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Identification of UDP glucosyltransferases from the aluminum-resistant tree *Eucalyptus camaldulensis* forming β-glucogallin, the precursor of hydrolyzable tannins

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ABSTRACT

In the highly aluminum-resistant tree *Eucalyptus camaldulensis*, hydrolyzable tannins are proposed to play a role in internal detoxification of aluminum, which is a major factor inhibiting plant growth on acid soils. To understand and modulate the molecular mechanisms of aluminum detoxification by hydrolyzable tannins, the biosynthetic genes need to be identified. In this study, we identified and characterized genes encoding UDP-glucose:gallate glucosyltransferase, which catalyzes the formation of 1-*O*-galloyl-β-D-glucose (β-glucogallin), the precursor of hydrolyzable tannins. By homology-based cloning, seven full-length candidate cDNAs were isolated from *E. camaldulensis* and expressed in *Escherichia coli* as recombinant N-terminal His-tagged proteins. Phylogenetic analysis classified four of these as UDP glycosyltransferase (UGT) 84A subfamily proteins (UGT84A25a, -b, UGT84A26a, -b) and the other three as UGT84J subfamily proteins (UGT84J3, -4, -5). *In vitro* enzyme assays showed that the UGT84A proteins catalyzed esterification of UDP–glucose and gallic acid to form 1-*O*-galloyl-β-D-glucose, whereas the UGT84J proteins were inactive. Further analyses with UGT84A25a and –26a indicated that they also formed 1-*O*-glucose esters of other structurally related hydroxybenzoic and hydroxycinnamic acids with a preference for hydroxybenzoic acids. The *UGT84A* genes were expressed in leaves, stems, and roots of *E. camaldulensis*, regardless of aluminum stress. Taken together, our results suggest that the UGT84A subfamily enzymes of *E. camaldulensis* are responsible for constitutive production of 1-*O*-galloyl-β-D-glucose, which is the first step of hydrolyzable tannin biosynthesis.

1. Introduction

Hydrolyzable tannins (HTs) are a class of polyphenols that occur in a range of dicotyledonous plants (Bate-Smith, 1984; Haslam, 2007). More than 500 HTs (gallotannins and ellagitannins) with diverse chemical structures have been isolated and characterized (Okuda and Ito, 2011; Yoshida et al., 2010). HTs often accumulate in high concentration in various plant tissues. For example, HTs constitute up to 17% dry wt in *Eucalyptus* leaves (Marsh et al., 2017), at least 3.5% dry wt in the inner barks of *Quercus robur* (Scalbert et al., 1988), and up to 70% dry wt in the galls of *Rhus javanica* leaves (Haslam, 2007). Hydrolyzable tannins are traditionally thought to function *in planta* as defense compounds against herbivory by insects (Agrawal et al., 2012; Barbehenn and Constabel, 2011) and mammals (DeGabriel et al., 2009; Takahashi and Shimada, 2008). Some studies also suggest that HTs may have roles in defense against infection by microbial pathogens (Buzzini et al., 2008).

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Recently, we proposed the detoxification of toxic metal ions as another physiological function of HTs (Tahara et al., 2014). Specifically, we hypothesized that HTs internally detoxify aluminum (Al) ions by formation of harmless complexes with Al in plant tissues. Aluminum toxicity is a primary factor limiting plant productivity on acid soils (Kochian et al., 2015), which comprise about 30% of the total ice-free land area of the world (von Uexküll and Mutert, 1995). Under acidic conditions below pH 5, Al is released into soil solution from minerals and inhibits plant root growth, leading to water and nutrient deficiencies (Kopittke et al., 2016). Therefore, solving the Al toxicity problem on acid soils is important in expanding the land area available for sustainable food and forest production. *Eucalyptus camaldulensis* Dehnh. (Myrtaceae) is a tree species that has extreme resistance to Al (Tahara et al., 2008). We recently found that *E. camaldulensis* contains a high concentration of the HT oenothein B that can bind with Al and detoxify Al in root symplast (Tahara et al., 2014). Oenothein B exhibits a high affinity for Al, probably due to its multiple *ortho*-diphenolic groups that can function as Al binding sites. It is known to form soluble 1:1 and 3:2 Al–oenothein B complexes and other insoluble complexes with Al (Tahara et al., 2017; Zhang et al., 2016).

For a genetic understanding and engineering of *in planta* functions of HTs including Al detoxification, biosynthetic genes for HTs need to be identified. Enzyme studies have revealed several committed steps in the biosynthetic pathway of HTs (for review, see Niemetz and Gross, 2005). The first step is esterification of gallic acid and UDP-glucose to form 1-*O*-galloyl-β-D-glucose (β-glucogallin) by UDP–glucose:gallate glucosyltransferase (EC 2.4.1.136) (Fig. 1; Gross, 1982, 1983). In turn, 1-*O*-galloyl-β-D-glucose is converted to 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose in a series of galloylation steps using 1-*O*-galloyl-β-D-glucose as a galloyl donor. Furthermore, two adjacent galloyl moieties of 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose are oxidized to yield hexahydroxydiphenoyl moieties, which define ellagitannins such as oenothein B. Although the enzymes for the above-mentioned reactions have been isolated and characterized, their genes have not been cloned. Recently, genes encoding UDP–glucose:gallate glucosyltransferases were identified in *Vitis vinifera* and *Quercus robur* (Khater et al., 2012; Mittasch et al., 2014). They are members of the UDP glycosyltransferase (UGT) superfamily, which transfer sugars from UDP-activated sugar donors to a wide range of acceptors, including specialized metabolites and hormones (Bowles et al., 2006).

Here, we report the identification and characterization of genes encoding UDP–glucose:gallate glucosyltransferase, which catalyzes the formation of 1-*O*-galloyl-β-D-glucose, the first committed step of HT biosynthesis, in *E. camaldulensis*. Candidate genes were expressed in *Escherichia coli* and the encoded enzymes were assayed for UGT activity toward gallic acid and related compounds. In *E. camaldulensis*, the effect of Al treatment on the expression of candidate genes was investigated. *Eucalyptus* species are suitable to elucidate the *in planta* functions of HTs because of their high accumulation capacity for HTs (Marsh et al., 2017) and the availability of genomic information and adapted techniques for genetic transformation (Hirakawa et al., 2011; Matsunaga et al., 2012; Myburg et al., 2014).

2. Results and discussion

2.1. Cloning of UGTs from E. camaldulensis

In the *E. camaldulensis* genome database (http://www.kazusa.or.jp/eucaly/), ca. 400 genes have been annotated as UGT genes by Pfam (ID PF00201). The number of putative UGT genes in *E. camaldulensis* is comparable with that of *Eucalyptus grandis* v2.0 genome (374 annotated UGT genes), but much larger than the

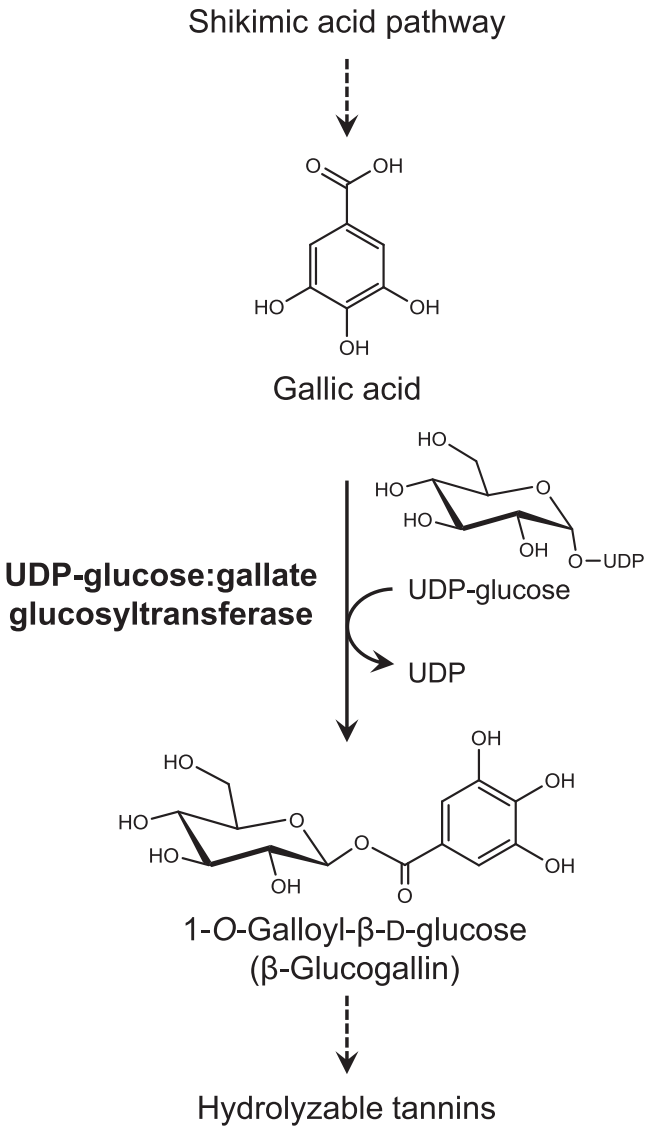

Fig. 1. Biosynthesis of 1-*O*-galloyl-β-D-glucose.

number of UGTs in *Arabidopsis thaliana* (115 genes), in *Oryza sativa* v7 (197 genes), and in *Populus trichocarpa* v3.0 (218 genes; http://phytozome.jgi.doe.gov/). Based on homology with UGT84A13, a previously identified 1-*O*-galloyl-β-D-glucose-forming UGT from *Quercus robur* (Mittasch et al., 2014), we selected four candidate UGT genes from the *E. camaldulensis* genome database (EcC009618.20, EcC011839.10, EcC05022.10, and EcC048085.30). The genes EcC009618.20, EcC011839.10, and EcC05022.10 were each represented by two closely related sequence variants. Therefore, in total, our homology-based reverse transcription polymerase chain reaction (RT-PCR) cloning approach resulted in the isolation of seven full-length candidate cDNAs from the roots of *E. camaldulensis*.

UGT superfamily proteins in plants have been grouped into 57 families (UGT71–99 and 701–728) at the time of writing, according to the UGT Nomenclature Guidelines (Mackenzie et al., 1997). UGTs that catalyze the esterification of glucose with acceptor molecules are known to cluster in phylogenetic group L consisting of UGT74, 75, and 84 families (Lim et al., 2001, 2002; Milkowski et al., 2000). Phylogenetic analysis of the deduced amino acid sequences of the seven candidate EcUGTs showed that four of these belong to