Identification and Expression Analysis of the Rehmannia WSD Acyltransferase Gene (Post-print)

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Abstract

Plant wax ester synthase catalyzes the synthesis of wax esters from long-chain alcohols and long-chain fatty acids, which plays a vital role in plant cuticular wax synthesis and resistance to environmental stresses including drought, pathogen attack, UV radiation, cold, and insect infestation. Cadmium is one of the most prevalent toxic heavy metals in the environment, posing severe threats to plant growth, development, quality, yield, and food safety. To investigate the expression of Rehmannia glutinosa wax ester synthase genes under cadmium stress, this study identified their members from full-length transcriptome sequencing data of R. glutinosa and analyzed the physicochemical properties, phylogenetic evolution, conserved domains, tissue expression, and cadmium stress expression of their encoded proteins using bioinformatics techniques and qRT-PCR. The results demonstrated that: (1) Two wax ester synthase genes, RgOATWSD1 and RgOATWSD2, were identified; the lengths, theoretical isoelectric points, and relative molecular weights of their encoded proteins were 463 aa and 473 aa, 8.86 and 9.34, and 51.31 kD and 52.49 kD, respectively, all of which were unstable proteins. (2) Both possessed the acyl_{{{WS}}_{{DGAT}}} conserved domain and DUF1289 superfamily, with the former comprising 92.65%–94.50% of their amino acid sequences. (3) Both were localized in the endoplasmic reticulum, with random coils and α-helices as the predominant secondary structures; RgOATWSD1 was a transmembrane protein, whereas RgOATWSD2 was not. (4) Both were differentially expressed in the roots, stems, and leaves of R. glutinosa. (5) Their expression was induced by cadmium stress, but their expression patterns differed. This study identified two wax ester synthase genes responsive to cadmium stress, laying a foundation for research on the cadmium stress expression and function of RgOATWSD in R. glutinosa.

Full Text

Identification and Expression Analyses of O-Acyltransferase WSD Genes in *Rehmannia glutinosa*

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Abstract

Wax ester synthase catalyzes the synthesis of wax esters from long-chain alcohols and fatty acids, playing crucial roles in plant wax synthesis and conferring resistance to various environmental stresses including drought, pathogenic bacteria, ultraviolet radiation, cold, and insect invasion. Cadmium (Cd) is one of the most prevalent toxic heavy metals in the environment, posing severe threats to plant growth, development, quality, yield, and food safety. To investigate the expression patterns of wax ester synthase genes in *Rehmannia glutinosa* under Cd stress, we identified candidate genes from full-length transcriptome sequencing data and analyzed their encoded proteins' physicochemical properties, phylogenetic relationships, and conserved domains using bioinformatics approaches. We further examined their tissue-specific and Cd stress-responsive expression via qRT-PCR. The results revealed: (1) Two wax ester synthase genes, designated *RgOATWSD1* and *RgOATWSD2*, were identified. Their encoded proteins consist of 463 and 473 amino acids, respectively, with theoretical isoelectric points of 8.86 and 9.34, and molecular weights of 51.31 kD and 52.49 kD. Both proteins were predicted to be unstable. (2) Both proteins contain the conserved acyl_{{{WS}}_{{DGAT}}} domain and DUF1289 superfamily, with the former covering 92.65%–94.50% of the amino acid sequences. (3) Both proteins localize to the endoplasmic reticulum, with secondary structures dominated by random coils and α-helices. *RgOATWSD1* is a transmembrane protein, whereas *RgOATWSD2* is not. (4) Both genes exhibit differential expression in roots, stems, and leaves of *R. glutinosa*. (5) Expression of both genes is induced by Cd stress, though with distinct expression patterns. This study identifies two Cd-responsive wax ester synthase genes, providing a foundation for further investigation of Cd stress responses and functional characterization of *RgOATWSD* genes.

Keywords: *Rehmannia glutinosa*, O-acyltransferase WSD, bioinformatics analysis, gene expression, cadmium stress

Introduction

Accelerated industrialization and urbanization in China have exacerbated heavy metal contamination in soils and food products, with cadmium (Cd) being the

primary pollutant. Cd-contaminated soils are difficult to remediate and severely affect plant growth, quality, and yield. Furthermore, Cd transfer through the food chain from plants to animals and humans poses significant health risks. Phytoremediation using naturally occurring or genetically modified plants to absorb, detoxify, and accumulate Cd from contaminated soils represents an environmentally and economically valuable approach (Chen et al., 2022; Luo et al., 2022; Yu et al., 2022). To date, several Cd-responsive genes have been identified, including the transcription factor *BpbZIP1* from paper mulberry (Chen et al., 2022), the NAS gene family in peanut (Luo et al., 2022), the *TaAlaAT* gene in wheat (Yu et al., 2022), the WRKY gene family in *Sedum plumbizincicola* (Wang et al., 2023), and *OsPT1* in rice (Jiang et al., 2023). However, numerous plant genes involved in Cd stress responses remain to be identified and functionally characterized, such as plant wax ester synthase (WS or WSD) genes.

Wax esters, synthesized from long-chain alcohols and fatty acids via esterification catalyzed by wax ester synthase, are widely distributed in animals, plants, and microorganisms, serving diverse biological functions. In plants, wax esters primarily exist in the cuticular layer of young shoots or in jojoba seed oil, protecting against water loss and drought (Xiao et al., 2020; Wang et al., 2023), pathogen attack and UV radiation (Xiao et al., 2020), cold stress (Zhu et al., 2022), and insect damage (Lin et al., 2020). Wax ester synthase genes have been cloned from various plants including wheat (Wen et al., 2021), sorghum (Wang et al., 2023), coconut (Zhu et al., 2022), *Arabidopsis* and jojoba (Xiao et al., 2020), *Stipa capillata* and *Stipa purpurea* (Yang et al., 2020), turnip (Yang et al., 2019), *Dianthus spiculifolius* (Zhou et al., 2018), grape (Nicolas et al., 2020), apple (Wang et al., 2022), and sunflower (Shalini & Martin, 2020). However, no studies have reported the cloning of WSD genes from *R. glutinosa* or their expression under Cd stress.

Rehmannia glutinosa, a perennial herb in the Scrophulariaceae family, is native to Liaoning, Inner Mongolia, and Henan provinces in China (Zhou et al., 2020). Its tuberous roots are a renowned traditional Chinese medicinal material with an annual demand of approximately 1.5$×10^{7}$ kg. Medicinally, it is classified into three types: fresh *Rehmannia*, dried *Rehmannia*, and processed *Rehmannia*. Fresh *Rehmannia* is sweet and bitter, used for clearing heat, promoting fluid production, cooling blood, and nourishing blood. Dried *Rehmannia* is sweet and cold, used for clearing heat and nourishing yin. Processed *Rehmannia* is slightly warm and sweet, used for nourishing blood and yin, and replenishing essence (Xu & Fu, 2017). While extensive research has been conducted on *R. glutinosa* germplasm resources, pharmacological effects, chemical constituents, cultivation, breeding, and omics, studies on Cd pollution and stress responses are limited. Only the *RgABCC* gene has been reported to respond to Cd stress in *R. glutinosa* (Zhao et al., 2009; Yang et al., 2021), indicating that further investigation of Cd-responsive genes in this species is warranted.

In this study, we utilized *R. glutinosa* as experimental material to identify wax

ester synthase (WS or WSD) and their encoding genes (*RgOATWSD*) from full-length transcriptome sequencing data. Using bioinformatics, pot cultivation, and real-time quantitative PCR, we analyzed the physicochemical properties, phylogenetic relationships, and conserved domains of the encoded proteins, as well as their tissue-specific and Cd stress-responsive expression patterns. The study aimed to address three questions: (1) whether wax ester synthase genes exist in *R. glutinosa*; (2) the size of these genes and characteristics of their encoded proteins; and (3) the spatial expression patterns and Cd stress responses of these genes.

Materials and Methods

1.1 Materials

The full-length transcriptome sequencing data for the *R. glutinosa* "Jinjiu" variety, including gene annotation tables and CDS tables, were obtained from NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA780233). Potted *R. glutinosa* "Jinjiu" plants were cultivated in a climate chamber at the College of Life Sciences, Henan Normal University, under a 14 h light/8 h dark photoperiod at (26$±$2)°C with a light intensity of 2,000 lx.

1.2.1 Identification of Acyltransferase WSD Gene Family Members

The gene annotation table was opened and searched using the terms "Wax ester synthase," "WS," or "WSD" to identify target enzymes and their corresponding gene names. These gene names were then used to retrieve the genes, their sizes, and nucleotide sequences from the CDS table, which were designated as *RgOATWSD* genes.

1.2.2 Bioinformatics Analysis of *RgOATWSD* Genes

The ORF Finder tool in NCBI (https://www.ncbi.nlm.nih.gov/home/analyze/) was used to deduce amino acid sequences, and the blastn tool was employed to identify homologous sequences. Multiple sequence alignment of amino acids was performed using DNAMAN 6.0 software. Phylogenetic trees were constructed using MEGA 6.0 software. Physicochemical properties of RgOATWSD proteins were predicted using the ExPASy-ProtParam online tool (https://web.expasy.org/protparam/). Subcellular localization was predicted using WoLF PSORT (https://wolfpsort.hgc.jp/). Transmembrane domains were analyzed using TMHMM 2.0. Conserved domains were analyzed using NCBI's Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Signal peptides were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). N-glycosylation sites were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). Phosphorylation sites were predicted using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/). Secondary and tertiary structures were predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=npsa_{sopma}.html) and

SWISS-MODEL (https://swissmodel.expasy.org/), respectively.

1.2.3 Cadmium Treatment of *R. glutinosa* Seedlings

Fresh *R. glutinosa* tuberous roots were cut into 2–3 cm pieces and planted in potting substrate (nutrient soil and vermiculite at a 1:1.5 ratio). Seedlings were cultivated in a climate chamber and watered every 2–3 days. After four weeks, uniformly growing seedlings were selected for treatment. Cadmium-treated seedlings (TC) were watered with 40 $mmol \cdot L^{-1}$ cadmium solution, while control seedlings (CK) received the same volume of water. Both groups had three replicates. After 12 h of treatment, roots, stems, and leaves were collected from both TC and CK groups, washed, dried, snap-frozen in liquid nitrogen, and stored at –80°C for further analysis (Yuan, 2023).

1.2.4 qRT-PCR Detection of Gene Expression

Based on the nucleotide sequences of *RgOATWSD1* and *RgOATWSD2*, qRT-PCR primers were designed using Primer Premier 5: (1) *RgOATWSD1* forward primer: 5'-AGCGAGTTGTTGCTGATGC-3', reverse primer: 5'-GTTGCCCCACTGACTTCCA-3'. (2) *RgOATWSD2* forward primer: 5'-TGATAAGTCGCCGATTAGGTC-3', reverse primer: 5'-CTTTTGATGGTTCGATGTGCT-3'. Primers were synthesized by Suzhou Genewiz Biotechnology Co., Ltd. Total RNA extraction and qRT-PCR were performed using methods established in our previous research, with the *TIP41* gene as an internal reference. Expression levels of both genes in roots, stems, and leaves of TC and CK groups were detected by qRT-PCR, and relative expression was calculated using the 2^(-ΔΔCt) method (Li et al., 2022).

Results

2.1 Identification of the *RgOATWSD* Gene Family

In the gene annotation table, we identified "Wax ester synthase-like Acyl-CoA acyltransferase domain" or "O-acyltransferase WSD1 (*Arabidopsis thaliana*)" and "O-acyltransferase WSD1-like isoform X2 [*Sesamum indicum*]" with corresponding gene names F01_{{{transcript}}*{{13342}}} and F01*{{{transcript}}_{{16419}}}. In the CDS table, these genes were found to be 1,392 bp and 1,422 bp in size, respectively [Figure 1: see original paper]. They were designated *RgOATWSD1* and *RgOATWSD2*, collectively referred to as *RgOATWSD* genes.

2.2 Amino Acid Sequence Derivation, Homology Alignment, and Phylogenetic Analysis

Based on the nucleotide sequences of *RgOATWSD1* and *RgOATWSD2*, DNAMAN 6.0 software was used to deduce the encoded proteins consisting of 463 and 473 amino acid residues, respectively, designated RgOATWSD1

and RgOATWSD2 [Figure 1: see original paper]. Multiple sequence alignment using DNAMAN 6.0 revealed that RgOATWSD1 shares high similarity with *Paulownia fortunei* (KAI3448120.1, 87.79%), *Sesamum indicum* (XP_{011092557}.1, 83.62%), and *Handroanthus impetiginosus* (PIM99635.1, 82.51%). RgOATWSD2 shows high similarity with *Paulownia fortunei* (KAI3457227.1, 83.01%), *Sesamum indicum* (XP_{011100361}.1, 78.69%), and *Phtheirospermum japonicum* (GFP86401.1, 77.73%). Phylogenetic analysis using MEGA 6.0 showed that RgOATWSD1 clusters with *Paulownia fortunei*, indicating the closest evolutionary relationship, while RgOATWSD2 clusters with *Phtheirospermum japonicum*. Notably, both *Paulownia fortunei* and *Phtheirospermum japonicum* belong to the Scrophulariaceae family [FIGURE:2, FIGURE:3].

2.3 Physicochemical Properties of RgOATWSD

Analysis using the ExPASy-ProtParam tool revealed that RgOATWSD1 comprises 463 amino acid residues including 20 amino acid types, with Ala (8.4%), Leu (11.0%), and Ser (8.2%) being most abundant. Its molecular weight is 51,310.89 Da, with a chemical formula of $C_{2283}H_{3693}N_{639}O_{649}S_{26}$, containing 50 negatively charged residues (Asp+Glu) and 57 positively charged residues (Arg+Lys). The theoretical isoelectric point is 8.86, instability index 45.19, aliphatic index 102.76, and grand average of hydropathicity 0.049, indicating an unstable, hydrophobic protein. RgOATWSD2 comprises 473 amino acid residues with Leu (11.6%), Lys (7.6%), and Ser (7.6%) being most abundant. Its molecular weight is 52.49 kD, chemical formula $C_{2353}H_{3791}N_{629}O_{679}S_{23}$, containing 44 negatively charged residues and 57 positively charged residues. The theoretical isoelectric point is 9.34, instability index 42.93, aliphatic index 92.96, and grand average of hydropathicity –0.140, indicating an unstable, hydrophilic protein.

NetPhos 2.0 analysis identified 42 and 44 phosphorylation sites with scores >0.5 in RgOATWSD1 and RgOATWSD2, respectively, distributed uniformly along the polypeptide chains. RgOATWSD1 contains 37 serine, 18 threonine, and 7 tyrosine phosphorylation sites, while RgOATWSD2 contains 36 serine, 31 threonine, and 12 tyrosine phosphorylation sites. NetNGlyc 1.0 analysis revealed no N-glycosylation sites in either protein.

TMHMM 2.0 analysis showed that RgOATWSD1 is a single-pass transmembrane protein, with amino acids 1–184 located inside the membrane, 185–207 spanning the membrane, and 208–463 outside the membrane. In contrast, RgOATWSD2 lacks transmembrane domains and is entirely extracellular. SignalP-4.1 analysis detected no signal peptide peaks in either protein, indicating they are not secreted proteins. WoLF PSORT predicted subcellular localization in the endoplasmic reticulum for both proteins. SOPMA secondary structure analysis revealed that RgOATWSD1 consists of 39.96% α-helices, 39.09% random coils, 17.71% extended strands, and 3.24% β-turns, with α-helices being most abundant. RgOATWSD2 comprises 39.32% α-helices,

41.01% random coils, 16.49% extended strands, and 3.17% β-turns, with random coils being most abundant [Figure 4: see original paper].

2.4 Conserved Domain Prediction

NCBI Conserved Domain Search revealed that both RgOATWSD1 and RgOATWSD2 contain the acyl_{{{WS}}*{{DGAT}}} conserved domain and DUF1289 superfamily. The acyl*{{{WS}}_{{DGAT}}} domain covers 92.65% of RgOATWSD1 and 94.50% of RgOATWSD2 amino acid sequences. This family represents an acyltransferase family similar to WS/diacylglycerol acyltransferase (DGAT) and monoacylglycerol acyltransferase (MGAT), exhibiting DGAT- and MGAT-like activities. The DUF1289 domain accounts for 7.35% and 5.5% of the proteins, respectively, with unknown function [Figure 5: see original paper].

2.5 Tertiary Structure Analysis

SWISS-MODEL tertiary structure analysis confirmed the presence of secondary structural elements consistent with the above predictions [Figure 4: see original paper]. However, the tertiary structures of RgOATWSD1 and RgOATWSD2 differed morphologically, with RgOATWSD1 exhibiting a more globular shape [Figure 6: see original paper].

2.6 Effects of Cadmium on *RgOATWSD* Gene Expression in Different *R. glutinosa* Tissues

qRT-PCR analysis revealed that both *RgOATWSD1* and *RgOATWSD2* are expressed in roots, stems, and leaves of both control and Cd-treated seedlings. *RgOATWSD1* showed highest expression in leaves, while *RgOATWSD2* was most highly expressed in roots. Under Cd stress, expression levels of both genes changed significantly compared to controls, but in opposite directions: *RgOATWSD1* was upregulated in roots, stems, and leaves, with the most pronounced change in roots, whereas *RgOATWSD2* was downregulated in all tissues, with the most significant decrease in stems [Figure 7: see original paper]. These results demonstrate differential expression patterns of the two genes in response to Cd stress in *R. glutinosa*.

Discussion

Plant fatty acid metabolism occurs primarily through the acyl reduction pathway, which produces wax esters, and the decarbonylation pathway, which forms alkanes, aldehydes, ketones, and secondary alcohols. While WS or WSD1 genes have been reported in jojoba and *Arabidopsis*, no studies have described WSD genes in *R. glutinosa*. This study identified two wax ester synthase genes, *RgOATWSD1* and *RgOATWSD2*, from *R. glutinosa* full-length transcriptome data. These genes show high homology with known plant wax ester synthase genes and likely possess similar functions (Kunst & Samuels, 2009). Both

RgOATWSD1 and RgOATWSD2 localize to the endoplasmic reticulum, consistent with *Arabidopsis* WSD1 localization (Xiao et al., 2020). Additionally, RgOATWSD1 exhibits transmembrane regions and hydrophobicity similar to jojoba WS (Xiao et al., 2020). Spatial expression analysis revealed that both genes are expressed in roots, stems, and leaves at varying levels, with expression in stems and leaves matching the pattern observed for *Arabidopsis WSD1* (Patwari et al., 2019). These findings suggest that *RgOATWSD* genes share structural features and spatial expression characteristics with known wax ester synthase genes from other plant species.

Plant cuticular waxes serve as protective barriers against environmental stresses, shielding plants from drought, salt, cold, UV radiation, pathogens, and insects. For instance, cuticular wax ester accumulation and biosynthesis are closely associated with drought resistance responses (Seo et al., 2011; Patwari et al., 2019). *Arabidopsis AtWSD1* contributes to wax ester accumulation during drought, with increased wax deposition in leaves and stems, indicating its key role in wax synthesis (Patwari et al., 2019). Apple *MdWSD1* regulates ester and alcohol synthesis, affecting cuticular wax content (Wang, 2016). Turnip *BrrWSD1* splice variants function in wax ester synthesis under drought stress (Yang et al., 2019). Cabbage *BoWSD1* shows varied expression patterns under different treatments, suggesting its involvement in responses to abiotic stresses including NaCl, drought, cold, and heat (Zeng et al., 2020). However, to our knowledge, no studies have reported Cd stress-responsive expression of plant WSD genes. Therefore, we investigated *RgOATWSD* expression under Cd stress concurrently with our team's studies on Cd stress in *R. glutinosa* and cotton (Wei, 2023; Yuan, 2023). We found significant responses of *RgOATWSD* genes to Cd stress, expanding the scope of abiotic stress responses for plant WSD genes. The mechanism underlying this Cd stress response remains to be elucidated.

The molecular mechanisms of plant Cd stress responses are not fully understood (Zhang et al., 2023), but several key findings have emerged. Cd stress activates plasma membrane Ca^{2+} signaling pathways (Zhang et al., 2023) and triggers physiological and biochemical regulatory networks, including maintenance of reactive oxygen and nitrogen species, antioxidant enzymes, non-enzymatic antioxidants, calcium signaling, hormones, endoplasmic reticulum processing, regulatory proteins, and gene expression changes (An et al., 2021). Regarding gene expression, families such as NRAMP (involved in Cd uptake, transport, and detoxification), HMA (Cd transport to shoots), MYB, ABA, ZIP, bHLH, and RCD1 have been implicated in Cd stress responses (Wei, 2023). While *RgOATWSD1* and *RgOATWSD2* responses to Cd stress have not been previously reported, their endoplasmic reticulum localization suggests their Cd stress response mechanism may involve endoplasmic reticulum processing and gene regulatory pathways similar to those described above (An et al., 2021).

In summary, this study successfully identified two wax ester synthase genes, *RgOATWSD1* and *RgOATWSD2*, from *R. glutinosa* full-length transcriptome data. Bioinformatics analysis revealed their physicochemical properties and

structural characteristics, while expression analysis demonstrated distinct spatial expression patterns in roots, stems, and leaves and differential responses to Cd stress. These findings expand the functional gene database for *R. glutinosa* and provide new insights into Cd-responsive wax ester synthase genes in plants, laying a foundation for further studies on their structure, function, and molecular mechanisms of Cd stress response.

References

AN TT, HUANG D, WANG H, et al., 2021. Research advances in plant physiological and biochemical mechanisms in response to cadmium stress[J]. Chin Bull Bot, 56(3): 347-362.

CHEN SS, XIE MH, CUI MK, et al., 2022. Identification of *Broussonetia papyrifera* transcription factor *BpbZIP1* and analysis of its response to cadmium stress[J]. Bull Bot Res, 42(3): 394-402.

JIANG N, SHI Y, ZHAO ZH, et al., 2023. Expression and functional analysis of *OsPT1* gene in rice under cadmium stress[J]. Biotechnol Bull, 39(1): 166-174.

KUNST L, SAMUELS L, 2009. Plant cuticles shine: advances in wax biosynthesis and export[J]. Curr Opin Plant Biol, 12(6): 721-727.

LI JJ, ZHU JL, LI HM, et al., 2022. The effects of NAA on the tuberous root yield and quality of *Rehmannia glutinosa* and its regulatory mechanism by transcriptome and metabolome profiling[J]. Curr Issues Mol Biol, 44(8): 3291-3311.

LIN N, FU LJ, SONG YQ, et al., 2020. Wax composition and concentration in jujube (*Ziziphus jujuba* Mill.) cultivars with different resistance to fruit cracking[J]. J Plant Physiol, 255(12): 153294.

LUO YF, WANG CH, ZHANG Z., et al., 2022. Genome-wide identification of peanut NAS family and their expression response to iron deficiency and cadmium stress[J]. Plant Physiol J, 58(6): 1119-1132.

NICOLAS D, RICCO T, WONG DCJ, et al., 2020. Drought stress modulates cuticular wax composition of the grape berry[J]. J Exp Bot, 71(10): 3126-3141.

PATWARI P, SALEWSKI V, GUTBROD K, et al., 2019. Surface wax esters contribute to drought tolerance in *Arabidopsis*[J]. Plant J, 98(4): 727-744.

SEO PJ, LEE SB, MI CS, et al., 2011. The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis*[J]. Plant Cell, 23(3): 1138-1152.

SHALINI T, MARTIN A, 2020. Identification, isolation, and heterologous expression of Sunflower wax synthase for the synthesis of tailored wax esters[J]. J Food Biochem, 44(10): e13433.

WANG CY, LI ZJ, WANG P, et al., 2023. Physiological and biochemical analysis of drought resistance in sorghum cuticular wax-deficient mutant sb1[J]. Biotechnol Bull, 39(5): 46-53.

WANG DY, 2016. The clone of *MdCER4*, *MdWSD1*, *MdMAH1* and their response to ethylene regulation[D]. Zibo: Shandong University of Technology: 16-25.

WANG HB, LV XY, LI YY, et al., 2022. Identification and expression analysis of WSD (Wax ester synthase/diacylglycerol acyltransferase) gene family in apple[J]. Erwerbs-Obstbau, 6: 1-12.

WANG JC, QIU WM, JIN KM, et al., 2023. Comprehensive analysis of WRKY transcription factor in *Sedum plumbizincicola* responding to cadmium stress[J]. J Nanjing Forestry Uni (Nat Sci Ed), 47(2): 49-60.

WEN HW, DANG YF, DONG FF, et al., 2021. Analysis on differentially expressed genes of flag leaf epidermis wax based on transcriptome sequencing in wheat[J]. J Agric Sci Technol, 49(12): 1462-1466.

WEI X, 2023. Function and molecular mechanism of *GhRCD1* gene in cotton under Cd stress[D]. Xinxiang: Henan Normal University: 16-25.

XIAO YF, PENG J, ZHANG Y, et al., 2020. Research progress and applications of wax ester synthase[J]. J Beijing Uni Chem Technol (Nat Sci Ed), 47(6): 1-11.

XU J, FU ZD, 2017. Discussion on the historical evolution and efficacy of processed products of *Rehmannia glutinosa*[J]. J Tradit Chin Med, 39(9): 1913-1916.

YANG YH, WANG CJ, LI RF, et al., 2021. Transcriptome-based identification and expression characterization of *RgABCC* transporters in *Rehmannia glutinosa*[J]. PLoS ONE, 16(6): e0253188.

YANG YQ, PU YN, YIN X, et al., 2019. A splice variant of *BrrWSD1* in turnip (*Brassica rapa* var. *rapa*) and its possible role in wax ester synthesis under drought stress[J]. J Agric Food Chem, 67(40): 11077-11088.

YANG YQ, ZHOU ZL, LI Y, et al., 2020. Uncovering the role of positive selection site of wax ester synthase/diacylglycerol acyltransferase in two closely related *Stipa* species in wax ester synthesis under drought stress[J]. J Exp Bot, 71(14): 4159-4170.

YU YA, ZHANG XB, ZHANG L, et al., 2022. Cloning of *TaAlaAT* gene in wheat (*Triticum aestivum* L.) and its expression analysis under cadmium stress[J]. Jiangsu Agric Sci, 50(18): 48-53.

YUAN P, 2023. Study on the tolerance of *Rehmannia Glutinosa* to heavy metal cadmium stress[D]. Xinxiang: Henan Normal University: 50-51.

ZENG AS, XU YY, SONG LX, et al., 2020. Validation of suitable reference genes for qRT-PCR in cabbage (*Brassica oleracea* L.) under different abiotic

stress experimental conditions[J]. J Plant Biochem Biotechnol, 30(1): 184-195.

ZHAO CJ, BAI L, OU YF, et al., 2009. Decontamination of organochlorine pesticides residue and heavy metal in *Rehmannia glutinosa* Libosch by SFE[J]. J Chromatogr Sci, 47(10): 919-924.

ZHOU A, LIU E, JIAO L, et al., 2018. Characterization of increased cuticular wax mutant and analysis of genes involved in wax biosynthesis in *Dianthus spiculifolius*[J]. Hortic Res, 5(40): 3-9.

ZHOU YQ, SHAO LY, GUO MM, et al., 2020. Cloning and bioinformatics analysis of C3H gene in *Rehmannia glutinosa*[J]. Guihaia, 40(9): 1281-1287.

ZHU C, WANG FY, SUN XW, et al., 2022. Bioinformatics and expression analysis of wax synthesis related genes in coconut[J]. Mol Plant Breed, 20(13): 4289-4295.

ZHANG YT, WANG ZQ, LIU YS, et al., 2023. Plasma membrane-associated calcium signaling modulates cadmium transport[J]. New Phytol, 238(1): 313-331.

Note: Figure translations are in progress. See original paper for figures.