

## Physiological, Biochemical and Molecular Responses of Barley Seedlings to Aluminum Stress

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**Abstract:** Barley (*Hordeum vulgare* L.) is one of the most Aluminum (Al) sensitive cereal species. In this study, the physiological, biochemical, and molecular response of barley seedlings to Al treatment was examined to gain insight into Al response and tolerance mechanisms. The results showed that superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activity were inhibited to different degrees following Al exposure. The MDA content also significantly increased with increasing Al concentrations. SRAP results indicated significant differences between Al treatments and controls in terms of SRAP profile, and the genomic template stability (GTS) decreased with increasing Al concentration and duration. These integrative results help to elucidate the underlying mechanisms that the barley response to Al toxicity.

**Keywords:** Barley; Al stress; Antioxidant enzymes; SRAP (Sequence-related amplified polymorphism); GTS (Estimation of genomic template stability)

### 1 Introduction

Aluminum (Al) accounts for 7.1% of crust and represents its most abundant metal element. Al toxicity is a major constraint to crop productivity in acid soils, occurring in up to 40% of the world's arable lands [1]. Typically, Al is adsorbed onto soil colloids and in combination with other minerals is non-toxic to plants. When soil acidification occurs, the levels of soluble Al in the soil dramatically increases, limiting crop growth and yield in acidic soil [2-4]. Previous studies have shown that Al toxicity due to soil acidification can damage the microscopic structure of plants, hinder the growth of plant roots, inhibit the absorption of nutrients, imbalance crop metabolism, inhibit chlorophyll synthesis, decreases photosynthesis, and alter the activity of key enzymes, ultimately reducing crop production [5-8]. Al toxicity also causes DNA damage, affects DNA composition, template activity, and chromatin structure in plants. Al restricts DNA replication through enhancing the rigidity of the double helix [9-10].

Barley (*H. vulgare* L.) is highly sensitive to Al toxicity [11] and as such, studies on the barley response to Al toxicity and the evaluation of Al stress has important practical significance. Studies have revealed morphological, physiological, biochemical, and cellular alterations induced by Al toxicity due to the increased production of reactive oxygen species (ROS), leading to damage to cellular organelles, membranes, and various biomolecules [12-16]. Although the physiological mechanisms of Al toxicity and tolerance have been clarified, the process of DNA/nuclear damage in response to Al stress remains poorly characterized in barley.

Sequence-related amplified polymorphism (SRAP) is a novel technique that amplifies open reading frames (ORFs) using unique primers [17]. Due to the variability of introns, promoters, and intervening sequences across species and individuals, SRAP has utility for comparative genomics, genetic diversity and genetic map construction [18]. Our recent studies have demonstrated the molecular basis of rice DNA damage using SRAP under Cadmium stress [19]. The effects of Al stress in barley plants are less well investigated.

In this study, we defined Al-induced physiological and biochemical changes, and DNA damage in barley seedlings under Al stress. These results help elucidate the mechanisms of Al tolerance in barley, and offer new perspectives for improving the barley tolerance in acidic soil.

## 2 Materials and Methods

### 2.1 Plant Materials, Growth Conditions, and Stress Treatments

The barley cultivar, Franklin, was assessed in this study. Seeds were surface sterilized in 0.2% NaClO for 20 min, rinsed with distilled (5 times) and germinated in moist sand. When the second leaf emerged, seedlings were selected for uniformity and transplanted into containers. The composition of the basic nutrient solution ( $\text{mg L}^{-1}$ ) was:  $(\text{NH}_4)_2\text{SO}_4$  48.2,  $\text{MgSO}_4$  154.88,  $\text{K}_2\text{SO}_4$  15.9,  $\text{KNO}_3$  18.5,  $\text{KH}_2\text{PO}_4$  24.8,  $\text{Ca}(\text{NO}_3)_2$  86.17, Fe-citrate 7,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.9,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.11,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.04,  $\text{HBO}_3$  2.9, and  $\text{H}_2\text{MoO}_4$  0.01. The pH of the solution was adjusted to 4.0. Growth inhibition tests were performed in barley plantlets exposed to 0, 50, 100, and 200  $\mu\text{mol L}^{-1}$  Al for 5 or 10 days. Containers were incubated in a growth chamber at  $26^\circ\text{C} \pm 1^\circ\text{C}$  and a 14/10 h day-night photoperiod with a light intensity of 10000 lux. Each treatment was repeated on three occasions. After harvesting, each plant was separated into the leaf and roots, washed thoroughly with distilled water, frozen in liquid nitrogen and maintained at  $-80^\circ\text{C}$  for biochemical and molecular analysis.

### 2.2 Cellular Antioxidant Enzyme Activities and MDA Content Measurement

Barley leaves (0.3 g) were homogenized 50  $\text{mmol L}^{-1}$  sodium phosphate buffer (pH 7.8). Homogenates were centrifuged ( $10000 \times g$ ) at  $4^\circ\text{C}$  for 20 min and supernatants were collected for enzymatic analysis. SOD, POD, CAT and Malondialdehyde (MDA) content were measured based on previous studies [19].

### 2.3 DNA Isolation, SRAP-PCR, Cloning and Sequencing of SRAP Fragments

For both control group and Al treatments groups, roots were collected 5 d and 7 d post-germination and flash frozen in liquid nitrogen prior to storage at  $-80^\circ\text{C}$ . Total DNA was extracted from  $\sim 100$  mg of fresh roots using a CTAB protocol [20]. SRAP amplification was performed using 30 primer combinations, including 9 forward and 8 reverse primers (Tab. 1). PCR amplification for SRAP was performed in a 20  $\mu\text{l}$  volume containing 80 ng of template DNA, 50  $\mu\text{M}$  forward primer, 50  $\mu\text{M}$  reverse primer, 1.0  $\mu\text{l}$  dNTPs (2.0  $\text{mmol/l}$  each), 2.0  $\mu\text{l}$   $\text{MgCl}_2$  (20  $\text{mmol/l}$ ), 1U Taq DNA polymerase, and 1  $\times$  PCR buffer. The reaction conditions were as follows: pre-denaturation at  $94^\circ\text{C}$  for 5 min, 5 cycles at  $94^\circ\text{C}$  for 1 min,  $35^\circ\text{C}$  for 50 sec,  $72^\circ\text{C}$  for 1 min, followed by an additional 30 cycles at  $94^\circ\text{C}$  for 1 min,  $50^\circ\text{C}$  for 50 sec,  $72^\circ\text{C}$  for 1 min, and a 10 min final extension at  $72^\circ\text{C}$ . Amplified products were analyzed by electrophoresis on 1.5% agarose gels.

**Table 1:** The sequence of SRAP primers

No.	Forward primer	No.	Reverse primer
Me1	TGAGTCCAAACCGGATA	em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGTAA	em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTCC	em7	GACTGCGTACGAATTCAA
Me8	TGAGTCCAAACCGGTGC	em8	GACTGCGTACGAATTCTG
Me9	TGAGTCCAAACCGGTAG		

### 2.4 Estimation of Genomic Template Stability (GTS)

Changes in the SRAP profiles were expressed as GTS which qualitatively measures evident changes. GTS was calculated using the formula:

$$GTS = \left(1 - \frac{a}{n}\right) \times 100$$

where  $a$  is the average number of changes in DNA profiles and  $n$  is the number of bands in control DNA profiles. Polymorphisms in the SRAP profiles included the disappearance and appearance of new PCR bands, in comparison to control SRAP profiles. Averages were calculated for each test group exposed to different Al treatments [19]. For the comparison of the sensitivity of each parameter (SOD, CAT, MDA, POD and GTS), changes were calculated as a percentage of their control value (100%).

### 2.5 Statistical Analysis

All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance, Duncan's new multiple range method and Student's  $t$  test were used to perform multiple comparison of physiological indices.

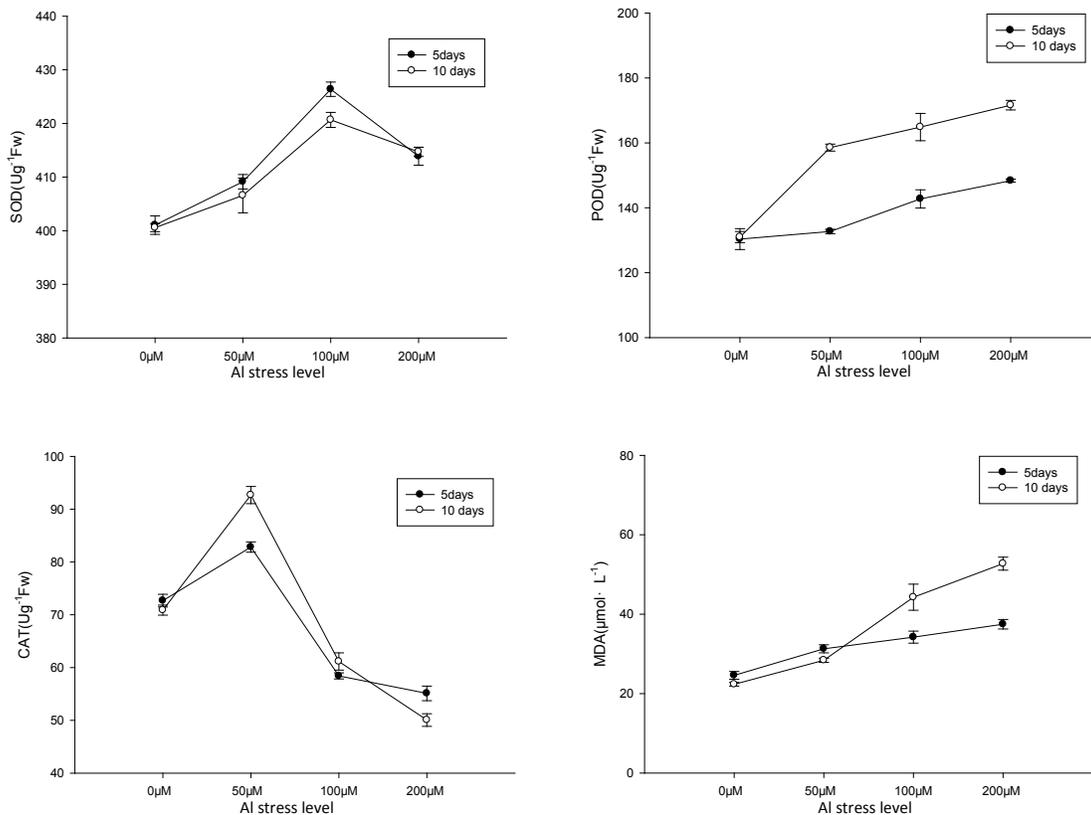
## 3 Results

### 3.1 Influence of Al Stress on Antioxidant Enzyme Activity of Barley Seedlings

SOD can remove contaminants and toxic material produced by cells [21]. At Al concentrations of 50 and 100  $\mu\text{M}$ , SOD activity increased. When the concentration of Al reached 200  $\mu\text{M}$ , SOD activity began to decline (Fig. 1(A)). When the Al concentration was lower than 100  $\mu\text{M}$ , SOD effectively eliminates oxygen free radicals. This represents plant defense responses, as plants under abiotic stress resist ROS induced stress and subsequent plant damage. SOD activity increases to remove ROS and reduce membrane lipid peroxidation. When the concentration of Al increased to 200  $\mu\text{M}$ , SOD activity gradually decreased, but was active in comparison to controls. These results suggest that enhanced membrane lipid peroxidation and permeability leads to the collapse of antioxidant defenses when Al stress increases [22].

The POD antioxidant system in plants coordinates with SOD to maintain low levels of free radicals, thereby preventing toxicity [21]. The influence of Al on the activity of POD in barley is shown in Fig. 1(B). POD activity increased as the concentration and time of Al exposure increased, indicating that POD deoxygenates excessive  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$  in plants, remove damaging ROS.

CAT is an enzyme scavenger that promotes  $\text{H}_2\text{O}_2$  decomposition into  $\text{O}_2$  and  $\text{H}_2\text{O}$ , removing hydrogen peroxide in plants, and decreasing  $\text{H}_2\text{O}_2$  toxicity. As shown in Fig. 1(C), when the concentration of Al increased to 50  $\mu\text{M}$ , the activity of CAT increased. However, upon further increases in Al concentrations, CAT activity was inhibited. These results suggest that as the concentration of Al rises,  $\text{H}_2\text{O}_2$  accumulates in barley cells to levels in which CAT is no longer effective, eventually leading to apoptotic induction.



**Figure 1:** Effects of increasing Al concentration (0, 50, 100 and 200 µmol L<sup>-1</sup>) on (A) SOD, (B) POD, (C) CAT activities and (D) MDA content in leaves of barley seedlings after 5d and 10d under Al stress. Error bars represent ± SE ( $n = 3$ )

### 3.2 Influence of Al Stress and MDA Content of Barley Seedlings

Under abiotic stress, plant membranes undergo lipid peroxidation. MDA is a final product of membrane lipid peroxidation and an important indicator of membrane damage. MDA is used as a measure of lipid peroxidation and can be used as a marker of plant stress conditions [23]. Under Al stress, MDA levels significantly change in barley (Fig. 1(D)). MDA increases in a time and concentration dependent manner in response to Al (Fig. 1(D)) suggesting that Al toxicity enhances lipid peroxidation.

### 3.3 Effect of Al Stress on SRAP Profiles of Root Genomes

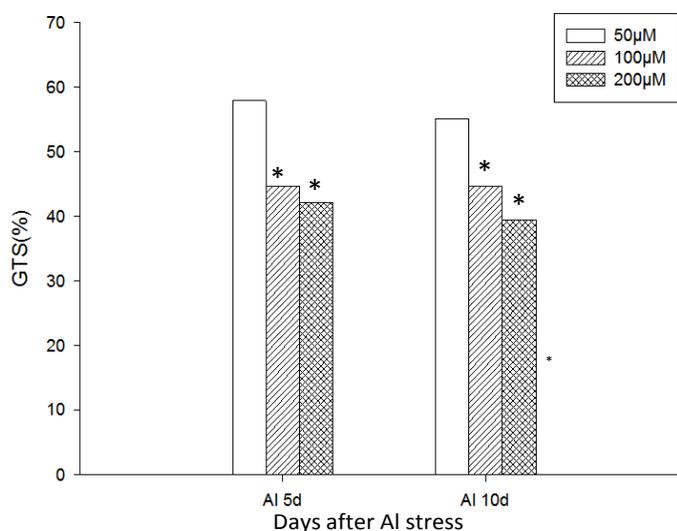
SRAP-PCR analysis was performed on each pool of genomic DNAs extracted from control and Al-treated roots. Each pool contained DNA from five plantlets to avoid intra-population genetic polymorphisms. In total, 72 10-mer priming oligonucleotides were used to analyze PCR products of which nine provided specific and stable data (Tab. 2). In all cases, the SRAP patterns generated by Al-exposed plantlets clearly differed to those obtained of control DNA. The results from 9 of the primers are shown in Tab. 2. The principal events observed following Al exposure were variations in the normal banding patterns in comparison to normal control seedlings. We observed 16, 21, 22, 17, 21, and 23 mutated loci amplified by only nine primers from 50 to 200 µmol L<sup>-1</sup> after 5 and 10 days, respectively. Al-treated plantlets were determined through the comparison of Al-treated SRAP fingerprints with controls (Tab. 2). DNA polymorphism values (SRPA strip increase or disappearance) of Al exposure after 5 days (GTS<sub>5</sub>) were 57.9%, 47.7% and 42.1%, respectively. The GTS<sub>10</sub> was 55.2%, 44.7% and 39.5%, respectively (Fig. 2). These results implied that the genomic stability of barley seedling root tip cells is

significantly weaker in increased Al concentrations. The simultaneous amplification of both the unaffected and affected regions of the barley genome confirmed the reliability and sensitivity of the SRAP technique.

**Table 2:** Changes in total bands and genomic template stability (GTS, %) in control (Con.) of barley root genome in different concentration of Al stress

Primers	Franklin						
	Con.	5d a/b*			10d a/b*		
		50	100	200	50	100	200
Me2-em1	3	1/1(33)	0/1(66)	1/0(66)	0/1(66)	2/0(33)	1/1(33)
Me7-em1	5	0/1(80)	1/3(20)	0/2(60)	1/2(40)	1/3(20)	1/3(20)
Me5-em4	7	0/4(43)	1/4(29)	0/4(43)	0/1(86)	1/4(29)	1/3(42)
Me1-em5	4	0/0(100)	0/2(50)	0/3(25)	2/1(75)	0/3(75)	1/2(75)
Me3-em5	3	2/1(0)	1/0(66)	1/1(33)	0/1(66)	1/1(33)	1/1(33)
Me2-em7	4	1/1(50)	2/1(25)	0/4(0)	2/2(0)	0/0(100)	0/2(50)
Me4-em7	3	0/0(100)	0/0(100)	1/0(66)	0/0(100)	1/0(66)	0/1(66)
Me6-em8	4	1/1(50)	1/2(25)	2/1(25)	1/2(25)	1/2(25)	1/2(25)
Me8-em6	5	0/2(60)	0/2(60)	0/2(60)	0/1(80)	0/1(80)	0/2(60)
Total average (GTS)	38(100)	16(57.9)	21(44.7)	22(42.1)	17(55.2)	21(44.7)	23(39.5)

Note: a indicates appearance of new bands, b indicates disappearance of normal bands. The value in () is GTS genome template stability.



**Figure 2:** Comparison of SRAP profile (GTS) in barley seedling root template genome stability exposed to 50, 100 and 200  $\mu\text{mol L}^{-1}$  Al for 5d and 10d under Al stress. Control values were considered as 100%. Asterisks indicate significant differences at  $P < 0.05$

#### 4 Discussion

The inhibition of Al stress is crucial to plant growth and development. However, the mechanisms through which Al ions lead to root cell damage remain controversial [24]. It has been proposed that Al

ions inhibit enzyme activity and DNA replication, inhibit cell transduction, lead to cell wall damage, block transportation processes at the cell surface, and disturb  $\text{Ca}^{2+}$  balance [25].

The normal metabolism of oxygen in chloroplasts and mitochondria generates ROS, which is accelerated by the heavy metal content in the soil, and other environmental stresses [19,26]. ROS accumulation leads to lipid peroxidation and increased MDA levels [19,23]. In this study, the activity of SOD, POD and CAT increased at low Al concentrations, but high Al concentrations for an extended period of time led to the inhibition of these enzymes. Plants that were protected produced a large number of metabolites to minimize heavy metal poisoning. However, excessive metabolic activity leads to toxicity. This study showed that the activity of SOD and CAT significantly increased when Al concentrations increased from 50 to 100  $\mu\text{M}$ . However, when the Al concentration increased to 200  $\mu\text{M}$ , SOD activity decreased. POD activity increased with increasing Al stress. These results suggest that in conditions of low Al, the major threat to the health of barley plants was  $\text{H}_2\text{O}_2$ , consistent with previous studies. However, the activity of SOD and CAT decreased at Al concentrations of 200  $\mu\text{M}$  and 50  $\mu\text{M}$ . This may have led to the collapse of protective enzyme function in barley leaves.

Under Al stress, single strand breaks in DNA occur, leading to base pair modifications and a loss of DNA repair. DNA damage leads to mutations in DNA, apoptosis induction, and a variety of toxic side-effects if insufficiently repaired. To date, RAPD has been used to detect the genetic toxicological effects of pollutant biomarkers [27]. However, RAPD technology is limited by low reproducibility and stability. SRAP is a new form of DNA fingerprinting based on PCR technology [28]. SRAP enabled the detection of ORFs permitting gene amplification in the absence of sequence information. SRAP is widely accepted due to its rapid and simple operation, low cost, good reliability, high reproducibility and ease of sequencing. SRAP has been successfully applied to plant genetic maps [18], and for the assessment of crop genetic diversity [29]. SRAP can detect DNA damage and mutations in response to environmental pollutants, characterized by DNA fragmentation. We assessed genome polymorphisms at the DNA level using SRAP, in which a series of random primers were used for amplification, allowing detection of the entire genome. In this study, the barley root genome SRAP bands were amplified under Al stress conditions and clear differences in the response to Al treatment were observed (Tab. 2). This might be related to DNA crosslinking (reduction in hyperchromic effect) under Al stress. Thus the priming sites of some oligonucleotides have a high affinity for primers, so the primers cannot bind to primer binding sites. This is the reason for the appearance of new SRAP bands. The SRAP marker is amplified by open reading frames (ORFs) using specifically designed primers and is very polymorphic. Thus it better reflects the changes in genes in response to Al toxicity. GTS dramatically declined under Al stress conditions. The differential band patterns in response to Al stress were likely due to genome rearrangements, point mutations, double chain fractures, DNA insertions/deletions, DNA-protein crosslinking, methylation, and other forms of DNA damage. In comparison with the molecular level of plants affected by heavy metals as reported in previous studies [9, 10, 19], the polymorphic changes were therefore more significant and reliable, and DNA polymorphisms therefore act as biomarkers for the detection of Al pollution in plants.

In summary, the physiological and biochemical changes in barley seedlings under Al stress correlated with the SRAP profiles. This was the first study to employ SRAP for the assessment of DNA damage in barley roots under Al stress. SRAP could detect plant damage more rapidly than other physiological and biochemical indicators including germination rates, chlorophyll content, and enzyme activity. SRAP in combination with plant physiology thus provides a rapid and reliable method to analyze DNA damage in response to environmental stress. These methods represent an effective tool to detect genetic toxicity in response to Al pollution. This enhances our understanding of the plant defense mechanisms employed by barley in response to Al toxicity.

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