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Research article

The *Arabidopsis Ethylene-Insensitive 2* gene is required for lead resistance

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ABSTRACT

The *Arabidopsis Ethylene-Insensitive 2* (*EIN2*) gene has been shown to be involved in the regulation of abiotic and biotic stresses, including ozone stress, high salt, oxidative stress and disease resistance. However, little is known about the role of *EIN2* gene in lead (Pb) resistance in *Arabidopsis*. In this study, we showed that *EIN2* gene is required for Pb(II) resistance in *Arabidopsis*. *EIN2* gene was induced by Pb(II) treatment, and the *ein2-1* mutant showed enhanced sensitivity to Pb(II). A higher Pb content was detected in *ein2-1* plants than in wild-type plants when subjected to Pb(II) treatment, which was associated, at least in part, with reduction in expression of *AtPDR12* gene, a pump excluding Pb(II) and/or Pb(II)-containing toxic compounds from the cytoplasm. Moreover, the *ein2-1* mutation also impaired glutathione (GSH)-dependent Pb(II) resistance, which was related to constitutive reduction of express of *GSH1* gene involved in GSH synthesis and consequently reduced GSH content. Taken together, all these results suggest that *EIN2* gene mediates Pb(II) resistance, at least in part, through two distinct mechanisms, a GSH-dependent mechanism and a GSH-independent AtPDR12-mediated mechanism.

1. Introduction

Heavy metals are important environmental pollutants with high toxicity to animals and plants [1–3]. Plants grown in contaminated agricultural fields contain the heavy metals and thus endanger animals who feed on them [4]. Among the heavy metals, lead (Pb) is particularly important, since it is toxic to many organisms, widespread in the human environment, and causes multiple serious health problems in growing children and adults [4].

Plants possess a range of potential cellular mechanisms that may be involved in the detoxification of heavy metals and thus tolerance to metal stress. These include roles for the following: for mycorrhiza and for binding to cell wall and extracellular exudates; for reduced uptake or efflux pumping of metals at the plasma membrane; for chelation of metals in the cytosol by peptides such as phytochelatins; for the repair of stress-damaged proteins; and for the compartmentation of metals in the vacuole by tonoplast-located transporters [5]. In addition, many transporters have been identified that detoxify heavy metals in diverse organisms. Since cells have to remove heavy metals from the cytoplasm to avoid toxicity, some transporters extrude them across the plasma membrane and others sequester them into inactive organelles [4]. These include P-type pumps such as ZntA from *Escherichia coli*, a Pb(II)/Cd(II)/Zn(II)-transporting ATPase that mediates resistance to toxic concentrations of Pb, Cd(II), and Zn(II) by pumping these metals out of cells [6,7]. CAD2 from yeast (Saccharomyces cerevisiae) has a similar role [8]. Recently, it was found that that *AtPDR12*, a member of the pleiotropic drug resistance subfamily of ABC transporters in *Arabidopsis* has a role in Pb(II) detoxification [4]. More interestingly, ACBP1 encoding acyl-CoA-binding protein was showed to be involved in mediating Pb(II) tolerance in *Arabidopsis* with accumulation of Pb(II) in shoots [9]. Although recent studies have unraveled some of the key players in Pb(II) detoxification [4,6–9], a lot of questions related to its mode of regulation and its modulation of signaling networks that control this process remain to be determined.

The plant hormone ethylene is involved in many aspects of the plant life cycle, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening [10]. Moreover, ethylene is also implicated in modulating the plant responses to environmental stimuli from biotic and abiotic stresses, such as wounding, hypoxia, ozone, chilling, or freezing [10]. The *Arabidopsis EIN2* gene is a central component of the ethylene

Abbreviations: *ACTIN*, *ACTIN11*; BSO, buthionine sulfoximine; *EIN2*, *Ethylene-Insensitive 2*; FW, fresh weight; MS, Murashige and Skoog; Pb, lead; RT-PCR, reverse transcription-PCR; WT, wild-type *Arabidopsis* ecotype Columbia Col-0.

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signaling pathway, and it is also a bifunctional transducer of ethylene and stress responses [11]. *EIN2* gene has been shown to be involved in mediating ozone stress, high salt, oxidative stress and disease resistance [11–14]. However, it is unclear whether it also plays a role in heavy metal Pb(II) resistance in *Arabidopsis*.

In this study, we demonstrated that *EIN2* gene mediates Pb(II) resistance, at least in part, through two distinct mechanisms, a glutathione (GSH)-dependent mechanism and a GSH-independent AtPDR12-mediated mechanism.

2. Materials and methods

2.1. Plant material and growth conditions

Wild-type *Arabidopsis* ecotype Columbia Col-0 (WT) and the *ein2-1* mutant were used in this study. Seeds were surface sterilized and plated on Murashige and Skoog (MS) media [15] containing 1% sucrose. Plates were stored for 3 days in the dark at 4 °C and then placed in a growth chamber set at 22 °C, 100 μmol m^{-2} s^{-1} light intensity and standard long-day conditions (16 h light/8 h dark).

2.2. Treatments and Pb(II) resistance test

For Pb(II) resistance test and buthionine sulfoximine (BSO) treatment, wild-type and *ein2-1* seeds were germinated and grown vertically on 1/2 MS media supplemented with indicated concentrations of $Pb(NO_3)_2$ or BSO (Sigma, St. Louis, MO, USA). After indicated days of growth, plants were sampled for root growth assay and measurements of fresh weight.

2.3. Measurement of Pb content

Two-week-old wild-type and *ein2-1* seedlings grown on MS media were treated with 0.5 mM $Pb(NO_3)_2$ (Sigma, St. Louis, MO) for 2 days, and then sampled for analysis of Pb content. Pb content was determined according to the method described by Lee et al. [4]. Briefly, plant tissues were digested with 11 N HNO_3 at 200 °C overnight. Digested samples were diluted with 0.5 N HNO_3 and analyzed using an atomic absorption spectrometer (M6, Thermo Elemental, USA).

2.4. Measurement of GSH content

Two-week-old wild-type and *ein2-1* seedlings grown on 1/2 MS media with or without 0.5 mM $Pb(NO_3)_2$ were sampled for analysis of GSH content. GSH was extracted from seedlings and quantitated according to Lee et al. [16].

2.5. RT-PCR analysis of gene expression

Two-week-old wild-type seedlings grown on 1/2 MS media with or without 0.5 mM $Pb(NO_3)_2$ were sampled for RT-PCR analysis. Seedlings were homogenized with a mortar and pestle in liquid nitrogen. RNA was extracted using Trizol Reagent (Invitrogen, CA), followed by chloroform extraction, isopropanol precipitation, and spectrophotometric quantification. cDNA was synthesized from DNase-treated RNA with Superscript reverse transcriptase (Invitrogen), following the manufacturer's instructions. The cDNA products were standardized for semi-quantitative RT-PCR using β-actin primers as a reference. For each transcript, sequence-specific 5′ and 3′ primers were designed with melting temperatures between 52 °C and 60 °C. The number of cycles was established empirivelly to amplify the target cDNA to allow the maximal detection of differences in transcript number. The following primers were designed for gene-specific transcript amplifications:

ACTIN11 (*ACTIN*, At3g12110): forward, 5′-GATTTGGCATCAC ACTTTCTACAATG-3′, and reverse, 5′-GTTCCACCACTGAGCAC AATG-3′;
EIN2 (AT5G03280): forward, 5′-GAAGACGAATCAATAGTGCGG-3′ and reverse, 5′- 5′-TGCGGAATGAAGGAGGAC-3′;
AtPDR12 (at1g15520): forward, 5′-GAAGCGGCTTTAGGAGTC GATTTCGC-3′, and reverse, 5′-CGTCCACTCGAATCCTATCAT AGCG-3′;
GSH1 (At4g23100): forward, 5′-ATCTACGCTTTGTCCCCATTC-3′, and reverse, 5′-ATATTCCCAGAGGTTCGGTG-3′.

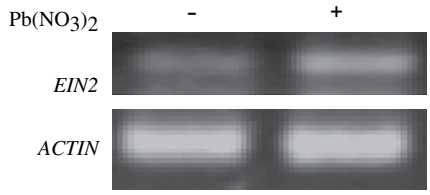

Fig. 1. Induction of *EIN2* gene by Pb(II) treatment. Two-week-old wild-type seedlings grown on 1/2 MS media with or without 0.5 mM $Pb(NO_3)_2$ were sampled for RT-PCR analysis. Three independent experiments were carried out and trends were similar. *ACTIN* was used as a loading control.

2.6. Statistical analysis

Data are the means ± S.E. of three independent replicates. The analyses of variance were computed on statistically significant differences ($P < 0.05$) determined based on the appropriate *F*-tests. The mean differences were compared utilizing Duncan's multiple range test.

3. Results and discussion

The *Arabidopsis EIN2* gene, which encodes a polypeptide of 1294 amino acids with a molecular mass of 141 kD and dimorphic structure, is a central component of the ethylene signaling pathway [11]. To determine whether *EIN2* is mediated by Pb(II) treatment, the expression pattern of *EIN2* was analyzed in response to Pb(II) treatment. As shown in Fig. 1, Pb(II) treatment significantly increased the transcript level of *EIN2*, indicating the possible involvement of *EIN2* in mediating Pb(II) resistance.

The fact that the transcript of *EIN2* gene is induced by Pb(II) stress prompted us to evaluate the role of the *EIN2* gene in plant Pb(II) resistance. Therefore, we tested the tolerance of the *ein2-1* mutant to Pb(II). At different developmental stages, when grown on 1/2 MS media, there was no significant difference between the wild type and the *ein2-1* mutant (Fig. 2A and B); however, when grown on 1/2 MS containing different concentrations of Pb(II), *ein2-1* seedlings were more sensitive to Pb(II) than wild-type seedlings (Fig. 2A and B).

Quantitative analyses confirmed that at each Pb(II) concentration, the root length of the *ein2-1* mutant was significantly ($P < 0.05$) shorter than wild type (Fig. 2C), and that the fresh weights of wild-type and *ein2-1* seedlings were similar in the 1/2 MS media but, in the Pb(II)-containing media, the fresh weight of the *ein2-1* seedlings was significantly ($P < 0.05$) lower than that of wild-type seedlings (Fig. 2D). These results suggest that *EIN2* is involved in mediating Pb(II) resistance.

To further determine whether enhanced Pb(II) sensitivity of *ein2-1* seedlings is associated with increased Pb content, we measured Pb content in *ein2-1* seedlings subjected to Pb(II) treatment. As shown in Fig. 3, higher Pb content was detected in *ein2-1*