ROS1-mediated decrease in DNA methylation and increase in expression of defense genes and stress response genes in Arabidopsis thaliana due to abiotic stresses

Liping Yang¹, Chenjing Lang¹, Yanju Wu¹, Dawei Meng¹, Tianbo Yang², Danqi Li³, Taicheng Jin¹* and Xiaofu Zhou¹*

Abstract

Background: Small interfering RNAs (siRNAs) target homologous genomic DNA sequences for cytosine methylation, known as RNA-directed DNA methylation (RdDM), plays an important role in transposon control and regulation of gene expression in plants. Repressor of silencing 1 (ROS1) can negatively regulate the RdDM pathway.

Results: In this paper, we investigated the molecular mechanisms by which an upstream regulator ACD6 in the salicylic acid (SA) defense pathway, an ABA pathway-related gene ACO3, and GSTF14, an endogenous gene of the glutathione S-transferase superfamily, were induced by various abiotic stresses. The results demonstrated that abiotic stresses, including water deficit, cold, and salt stresses, induced demethylation of the repeats in the promoters of ACD6, ACO3, and GSTF14 and transcriptionally activated their expression. Furthermore, our results revealed that ROS1-mediated DNA demethylation plays an important role in the process of transcriptional activation of ACD6 and GSTF14 when Arabidopsis plants are subjected to cold stress.

Conclusions: This study revealed that ROS1 plays an important role in the molecular mechanisms associated with genes involved in defense pathways in response to abiotic stresses.

Keywords: DNA methylation, Salicylic acid, ROS1-mediated DNA demethylation, Abiotic stresses

Background

DNA methylation is one of the most common forms of covalent DNA modification in the genomes of eukaryotes and plays an important role in the growth and development of plants and in responses to various abiotic stresses. RNA silencing is a conserved pathway that results in the blockage of gene expression in both the cytoplasm and nucleus of eukaryotic organisms [1]. In plants, small interfering RNAs (siRNAs) target homologous sequences for DNA methylation, a process known as RNA-directed DNA methylation (RdDM); this process plays an important role in regulating gene expression, controlling the activity of transposable elements, and defending against foreign DNAs, such as DNA viruses [2–4]. These siRNAs are synthesized by RNA polymerase IV (Pol IV), RNA-dependent RNA polymerase (RDR2), and Dicer-like 3 (DCL3) [5]. Argonaute protein 4 (AGO4) and the DNA methyltransferases DRM1/2, MET1, and CMT3 perform de novo methylation and maintain methylation of the target DNA [6]. DNA methylation can be reversed by DNA glycosylases/lyases in Arabidopsis plants, and this process is known as active demethylation [7]. Repressor
of silencing 1 (ROS1) can negatively regulate the RdDM pathway [8, 9]. ROS1-mediated DNA demethylation helps determine genomic DNA methylation patterns and protects active genes from being silenced [10].

Abiotic stresses mainly include drought, cold, and salt stresses, which severely threaten plant growth and crop yields [11, 12]. Abiotic stresses can induce the accumulation of endogenous abscisic acid (ABA), triggering ABA signal transduction to cope with adverse environmental factors [13–15]. When plants are under cold stress, ABA can regulate the expression of cold resistance genes in plants in response to stress [16–18]. Abiotic stress also affects dynamic changes in DNA methylation in plants. Changes in methylation levels and patterns regulate the expression of stress-responsive genes, thereby improving the resistance of plants to stress [19]. In Arabidopsis, the ros1 mutant is hypersensitive to ABA, and ROS1 participates in the ABA response by regulating the expression of NICOTINAMIDASE 3 (NIC3) [20]. Soybean has been found to show abnormal expression of approximately 49 transcription factors under salt stress, and the expression profiles of the MYB, b-ZIP, and AP2/DREB transcription factor families are reportedly significantly correlated with the DNA methylation of their gene sequences [21]. Abiotic stress can regulate the expression of stress-responsive genes by inducing dynamic changes in DNA methylation, thereby improving the adaptability of plants to the environment.

Salicylic acid (SA) is an important signaling molecule in plant defense responses and can induce the expression of defense genes and the development of systemic resistance [22]. At least three types of SA regulators have been described [23]: type I regulators, including enzymes involved in SA biosynthesis, e.g., SA INDUCTION-DEFICIENT 2 (SID2) [24], type II regulators such as accelerated cell death 6 (ACD6), which are upstream from SA [25–27], and type III regulators, which transduce signals downstream from SA, e.g., NONEXPRESSOR OF PR GENES 1 (NPR1) [28]. A gain-of-function mutant of ACD6 (acd6–1) has been reported to increase the expression of defense genes in the SA pathway [29]. Plants respond to pathogens via the SA, jasmonic acid (JA), and ethylene (ET) pathways [2]. The role of SA in plant tolerance to various abiotic stresses has been extensively studied [30]. SA also plays an important role in modulating plant responses to many abiotic stresses, including salt, drought, and chilling [31]. For example, salinity induces increases in endogenous SA levels and the activity of the SA biosynthesis enzyme in rice seedlings [32]. Our previous study revealed the molecular mechanisms underlying the induction of defense genes in the SA pathway by biotic stresses in Arabidopsis plants [4, 33]. However, the regulatory mechanisms of genes involved in defense pathways in response to abiotic stresses remain unclear.

In this study, we determined the molecular mechanisms underlying the functioning of the upstream regulator ACD6 of the SA pathway, the endogenous gene GSTF14 in the glutathione S-transferase (GST) superfamily, and aconitate hydratase 3 (ACO3) in response to abiotic stresses. The results showed that the expression levels of defense genes (ACD6, NPR1, and PR5) in the SA pathway, ACO3, and GSTF14 significantly increased after exposure to water deficit, cold, and salt stresses. Sequencing results confirmed that abiotic stresses induced demethylation of the repeats in the promoters of ACD6, ACO3, and GSTF14 and transcriptionally activated their expression. Further experiments revealed that ROS1-mediated DNA demethylation plays an important role in the mechanisms of these defense genes in response to abiotic stresses.

Results

Induction of SA pathway-related defense genes by abiotic stresses

Our previous studies verified an upstream regulator (ACD6) in the SA pathway, and GSTF14, an endogenous gene of the glutathione S-transferase superfamily that is implicated in numerous stress responses, which revealed the molecular mechanism underlying the induction of defense gene expression in the SA pathway by biotic stresses [4, 33]. To investigate whether abiotic stress can induce the expression of ACD6, GSTF14, and an ABA pathway-related gene (ACO3), the wild-type Columbia (Col-0) line of Arabidopsis thaliana was selected for water deficit treatment, cold stress treatment, and salt stress treatment. On days 5–7, the leaves of Col-0 plants treated with water deficit stress turned slightly yellow and shrunk (Fig. 1B, C) compared to those of untreated Col-0 plants (Fig. 1A). On day 14, anthocyanin accumulation in the leaves of Col-0 plants treated with water deficit stress clearly increased, and the leaves turned severely yellow and withered (Fig. 1D). No significant phenotypic changes were observed in plants treated with cold stress (4°C) for 24 h or salt stress (150 mM) for 3 days. Abiotic stresses make a large amount of Reactive Oxygen Species (ROS) accumulate in plant cells. ROS content can be served as a kind of stress makers, including hydrogen peroxide content and superoxide anion. To confirm that Col-0 plants treated under different conditions were indeed stressed, we performed the measurements of hydrogen peroxide content by spectrophotometry. The results showed that hydrogen peroxide content significantly increased in Col-0 plants after cold stress (4°C) for
24 h, water deficit for 7 days or salt stress (150 mM) for 3 days (Fig. 1E) and confirmed the treated plants were under the specific stress conditions.

We extracted total RNA from wild-type Arabidopsis Col-0 plants on the 7th day of water deficit treatment for comparative analysis of gene expression. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis confirmed that ACD6, GSTF14, and ACO3 were significantly upregulated after water deficit treatment, and the upregulation of GSTF14 expression was more pronounced (Fig. 1F). Since ACD6 is an upstream regulator of the SA pathway, the increase in ACD6 expression may upregulate the expression of the defense genes NPR1 and PR5 (Fig. 1F). To further investigate whether cold or salt stress can also induce the expression of defense genes in the SA pathway, we analyzed the expression of related genes in untreated Col-0 plants and Col-0 plants treated under different conditions. The results showed that compared with controls, Col-0 plants treated with cold or salt stress had significantly higher expression levels of defense genes ACD6, NPR1, PR5, and stress response genes GSTF14 and ACO3 and confirmed that cold stress and salt stress activated ACD6 expression, which was significantly increased after 24 h of cold stress treatment (Fig. 1G, H).

**Direct correlation between the increased expression of defense and stress resistance genes and the reduction in promoter DNA methylation**

To determine whether upregulation of these genes is correlated with a decrease in methylation at these genes’ promoters, the DNA methylation data of these genes were first searched at http://epigenomics.mcdb.ucla.edu/BS-Seq/[33, 34]. Extensive methylation was found in the promoter sequences corresponding to the promoter regions of ACD6 and ACO3, and strong methylation corresponding to the promoter region of GSTF14 was found through a methylation pattern search. Col-0 plants were treated with abiotic stresses, and cytosine methylation in the gene promoter regions were analyzed with bisulfite treatment. The DNA methylation levels in the region of the ACD6 promoter was reduced by 16.27% (a change from 78.30 to 62.03%) in CG sites, by 13.56% (a change from 21.67 to 8.11%) in CNG sites, and by 7.71% (a change from 13.51 to 5.80%) in CHH sites in Col-0 plants treated with water deficit stress. The DNA methylation levels in the region of the ACD6 promoter was reduced by 20.55% (a change from 78.32 to 57.77%) in CG sites, by 14.11% (a change from 21.67 to 7.56%) in CNG sites, and by 8.15% (a change from 13.51 to 5.36%) in CHH sites in Col-0 plants treated with cold stress. The DNA methylation levels in the region of the ACD6 promoter was reduced by 14.86% (a change from 78.32 to 63.46%) in CG sites, by 13.41% (a change from 21.67 to 8.26%) in CNG sites, and by 8.26% (a change from 13.51 to 5.25%) in CHH sites in Col-0 plants treated with salt stress (Fig. 2A).

The data revealed that CG methylation of the repeats in the ACO3 promoter was not significantly altered, while DNA methylation in the ACO3 promoter was reduced by 32.56% (a change from 65.89 to 33.33%) in CNG sites and by 33.33% (change from 42.22 to 8.89%) in CHH sites in Col-0 plants treated with water deficit stress. The DNA methylation levels in the ACO3 promoter was significantly reduced by 32.56% (a change from 65.89 to 20%) in CNG sites and by 33.33% (change from 42.22 to 8.16%) in CHH sites in Col-0 plants treated with cold stress. DNA methylation in the ACO3 promoter was significantly reduced by 44.46% (a change from 65.89 to 21.43%) in CNG sites and by 33.03% (a change from 42.22 to 9.19%) in CHH sites in Col-0 plants treated with salt stress (Fig. 2B). DNA methylation in the GSTF14 promoter was also analyzed using bisulfite sequencing. DNA methylation of the repeats in the GSTF14 promoter was reduced by 14.81% (a change from 90.30 to 75.49%) in CG sites, by 15.43% (a change from 64.04 to 48.61%) in CNG sites, and by 12.06% (a change from 20.78 to 8.72%) in CHH sites in Col-0 plants treated with cold stress. DNA methylation of the repeats in the GSTF14 promoter was reduced by 17.27% (a change from 90.30 to 73.03%) in CG sites, by 12.58% (a change from 64.04 to 51.46%) in CNG sites, and by 12.58% (a change from
Fig. 1 (See legend on previous page.)
20.78 to 9.63%) in CHH sites in Col-0 plants treated with cold stress. DNA methylation in the GSTF14 promoter was reduced by 14.80% (a change from 90.30 to 75.50%) in CG sites, by 11.29% (a change from 64.04 to 52.75%) in CNG sites, and by 12.43% (a change from 20.78 to 8.35%) in CHH sites in Col-0 plants treated with salt stress (Fig. 2C).

Role of ROS1 in regulation of the SA pathway in response to abiotic stresses

To further study the molecular mechanisms underlying the functioning of defense genes of the SA pathway in response to abiotic stresses, we used RNA gel blotting to analyze the expression of related genes in plants mutated at key functional elements of the RdDM pathway. The results showed that ACD6 and GSTF14 expression clearly increased in the mutant ago4 and DNA methyltransferase mutants met1, drm1/2, and cmt3 with Col-0 ecotypes as controls (Fig. 3A). RT-qPCR results further confirmed that ACD6, GSTF14, and ACO3 were upregulated in the ago4 mutant (Fig. 3B), indicating that RdDM has an important role in maintaining the low transcription levels of ACD6, GSTF14, and ACO3 in wild-type plants; however, these mutants showed increased transcript levels for those genes.

ROS1 can negatively regulate the RdDM pathway [8, 9]. To determine whether ROS1 plays a role in the responses of these genes to abiotic stress, we applied cold stress...
treatment to loss-of-function Arabidopsis ros1 mutants and Arabidopsis Col-0 plants. Under normal growth conditions, the ros1 mutants showed no obvious developmental defects compared to Col-0 plants. Compared with the cold stress-treated Col-0 plants, the cold stress-treated ros1 mutants appeared to exhibit more severely deformed leaves and increased anthocyanin accumulation on the 7th day (Fig. 3C), indicating that the ros1 mutants exhibited increased susceptibility to cold stress. We further compared the expression of the ACD6 gene between cold stress-treated ros1 mutants (ros1 + cold) and cold stress-treated Col-0 (Col-0 + cold) plants. The results showed that ACD6 expression in the cold stress-treated Col-0 plants significantly increased, compared with that in untreated Col-0 plants. However, the increase in ACD6 expression in the cold stress-treated ros1 mutants and loss-of-function ros1 dml2 dml3 (rdd) mutants was partially inhibited compared with that in the cold stress-treated Col-0 plants (Fig. 3D). ROS1 plays an important role in the activation of defense genes in response to abiotic stress, which was confirmed by the expression levels of GSTF14 and ACO3. When cold stress-treated Col-0 plants were used as the control, the increase in GSTF14 and ACO3 expression was partially inhibited in the cold stress-treated ros1 mutants (Fig. 3D).

ROS1-mediated decrease in DNA methylation of genes under abiotic stresses

Sequencing analysis demonstrated that the DNA methylation levels of the repeats in the ACD6 promoter in cold stress-treated Col-0 plants were significantly reduced compared with untreated Col-0 plants, including the CG, CNG, and CHH sites, while the decrease in DNA methylation levels of the repeats in the ACD6 promoter in cold stress-treated ros1 mutants was partially inhibited
(Fig. 4A). When Col-0 plants were used as the control, the DNA methylation levels of the repeats in the ACD6 promoter in ros1 mutants were not significantly altered (Fig. 4A).

The results demonstrated that DNA methylation at CNG and CHH sites in the ACO3 promoter in cold stress-treated Col-0 plants was significantly decreased compared with untreated Col-0 plants, while the decrease in DNA methylation at CNG and CHH sites in the ACO3 promoter in cold stress-treated ros1 mutants was partially inhibited (Fig. 4B). When Col-0 plants were used as the control, the DNA methylation levels of the repeats in the ACO3 promoter in ros1 mutants were not significantly altered (Fig. 4B).

The results further confirmed that the DNA methylation levels in the GSTF14 promoter in cold stress-treated Col-0 plants were significantly decreased compared with untreated Col-0 plants, including the CG, CNG, and CHH sites, while the decrease in DNA methylation in the GSTF14 promoter in cold stress-treated ros1 mutants was partially inhibited (Fig. 4C). When Col-0 plants were used as the control, the DNA methylation levels of the repeats in the GSTF14 promoter in ros1 mutants were not significantly altered (Fig. 4C). These results revealed that activation of the expression of the defense gene ACD6 in the SA pathway, the stress response genes GSTF14 and ACO3 was related to ROS1-mediated DNA demethylation.

**Discussion**

Scientists began to focus on the important role of hormones in the regulation of plant growth and development and resistance to abiotic stresses in 1930. In this field, the ABA pathway has been well studied. ABA is a key hormone regulating the response of plants to abiotic stresses, such as drought. A total of 40 stress-inducible transcription factor genes have been found in *Arabidopsis* [35]. For example, MYB transcription factors are indispensable to the adaptation of plants to cold stress and can affect plant resistance to drought by controlling...
stress-induced ABA synthesis [36]. We know less about the role of the SA defense pathway in the response of plants to abiotic stresses and the related molecular mechanisms.

In this study, we demonstrated that abiotic stresses, including water deficit, cold, and salt stresses, induced DNA demethylation of repeats in the promoters of ACD6, ACO3, and GSTF14 and transcriptionally activated their expression. Furthermore, our results confirmed that ROS1-mediated DNA demethylation plays a role in the process of transcriptional activation of the target genes (ACD6, ACO3, and GSTF14) regulated by RNA-directed DNA methylation (RdDM) when Arabidopsis Col-0 plants are subjected to cold stress.

Sequencing results confirmed that the increase in the expression of ACD6, GSTF14, and ACO3 was related to the reduction in the DNA methylation levels of the promoters of these genes. Under the same stress conditions, different genes differ in the levels and patterns of DNA methylation (Fig. 2), suggesting that complex molecular mechanisms regulate the expression of these genes. Our results revealed that abiotic stresses (water deficit, cold, and salt stresses) induced DNA demethylation of the ACD6, ACO3 and GSTF14 promoters and transcriptionally activated the expression of the defense genes ACD6, NPR1 and PR5 in the SA pathway and stress response genes ACO3 and GSTF14, thereby enhancing the adaptability of plants to abiotic stresses.

ROS1 can negatively regulate the RdDM pathway [8, 9]. Recent research has shown that ROS1-mediated DNA demethylation can act on three DNA methylation sites: CG, CNG, and CHH [37]. DNA methylation sequencing of ros1 mutants has revealed that ROS1 generally targets genes containing CG, CNG, and CHH methylation sites in transposable elements and repeats but does not target genes containing only CG methylation sites [38]. Furthermore, our results confirmed that the RdDM pathway has an important role in maintaining the low transcription levels of ACD6, GSTF14, and ACO3 in wild-type Col-0 plants (Fig. 3A, B). When cold stress-treated Col-0 plants were used as the control, the increase in the expression of ACD6, GSTF14, and ACO3 in ros1 mutants treated with cold stress for 24 h was partially inhibited (Fig. 3D). Furthermore, after 24 h of cold stress treatment in Col-0 plants, DNA methylation levels in the repeats of the ACD6, ACO3 and GSTF14 promoters were significantly reduced, while the decrease in DNA methylation levels in the repeats of the ACD6 and ACO3 promoters in cold stress-treated ros1 mutants was partially inhibited (Fig. 4). These data analyses indicate that ROS1 is only partially responsible for changes in expression of and levels of methylation of the target genes under cold stress.

This study has revealed the role of ROS1 in the regulation of defense genes ACD6, NPR1 and PR5 in the SA pathway and ACO3 and GSTF14 in response to abiotic stresses. Due to the complexity of the dynamic regulation of DNA methylation, the molecular mechanisms by which plants adapt to various adverse environmental factors and how different signaling pathways interact still require in-depth study.

Conclusions
Our study reveals the molecular mechanism by which plant defense genes in the SA pathway and stress resistance genes are involved in responses to various abiotic stresses. The results show that the RdDM pathway has an important role in maintaining the low transcription levels of ACD6, GSTF14, and ACO3 in wild-type Col-0 plants. Further studies revealed that abiotic stresses induced DNA demethylation of the ACD6, ACO3, and GSTF14 promoters and transcriptionally activated the expression of defense genes and stress resistance genes. Moreover, ROS1-mediated DNA demethylation plays an important role in this process.

Methods
A) Abiotic stress treatments and hydrogen peroxide content measurements

thaliana ecotype Columbia (Col-0) and mutant plants were used for this work. The ago4 mutant seeds (original source) [39] and ros1 and rdd mutant seeds (original source) [40] were provided by Chengguo Duan in Shanghai Center for Plant Stress Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (CAS). Col-0, met1, drm1/2, and cmt3 mutant seeds were provided by the Institute of Genetics and Developmental Biology, CAS. Seeds were surface-sterilized with 30% bleach, washed three times with sterile water, and sown on Murashige and Skoog (MS) plates. The seedlings were grown for approximately 2 weeks and then transferred to a 22°C environment with a 16-h light/8-h dark cycle for 2 weeks. Arabidopsis plants were transferred to soil in a greenhouse (22°C, with a 16-h light/8-h dark cycle) and treated with abiotic stresses, including cold stress (4°C, 24h), salt stress (150 mM NaCl, 3 days), and water deficit stress (not watered, 7 days). The measurements of hydrogen peroxide content were performed by spectrophotometry [41] after Col-0 plants were treated with cold stress, water deficit or salt stress, respectively [34]. Each experiment consisted of three biological replicates and was repeated twice. The significant experimental details are as follows.
RT-qPCR analysis and RNA gel blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocols. Total RNA was subsequently used for RT-qPCR analysis. For RT-qPCR, total RNA was extracted from the treated plants and subsequently used for reverse transcription. Complementary DNA synthesis was performed using a reverse transcription kit (Takara). Quantitative RT-PCR was performed using SYBR green mix (Qiagen). Each experiment consisted of three biological replicates and was repeated twice. For the high-molecular-weight RNA gel blot analyses, 10 μg of total RNA was extracted from the treated plants and separated on 1% agarose-formaldehyde gels, transferred to Hybond-N+ membranes, and hybridized as described previously [4]. ACD6 (AT4G14400) and GSTF14 (AT1G49860) probe primer pairs were as follows: F (ACD6), 5′-TCTCCCTGTTGAAGATGTCG-3′ and R (ACD6), 5′-TTACCGATGCAAACAGAGCC-3′; F (GSTF14), 5′-AGGCGATGCTCCCATACTTGG-3′ and R (GSTF14), 5′-TTATAGGCAAAACGACGCTGC-3′; F (ACO3), 5′-AGAAGTCATGAAAGCCA-3′ and R (ACO3), 5′-GAATCTCCATTACGTCAACCGC-3′.

Bisulfite sequencing

Total DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) buffer as previously described [23] and purified using a DNA purification kit (Promega). The purified DNA was used for bisulfite treatment using the EpiTect bisulfite kit (Qiagen, http://www.qiagen.com/default.aspx) according to the manufacturer’s instructions. The purified bisulfite-treated DNA was amplified by ACD6 (AT4G14400) and GSTF14 (AT1G49860) promoter-specific primer pairs as follows:

F (ACD6), 5′-AATTTATTTTAGTAAAGGAG-3′ and R (ACD6), 5′-TTTACCTT (G/A) TCCTCATCACA-3′; F (GSTF14), 5′-TTTGAAGGTTGTGTATTTAAA-3′ and R (GSTF14), 5′-CCCATACTCATATATTTCAT-3′; F (ACO3), 5′-GTTATATATTGAAAGGAGTGT-3′ and R (ACO3), 5′-CAGACTTCTTATTATTTTGTATA-3′. PCR included 40 cycles of 95°C for 30 s, 55°C for 30 s, 50°C for 30 s, and 62°C for 2 min. Cytosine methylation analysis was provided by https://www.cymate.org/cymate.html, as described previously [42]. Each experiment consisted of three biological replicates and was repeated twice.

RT-qPCR: Reverse transcription-quantitative PCR; Col-0: Columbia; RT-sqPCR: Reverse transcription-semi quantitative PCR; rdd: Ros1 dml2 dml3.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03473-4.

Additional file 1.

Additional file 2.

Additional file 3.

Acknowledgments

We thank Prof. Chengguo Duan, Shanghai Center for Plant Stress Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (CAS), for providing the ros1 and ros1 dml2 dml3 mutant seeds and Prof. Xiaofeng Cao, Institute of Genetics and Developmental Biology, CAS, for providing the ago4–1, met1, and cmt3 mutant seeds. This work was supported by the Key Laboratory of Jilin Province for Plant Resources Science and Green Production, China.

Authors’ contributions

YLP performed the important experiments and prepared the manuscript; HH, LCJ, WYJ, MDW, YTB, LDQ participated in the research work; JTC and ZXF edited the manuscript and gave advices regarding the work. The authors have read and approved the manuscript.

Funding

This study was supported by grants from the National Natural Science Foundation of China (Grant Number 31301043) and the Department of Finance of Jilin Province (Grant Number JJKH20191013KJ). These founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. About genes database could download from NCBI by their accession number. The accession numbers of these genes are as follows: ACD6 (AT4G14400) and GSTF14 (AT1G49860).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

Author details

1 The School of Life Sciences, Jilin Normal University, Siping, China. 2 The School of Life Sciences, Northwest A&F University, Xianyang, Shaanxi, China. 3 The School of Life Sciences, Jilin University, Changchun, China.

Received: 16 October 2020 Accepted: 15 February 2022

Published online: 07 March 2022
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