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Research article

The interaction of salicylic acid and Ca^{2+} alleviates aluminum toxicity in soybean (*Glycine max* L.)

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ABSTRACT

Both calcium ion (Ca^{2+}) and salicylic acid (SA) influence various stress responses in plants. In acidic soils, aluminum (Al) toxicity adversely affects crop yield. In this study, we determined the influences of Ca^{2+} and SA on root elongation, Al accumulation, and citrate secretion in soybean plant. We also investigated the activity of antioxidative enzymes in Al-exposed soybean roots. Root elongation was severally inhibited when the roots were exposed to 30 μM Al. The Al-induced inhibition of root elongation was ameliorated by Ca^{2+} and SA but aggravated by Ca^{2+} channel inhibitor (VP), CaM antagonists (TFP), Ca^{2+} chelator (EGTA), and SA biosynthesis inhibitor (PAC). Furthermore, 1.0 mM $CaCl_2$ and 10 μM SA reduced the accumulation of Al in roots, but their inhibitors stimulated the accumulation of Al in roots. Citrate secretion from these roots increased with the addition of either 1.0 mM $CaCl_2$ or 10 μM SA but did not increase significantly when treated with higher Ca^{2+} concentration. Enzymatic analysis showed that Ca^{2+} and SA stimulated the activities of superoxidase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) in Al-treated roots. In addition, SA restored the inhibition of Ca^{2+} inhibitors on root elongation and Al content. Thus, both Ca^{2+} and SA contribute to Al tolerance in soybean. Furthermore, Ca^{2+} supplements rapidly increased Al-induced accumulation of free-SA or conjugated SA (SAG), while Ca^{2+} inhibitors delayed the accumulation of SA for more than 8 h. Within 4 h of treatment, SA increased cytosolic Ca^{2+} concentration in Al-treated roots, and upregulated the expression of four genes that possibly encode calmodulin-like (CML) proteins. These findings indicate that SA is involved in Ca^{2+}-mediated signal transduction pathways in Al tolerance.

1. Introduction

Aluminum (Al) is the most abundant metal in the earth's crust. Al^{3+} ions formed under acidic condition are highly toxic on plant roots and reduce the yield of crops (Kochian, 1995; Ma et al., 2001). Plants combat Al toxicity by external exclusion and internal detoxification (Kochian, 1995). Several signaling molecules, including Ca^{2+}, abscisic acid (ABA), and SA are shown to alleviate Al toxicity (Liu et al., 2012).

As a secondary messenger, intracellular Ca^{2+} can directly or indirectly modulate various biological processes, including cellular response to abiotic stress. Moreover, Ca^{2+} can effectively resist cascade reactions (Reddy, 2001). In plants, the assimilation and transportation of Ca^{2+} depends on the interaction between Ca^{2+} and Al^{3+}, which in turn may lead to Al-induced injury (Jones et al., 1998). Al influences intracellular Ca^{2+} signal transduction by increasing the concentration of cytoplasmic free Ca^{2+} in wheat (Zhang and Rengel, 1999). Hossain et al. (2005) found that even in the presence of toxic Al levels that inhibit root elongation, an enhanced Ca^{2+} supplement elicited the normal synthesis of hemicellulose polysaccharides and phenolic compounds. Furthermore, previous studies have reported that Ca^{2+} can induce citrate secretion in plants. For example, in the presence of Al^{3+}, citrate concentration in soybean roots increased when Ca^{2+} were added to the plant roots (Silva et al., 2001). During an intracellular Ca^{2+} signal transduction, Ca^{2+} is bound to calcium-binding proteins. Ca^{2+} signals can be detected by Ca^{2+} sensor proteins, such as calmodulin (CaM) or CaM-like proteins (CMLs) (Bender et al., 2014).

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CMLs, such as CML37, CML38 and CML39, probably act as sensors in Ca^{2+}-mediated developmental pathways, which are associated with stress response in *Arabidopsis* (Vanderbeld and Snedden, 2007). In blueberry leaves, the CaM gene *VcCaM1* regulates Ca^{2+} homeostasis and antioxidant systems, improving plant performance under toxic Al levels (Inostroza-Blancheteau et al., 2013). Although the properties and physiological functions of CaM are reasonably known in plants, the physiological roles of most CMLs are still largely unknown (Dobney et al., 2009).

SA is an endogenous signaling molecule that modulates diverse processes in plants subjected to both biotic and abiotic stresses (Shah and Klessing, 1999). Emerging evidence has shown that SA plays an important role in Al tolerance. For example, SA reduces Al uptake in soybean roots under Al stress and suppresses Al-induced inhibition of root elongation (Liu et al., 2012). Kawano et al. (1998) discovered that when a suspension of tobacco cells is treated with SA, the concentration of superoxide anions increases in a rapid and transient manner. Consequently, there is an increase in the cytosolic concentrations of Ca^{2+}. Interestingly, simultaneous addition of SA and Ca^{2+} reduces the NaCl-induced oxidative stress in wheat more effectively by preventing the accumulation of malondialdehyde (MDA) (Al-Whaibi et al., 2012). Raz and Fluhr (1992) reported that in the presence of an exogenous Ca^{2+}, SA could induce chitinase formation in tobacco leaves that lack CaM. These facts suggest that both SA and Ca^{2+} are involved in mitigating Al toxicity, and the effect of SA and Ca^{2+} on Al toxicity vary with plant species and experimental conditions. Thus, it is necessary to further investigate the interaction between Ca^{2+}- and SA-mediated responses to Al and their mechanisms in Al tolerance.

Various agronomic and/or genetic interventions have been applied to overcome negative effects of Al^{3+} that inhibit soybean production. Soybean is an acid-soil-sensitive plant (Marschner, 1995). To better understand the relationship between SA and Ca^{2+} under Al stress, in the present study, we investigated the interaction between Ca^{2+} and SA in Al tolerance in soybean with a number of physiological and molecular indicators.

2. Materials and methods

2.1. Soybean cultivation

Seeds of soybean (cv. Jiyu 70) were germinated in peat moss within 3 days in darkness. After germination, soybean seedlings were transferred into 0.5 mM $CaCl_2$ solution (pH 4.5) for 24 h and used for evaluation of Al toxicity by measuring root elongation. The seedlings used for other experiments were cultured in nutrient solution described by Horst et al. (1992) (pH 4.5) with following composition (in μM): KNO_3, 750; $Ca(NO_3)_2$, 250; $MgSO_4$, 325; KH_2PO_4, 10; Fe-EDTA, 20; H_3BO_3, 8; $CuSO_4$, 0.2; $ZnSO_4$, 0.2; $MnCl_2$, 0.2; $(NH_4)_6Mo_7O_{24}$, 0.2. The pH of solution was adjusted to 4.5 with 0.1 M HCl and replaced on each other day. All the experiments were performed in a controlled growth chamber in which a 14 h/10 h light/dark period was maintained under a light intensity of 500 μM photons^{-1} m^{-2}; the temperature of the chamber was maintained between 22 and 25 °C, while its relative humidity was kept intact at 70%.

2.2. Measurement of root elongation

Four-day seedlings were transferred into 0.5 mM $CaCl_2$ solution (pH 4.5) overnight. The root length of seedlings was measured before performing the experiment. Twelve hours after conducting various treatments, the root length of seedlings were used a scale to measure again and evaluate the relative elongation.

2.3. Measurement of Al content

After germination, the seven-day-old roots were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing the following reagents for 12 h: 30 μM $AlCl_3$, 10 μM SA, 1.0 mM $CaCl_2$, 100 μM verapamil (VP), 100 μM trifluoperazine (TFP), 0.5 mM ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 100 μM paclobutrazol (PAC). The root tips were washed thrice with deionized water and excised (0–1 cm). Al was extracted from the root tips by soaking them in 1 ml of 2 M HCl for 48 h. The Al content was determined with an atomic absorption spectrophotometer equipped with a graphite furnace atomizer (PerkinElmer Analyst 700, Norwalk, CT, USA).

2.4. Collection of root exudates and measurement of citrate secretion from soybean

Fourteen-day-old seedlings were cultured in 0.5 mM $CaCl_2$ solution (pH 4.5) for 12 h in the dark. Then the seedlings were exposed to $AlCl_3$ (0, 30 μM) with $CaCl_2$ (0, 1.0 mM), SA (0, 10 μM), VP (0, 100 μM), TFP (0, 100 μM), EGTA (0, 0.5 mM), PAC (0, 100 μM) in 0.5 mM $CaCl_2$ solution (pH 4.5) for 12 h. The root exudates were collected at 3 intervals within 12 h. Citrate in these exudates was detected by high performance liquid chromatography (HPLC) (LC20 AT, Shimadzu, Tokyo, Japan) that was equipped with a reverse-phase C18 column (VP-ODS, 150 mm × 4.6 mm) and a UV detector (210 nm).

2.5. Measurement of free-SA and conjugated- SA (SAG) content in soybean roots

Seven-day-old seedlings were transferred to 0.5 mM $CaCl_2$ solution (pH 4.5) for 12 h in the dark. Then the seedlings were subjected to $AlCl_3$ (0, 30 μM), $CaCl_2$ (0, 1.0 mM), VP (0, 100 μM), TFP (0, 100 μM) and EGTA (0, 0.5 mM) in 0.5 mM $CaCl_2$ solution (pH 4.5) for 12 h. Root tips (0–3 cm) were excised, frozen immediately in liquid nitrogen, and stored at −80 °C for free-SA and SAG determination. The content of SA were measured according to Zhang et al. (2004) with minor modifications.

1.0 g of the roots was ground with 4 ml of 5% trichloroacetic acid (TCA) and 16 ml of double distilled H_2O. The homogenate was dissolved in 30 ml of diethyl ether and transferred into a 50 ml of centrifugation tube. After shaking and extracting for 12 h, the homogenate was centrifuged at 10,000 *g* for 5 min at 4 °C. The supernatant was evaporated to dryness on a rotary evaporator, and then the residue was dissolved in 1.0 ml of 50:50 methanol-acetic acid buffer solution (pH 3.2) for performing free-SA analysis.

Conjugated SA (SAG) content was indirectly determined by acid hydrolysis. 18.5% HCl was added to the aqueous phase, which was obtained by extracting with diethyl ether; the final concentration of HCl was 3.2%. The samples were incubated in a water bath at 80 °C for 1 h. After cooling, three rounds of extraction were performed using ethyl ether. The ethyl-ether phase supernatants were merged and condensed by rotary evaporation at 40 °C. One milliliter of methanol-acetic acid solution (1:1, v/v; pH 3.2) was added to a 50 ml rotary evaporator flask. Then the dissolved fraction containing SAG was transferred to 1.5-ml Eppendorf tubes. Quantitative analysis of free-SA and SAG were performed by HPLC (LC20 AT, Shimadzu, Tokyo, Japan) that was equipped with a reverse-phase C18 column (VP-ODS; 150 mm × 4.6 mm) and a fluorescence detector (excitation wavelength 310 nm; emission wavelength 415 nm). The samples were filtered through 0.3 μM microporous filters and injected into the HPLC column.