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Overexpression of *Sedum alfredii* cinnamyl alcohol dehydrogenase increases the tolerance and accumulation of cadmium in Arabidopsis

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ABSTRACT

Plant cell walls protect plants from heavy metal stress by resistance, transport and the adsorption process. Cell walls are impregnated with lignin, which enables structural integrity, long-distance water transport and protects plants from many types of stress. Cinnamyl alcohol dehydrogenase (CAD) is an essential enzyme that participates in the final step of the phenylpropanoid lignin biosynthetic pathway and plays an important role in the stress resistance process. In this study, we demonstrated that a CAD-encoding gene in the hyper-accumulating *Sedum alfredii* Hance, *SaCAD,* is constitutively expressed in all tissues. While the abundance of the *SaCAD* transcript is affected by cadmium (Cd) stress, it is upregulated in roots, stems and leaves during Cd treatment. Overexpression of *SaCAD* in transgenic *Arabidopsis thaliana* significantly increased CAD activities. Compared with the wild type (WT) plants, the Cd concentrations of *SaCAD*-overexpressing plants increased in the leaves and roots under Cd stress. The increased fixation of Cd to the thickened cell wall in the *SaCAD*-overexpressing *A. thaliana* plants resulted in better growth when the plants were grown in Cd stress conditions. In agreement with these data, *SaCAD*-overexpressing plants exhibited higher Cd tolerance compared to the wild type (WT) with higher chlorophyll and proline (Pro) contents and antioxidant enzyme activity, as well as a lower methane dicarboxylic aldehyde (MDA) content, electric conductivity and reactive oxygen species when exposed to Cd stress due to a lower amount of Cd distributed in the cytoplasm which is the most site of cytosolic metabolism. SaCAD was found to localize to the cytoplasm of tobacco cells. NMT analysis of the root tips from transgenic *A. thaliana* lines during Cd stress confirmed that *SaCAD*-overexpressing plants were capable of retaining more Cd in the cell wall in the Cd-supplied growth medium, which provided additional evidence for the potential role of *SaCAD* in heavy metal ion compartmentation and detoxification. In summary, we concluded that *SaCAD* performs critical functions in plants: Cd absorption and fixation to lignified cell wall during stress conditions.

1. Introduction

Plant cell walls are chemically complex, consisting of an extra-cytosolic matrix comprising extensively cross-linked polysaccharides, cellulose, hemicelluloses, and pectin that are impregnated with lignin and proteins (Somerville et al., 2005; Popper, 2008; Achyuthan et al., 2010; Gilbert, 2010; Lionetti et al., 2010; Seifert and Blaukopf, 2010). Cell wall lignification is one of the most important evolutionary adaptations that plants evolved to facilitate their move to terrestrial habitats (Weng and Chapple, 2010). In addition to contributing to structural integrity, mechanical support and the robustness of plant stems, lignin waterproofs the cell wall enabling long-distance water transport and protects plants from microorganisms, herbivores and the oxidative stress caused by abiotic stress (Koehler and Telewski, 2006; Robinson and Mansfield, 2009; Tronchet et al., 2010). Lignin is chemically heterogeneous due to its biosynthesis by an oxidative polymerization process, which links various phenolic monomers with carbon-carbon as well as ether linkages, making lignin structurally the most recalcitrant and cohesive component of the cell wall matrix (Boerjan et al., 2003; Torney et al., 2007; Nadji et al., 2009). This cross-coupling within

Abbreviations: (CAD), cinnamyl alcohol dehydrogenase; (Q-PCR), real-time quantitative RT-PCR; (ROS), reactive oxygen species; (SOD), superoxide dismutase; (POD), peroxidase

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lignin polymers, as well as with cell wall polysaccharides, contributes to the structural stiffness and strength to the cell wall, allowing plants to attain their remarkable upright statures. The lignified cell wall was proposed to potentially immobilize toxic heavy metal ions, as functional groups presenting in pectin, lignin, etc. could bind those ions to lower the free toxic ions in the cytoplasm (Krzesłowska, 2011). This tough structural component makes the generation of lignin-sufficient and lignin-altered plants a target of plant breeders and genetic engineers, as plants with altered lignin content and/or composition/structure, may have strengthened cell wall recalcitrance, allowing for more cost-effective and efficient phytoremediation for compartmentation and detoxification (Chen and Dixon, 2007; Himmel et al., 2007; Vanholme et al., 2010).

CAD is a multifunctional enzyme that regulates the final step of lignin biosynthesis by catalyzing the NADPH-dependent reduction of p-coumaraldehyde, coniferaldehyde and sinapaldehyde to their corresponding hydroxycinnamyl alcohol (monolignols), which are further incorporated into lignin as p-hydroxyphenyl (or H units), guaiacyl (or G units) and syringyl (or S units), respectively (Kim et al., 2002; Raes et al., 2003; Saballos et al., 2009).

Upon perceiving stress, plants undergo large-scale transcriptional, translational and post-translational reprogramming that directly influences a network of processes spanning the various levels of biological organization from the cellular to the organismic levels (Hahlbrock et al., 2003; Molinier et al., 2006; Nakashima et al., 2009; Spoel et al., 2009; Walley and Dehesh, 2010). Lignification is also known to be stress-inducible and influenced by environmental stresses such as ozone, UV-B, heavy metal exposure, drought, pathogen infection, nitrogen fertilization, as well as by mechanical wounding, herbivore attack, thigmomorphogenetic responses (wind and mechanical stimulation) and gravity (Pomar et al., 2004; Koehler and Telewski, 2006; Saidi et al., 2009; Moura et al., 2010; Zhao and Dixon, 2010).

Cadmium is one of the most significant heavy metal pollutants that is widespread in our environment because of rapid industrialization and urbanization. Plants have to develop a broad range of complex defense systems to survive harsh environmental elements, such as heavy metal pollution, because they are confined to the place where they grow (He et al., 2011). Under high Cd exposure, plants display chlorosis in their young leaves, decreased root growth, changed growth patterns of their roots, and finally growth retardation and small biomass (Di Toppi and Gabbrielli, 1999; Wu et al., 2012). Plants have evolved a number of defense systems, such as cell wall binding; chelation with phytochelatins, metallothioneins, or organic acids; compartmentation in the vacuole to protect the cell from the damage caused by cadmium (Yadav, 2010; Ding et al., 2017), and enrichment in leaf trichomes to counteract cadmium stress (Clemens, 2006; DalCorso et al., 2008). However, the underlying physiological mechanisms remain unclear. As the first barrier to direct contact with cadmium and to prevent it from entering into the cell, the cell wall is a pivotal site for cadmium storage in plants (Lozano-Rodriguez et al., 1997; Carrier et al., 2003), and the deposition of cadmium in the cell wall is considered to be a critical mechanism for cadmium tolerance (Xiong et al., 2009). Either the change of the cell wall compositions or the modification of certain cell wall components affects the binding capacity of the cell wall to metal ions. Elucidation of the mechanisms mediating heavy metal uptake, transport and tolerance would thus be of great importance to reduce heavy metal translocation into food chains or to perform the phytoremediation of polluted soils (He et al., 2015), since hyper-accumulators are widely accepted as ideal model plants for this type of research . In previous study, *Sedum alfredii* Hance was found to hyper-accumulate cadmium and zinc in both the natural habitat and laboratory cultivation systems (Yang et al., 2004) . It grows without any significant adverse effect following treatments of up to 1 mM $CdCl_2$, and most cadmium accumulated in the leaves and was found in the apoplastic cell wall, chelated by either proteins or pectates (Peng et al., 2017; Tao et al., 2016), which would be an excellent model system for the study of cadmium hyperaccumulation if the genetic transformation system had been development. However, it remains unknown that how originally cell walls contribute to cadmium hyperaccumulation in *S. alfredii.* More attention should be paid to the role of cell wall associated genes of *S. alfredii*. In this study, we characterized the expression and subcellular localization of *SaCAD*, the Arabidopsis ortholog of *AtCAD7*. Ectopic expression of *SaCAD* in the model plant *A. thaliana* resulted in enhancing Cd tolerance and accumulation. In order to clarified the role of SaCAD against cadmium toxicity, a study on the effects of Cd exposure on cell wall properties was conducted. Additionally, the role of important antioxidant is of considerable interest, due to the fact that oxidative stress is a major mechanism underlying Cd toxicity. Our results indicated that *SaCAD* is a Cd-responsible cinnamyl alcohol dehydrogenase. It performs critical functions in *S. alfredii* hyper-accumulation and tolerance of heavy metals such as cadmium.

2. Results

2.1. Characterization and expression pattern of SaCAD

An RNA-Seq transcriptomic assay was used to identify tolerance and accumulation-related genes of *S. alfredii* in response to cadmium supply. Among the differentially expressed ESTs, a truncated cDNA, similar to other plant cinnamyl alcohol dehydrogenase gene mRNA sequences, showed more than a 10-fold transcriptional increase in the roots of *S. alfredii* treated with cadmium stress compared to the untreated control. The full-length cDNAs of *S. alfredii* were isolated through the 5′-and 3′-rapid amplification of its cDNA ends (RACE). The full-length cDNA of *SaCAD* is 1371 bp, and its longest open reading frame codes for a protein of 362 amino acids with a predicted molecular mass of approximately 38.65 kDa. The 5′-untranslated region (UTR) is 113 bp, and the 3′-UTR is 169 bp with a polyadenylation tail. SaCAD belonged to the cinnamyl alcohol dehydrogenase family and contained no transmembrane domains (Fig. 1). The phylogenetic analysis indicated that *SaCAD* clustered closely with *AtCAD7* and *AtCAD8* (Supplementary Fig. S1). The multiple alignment analysis among SaCAD and the AtCADs demonstrated that SaCAD contained all of the three key identifying structures of CAD, (1) the Zn1 binding site $GHE(X)_2(X)_5G(X)_2V$, (2) the Zn2 binding site $GD(X)_{9.10}C(X)_2C(X)_2C(X)_7C$, and (3) the glycine-rich NADPH binding site $GXG(X)_2G$, and these structures were almost conserved in SaCAD compared with other CADs, suggesting a functional similarity among these species (Deng et al., 2013; Fig. 1).

Q-PCR analytical results showed that the transcriptional level of *SaCAD* was higher in the *S. alfredii* roots than in the untreated control. The Q-PCR results agreed with the transcriptomic analytical trend. The transcriptional level of *SaCAD* was markedly increased in the *S. alfredii* stems after treatment with cadmium, while the transcriptional induction in the leaves was relatively weaker (Fig. 2). In addition, *SaCAD* transcriptional induction levels were significantly higher in the roots than in the stems and leaves (Fig. 2B). Additionally, the tissue expression analysis showed that the transcriptional level of *SaCAD* was the highest in the roots, intermediate in the leaves, and the lowest in the stems of *S. alfredii* during both treatment and the untreated control (Fig. 2A). These results suggested that SaCAD might participate in the *S. alfredii* response to cadmium stress.

Monolignol biosynthesis in Arabidopsis has been shown to be intracytoplasmic and transported into the apoplast (Vanholme et al., 2010). To determine the subcellular localization of SaCAD, the *SaCAD* gene was cloned downstream of the constitutive cauliflower mosaic virus 35S promoter to create the SaCAD-GFP fusion construct. GFP signals were observed in the cytoplast expressing the construct (Fig. 3), suggesting that SaCAD is likely to be a cytoplasmic protein. The precise subcellular localization needed extracted protoplast of *S. alfredii.*