible that LOOH-derived aldehydes are involved in Al toxicity. In this study, I verified the hypothesis that LOOH-derived aldehydes, especially highly electrophilic  $\alpha,\beta$ -unsaturated aldehydes (2-alkenals), participate in Al toxicity. Transgenic tobaccos overexpressing *Arabidopsis thaliana* 2-alkenal reductase (AER-OE plants), wild-type and an empty vector-transformed control line (SR-Vec) were exposed to Al on their roots. Compared with the two control plants, AER-OE plants suffered less retardation of root elongation under Al treatment and showed rapid regrowth upon Al removal. Under Al treatment, the roots of AER-OE plants accumulated Al and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to the same levels as did the sensitive controls, while they accumulated lower level of aldehydes and suffered less cell death than SR1 and SR-Vec roots. In SR1 roots, Al treatment markedly increased the contents of the highly reactive 2-alkenals acrolein, 4-hydroxy-(*E*)-2-hexenal, and 4-hydroxy-(*E*)-2-nonenal and other aldehydes such as malondialdehyde and formaldehyde. In AER-OE roots, accumulation of these aldehydes was significantly less. Growth of the roots exposed to 4-hydroxy-(*E*)-2-hexenal and (*E*)-2-hexenal were retarded more in SR1 than in AER-OE plants. Thus, the lipid peroxide-derived aldehydes, formed in the downstream of ROS, injured root cells directly. Their suppression by AER provides a new defense mechanism against Al toxicity.

This study indicates that Al toxicity induced irreversible oxidative damage in tobacco and *Arabidopsis*. Plants with overexpressed antioxidant enzyme genes DHAR and GR showed enhanced Al tolerance in tobacco and *Arabidopsis*. However, MDAR showed no protective effect on improving Al tolerance in tobacco. Both DHAR-OX and GR-OX plants showed increased AsA level and APX activity in their roots as compared with wild-type plants, indicating AsA and APX play a paramount role in Al tolerance.

Furthermore, tobacco plants with overexpressed AER gene showed improving tolerance to Al. AER-OE plants accumulated less LOOH-derived aldehydes, especially 2-alkenals, than that in wild-type plants, indicating the LOOH-derived aldehydes are the cause of Al-induced injury, and enhanced aldehydes scavenging capacity could alleviate Al toxicity. Taken together, oxidative injuries caused both by reactive oxygen species and LOOH-derived aldehydes, are the important causes of Al toxicity. Our study provide a new mechanism for understanding Al toxicity in plants, meanwhile, new strategies for breeding Al tolerant plants are suggested. This will benefit improving plant productivity on acid soils in the world.

# Chapter 1

## General introduction

### 1.1 Al toxicity in the world

Aluminum (Al) is the most abundant metal and the third most common elements in the earth's crust (Kochian, 1995). In acid soils (pH < 5.5), the phototoxic species  $Al^{3+}$  is solubilized to levels that inhibit root growth, and the inhibition of root elongation is thought to be the most important symptoms of Al toxicity and the cause of crop production limitation (Kochian, 1995; Larsen et al., 1998). Approximately 30% of the world's total land area consists of acid soils, and as much as 50% of the world's potentially arable lands are acidic (von Uexküll and Mutert, 1995; Kochian et al., 2004). The production of staple food crops, and in particular grain crops, is negatively impacted by acid soils (Kochian et al., 2004). For example, 20% of the maize and 13% of the rice production worldwide is on acid soils (von Uexküll and Mutert, 1995; Kochian et al., 2004). Furthermore, the tropics and subtropics account for 60% of the acid soils in the world, and as a consequence of a rapid industrial development and environmental pollution, this area increases from year to year especially in developing countries (Tamás et al., 2003). Thus, acid soils limit crop yield in many developing countries where food production is critical. Despite the agronomic importance and extensive investigation of this problem, little is known about fundamental mechanisms of Al toxicity and tolerance (Larsen et al., 1998).

## 1.2 Al toxicity and oxidative stress

There are two strategies that plants can use to deal with Al toxicity: exclusion of Al from the root apex (apoplastic Al detoxification or external resistance), or development of the ability to tolerate Al once it enters the plants symplasm (internal resistance) (Taylor, 1991; Kochian, 1995; Ma et al., 1997; Larsen et al., 1998). Among several Al toxicity mechanisms, there is increasing evidence that the injury of the plasma membrane is the primary site of Al toxicity as a result of disintegration of reactive oxygen species (ROS) metabolism in the cells (Tamás et al., 2003). Cakmak and Horst (1991) first reported the Al induced inhibition of root elongation was correlated with enhanced lipid peroxidation in soybean. It has been recently suggested that Al-enhanced oxidative stress is a decisive event for inhibition of cell growth (Yamamoto et al., 2002, 2003). Genes related to oxidative stress also induced by Al treatment, including peroxidase and glutathione S-transferase (GST) (Richards et al., 1998; Ezaki et al., 2000). Furthermore, genes induced by Al also induced by oxidative stress, such as auxin-induced gene (*parA*), GST, peroxidase and SOD gene in tobacco and blue copper-binding protein (BCB) and oxygen oxidoreductase gene in *Arabidopsis thaliana*, and so on (Sharma and Davis, 1994; Willekens et al., 1994; Ezaki et al., 1995; Richards et al., 1998). These studies demonstrate that there is strong correlation between Al toxicity and oxidative stress.

Oxidative stress can be divided into two phases. During the first phase, ROS directly react with proteins, amino acids, and nucleic acids, and cause peroxidation of membrane lipids (Halliwell and Gutteridge, 1989; Sunkar et al., 2003). The second phase is characterized by lipid peroxidation chain reaction, resulting in chemically reactive

cleavage products including alkanes, alkenes, aldehydes, ketones, and hydroxyl acids (Esterbauer et al., 1991; Witz, 1989; Sunkar et al., 2003). Given their potentially toxic nature, cellular strategies have evolved to detoxify both ROS and toxic products, especially aldehydes, which comprise a major portion of the lipid peroxidation products (Sunkar et al., 2003).

### **1.3 Antioxidant and antioxidant enzymes in plants**

Ascorbate acid (AsA) is an important antioxidant present in cytosol, chloroplast, vacuoles, mitochondria, and apoplast (Potters et al., 2002; Pignocchi and Foyer, 2003). It reacts chemically with a range of ROS, while ascorbate peroxidases catalyse the specific ascorbate-dependent detoxification of  $H_2O_2$  in plant cells (Horemans et al., 2000). In addition to its role in antioxidant defence, it also works by regulation of gene transcription and translation (Noctor et al., 2000), cell division and elongation (Kerk and Feldman, 1995), modulation of enzyme activity and action as an enzyme co-factor (Smirnoff and Wheeler, 2000). AsA is synthesized in the mitochondria and consequently transported to other compartments of plant cells (Horemans et al., 2000a). Most of AsA is reported to be localized in cytoplasm (Pignocchi et al., 2003), up to 10% is localized in the apoplast (Noctor and Foyer, 1998) and 12–30% could accumulate in chloroplasts (Horemans et al., 2000a). AsA serves as an electron donor for  $H_2O_2$  detoxification. APX uses two molecules of AsA to reduce  $H_2O_2$  to water with two molecules of monodehydroascorbate (MDHA) being generated in this reaction. In the chloroplast stroma, MDHA is reduced enzymatically to AsA by MDAR using both NADH and NADPH as electron donors. Being an unstable radical due to its short lifetime, MDHA spontaneously

disproportionates to AsA and dehydroascorbate (DHA) if not rapidly reduces to AsA (Noctor and Foyer, 1998). DHAR catalyzes the reduction of DHA to AsA using reduced GSH. With its ability to directly regenerate AsA, DHAR and MDAR are the key enzymes in maintaining reduced pool of AsA (Eltayeb et al., 2007). Overexpression of DHAR or MDAR genes have been showed to have increased tolerance to ozone, salt and osmotic stresses (Eltayed et al., 2006; 2007).

Glutathione ( $\gamma$ -Glu-Cys-Gly, GSH) and glutathione reductase (GR, EC 1.6.4.2) are important components of the cell's scavenging system for ROS (Foyer and Halliwell, 1976; Kunert and Foyer, 1993; Pilon-Smits et al., 2000). GSH is a substrate for the hydrogen peroxide-removing enzyme, GSH peroxidase, and for dehydroascorbate reductase (Yamaguchi et al., 1999). Most of the metabolic functions of GSH involve the oxidation of GSH to glutathione disulfide (GSSG) (Pilon-Smits et al., 2000). GR mediates the reduction of GSSG to GSH. An increase in GSH contents and GR activity can confer tolerance to various stresses including Al stress (Yamaguchi et al., 1999). Yamaguchi et al. (1999) reported that endogenous GSH protect suspension-cultured tobacco cells from Al toxicity. Devi et al. (2003) reported that an Al-tolerant tobacco cell line had higher contents of AsA and GSH than the isogenic Al-sensitive cell line; and the higher AsA and GSH contents in the tolerant cell line could be related to the protection of cells from ROS generation and lipid peroxidation. Studies using transgenic plants have shown that GR plays an important role in resistance to oxidative stress caused by photoinhibition (Aono et al., 1993; Foyer et al., 1995) and paraquat (Aono et al., 1995). These findings suggest that maintaining a high GSH level is important in achieving oxidative tolerance including Al tolerance.

#### **1.4 The toxicity of lipid-peroxide derived aldehydes induced by stresses in plants**

Aldehyde molecules are common intermediates in most cellular pathways such as carbohydrate, amino acid, protein, lipid or steroid metabolism (Yoshida et al., 1998; Kirch et al., 2004; Kotchoni et al., 2006). However, when produced in excessive amounts, they can have detrimental effects on cellular metabolism because their chemical reactivity (Lindahl, 1992). Aldehydes can cause genotoxic effects including chromosomal aberrations and DNA adducts (Karlhuber et al., 1997; Subramaniam et al., 1997; Comporti, 1998; Wacker et al., 2001), lipid peroxidation resulting in the loss of membrane integrity or modification of proteins subsequently causing cellular and developmental arrest (Lindahl, 1992; Chen et al., 1998; Chen and Murata, 2002; Chen et al., 2002). On the other hand, recent findings suggest that some aldehydes at low concentrations, can act as signaling molecules for inducing stress defense genes (Alméras et al., 2003; Weber et al., 2004; Mano et al., 2009), while at high concentrations they may exert cytotoxicity (Mano et al., 2009). In animal studies, aldehydes have been implicated as being causally involved in the pathogenesis of a number of inflammatory and degenerative diseases; moreover, increased levels of aldehydes (HNE) have been observed in a wide range of human diseases including cancer, heart disease, Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, deep venous thrombosis, diabetes mellitus, and mitochondrial complex 1 deficiency (Poli and Schaur, 2000; Nair et al., 2007).

Plants have several enzymes for detoxifying aldehydes and overexpression of those enzymes leads to enhanced various environmental stresses. NADPH-dependent

aldose/aldehyde reductase (ALR) can catalyze the reduction of aldehydes to alcohols (Oberschall et al., 2000). Overexpression of an alfalfa ALR gene in tobacco leads to reduced accumulation of malondialdehyde and improves tolerance to stresses from drought, UV-B radiation, low temperature, and heavy metals ( $\text{Cu}^{2+}$  and  $\text{Cs}^{2+}$ ) (Oberschall et al., 2000; Hideg et al., 2003; Hegedüs et al., 2004). Aldehyde dehydrogenase (ALDH; EC 1.2.1.3) catalyze the oxidation of aldehydes to carboxylic acids (Yoshida et al., 1998; Sunkar et al., 2003). *Arabidopsis* plants overexpressing the *Arabidopsis* cytoplasmic ALDH3 gene show improved tolerance to drought, salinity, heavy metals, methyl viologen and  $\text{H}_2\text{O}_2$  (Ramanjulu et al., 2003). Kotchoni et al. (2006) reported that overexpression of the chloroplastic ALDH7 gene in *Arabidopsis* confers tolerance to salt and dehydration stress, and suppression of this gene causes increased sensitivity to these stresses. NADPH-dependent 2-alkenal reductase (AER; EC 1.3.1.74) from *A. thaliana* catalyzes reduction of the  $\alpha,\beta$ -unsaturated bond of 2-alkenals to form saturated aldehydes and has a high specificity for HNE (Mano et al., 2002). Overexpression of the *Arabidopsis* AER gene improves resistance to HNE-induced necrosis and photooxidative injury in tobacco (Mano et al., 2005) and salt stress in *A. thaliana* (Papdi et al., 2008). The results of these studies indicate that LOOH-derived aldehydes are commonly involved in the damage to plant cells by various environmental stresses. However, nothing is known about LOOH-derived aldehydes toxicity under Al stress.

## 1.5 Objectives

Above all, it is obviously that oxidative stress is involved in Al toxicity, but the detailed mechanism in enhancing Al tolerance through improving antioxidant capacity is still



obscure; moreover, the injuring species that caused by Al ions remain unidentified. The objectives of this study were, therefore: (1) to characterize the functions of three key antioxidant enzymes, DHAR, MDAR and GR, in Ascorbate-Glutathione cycle under Al stress using transgenic plants; and (2) to identify Al-induced injuring molecules using 2-alkenal reductase overexpressed tobacco plants. The findings of our study provide a new defense mechanism against Al toxicity in plants, and suggest a new strategy for breeding of Al tolerance plants and improving crop productivity in the world.

## Chapter 2

# Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco

### 2.1 Abstract

Aluminum (Al) inhibits plant growth partly by causing oxidative damage that is promoted by reactive oxygen species and can be prevented by improving antioxidant capacity. Ascorbic acid (AsA), the most abundant antioxidant in plants, is regenerated by the action of monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR). We investigated the role of MDAR and DHAR in AsA regeneration during Al stress using transgenic tobacco (*Nicotiana tabacum*) plants overexpressing *Arabidopsis* cytosolic MDAR (MDAR-OX) or DHAR (DHAR-OX). DHAR-OX plants showed better root growth than wild-type (SR-1) plants after exposure to Al for 2 weeks, but MDAR-OX plants did not. There was no difference in Al distribution and accumulation in the root tips among SR-1, DHAR-OX, and MDAR-OX plants after Al treatment for 24 h. However, DHAR-OX plants showed lower hydrogen peroxide content, less lipid peroxidation and lower level of oxidative DNA damage than SR-1 plants, whereas MDAR-OX plants showed the same extent of damage as SR-1 plants. Compared with SR-1 plants, DHAR-OX plants consistently maintained a higher AsA level both with and without Al exposure, while MDAR-OX plants maintained a higher AsA level only without Al exposure. Also, DHAR-OX plants maintained higher APX activity under Al stress. The

higher AsA level and APX activity in DHAR-OX plants contributed to their higher antioxidant capacity and higher tolerance to Al stress. These findings show that the overexpression of DHAR, but not of MDAR, confers Al tolerance, and that maintenance of a high AsA level is important to Al tolerance.

**Keywords** aluminum · ascorbic acid · dehydroascorbate reductase · monodehydroascorbate reductase · reactive oxygen species · aluminum tolerance

## Abbreviations

Al	Aluminum
APX	Ascorbate peroxidase
AsA	Ascorbic acid
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
8-OHdG	8-Hydroxy-2'-deoxyguanosine
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HS	Hoagland solution
MDA	Monodehydroascorbate
MDAR	Monodehydroascorbate reductase
OX	Overexpressor
ROS	Reactive oxygen species

## 2.2 Introduction

Aluminum (Al) is the most abundant metal in the earth's crust and is a major factor limiting plant growth and productivity in acid soils (Kochian, 1995). The primary site of Al accumulation and toxicity is the root meristem, and the inhibition of root elongation is the most notable symptom of Al toxicity (Delhaize and Ryan, 1995; Yamamoto et al., 2003). In roots, Al triggers the sustainable accumulation of reactive oxygen species (ROS), such as hydrogen peroxide ( $H_2O_2$ ) and superoxide anion ( $O_2^{\cdot-}$ ), and Al-induced inhibition of root elongation shows a strong positive correlation with Al-induced ROS generation (Jones et al., 2006; Tahara et al., 2008; Xue et al., 2008). Lipid peroxidation, is an important symptom of oxidative stress associated with Al exposure in several species (Yamamoto et al., 2001; Basu et al., 2001; Ma et al., 2007). DNA damage is also associated with Al exposure (Achary et al., 2008) and can lead to terminal differentiation and irreversible root growth (Rounds and Larsen, 2008).

In plants, oxidative damages can be alleviated through enhanced antioxidant capacity. Under Al stress, several antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are up-regulated (Chen et al., 2005). ROS scavenging genes are also induced by Al, such as peroxidase, glutathione S-transferase (GST), SOD, and blue copper binding (BCB) protein (Richards et al., 1998; Houde and Diallo, 2008). Overexpression of four Al-induced genes [*Arabidopsis* blue-copper-binding protein (*AtBCB*), *N. tabacum* glutathione S-transferase (*parB*), *N. tabacum* GDP-dissociation inhibitor gene (*NtGDII*) and *N. tabacum* peroxidase gene (*NtPox*)] ameliorated Al toxicity in *Arabidopsis* (Ezaki et al., 2000). Three of these genes also

provided protection against oxidative stress. Overexpression of genes encoding antioxidant enzymes (MnSOD) in canola also conferred Al tolerance (Basu et al., 2001). In *Melaleuca* trees, the Al tolerant cultivar showed higher activity of antioxidant enzymes than the sensitive one under Al stress (Tahara et al., 2008). These findings demonstrate a strong link between Al toxicity and oxidative stress, and enhanced antioxidant capacity can enhance Al tolerance.

Ascorbic acid (AsA) is the major redox buffer in plants and is present in the cytoplasm, chloroplasts, mitochondria, and apoplast. It is an important antioxidant that serves as an electron donor and reacts with ROS (Pignocchi and Foyer, 2003). AsA also works as a cofactor of many enzymes, as a regulator of cell division and expansion, and as a signal transduction molecule in plants (Green and Fry, 2005). An increase in AsA contents can confer tolerance to various stresses including salt, ozone and chilling stress (Chen and Gallie, 2005; Eltayeb et al., 2007; Stevens et al., 2008). Devi et al. (2003) reported that an Al-tolerant tobacco cell line had higher contents of AsA and glutathione (GSH) than the isogenic Al-sensitive cell line; and the higher AsA and GSH contents in the tolerant cell line could be related to the protection of cells from ROS generation and lipid peroxidation. In rice, enhanced AsA level alleviated Al induced inhibition of root growth (Guo et al., 2005), and exogenous AsA treatment recovered root elongation under Al stress (Wang and Kao, 2007). These findings suggest that maintaining a high AsA level is important in achieving Al tolerance.

Regulation of AsA levels in plants is tightly controlled by the level of its synthesis, recycling, degradation, and transportation; the AsA recycling pathway, in particular, plays

an important role in the stress response and adaptation (Stevens et al., 2008). Under drought stress, AsA regeneration is more important than AsA synthesis in maintaining a high AsA level (Bartoli et al., 2005). In plants, APX uses two molecules of AsA to reduce  $H_2O_2$  to water with generation of two molecules of monodehydroascorbate (MDHA). MDHA is reduced to AsA by MDAR using NADH/NADPH as electron donors. Since MDHA is an unstable radical due to its short lifetime, if it is not rapidly reduced to AsA by MDAR, it spontaneously disproportionates to AsA and dehydroascorbate (DHA). DHA is reduced to AsA by DHAR using GSH as a reducing substance. Therefore, DHAR and MDAR are the key components in maintaining the reduced pool of AsA and are of paramount importance in oxidative stress tolerance (Eltayeb et al., 2007).

There is growing evidence that overexpression of DHAR enhances tolerance to environmental stresses. Enhancement of the human DHAR gene in tobacco increased tolerance to low temperature and salt stress (Kwon et al., 2003). Overexpression of wheat DHAR conferred protection against ozone in tobacco (Chen and Gallie, 2005). Overexpression of rice DHAR increased salt tolerance in *Arabidopsis* (Ushimura et al., 2006). Overexpression of *Arabidopsis* cytosolic DHAR increased tolerance to drought and ozone stresses in tobacco (Eltayeb et al., 2006). By comparison, the function of MDAR in protecting plants against oxidative stress has been less investigated. Recently, Eltayeb et al. (2007) showed that overexpression of MDAR increased tolerance to salt and osmotic stresses in tobacco; and Stevens et al. (2008) reported that the increased MDAR activity contributed to chilling tolerance in tomato fruit. Taken together, these findings suggest that overexpression of both DHAR and MDAR can regenerate AsA and improve plant tolerance to oxidative stress. Oxidative stress is involved in Al toxicity, but the

mechanisms underlying the regeneration of AsA and the protection of plants against Al damage by DHAR and MDAR are unknown. Here, we investigated these mechanisms by developing transgenic tobacco plants overexpressing *Arabidopsis* cytosolic DHAR (DHAR-OX) and MDAR (MDAR-OX) (Eltayeb et al., 2006; 2007) and characterized the effect of DHAR and MDAR overexpression on tolerance to Al stress.



## 2.3 Materials and methods

### Plant materials and growth conditions

Transgenic tobacco plants overexpressing *Arabidopsis thaliana* cytosolic DHAR (AtDHAR2; At1g75270) or MDAR (AtMDAR1; At3g52880) were generated by Eltayeb et al. (2006; 2007). Seeds of tobacco (*Nicotiana tabacum*: wild-type SR-1 and transgenic DHAR2, DHAR5, DHAR7, MDAR1, MDAR3, and MDAR4) were surface sterilized in 1% (w/v) sodium hypochlorite for 20 min, grown on MS (Murashige and Skoog, 1962) agar plates (pH 5.7) for 4 weeks and then transferred to aerated 1/6 Hoagland solution (HS) (pH 5.7), and maintained in a growth chamber (constant 25 °C, 16-h photoperiod at 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation [PAR]) for another 4 weeks. Uniform plants were selected and precultivated for 24 h at pH 4.3 in 1/6 HS. Thereafter, the plants were exposed to 0  $\mu\text{M}$  (control) or 400  $\mu\text{M}$   $\text{AlCl}_3$  for 24 h in the same solution at pH 4.3. Roots were used for histochemical staining, for determining the levels of Al accumulation, malondialdehyde (MDA), oxidative DNA damage (8-OHdG),  $\text{H}_2\text{O}_2$ , AsA and GSH, and for enzyme assays. It is worth noting that the main part of the root that is injured by Al toxicity is the root tip (0-2 mm). However, to obtain enough tissue for experimental analyses, the first 1 or 3 cm of the root tips, which also included non-injured tissue, was collected.

### Western blot analysis

Proteins were extracted from SR-1, DHAR-OX and MDAR-OX roots exposed to 0 or 400

$\mu\text{M}$  Al for 24 h. Antibodies raised against DHAR or MDAR were used to carry western blot analysis as described by Eltayeb et al. (2006; 2007). It was confirmed that *Arabidopsis* DHAR and MDAR were overexpressed in DHAR-OX and MDAR-OX tobacco plants both with and without Al exposure (Supplementary Fig. 1).

### Effect of Al on root growth

Surface sterilized seeds of SR-1, DHAR-OX and MDAR-OX plants were grown onto 1/6 MS square agar plates (pH 4.3) containing 0, 300, 400, or 500  $\mu\text{M}$  Al following the procedure of Ezaki et al. (2007). The plates were positioned vertically in a growth chamber (constant 25 °C, 16-h photoperiod at 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) for 4 days, during which, the seeds had germinated and there was no difference of germination among SR-1, DHAR-OX and MDAR-OX. Then they were turned by 45°, and the seedlings were allowed to grow for another 10 days. Thereafter, root growth was measured. Root elongation values under different levels of Al are presented as a percentage of the values obtained under control conditions (without Al).

### Al distribution and accumulation in root tips

Localization of Al ions in roots was determined by staining with morin (Sigma, St. Louis, MO, USA) (Tice et al., 1992). Root tips were excised following exposure to 0 or 400  $\mu\text{M}$  Al for 24 h, washed in 5 mM  $\text{NH}_4\text{OAc}$  buffer (pH 5.0) for 10 min, stained with 100  $\mu\text{M}$  morin in 5 mM  $\text{NH}_4\text{OAc}$  buffer (pH 5.0) for 1 h, and finally washed in 5 mM  $\text{NH}_4\text{OAc}$  buffer (pH 5.0) for 10 min. Stained root tips were observed through an Olympus BX51

microscope (Olympus, Tokyo, Japan) equipped with a BP 400–440 excitation filter and an LP 470 barrier filter. A total of five to eight individual roots from five seedlings were examined, and the experiment was repeated three times. For determination of the Al content, the last 1 cm of root tips (20 mg) was washed three times with  $\text{NH}_4\text{OAc}$  buffer and dried, then digested with a concentrated acid mixture ( $\text{HNO}_3:\text{H}_2\text{SO}_4$ , 1:1, v/v) at  $160^\circ\text{C}$  for 3 h. The Al content was determined by an inductively coupled plasma atomic emission spectrometer (ICP-AES, Ciros CCD, Rigaku, Japan).

#### Visualization of lipid peroxidation and measurement of MDA content

Lipid peroxidation was histochemically detected by Schiff's reagent (Yamamoto et al., 2001) which detects aldehydes that originate from lipid peroxides. Root tips exposed to 0 or  $400\ \mu\text{M}$  Al for 24 h were excised and stained immediately with Schiff's reagent (Wako, Osaka, Japan) for 20 min, rinsed with a freshly prepared sulfite solution (0.5% [w/v]  $\text{K}_2\text{S}_2\text{O}_5$  in 0.05 M HCl), and then kept in the sulfite solution for observation. Stained roots were observed under a light stereomicroscope (Olympus SZX7, Tokyo, Japan). A total of five to eight individual roots from five seedlings were examined, and the experiment was repeated three times.

The amount of MDA, the end product of lipid peroxidation, was assessed by the TBARS method (Heath and Packer, 1968). The last 1 cm of root tips (50 mg) was frozen in liquid nitrogen and homogenized by mortar and pestle in 5 mL pre-cooled 10% (w/v) trichloroacetic acid (TCA) at  $4^\circ\text{C}$ . The homogenate was centrifuged at  $5000 \times g$  for 10 min. The supernatant was used for MDA assay and MDA content was calculated using  $155\ \text{mM}^{-1}\text{cm}^{-1}$  as extinction coefficient.

## Visualization of plasma membrane integrity

Root tips exposed to 0 or 400  $\mu\text{M}$  Al for 24 h were excised and stained immediately with Evans blue (Sigma) solution (0.025% [w/v] Evans blue in water) for 10 min (Yamamoto et al., 2001). Stained roots were washed three times with water, after which the dye no longer eluted from the roots, and then examined. Stained roots were observed under a light stereomicroscope (Olympus SZX7, Tokyo, Japan). A total of five to eight individual roots from five seedlings were examined, and the experiment was repeated three times.

The loss of plasma membrane integrity was also measured spectrophotometrically as Evans blue uptake (Schützendübel et al., 2001). Ten root tips (0 to 1 cm) were excised and incubated in Evans blue solution for 30 min. After washing the roots for 15 min with water, the trapped Evans blue was released from the roots by homogenizing root tips with a microhomogenizer in 1 mL of a measuring solution (50% [v/v] methanol and 1% SDS). The homogenate was incubated for 15 min in a water bath at 50 °C and centrifuged at 14,000g for 15 min. The absorbance of the supernatant was determined at 600 nm.

## Oxidative DNA damage analysis

Oxidative DNA damage was evaluated by the amount of 8-OHdG according to Watanabe et al. (2006). Briefly, the last 3 cm of root tips (0.5 g) exposed to 0 or 400  $\mu\text{M}$  Al for 24 h, was frozen in liquid nitrogen, ground to powder in pre-cooled mortar and homogenized with 3 mL extraction buffer [100 mM Tris-HCl buffer (pH 7.0) containing 2% hexadecyltrimethylammonium bromide (CTAB) (w/v), 1.4 M NaCl, 20 mM EDTA]. The

homogenate was incubated for 30 min at 65 °C and then centrifuged at 10,000g for 5 min. A 900 µL aliquot of the upper phase was mixed with 600 µL cold isopropyl alcohol and the mixture was kept at -80 °C for 10 min to precipitate the DNA. After centrifugation, the pellet was washed with 0.8 mL 70% ethanol, dried and dissolved in 200 µL TE buffer [10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA]. Then, 10 µL RNase A (10 mg mL<sup>-1</sup>) was added to the solution. Following an incubation at 37 °C for 30 min, the DNA was precipitated by mixing with 120 µL of a 20% (w/v) solution of polyethylene glycol 6000 containing 2.5 M NaCl. The DNA obtained was washed with ethanol, dried, and redissolved with 200 µL TE buffer. Then 60 µL of 3 M sodium acetate and 1320 µL of cold 100% ethanol were added. Following storage at -80 °C overnight, the DNA was precipitated by centrifugation at 10,000g for 30 min at 4 °C, washed with ethanol and dried. DNA obtained (50 µg) was then dissolved in 50 µL of 10 mM Tris-HCl buffer (pH 7.0), and 6 U of nuclease P1 (Wako Pure Chemical Ind.) in 10 µL of 0.5 M sodium acetate was added. Following incubation at 37 °C for 30 min, 7.5 U of alkaline phosphatase (Nippon Gene Co.) and 72.5 µL of 0.4 M Tris-HCl buffer (pH 7.5) were added and the mixture was incubated at 37 °C for 30 min. Then DNA was dried and dissolved in 100 µL HPLC buffer (5% methanol in 10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer) prior to HPLC analysis.

The amount of 8-OHdG in the DNA was measured using HPLC-electrochemical detector system. A CAPCELL PAK C18 MG column (4.6 × 250 mm; Shiseido Co. Ltd, Tokyo, Japan) was used as the analytical column. HPLC was carried out with a 880-PU solvent delivery system (Japan Spectroscopic Co., Tokyo, Japan) equipped with an electrochemical detector (model: Coulochem II; ESA, Chelmsford, MA. USA). The eluent buffer contained 5% (v/v) methanol and 10 mM NaH<sub>2</sub>PO<sub>4</sub> at a flow rate of 1 mL

min<sup>-1</sup>. The amount of 8-OHdG was calculated by calibration against curves established from HPLC runs of standard samples containing known amounts of authentic 8-OHdG, and expressed as the amount of 8-OHdGs per µg DNA.

#### Assaying H<sub>2</sub>O<sub>2</sub> content

Hydrogen peroxide levels were determined according to Velikova et al. (2000). The last 3 cm of root tips (0.5 g) was frozen in liquid nitrogen, ground to powder in pre-cooled mortar and homogenized with 2 mL cold 0.1% (w/v) TCA. The homogenate was centrifuged at 12 000g for 30 min at 4 °C and 0.4 mL of the supernatant was added to 0.4 mL 10 mM potassium phosphate buffer (pH 7.0) and 0.8 mL 1 M potassium iodide (KI). The absorbance of supernatant was read at 390 nm. The content of H<sub>2</sub>O<sub>2</sub> was calculated against a calibration curve using H<sub>2</sub>O<sub>2</sub> standards.

#### Enzyme analysis

The last 3 cm of root tips (0.5 g) from SR-1, DHAR-OX and MDAR-OX exposed to 0 or 400 µM Al for 24 h was frozen in liquid nitrogen, ground to fine powder in pre-cooled mortar and homogenized in 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM ascorbate. The slurry was then centrifuged at 12 000g for 30 min at 4 °C, and the supernatant was used for enzyme assays at 25 °C.

DHAR (EC 1.8.5.4) activity was assayed by the method of Nakano and Asada (1981). The assay was performed in a reaction mixture containing 50 mM potassium phosphate

buffer (pH 7.8), 2 mM dehydroascorbate, 5 mM reduced GSH, 0.1 mM EDTA, and crude extract. DHAR activity was calculated from the increase in the AsA content by measuring the absorbance at 290 nm and using the  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$  extinction coefficient.

MDAR (EC 1.6.5.4) activity was assayed in a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 0.2 mM NADH, 2.5 mM ascorbate, 1 unit ascorbate oxidase, and crude extract. MDAR activity was calculated from the change in NADH oxidation by measuring the decrease in absorbance at 340 nm and using the  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$  extinction coefficient (Hossain et al., 1984).

APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) by measuring the decrease in absorbance at 290 nm as ascorbate is oxidized. GR (EC1.6.4.2) activity was calculated from the decrease in absorbance of NADPH at 340 nm (Foyer and Halliwell 1976). Protein concentration was determined by Bradford's (1976) method, using bovine serum albumin as a standard.

#### Determination of AsA and GSH levels

AsA and DHA were measured by HPLC according to Eltayeb et al. (2006). The last 3 cm of root tips (0.5 g) was frozen in liquid nitrogen and homogenized in 5 mL of ice-cold 5% metaphosphoric acid. The homogenate was centrifuged at  $10\,000 \times g$  for 15 min at  $4^\circ\text{C}$ . The supernatant was filtered through a  $0.45\text{-}\mu\text{m}$  MILLEX-HV filter unit (Millipore, Bedford, MA, USA). For determination of reduced AsA, a  $5\text{-}\mu\text{L}$  sample was resolved on a CAPCELL PAK C18 120 (Shiseido Co. Ltd, Tokyo, Japan) column, with a mobile phase

of 80% acetonitrile and 20% 0.01 M potassium phosphate (pH 3.0) at a rate of 0.5 mL min<sup>-1</sup>. AsA was detected by a Tosoh UV-8010 absorbance detector (Tosoh Co., Tokyo, Japan) set at 258 nm. The AsA standard curve was linear in the range of 50 – 350 ng. DHA was reduced to AsA by neutralizing metaphosphoric acid in samples with 5 M KOH and then adding dithiothreitol (DTT) to a final concentration of 20 mM. The resulting solution was incubated in darkness at 25 °C for 30 min. The amount of DHA in samples was calculated from the difference between the total AsA (reduced plus oxidized) and reduced AsA.

To determine the contents of GSH and GSSG, the last 3 cm of root tips (0.5 g) was frozen in liquid nitrogen and homogenized in 5 % TCA and then centrifuged at 10 000 g for 30 min at 4°C. The supernatant was used to determine total glutathione (GSH plus GSSG) and GSSG contents according to Anderson (1985). The assay was based on sequential oxidation of GSH by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and the reduction of GSSG by NADPH in the presence of GR. To determine GSSG content, 2-vinylpyridine was added to the supernatant. GSH content was obtained from the difference between the total glutathione and GSSG.

### Statistical analyses

Statistical analysis was carried out using the Statistical Analysis System (SAS 8.0) software. Data are expressed as means and SE. Means were compared using an ANOVA protected least significant difference (LSD) test ( $P < 0.05$ ).



## 2.4 Results

### Effect of Al on root growth

Both wild-type and transgenic tobacco plants grown on agar plates with different concentrations of Al exhibited concentration-dependent inhibition of root growth (Fig. 1). Treatment with 300  $\mu\text{M}$  Al resulted in a marked reduction in root growth of SR-1 and MDAR-OX plants by 47 and 43%, respectively. By comparison, the DHAR-OX plants showed only a 28% reduction in root growth under the same conditions. Moreover, with exposure to 400 and 500  $\mu\text{M}$  Al, the root growth of SR-1 plants was reduced by 57 and 62%, respectively. The respective reduction in MDAR-OX plants was 54 and 58%, and that of DHAR-OX plants was 33 and 40%. DHAR-OX plants maintained 20% higher root growth than SR-1 and MDAR-OX plants under Al treatment.

### Al distribution and accumulation in root tips

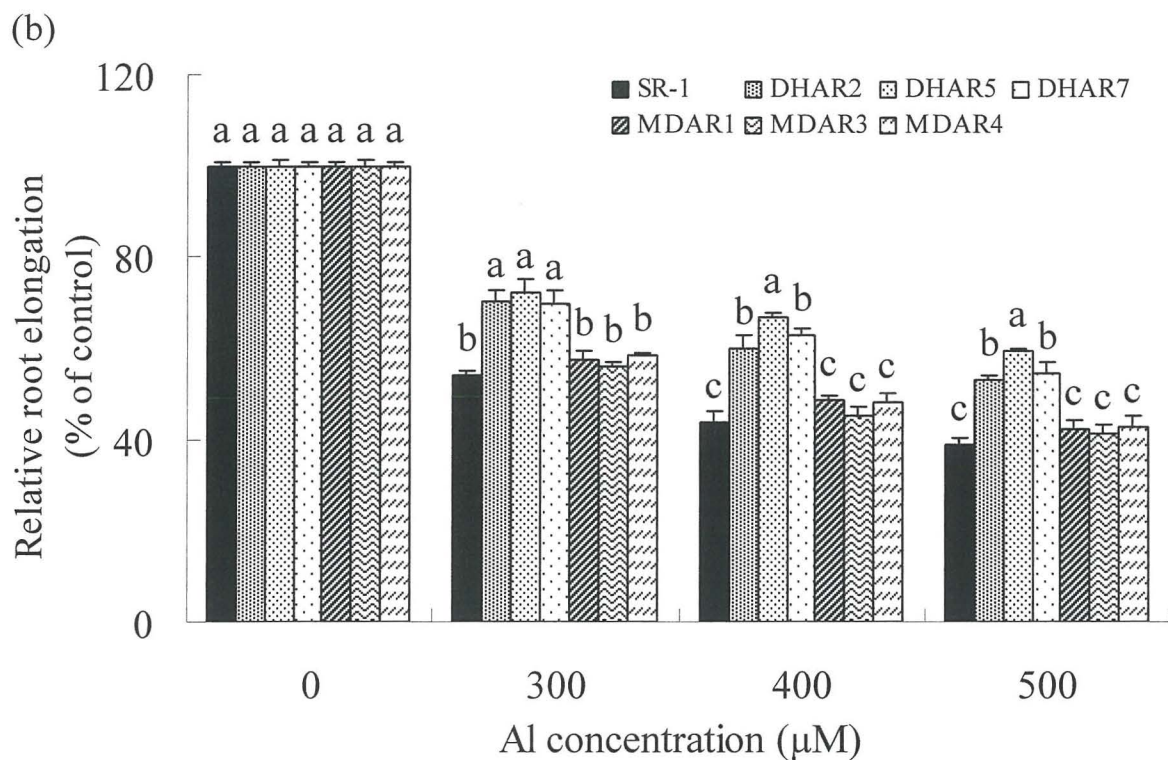
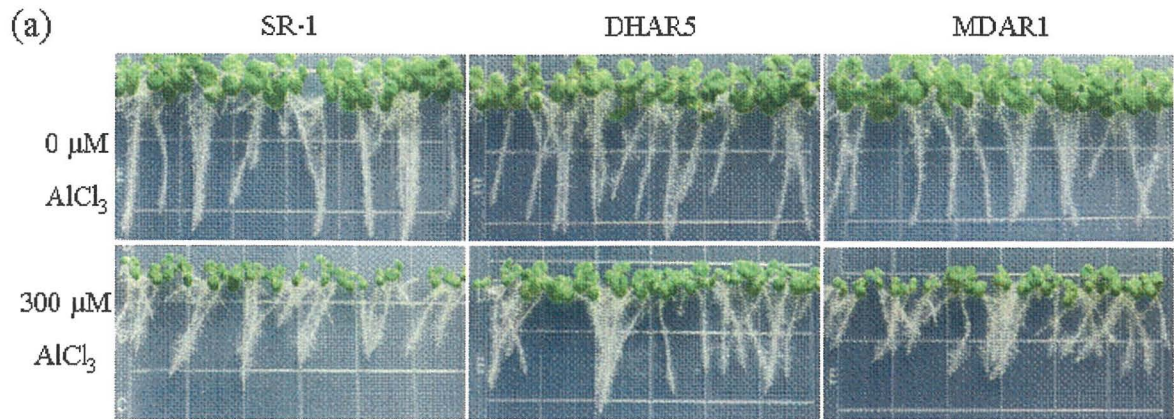
Morin is a fluorochrome with high sensitivity to Al and is widely used to detect the presence of Al in plant tissue (Ezaki et al., 2000; 2007; Jones et al., 2006). Roots without exposure to Al showed an extremely low level of morin fluorescence (Fig. 2a). Following exposure to 400  $\mu\text{M}$  Al for 24 h, a marked increase in Al-induced morin fluorescence was observed, particularly in the elongation zone, and no difference in Al distribution was observed in the root tips of SR-1, DHAR-OX and MDAR-OX plants. The Al content in the roots, as determined with a plasma atomic emission spectrometer, also showed no difference among all tested plants (Fig. 2b). Thus, Al distribution and accumulation in the

roots were not affected by overexpression of DHAR or MDAR.

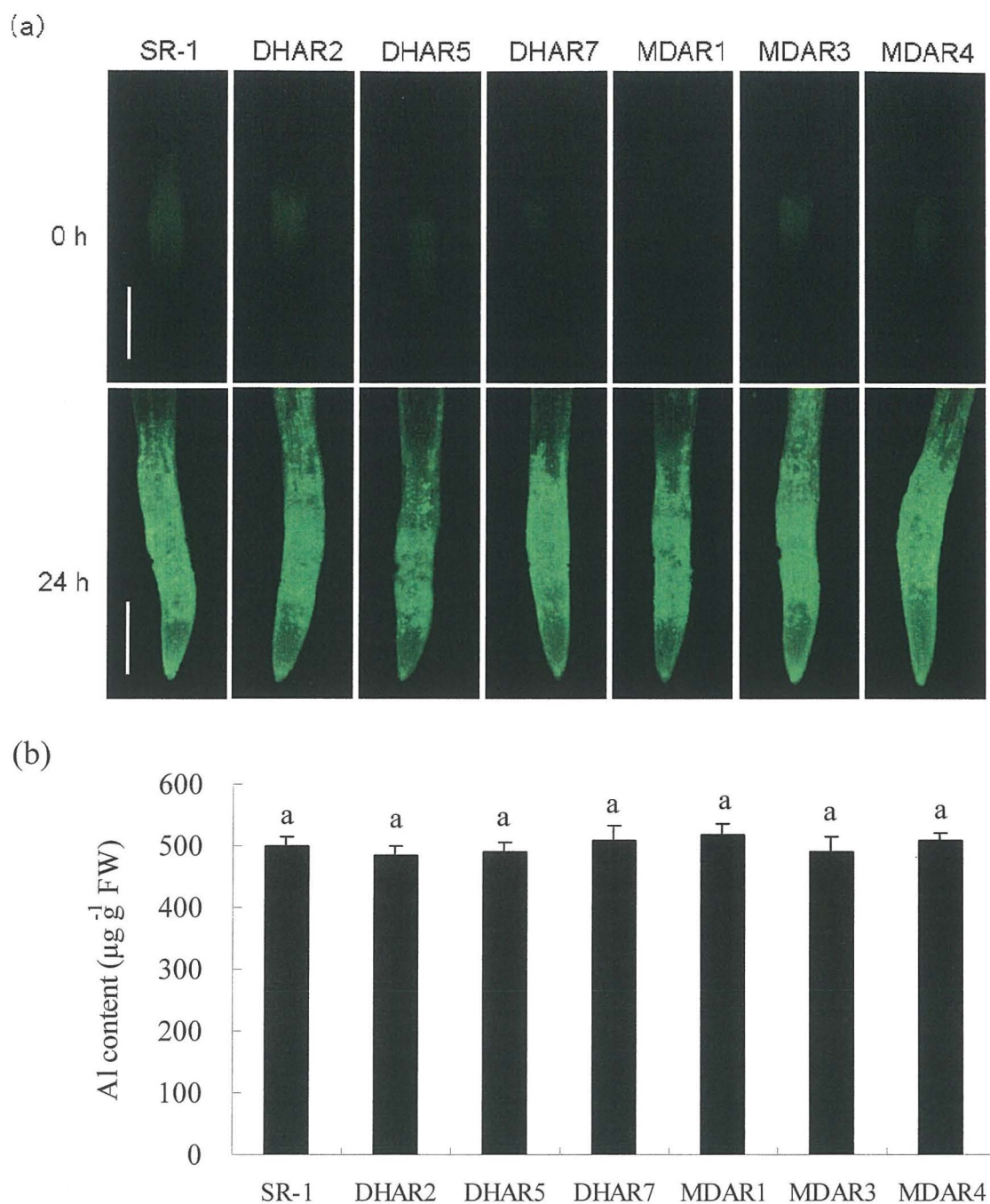
#### Lipid peroxidation and plasma membrane integrity

Schiff's reagent staining and MDA determination have been widely used in observation and determination of Al-induced lipid peroxidation in plants (Yamamoto et al., 2001; Ma et al., 2007). Observation of lipid peroxidation by staining roots with Schiff's reagent was based on the intensity of the developed pink color (Fig. 3a). In the absence of Al, no pink color was detected. Upon exposure to Al for 24 h, the root tips showed a clear pink color. However, the degree of lipid peroxidation was lower, as indicated by the paler pink, in the roots of DHAR-OX plants than in the roots of SR-1 and MDAR-OX plants. The amount of MDA present in root tips of SR-1, DHAR-OX and MDAR-OX also indicated that the level of peroxidation was increased by exposure to Al (Fig. 3b). The increase was significantly higher in SR-1 and MDAR-OX plants than in DHAR-OX plants.

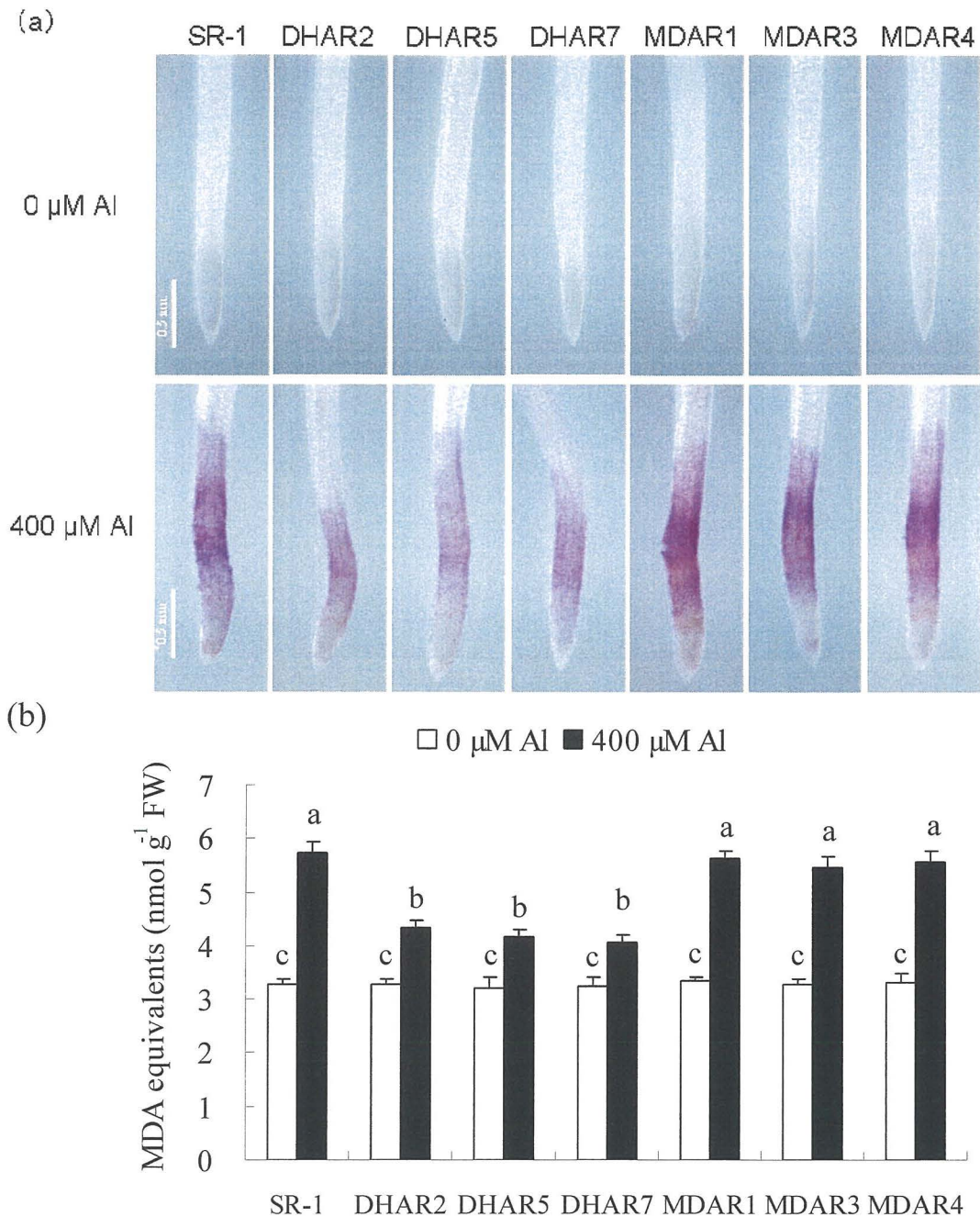
Evans blue staining reveals the loss of plasma membrane integrity induced by Al (Yamamoto et al., 2001). Upon exposure to Al, the plasma membrane integrity in the root tip showed distinct damage as revealed by both Evans blue staining (Fig. 4a) and uptake analysis (Fig. 4b). The maintenance of plasma membrane integrity in the roots of DHAR-OX plants was better than in the roots of SR-1 plants, and there was no difference between those of MDAR-OX and SR-1 plants.



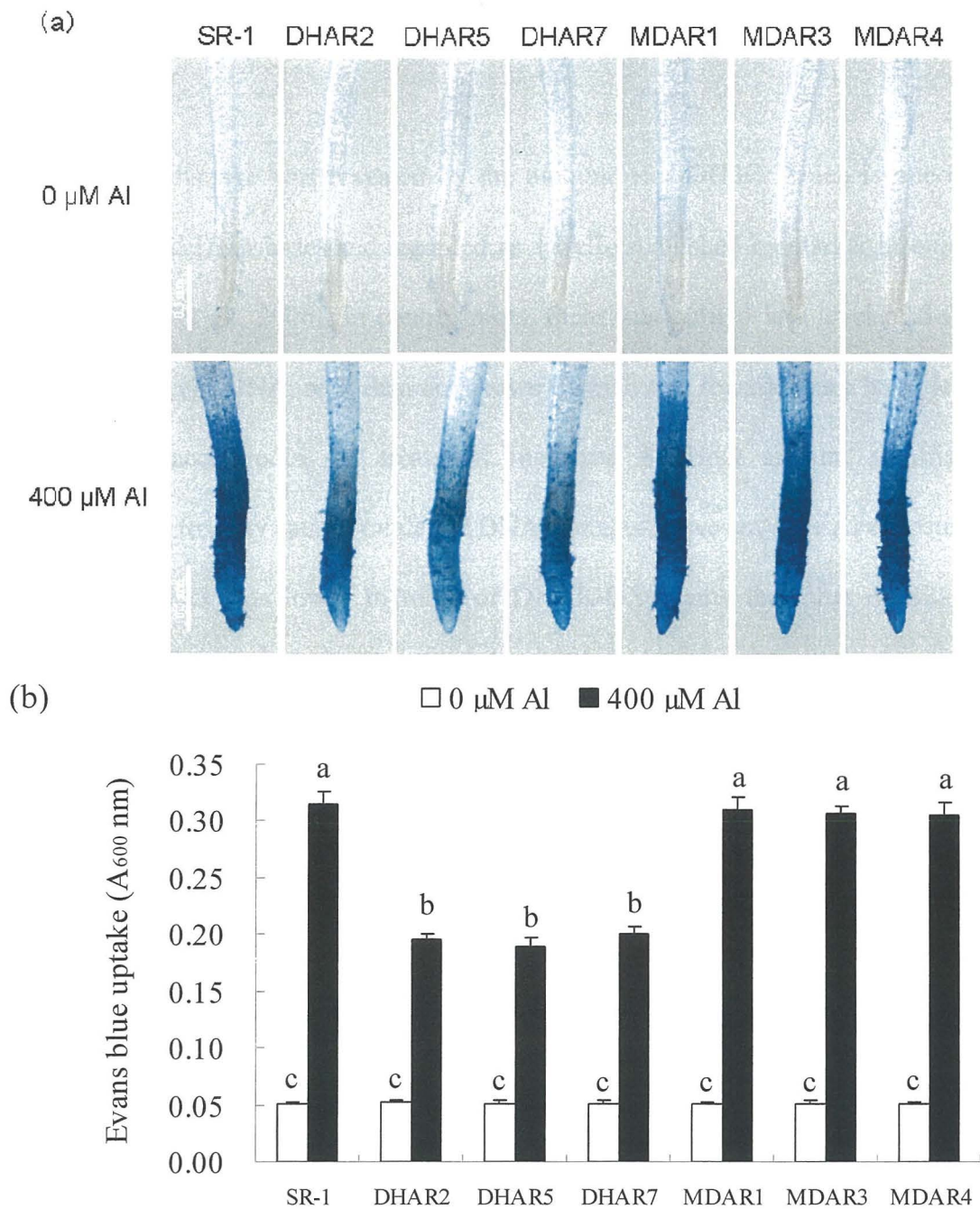
**Figure 1** Relative root growth of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX) under 0, 300, 400, or 500  $\mu\text{M}$  Al treatment for 2 weeks. (a) Root growth of SR-1, DHAR5 and MDAR1 under 300 $\mu\text{M}$  Al. (b) Relative root elongation was estimated by comparing root length between the Al treatments (300, 400, or 500  $\mu\text{M}$  Al) and the control treatment (0  $\mu\text{M}$  Al). Data are mean  $\pm$  SE ( $n = 4$ ) (each replication included ten plants). Statistical analysis was done for each Al concentration independently. Data followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).



**Figure 2** Detection of Al localization and Al content in root tips of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX). Plants were exposed to 0 or 400  $\mu\text{M}$  Al for 24 h. (a) Al localization. The roots were stained with morin, a stain that fluoresces when complexed with Al. Bar indicates 0.5 mm. (b) Al contents. Data are mean  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).



**Figure 3** Lipid peroxidation damage in the roots of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX) under 0 or 400  $\mu\text{M}$  Al treatment for 24 h. (a) Histochemical detection of lipid peroxidation by Schiff's reagent. The positive staining in the photomicrographs shows as pink. Bar indicates 0.5 mm. (b) MDA content. Data are mean  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).



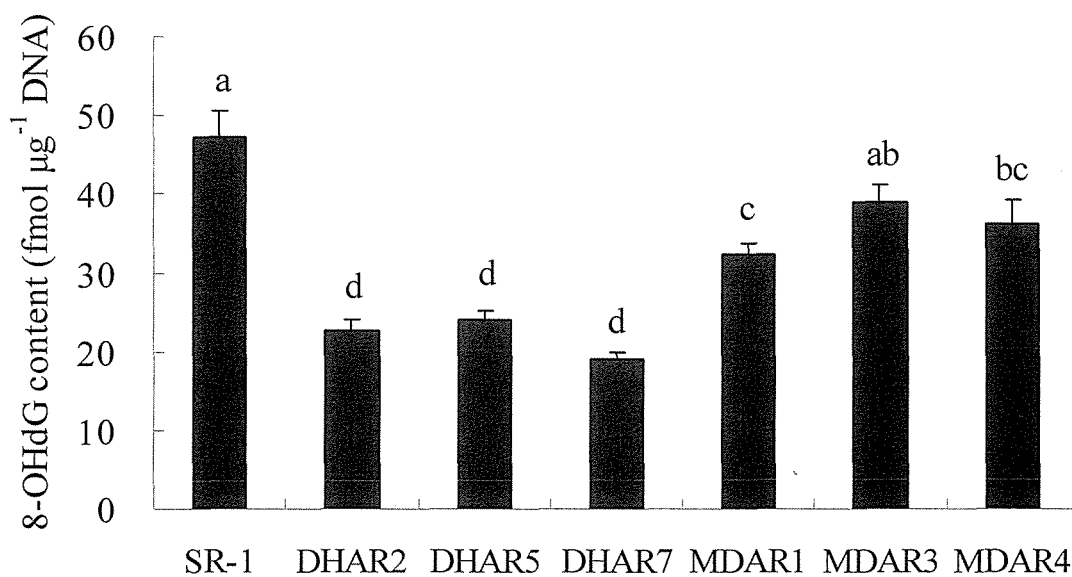
**Figure 4** The loss of plasma membrane integrity in the roots of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX) under 0 or 400  $\mu\text{M}$  Al treatment for 24 h. (a) The roots were stained with Evans blue. Positive staining is shown as blue image in the photomicrographs. Bar indicates 0.5 mm. (b) Quantified assay of Evans blue uptake. Data are mean  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).

## Oxidative DNA damage

Oxidative DNA damage was revealed by the amount of 8-OHdG, which is one of the abundant oxidized DNA bases and regarded as a well-established biomarker of oxidative stress (Watanabe et al., 2006). In control roots, there was quite a low level of 8-OHdG ( $12.5 \pm 1.5 \text{ fmol } \mu\text{g}^{-1} \text{ DNA}$ ,  $n=3$ , data not shown), which was regarded as a basal level of 8-OHdG in tobacco roots. Al treatment increased 8-OHdG amount significantly, indicating that Al toxicity caused oxidative DNA damage. However, after Al exposure, the amount of 8-OHdG was lower in roots of DHAR-OX plants than that of SR-1 and MDAR-OX plants under Al treatment (Fig. 5).

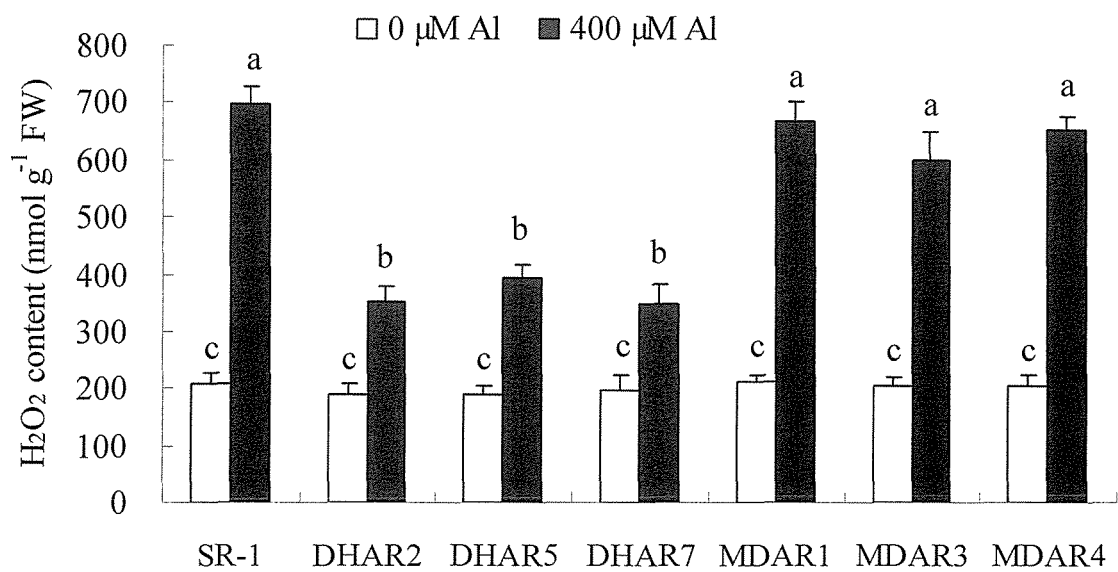
## Hydrogen peroxide content

No difference in the content of  $\text{H}_2\text{O}_2$  in the roots of SR-1, DHAR-OX and MDAR-OX plants was observed without Al exposure. Al treatment increased  $\text{H}_2\text{O}_2$  content in all plants. The increase was over threefolds in roots of SR-1 and MDAR-OX plants, while it was only twofolds in DHAR-OX plants (Fig. 6).



**Figure 5** Formation of 8-OHdG in the roots of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX) by 400  $\mu\text{M}$  Al treatment for 24 h. Data are mean  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).





**Figure 6** Effect of Al on H<sub>2</sub>O<sub>2</sub> content in the roots of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX). Plants were exposed to 0 or 400  $\mu\text{M Al}$  for 24 h. Data are mean  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).

## Enzyme activities

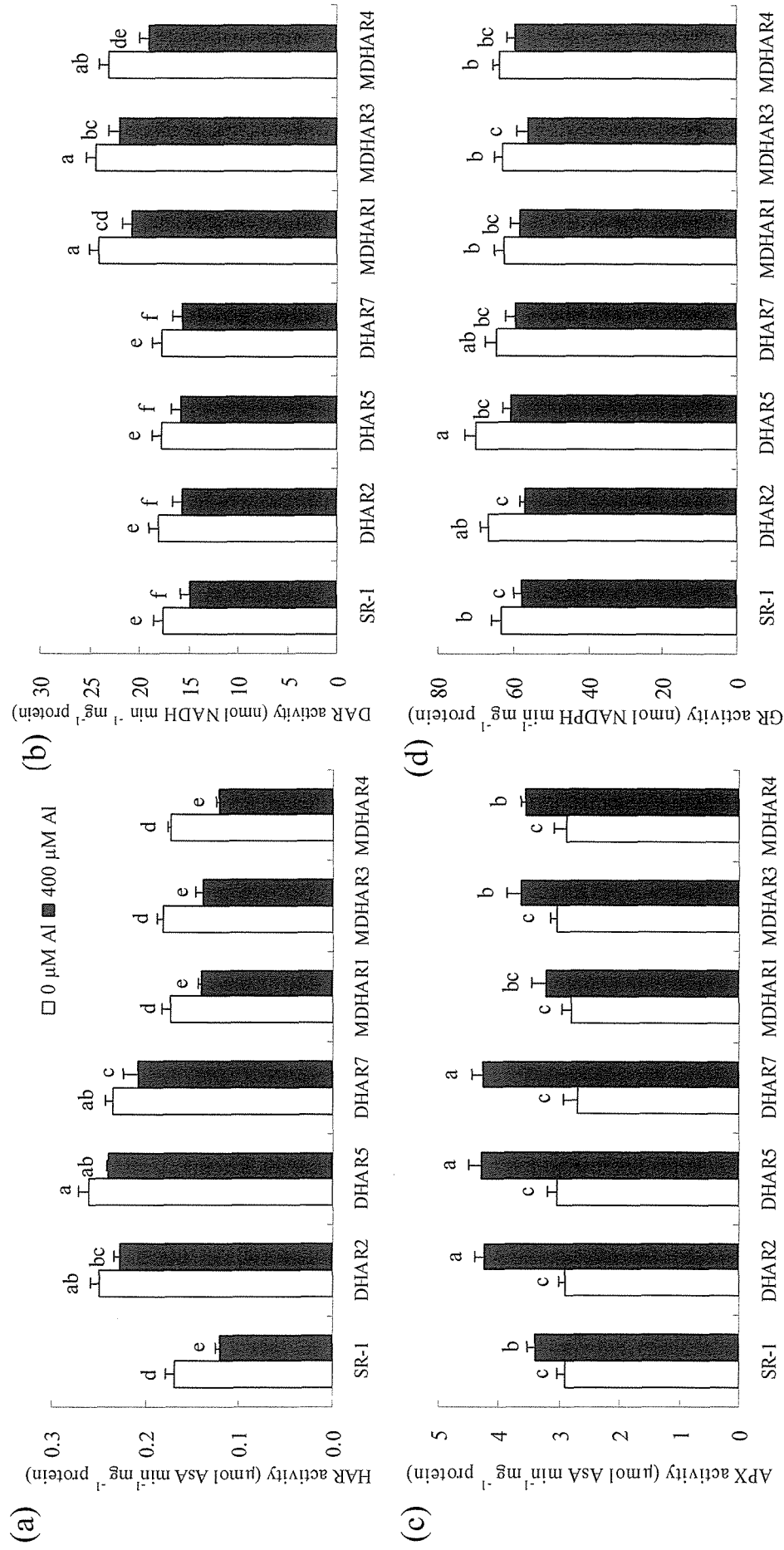
In the absence of Al, the average DHAR activity in DHAR-OX plants was 1.5-folds (Fig. 7a) and MDAR activity in MDAR-OX plants was 1.7-folds compared to SR-1 plants (Fig. 7b). Overexpression of DHAR did not affect MDAR activity in DHAR-OX plants and, similarly, overexpression of MDAR did not affect DHAR activity in MDAR-OX plants. Exposure to 400  $\mu$ M Al decreased the activity of both DHAR and MDAR, but DHAR-OX plants maintained twofolds higher DHAR activity, and MDAR-OX plants maintained 1.8-folds higher MDAR activity than SR-1 plants. These results show that regardless of Al exposure, the DHAR-OX and MDAR-OX plants had higher DHAR and MDAR activity, respectively, than the SR-1 plants.

In the absence of Al exposure, APX activity was similar in SR-1, DHAR-OX and MDAR-OX plants (Fig. 7c). Upon exposure to Al, an increase in APX activity up to 36% was observed in DHAR-OX plants compared to only 10 and 13% in SR-1 and MDAR-OX plants, respectively. Thus, DHAR-OX plants, but not MDAR-OX plants, maintained higher APX activity than SR-1 plants under Al treatment. GR activity was decreased by Al exposure in all plants, and there was no difference between the plants (Fig. 7d).

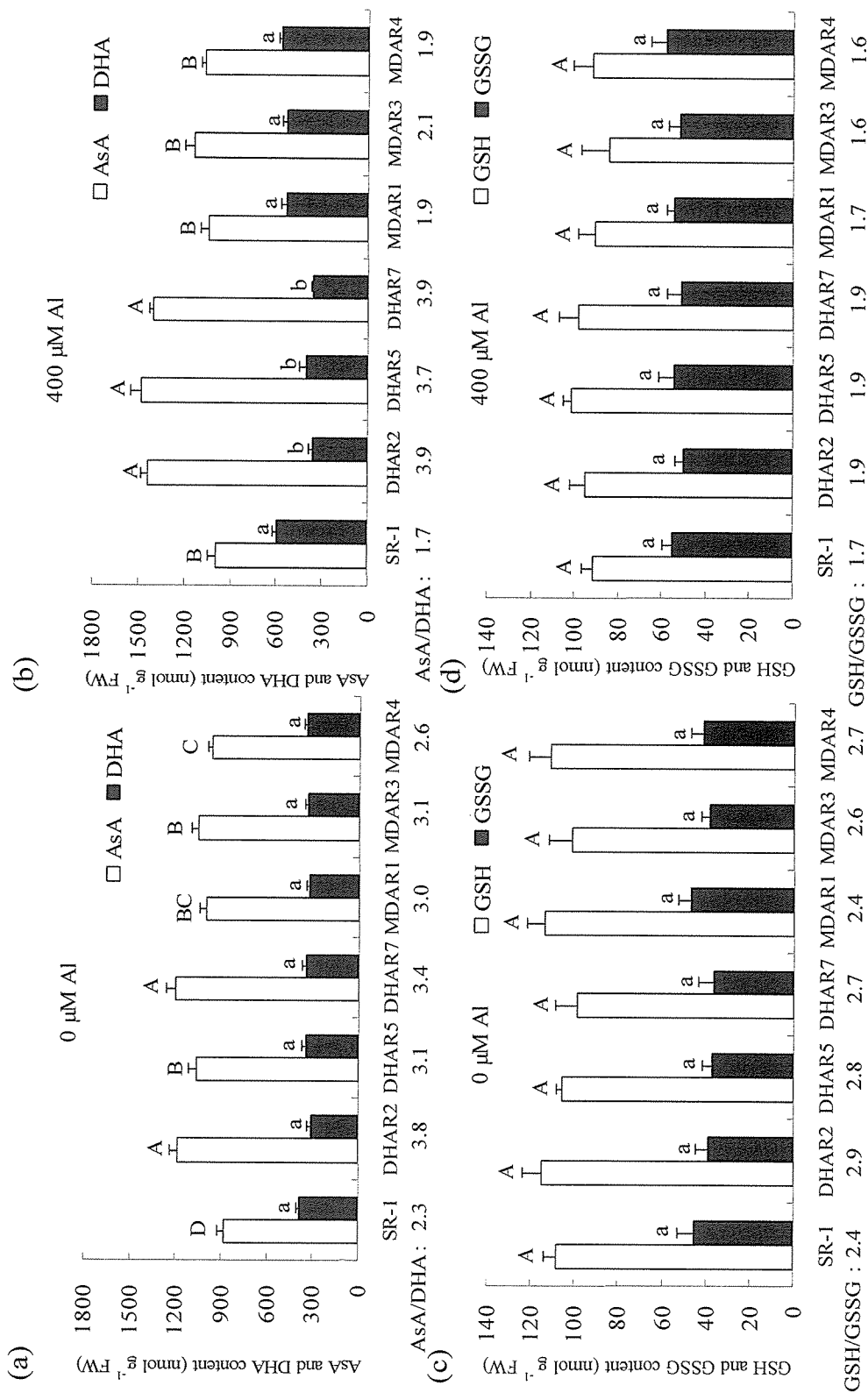
## AsA and GSH levels

In the absence of Al, DHAR-OX and MDAR-OX plants showed higher AsA level and AsA/DHA ratio than SR-1 plants (Fig. 8a). With 400  $\mu$ M Al treatment, there was no

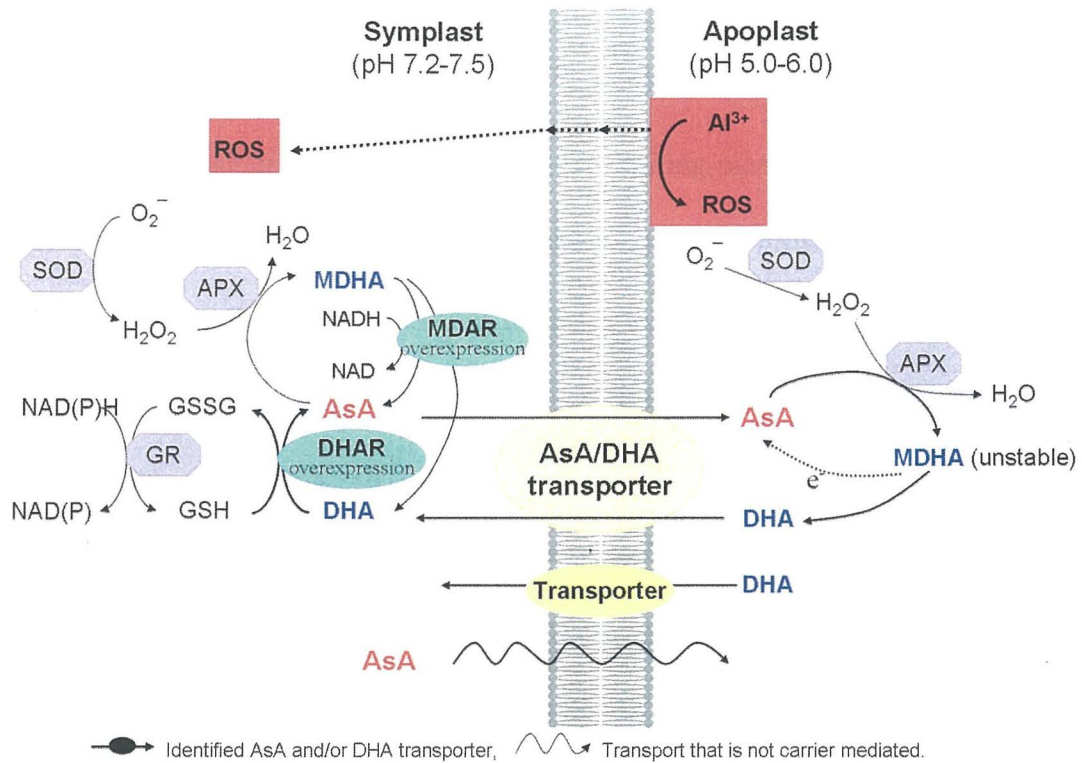
difference in AsA level between SR-1 and MDAR-OX plants, while DHAR-OX plants showed a significantly higher AsA level compared to both SR-1 and MDAR-OX plants (Fig. 8b). Also, upon exposure to Al, MDAR-OX plants showed higher DHA contents and lower AsA/DHA ratio than that in DHAR-OX plants. No difference in GSH and GSSG contents was observed among SR-1, DHAR-OX and MDAR-OX plants both with and without Al treatment (Fig. 8c, d).



**Figure 7** Effect of Al on activities of DHAR (a), MDAR (b), APX (c) and GR (d) in the roots of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX). Plants were exposed to 0 or 400  $\mu\text{M Al}$  for 24 h. Data are mean  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).



**Figure 8** Effect of Al on levels of AsA (a and b) and GSH (c and d) redox state in the roots of wild-type (SR-1) and transgenic plants (DHAR-OX and MDAR-OX). Plants were exposed to 0 or 400 μM Al for 24 h. Data are mean ± SE ( $n = 3$ ). Values followed by the same letter are not significantly different (LSD test,  $P < 0.05$ ).



**Figure 9** Overview of the hypothetical interactions between Al-induced ROS and the depletion/regeneration of AsA. Al-induced ROS are mainly in apoplast, and they deplete apoplastic AsA with generation of MDHA. In the apoplast, transportation system for MDHA remains unknown. Since MDHA is rather unstable in the acidic condition, if not transported, it would spontaneously oxidize to DHA. Thus, overexpression of cytosolic MDAR can have no effect on apoplastic AsA regeneration under Al stress. However, apoplastic generated DHA can be transported into the symplast by an AsA/DHA transporter, thus, can be re-reduced by the activity of overexpressed DHAR using GSH as an electron donor. Symplastic regenerated AsA can be transported or diffused into the apoplast for further ROS detoxification. Therefore, in this model, overexpression of DHAR can maintain high levels of AsA under Al stress and can confer tolerance to Al. This scenario would explain as to why MDAR-OX plants showed no amelioration of Al-induced stress, but DHAR-OX plants showed enhanced tolerance to Al.

## 2.5 Discussion

Al toxicity is one of the most important factors limiting plant productivity in acid soils. The primary symptom of Al toxicity is the inhibition of root growth, which has become a widely accepted indicator for assessing the Al tolerance in plants (Delhaize and Ryan, 1995; Ezaki et al., 2000; Tahara et al., 2008). In this study, transgenic plants overexpressing *Arabidopsis* DHAR showed better root growth than wild-type plants under Al stress, while transgenic plants overexpressing *Arabidopsis* MDAR showed root growth similar to that of wild-type plants. These results indicate that Al tolerance was elevated in DHAR-OX plants, but not in MDAR-OX plants (Fig. 1).

In various plant species, a decrease in Al accumulation in the root tip has been considered as an important mechanism conferring Al tolerance (Ma et al., 2001). In this study, we observed no difference in Al localization and accumulation in root tips of SR-1, DHAR-OX, and MDAR-OX plants after 24 h Al exposure (Fig. 2), which indicates that overexpression of DHAR and MDAR had no effect on alleviation of Al accumulation. Similarly, Devi et al. (2003) reported that an Al-tolerant tobacco cell line accumulated Al to the same extent as its isogenic Al-sensitive cell line, and the tolerant line was also more tolerant to oxidative stress caused by H<sub>2</sub>O<sub>2</sub>, copper and iron, suggesting that Al-tolerance is also related to tolerance to oxidative stress. Recently, ROS have been recognized as an important cause of damage to the root under Al stress (Ma et al., 2007; Tahara et al., 2008), and high ROS scavenging ability can result in an enhanced tolerance to Al (Ezaki et al., 2008). We therefore postulated that the higher Al tolerance ability in DHAR-OX plants could be associated to its higher antioxidant capacity.

In our study, Al treatment triggered H<sub>2</sub>O<sub>2</sub> production, lipid peroxidation, and the loss of plasma membrane integrity (Figs. 3, 4, 5), which is in agreement with previous results (Yamamoto et al., 2001; Ma et al., 2007; Achary et al., 2008). The present study also showed that the amount of 8-OHdG, as marker of DNA oxidative damage (Watanabe et al., 2006), was increased significantly in Al-treated roots and provides evidence that Al toxicity results in oxidative DNA damage. However, DHAR-OX plants showed lower H<sub>2</sub>O<sub>2</sub> production and lipid peroxidation, better maintenance of plasma membrane integrity and less oxidative DNA damage under Al stress compared to SR-1 plants. These differences can be attributed to the higher AsA level in DHAR-OX plants as compared to SR-1 plants (Fig. 8b). The higher AsA level also contributed to maintain higher APX activity, since APX can be inactivated by H<sub>2</sub>O<sub>2</sub> in the absence of AsA (Hossain and Asada, 1984). There was no difference in GSH content and GR activity in all plants after Al treatment (Figs. 6d, 7d), which indicates that the GSH level is not a limiting factor for AsA regeneration. In addition, DHAR-OX plants showed lower DHA levels and higher AsA levels than SR-1 plants under Al stress. This indicates the efficient regeneration of DHA to AsA by DHAR. The high AsA content and APX activity in DHAR-OX plants decreased H<sub>2</sub>O<sub>2</sub> level, and resulted in lower level of lipid peroxidation, loss of plasma membrane integrity and oxidative DNA damage. In MDAR-OX plants, no increase in AsA level and APX activity was observed under Al stress, and these plants showed Al injuries similar to SR-1 plants. Thus, the higher amount of AsA in DHAR-OX plants is associated with higher Al tolerance, which suggests that this enzyme plays an important role in AsA regeneration during Al exposure.



Our study also showed that both DHAR-OX and MDAR-OX plants exhibited a higher total AsA (AsA plus DHA) level than SR-1 plants in the absence of Al. It appears that the transgenic plants were more efficient in converting DHA or MDHA to AsA (Eltayeb et al., 2006, 2007). In the absence of such conversion, MDHA is easily oxidized to DHA and DHA is easily hydrolyzed to 2,3-diketogulonic acid. Previous studies (Chen et al., 2003; Eltayeb et al., 2006, 2007) have also shown that overexpression of DHAR or MDAR resulted in increased total AsA level. Our study also showed a small increase in total AsA level in all plants after Al treatment (Fig. 7 a, b). A similar increase in total AsA level was also found in citrus leaves under Al stress (Chen et al., 2005), and in cowpea under manganese stress (Fecht-Christoffers and Horst, 2005). We cannot exclude the possibility that Al stress may also induce the synthesis of AsA by a yet unknown mechanism to provide protection from Al toxicity.

Aluminum has a low mobility in plants. Over 85% of accumulated Al is bound to the cell wall and plasma membrane in the apoplast, and therefore, the Al effect is mainly derived from its external association with cells (Yamamoto et al., 2003; Wang et al., 2004). Recently, it was reported that Al triggered ROS ( $O_2^-$ ,  $H_2O_2$  and  $\cdot OH$ ) generation in the apoplast within 4 h under both low (1  $\mu M$ ) and high (100  $\mu M$ ) Al concentrations (Achary et al., 2008). Histochemical detection also showed a high level of  $H_2O_2$  accumulation in the apoplast (Xue et al., 2008). Therefore, scavenging of ROS in the apoplast is important for improving Al tolerance. In the apoplast, AsA is the most important antioxidant molecule (Pignocchi and Foyer, 2003; Conklin and Barth, 2004), and 90% of AsA degradation occurs in the apoplast via the production of DHA (Green and Fry, 2005). Moreover, apoplastic AsA is important in elongation growth of cell walls and in the

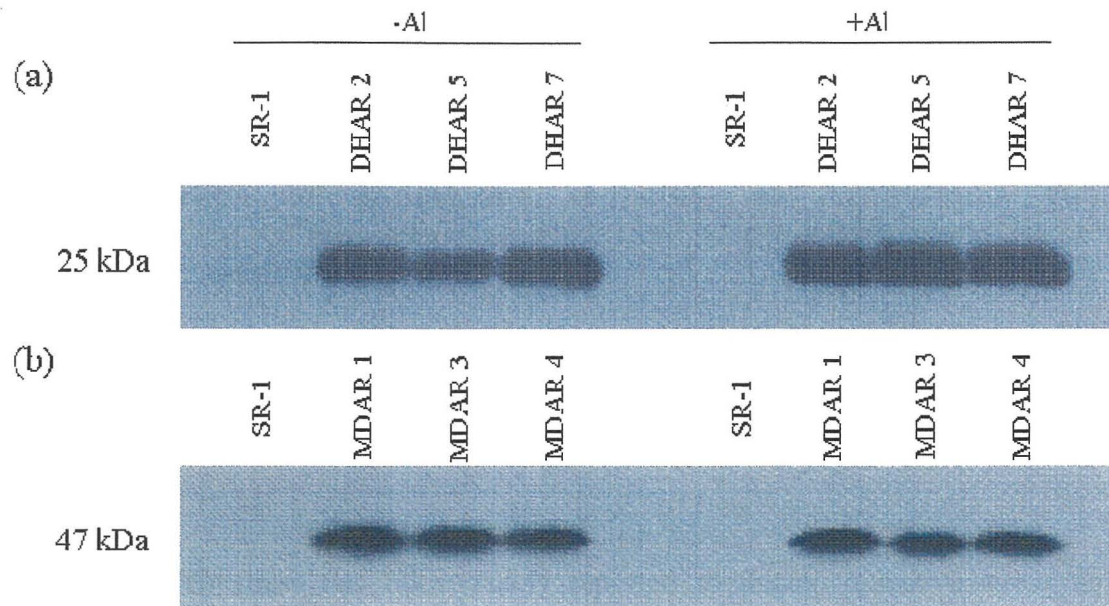
signal transduction pathways that regulate cell growth (Pignocchi and Foyer, 2003; Green and Fry, 2005; Houde and Diallo, 2008). Therefore, maintaining a high AsA level in the apoplast is important for the defense against Al-induced oxidative damage.

DHAR is located in the symplast, including cytoplasm, plastids, mitochondria, and chloroplasts (Conklin and Barth, 2004; Mittler et al., 2004). Thus, oxidized AsA (MDHA and DHA) in the apoplast must be regenerated either by a plasma membrane MDAR as proposed by Bérczi and Møller (1998) or after transportation across the plasma membrane. Previous studies have demonstrated the existence of an AsA/DHA transporter in the plasma membrane (Horemans et al., 1997, 2000). The apoplastic DHA is actively transported by an AsA/DHA transporter across the plasma membrane and is reduced by DHAR in the symplast, with concurrent export of AsA to the apoplast (Horemans et al., 2000). Furthermore, it has been reported that the rate of transport of AsA/DHA is not a limiting factor suggesting that the symplastic recycling of DHA back to AsA could be efficiently regenerated provided that sufficient DHAR enzyme is available (Conklin and Barth, 2004). Therefore, the cytosolic DHAR can regulate apoplastic AsA content through the function of AsA/DHA transporter (Fig. 8). In addition, when ROS generated in the apoplast penetrate into the symplast, or are generated within symplast in response to Al treatment, they oxidize AsA to form DHA. The DHAR in the symplast can reduce DHA to AsA directly (Fig. 8). Thus, it is likely that the DHAR in the symplast can efficiently recycle both apoplastic and symplastic DHA back to AsA, and can protect the cells from oxidative damage caused by Al-triggered ROS. Chen and Gallie (2005) have shown that overexpression of cytosolic DHAR regulated both symplastic and apoplastic AsA levels. Yoshida et al. (2006) proved that cytosolic DHAR was important for

reducing ROS from extracellular sources, such as ozone, and in elevating ozone tolerance through increasing apoplastic AsA content. To confirm this, the apoplastic or symplastic AsA and DHA levels have to be measured in the root tip. However, it is difficult to separate apoplastic and symplastic AsA in tobacco root, especially in the Al-damaged region (last 0–2 mm of root tip). Taken together, our findings suggest that Al-induced  $H_2O_2$  can be detoxified by reaction with AsA, catalyzed by APX, with a consequent production of DHA. Because there is high activity of DHAR in the symplast in DHAR-OX plants, and the apoplastic DHA can be transported into the symplast, both symplastic and apoplastic DHA are effectively reduced to AsA by cytosolic DHAR, and the recycled AsA can return to the apoplast, through a transporter in the plasma membrane, for further detoxification of Al-induced  $H_2O_2$  in the apoplast (Fig. 8).

In contrast to DHA, MDHA is an unstable radical, especially under acidic conditions (apoplast pH is 5–6); if not rapidly reduced, the apoplastic MDHA will spontaneously be oxidized to DHA (Asada, 1999). In our study, overexpression of cytosolic MDAR increased MDAR activity but showed no effect on accelerating the AsA regeneration under Al stress. Since Al induces ROS generation mainly in the apoplast, MDHA may be mainly produced in the apoplast. MDAR is located in the symplast, and if apoplastic generated MDHA cannot be rapidly transported into the symplast, MDAR would have no effect on apoplastic MDHA regeneration. However, there is no known system for the transportation of MDHA from the apoplast to the symplast. Thus, the apoplastic MDHA will have to be directly oxidized to DHA. The overexpressed cytosolic MDAR would, therefore, have no effect on AsA regeneration under Al stress (Figs. 8, 9).

In summary, we have characterized the functions of DHAR and MDAR in the alleviation of Al-stress. DHAR-OX plants had a better tolerance to Al stress, but MDAR-OX plants did not. Al tolerance in DHAR overexpressing plants is ascribed to the alleviation of ROS damage by maintaining a high AsA level and APX activity. In contrast, plants overexpressing MDAR are not able to maintain a high AsA level and APX activity under Al stress and therefore show no Al tolerance. These results show that the higher DHAR activity, which results in maintenance of a high level of AsA and APX activity, can contribute to Al tolerance in tobacco.



Supplemental Figure. 1

Western blot analysis of the proteins encoded by the DHAR (a) and MDAR (b) genes. Proteins were extracted from SR-1, DHAR-OX and MDAR-OX roots exposed to 0 or 400  $\mu$ M Al for 24 h. A 20- $\mu$ g protein sample from root was separated by SDS-PAGE, transferred to Hybond ECL nitrocellulose membrane, and immunoblotted with antibody against *Arabidopsis* DHAR or MDAR as described by Eltayeb et al. (2006, 2007).

## Chapter 3

# Overexpression of glutathione reductase in *Arabidopsis* confers tolerance to aluminum stress

### 3.1 Abstract

Aluminum (Al) stress has been widely reported to induce oxidative stress through formation of reactive oxygen species in plant cells. Glutathione (GSH) is a cellular antioxidant that protects cells from oxygen stress. Glutathione reductase (GR) is an important enzyme for GSH regeneration. To examine the protective effect of GR against Al stress, we developed transgenic *Arabidopsis* plants overexpressing cytosolic *AtGR1* gene (accession No. At3g24170). GR transgenic plants exhibited better root elongation, lower hydrogen peroxide content and less lipid peroxidation compared to wild-type plants under Al stress. Although no difference in Al accumulation and the activities of superoxide dismutase (SOD), catalase (CAT) and dehydroascorbate reductase (DHAR) were observed in roots of transgenic and wild-type plants after 24-h Al treatment, GR transgenic plants showed higher activities of GR and ascorbate peroxidase (APX), and higher levels of GSH and ascorbate than wild-type plants. Our results demonstrate that overexpression of GR improves the antioxidant capacity, and leads to enhanced tolerance to Al stress.

Key words: Aluminum tolerance; antioxidant enzymes; glutathione; glutathione reductase; reactive oxygen species

**Abbreviations:**

APX	ascorbate peroxidase
CAT	catalase
DHAR	dehydroascorbate reductase
GR	glutathione reductase
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
MDA	malondialdehyde
OX	overexpression
ROS	reactive oxygen species
SOD	superoxide dismutase

### 3.2 Introduction

Aluminum (Al) is the most abundant metal in the earth's crust and is a major factor limiting plant growth and productivity in acid soils (Kochian, 1995). The primary site of Al accumulation and toxicity is the root meristem, and the inhibition of root elongation is the most notable symptom of Al toxicity (Delhaize and Ryan, 1995; Yamamoto et al., 2003). In roots, Al triggers the sustainable production of reactive oxygen species (ROS), such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sub>2</sub><sup>-</sup>), and Al-induced inhibition of root elongation shows a strong positive correlation with Al-induced ROS generation (Jones et al., 2006; Tahara et al., 2008; Xue et al., 2008). Lipid peroxidation, which is an important symptom of oxidative stress, is associated with Al exposure in several species (Yamamoto et al., 2001; Basu et al., 2001; Ma et al., 2007).

In plants, oxidative damages can be alleviated through enhanced antioxidant capacity. Under Al stress, several antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) are up-regulated (Chen et al., 2005). ROS scavenging genes are also induced by Al, such as peroxidase, glutathione S-transferase (GST), SOD, and blue copper binding (BCB) protein (Richards et al., 1998; Houde and Diallo, 2008). Overexpression of four Al-induced genes [*Arabidopsis* blue-copper-binding protein (*AtBCB*), *N. tabacum* glutathione S-transferase (*parB*), *N. tabacum* GDP-dissociation inhibitor gene (*NtGDII*) and *N. tabacum* peroxidase gene (*NtPox*)] ameliorated Al toxicity in *Arabidopsis* (Ezaki et al., 2000). Three of these genes also provided protection against oxidative stress. Overexpression of genes encoding antioxidant enzymes (MnSOD and DHAR) in canola and tobacco also conferred Al



tolerance (Basu et al., 2001; Yin et al., 2009). In *Melaleuca* trees, the Al tolerant cultivar showed higher activity of antioxidant enzymes than the sensitive one under Al stress (Tahara et al., 2008). These findings demonstrate a strong link between Al toxicity and oxidative stress, and enhanced antioxidant capacity can enhance Al tolerance.

Glutathione ( $\gamma$ -Glu-Cys-Gly, GSH) and glutathione reductase (GR, EC 1.6.4.2) are important components of the cell's scavenging system for ROS (Foyer and Halliwell, 1976; Kunert and Foyer, 1993; Pilon-Smits et al., 2000). GSH is a substrate for the hydrogen peroxide-removing enzyme, GSH peroxidase, and for dehydroascorbate reductase (Yamaguchi et al., 1999). Most of the metabolic functions of GSH involve the oxidation of GSH to glutathione disulfide (GSSG) (Pilon-Smits et al., 2000). GR mediates the reduction of GSSG to GSH.

An increase in GSH contents and GR activity can confer tolerance to various stresses including Al stress (Yamaguchi et al., 1999). Yamaguchi et al. (1999) reported that endogenous GSH protect suspension-cultured tobacco cells from Al toxicity. Devi et al. (2003) reported that an Al-tolerant tobacco cell line had higher contents of AsA and glutathione (GSH) than the isogenic Al-sensitive cell line; and the higher AsA and GSH contents in the tolerant cell line could be related to the protection of cells from ROS generation and lipid peroxidation. Studies using transgenic plants have shown that GR plays an important role in resistance to oxidative stress caused by photoinhibition (Aono et al., 1993; Foyer et al., 1995) and paraquat (Aono et al., 1995). These findings suggest that maintaining a high GSH level is important in achieving oxidative tolerance including Al tolerance.

Oxidative stress is involved in Al toxicity, but the mechanisms underlying the regeneration of GSH and the protection of plants against Al damage by GR are unknown. Here, we investigated these mechanisms by developing transgenic Arabidopsis plants overexpressing *AtGR1* gene (GR-OE) and characterized the effect of *AtGR1* overexpression on tolerance to Al stress.

### 3.3 Materials and methods

#### Construction of plant expression vector using Gateway cloning technology

The coding region of the *A. thaliana* cytosolic AtGR1 gene (accession No. At3g24170) was amplified from cDNA with the primer AtGRGW-1 5'-GGCTTCACCATGGCGAGGAAGATGATGCTTGTTGATGGTGA-3' and AtGRGW-2 5'-GAAAGCTGGGTCTCATAGATTTGTCTTAGGTTTGGGTTTGT-3'. The PCR conditions were: 94 °C for 5 min, 94 °C for 30s, 55 °C for 45s, 72 °C for 1 min, 72 °C for 7 min, 30 cycles (Takara Thermal Cycler, TP600, TAKARA SHIZO CO.LTD., Ohtsu, Japan). The second PCR was performed with primers 5'-GGGGACAAGTTTGTACAAAAAGCAGGGCTTCACCATG-3' and 5'-AGATTGGGGACCACTTTGTACAAGAAAGCTGGGTC-3' which contained *attB* sites and the PCR products from the first reactions as template, and the PCR condition was the same as above. The linear fragments flanked by *attB* sequences were subjected to site-specific recombination with the entry vector pDONR<sup>TM</sup>/Zeo (Invitrogen), containing the *ccdB* gene, flanked by *attP* sites and catalyzed by BP Clonase, yielding entry clones that were used to transform *E. coli* competent DH5a cells. Transformants were grown on LB agar plates containing  $\mu\text{g ml}^{-1}$  zeocin. Colonies were picked from each plate for colony PCR using Taq polymerase and outer pDONR primers (Invitrogen); and grown in liquid culture for subsequent plasmid preparation. The entry clones were subjected to another round of site-specific recombination catalyzed by the LR Clonase enzyme mix in order to subclone the genes of interest into a destination vector, i.e. plant expression vector pEarleyGate vector 201 (Earley et al., 2006) containing one cauliflower mosaic

virus 35S promoter and the *ccdB* gene flanked by *attR* sites, to generate expression clones. The resulting expression construct was used to transform *E. coli* DH5 $\alpha$  strains. Transformants were selected on LB agar plates containing  $\mu\text{g mL}^{-1}$  kanamycin. The construct (pEarleyGate201-AtGR1) was then cloned into *Agrobacterium tumefaciens* strain C58C1 by Free and Thaw method (Xu and Li, 2008).

#### Agrobacterium-mediated transformation of Arabidopsis

*Agrobacterium tumefaciens* strain C58C1 carrying the binary plasmid pEarleyGate201-AtGR1 was used and transformed according to Clough and Bent (1998). Bacteria were grown to stationary phase in liquid culture at 25-28°C, 250 rpm in sterilized LB (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter water) carrying added kanamycin ( $50 \mu\text{g mL}^{-1}$ ). Cultures were typically started from a 1:100 dilution of small overnight cultures and grown for roughly 18-24 h. Cells were harvested by centrifugation for 20 min at room temperature at 5500 g and then resuspended in infiltration medium to a final OD<sub>600</sub> of approximately 0.8 prior to use. The revised floral dip inoculation medium contained 5% sucrose and 0.05% Silwet L-77 (OSi Specialties, Inc., Danbury, CT, USA).

For floral dip, the inoculum was added to a beaker, plants were inverted into this suspension such that all above-ground tissues were submerged, and plants were then removed after 3-5 sec of gentle agitation. Dipped plants were removed from the beaker, placed in a plastic tray and covered with a tall clear-plastic dome to maintain humidity. Domes were removed approximately 12-24 h after treatment. Plants were grown for a

further 3-5 weeks until siliques were brown and dry, keeping the bolts from each pot together and separated from neighboring pots using transparent plastic cover. Seeds were stored in microfuge tubes and kept at 4°C under desiccation.

#### Growth conditions

Three transgenic *Arabidopsis* (*Arabidopsis thaliana*) lines, line 2, line 9 and line 11, that overexpress *A. thaliana* GR (GR-OX lines), the wild type Columbia (Col) were used. All the experiments were performed in a growth chamber kept at 25°C during the day and night, with a 14-h photoperiod at 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation.

#### Western blot analysis

Proteins were extracted from Col control and GR-OE plants by extracting buffer (50 mM Tris-HCl (pH7.5) containing 100 mM NaCl, 1 mM EDTA, 0.1% SDS and 1% protease inhibitor (Sigma)). Protein samples (20  $\mu\text{g}$ ) were separated in 10 % SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) by ATTO semidry transfer cell (ATTO Corporation, Tokyo, Japan). Immunodetection was performed using diluted (1 : 3000) antibodies against HA protein as the first antibody, and a diluted (1 : 5000) horseradish peroxidase-conjugated anti-rat (Roche, Mannheim, Germany) as the second antibody.

#### Al treatment

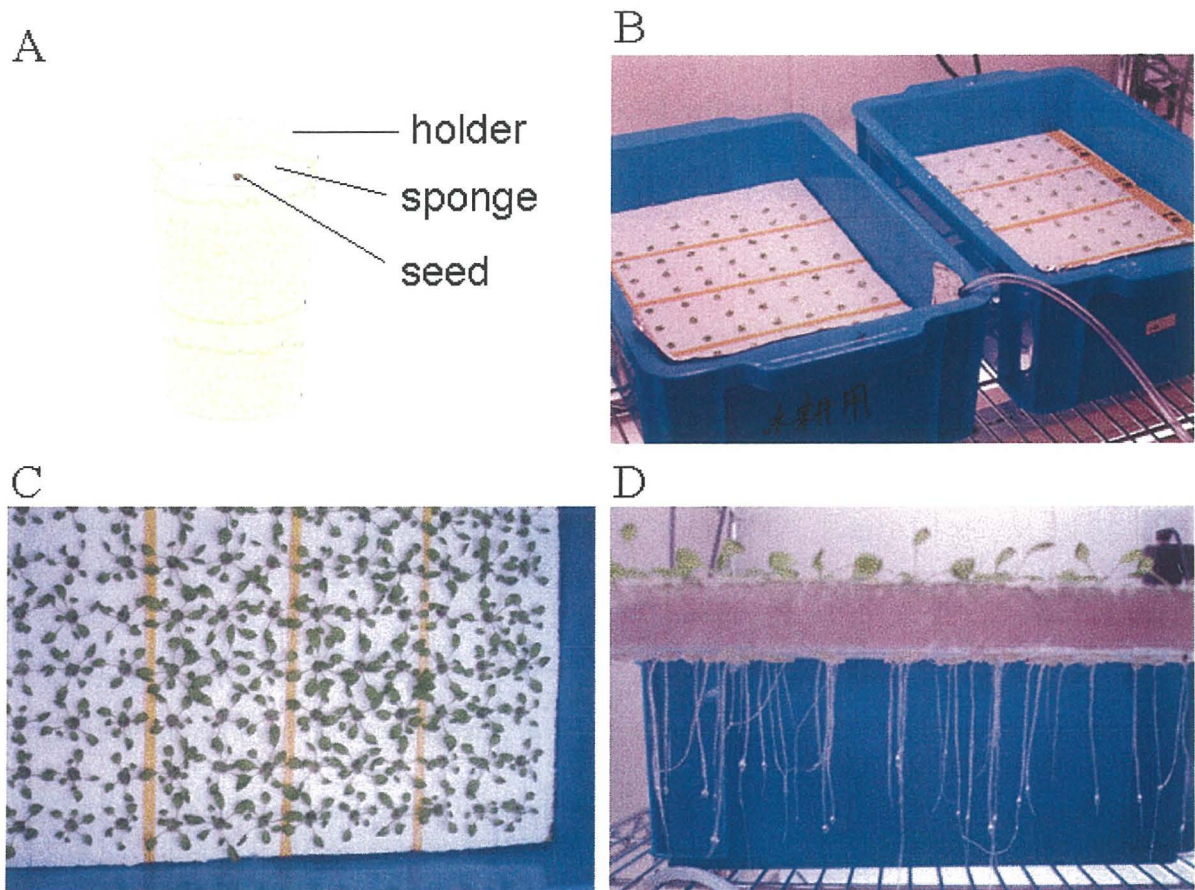
Seeds were sterilized in 2% (w/v) sodium hypochlorite for 20 min and incubated in 4°C for 2 d. Then, seeds were sown on the surface of 1/6 MS (Murashige and Skoog, 1962) agar (0.6 % agar, 1% sucrose) plate containing 0 $\mu$ M AlCl<sub>3</sub> at pH 5.7 (pH control), or containing 0, 50, 100, or 200  $\mu$ M AlCl<sub>3</sub> at pH 4.2, in square Petri dishes according to Yin et al. (2009). The Petri dishes were placed in the growth chamber and positioned vertically for 2 days, by which time the seeds had germinated; there was no difference in germination between the Col and GR-OE plants. The Petri dishes were then tilted backward 45° and the plants were allowed to grow for an additional 4 days. At the end of that period, the root length was measured for each treatment. Root elongation values under different levels of Al were presented as percentage of the value under control (no Al).

For further assay of Al tolerance, the plants were grown in a hydroponic system. Sterilized seeds were sown on sponges with a holder in aerated 1/6 Hoagland solution (HS) (pH 5.7) and cultured for 21 d (Fig. 1). The uniformly grown plants were selected and pre-cultured for 24 h in 1/6 HS (pH 4.2). They were then exposed to 0  $\mu$ M (pH 5.7), 0  $\mu$ M (pH 4.2) or 50  $\mu$ M AlCl<sub>3</sub> in 1/6 HS (pH 4.2) for 24 h. Roots were sampled and used for determining the contents of Al, H<sub>2</sub>O<sub>2</sub> and MDA, enzyme activities and antioxidant amount.

#### Al content

For determination of the Al content, 10 mg root tip (0 to 1 cm) was washed three times with distilled water and dried, then digested with a concentrated acid mixture

( $\text{HNO}_3:\text{H}_2\text{SO}_4$ , 1:1, v/v) at  $160^\circ\text{C}$  for 3 h. The Al content was quantified by using an inductively coupled plasma atomic emission spectrometer (ICP-AES, Ciros CCD, Rigaku, Japan).



**Figure 1.** Hydroponic system for growing multiple *Arabidopsis* plants. A, A carrier of the culture apparatus. B, Seeds were sown on the sponge and cultured in well aerated 1/6 HS. C, Up view of 21-d seedlings. D, Roots of 21-d seedlings.

## H<sub>2</sub>O<sub>2</sub> content

Hydrogen peroxide content was determined according to the method of Ryan et al. (2009). Roots (0.1 g) were frozen in liquid nitrogen, ground to powder in pre-cooled mortars, and homogenized with 1.5 ml cold 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12000g for 30 min at 4 °C, and 0.4 ml of the supernatant was added to 0.4 ml 10 mM potassium phosphate buffer (pH 7.0) and 0.8 ml 1 M KI. The absorbance of the mixture was read at 390 nm, which developed within 25 min and was stable for at least 2 h. The content of H<sub>2</sub>O<sub>2</sub> was calculated against a calibration curve using H<sub>2</sub>O<sub>2</sub> standards.

## MDA determination

The amount of MDA, the end product of lipid peroxidation, was assessed by using the TBARS method (Heath and Packer, 1968). Roots (0.15 g) were frozen in liquid nitrogen and homogenized by mortar and pestle in 2 mL pre-cooled 50 mM potassium phosphate (pH 7.8) at 4 °C. The homogenate was centrifuged at 5000g for 10 min. The supernatant was used for MDA assay and MDA content was calculated using 155 mM<sup>-1</sup>cm<sup>-1</sup> as extinction coefficient.

## Enzyme activities

Root tissues were frozen in liquid nitrogen, ground to fine powder in pre-cooled mortar and homogenized in 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM



ascorbate. The slurry was then centrifuged at 12 000g for 30 min at 4 °C, and the supernatant was used for enzyme assays at 25 °C.

Glutathione reductase (GR, EC1.6.4.2) activity was determined spectrophotometrically by monitoring GSSG (glutathione oxidized)-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (Foyer and Halliwell, 1976). The reaction mixture (1 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.2 mM GSSG, 0.2 mM NADPH and 0.2 mL enzyme extract.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by the inhibition of cytochrome c reduction of xanthine-xanthine oxidase for at 550 nm (McCord and Fridovich, 1969). The reaction mixture (1 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.01 mM cytochrome C, 0.01 unit xanthine oxidase and 0.2 mL enzyme extract.

Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the rate of disappearance of H<sub>2</sub>O<sub>2</sub> at 230 nm (Cakmak and Marschner, 1992). The reaction mixture (1 mL) consisted of 100 mM potassium phosphate buffer (pH 7.8), 10 mM H<sub>2</sub>O<sub>2</sub> and 0.2 mL of enzyme extract.

APX (EC 1.11.1.11) activity was determined by measuring the decrease in absorbance at 290 nm as ascorbate is oxidized according to Nakano and Asada (1981). The reaction mixture (1 mL) consisted of 100 mM potassium phosphate buffer (pH 7.8), 0.5 mM ascorbate, 0.5 mM H<sub>2</sub>O<sub>2</sub> and 0.2 mL of enzyme extract.

DHAR (EC 1.8.5.4) activity was determined by measuring the increase in absorbance at 290 nm as dehydroascorbate is reduced (Nakano and Asada, 1981). The reaction mixture (1 mL) consisted of 50 mM potassium phosphate buffer (pH 7.8), 2 mM

dehydroascorbate, 5 mM reduced GSH, 0.1 mM EDTA, and 0.2 mL enzyme extract.

The protein content in the enzyme extracts was determined according to Bradford (1976) method using bovine serum albumin as a standard.

#### Determination of Asc and GSH level

Roots (0.1 g) were frozen in liquid nitrogen and homogenized in 5% TCA and then centrifuged at 10 000g for 30 min at 4°C. The supernatant was used to determine total Asc and Asc contents according to Arakawa et al. (1981), and total glutathione (GSH + GSSG) and GSSG contents according to Anderson (1985).

The assay of Asc was based on the reduction of Fe (III) to Fe(II) by Asc, followed by the determination of Fe(II) by complexation with 4,7-diphenyl-1,10-phenanthroline. To determine DHA content, DHA was reduced to total Asc by DTT. DHA content was obtained from the difference between the total Asc and Asc.

The assay of total glutathione was based on sequential oxidation of GSH by 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and the reduction of GSSG by NADPH in the presence of GR. To determine GSSG content, 2-vinylpyridine was added to the supernatant. GSH content was obtained from the difference between the total glutathione and GSSG.

#### Statistical analysis

Statistic analysis was carried out using the Statistical Analysis System (SAS 8.0) software.

Data were subject to ANOVA, and means were compared by Tukey test ( $P < 0.05$ ).

### 3.4 Results

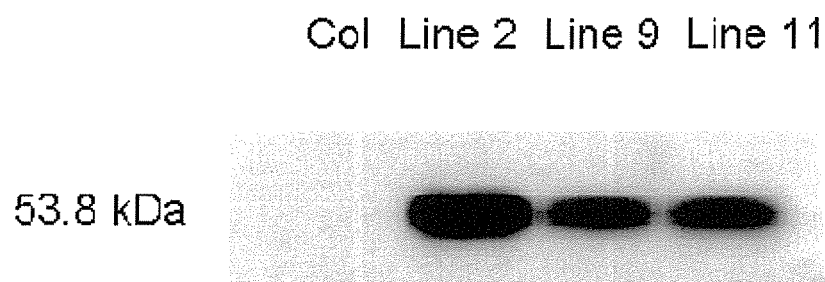
#### Effect of Al on root elongation

Overexpression of *AtGR1* gene was first confirmed by western blot analysis in GR-OX *Arabidopsis* plants (Fig. 2). To further examine whether overexpression of *AtGR1* improves Al tolerance in *Arabidopsis* plants, root elongation was compared between the GR-OX lines (Line 2, Line 9 and Line 11) and wild type Col. All lines showed similar root elongation on MS agar plates under pH 5.7 without Al treatment. Under acidic condition (pH 4.2 without  $\text{AlCl}_3$ ), wild type Col showed slightly slow root elongation compared with GR-OX lines. However, when  $\text{AlCl}_3$  was added to the plates, root elongation of all lines was inhibited significantly, and this inhibition increased with increasing  $\text{AlCl}_3$  concentration (Fig. 3). The inhibition was more severe in Col plants than in GR-OX plants. When exposed to 50  $\mu\text{M}$   $\text{AlCl}_3$ , the wild type Col showed a 38% decrease in root elongation, whereas GR-OX plants showed only a 23% reduction. Following 100  $\mu\text{M}$   $\text{AlCl}_3$  exposure, Col plants showed 47% inhibition of root elongation, whereas in GR-OX plants the inhibition was 33%. Especially, when exposed to 200 $\mu\text{M}$   $\text{AlCl}_3$ , root growth in wild type plants was inhibited by 80%, while roots of all three transgenic lines still showed 50% elongation (Fig. 3). These results indicate that overexpression of *AtGR1* could confer tolerance to Al in *Arabidopsis* plants.

#### Al accumulation in root tips

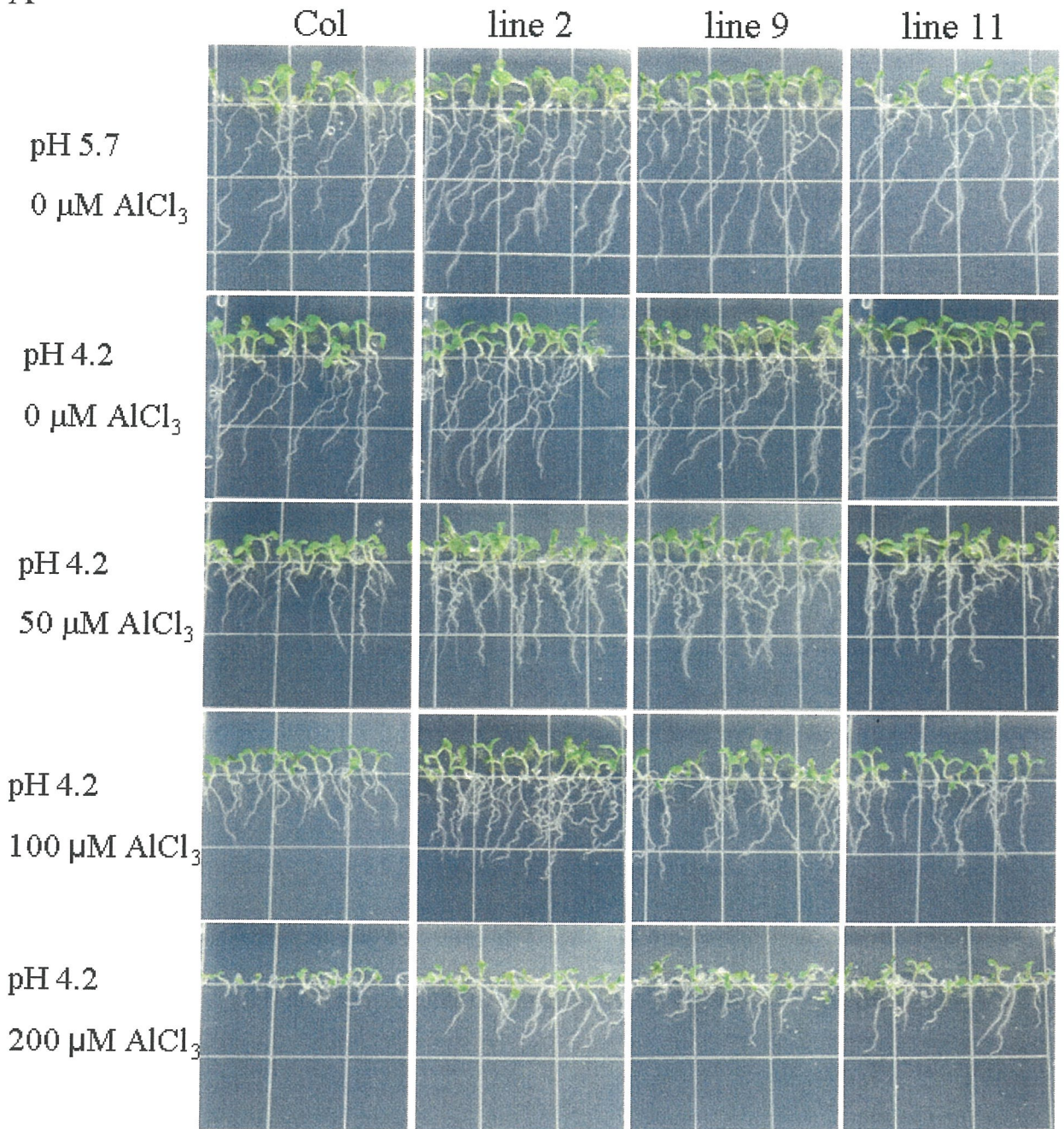
Al content was determined in 0-1 cm root tips of wild type Col and GR-OX lines (Fig. 4). There was no difference in Al contents in root tips of Col and GR-OX plants both with

and without (data not shown)  $\text{AlCl}_3$  treatment, indicating that overexpression of AtGR1 did not affect Al accumulation in Arabidopsis plants.

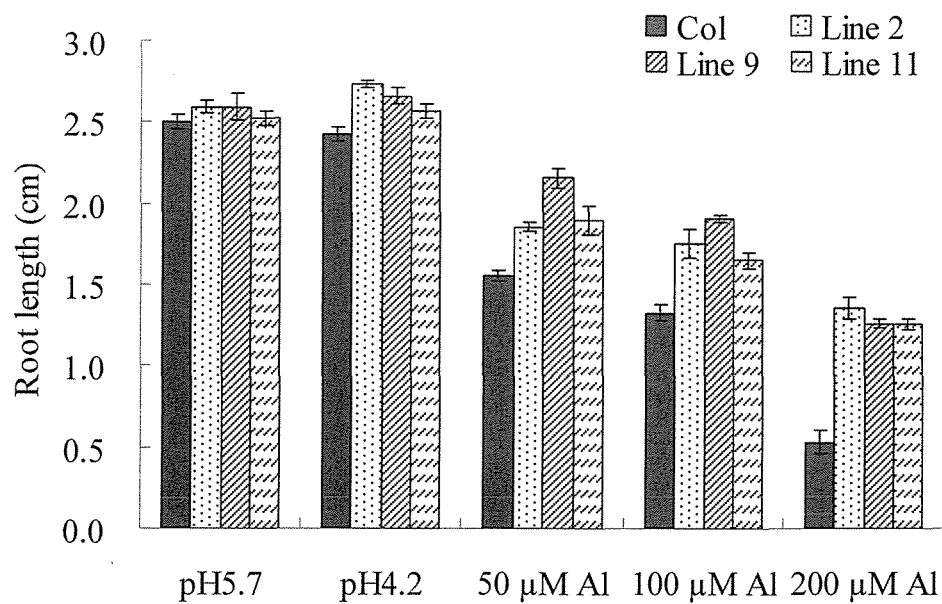


**Figure 2.** Western blot analysis confirmed the overexpression of *AtGR1* in Arabidopsis. A 20- $\mu\text{g}$  protein sample from root was separated by SDS-PAGE, transferred to Hybond ECL nitrocellulose membrane, and immunoblotted with antibody against HA as described in Material and methods.

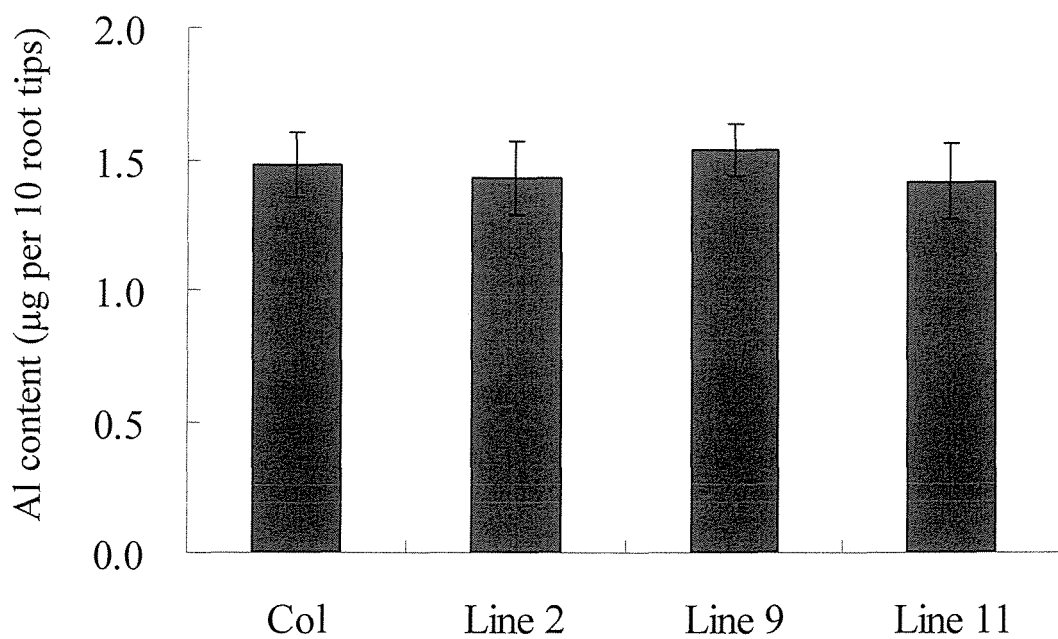
A



B



**Figure 3** A, Root growth of wild-type Columbia (Col) and transgenic GR-OX plants (Line 2, Line 9 and Line 11) under different AlCl<sub>3</sub> treatment. Seeds were grown on 1/6 MS agar plate containing 0 (pH 5.7 or 4.2), 50, 100 and 200 μM AlCl<sub>3</sub> for 7 d. B, Root length was measured after taking a photograph. Data are means ± SE of three replications (each replication included ten plants). Statistic analysis was done for each Al concentration independently. Data followed by the same letter are not significantly different according to Tukey test ( $P < 0.05$ ).



**Figure 4.** Al content in root tips (0-1 cm) of wild-type Columbia (Col) and GR-OX lines (Line 2, Line 9 and Line 11) after exposing to 50  $\mu\text{M}$   $\text{AlCl}_3$  for 24 h. Data are means  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different according to Tukey test ( $P < 0.05$ ).

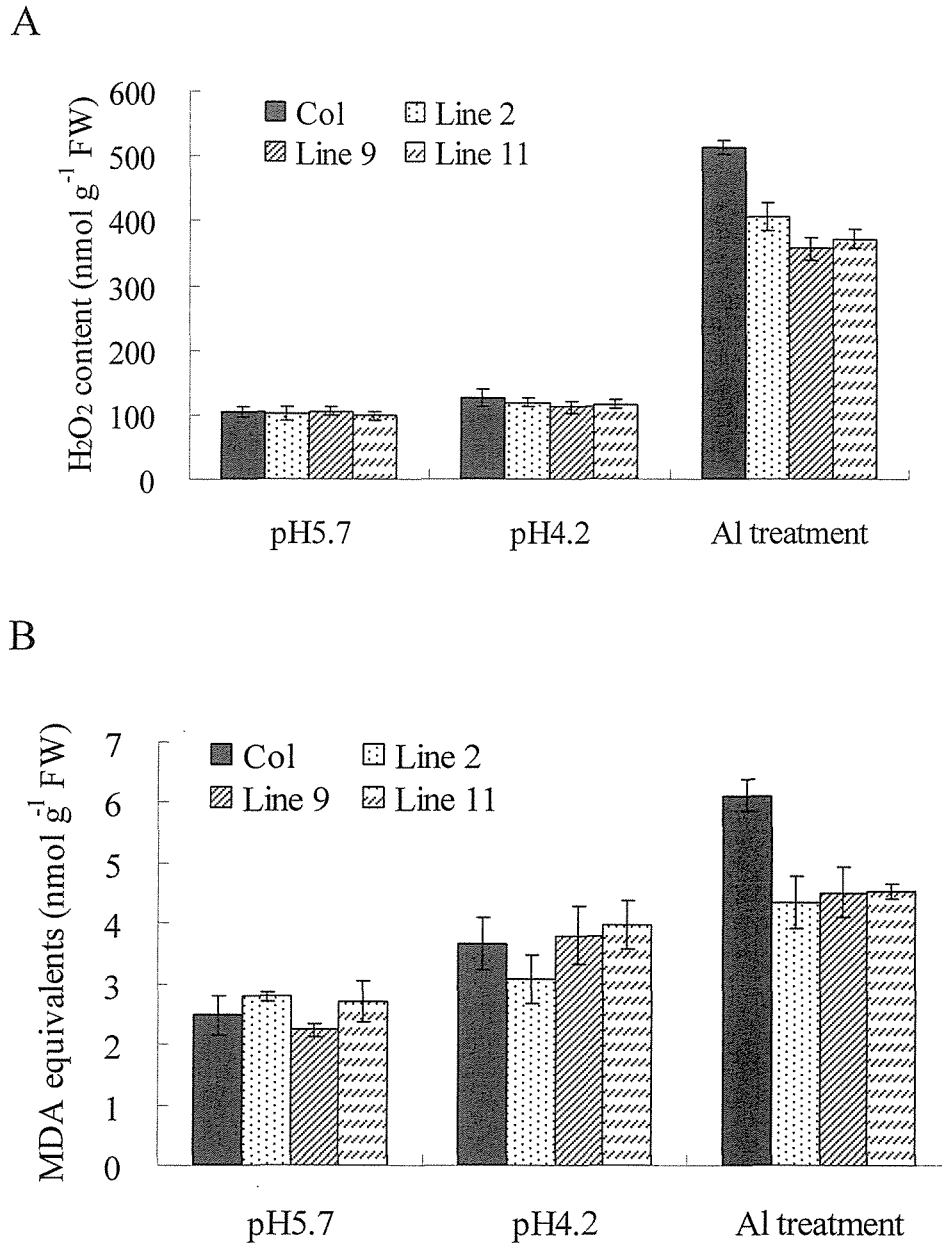


### Effect of Al on lipid peroxidation and hydrogen peroxide generation

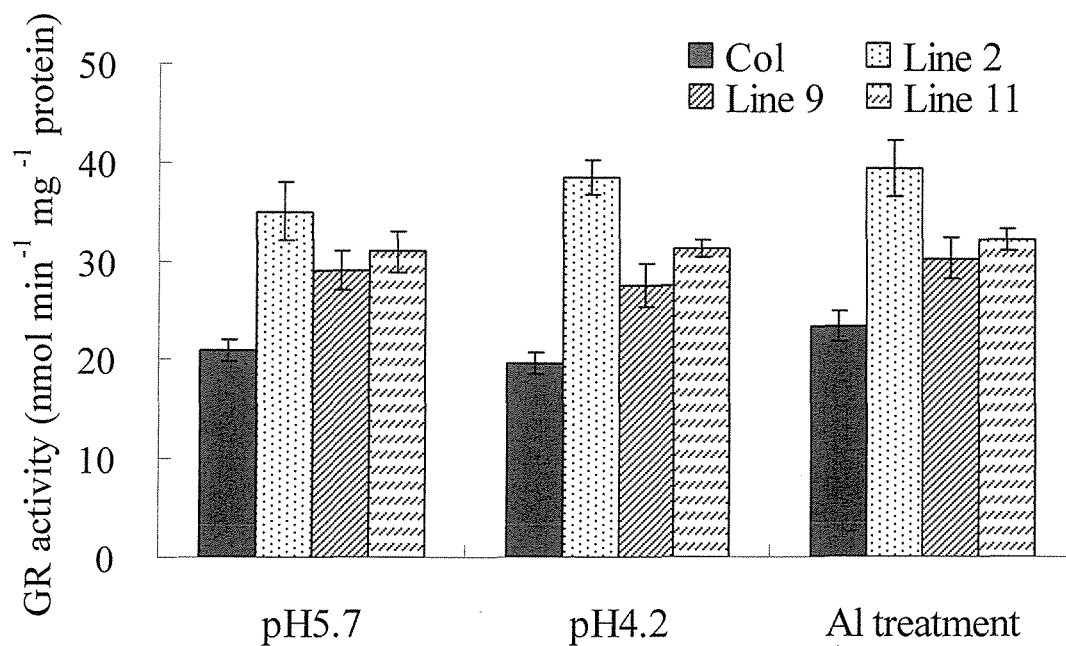
Lipid peroxidation was measured in terms of MDA content in roots of Col and GR-OX plants (Fig. 5a). No significant difference in MDA levels was observed between Col and GR-OX plants without Al treatment except at pH4.2 where MDA contents increased but non-significantly. Al treatment increased MDA content significantly in all lines. The increase was markedly higher in Col (2.5 folds) than in GR-OX plants (1.8 folds). It should be noted that the observed increase might be an under estimation of the actual values because of the technical limitation of inducing the non-injured tissue in the samples for MDA analysis. Thus, the actual increase of the MDA contents in the injured cells must be much greater than it appears in Fig. 4a. Moreover, under acidic condition without Al treatment, MDA contents also increased slightly. Similar with the change of MDA production under Al stress, H<sub>2</sub>O<sub>2</sub> contents increased significantly by AlCl<sub>3</sub> in all lines, but it increased more in wild type Col, which increased by 4.9 times than in GR-OX plants, which increased by 3.7 times (Fig. 5b).

### Effect of Al on GR activities

Regardless of Al exposure, GR activity was 1.4–2.0 times in the roots of GR-OX plants as compared with the wild type Col plants (Fig. 6). Both Al and acid treatment did not affect GR activity in Col and GR-OX plants.



**Figure 5.** Effect of Al on the levels of H<sub>2</sub>O<sub>2</sub> (A) and MDA (B) in roots of wild-type Columbia (Col) and GR-OX lines (Line 2, Line 9 and Line 11). Plants were exposed to 0 (pH 5.7 or 4.2) or 50  $\mu$ M AlCl<sub>3</sub> for 24 h. Data are means  $\pm$  SE (n = 3). Values followed by the same letter are not significantly different according to Tukey test ( $P < 0.05$ ).



**Figure 6.** Effect of Al on GR activity in roots of wild-type Columbia (Col) and GR-OX lines (Line 2, Line 9 and Line 11). Plants were exposed to 0 (pH 5.7 or 4.2) or 50  $\mu\text{M}$   $\text{AlCl}_3$  for 24 h. Data are means  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different according to Tukey test ( $P < 0.05$ ).

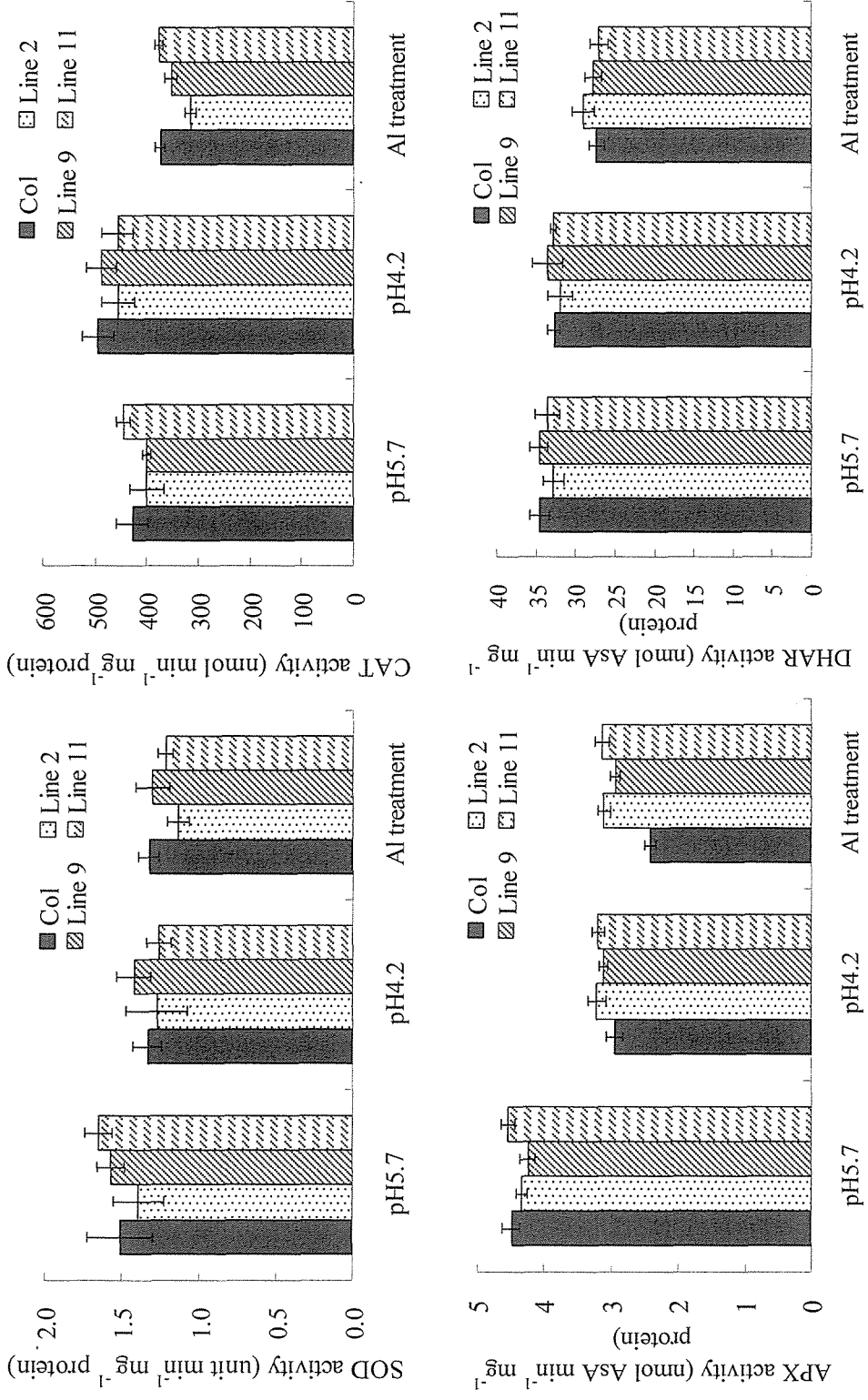
### Effect of Al on other antioxidant enzyme activities

In the absence of Al under pH 5.7 condition, no difference in the activities of CAT, SOD, APX and DHAR can be observed in roots of wild type and transgenic plants (Fig. 7). Likewise, under acidic conditions (without Al), the activities of those antioxidant enzymes also showed no difference between wild type and transgenic plants except APX showed a slightly increase in GR-OE plants. Al treatment decreased the activities of CAT, SOD and DHAR in all lines, and still showed no difference between wild type and transgenic plants. APX was very sensitive to  $AlCl_3$ , its activity decreased significantly in all lines under Al stress. It decreased more in wild type plants (46% decreased) than in the transgenic ones, which still maintained 70% activity.

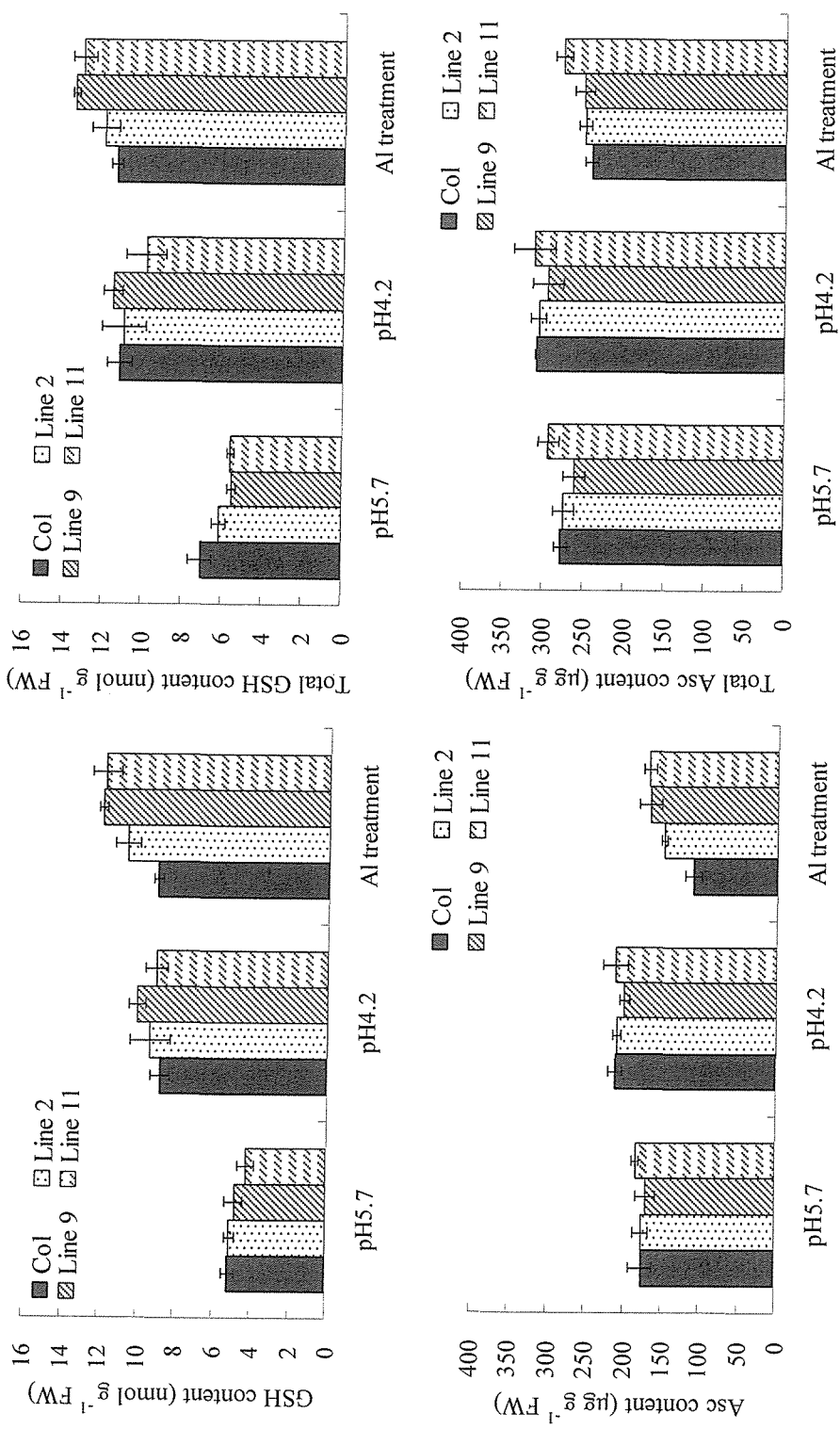
### Effect of Al on contents of AsA and GSH

In the absence of Al under pH5.7 and 4.2 conditions, although acidic treatment increased AsA content slightly, it showed no difference between wild type and transgenic plants (Fig. 8). Al treatment decreased AsA contents significantly in wild type plants, but not in transgenic Arabidopsis.

GSH content also showed no difference between wild type and transgenic plants in the absence of Al, although it increased by acidic treatment (Fig. 8). A significant difference was found under Al stress that GR-OX plants maintained higher GSH and lower GSSH levels than that in wild type Col. Moreover, it is worth to note that the level of GSH was increased by acidic treatment in all lines.



**Figure 7.** Effect of Al on antioxidant enzyme activities (SOD, CAT, APX and DHAR) in roots of wild-type Columbia (Col) and GR-OX lines (Line 2, Line 9 and Line 11). Plants were exposed to 0 (pH 5.7 or 4.2) or 50  $\mu\text{M}$   $\text{AlCl}_3$  for 24 h. Data are means  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different according to Tukey test ( $P < 0.05$ ).



**Figure 8.** Effect of Al on the levels of GSH and AsA in roots of wild-type Columbia (Col) and GR-OX lines (Line 2, Line 9 and Line 11). Plants were exposed to 0 (pH 5.7 or 4.2) or 50 μM AlCl<sub>3</sub> for 24 h. Data are means ± SE (n = 3). Values followed by the same letter are not significantly different according to Tukey test (*P* < 0.05).

### 3.5 Discussion

Al toxicity is one of the most important factors limiting plant production in acid soils. The primary symptom of Al toxicity is the inhibition of root growth, which has become a widely accepted indicator for assessing the Al tolerance in plants (Delhaize and Ryan, 1995; Ezali et al., 2008; Tahara et al., 2008). In this study, transgenic *Arabidopsis* overexpressing AtGR1 gene showed rapid root elongation than wild type plants under various concentrations of Al treatment, indicating that Al tolerance in the transgenic plants was elevated significantly.

In general, there are two strategies to deal with Al toxicity in plants: exclusion of Al from the root apex or development of Al tolerance once it enters the plant symplasm (Delhaize and Ryan, 1995; Kochian, 1995; Deng et al., 2006). In this study, we observed no difference in Al accumulation in root tips of wild type and transgenic plants after 24 h Al exposure (Fig. 3), indicating that their difference in Al tolerance ability was not ascribed to the different Al accumulation, and overexpression of AtGR1 had no effect on alleviation of Al accumulation in root. Previous studies indicated spatial and temporal correlations between Al toxicity, ROS generation, lipid peroxidation and root elongation inhibition. Since oxidative stress has been implicated in Al injury, we would postulate that plants overexpressing the antioxidant enzymes gene AtGR1 should also show enhanced tolerance to Al stress. Basu et al. (2001) reported that increased SOD activity by wheat *MnSOD1* overexpression in canola decreased lipid peroxidation and conferred Al resistance. We observed that Al treatment triggered great generation of H<sub>2</sub>O<sub>2</sub> and lipid peroxidation, and our transgenic *Arabidopsis* showed significantly lower levels of H<sub>2</sub>O<sub>2</sub>

and lipid peroxidation and less inhibition of root elongation with Al exposure as compared with wild type plants (Fig. 5). The data clearly suggest a correlation between decreased H<sub>2</sub>O<sub>2</sub> generation and lipid peroxidation and increased tolerance to Al.

Compared with wild type plants, the transgenic plants developed in this study showed a 1.4- to 2.0-fold increase in GR activity regardless of Al exposure (Fig. 6). Under Al stress, the transgenic plants showed higher activity of APX and higher level of AsA and GSH (Fig. 5 and 6), which could benefit scavenging of H<sub>2</sub>O<sub>2</sub> and decreasing lipid peroxidation. Furthermore, it has been reported that the higher AsA level also contributed to maintain higher APX activity, since APX can be inactivated by H<sub>2</sub>O<sub>2</sub> in the absence of AsA (Hossain and Asada, 1984). Thus, the higher Al tolerance ability in AtGR1 transgenic plants can be ascribed to its higher antioxidant capacity through the maintenance higher AsA and GSH level, and as well as APX and GR activities.

In our previous study, it was shown that overexpression of AtDHAR1 conferred Al tolerance through increasing AsA level and maintaining high APX activity (Yin et al., 2010). Interestingly, in the present study it was also found that the transgenic maintained higher AsA level and APX activity under Al stress. It suggests that AsA and APX play a paramount role in detoxification of Al-induced injury in plant cells, and improvement of Al tolerance.

Our results demonstrate that Al inhibits root growth, increases generation of H<sub>2</sub>O<sub>2</sub> and increases lipid peroxidation in the roots of wild type Arabidopsis. Transgenic plants showed less inhibition of root elongation and lower levels of both H<sub>2</sub>O<sub>2</sub> and MDA



compared with wild type plants. Al tolerance in AtGR1 overexpressing plants is ascribed to the alleviation of oxidative damage by maintaining a high AsA and GSH level, and as well as APX and GR activity under Al stress.

## Chapter 4

### The involvement of lipid peroxide-derived aldehydes in aluminum toxicity of tobacco roots

#### 4.1 Abstract

Oxidative injury of the root elongation zone is a primary event in aluminum (Al) toxicity in plants, but the injuring species remain unidentified. We verified the hypothesis that lipid peroxide-derived aldehydes—especially highly electrophilic  $\alpha,\beta$ -unsaturated aldehydes (2-alkenals)—participate in Al toxicity. Transgenic tobaccos overexpressing *A. thaliana* 2-alkenal reductase (AER-OE plants), wild-type SR1, and an empty vector-transformed control line (SR-Vec) were exposed to AlCl<sub>3</sub> on their roots. Compared with the two controls, AER-OE plants suffered less retardation of root elongation under AlCl<sub>3</sub> treatment and rapid growth upon Al removal. Under AlCl<sub>3</sub> treatment, the roots of AER-OE plants accumulated Al and H<sub>2</sub>O<sub>2</sub> to the same levels as did the sensitive controls, while they accumulated lower levels of aldehydes and suffered less cell death than SR1 and SR-Vec roots. In SR1 roots, AlCl<sub>3</sub> treatment markedly increased the contents of the highly reactive 2-alkenals acrolein, 4-hydroxy-(*E*)-2-hexenal, and 4-hydroxy-(*E*)-2-nonenal and other aldehydes such as malondialdehyde and formaldehyde. In AER-OE roots, accumulation of these aldehydes was significantly less. Growth of the roots exposed to 4-hydroxy-(*E*)-2-nonenal and (*E*)-2-hexenal were retarded more in SR1 than in AER-OE plants. Thus, the lipid peroxide-derived aldehydes, formed downstream of reactive oxygen species, injured root cells directly. Their suppression by

AER provides a new defense mechanism against Al toxicity.

**Key words:** Aldehyde, 2-alkenal reductase, Aluminum, Lipid peroxidation, Oxidative stress, *Nicotiana tabacum*.

### Abbreviations

AER	2-alkenal reductase
ALDH	aldehyde dehydrogenase;
BHA	butylated hydroxyanisole;
DCF-DA	2',7'-dichlorofluorescein diacetate;
DNP-derivative	2,4-dinitrophenylhydrazo derivative;
HHE	4-hydroxy-( <i>E</i> )-2-hexenal;
HNE	4-hydroxy-( <i>E</i> )-2-nonenal;
LOOH	lipid peroxide;
ROS	reactive oxygen species.

## 4.2 Introduction

Aluminum (Al) is the most abundant metal in the earth's crust and is a major factor limiting plant growth and productivity in acid soils, which cover about 50% of the world's potentially arable land surface (Kochian 1995; Kochian et al., 2004). The primary site of Al accumulation and toxicity is the root meristem, and inhibition of root elongation is the most notable symptom of Al toxicity (Delhaize and Ryan, 1995; Yamamoto et al., 2003). Al causes various adverse effects, such as disruption of signal transduction pathways, inhibition of cell division and ion fluxes, disruption of cytoskeletal dynamics, induced generation of reactive oxygen species (ROS) and disturbance of plasma membrane stability and function (Jones and Kochian, 1995; Blancaflor et al., 1998; Yamamoto et al., 2001, 2002; Kochian et al., 2004; Ma et al., 2007). Of all these toxic effects, the generation of ROS is observed rapidly and sustainably in roots after Al exposure. Al-induced generation of ROS has been shown in maize and *Allium* roots (Jones et al., 2006; Achary et al., 2008). Tahara et al. (2008) showed that ROS generated to a greater degree in Al-sensitive species than in Al-tolerant species. Yamamoto et al. (2002, 2003) have shown a correlation between ROS level and inhibition of growth capacity in cultured tobacco cell. Furthermore, ROS generation increases with increasing Al concentration (Achary et al., 2008; Xue et al., 2008). Generation of ROS appears to be a cause, rather than a result, of Al-induced cell injury, because high ROS scavenging ability resulted in enhanced Al tolerance (Devi et al., 2003; Ezaki et al., 2008). In addition, overexpression of genes encoding antioxidant enzymes (peroxidase and superoxide dismutase) conferred Al tolerance to the transgenic plants (Ezaki et al., 2000; Basu et al., 2001). Thus ROS appears to be the primary factors that cause growth inhibition in

Al-stressed roots.

Downstream of ROS generation, lipid peroxidation, is a common symptom of Al toxicity (Yamamoto et al., 2001), and it increases with increasing Al concentration (Achary et al., 2008). From animal cell studies, it is now recognized that the toxicity of lipid peroxide (LOOH) is largely ascribable to LOOH-derived aldehydes. In particular,  $\alpha,\beta$ -unsaturated aldehydes such as 4-hydroxy-(*E*)-2-nonenal (HNE) and acrolein are strong electrophiles and readily modify proteins and nucleic acids (Esterbauer et al., 1991; Taylor et al., 2002; O'Brien et al., 2005; Møller et al., 2007). HNE causes depletion of glutathione, a decrease in protein thiols, disturbance of calcium homeostasis, inhibition of DNA, RNA, and protein synthesis, lactate release, morphological changes of cells, and finally leading to cell death (Esterbauer et al., 1991; Burcham, 1998). Increased of HNE has been observed in a wide range of human diseases, including Alzheimer's disease, Parkinson's disease, and mitochondrial complex 1 deficiency (Poli and Schaur, 2000).

In plants, too, a close correlation between the level of LOOH-derived aldehydes (determined as thiobarbituric acid-reactive substances (TBARS)) and cellular damage has been shown under environmental stresses caused by heat, chilling, UV-B radiation, salinity, heavy metals and Al (Ma et al., 2007, Ezaki et al., 2008). Their involvement in cellular damage has been demonstrated by the protective effects of the aldehyde-scavenging enzymes aldehyde dehydrogenase (Sunkar et al., 2003; Kotchoni et al., 2006) and aldehyde reductase (Oberschall et al., 2000; Hideg et al., 2003; Hegedüs et al., 2004) to confer tolerance against various environmental stresses when they were overexpressed in plants. Occurrence of HNE in plants under oxidative stress has been

deduced by detection of modified proteins in the mitochondria of *A. thaliana* leaves (Winger et al., 2007). HNE rapidly inhibited respiration in isolated potato mitochondria by inactivating pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, NAD-malic enzyme (Millar and Leaver, 2000), and alternative oxidase (Winger et al., 2005). HNE and other 2-alkenals also inactivated photosynthesis in isolated chloroplasts (Mano et al., 2009). *Arabidopsis thaliana* contain 2-alkenal reductase (AER; E.C. 1.3.1.74) that catalyzes the reduction of the  $\alpha,\beta$ -unsaturated bond of 2-alkenals to produce *n*-alkanals (Mano et al., 2002). Overexpression of AER in tobacco (Mano et al., 2005) and in *A. thaliana* (Papdi et al., 2008) improved the tolerance to photooxidative stress and NaCl stress, respectively. Thus, accumulated observation indicates that LOOH-derived aldehydes, especially 2-alkenals, are commonly involved in oxidative damage in plant cells. Considering the critical importance of ROS in Al toxicity to roots, it is expected that 2-alkenals are produced and mediate damage in the stressed root cells.

To evaluate the roles of LOOH-derived aldehydes in root injury under Al stress, we employed transgenic tobacco plants that overexpress the AER gene (AER-OE plants; Mano et al., 2005). With Al treatment, the roots of AER-OE accumulated Al and H<sub>2</sub>O<sub>2</sub> to the same levels as those of the wild type, but they showed resistance to inhibition of elongation. Aldehyde analysis revealed that the Al-induced increases in contents of several toxic aldehydes, including HNE and acrolein in wild-type plants, but these aldehydes were significantly suppressed in the AER-OE plants. On the basis of these results we propose that the inhibition of root growth by aluminum ions is induced by toxic aldehydes generated with ROS.

### 4.3 Materials and methods

#### Plant materials and growth conditions

Two transgenic tobacco (*Nicotiana tabacum*) lines, P1#14 and P1#18, that overexpress *A. thaliana* AER (AER-OE lines), the wild type Petit Havana SR1, and the empty vector-transformed line SR-Vec (Mano et al., 2005) were used. Plants were cultured in a growth chamber kept at 25°C with a 14-h photoperiod at 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation.

#### Treatment with $\text{AlCl}_3$

Seeds were sterilized in 1% (w/v) sodium hypochlorite for 20 min and sown on the surface of 1/6 MS (Murashige and Skoog, 1962) agar (pH 4.2), containing 0, 300, 400, or 500  $\mu\text{M}$   $\text{AlCl}_3$ , in square Petri dishes (Ezaki et al., 2007). The Petri dishes were placed in the growth chamber and positioned vertically for 4 d, by which time the seeds had germinated; there was no difference in germination between the SR1, SR-Vec, and AER-OE plants. The Petri dishes were then tilted backward 45° and the plants were allowed to grow for an additional 10 d. At the end of that period, the root length was measured for each treatment. Root elongation values under different levels of Al were presented as percentage of the value under control (no Al).

For further assay of Al tolerance, the plants were grown in a hydroponic system. Seeds of tobacco were first grown on MS agar plates (pH 5.7) for 28 d and then

transferred to aerated 1/6 Hoagland solution (HS) (pH 5.7) and cultured for an additional 28 d. The uniformly grown plants (6-8 leaves) were selected and pre-cultured for 24 h in 1/6 HS (pH 4.2). They were then exposed to 0  $\mu\text{M}$  (control) or 500  $\mu\text{M}$   $\text{AlCl}_3$  in 1/6 HS (pH 4.2) for 24 h. Thereafter, one set of seedlings was re-transplanted into well aerated 1/6 HS without  $\text{AlCl}_3$  and kept for 3 d, and the root morphology and fresh weight were observed. The other set of seedlings was used for determination of Al and  $\text{H}_2\text{O}_2$  content, electrolyte leakage, AER activity. Those seedlings (6-8 leaves) were treated by Al and BHA for aldehydes analysis.

#### Al distribution and accumulation

Root tips were excised and incubated in 5 mM ammonium acetate ( $\text{NH}_4\text{OAc}$ ) buffer (pH 5.0) for 10 min, then stained in 100  $\mu\text{M}$  morin (Sigma, St. Louis, USA) in  $\text{NH}_4\text{OAc}$  buffer for 1 h, and finally washed with  $\text{NH}_4\text{OAc}$  buffer for 10 min (Tice et al., 1992). Stained root tips were observed under an Olympus BX51 microscope (Olympus, Tokyo, Japan) (excitation wavelength 420 nm and emission 510 nm). A total of five to eight individual roots from five seedlings were examined for each time point, and the experiment was repeated three times. For determination of the Al content, 0.1 g root tip (0 to 10 mm) was washed three times with distilled water and dried, then digested with a concentrated acid mixture ( $\text{HNO}_3:\text{H}_2\text{SO}_4$ , 1:1, v/v) at 160°C for 3 h. The Al content was quantified by using an inductively coupled plasma atomic emission spectrometer (ICP-AES, Ciros CCD, Rigaku, Japan).

#### $\text{H}_2\text{O}_2$ detection and determination



H<sub>2</sub>O<sub>2</sub> distribution in the root tips was detected by 2',7'-dichlorofluorescein diacetate (DCF-DA) (Wako Pure Chemical, Osaka, Japan) (Jones et al., 2006). Root tips were excised and placed into a solution containing 200 μM CaCl<sub>2</sub> (pH 4.4) and 10 μM DCF-DA for 15 min. The DCF-DA fluorescence was then detected under an Olympus BX51 microscope (excitation 488 nm and emission 530 nm). H<sub>2</sub>O<sub>2</sub> content was determined according to the method of Ryan et al. (2009). Root tips (0.3 g, 0-20 mm) were frozen in liquid nitrogen, ground to powder in pre-cooled mortars, and homogenized with 2 mL cold 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12000g for 30 min at 4 °C, and 0.4 mL of the supernatant was added to 0.4 mL 10 mM potassium phosphate buffer (pH 7.0) and 0.8 mL 1 M KI. The absorbance of the mixture was read at 390 nm, which developed within 25 min and was stable for at least 2 h. The content of H<sub>2</sub>O<sub>2</sub> was calculated against a calibration curve using H<sub>2</sub>O<sub>2</sub> standards.

#### Visualization of lipid peroxidation and cell death

Aldehydes that originated from LOOH in the roots were visualized with Schiff's reagent as described by Yamamoto et al. (2001). Root tips were excised and stained with Schiff's reagent (Wako Pure Chemical) for 20 min, rinsed with a freshly prepared sulfite solution (0.5% [w/v] K<sub>2</sub>S<sub>2</sub>O<sub>5</sub> in 0.05 M HCl), and then kept in the sulfite solution and observed instantly under a light stereomicroscope (Olympus SZX7, Tokyo, Japan). Cell death was detected by staining roots with Evans blue (Sigma) solution (0.025% [w/v] Evans blue in 100 μM CaCl<sub>2</sub>, pH 5.6) for 10 min (Yamamoto et al., 2001). Stained roots were washed three times with 100 μM CaCl<sub>2</sub> (pH 5.6) and then observed under a light microscope

(Olympus BX51, Tokyo, Japan). A total of five to eight individual roots from five seedlings were examined, and the experiment was repeated three times.

#### Electrolyte leakage assay

Loss of plasma membrane integrity was studied in terms of electrolyte leakage (EL) by measuring changes in electrical conductivity (Singh et al., 2007). Root tips (0.1 g, 0-20 mm) were incubated in distilled water at 25°C for 2 h in tubes and the initial electrical conductivity (E1) of the medium was measured. The tubes containing the root material were then boiled for 30 min to release all the electrolytes, then cooled to 25°C and the final electrical conductivity (E2) was measured. The EL was calculated as follows:  $EL = (E1/E2) \times 100$ .

#### Assessment of enzyme activity

Roots were frozen and ground in liquid nitrogen with a pre-cooled mortar and pestle, and then 50 mM potassium phosphate (pH 7.0) containing 1% protease inhibitor cocktail (P9599, for plant cell and tissue extraction, Sigma) was added. Homogenates were centrifuged at 8000 g for 10 min at 4°C and the supernatant was concentrated on a Microcon filter (YM-10, Millipore) at 8000 g for 10 min. AER activity was assayed by the rate of oxidation of NADPH at 340 nm in a reaction mixture containing 50 mM MES-NaOH (pH 6.0), 0.1 mM NADPH, and 0.1 mM diamide as electron acceptors (Mano et al., 2002; 2005). Protein was determined by the Bradford (1976) method, using bovine serum albumin as a standard.

## Aldehyde identification and quantitation by HPLC

Seedlings (6-8 leaves) were treated by 0 or 500  $\mu\text{M}$   $\text{AlCl}_3$  under 0 or 10  $\mu\text{M}$  BHA for 24 h. Then, roots of seedlings were used for aldehyde analysis. Aldehydes were extracted from the roots and derivatized with 2,4-dinitrophenylhydrazine, then identified and quantified by reverse-phase HPLC according to the method of Matsui et al. (2009), with a slight modification. Root tips (0.3 g, 0-20 mm) were frozen in liquid nitrogen, ground to fine powder by pre-cooled motor and pestle, and then homogenized in 3 mL acetonitrile containing 1.5 nmol 2-ethylhexanal (as an internal standard) and 0.005% (w/v) butylhydroxytoluene. The slurry was incubated in a screw-capped glass tube at 60°C for 30 min. Then an extract was collected through a glass filter in another glass tube. 2,4-Dinitrophenylhydrazine (final concentration of 0.5 mM) and formic acid (final concentration 0.5 M) were added and the solution was mixed well and incubated at 25°C for 60 min. Then, 3 mL saturated NaCl solution and 0.3 g  $\text{NaHCO}_3$  were added to neutralize the formic acid. After centrifugation, the upper acetonitrile layer was collected and dried in vacuo. The residue was dissolved in 500  $\mu\text{L}$  acetonitrile and passed through a BondEluteC18 cartridge (sorbent mass 200 mg, Varian), which had been pre-washed with 2 mL acetonitrile. The material passed through the cartridge was collected and 10- $\mu\text{L}$  aliquots were subjected to HPLC in a Wakosil DNPH-II column (4.6  $\times$  150 mm, Wako Pure Chemical). Wakosil DNPH-II Eluents A and B (Wako) were used to separate out the compounds, with 100% A (0 to 5 min), a linear gradient from 100% A to 100% B (5 to 20 min), and subsequently 100% B (20 to 25 min) at a flow rate of 1.0  $\text{mL min}^{-1}$ . Dinitrophenylhydrazo (DNP)-derivatives of aldehydes were detected at 340 nm.

Aldehydes were identified by their retention times, as compared with those of DNP derivatives of authentic aldehydes (Matsui et al., 2009). To determine the content of an aldehyde ( $\text{nmol g FW}^{-1}$ ) from its peak area, the ratio of the peak area to the peak area of the internal standard was first determined. The amount of an aldehyde was obtained by multiplying this ratio by the added amount of internal standard i.e.,  $1.5 \text{ nmol } 0.3 \text{ g FW}^{-1}$ . For identified aldehydes, the amount was further corrected for the DNP-derivatization efficiency of the aldehyde and the extraction efficiency and absorption coefficient of the derivative relative to those of the internal standard (Matsui et al., 2009).

#### Effect of exogenous application of 2-alkenal on root growth

Two aldehydes species were exogenously applied to evaluate the effect of 2-alkenals on root growth in tobacco. For HNE treatment, seeds were sown on a sponge with a holder in 1/6 HS directly, and 21-d old seedlings were treated by 0 or  $10 \mu\text{M}$  HNE (Alexis Biochemicals, San Diego, USA) in the same medium for 24 h, then they were exposed to well aerated 1/6 HS without HNE and kept for 5 d for recovery. After that, the maximum root length was measured. For (*E*)-2-hexenal treatment, seeds were first grown on MS agar plates (pH 5.7) for 28 d and then transferred to aerated 1/6 HS (pH 5.7) and cultured for an additional 21 d. The uniformly grown plants were selected and exposed to 0, 10, 100 or  $300 \mu\text{M}$  (*E*)-2-hexenal (Tokyo Chemical Industry, Tokyo, Japan) for 24 h, and then they were transplanted into well aerated 1/6 HS without (*E*)-2-hexenal for 5 d for recovery. After that, root fresh weight and the maximum root length were measured.

#### Effect of BHA on root elongation under Al stress

When the seedlings were treated with  $\text{AlCl}_3$  in the presence of BHA (Wako Pure Chemical, Osaka, Japan), seeds were first sown on the surface of 1/6 MS agar plate and grown for 14 d. Then seedlings with same root length (8-10 mm) were transferred into sterilized filter paper soaked by following four solutions: (1) 1/6 HS, (2) 10  $\mu\text{M}$  BHA in 1/6 HS, (3) 500  $\mu\text{M}$   $\text{AlCl}_3$  in 1/6 HS, and (4) 500  $\mu\text{M}$   $\text{AlCl}_3$  + 10  $\mu\text{M}$  BHA in 1/6 HS in petri dishes for 5 d. At the end of treatment, root elongation was measured.

#### Statistical analyses

Experiments were performed three times. Data were analyzed by using the programs of Statistical Analysis System (SAS 8.0). Data were subject to ANOVA, and means were compared by Tukey-Kramer test ( $P < 0.05$ ).

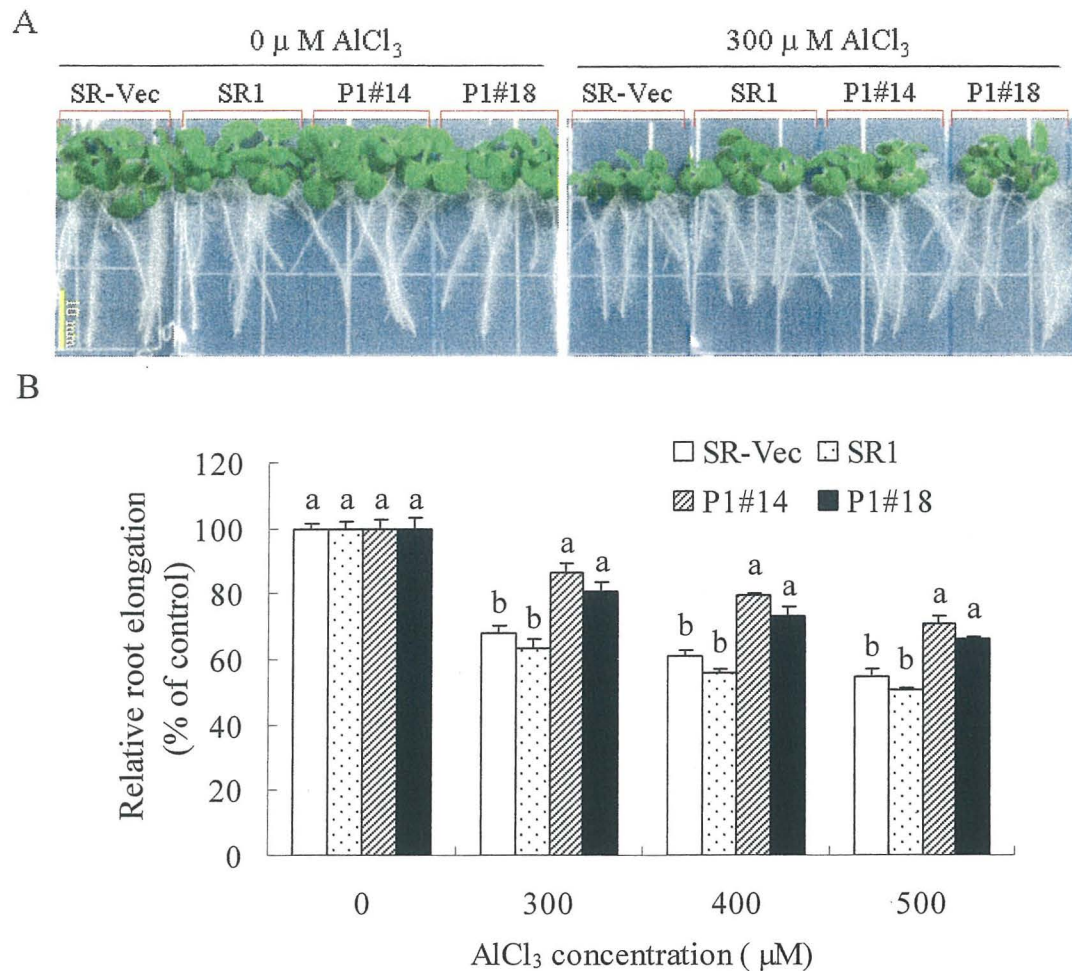
#### 4.4 Results

##### AER-overexpressing plants show Al tolerance

To examine whether AER overexpression improves Al tolerance in tobacco plants, root elongation was compared between the AER-OE lines (P1#14 and P1#18) and the two control lines (SR1 and SR-Vec). All lines showed similar root growth on MS agar plates in the absence of AlCl<sub>3</sub>. When AlCl<sub>3</sub> was added to the plates, root growth of the seedlings was inhibited; this inhibition increased with increasing Al concentration (Fig. 1). Notably, the inhibition was more severe in SR1 and SR-Vec plants than in AER-OE plants. When exposed to 300 μM AlCl<sub>3</sub>, the SR1 and SR-Vec plants showed a 35% decrease in root elongation, whereas AER-OE plants showed only a 16% reduction. Following 400- and 500-μM AlCl<sub>3</sub> exposure, SR1 and SR-Vec plants showed 40% and 46% inhibition of root elongation, whereas in AER-OE plants the inhibition was 20% and 28%, respectively. Thus, with respect to the root elongation, the AER-OE plants showed tolerance to Al.

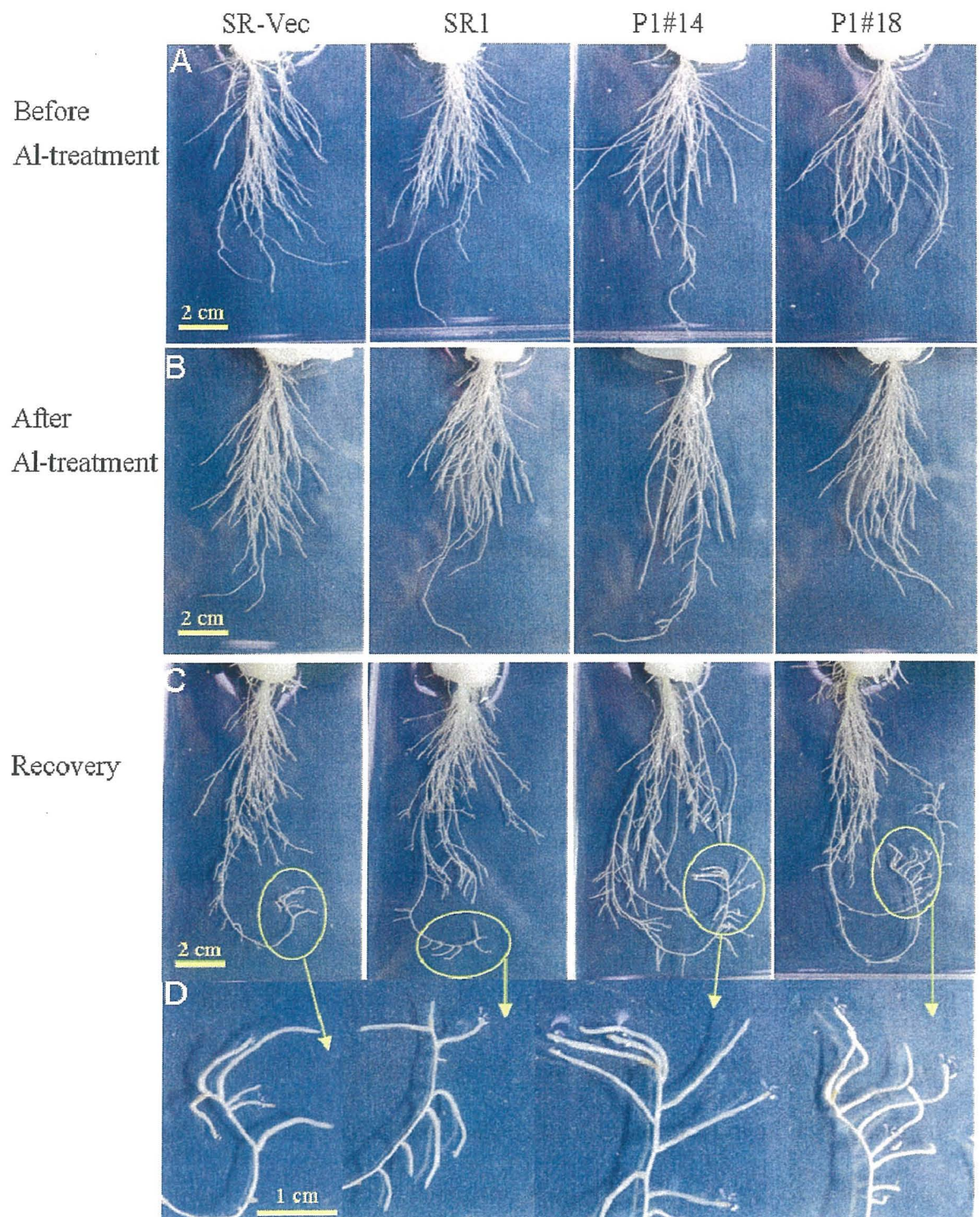
To evaluate the recovery of root growth after Al stress, hydroponically cultured plants were transiently treated with AlCl<sub>3</sub> and then transferred to Al-free conditions. After treatment with 500 μM AlCl<sub>3</sub> for 24 h, the root fresh weight per plant showed no difference among lines. After 3-d recovery, the root fresh weight of AER-OE plants was 130% of that of SR1 and SR-Vec plants, indicating the less damage and quick recovery of AER-OE plants from Al stress (Fig. 2E). Furthermore, after recovery, several new, white adventitious roots emerged, and these new roots grew longer and thicker in AER-OE plants than in SR1 and SR-Vec plants (Fig. 2 C and D).

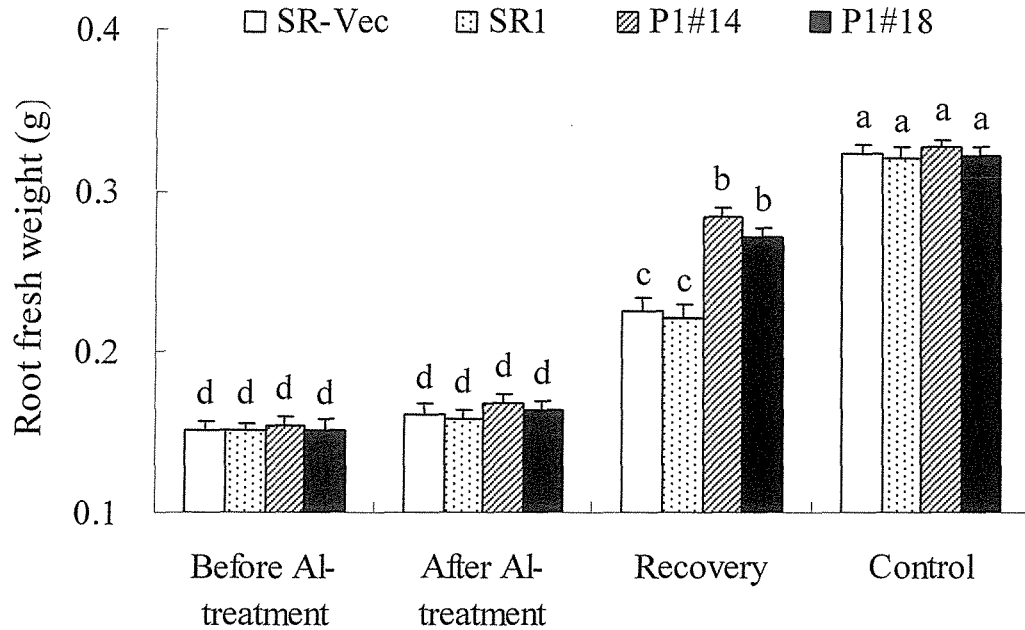
AER-OE plants showed higher AER activities in the roots as compared with SR1 and SR-Vec plants (Fig. 3). AER activity in the roots of AER-OE plants was 400% to 600% of that in the SR1 and SR-Vec plants, irrespective of AlCl<sub>3</sub> treatment. These results indicated that AER overexpression released from the Al-induced retardation of root growth.



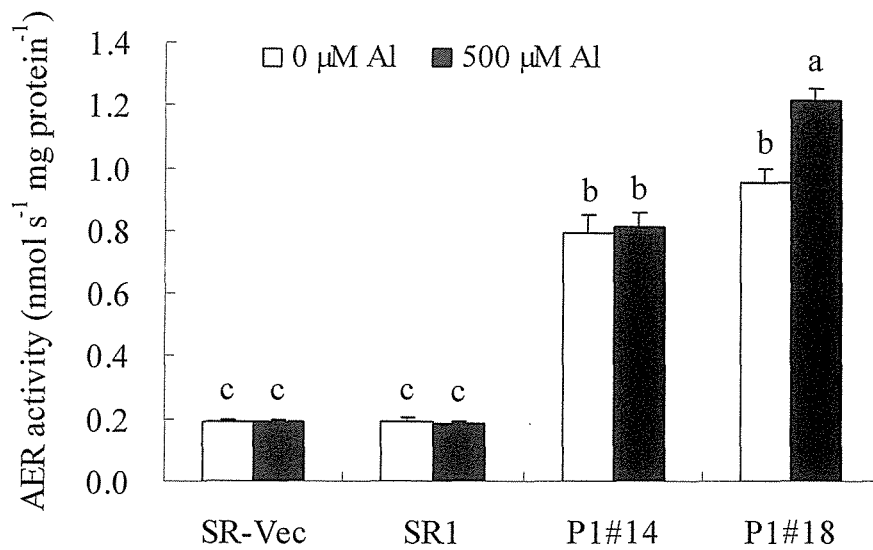
**Figure 1.** A, Root growth of SR1 and AER-OE lines (P1#14). Seeds were grown for 14 d on 1/6 MS agar plate (pH 4.2) containing 0 or 300  $\mu\text{M}$   $\text{AlCl}_3$ . B, Effect of increasing  $\text{AlCl}_3$  concentration on root elongation. Seeds were grown on 1/6 MS medium (pH 4.2) containing 0, 300, 400, or 500  $\mu\text{M}$   $\text{AlCl}_3$ . Root length was measured after 14 d. Root elongation values at different levels of  $\text{AlCl}_3$  were represented as percentages of the values observed without  $\text{AlCl}_3$ . Data are means  $\pm$  SE of three replications (each replication included 10 plants). Values followed by the same letter in the same  $\text{AlCl}_3$  concentration are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).





**E**

**Figure 2.** Root morphology (A–D) and weight (E) of SR-Vec, SR1 and AER-OE lines (P1#14 and P1#18). Seeds were sown on MS agar plates and the seedlings were cultured for 28 d, then transplanted into hydroponic medium and cultured for another 28 d. Seedlings were treated with 500  $\mu\text{M}$   $\text{AlCl}_3$  in 1/6 HS for 24 h and then cultured in Al-free well aerated 1/6 HS for 3 d to recover. Root morphology was recorded before (A) and after (B) Al treatment and 3 d after removal of  $\text{AlCl}_3$  (C and D). For fresh weight determination (E), roots were collected from the plants either before or after Al treatment, after the 3-d recovery or without Al treatment. Data are means  $\pm$  SE ( $n = 8$ ). Values followed by the same letter are not significantly different according to Tukey-Kramer ( $P < 0.05$ ).



**Figure 3.** Activity of AER in roots. Seedlings were grown on MS agar plates for 28 d and then in hydroponic medium for 28 d. Seedlings were treated with or without 500  $\mu\text{M AlCl}_3$  for 24 h. Proteins were extracted from the roots and AER activity in the extract was determined, as in Materials and Methods. Data are means  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).

## AER-overexpressing plants accumulate Al and H<sub>2</sub>O<sub>2</sub>

We then examined whether overexpression of AER affected Al accumulation and distribution in the roots. Localization of Al ions in the roots was determined with the fluorescent probe morin (Tice et al., 1992), which has a high specificity for Al<sup>3+</sup> and is used widely to detect Al in plant tissues (Larsen et al., 1996; Jones et al., 2006; Ezaki et al., 2007). Roots without AlCl<sub>3</sub> exposure showed no fluorescence (data not shown). Following exposure to 500 μM AlCl<sub>3</sub> for 24 h, a marked increase in Al-induced morin fluorescence was observed, particularly in the region of 0 to 1 mm from the root tip (Fig. 4A). All the Al-treated plants showed an intense fluorescence signal in the root tips, and there was no difference among the SR1, SR-Vec and AER-OE plants. The Al content in the root tips (0-10 mm), as determined with a plasma atomic emission spectrometer, also showed no difference among SR1, SR-Vec, and AER-OE plants (Fig. 4B). Thus, accumulation and distribution of Al in the roots were not affected by overexpression of AER.

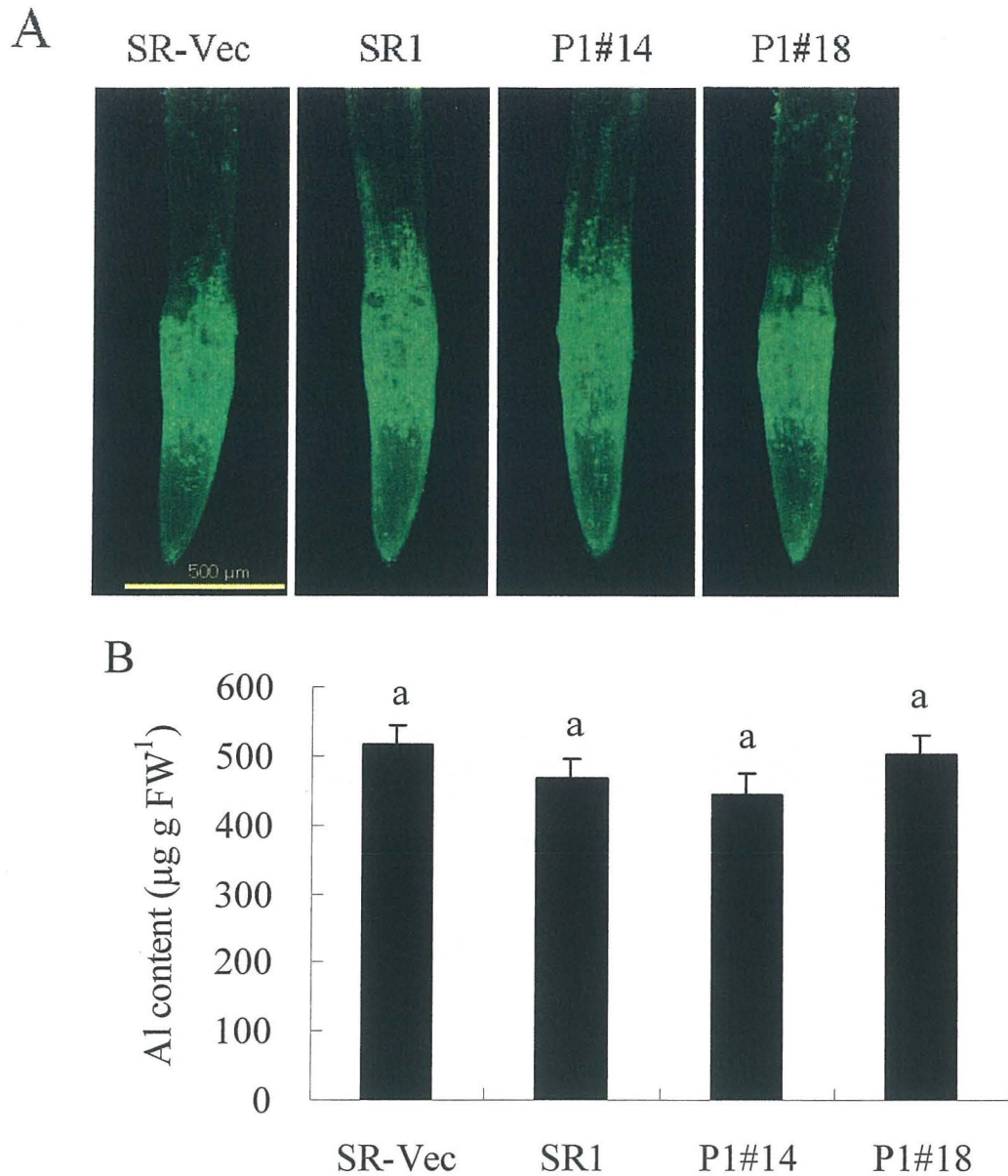
To evaluate ROS production in the roots, we used DCF-DA fluorescence, which indicates H<sub>2</sub>O<sub>2</sub> accumulation. DCF-DA fluorescence was similarly low without AlCl<sub>3</sub> treatment, and it was markedly increased by AlCl<sub>3</sub> treatment in root apex, especially at the elongation zone (Fig. 5A). The increased levels and the position of H<sub>2</sub>O<sub>2</sub> formation appeared similar among the four lines. Quantitative analysis of H<sub>2</sub>O<sub>2</sub> in root tips (0-20 mm) by iodide oxidation assay confirmed that H<sub>2</sub>O<sub>2</sub> content was increased by AlCl<sub>3</sub> treatment (Fig. 5B); the levels did not differ among all the lines either before or after the treatment. These results showed that the Al-tolerant AER-OE lines accumulated Al and

subsequently produced  $H_2O_2$  at the root apex, to the same extent as the Al-sensitive control lines. In addition, overexpression of AER did not affect ROS-scavenging enzyme activities (SOD, APX and catalase; Mano et al., 2005). Therefore, the tolerance of the AER-OE lines is attributable to a difference in some factor(s) downstream of ROS production.

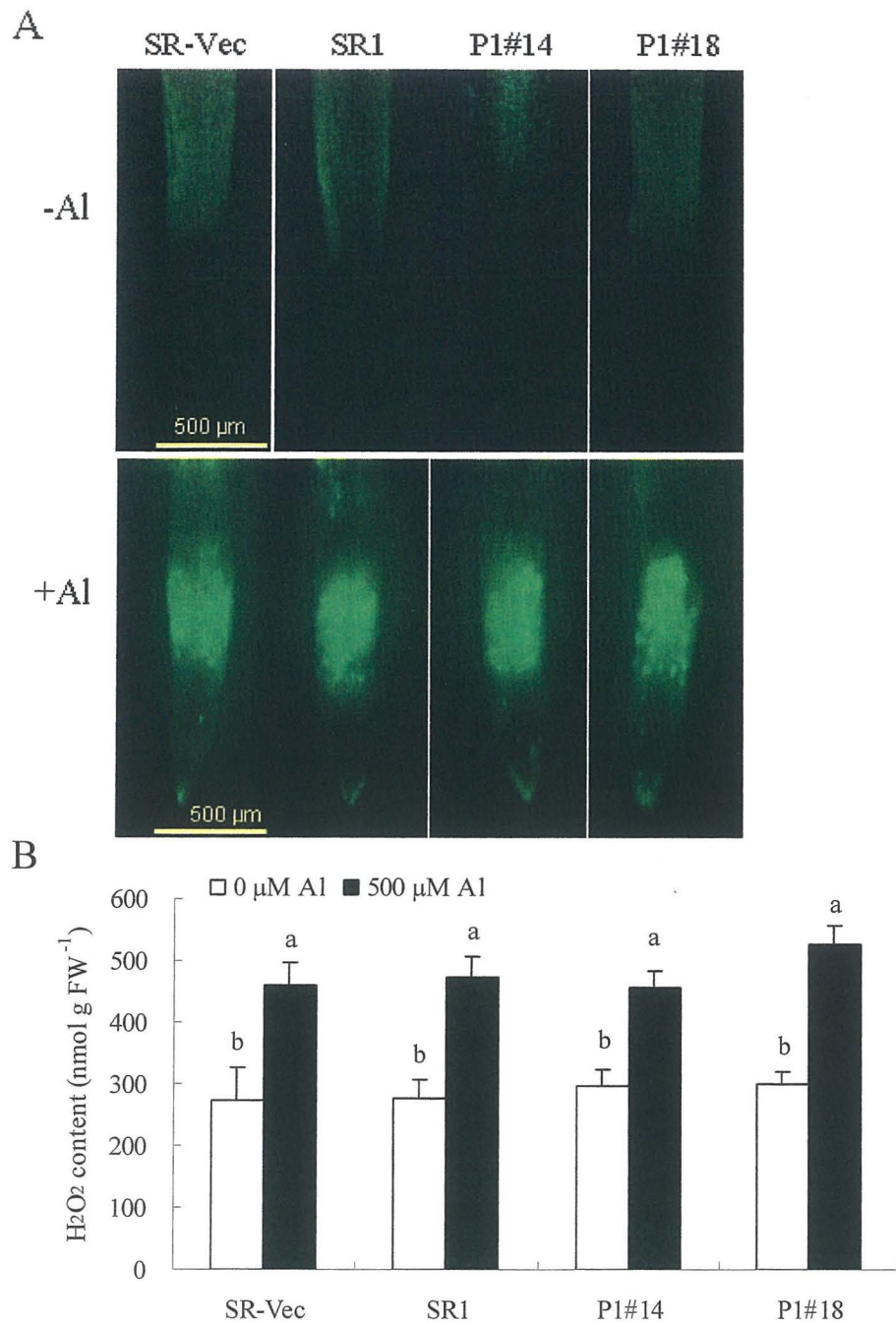
Differential aldehyde levels are correlated with differences in cell death

LOOH-derived aldehydes in plant tissues can be visualized with Schiff's reagent by the development of a pink dye (Yamamoto et al., 2001; Han et al., 2008). Without  $AlCl_3$  treatment, pink staining was barely observed in any of the lines (Fig. 6A). After exposure to  $AlCl_3$  for 24 h, the roots were clearly pink, mainly around the elongation zone, indicating aldehydes were produced at the same site of  $H_2O_2$ . The roots of AER-OE plants appeared a paler pink than those of SR1 and SR-Vec plants, indicating lower aldehyde contents in the former.

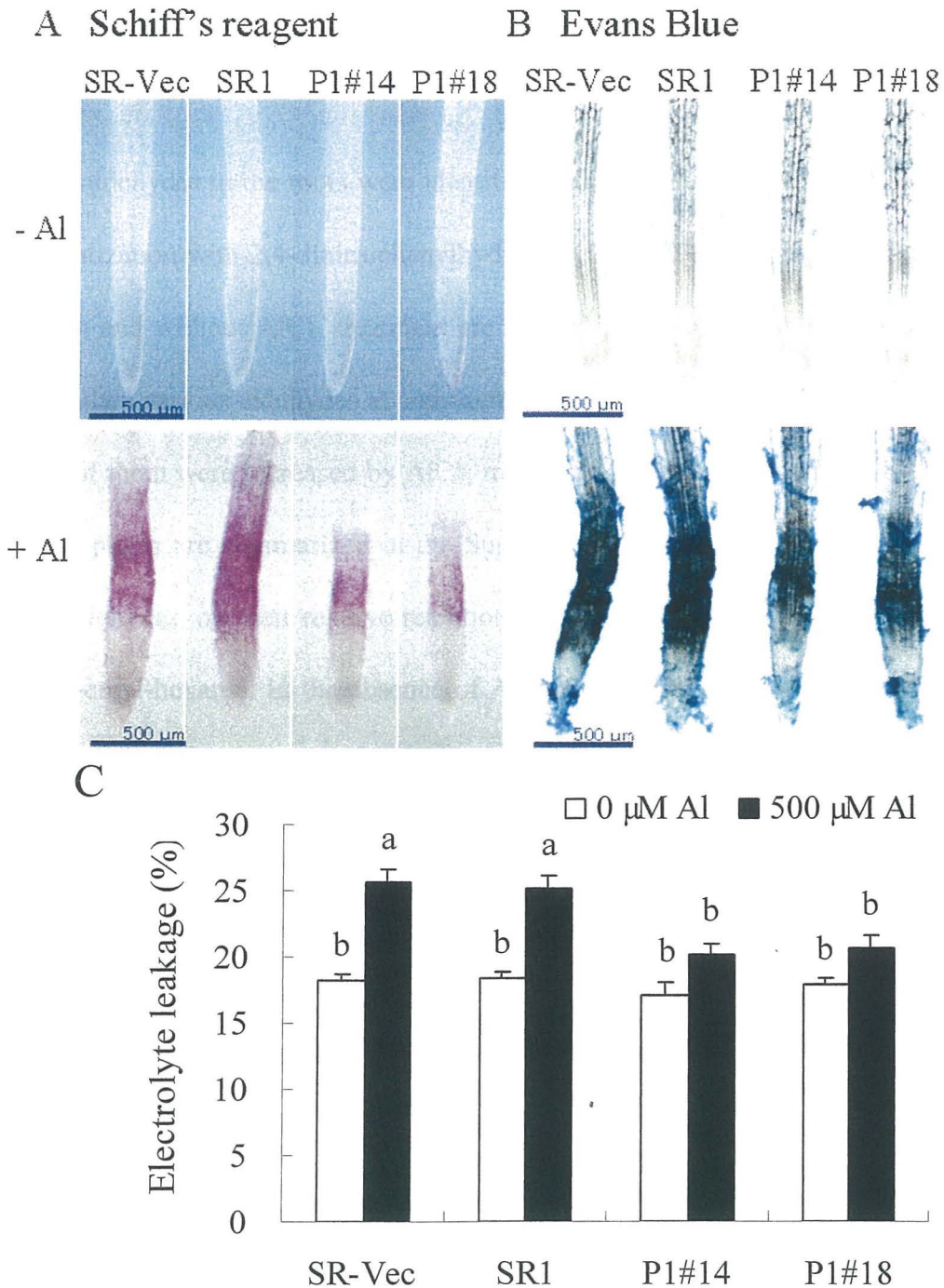
Evans blue staining showed that extensive cell death was induced by  $AlCl_3$  treatment around the roots, and especially at the elongation zone (Fig. 6B), as reported previously (Yamamoto et al., 2001). As with the results of Schiff's reagent staining, stronger Evans blue staining was observed in the SR1 and SR-Vec lines than in the AER-OE lines. Electrolyte leakage assay confirmed that the membrane injury due to  $AlCl_3$  treatment was significantly suppressed in the AER-OE plants than in the SR1 and SR-Vec plants (Fig. 6C). These results revealed a close correspondence between Al-induced damage of the root and aldehyde accumulation therein.



**Figure 4.** Aluminum distribution and accumulation in roots. Seedlings were grown on MS agar plates for 28 d and then in hydroponic medium for 28 d. Seedlings were treated with or without 500  $\mu\text{M}$   $\text{AlCl}_3$  for 24 h, and then the roots were stained with morin (A) and no fluorescence in the roots prior to Al treatment. Al content in the root tips (0-10 mm) was measured by ICP-AES (B), as described in Materials and Methods. Data are means  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).



**Figure 5.** H<sub>2</sub>O<sub>2</sub> distribution and accumulation in roots. Seedlings were grown on MS agar plates for 28 d and then in hydroponic medium for 28 d. Seedlings were treated with or without 500  $\mu\text{M AlCl}_3$  for 24 h. Roots were treated with DCF-DA (A) or used for determination of H<sub>2</sub>O<sub>2</sub> content in the tip regions (0-20 mm) by iodide oxidation (B), as described in Materials and Methods. Data are means  $\pm$  SE ( $n = 3$ ). Values followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).



**Figure 6.** Lipid peroxidation and membrane injury in roots. Seedlings were grown on MS agar plates for 28 d and then in hydroponic medium for 28 d. Seedlings were treated with or without 500 μM AlCl<sub>3</sub> for 24 h and then stained with Schiff's reagent to visualize lipid peroxidation (A) or with Evans blue to detect cell death (B), as described in Materials and Methods. (C) Electrolyte leakage. Data are means ± SE (*n* = 3). Values followed by the same letter are not significantly different according to Tukey-Kramer test (*P* < 0.05).



## Specific aldehydes are suppressed in AER-overexpressing plants

Individual aldehydes in the roots were identified and quantified in a reverse-phase HPLC after derivatization with 2,4-dinitrophenylhydrazine. Typical chromatograms for SR1 and P1#18 with and without AlCl<sub>3</sub> treatment are shown in Fig. 7. It was found that tobacco roots contained various aldehydes at considerable levels, even in the absence of Al stress, and some of them were increased by AlCl<sub>3</sub> treatment. Aldehyde contents of SR1, P1#14, and P1#18 plants are summarized in the Supplemental tables 1-3. We distinguished 35 peaks of aldehydes, on their relative retention time as compared with that of the internal standard 2-ethyl-hexanal. In the absence of Al stress, the aldehyde contents of the roots did not differ significantly among SR1 and the two AER-OE lines. The most abundant aldehyde was formaldehyde (ca. 50 nmol g FW<sup>-1</sup>) and the second were malondialdehyde and *n*-heptanal (2 to 4 nmol g FW<sup>-1</sup>). In addition to these, more reactive 2-alkenals such as HNE, acrolein and HHE were present at ca. 1 nmol g FW<sup>-1</sup>. These values can be regarded as the basal physiological levels of these aldehydes (see Discussion).

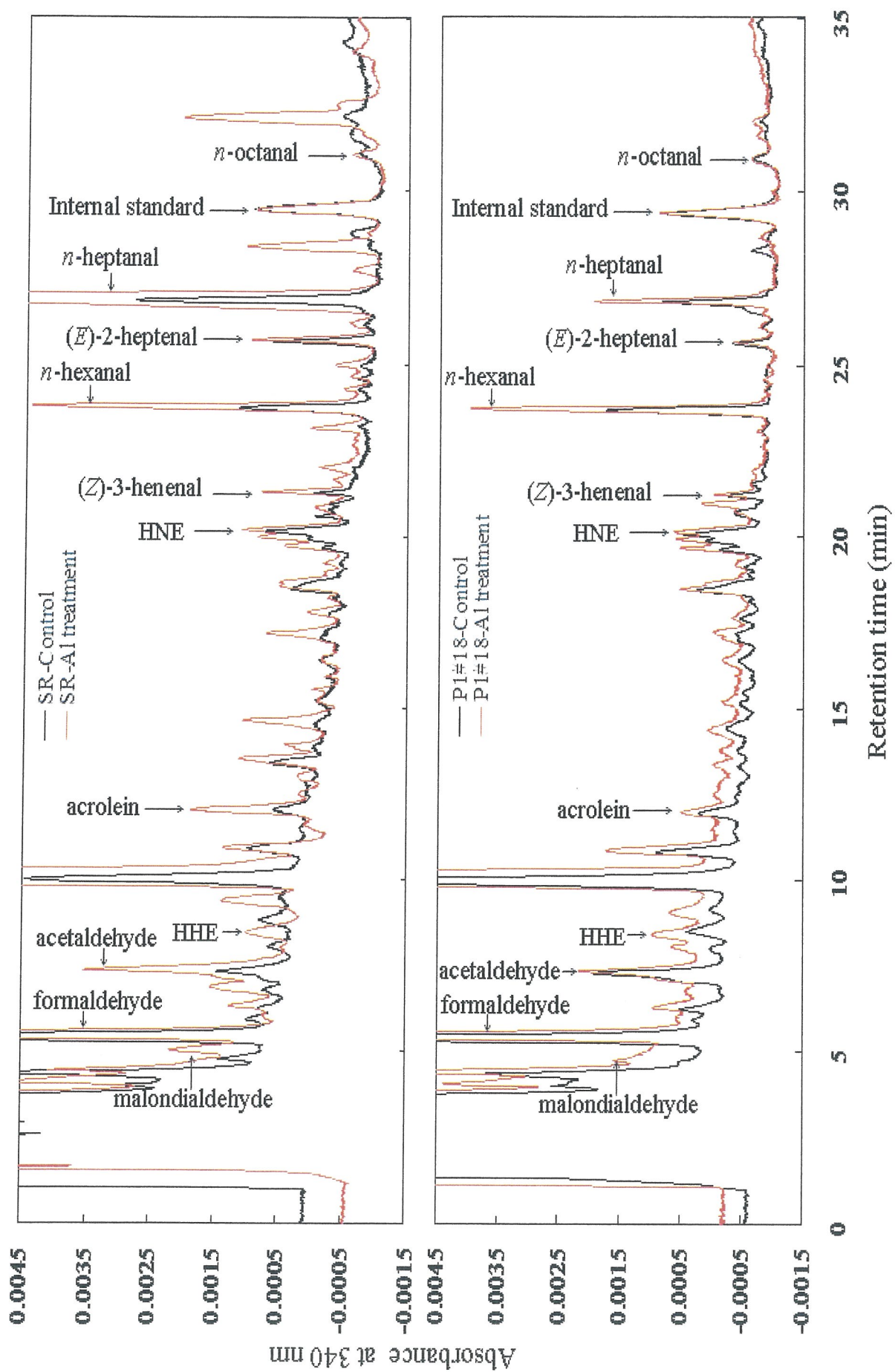
With AlCl<sub>3</sub> treatment, the content of each aldehyde changed differently, and according to the mode of change, aldehydes were grouped into three groups: Group 1 (Supplemental Table 1) included aldehydes of which the contents were not affected by AlCl<sub>3</sub> in any of the tested lines. Identified in this group were (*E*)-2-pentenal, *n*-pentanal, (*E*)-2-octenal, and *n*-nonanal, listed in order of elution. Group 2 (Supplemental Table 2) included aldehydes of which the contents were increased by AlCl<sub>3</sub> treatment in all lines. Four compounds were found in this group, and identified was crotonaldehyde only. These aldehydes did not appear to be relevant to the protective effect of AER. Group 3

(Supplemental Table 3) included aldehydes of which the contents were significantly increased by AlCl<sub>3</sub> treatment only in SR1 but not in the two AER-OE lines P1#14 and P1#18; identified in this group were malondialdehyde, formaldehyde, acetaldehyde, HHE, acrolein, butyraldehyde, phenylacetaldehyde, HNE, (*Z*)-3-hexenal, *n*-hexanal, (*E*)-2-heptenal, *n*-heptanal, and *n*-octanal.

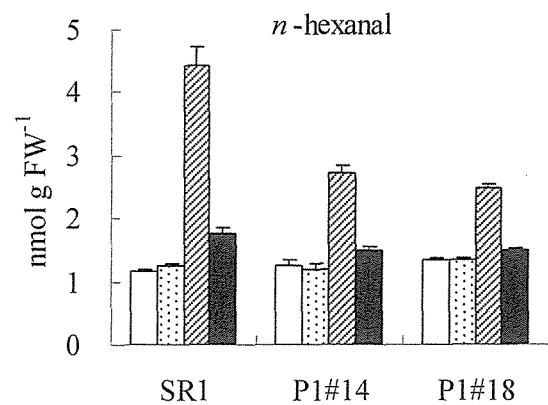
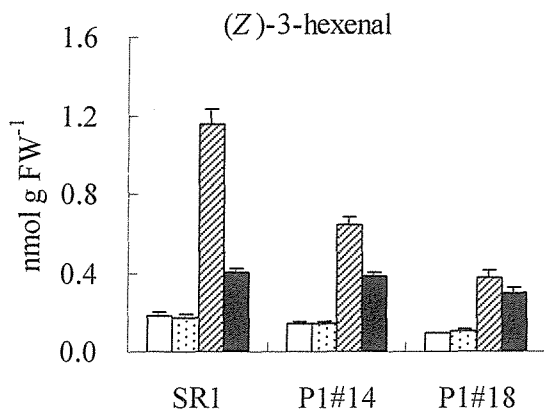
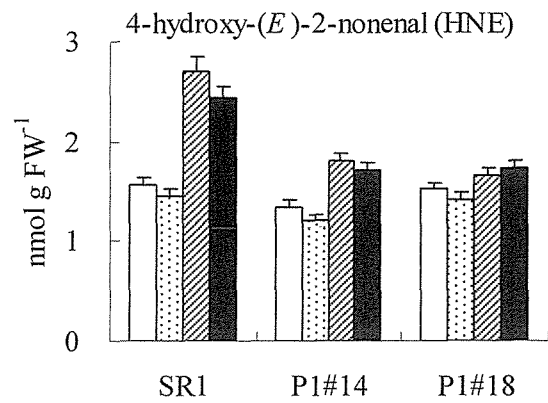
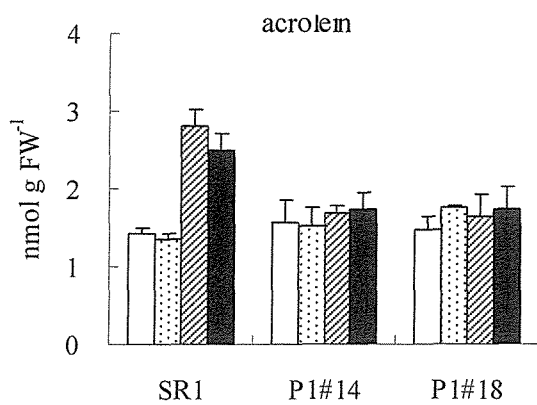
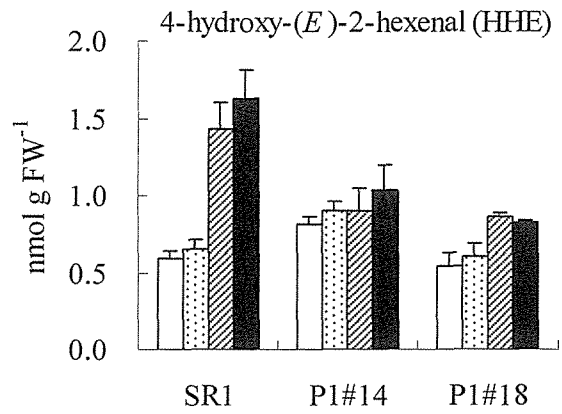
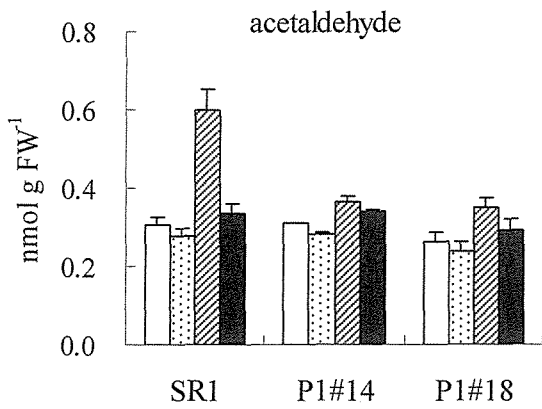
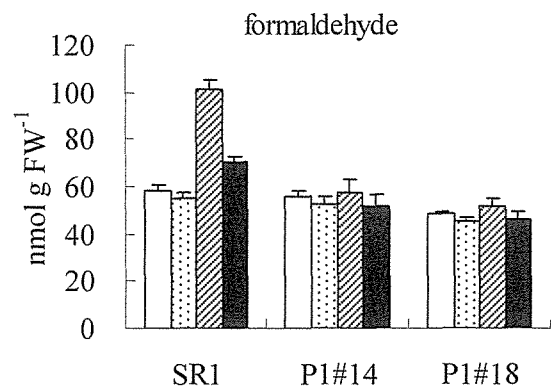
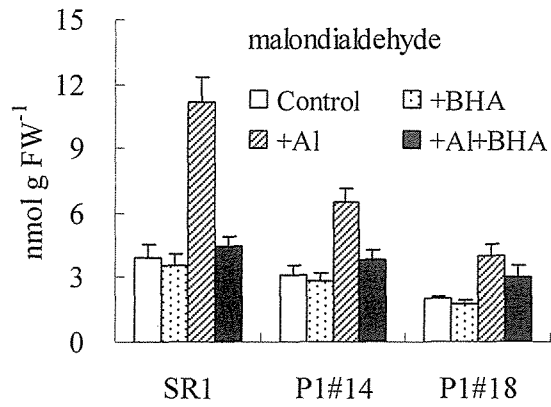
The Group 3 aldehydes are candidates for the damage-causing molecules, and indeed they include highly reactive 2-alkenals such as acrolein, HHE, and HNE. Their changes are represented in Fig. 8. The increase in the contents of these aldehydes by AlCl<sub>3</sub> treatment in SR1 ranged from 50% (*n*-octanal) to 540% ((*Z*)-3-hexenal). The greatest absolute increase with AlCl<sub>3</sub> treatment in SR1 plants was that of formaldehyde (40 nmol g FW<sup>-1</sup>; increased 75%), and the second highest was that of malondialdehyde (7.3 nmol g FW<sup>-1</sup>; increased 184%). For HNE, acrolein, and HHE, the Al-induced increases in SR1 were 1.2 nmol g FW<sup>-1</sup> (increased 75%), 1.4 nmol g FW<sup>-1</sup> (increased 100%), and 0.8 nmol g FW<sup>-1</sup> (increased 140%), respectively. In contrast, in the AER-OE plants, the increases in the contents of these aldehydes were lower than in SR1 plants (see Supplemental Table 3 for statistical analysis). It should be noted that the observed increases here are ‘diluted’ results because of the technical limitation; although the major injured part of Al toxicity was the root tip (0-2 mm), we had to include non-injured tissue also in the samples for the aldehydes analysis (0-20 mm from root tip) in order to collect the required amount (0.3 g for one analysis). Thus, the actual increase of the aldehyde contents in the Al-injured cells must be much greater than it appears in Fig. 8.

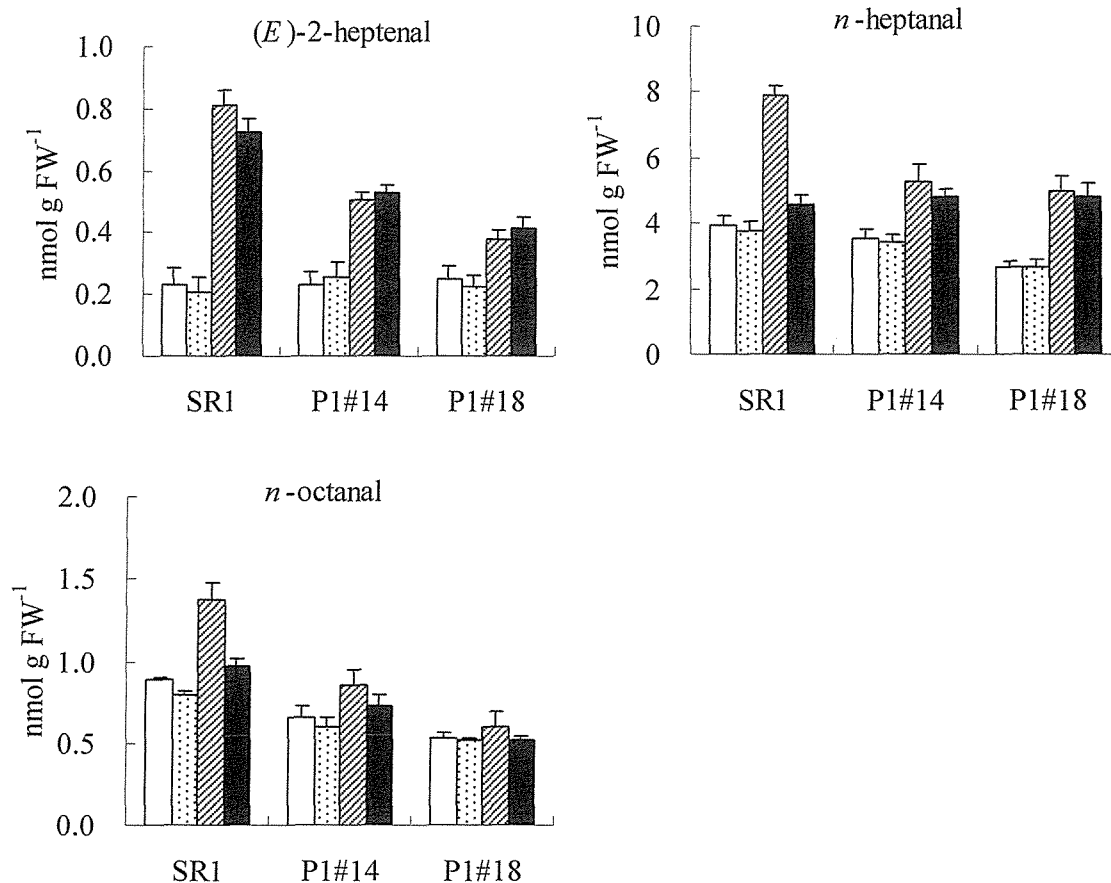
Of these Group 3 aldehydes, only HNE, acrolein, and HHE are substrates for AER

(Mano et al., 2002). Therefore, suppression of the other aldehydes in the AER-OE plants was an indirect effect of AER activity, probably through the scavenging of some precursor 2-alkenals (see Discussion). There were 13 unidentified aldehydes in Group 3, and they could be candidates for such precursors. It is also possible that some strongly toxic unknown aldehydes are included. All of these aldehydes are potentially toxic, and increases in their contents could cause Al-induced damage of root tissues. Thus overexpression of AER suppressed the increases in contents of these aldehydes, via the direct enzymatic activity of AER or via indirect effects, thereby improving the tolerance of root tissues to Al toxicity.



**Figure 7.** Typical chromatograms of DNP derivatives of aldehydes in the root tips (0-20 mm) of SR1 and P1#18 with (red lines) and without (black lines)  $\text{AlCl}_3$  (500  $\mu\text{M}$ ) treatment. Identified aldehydes are labeled at the top of each peak. HPLC conditions are described in the Materials and Methods and DNP-derivatives of aldehydes were detected at 340 nm.





**Figure 8.** Contents of aldehydes in the root tips (0-20 mm) of SR1 and AER-OE lines P1#14 and P1#18. Seedlings were grown on MS agar plates for 28 d and then in hydroponic medium for 28 d, then they were treated by following solutions: (1) 1/6 HS (Control), (2) 10  $\mu$ M BHA in 1/6 HS (+BHA), (3) 500  $\mu$ M AlCl<sub>3</sub> in 1/6 HS (+Al), and (4) 500  $\mu$ M AlCl<sub>3</sub> + 10  $\mu$ M BHA in 1/6 HS (+Al+BHA) for 24 h as described in Materials and Methods. Data are means  $\pm$  SE (n=3).

## 2-Alkenals inhibit root growth

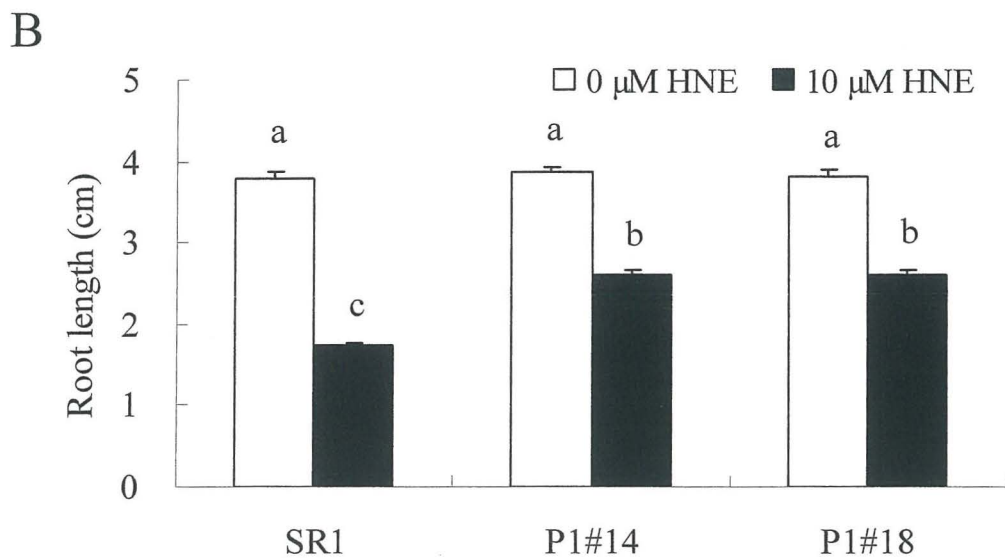
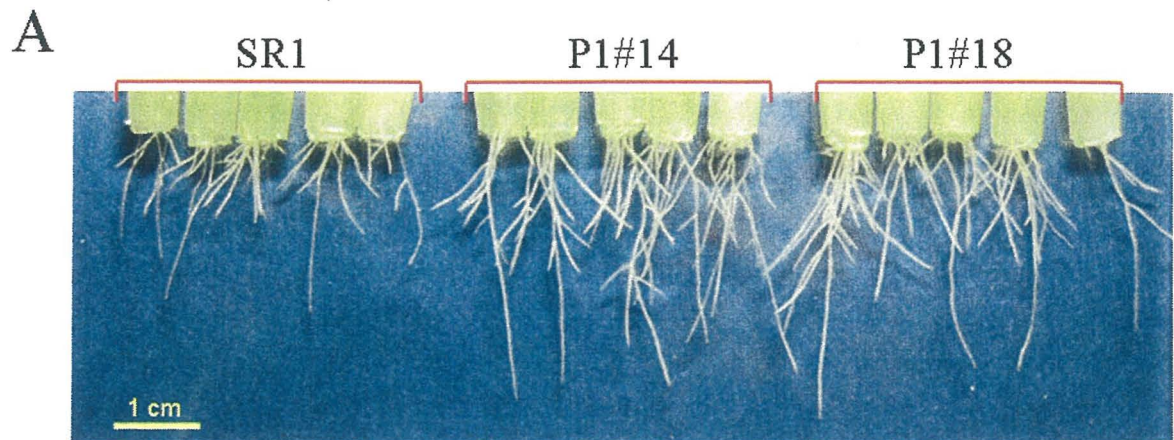
Toxicity of 2-alkenals to roots was verified by examining the effects of HNE and (*E*)-2-hexenal on root elongation. HNE at 10  $\mu$ M inhibited root growth significantly, and the inhibition was severer in SR1 plants (55%) than in AER-OE plants (32%) (Fig. 9). Similar results were obtained for (*E*)-2-hexenal (Supplementary Fig. 1); with increasing concentration from 10 to 300  $\mu$ M, root growth inhibition was increased, and AER-OE plants suffered less. These results confirmed that 2-alkenals can be cause of root growth inhibition and they were effectively detoxified in AER-OE plants.

## BHA partly protects roots from Al injury

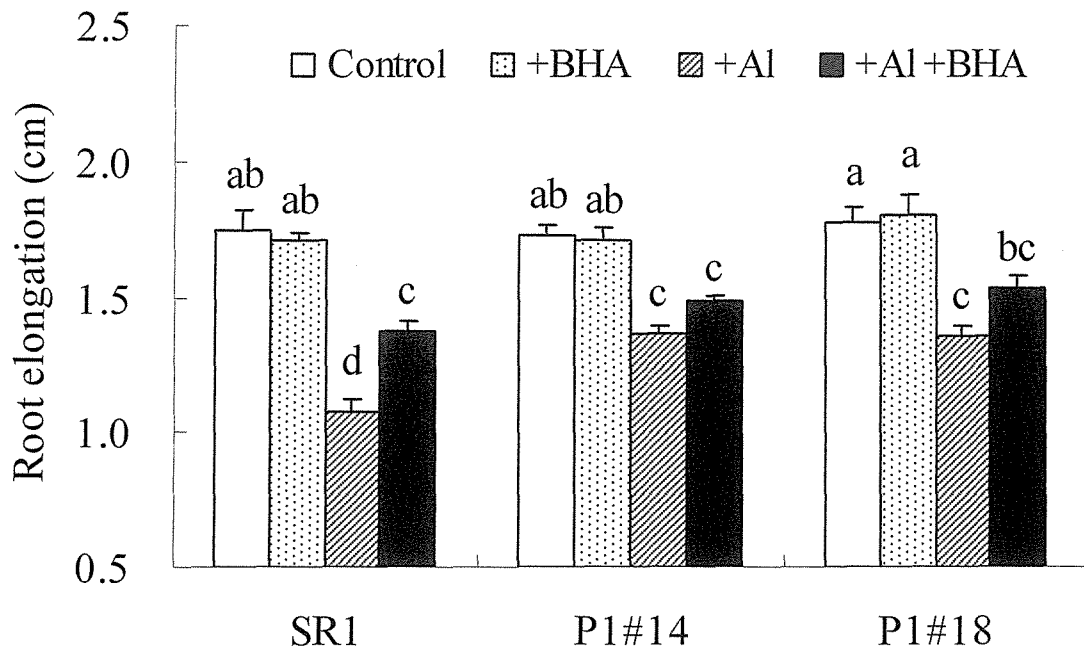
Yamamoto et al. (2001) previously suggested that lipid peroxidation was not the primary cause of elongation inhibition in pea roots under Al stress, which based on the result that BHA (butylated hydroxyanisole) suppressed the Al-induced increase in TBARS in roots, but did not prevent the inhibition of root elongation in pea. In cultured tobacco cells, however, BHA could prevent Al-induced growth inhibition (Yamamoto et al., 2002). In order to investigate the effect of BHA in tobacco plants, BHA was applied in SR1 and AER-OE plants under Al stress. Our result showed that BHA could suppress the H<sub>2</sub>O<sub>2</sub> production (Supplementary Fig. 2), and alleviated Al-induced root growth inhibition by 18% in SR1 plants (Fig. 10). Because BHA alleviated root elongation and growth capacity in both tobacco plants and cultured cells under Al stress, the effect of BHA in alleviating Al stress in tobacco might be different from that in pea (Yamamoto et al., 2001).



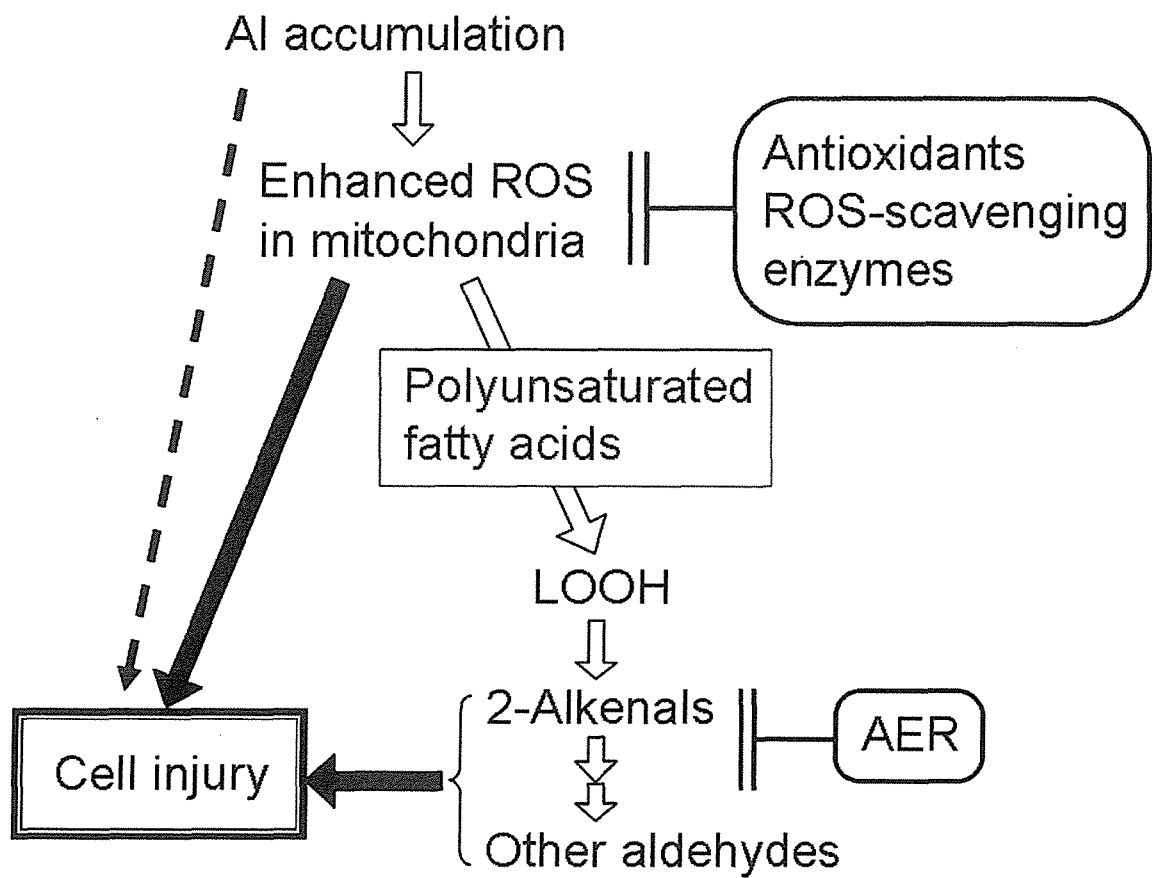
To investigate further the effect of BHA on root growth under Al stress in tobacco, individual aldehydes in AlCl<sub>3</sub>- and/or BHA-treated roots were identified and quantified as described above. With BHA treatment under Al stress, the content of each aldehyde changed differently (Fig. 8 and Supplemental Table 1-3). Some aldehydes contents were decreased by BHA, including malondialdehyde, formaldehyde, acetaldehyde, crotonaldehyde, (*Z*)-3-hexenal, *n*-hexanal, *n*-heptanal and *n*-octanal. Some aldehydes contents was not affected by BHA, including HHE, acrolein, HNE, (*E*)-2-pentenal, (*E*)-2-heptenal, *n*-pentanal, (*E*)-2-octenal and *n*-nonanal.



**Figure 9.** Effect of HNE on root growth. 21-d old seedlings were treated with or without 10  $\mu\text{M}$  HNE in 1/6 HS for 24 h and then cultured in HNE-free well aerated 1/6 HS for 5 d to recover. A, Root growth of SR1 and AER-OE lines (P1#14 and P1#18) under HNE treatment. B, Root length was measured after recovery. Data are means  $\pm$  SE ( $n = 20$ ). Values followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).



**Figure 10.** Effect of  $\text{AlCl}_3$  and BHA on root growth of SR1 and AER-OE lines (P1#14 and P1#18). Seeds were sown on MS agar plates and the seedlings were cultured for 14 d, then seedlings were transferred into sterilized filter paper soaked by following solutions: (1) 1/6 HS (Control), (2) 10  $\mu\text{M}$  BHA in 1/6 HS (+BHA), (3) 500  $\mu\text{M}$   $\text{AlCl}_3$  in 1/6 HS (+Al), and (4) 500  $\mu\text{M}$   $\text{AlCl}_3$  + 10  $\mu\text{M}$  BHA in 1/6 HS (+Al+BHA) for 5 d as described in Materials and Methods. At the end of treatment, root elongation was measured. Data are means  $\pm$  SE of three replications (each replication included 10 plants). Values followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).



**Figure 11.** Summary scheme of how LOOH-derived aldehydes act downstream of the formation of ROS and AER prevents Al-induced cell injury. In some Al-sensitive species like pea, ROS-independent Al toxicity can be critical. Abbreviations are as follows: ROS, reactive oxygen species; LOOH, lipid peroxide; AER, 2-alkenal reductase.

## 4.5 Discussion

The overexpressed AER suppressed the LOOH-derived aldehyde levels without affecting the accumulation of Al and H<sub>2</sub>O<sub>2</sub> (Figs. 4 and 5). Taking advantage of this, we could investigate the toxicity of aldehydes separately from that of the upstream ROS. Compared with the two types of control plants, AER-OE plants showed significantly higher relative rate of root elongation in the presence of AlCl<sub>3</sub>, as well as rapid root growth recovery after the removal of AlCl<sub>3</sub> and effective maintenance of membrane integrity in the roots (Figs. 1, 2, 6). Thus overexpression of AER alleviated the Al-induced irreversible damage to root cells, especially to the elongation zone. This tolerance is attributed exclusively to suppression of the Al-induced increase in aldehyde contents. Increases in the contents of not only 2-alkenals, which are directly scavenged by AER, but also many other aldehydes that are incompatible with AER, were suppressed in the AER-OE plants (Fig. 8). Aldehydes accumulated around the root elongation zone, where cell death was most prominent, and the suppression of aldehyde accumulation at the elongation zone closely paralleled the alleviation of cell death (Fig. 6). When added exogenously, 2-alkenals inhibited the root elongation and its inhibition was alleviated by the overexpression of AER (Fig. 9 and Supplemental Fig.1). These results clearly indicate that, in Al stress, LOOH-derived aldehydes are involved in root cell injury.

Thus far, the alleviation of Al toxicity by the enhanced endogenous antioxidant levels (Ezaki et al., 2000; Basu et al., 2001) and by an exogenously added antioxidant (Yamamoto et al. 2002) has been explained as the detoxication of ROS. However, taking the formation and effects of aldehydes into consideration, the protection due to enhanced

antioxidant levels can be partly explained as an indirect suppression of the downstream aldehyde production (Fig. 11). Of course, the above conclusion does not exclude the direct participation of ROS in the Al toxicity. Furthermore, our results also suggest the participation of some factors other than aldehydes in the root injury; in AER-OE roots, the increase of toxic 2-alkenals was totally suppressed but the growth inhibition was only partially alleviated (Figs. 1 and 8). The protective effect of BHA in alleviating root growth inhibition in AER-OE plants also demonstrates that both ROS and aldehydes are involved in the Al toxicity (Fig. 10; Supplemental Fig. 2).

It was previously stated that lipid peroxidation was not the primary cause of elongation inhibition in pea roots under Al stress (Yamamoto et al., 2001). This conclusion was derived solely from the observation that in pea plants the antioxidant BHA failed to alleviate Al-induced inhibition of root elongation although it effectively suppressed an increase in TBARS. While in tobacco cultured cells, BHA could protect from Al toxicity through inhibition of ROS generation (Yamamoto et al., 2002). Furthermore, it was reported that the major decomposition product of Al-induced lipid peroxidation was HNE but not TBARS in barley roots and soybean liposomes, and assay of HNE production is essential to quantify the lipid peroxidation of plant membranes (Sakihama and Yamasaki, 2002). We here observed, in tobacco plants, BHA could suppress both ROS generation and TBARS increase, and the inhibition of root elongation. Based on these apparent protecting effects of BHA, the Al toxicity in tobacco is at least partially ascribed to oxidative stress, in which LOOH-derived aldehydes are possibly involved. The contrasting effectiveness of BHA against Al toxicity in pea and tobacco

might be due to differential sensitivity to Al of the two species. In pea plants, 10  $\mu\text{M}$   $\text{AlCl}_3$  inhibited root elongation by 67% (Yamamoto et al., 2001). In tobacco plants, 500  $\mu\text{M}$   $\text{AlCl}_3$  in the agar and hydroponic medium inhibited root growth 46% and 40%, respectively (Fig. 1 and 10). Thus pea is more sensitive to Al. Because oxidative stress is not the only cause of Al toxicity, it is possible that in pea cells, Al ions at low concentration exerted a specific toxicity. In more tolerant tobacco cells, oxidative stress induced by Al ions at higher concentrations could be the major cause of toxicity.

Among the detected aldehydes, malondialdehyde is a commonly studied marker of oxidative stress and has been shown to modify proteins by Schiff base addition (Fenaille et al., 2002; Taylor et al., 2004). Acrolein has DNA-damaging effects and inhibits enzymes with functional SH groups (Esterbauer et al., 1991). Recently, it was shown that acrolein inactivates the Calvin cycle enzymes phosphoribulokinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, aldolase and Rubisco, and causes a rapid drop in the glutathione pool in chloroplasts *in vitro* (Mano et al., 2009). HNE is the most cytotoxic and abundant aldehyde generated through ROS-mediated lipid peroxidation; it is a highly reactive electrophile that forms Michael adducts via the C-3 atom and Schiff adducts via the C-1 aldehyde group; it modifies amino acids and forms cross-links in proteins, thus causing serious damage in cells (Winger et al., 2007). The toxicity of other aldehydes to plant components has been investigated less thoroughly.

The *in vivo* effect of an aldehyde depends on both its chemical reactivity and its intracellular concentration. Highly reactive 2-alkenals such as acrolein, HNE, and HHE

affect cellular metabolism even at low levels, whereas less reactive aldehydes such as malondialdehyde and formaldehyde can be toxic only when their levels are much higher than those of the 2-alkenals (Esterbauer et al., 1991). We found that the level of HNE in SR1 increased by 1.2 nmol g FW<sup>-1</sup> with Al treatment, whereas that of malondialdehyde increased by 7.3 nmol g FW<sup>-1</sup> (Fig. 8). In light of the fact that the reactivity of malondialdehyde is one-tenth that of HNE (Esterbauer et al., 1975), the extent of the damage caused by malondialdehyde may be almost the same as that caused by HNE. In SR1 a large increase was also found in the content of formaldehyde, the content of which was 100 times that of acrolein under Al exposure. If we assume that formaldehyde is 400 times weaker than acrolein (from toxicity data observed in lettuce seed germination; Reynolds, 1977), the damaging effect of formaldehyde in Al-stressed roots should be one-fourth that of acrolein.

Several targets of aldehydes in plant cells have been identified. Mitochondrial lipoate enzymes such as H-subunit of glycine decarboxylase and pyruvate dehydrogenase are highly sensitive to HNE (Taylor et al., 2002), and most probably to other 2-alkenals. Winger et al. (2007) revealed that oxidative stress to *A. thaliana* increased the HNE-modification on various proteins including ATP synthase  $\beta$  subunit and malate dehydrogenase. They also showed that several enzymes were inactivated by the HNE-modification. In Al-stressed roots, if these susceptible targets in the cells of elongation zone are attacked by HNE and other 2-alkenals, the energy metabolism will be stopped, resulting in the inhibition of growth. In addition, LOOH-derived aldehydes such as malondialdehyde and HNE can cause secondary membrane damage via avid binding to membrane proteins, eventually resulting in loss of membrane integrity (Esterbauer et al.,

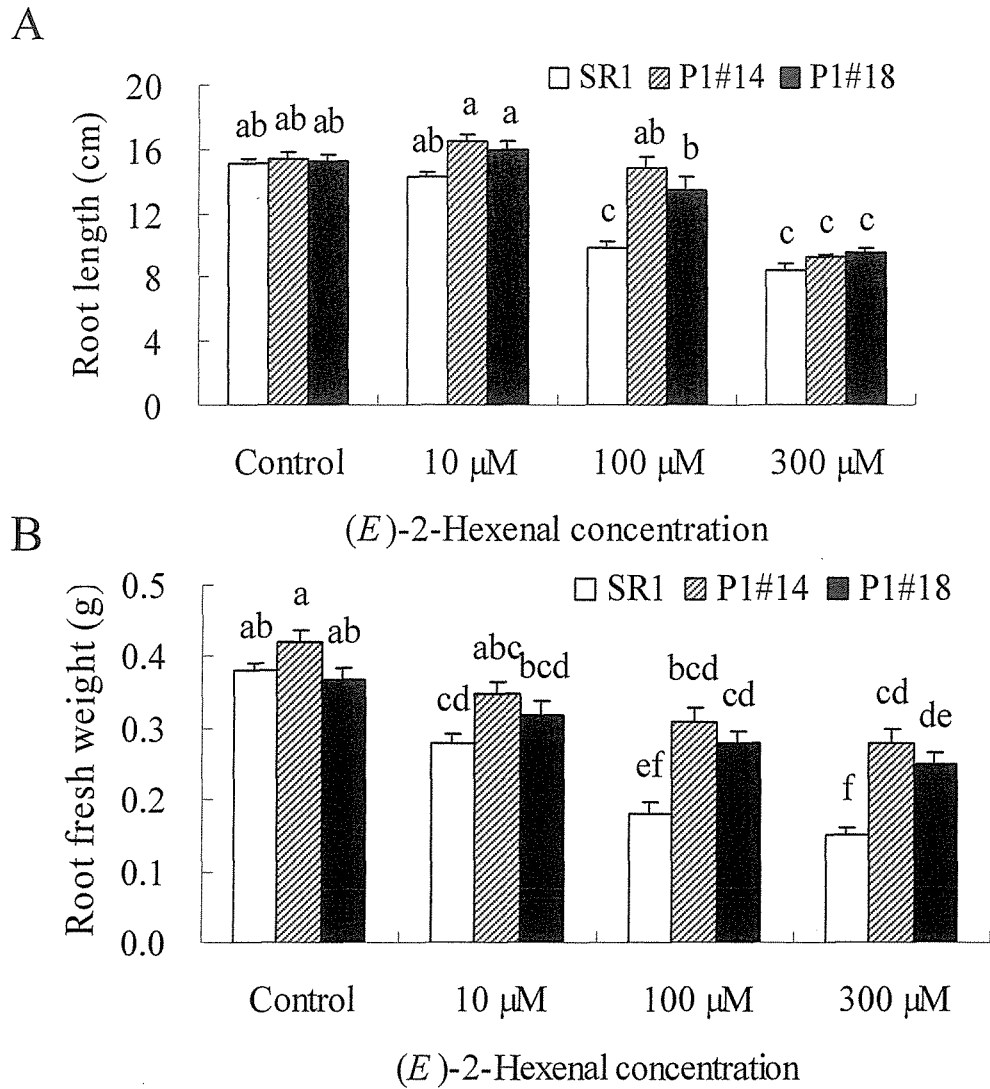


1991; Mueller, 2004; Halliwell, 2006). We found that the overexpressed AER alleviated membrane leakiness and cell death under AlCl<sub>3</sub> stress in parallel with the suppression of aldehyde levels (Fig. 6B and C); this suggests that the aldehydes affected membrane integrity under AlCl<sub>3</sub> stress.

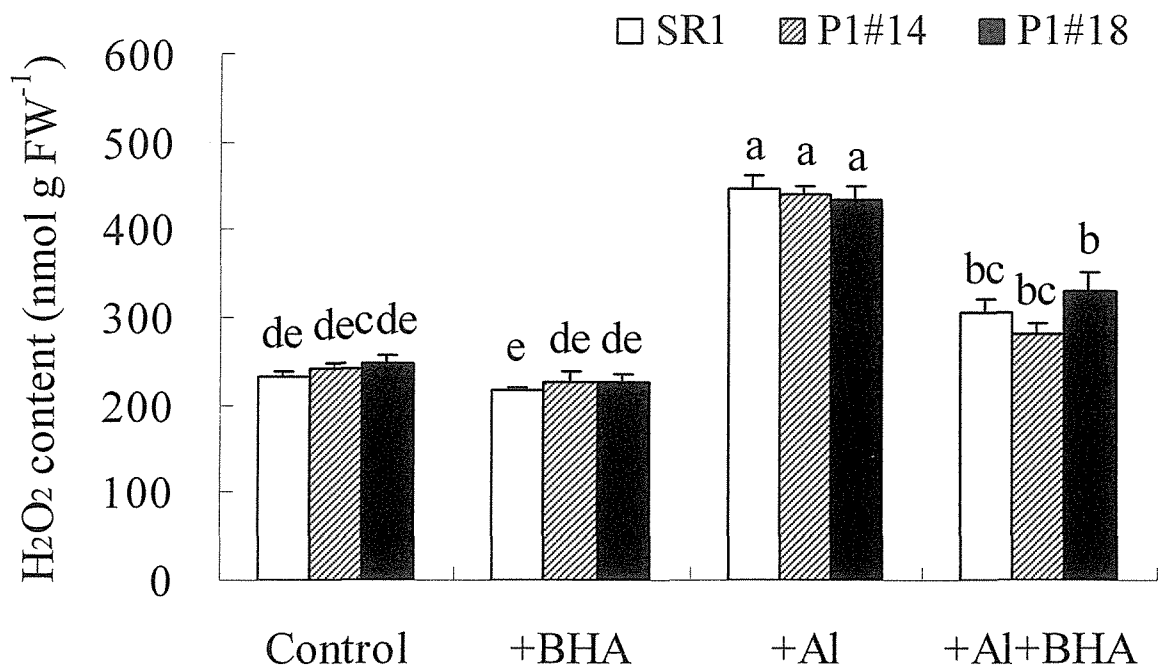
Overexpression of AER could lead to suppression of the production of a wide range of aldehydes, including malondialdehyde, formaldehyde, acetaldehyde, (*Z*)-3-hexenal, *n*-hexanal, (*E*)-2-heptenal, *n*-heptanal, and *n*-octanal, as well as the AER-substrate 2-alkenals such as HHE, acrolein, and HNE (Fig. 8). This can be explained as a secondary effect of AER activity, as follows. There are multiple enzymatic pathways from polyunsaturated fatty acids to aldehydes that sometimes overlap each other (Blée, 1998), and many more reactions for non-enzymatic aldehyde formation are possible (Grosch, 1987). In these reactions, aldehydes are generally produced from the longer chain peroxides, which sometimes contain the  $\alpha,\beta$ -unsaturated carbonyl structures (Esterbauer et al., 1991). AER could scavenge such long-chain precursors, thus suppressing generation of the descendant aldehydes. Although the substrate specificity of AER for long-chain compounds has not been tested extensively and the supposed precursors have yet to be identified, the enzyme prefers hydrophobic rather than that hydrophilic aldehydes and can utilize C18 ketones as substrates (Mano et al., 2005). These results suggest that AER can act primarily at the upstream sites of aldehyde-production pathways and regulate the global aldehyde composition of the cell.

In summary, tobacco plants overexpressing the *A. thaliana* AER gene showed increased ability to tolerate Al stress. We ascribe this greater tolerance to a decrease in the

production of aldehydes, which in turn resulted in reduced membrane damage and cell death in the roots, permitting improved root growth under Al stress. To the best of our knowledge, this is the first detailed report of the production of aldehydes under Al stress and the significance of aldehyde detoxication in enhancing Al tolerance in plants. Our findings should contribute to a better understanding of Al-induced aldehyde toxicity and provide a new strategy for improving Al stress tolerance in plants.



**Supplemental Figure 1.** Root growth of SR1 and AER-OE lines (P1#14 and P1#18) under (*E*)-2-hexenal treatment. Seeds were sown on MS agar plates and the seedlings were cultured for 28 d, then transplanted into hydroponic solution and cultured for another 21 d. Seedlings were treated with 0, 10, 100 or 300 μM (*E*)-2-hexenal in 1/6 HS for 24 h and then cultured in (*E*)-2-hexenal-free well aerated 1/6 HS for 5 d to recover. Then root length (A) and root fresh weight (B) were measured. Data are means ± SE ( $n = 8$ ). Values followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).



**Supplemental Figure 2.** H<sub>2</sub>O<sub>2</sub> content in root tips (0-20 mm) of SR1 and AER-OE lines (P1#14 and P1#18). Seedlings were grown on MS agar plates for 28 d and then in hydroponic solution for 28 d. Seedlings were subjected to four treatments: (1) 1/6 HS (Control), (2) 10 μM BHA in 1/6 HS (+BHA), (3) 500 μM AlCl<sub>3</sub> in 1/6 HS (+Al), and (4) 500 μM AlCl<sub>3</sub> + 10 μM BHA in 1/6 HS (+Al+BHA) for 24 h. Data are means ± SE (*n* = 3). Values followed by the same letter are not significantly different according to Tukey-Kramer test (*P* < 0.05).

**Supplemental Table 1.** Aldehydes, whose content was not affected by AI treatment, and showed no difference between wild type (SR1) and AER-OE plants (P1#14, P1#18).

Aldehyde	Rt	Aldehyde contents (nmol g fresh weight <sup>-1</sup> )												
		SR1				P1#14				P1#18				
		Control	+BHA	+AI	+AI+BHA	Control	+BHA	+AI	+AI+BHA	Control	+BHA	+AI	+AI+BHA	
u.i. 0.084	0.084	9.29 a	9.26 a	<b>6.46 a</b>	6.65 a	8.90 a	9.00 a	<b>10.61 a</b>	10.33 a	8.03 a	7.93 a	<b>9.25 a</b>	+AI+BHA	9.20 a
u.i. 0.149	0.149	9.32 abc	8.88 bc	<b>13.53 a</b>	12.04 ab	8.98 bc	8.60 bc	<b>9.76 abc</b>	8.54 bc	6.96 c	6.62 c	<b>9.72 abc</b>	+AI	8.74 bc
u.i. 0.215	0.215	0.45 ab	0.23 b	<b>0.86 a</b>	0.19 b	0.80 a	0.11 b	<b>0.67 a</b>	0.14 b	0.73 a	0.15 b	<b>0.73 a</b>	+AI	0.21 b
( <i>E</i> )-2-pentenal	0.650	1.57 a	1.53 a	<b>1.83 a</b>	1.87 a	1.49 a	1.47 a	<b>1.67 a</b>	1.69 a	1.37 a	1.34 a	<b>1.63 a</b>	+AI+BHA	1.57 a
<i>n</i> -pentanal	0.697	0.32 a	0.33 a	<b>0.39 a</b>	0.38 a	0.29 a	0.28 a	<b>0.36 a</b>	0.40 a	0.29 a	0.29 a	<b>0.36 a</b>	+AI	0.41 a
( <i>E</i> )-2-octenal	1.054	0.58 abc	0.56 abc	<b>0.63 abc</b>	0.66 abc	0.37 c	0.40 c	<b>0.90 a</b>	0.81 ab	0.54 abc	0.52 bc	<b>0.83 ab</b>	+AI+BHA	0.73 abc
<i>n</i> -nonanal	1.208	0.58 a	0.61 a	<b>0.76 a</b>	0.85 a	0.54 a	0.60 a	<b>0.72 a</b>	0.87 a	0.57 a	0.73 a	<b>0.62 a</b>	+AI	0.86 a

Unidentified aldehydes (u.i.) were represented by relative retention time (rRt) as compared with internal standard.

Data are means for three replications. Data in lines followed by the same letter are not significantly different according to Tukey-Kramer test ( $P$

< 0.05).

**Supplemental Table 2.** Aldehydes, whose content was increased by AI treatment, but showed no difference between wild type (SR1) and AER-OE plants (P1#14, P1#18).

Aldehyde	Aldehyde contents (nmol g fresh weight <sup>-1</sup> )											
	SR1				P1#14				P1#18			
Rt	Control	+BHA	+AI	+AI+BHA	Control	+BHA	+AI	+AI+BHA	Control	+BHA	+AI	+AI+BHA
u.i. 0.124	8.46 b	8.24 b	<b>12.16 a</b>	11.02 ab	9.53 ab	9.27 ab	<b>8.77 ab</b>	8.42 b	10.25 ab	9.91 ab	<b>10.10 ab</b>	+AI+BHA
u.i. 0.239	2.07 b	1.87 b	<b>3.78 a</b>	2.99 ab	2.52 ab	2.29 ab	<b>3.07 ab</b>	2.46 ab	2.86 ab	2.58 ab	<b>2.63 ab</b>	2.42 ab
crotonaldehyde (CRA)	4.74 d	4.28 d	<b>8.82 a</b>	5.23 cd	4.15 d	3.77 d	<b>8.06 ab</b>	5.63 bcd	3.21 d	3.37 d	<b>7.34 abc</b>	4.76 d
u.i. 0.674	0.57 bc	0.50 bc	<b>1.74 a</b>	0.83 b	0.52 bc	0.47 c	<b>1.41 a</b>	0.70 bc	0.75 bc	0.59 bc	<b>1.56 a</b>	0.70 bc

Unidentified aldehydes (u.i.) were represented by relative retention time (rRt) as compared with internal standard.

Data are means for three replications. Data in lines followed by the same letter are not significantly different according to Tukey-Kramer test ( $P < 0.05$ ).

**Supplemental Table 3.** Aldehydes, whose increase by AI treatment was greater in wild type (SR1) than in AER-OE plants (P1#14, P1#18).

Aldehyde	Rt	Aldehyde contents (nmol g fresh weight <sup>-1</sup> )											
		SR1				P1#14				P1#18			
		Control	+BHA	+AI	+AI+BHA	Control	+BHA	+AI	+AI+BHA	Control	+BHA	+AI	+AI+BHA
u.i. 0.055	0.055	18.69 cd	16.88 cd	<b>51.60 a</b>	25.52 bc	19.93 cd	18.09 cd	<b>33.48 b</b>	16.69 cd	11.95 d	12.95 d	<b>32.16 b</b>	16.40 cd
u.i. 0.065	0.065	3.68 cd	3.51 cd	<b>11.52 a</b>	4.12 cd	2.35 d	2.49 d	<b>5.92 bc</b>	3.55 cd	5.07 bcd	3.55 cd	<b>7.18 b</b>	2.87 d
u.i. 0.069	0.069	11.29 b	10.47 b	<b>25.08 a</b>	9.91 b	13.99 b	13.23 b	<b>11.51 b</b>	9.40 b	10.26 b	10.06 b	<b>11.83 b</b>	9.46 b
u.i. 0.073	0.073	10.54 d	9.62 d	<b>34.29 a</b>	23.74 b	10.53 d	8.43 d	<b>18.76 bc</b>	14.03 cd	8.86 d	8.79 d	<b>21.51 b</b>	16.12 bcd
u.i. 0.092	0.092	9.91 c	9.28 c	<b>27.58 a</b>	10.11 c	11.72 c	8.94 c	<b>21.35 b</b>	10.27 c	11.90 c	9.15 c	<b>18.98 b</b>	9.46 c
u.i. 0.101	0.101	2.78 d	2.92 d	<b>10.56 a</b>	4.17 bcd	4.43 bcd	4.25 bcd	<b>6.38 bc</b>	3.50 cd	3.37 cd	3.21 cd	<b>7.16 b</b>	3.90 bcd
malondialdehyde	0.160	3.94 bc	3.56 c	<b>11.20 a</b>	4.44 bc	3.11 c	2.82 c	<b>6.47 b</b>	3.87 bc	2.01 c	1.82 c	<b>3.79 bc</b>	3.40 c
formaldehyde	0.186	58.57 bc	55.25 bc	<b>101.67 a</b>	70.39 b	55.64 bc	52.76 c	<b>57.39 bc</b>	51.51 c	48.35 c	45.54 c	<b>51.56 c</b>	46.36 c
acetaldehyde	0.248	0.31 b	0.28 b	<b>0.60 a</b>	0.34 b	0.31 b	0.28 b	<b>0.37 b</b>	0.34 b	0.26 b	0.24 b	<b>0.35 b</b>	0.29 b
4-hydroxy-(E)-2-hexenal	0.269	0.60 c	0.66 c	<b>1.44 ab</b>	1.63 a	0.81 c	0.90 c	<b>0.91 c</b>	1.04 bc	0.55 c	0.60 c	<b>0.87 c</b>	0.82 c
u.i. 0.301	0.301	0.36 c	0.18 c	<b>3.25 a</b>	0.25 c	0.36 c	0.18 c	<b>1.69 b</b>	0.25 c	0.25 c	0.13 c	<b>2.07 b</b>	0.18 c
Acrolein	0.367	1.43 c	1.36 c	<b>2.82 a</b>	2.51 ab	1.58 bc	1.51 bc	<b>1.68 bc</b>	1.74 bc	1.48 c	1.76 bc	<b>1.64 bc</b>	1.74 bc
u.i. 0.441	0.441	0.82 b	0.70 bc	<b>1.34 a</b>	0.80 b	0.51 bcd	0.48 bcd	<b>0.61 bcd</b>	0.62 bcd	0.34 d	0.33 d	<b>0.38 cd</b>	0.42 cd
butyraldehyde	0.557	0.34 e	0.30 e	<b>0.96 bc</b>	1.71 a	0.36 e	0.32 e	<b>0.78 cd</b>	1.20 b	0.54 de	0.43 de	<b>0.82 bcd</b>	1.23 b
u.i. 0.604	0.604	0.26 d	0.28 d	<b>1.01 a</b>	0.95 ab	0.31 d	0.30 d	<b>0.65 bc</b>	0.59 cd	0.29 d	0.31 d	<b>0.40 cd</b>	0.42 cd

u.i. 0.618	0.618	0.42 bc	0.38 bc	<b>1.11 a</b>	0.55 b	0.37 bc	0.34 c	<b>0.53 bc</b>	0.45 bc	0.36 bc	0.37 bc	<b>0.45 bc</b>	0.40 bc
Phenylacetaldehy	0.628	0.51 d	0.54 d	<b>1.71 b</b>	2.33 a	0.53 d	0.51 d	<b>1.10 c</b>	2.16 ab	0.57 d	0.61 cd	<b>0.79 cd</b>	2.24 a
de													
4-hydroxy-( <i>E</i> )-2-	0.682	1.56 bcd	1.46 bcd	<b>2.71 a</b>	2.44 a	1.34 cd	1.22 d	<b>1.80 b</b>	1.71 bc	1.52 bcd	1.42 bcd	<b>1.66 bc</b>	1.74 bc
nonenal													
( <i>Z</i> )-3-hexenal	0.706	0.18 de	0.18 de	<b>1.16 a</b>	0.40 c	0.14 de	0.14 de	<b>0.65 b</b>	0.39 c	0.10 e	0.11 e	<b>0.37 c</b>	0.30 cd
<i>n</i> -hexanal	0.810	1.18 d	1.24 cd	<b>4.44 a</b>	1.76 c	1.27 cd	1.21 d	<b>2.73 b</b>	1.50 cd	1.36 cd	1.33 cd	<b>2.49 b</b>	1.49 cd
( <i>E</i> )-2-heptenal	0.889	0.23 d	0.21 d	<b>0.72 a</b>	0.81 ab	0.23 d	0.25 d	<b>0.51 c</b>	0.53 bc	0.25 d	0.22 d	<b>0.38 cd</b>	0.42 cd
<i>n</i> -heptanal	0.922	3.97 bcd	3.78 bcd	<b>6.89 a</b>	4.58 bc	3.57 cd	3.42 cd	<b>5.30 b</b>	4.82 bc	2.66 d	2.67 d	<b>4.83 bc</b>	5.00 bc
<i>n</i> -octanal	1.066	0.88 bc	0.80 bcd	<b>1.37 a</b>	0.98 b	0.66 cd	0.60 cd	<b>0.85 bc</b>	0.73 bcd	0.53 d	0.51 d	<b>0.61 cd</b>	0.52 d
u.i. 1.095	1.095	0.73 bc	0.59 bc	<b>1.39 a</b>	0.69 bc	0.64 bc	0.52 c	<b>0.98 b</b>	0.61 bc	0.44 c	0.35 c	<b>0.72 bc</b>	0.42 c

Unidentified aldehydes (u.i.) were represented by relative retention time (rRt) as compared with internal standard.

Data are means for three replications. Data in lines followed by the same letter are not significantly different according to Tukey-Kramer test ( $P$

< 0.05).



## Chapter 5

### General discussion

A range of biotic and abiotic stresses, and normal metabolism (photosynthesis and respiration) increased levels of ROS in plants. These ROS include hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), superoxide ( $\text{O}_2^-$ ) and the hydroxyl radical ( $\text{OH}^\cdot$ ). Under normal conditions, the production of ROS in cells is low ( $240 \mu\text{MS}^{-1} \text{O}_2^-$  and a steady-state level of  $0.5 \mu\text{M} \text{H}_2\text{O}_2$  in chloroplast) (Polle, 2001), which can act as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense and systemic signaling (Mittler, 2002). While under stress conditions, the cellular homeostasis of ROS production is disrupted, and the enhanced production of ROS can pose a threat to cells. The production of ROS during these stresses results from pathways such as photorespiration and the photosynthetic apparatus in chloroplast and from mitochondrial respiration (Mittler, 2002).

We observed markedly increased level of  $\text{H}_2\text{O}_2$  in the roots of both tobacco and *Arabidopsis* under Al stress, and the tolerant lines showed less  $\text{H}_2\text{O}_2$  generation, indicating that ROS is one of the Al-induced injuring molecules in these plants. The similar results were also reported by Yamamoto et al. (2002) that in cultured tobacco cells and pea roots, Al triggered  $\text{O}_2^-$  generation inside cells, respiration inhibition, and ATP depletion, which are well correlated with the inhibition of cell growth and root elongation, respectively. Recently, it was shown that Al triggered ROS generation in the roots of rice (Ma et al., 2007), *Allium* (Achary et al., 2008), *Miscanthus* and *Andropogon* (Ezaki et al.,

2008), Melaleuca tree (Tahara et al., 2008), and cassia (Xue et al., 2008). Furthermore, these ROS generated less in tolerant cultivars (Ma et al., 2007; Tahara et al., 2008).

Plants have developed various ROS-scavenging pathways to tightly control the ROS production in the cells. The major ROS-scavenging pathways of plants include SOD, found in almost all cellular compartments, the water-water cycle in chloroplasts, the ascorbate-glutathione (AsA-GSH) cycle in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes, glutathione peroxidase and CAT in peroxisomes. The finding of the AsA-GSH cycle in almost all cellular compartments tested to date, as well as the high affinity of APX for H<sub>2</sub>O<sub>2</sub>, suggests that this cycle plays a crucial role in controlling the level of ROS in these compartments (Mittler, 2002). The antioxidants in this cycle such as AsA and GSH, are also found at high concentrations in chloroplasts and other cellular compartments, suggesting their paramount functions for plant defense against oxidative stress (Noctor and Foyer, 1998). It is generally believed that maintaining a high reduced per oxidized ratio of AsA and GSH is essential for the proper scavenging of ROS in cells. This ratio is maintained by DHAR, MDAR and GR using NAD(P)H as reducing powder (Noctor and Foyer, 1998; Mittler, 2002). Thus, those enzymes play an important role in controlling reduced AsA and GSH level in cells, and as well as in oxidative stress including Al toxicity.

In our study, the increased activities of DHAR or GR lead to Al tolerance, but not MDAR. Both DHAR and GR overexpressing plants maintained a high reduced AsA level in their roots under Al stress, while MDAR overexpressing plants could not. These results indicate that the reduced AsA level is of great important to obtain Al tolerance in plants. It

is known that Al mainly accumulated in the apoplast (Wang et al., 2004), and AsA is the major and probably the only antioxidant buffer in the apoplast (Pignocchi and Foyer, 2003), therefore, it is reasonable that this antioxidant plays an unsubstitutable role in Al tolerance. Furthermore, the high level of AsA also contributes to maintain high activity of APX, which catalyzes detoxification of  $H_2O_2$ , because APX can be inactivated by  $H_2O_2$  in the absence of AsA (Hossain and Asada, 1984). We observed that the Al-tolerant DHAR and GR overexpressing plants also maintained higher APX activity, while MDAR overexpressing plants did not. Thus, the high level of AsA and highly maintained APX activity are the key point for obtaining Al tolerance.

If Al-induced ROS can not be effectively scavenged in cells, they will further initiate lipid peroxidation, which lead to the generation of toxic aldehyds. These aldehydes are highly toxic, which can cause membrane damage in return. Here, we used AER overexpressing tobacco plants to investigate whether Al stress induced aldehydes generation, and whether detoxification of aldehydes, especially the most toxic 2-alkenals could lead to improved Al tolerance. Our results showed that Al indeed induced toxic aldehydes, and the increase of aldehydes was higher in wild-type SR1 than in AER-OE plants. These results showed that aldehydes, especially 2-alkenals are toxic targets of Al injury, and suppression of them could alleviate Al-caused root damage.

Although a large amount of the nowadays researches are focus on the functions of organic acid exclusion in Al tolerance, our study clear shows that both ROS and lipid peroxide-induced aldehydes are the primary molecules of Al toxicity. Moreover, in acid soils, not only Al ion is the toxic source, the acid condition itself also cause stress to plant.

In our study, we found that Arabidopsis is a relative acid-sensitive plant, whereas tobacco is a relative acid-tolerant plant. Because only acid without Al ion slows the root growth of Arabidopsis, while not slows the root growth of tobacco. Thus, for the acid-sensitive plant growing in acid soils, not only obtain Al tolerance, but also acid-tolerance is important for their growth. Fortunately, both of Al and acid stress cause oxidative damage, so we can improve both Al and acid tolerance through improving antioxidant capacity or decreasing the generation of oxidative stress-induced toxic targets, and this seems more effective than only organic acid exclusion in Al tolerance.

## Chapter 6

### Conclusion

Our study indicates that Al toxicity causes cell damage through induction of ROS and lipid peroxide-derived aldehydes that generated from oxidative stress. Suppression of Al-induced ROS or lipid peroxide-derived aldehydes effectively alleviates cell injury and root growth (Fig. 1). Our study provides a novel mechanism of Al toxic mechanism, and as well as a new strategy for improving plant, especially crop productivity in acid soils.

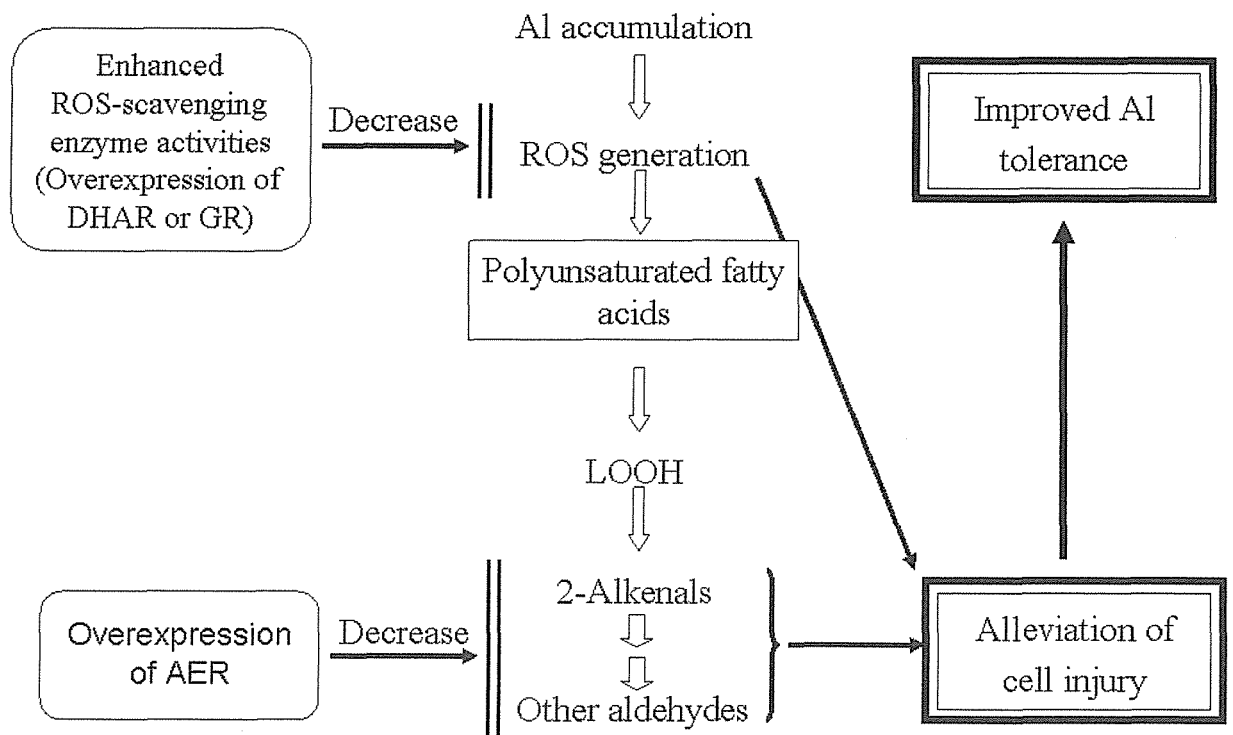


Figure 1. Summary scheme of how to improve Al tolerance through enhancing antioxidant capacity.

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## 和文要旨

アルミニウム障害は、酸性土壌における植物の成長や生産性を制限する最大の要因である。アルミニウムイオンは酸化的障害を引き起こし、植物の成長を阻害する。この酸化的障害は活性酸素種により促進され、抗酸化能の向上により軽減される。アスコルビン酸 (AsA) およびグルタチオン (GSH) は植物の主要な抗酸化物質であり、モノデヒドロアスコルビン酸レダクターゼ (MDAR), デヒドロアスコルビン酸レダクターゼ (DHAR), グルタチオンレダクターゼ (GR) の作用によって再生されることが明らかにされている。しかし、アルミニウム耐性における DHAR, MDAR および GR の機能は未解明のままである。そこで本研究では、アルミニウムストレス期間中の AsA および GSH の再生における DHAR, MDAR および GR の役割を明らかにすることを目的とした。供試材料として、AtMDAR もしくは AtDHAR を過剰発現させた遺伝子組換えタバコ (それぞれ MDAR-OX 株もしくは DHAR-OX 株), および AtGR を過剰発現させた遺伝子組換えシロイヌナズナ (GR-OE 株) を用い、対照植物であるタバコ野生株 SR-1 およびシロイヌナズナ野生株 Columbia (Col) と比較した。

寒天培地上で 14 時間  $AlCl_3$  に曝したところ、DHAR-OX 株は野生株 SR-1 よりも根の成長が早かったが、MDAR-OX 株では同等であった。水耕培地で 24 時間  $AlCl_3$  処理 (500  $\mu M$ ) したところ、根端におけるアルミニウムの分布や集積について、SR-1, DHAR-OX 株および MDAR-OX 株の間に差異は認められなかった。しかし、DHAR-OX 株は SR-1 よりも過酸化水素含量が少なく、脂質過酸化や酸化的 DNA 障害レベルが小さかった。一方、MDAR-OX 株は SR-1 と同程度の障害を示した。SR-1 と比較したところ、DHAR-OX 株はアルミニウムの有無に関わらず AsA レベルをより高く維持していたが、MDAR-OX 株はアルミニウム不在の場合のみ AsA レベルを高く保った。また、DHAR-OX 株はアルミニウムストレス下でアスコルビン酸ペルオキシダーゼ (APX) 活性を高く維持した。これ

らの結果より，DHAR-OX 株の高い抗酸化能およびアルミニウムストレス耐性は，AsA レベルおよび APX 活性の維持に起因していることが明らかとなった．また，アルミニウム耐性の付与には MDAR ではなく DHAR の過剰発現が関与しており，また AsA レベルを高く維持することが重要であることが示された．

GR を過剰発現させたシロイヌナズナも，野生株 Col に比べて高いアルミニウムストレス耐性を示した．アルミニウムストレス下において，GR-OE 株は野生株 Col に比べて，根の伸長も良好に保たれ，過酸化水素含量および脂質過酸化が少なかった．アルミニウムを 24 時間処理したところ，根におけるアルミニウムの蓄積およびスーパーオキシドディスムターゼ (SOD)，カタラーゼ (CAT)，デヒドロアスコルビン酸レダクターゼ (DHAR) の活性について，GR-OE 株と野生株 Col との間に差異は認められなかった．しかし，GR-OE 株は Col よりも GR および APX の活性がともに高く，GSH および AsA のレベルも高かった．GR の過剰発現はアルミニウム蓄積やその他の抗酸化酵素の活性には影響しなかった．これらの結果より，シロイヌナズナで GR を過剰発現させると，細胞中の GSH および AsA レベルが増加し，これにより抗酸化能が向上して過酸化水素の生成と脂質の過酸化が抑制され，最終的にアルミニウムストレス耐性が高まることが明らかとなった．

活性酸素の下流で生じる脂質過酸化はアルミニウム障害の共通した反応であり，これはアルミニウム濃度が高まるにつれて増加する．動物細胞における研究では，現在のところ，脂質過酸化 (LOOH) 障害の大部分はそれに由来するアルデヒドに起因すると考えられている．植物では，高温，低温，紫外線-B，塩，重金属およびアルミニウムによって生じる環境ストレス条件において，LOOH 由来アルデヒドのレベル (チオバルビツール酸反応性物質として測定される) と細胞障害との間に密接な関係があることが報告されている．したがって，LOOH 由来アルデヒドがアルミニウム障害と関係している可能性がある．本実験では，LOOH 由来アルデヒド，特に高求電子性の  $\alpha$ ,  $\beta$ -不飽和アルデヒド (2-アルケ

ナール) がアルミニウム障害に関与しているという仮説を実証することを目的とした。*Arabidopsis thaliana* 2-アルケナールレダクターゼを過剰発現した遺伝子組換えタバコ (AER-OE 株), 野生株 SR-1 および空ベクター導入株 (SR-Vec) の根をアルミニウムに曝した。その結果, 2つの対照株に比べ, AER-OE 株はアルミニウム処理による根の伸長の遅延の程度が小さく, アルミニウムが除去された後の再伸長も早かった。また, アルミニウム処理によって AER-OE 株の根においてもアルミニウムが蓄積し, その程度は過酸化水素に感受的な対照株と同等であったが, 対照株よりもアルデヒドの蓄積量や細胞死が少なかった。SR-1 の根では, アルミニウム処理によって高活性 2-アルケナールであるアクロレイン, 4-ヒドロキシ-(E)-2-ヘキセナール, 4-ヒドロキシ-(E)-2-ノネナール, およびマロンジアルデヒドやホルムアルデヒドのようなその他のアルデヒドの量が顕著に増加した。一方, AER-OE 株の根ではこれらのアルデヒドの蓄積が有意に少なかった。4-ヒドロキシ-(E)-2-ヘキセナールおよび(E)-2-ヘキセナールに曝された根の成長阻害は, SR-1 よりも AER-OE 株のほうが小さかった。これらの結果より, 活性酸素の下流で産生される LOOH 由来アルデヒドは直接に根の細胞を損傷することが明らかとなった。また, AER による損傷の抑制はアルミニウム障害に対する新たな防御機構と考えられた。

本研究の結果, タバコおよびシロイヌナズナでは, アルミニウム障害が不可逆的な酸化障害を引き起こすことが明らかとなった。抗酸化酵素遺伝子である DHAR および GR を過剰発現させたタバコおよびシロイヌナズナではアルミニウム耐性が高まることが示された。しかし, タバコにおいて MDAR を過剰発現させたところ, アルミニウム耐性を向上させる効果は認められなかった。DHAR-過剰発現タバコおよび GR-発現シロイヌナズナは野生種に比べ, 根における AsA レベルおよび APX 活性が高く, これらがアルミニウム耐性において非常に重要な役割を果たしていることが明らかとなった。さらに, AER 遺伝子を過剰発現させたタバコもアルミニウム耐性が向上することが示された。AER 過

剩發現タバコは野生種よりも LOOH 由来アルデヒド, 特に 2-アルケナールの蓄積が少なかった. LOOH 由来アルデヒドはアルミニウム障害の要因であり, アルデヒド消去能はアルミニウム障害を軽減することが分かった. 以上の結果, 活性酸素および LOOH 由来アルデヒドによって生じた酸化損傷はアルミニウム障害の重要な要因であることが明らかとなった. 本研究によって植物のアルミニウム障害の新たな機構が解明されるとともに, アルミニウム耐性植物の育成に対する新たな研究戦略が示された. これらは世界の酸性土壌地域の植物生産性の向上に寄与すると考えられる.

## List of Publication

1. **Title:** Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco

**Authors:** Lina Yin, Shiwen Wang, Amin Elsadig Eltayeb, Md. Imtiaz Uddin, Yoko Yamamoto, Wataru Tsuji, Yuichi Takeuchi, Kiyoshi Tanaka

**Journal:** Planta

**Date:** 2010, 2

This paper covers Chapter 2 in the thesis.

2. **Title:** The involvement of lipid peroxide-derived aldehydes in aluminum toxicity of tobacco roots

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