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Differential expression of vacuolar and defective cell wall invertase genes in roots and seeds of metalliferous and non-metalliferous populations of *Rumex dentatus* under copper stress

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ABSTRACT

Acid invertase activities in roots and young seeds of a metalliferous population (MP) of *Rumex dentatus* were previously observed to be significantly higher than those of a non-metalliferous population (NMP) under Cu stress. To date, no acid invertase gene has been cloned from *R. dentatus*. Here, we isolated four full-length cDNAs from the two populations of *R. dentatus*, presumably encoding cell wall (*RdnCIN1* and *RdmCIN1* from the NMP and MP, respectively) and vacuolar invertases (*RdnVIN1* and *RdmVIN1* from the NMP and MP, respectively). Unexpectedly, *RdnCIN1* and *RdmCIN1* most likely encode special defective invertases with highly attenuated sucrose-hydrolyzing capacity. The transcript levels of *RdmCIN1* were significantly higher than those of *RdnCIN1* in roots and young seeds under Cu stress, whereas under control conditions, the former was initially lower than the latter. Unexpected high correlations were observed between the transcript levels of *RdnCIN1* and *RdmCIN1* and the activity of cell wall invertase, even though *RdnCIN1* and *RdmCIN1* do not encode catalytically active invertases. Similarly, the transcript levels of *RdmVIN1* in roots and young seeds were increased under Cu stress, whereas those of *RdnVIN1* were decreased. The high correlations between the transcript levels of *RdnVIN1* and *RdmVIN1* and the activity of vacuolar invertase indicate that *RdnVIN1* and *RdmVIN1* might control distinct vacuolar invertase activities in the two populations. Moreover, a possible indirect role for acid invertases in Cu tolerance, mediated by generating a range of sugars used as nutrients and signaling molecules, is discussed.

1. Introduction

Extensive mining, industrial, agricultural and military operations have released enormous amounts of heavy metals (HMs) into the environment (Kavamura and Esposito, 2010; Nagajyoti et al., 2010; Rehman et al., 2017). Elevated concentrations of HMs in the soil pose a serious threat to plants, though some HMs are essential for plant physiological and biochemical processes (Nagajyoti et al., 2010; Miransari, 2011; Rizwan et al., 2016). Some higher plant species called metallophytes have evolved tolerance mechanisms and can survive and thrive on metal-contaminated soils (Baker et al., 2010; Kavamura and Esposito, 2010). These mechanisms include immobilization, exclusion, complexation and compartmentalization of metal ions at both the molecular and cellular levels (Leitenmaier and Küpper, 2013; Sharma and Chakraverty, 2013; Kushwaha et al., 2015; Sharma et al., 2016). Few studies of the responses of metallophytes to HMs have addressed plant sucrose (Suc) metabolism, which is known to play crucial roles in the plant stress response mainly by producing a range of sugars used as nutrients and signaling molecules (Ruan, 2014).

Suc is the major product of photosynthesis in higher plants and is transported from source leaves to heterotrophic sinks such as flowers, fruits, seeds and roots (Ruan, 2014). For sink organs, Suc and its hydrolysis products, glucose (Glc) and fructose (Fru), are not only carbon and energy resources but also important metabolic signals that affect the expression of various genes (Ruan, 2012, 2014; Liu et al., 2013). It has become evident that Suc metabolism plays crucial roles in sink growth, development and stress responses (Koch, 2004; Ruan et al., 2010; Albacete et al., 2011; Ruan, 2014). In higher plants, there are two enzymes that catalyze the hydrolysis of Suc: Suc synthase (SUS, EC 2.4.1.13) and invertase (EC 3.2.1.26; Roitsch and González, 2004). The former catalyzes reversible hydrolysis to UDP-Glc and Fru, whereas the latter irreversibly hydrolyzes Suc to Glc and Fru. Plants possess three isoforms of invertases with different biochemical properties and subcellular locations: neutral/alkaline invertases in the cytoplasm and acid

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invertases in the extracellular space (cell wall invertase, CWIN) and the vacuole (vacuolar invertase, VIN; Roitsch and González, 2004). CWINs and VINs are classified in plant glycoside hydrolase family 32 (GH32) with plant fructan exohydrolases (FEHs) and fructosyltransferases (FTs; Van den Ende et al., 2009). FEHs are believed to have evolved from CWIN ancestor genes (Van den Ende et al., 2009). They are very similar at the molecular and structural levels but are functionally different (De Coninck et al., 2005). FEHs do not hydrolyze Suc and only hydrolyze terminal Fru-Fru bonds in fructans. Some special FEHs, which were later renamed as defective CWINs with remaining FEH side activities, have previously been reported in non-fructan plants (Van den Ende et al., 2003; De Coninck et al., 2005). More recently, tobacco Nin88 was shown to be a defective CWIN lacking both invertase and FEH activity instead of an active CWIN as previously designated (Le Roy et al., 2013). As acid invertases are key enzymes in Suc metabolism, and their substrates and hydrolytic products are both nutrients and signaling molecules, increasing evidence has shown that acid invertases play a pivotal role in various aspects of plant growth and development, as well as in defense responses to various abiotic and biotic stresses such as heat, cold, drought, wounding, hypoxia, salinity and pathogen infection (Roitsch and González, 2004; Ruan et al., 2010; Albacete et al., 2011; Ruan, 2012, 2014). However, the role of defective CWINs remains unclear and opens a completely new research area (Van den Ende et al., 2003; De Coninck et al., 2005; Le Roy et al., 2013)

Root growth and seed development are crucial for plants to survive and reproduce in metal-contaminated soil (Keller et al., 2003; Huang et al., 2011a). Roots are directly exposed to HMs in the soil and are the first organs subjected to HM stress. Acid invertase-mediated Suc import and degradation might be crucial for maintaining root growth under HM stress (Albacete et al., 2011). Reproductive development, especially the seed and fruit set established around fertilization, is generally much more sensitive to biotic and abiotic stress than the vegetative stage (Ruan, 2014). There is compelling evidence that the high sensitivity of reproductive development relates to reduced acid invertase activity and to disruption of Suc metabolism under stresses including cold (Thakur et al., 2010), drought (Boyer and McLaughlin, 2006; Ji et al., 2010) and heat (Li et al., 2011; Snider et al., 2011). To our knowledge, Suc metabolism, and invertase expression and activity in plant reproductive organs under HM stress have rarely been reported.

In our previous studies, two populations of *R. dentatus*, one from a Cu mine and the other from an uncontaminated site, were compared with respect to root growth and phenology and reproductive traits under Cu stress (Huang et al., 2011a; Cai et al., 2013). Compared to the NMP plants, MP *R. dentatus* displayed a higher root biomass and root/shoot biomass ratio under Cu stress (Cai et al., 2013). The reproductive development of the MP showed a higher resistance to Cu stress than that of the NMP, with a shorter life cycle, a larger reproductive biomass and a higher capacity for seed set under Cu stress (Huang et al., 2011a). Corresponding higher acid invertase activities and sugar content were also observed in roots and young seeds of MP *R. dentatus* compared to the NMP under Cu stress (Cai et al., 2013; Huang et al., 2013). Here, we describe the isolation of full-length cDNAs presumably encoding acid invertases from the two populations of *R. dentatus* and compared their expression patterns in roots and young seeds under optimal and Cu stress conditions.

2. Materials and methods

2.1. Plant material and cultivation

In this study, two natural populations of *R. dentatus* were selected. One population (MP) was collected from Tonglushan Hill, an operational Cu mine with a 3000-year history in Hubei Province, China, and the other (NMP) was from the campus of Wuhan University, approximately 75 km northwest of the Cu mine. Seed germination and seedling hydroponic cultivation were performed as described by Cai et al. (2013). Cu treatments were conducted by adding 10 μM $CuCl_2{\cdot}2H_2O$ to the culture solutions as described by Cai et al. (2013). Fresh root samples were harvested after treatment for 0 h, 6 h, 72 h and 144 h. Potted plants were grown in soil with a Cu concentration of 0 or 500 mg kg^{-1} as described by Huang et al. (2011a). Seeds were harvested 4 days after anthesis.

2.2. RNA isolation and reverse transcription

Total RNA was extracted from approximately 100 mg root or seed samples using RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Reverse transcription was performed according to the manufacturer's instructions for the Reverse Transcription System (Promega, Madison, USA).

2.3. Cloning of acid invertase genes

The degenerate oligonucleotide primers used for acid invertase internal fragment amplification were designed based on the conserved amino acid sequences NW(I/M)NDPN(G/A)P and G(M/N)WEC(V/P)DF from other plant species. The primers CWInv-F and CWInv-R were used for CWIN gene fragment cloning, and VInv-F and VInv-R were used for VIN (Table 1). The amplified fragments were cloned into the pMD18-T vector (TaKaRa, Kyoto, Japan) and sequenced.

Using the sequence information of internal fragments, the specific primers CWInv-3′GSP1, CWInv-3′GSP2, VInv-3′GSP1 and VInv-3′GSP2 (Table 1) were designed to amplify the 3′-end of the cDNA by rapid amplification of 3′-cDNA ends (3′-RACE) according to the protocol for the 3′-Full RACE Core Set Ver.2.0 (TaKaRa, Kyoto, Japan). Additionally, CWInv-5′GSP1, CWInv-5′GSP2, CWInv-5′GSP3, VInv-5′GSP1, VInv-5′GSP2 and VInv-5′GSP3 (Table 1) were used to obtain the 5′ -end of the cDNA according to the protocol for the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA).

The full-length cDNA sequences of acid invertase genes were obtained by selecting the specific primers CWInv-full F/R and VInv-full F/

Table 1
Sequences of primers used in this study.

Primers	Sequences (5′ – 3′)
Degenerate primers for internal fragments cloning	
CWInv-F	AAYTGGATNAAYGAYCCNAAYGSNCC
CWInv-R	AARTCNGGRCAYTCCCAVWTNCC
VInv-F	AAYTGGATGAAYGAYCCNAAYGGNCC
VInv-R	AARTCNACRCAYTCCCACATNCC
Specific primers for 3′ and 5′-end cloning	
CWInv-3′GSP1	CATAAACGGATGTTGGTCAGG
CWInv-3′GSP2	ACCCTCTTATGACACCAAATGTG
VInv-3′GSP1	AGACGGACTCTGGCGGGTTAC
VInv-3′GSP2	TACGAGCTGATTGAGGACCACG
CWInv-5′GSP1	GCTGAGCCTGACCAACA
CWInv-5′GSP2	AATGGCGATTGGTTCTTCAGTC
CWInv-5′GSP3	GTTGAATGTCCCCAAACAATCTG
VInv-5′GSP1	ATGGCTAAAGGGAGGTA
VInv-5′GSP2	GATATTGCCCCACACAGCGGAG
VInv-5′GSP3	CAAACGAATCATTGTCATCCCTC
Primers for full-length cDNA cloning	
CWInv-full F	ATGGCTTCCTCTTCTCATAAAGTTGC
CWInv-full R	TTACAATTCAGCTGTTACAATCTTTTC
VInv-full F	CTAATCACAAATCTCCGATGGCGA
VInv-full R	GAGCGGCTTCAAAAAAATCAATCAC
Primers for Real-time PCR	
GAPDH-F	AGACGGACTCTGGCGGGTTAC
GAPDH-R	CGTGGTCCTCAATCAGCTCGTA
RT-CWInv-F	CATAAACGGATGTTGGTCAGG
RT-CWInv-R	CACATTTGGTGTCATAAGAGGGT
RT-VInv-F	GACTTTGTTGGTGACAGCAGGTC
RT-VInv-R	CTGTAACCCCACTCGTTGTCG

Y = C/T; N = A/G/C/T; R = A/G. All primers were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).