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Research Article

Enhancement of Cold Tolerance Promotes Resistance to Aluminum Stress

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Abstract

Aluminum (Al) toxicity is one of the major factors that limit crop productivityin acid soils. Soil acidification enhances the release of Al^{3+}, which enters into root tip and prevents root growth. The improvement of Al resistance is important for increasing crop productivity. In this study, we determined that Al levels were decreased during cold acclimation. According to neutron activation analysis, the levels of ^{24}Na, ^{38}Cl, ^{42}K, ^{49}Ca, and ^{56}Mn were temporally decreased, and the levels were restored during cold acclimation. In contrast, ^{28}Al levels were decreased despite acclimation to cold stress. Furthermore, enhancement of cold tolerance by overexpression of *ICE1* in *Arabidopsis* and tomatoes improved Al resistance. These results suggest a relationship between cold tolerance and Al resistance.

INTRODUCTION

Environmental stresses such as cold, drought, and salinity restrict plant growth and crop yield. Thus, plants are required to adapt to such stresses for survival. Cold stress is one of the key factors that influences plant development and limits the geographical distribution of cold-sensitive plant species. Plants have evolved a mechanism called cold acclimation to enhance tolerance to freezing stress [1]. The molecular mechanismsinvolved in cold acclimation have been extensively investigated, and several molecules have been identifiedthat are important for cold tolerance [2].

Among several cold-signaling pathways, the ICE1-CBF/DREB1-dependent cold-signaling pathway appears to be the most important pathway for the control of cold-regulated genes and cold tolerance [3]. ICE1, a MYC-type basic helix-loop-helix (bHLH) transcription factor that binds to the MYC-recognition *cis*-elements (CANNTG) in the promoter of *CBF3/DREB1A*, is a positive regulator of cold signaling [4]. According to microarray data, *ICE1* regulates approximately 40% of cold-regulated (COR) genes and 46% of cold-regulated transcription factor genes [5]. ICE1 is likely to be an important regulator that controls *CBF3/DREB1A* and many *COR* genes. Overexpression of *ICE1* improves

tolerance to cold stress in *Arabidopsis* [4, 6], tomatoes [7, 8], and cucumber [9].

Aluminum (Al) is the most abundant metal in the earth's crust and constitutes approximately 8% by weight [10]. Because many plant species are sensitive to micromolar concentrations of Al ions, and low pH enhances the solubilization of Al, Al ion is toxic to plants and is a major factor in theinhibition of plant growth and crop production in acid soils [11]. Soil acidification occurs naturally when basic cations such as calcium, magnesium, and potassium are leached from soils. It is also accelerated by nitrogenous fertilizer and acid rain. More than 30% of the world's land is affected by acid soils, meaning approximately half of the potential arable lands is affected [12]. Al inhibits root growth and uptake of water and nutrients, resulting in the retardation of plant growth and the loss of crop production [11]. The root apex is the most sensitive zone, especially the distal transition zone, to Al stress [13,14]. In this zone, a region 1-3 mm behind the root tip, a transition occurs from cell division to cell elongation. The expression of *SbMATE*, which encodes for an Al-activated root citrate transporter for the extrusion of toxic compounds in sorghum [15], was specifically observed in the epidermal and outer cortical cell layers of the distal transition zone[14]. Al toxicitycauses damage to the membrane due to negative charges and the irreversible binding to Al, together with rigidification of the cell wall due to displacement of Ca^{2+} with Al^{3+} [16]. And a quick burst of mitochondrial ROS was also triggered by treatment with Al in *Arabidopsis* [17].

Al-resistant plants have evolved mechanisms for their detoxification. For example, plants useorganic acidsto expel Al from the root apex or detoxifyAl by chelating it with organic acids [18]. Exudation of organic acid anions, such as citrate, oxalate, and malate, enhances Al tolerance by forming stable complexes with Al [19]. These organic acid anions also function in chelating Al in the cytosol. Thus, overexpression of citrate synthase in tobacco, canola, and alfalfaand an increase in malate synthesis in tobacco and alfalfa enhanced Al tolerance [20-24]. Phosphorus is also reported to alleviate Al toxicity in *Citrus* by increasing immobilization of Al in roots [25]. MATE (multidrug and toxic compound extrusion), responsible for citrate exudation, was activated by Al in the Al-resistance barley cultivar [26]. Genes for antioxidant defense mechanisms, such as superoxide dismutase (SOD) andperoxidase, were upregulated by Al treatment in *Arabidopsis* [27]. Overexpression of the mitochondrial *SOD* gene conferred *Brassica napus* resistance to Al [28].

Here, we introduce a new molecular strategy to increase Al resistance. According to the neutron activation analysis, several elements were transiently decreased after cold shock, but the levels were mostly restored during cold acclimation. However, the levels of Al decreased despite the fact thatthe plants acclimated to the cold stress. Furthermore, the resistance to Al stress was enhanced in *ICE1*-overexpressing *Arabidopsis* or tomato plants, which showed cold tolerance [8,29]. These results suggest a relationship between cold tolerance and Al resistance.

MATERIALS AND METHODS

Plant Culture and Treatments

Wild-type and *AtICE1*-overexpressing *Arabidopsis thaliana* plants used were in the Col-0 background [6, 29]. Seeds were surface-sterilized and germinated on basal media containing a halfMurashige and Skoog (MS) mineral salts, 1% sucrose, and 0.8% agar with pH 4.5. Petri dishes were vertically positioned and maintained for 3 days. Then, the seedlings were transferred onto the media containing 50 μM or 100 μM $AlCl_3$ and grown for an additional 6 days. Root growth was measured as previously described [41].

The tomato (*Solanum lycopersicum*) cultivar Micro-Tom and the *SlICE1*-overexpressing tomatoes [7,8] were used for the treatment of aluminum stresses. These tomatoes were grown for 3 weeks on rockwool with 1/10 Hoagland's solution. Then, 1/10 Hoagland's solution with 0, 50, or 100 μM $AlCl_3$ was provided and tomatoes were grown for an additional 3 weeks. The weight of each tomato shoot was subsequently measured.

Measurement of Elemental Profiles

Wild-type *Arabidopsis* (ecotype Col-0) plants were grown at 23°C for 3 weeks and were incubated at 4°C for the indicated days. The relative amount of selected elements contained in each sample was measured by neutron activation analysis performed at JRR-3 located at the Japan Atomic Energy Agency. The samples were dried at 70°C for 2 days and then doubly sealed in a polyethylene vinyl bag. After irradiation for 10 seconds for ^{24}Na, ^{28}Al, ^{38}Cl, ^{42}K, ^{49}Ca and^{56}Mn in the research reactor with a thermal neutron flux of 6.0 x 10^{13} n cm^{-2} s^{-1}, gamma-rays emitted from samples were measured by a Ge(Li) detector. The gamma-rays used to determine ^{24}Na, ^{28}Al, ^{38}Cl, ^{42}K, ^{49}Ca and^{56}Mn were 1.369, 1.779, 2.168, 1.525, 3.084 and 1.810 MeV, respectively.

Measurement of H_2O_2 Concentration and Peroxidase Activity

Tomato plants were grown for 2 weeks on rockwool with 1/5 Hoagland's solution. Then, the plants were incubated with 1/5 Hoagland's solution with or without 50 μM $AlCl_3$ for 3 weeks. Leaf samples were harvested with 4 replicates for each treatment. The concentration of H_2O_2 in the tissue was determined by the following method as described [30] with slightly modification. Briefly, 0.1 g of leaf tissue was grinded in liquid nitrogen and 1 mL of cold acetone and 0.5 mL of cold water was added. 0.1 mL of 5% (w/v) titanium sulfate solved with 25% H_2SO_4 and 0.2 mL of ammonium hydroxide solution was added to precipitate the peroxide-titanium complex. The precipitate was collected by centrifugation at 21,500xg for 10 min. After washing with cold acetone, the precipitate was dissolved with 1.6 mL of 1M H_2SO_4. The absorbance at 420 nm was measured with a spectrophotometer (DU800, Beckman, USA). A standard curve was prepared with the several concentration of H_2O_2 solution.

Measurement of peroxidase activity was performed by the following procedure (http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-peroxidase.html). Briefly, after grinding 0.1 g of tomato leaves, 1 mL of 100 mM phosphate buffer (pH 6.8) was added. After centrifugation at 21,500xg for 15 min, supernatant was added to reaction mixture. One minute after reaction, sulfuric acid was added to stop reaction. The enzyme unit was calculated according to the procedure.

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated from tomato leaves. cDNA synthesis, real-time PCR, and comparative C_T analyses were performed as described [31] with gene-specific primers for *UBI3* [8], *SlPOD1*(5'-ATTGTCCACGTAGTGGAGGTGATTCC-3' and 5'-TCCTCCACTAAATAGTGCTTGATCAG-3').

RESULTS AND DISCUSSION

Decrease of Al content during cold acclimation

To investigate what types of minerals are altered during cold acclimation, the relative amount of several elements was measured twice by neutron activation analysis and representative one was shown in Table 1. According to these data, the concentration of ^{24}Na, ^{38}Cl, ^{42}K, ^{49}Ca, and ^{56}Mn showeda decrease on day 1 followed by the levels of these elements increasingon day 3. This temporal decrease may be due to a cold stress response, and the restoration of these elements is likely correlated with cold acclimation. Alternatively, ^{28}Al showed a specific profile during cold treatment. The level of ^{28}Al decreased even though cold acclimation occurred. These results suggest that cold acclimation decreases the Al concentration.

Overexpression of *ICE1* increased Al resistance

According to the results (Table 1), it is assumed that enhancement of cold tolerance increases Al resistance. Thus, *ICE1* overexpressing *Arabidopsis* [6] and tomato plants [8] were subjected to Al stress. The root growth of wild-type seedlings was inhibited by approximately 50% when the seedlings were treated with 100 μM $AlCl_3$ for 6 days, but onlyapproximately 20% of the root growth of *AtICE1*-overexpressing seedlings was inhibited (Figure 1). No significant growth change was observed in *AtICE1*-overexpressing seedlings treated with 50μM $AlCl_3$, compared to *AtICE1*-overexpressing seedlings without aluminum treatment (Figure 1C).

According to the microarray data (http://bar.utoronto.ca/), *MATE* (At1g51340) and several *MATE*-like genes were upregulated in roots by treatment with cold stress (Figure 1D).*MATE* encodes multidrug and toxic compound extrusion, which is Al-induced citrate transporter [32]. The expression of *MATE* facilitated the Al-activated efflux of citrate and enhanced tolerance to Al stress [32-35]. Up-regulation of these genes by cold stress may be involved in decrease in Al content. Expression of *SOS1*, which encodes Na^+ transporter mediating Na^+ efflux [36], was not changed after cold treatment, but *HKT1*, which is involved in uptake of Na^+ [37], was down-regulated by cold stress (Figure 1D). Decrease in influx and unchanged efflux may not affect content of Na.

SlICE1-overexpressing tomato plants also showed Al resistance (Figure 1E-H). Three-week-old tomato plants were treated with 0, 50, or 100 μM $AlCl_3$ for 3 weeks. Following the Al treatment with 100 μM $AlCl_3$, the leaves of wild-type plants appeared yellow (Figure 1G), and growth retardation was observed (Figure 1H). Alternatively, transgenic tomato plants had healthy leaves even though they were treated with 100 μM $AlCl_3$. The fresh weight of shoots in the transgenic plants was not altered with or without $AlCl_3$ treatment (Figure 1H). Together, these results suggest that an increase in cold tolerance by overexpression of *ICE1* enhanced the resistance to Al stress.

Table 1: Elemental profiles during cold treatment in *Aradisopsis* measured by neutron activation analysis.

	Day 0	Day 1	Day 2	Day 3
Na-24	1182.3	911.1	1237.8	1737.2
Al-28	740	406.6	275.8	169.9
Cl-38	1731.1	1532	1505.8	2144.5
K-42	873	539.8	546	669
Ca-49	3381.8	2223.1	2403.1	2920.2
Mn-56	1253.9	887.1	889.5	955.9

Overexpression of *SlICE1* decreases accumulation of H_2O_2 and enhances peroxidase activity

Generally, cold stress decreases the rate of metabolism, leading to the delay of energy dissipation and the introduction of oxidative damage [2]. To acclimate to cold stress, the synthesis of cryoprotective molecules is enhanced in plants. These compounds function in stabilizingthe membrane, protecting from dehydration, and scavenging reactive oxygen species (ROS). The level of H_2O_2 was also increased after treatment of aluminum stress in wild-type tomato leaves (Figure 2A). On the other hand, the level of H_2O_2 in *SlICE1*-overexpressing tomato leaves was not altered with or without aluminum stress (Figure 2A). Because the antioxidant activity in *SlICE1*-overexpressing plants is increased [7], the level of H_2O_2 was lower than that in wild type (Figure 2A).

The activity of antioxidant enzymes, including superoxide dismutase, catalase, glutathione peroxidase, and ascorbate peroxidase, and the accumulation of non-enzymatic antioxidants, such as ascorbic acid, glutathione, and carotenoids, are enhanced by abiotic stresses[38]. Overexpression of *SlICE1* enhances antioxidant activity and increases the accumulation of antioxidants, including ascorbic acid, β-carotene, and lycopene [7]. In addition, overexpression of *SlICE1* also enhanced peroxidase activity without aluminum stress (Figure 2B). The activity was increased after treatment of aluminum in wild type, but the level was not altered in *SlICE1*-overexpressing plants (Figure 2B). The increase in antioxidant activity and peroxidase activity may inhibit accumulation of ROS under aluminum stress. The mRNA expression of the aluminum-inducible gene *SlPOD1* (peroxidase, Solyc10g076240) was up-regulated by aluminum stress in wild-type tomato leaves and the level of gene was higher in *SlICE1*-overexpressing plants without aluminum treatment (Figure 2C). Proteomics revealed that the gene product was up-regulated by aluminum [39]. The mRNA expression of *SlPOD1* (Figure 2C) and peroxidase activity (Figure 2B) were similar, suggesting that peroxidase is one of factors to ROS.

Al stress causes an increase in the production of ROS (Figure 2A), causing peroxidation of membrane lipids. Detoxification of Al-induced ROS through antioxidant defense mechanisms, such as chelation by organic acid anions and amino acids, is one way to prevent Al-induced damage in cells and tissues [40,41]. High antioxidant status confers Al resistance to plants [42,43]. Taken together, the enhancement of antioxidant activity by cold stress may decrease Al levels.Overexpression of *ICE1* enhances the

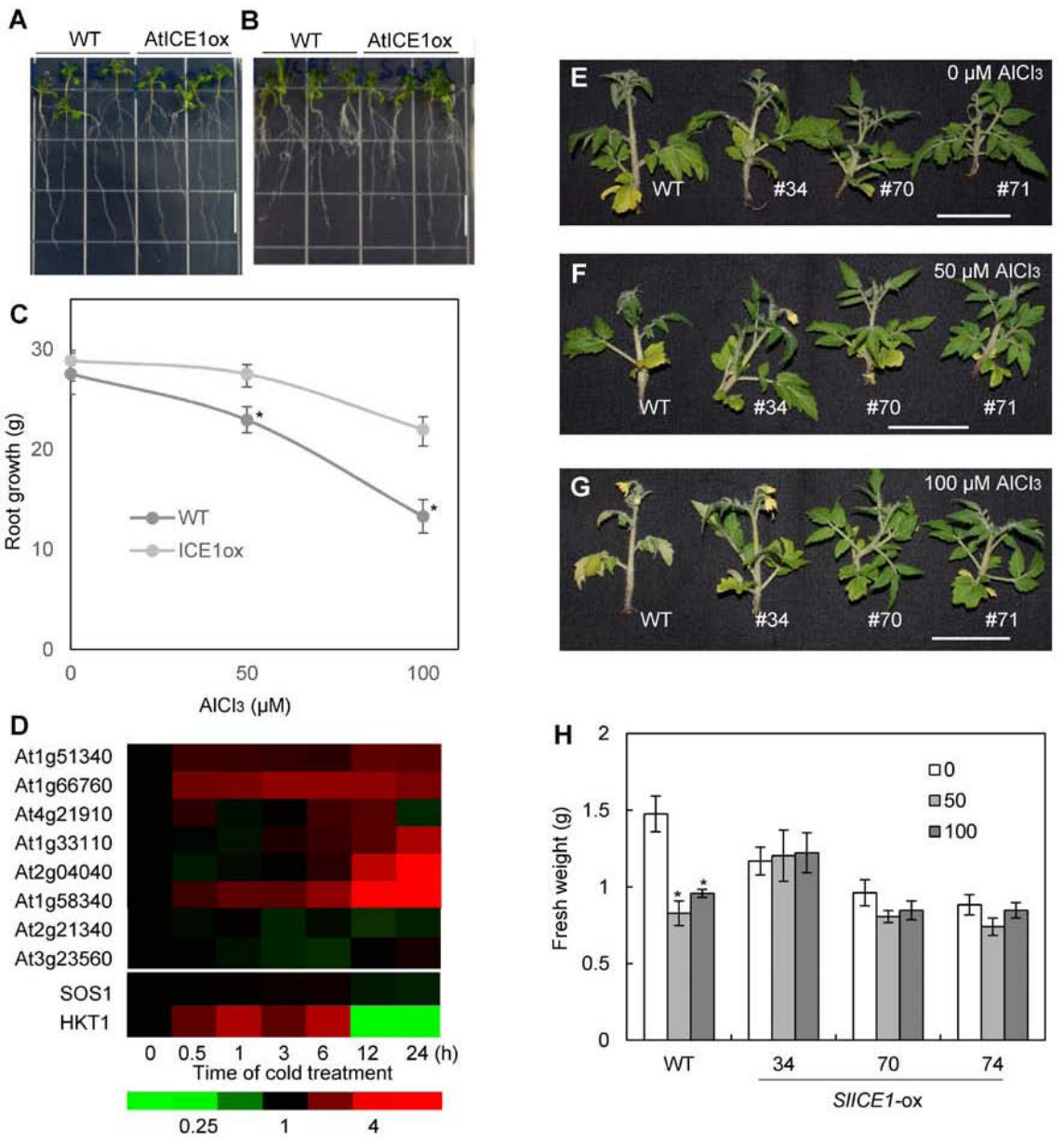

Figure 1 *ICE1* overexpression enhances tolerance to aluminum stress. Wild-type and *AtICE1*-overexpressed seedlings were grown on MS plates in the absence (A) or the presence (B) of 100 μM $AlCl_3$. (C) Root growth of wild-type and *AtICE1* overexpression seedlings grown on media with or without Al^{3+}. Data are the mean ± SE (n = 12). The asterisk shows a significant difference in root growth between wild-type and *AtICE*-overexpressed seedlings at $P < 0.05$ by Student's t-test.(D) Gene expression of *MATE* (At1g51340), *MATE-like*, *SOS1* (At2g01980), and *HKT1* (At4g10310) genes. Data was obtained from the microarray data (http://bar.utoronto.ca/). (E-G) Wild-type and *SlICE1*-overexpressing tomato plants were grown with Hoagland's solution without (E), with 50 μM$AlCl_3$(F), or with 100 μM $AlCl_3$ (G). (H) The fresh weight of shoots was measured. Data are the mean ± SE (n = 12). The asterisk shows a significant effect of Al at $P < 0.05$ by Student's t-test.

accumulation of antioxidant compounds, most likely leading to Al resistance.

CONCLUSIONS

Cold acclimation showedthedecrease Al levels (Table 1). Furthermore, overexpression of *ICE1* enhanced Al resistance in *Arabidopsis* (Figure 1) and tomato plants (Figure 2). These results suggest that enhancement of cold tolerance is correlated with an improvement in Al resistance. Because Al resistance results fromincreasing antioxidant activity, and antioxidant activity is enhanced by cold stress, antioxidant activity may be involved in the enhancement of Al resistance in *ICE1*-overexpressing *Arabidopsis* and tomatoes.

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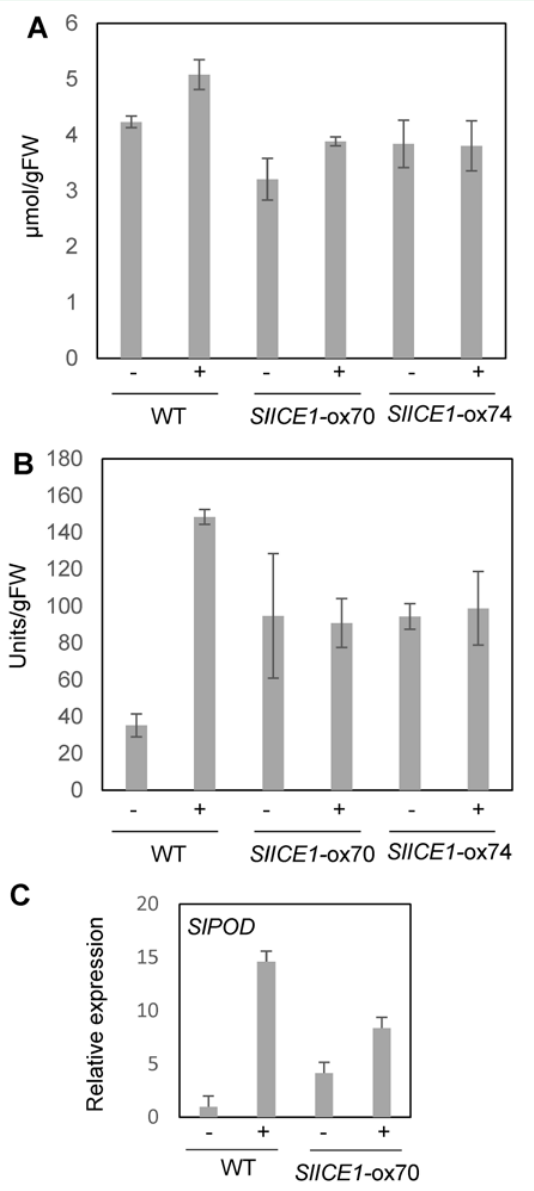

Figure 2 Overexpression of *SlICE1* exhibits lower H_2O_2 accumulation after treatment of aluminum stress (A) and higher peroxidase activity without aluminum treatment (B). Wild-type and *SlICE1* over expressing tomato plants were grown for 3 weeks with Hoagland's solution with 0 (-) or 50 (+) µM $AlCl_3$. The concentration of H_2O_2 (A) and the peroxidase activity (B) in leaf tissues was measured. Data are means ± SE (n = 4). (C) Total RNA was extracted from tomato leaves with 0 (-) or 50 (+) µM $AlCl_3$. Relative mRNA levels of *SlRAF*(rapid alkalization factor 1; Solyc09g074890.1.1) and *SlXTH9* (xyloglucan endotransglucosylase /hydrolase 9; Solyc11g066270.1.1) were determined by quantitative RT-PCR analyses. Data are means ± SD (n = 3).

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