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Endogenous salicylic acid is required for promoting cadmium tolerance of *Arabidopsis* by modulating glutathione metabolisms

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HIGHLIGHTS

- The role of endogenous SA in mediating Cd tolerance was explored using *sid2* mutants.
- Cd stress induces SA accumulation in a SID2 dependent way.
- Depletion of SA causes negative effects on Cd tolerance.
- Endogenous SA is required for promoting Cd tolerance by modulating GSH metabolism.
- Possible mode of SA signaling through GR/GSH pathway under Cd toxicity was discussed.

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ABSTRACT

A few studies with *NahG* transgenic lines of *Arabidopsis* show that depletion of SA enhances cadmium (Cd) tolerance. However, it remains some uncertainties that the defence signaling may be a result of catechol accumulation in *NahG* transgenic lines but not SA deficiency. Here, we conducted a set of hydroponic assays with another SA-deficient mutant *sid2* to examine the endogenous roles of SA in Cd tolerance, especially focusing on the glutathione (GSH) cycling. Our results showed that reduced SA resulted in negative effects on Cd tolerance, including decreased Fe uptake and chlorophyll concentration, aggravation of oxidative damage and growth inhibition. Cd exposure significantly increased SA concentration in wild-type leaves, but did not affect it in *sid2* mutants. Depletion of SA did not disturb the Cd uptake in either roots or shoots. The reduced Cd tolerance in *sid2* mutants is due to the lowered GSH status, which is associated with the decreased expression of serine acetyltransferase along with a decline in contents of non-protein thiols, phytochelatins, and the lowered transcription and activities of glutathione reductase1 (GR1) which reduced GSH regeneration. Finally, the possible mode of SA signaling through the GR/GSH pathway during Cd exposure is discussed.

1. Introduction

Anthropogenic pollution by cadmium (Cd) poses a serious threat to human and environmental health. Due to the high affinity for sulfhydryl groups in proteins, Cd interfering with various physiological processes such as photosynthesis, respiration and transpiration. Plants exposed to toxic concentrations of Cd display visible symptoms of growth inhibition, chlorosis, and finally death [1].

In plants, the primary strategy in response to Cd toxicity is sequestration of Cd into less sensitive organelles of cells (e.g. vacuoles) [2]. Glutathione (γ-Glu-Cys-Gly, GSH) is a key molecular compound for Cd chelation by itself or as a basic component for phytochelatins (PCs). It is also a powerful reductant, acting to protect plant cells against Cd-induced oxidative damage [2]. Due to the high demand for detoxification, Cd exposure rapidly depletes GSH and subsequently induces transcripts of GSH biosynthesis enzymes,

Abbreviations: Cd, cadmium; CAT, catalase; ICS, isochorismate synthase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSH1, γ-glutamylcysteine synthetase; GSH2, glutathione synthetase; GSSG, oxidized glutathione; MDA, malondialdehyde; NPT, total non-protein thiols; PCs, phytochelatins; POD, peroxidase; SA, salicylic acid; SAT, serine acetyltransferase; SOD, superoxide dismutase; TRX, thioredoxin; *wt*, wild-type plants.

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including serine acetyltransferase (SAT), γ-glutamylcysteine synthetase (GSH1) and glutathione synthetase (GSH2). SAT catalyzes the formation of O-acetyl-Ser and leads to synthesis of cysteine [3]. GSH1 catalyzes the formation of a peptide bond between L-Glu and L-Cys at the first stage, and then Gly is added to the dipeptide under the regulation of GSH2 [2]. Furthermore, GSH metabolic products are also involved in regeneration and degradation enzymes, such as glutathione reductase (GR) and glutathione peroxidase (GPX) respectively [4].

Recently, the regeneration and biosynthesis of GSH were identified as the link with salicylic acid (SA), an endogenous signal molecular with broad function in plants [5,6]. It has been found that GR1, the enzyme responsible for regeneration and maintaining GSH in its reduced state, is signaled through SA pathways [7]. Enhancement of free SA levels in *Arabidopsis* leaves stimulated the specific activity of SATs, leading to elevated GSH biosynthesis and increased Ni tolerance [8]. However, the mechanism of SA in mediating Cd tolerance through modulation of GSH metabolism remains poorly understood.

Whilst a few studies have reported that preliminary treatment with SA mitigates the Cd toxicity in many plant species, including rice [9–11], barley [12], melon [13], wheat [14], the mechanisms still need to be further elucidated. Pretreatment with SA by spraying and presoaking may influence the endogenous SA level and consequent response of signal transduction. However, this exogenous effect is concentration dependent because the basal levels of SA differ widely among plant species. For example, the total free SA in *Arabidopsis* is 0.25–1.0 $\mu g\,g^{-1}$ fresh weight, whereas potato might contain up to 10 $\mu g\,g^{-1}$ fresh weight [6]. When SA application exceeds the appropriate range, it may result in oxidative toxicity to plants. According to Rao et al. [15], high levels of SA act as a prooxidant because SA is able to oxidize proteins in *Arabidopsis* leaves and multiplies H_2O_2 generation in plants. Elsewhere, it has been shown that excessive SA pretreatment aggravates Cd-induced oxidative damage in perennial ryegrass [16] and soybean [17].

Comparison of wild-type plants with SA-deficient mutants, e.g. *NahG* and *sid2*, is an effective way to evaluate the endogenous role of SA in response to Cd toxicity. Surprisingly, although the two mutants have SA deficiency in common, *NahG* and *sid2* showed conflicting results when exposed to abiotic stress. The *NahG* transgenic lines, encoding a bacterial SA-decomposing salicylate hydroxylase, manifested higher tolerance than the wild-type plants to chilling [18], salt stress [19,20], and Cd toxicity [21,22]. By contrast, *sid2* mutants with a mutation in the gene encoding isochorismate synthase (ICS) are more sensitive to drought [23] and salt stress [24]. The study reported here (Table 2) with other recent studies [20,25] showed that the *NahG* genotype is not suitable for evaluating SA function because the defence signaling results from catechol accumulation, which acts as an antioxidant that compromises the inhibitory effects of stresses.

In this study, we carried out a set of hydroponic assays with *sid2* mutants and wild-type *Arabidopsis* to systematically examine the endogenous roles of SA on Cd tolerance. We sought to elucidate the relationship among endogenous SA, H_2O_2, antioxidant system, and GSH cycling by investigating the following specific questions. (1) Does depletion of SA aggravate Cd toxicity, including oxidative damage to plants? (2) What are the possible roles of SA in mediating Cd tolerance via modulating GSH biosynthesis and regeneration in *Arabidopsis*.

2. Materials and methods

2.1. Plant growth conditions

The Columbia-0 (wild-type, *wt*) and SA-deficient mutant (*sid2*) seeds were sterilized and then kept in dark at 4 °C for 2 days. Subsequently, the sterilized seeds were sown in 0.5 mL centrifuge tubes (Eppendorf, Co., Ltd., Germany), from which the bottom had been removed and which had been filled with 8 $g\,L^{-1}$ sucrose-free agar [26]. The centrifuge tubes were fitted in the floater microtube rack of hydroponic tanks, which contained 1/3 strength Hoagland's nutrient solution (pH 5.8, renewed twice a week). The hydroponic tanks were placed in a growth cabinet (22 °C/18 °C, 12 h/12 h light/dark, 75% relative humidity, 150 $\mu mol\,m^{-2}\,s^{-1}$ photon flux). After a three-week growth period, half of the wild-type and *sid2* mutant plants were subjected to Cd treatment under the same conditions by feeding with a hydroponic solution with 5 μM $CdCl_2$. The concentration had been confirmed to have an inhibitory effect on plant growth [27]. Thus four treatments were conducted in the experiment, namely (1) *wt*, (2) *sid2*, (3) *wt* + Cd, and (4) *sid2* + Cd. Each treatment had 20 replicates. After 0, 4, 8, and 12 days of Cd exposure, each treatment with 5 replicates was harvested. Six independent experiments were performed for determining (1) fresh weight, chlorophyll and SA concentrations; (2) element concentrations; (3) enzyme activities, i.e. superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and GR; (4) H_2O_2 and malondialdehyde (MDA) concentrations; (5) total non-protein thiols (NPT), GSH, oxidized glutathione (GSSG), and PCs concentrations; (6) transcription of GSH related genes. The plant materials were washed thoroughly with distilled water, and measured immediately after harvest.

Another experiment was conducted to investigate whether catechol, the SA-decomposition product by *NahG*, is involved in Cd tolerance. For this, the *wt* plants, *sid2* mutants and *NahG* transgenic lines were treated under the same condition as in the Cd treatment experiment with or without 10 μM catechol. The concentration for catechol had been confirmed to be nontoxic but have alleviating effect on plant growth under stress [20]. The plant materials were harvested after 12 days of Cd exposure for determining fresh weights and MDA concentrations.

2.2. Assays of elements in Arabidopsis tissues

The plant shoots and roots were dried at 70 °C to constant weight, ground and digested in 5 mL HNO_3 at 160 °C for 8 h. Elements including Cd, macroelements (K, S, Mg and Ca) and microelements (Fe, Zn, Cu and Mn) were estimated by Inductively Coupled Plasma Mass Spectroscopy (Agilent 7500a).

2.3. Assays of SA and chlorophyll contents in Arabidopsis leaves

Salicylic acid content in *Arabidopsis* leaves was extracted with 90% methanol and re-extracted by ethyl acetate, and then determined by HPLC (Diane, Ultimate 3000) according to the method described by Malamy et al. [28].

Chlorophyll content in *Arabidopsis* leaves was extracted with 80% ice-cold acetone and analyzed by the method described by Arnon [29].

2.4. Assays of antioxidant system in Arabidopsis leaves

2.4.1. H_2O_2, MDA concentrations and DAB staining

The H_2O_2 in *Arabidopsis* leaves was extracted with chilled acetone, reacted with titanium sulphate by forming yellow pellet, and measured spectrophotometrically at 415 nm [9]. The MDA was extracted with trichloroacetic acid, reacted with thiobarbituric acid by forming pink compounds, and assayed spectrophotometrically at 600, 532 and 450 nm [9]. The DAB staining was according to the method described by Thordal-Christensen et al. [30].

2.4.2. Activities of antioxidant enzymes (SOD, POD, CAT and GR)

Fresh leaves were homogenized with 50 mM KPO_4 buffer (pH 7.8, containing 1% polyvinylpyrrolidone) at 4 °C. The supernatant