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Overexpression of *Populus euphratica* xyloglucan endotransglucosylase/hydrolase gene confers enhanced cadmium tolerance by the restriction of root cadmium uptake in transgenic tobacco

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ABSTRACT

Cadmium (Cd^{2+}) is a toxic heavy metal impairing plant growth and development. Xyloglucan endotransglucosylase/hydrolase gene (*XTH*) is involved in the plant response to heavy metal toxicity, in addition to controlling cell wall extensibility. However, the link between XTH and Cd^{2+} stress has not yet been established in higher plants. *PeXTH* expression was up-regulated by 1.2–2.1-fold in *Populus euphratica* roots and leaves upon Cd^{2+} exposure (40–80 μM $CdCl_2$). Cellular Cd^{2+} analysis and flux data showed that the cadmium-elicited expression of *PeXTH* markedly restricted Cd^{2+} uptake and accumulation in *P. euphratica* roots. Moreover, tobacco plants overexpressing *PeXTH* were more tolerant to Cd^{2+} stress (80 μM $CdCl_2$) than wild-type tobacco in terms of root and shoot growth. Transgenic lines accumulated 49–58% less Cd^{2+} in root apical and mature regions, as compared to the wild type. The less buildup of Cd^{2+} in roots of transgenic lines was the result of lower influx of Cd^{2+} under Cd^{2+} stress. It is noting that transgenic plants displayed 56–87% higher xyloglucan degradation activity (XDA) than the wild type, leading to a 25–27% decline of xyloglucan content in the root cell walls. Therefore, overexpression of *PeXTH* increased the activity of XDA in transgenic plants, which enhanced the degradation of xyloglucan in the wall. The down-regulated amount of xyloglucan led to less binding sites for Cd^{2+} and thus reduced the root Cd^{2+} uptake and buildup in transgenic plants. Consequently, the Cd^{2+} toxicity was eventually alleviated in transgenic tobacco.

1. Introduction

Cadmium (Cd^{2+}) is one of the most deleterious heavy metals and has detrimental effect on herbaceous (DalCorso et al., 2008) and woody species (Elobeid et al., 2012; Polle et al., 2013). Excess Cd^{2+} directly or indirectly interferes with a series of physiological processes, including photosynthesis, transpiration, and nutrient balance, resulting in growth retardation and eventually plant death (di Toppi and Gabbrielli, 1999; DalCorso et al., 2008). Higher plants have developed various detoxification mechanisms to avoid Cd^{2+} toxicity at the tissue and cellular levels, such as root exclusion, root-to-shoot translocation restriction, cell wall binding and immobilization, chelation by phytochelatins, and vacuolar compartmentation (di Toppi and Gabbrielli, 1999; Lux et al., 2011; He et al., 2013). Among all these protective strategies, restricting entry of Cd^{2+} is the most efficient approach against Cd^{2+} toxicity (Zhu et al., 2012a).

Cell walls of root act as the first barrier against Cd^{2+} stress in immobilizing excesses of Cd^{2+} (Inouhe et al., 2012). It has shown that a large amount of root-absorbed Cd^{2+} was located in the cell walls of *Athyrium yokoscense* (Nishizono et al., 1987) and rice (*Oryza sativa*; He et al., 2008). The cell wall is mainly composed of cellulose and matrix polysaccharides, i.e., pectins and hemicelluloses (Cosgrove, 2005). Cellulose theoretically cannot tightly bind to heavy metals due to the unbranched structure (Yang et al., 2008). Recent studies demonstrated that the content of pectin and hemicellulose was associated with Cd^{2+} toxicity and resistance (Xiong et al., 2009; Zhu et al., 2012a). However, attempts to correlate pectin and hemicelluloses and Cd^{2+} tolerance in herbaceous plants have

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yielded conflicting results. The decreased pectin and hemicelluloses contents induced by phosphorus-deficiency enhanced Cd^{2+} exclusion in *Arabidopsis thaliana* roots (Zhu et al., 2012a). In contrast, exogenous NO increased pectin and hemicellulose content in the cell wall of rice roots, which enhanced increasing Cd^{2+} accumulation in root cell wall and decreasing Cd^{2+} accumulation in soluble fraction of leaves (Xiong et al., 2009).

Xyloglucan is an abundant hemicellulose in the walls of monocotyledons and dicotyledons, and is proposed to crosslink cellulose microfibrils non-covalently to form the cellulose-xyloglucan network (Cosgrove, 2005). Modification of the network is regulated by a variety of enzymes, including xyloglucan endotransglucosylases/hydrolases (XTHs; Fry et al., 1992). Via xyloglucan endotransglucosylase (XET) activity (Thompson and Fry, 2001) and/or xyloglucan endohydrolase (XEH) activity (Rose et al., 2002), XTHs can catalyze the cleavage and/or religation of xyloglucan chains in the cellulose-xyloglucan framework of plant cell walls, and thus regulate cell wall strength and extensibility (Rose et al., 2002; Vissenberg et al., 2005). Large multigene families of XTHs have been identified in a variety of species, such as *Arabidopsis* (33 members; Yokoyama and Nishitani, 2001), tomato (25 members; Saladié et al., 2006), rice (29 members; Yokoyama et al., 2004), and poplar (41 members; Geisler-Lee et al., 2006). XTHs have multifunctions in primary root elongation, flower opening, fruit ripening, petal abscission, and wood formation (Osato et al., 2006; Miedes and Lorences, 2009; Nishikubo et al., 2011; Singh et al., 2011; Harada et al., 2011). Recently, XTH is shown to have a novel function against heavy metal toxicity, due to the XET action and xyloglucan degrading activity (XDA, an indicator of extractable XET- and/or XEH-active XTH protein concentration; Zhu et al., 2012b). In *Arabidopsis*, one member of the XTH multigene family AtXTH31, was found to regulate aluminum sensitivity by modulating xyloglucan content in the cell wall (Zhu et al., 2012b). However, the link between XTH and Cd^{2+} stress has not yet been established in higher plants.

Populus euphratica is model tree species to explore the salt-tolerant physiology in woody plants (Ottow et al., 2005; Sun et al., 2009, 2010). However, *P. euphratica* is shown to be sensitive to Cd^{2+} stress (Polle et al., 2013). It is suggested that the higher Cd^{2+} susceptibility of *P. euphratica* is due to the failure to activate early protective responses upon Cd^{2+} exposure (Polle et al., 2013). Unexpectedly, in the present study we found that the expression of *PeXTH* was up-regulated in *P. euphratica* upon lower concentrations of Cd^{2+}, e.g., 40 μM. This implies that this Cd^{2+}-susceptible poplar can trigger protective responses upon Cd^{2+} exposure, but must at low concentrations. Previous studies show that PeXTH localized exclusively to the endoplasmic reticulum and cell wall (Han et al., 2013). *PeXTH* expression was up-regulated by NaCl in *P. euphratica* leaves and is suggested contributing to the development of leaf succulence (Han et al., 2013). We hypothesize that the Cd^{2+}-elicited expression of *PeXTH* may benefit *P. euphratica* to tolerate the heavy metal stress. The present study aimed to evaluate the role of PeXTH in Cd^{2+} tolerance. We investigated the correlations between *PeXTH* expression and Cd^{2+} flux and accumulation in *P. euphratica* roots. Moreover, *PeXTH* gene was introduced into the model plant, tobacco (*Nicotiana tabacum* cv. Wisconsin 38), demonstrating that the *PeXTH* overexpression could improve Cd^{2+} tolerance by the restriction of root uptake in transgenic tobacco plants.

2. Materials and methods

2.1. Plant material and cadmium treatment

In April, 1-year-old *P. euphratica* seedlings from the Xinjiang Uygur Autonomous Region of Northwest China were planted in individual pots (10 L) containing loam soil and placed in a greenhouse at Beijing Forestry University. The temperature in the greenhouse was 20–25 °C with a 12-h light/12-h dark photoperiod and 150 μmol m^{-2} s^{-1} of photosynthetically active radiation. Potted seedlings were well irrigated and watered with 1 L full-strength Hoagland nutrient solution every two weeks. In June, 20–30 uniform plants were washed free of soil and cultivated hydroponically in individual pots containing 1.5 L half-strength Hoagland nutrient solution for 14 days. The hydroponic culture was refreshed every 2 days during the 2 weeks of acclimation. For cadmium treatment, required amounts of $CdCl_2$ salt were added to the half-strength Hoagland nutrient solution. The concentration started at 40 μM and increased stepwise by weekly 40 μM, reaching 120 μM in the third week. Leaves and roots were harvested during the period of increasing $CdCl_2$ stress, quickly frozen in liquid nitrogen, and stored at −80 °C for further RNA extraction.

In addition, *P. euphratica* seedlings treated with $CdCl_2$ were also used to examine cellular Cd^{2+} concentration, transient and steady-state Cd^{2+} fluxes. In brief, *P. euphratica* seedlings were treated with 0 or 40 μM $CdCl_2$ for 1 week, and then exposed to 80 μM $CdCl_2$ for transient kinetics recording. Cd^{2+} fluxes were measured at the apex, ca. 300 μm from the root tip. For steady-state flux recording, *P. euphratica* plants pre-treated with or without 40 μM $CdCl_2$ (1 week) were subjected to 80 μM $CdCl_2$ for 3 days. Cd^{2+} fluxes were measured along the root axis at the meristematic zone, elongation zone, and mature zone. Moreover, cellular Cd^{2+} concentration within root cells was examined using Cd^{2+}-specific fluorescent probe (see below).

2.2. RNA isolation and expression analysis

Total RNA from *P. euphratica* tissues and tobacco roots (see below) was prepared using the Plant RNA Kit (QBio Technologies Inc., Beijing, China) and the Trizol reagent (Invitrogen, Carlsbad, CA), respectively. Extracted RNA was treated with RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized by the reverse transcription reaction using M-MLV reverse transcriptase (Promega, Madison, WI, USA).

The expression levels of *PeXTH* in *P. euphratica* leaves and roots were assessed by quantitative real-time PCR using SYBR Green Master Mix on the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corp., Carlsbad, CA, USA). *PeXTH* was amplified using specific primers (forward, 5′-AAAGGGTCTGCGTGGGATG-3′; reverse, 5′-CGGGAGGAGAAGTGGTTG-3′). The *Populus* housekeeping gene *PeACT7* (NCBI RefSeq acc. XM_002322628) was used as the internal control. *PeACT7* forward and reverse primers were 5′-ATGCTGCTAGGAGCCAGTGC-3′ and 5′-TTGTGCTCAGTGGTGGCTCTAC-3′, respectively. Each sample was run in triplicate and relative expression levels were calculated through the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen,

2.3. PeXTH overexpression in tobacco

The overexpression of *PeXTH* in *Nicotiana tabacum* cv. Wisconsin 38 using the CaMV 35S promoter was described previously (Han et al., 2013). Five positive transgenic lines, L5, L6, L8, L11, L14, were confirmed by Semi-quantitative PCR. Total RNA was isolated from roots of three-week-old tobacco plants transferred with or without *PeXTH*. Semi-quantitative PCR was performed in a 25-μL final volume containing 1 μL cDNA, 2 μL dNTP mixture (2.5 mM), 1 U Taq polymerase (Takara, Dalian, China), 2.5 μL 10× buffer, and 0.5 μL of each primer (10 μM). Forward and reverse primers of *PeXTH* were 5′-TTAGCCAAGGCAAAGGCAAC-3′ and 5′-AGCCCAGTCATCAGCATTCC-3′. Tobacco housekeeping gene *NtEF1α*