

Mediation of the salicylic acid pathway by ROS1 in response to abiotic stresses

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

Posted Date: September 9th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-34136/v2>

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Abstract

Background: DNA methylation plays an important role in the growth and development of plants in response to various abiotic stresses. Salicylic acid (SA) is an important signaling molecule that is synthesized by plants and induces the expression of defense genes.

Results: In this paper, we investigated the molecular mechanisms by which an upstream regulator (*ACD6*) in the SA pathway, an ABA pathway-related gene (*ACO3*), and a stress resistance gene (*GSTF14*) were induced by various abiotic stresses. The results demonstrated that abiotic stresses, including drought, cold, and salt stresses, induced the 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 were under cold stress.

Conclusion: Our results confirmed that ROS1 plays an important role in the process of defense genes in the SA pathway and stress resistance gene *GSTF14* in response to abiotic stresses.

1. Introduction

DNA methylation is one of the most common forms of DNA covalent modification in the genome of eukaryotes. It plays an important role in the growth and development of plants and in response 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]. This type of small interfering RNA (siRNA) is synthesized by RNA polymerase IV (Pol IV), RNA-dependent RNA polymerase (RDR2), and Dicer-like 3 (DCL3) together [5]. The AGO4 protein 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 removed by DNA glycosylases/lyases in *Arabidopsis*, 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 or crop yield [11, 12]. Abiotic stresses can induce 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-resistant genes in plants in response to stress [16-18]. Abiotic stress also affects the 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]. Aluminum, salt, and cold stresses induce the demethylation of the

coding sequence of the NtGPDH gene in tobacco, thereby promoting the expression of this gene [20]. Soybean has been found to show abnormal expression of approximately 49 transcription factors under salt stress, with expression profiles of the MYB, b-ZIP, and AP2/DREB transcription factor families significantly correlated with the DNA methylation of their gene sequences [21]. Variation in DNA methylation of four potato cultivars before and after cryopreservation has indicated that DNA methylation patterns can change in cryopreserved materials [12]. 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. Changes in methylation status caused by stress can be passed on to offspring, namely, stress memory [22].

Salicylic acid (SA) is an important signaling molecule in plant defense responses and can induce the expression of defense genes and acquisition of systemic resistance [23]. There are at least three upstream regulators of SA, and accelerated cell death 6 (*ACD6*) belongs to the second class of SA upstream regulators. The gain-of-function mutant of *ACD6*, *acd6-1*, can increase the expression of the genes *ACD6-1*, *EDS1*, *PAD4*, and *NPR1* and induce an increase in SA accumulation [24-29]. Plants respond to pathogens via the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathways [2]. Our previous study revealed the molecular mechanisms underlying the induction of defense genes in the SA pathway by biotic stresses [4], but the regulatory mechanism of the SA defense pathway in response to abiotic stresses remains unclear.

In this study, we determined the molecular mechanisms underlying functioning of the upstream regulator *ACD6* of the SA pathway, the stress resistance gene *GSTF14* 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, the ABA pathway-related gene *ACO3*, and the stress resistance gene *GSTF14* significantly increased after treatment with drought, cold, and salt stresses. Sequencing results confirmed that abiotic stresses induced the 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 process.

2. Results

2.1 Activation of the expression of the upstream regulator *ACD6* of the SA pathway by drought stress

Our previous studies have shown the molecular mechanism underlying the induction of defense gene expression in the SA pathway by biotic stresses [4, 30]. To investigate whether abiotic stress could induce the expression of the regulator *ACD6* and stress resistance genes *GSTF14* and *ACO3* in the SA pathway, the wild-type Columbia (Col-0) line of *Arabidopsis* was selected for drought-stress treatment, cold-stress treatment, and salt-stress treatment. There were no significant phenotypic changes in plants treated with cold stress (4 °C) for 24 h or salt stress (150 mM) for 3 days. On days 5-7, the leaves of *Arabidopsis* plants treated with drought stress turned slightly yellow and shrunk (Figure 1B, C) in comparison to untreated Col-0 plants (Figure 1A). On day 14, anthocyanin accumulation in the leaves of *Arabidopsis*

plants treated with drought stress clearly increased, and the leaves turned severely yellow and withered (Figure 1 D).

We extracted the total RNA from *Arabidopsis thaliana* plants on the 7th day of drought-stress treatment for comparative analysis of gene expression. The results of the reverse transcription–semiquantitative polymerase chain reaction (RT-sqPCR) assay showed significantly higher expression levels of the regulator *ACD6* of the SA pathway, the stress resistance gene *GSTF14*, and *ACO3* in the plants after drought-stress treatment than in untreated Col-0 plants; *GAPDH* acted as a reference gene in this study (Figure 1E). Consistent with the RT-sqPCR results, the quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis confirmed that *ACD6*, *GSTF14*, and *ACO3* were significantly upregulated after drought-stress treatment, and the upregulation of *GSTF14* expression was more significant (Figure 1F). Since *ACD6* is an upstream regulator of the SA pathway, the increase in *ACD6* expression could upregulate the expression of the defense genes *NPR1* and *PR5* (Figure 1G).

2.2 Induction of SA pathway-related defense genes by cold and salt stress

To further investigate whether cold stress could also induce the expression of defense genes in the SA pathway, we extracted total RNA from wild-type *Arabidopsis* (Col-0) plants treated under different conditions and detected the related defense genes. RT-sqPCR results showed that compared with controls, *A. thaliana* plants treated with cold or salt stress had significantly higher expression levels of defense genes *ACD6*, *NPR1*, and *PR5* and ABA pathway-related gene *ACO3* (Figure 2A, B). Consistent with the RT-sqPCR results, the RT-qPCR results further confirmed that cold stress and salt stress activated the expression of *ACD6*, which was significantly increased after 24 h of cold-stress treatment (Figure 2C, D). We also compared the expression of the stress resistance gene *GSTF14*. The results showed that the upregulation of *GSTF14* was the most significant in the plants treated with cold stress for 24 h (Figure 2C, D).

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

To investigate whether the increase in the expression of these defense and stress resistance genes was related to the changes in their promoter DNA methylation, the DNA methylation of the plants under stress treatments was detected and compared. Untreated *Arabidopsis* Col-0 plants were used as the controls. After drought-stress treatment, the CG, CNG, and CHH methylation of the repeats in the *ACD6* promoter decreased from 78.30% to 62.03%, from 21.67% to 8.11%, and from 13.51% to 5.80%, respectively. After cold-stress treatment, the CG, CNG, and CHH methylation of the repeats in the *ACD6* promoter decreased from 78.32% to 57.77%, from 21.67% to 7.56%, and from 13.51% to 5.36%, respectively. After salt-stress treatment, the CG, CNG, and CHH methylation of the repeats in the *ACD6* promoter decreased from 78.32% to 65.88%, from 21.67% to 8.26%, and from 13.51% to 6.85%, respectively (Figure 3A).

Similarly, we used untreated Col-0 as a control to perform DNA methylation sequencing of the repeats in the *ACO3* promoter in plants under drought-, cold-, and salt-stress treatments. After drought-stress

treatment, the CG methylation of the repeats in the *ACO3* promoter did not change significantly, while the CNG and CHH methylation of the repeats in the *ACO3* promoter decreased significantly, from 65.89% to 33.33% and from 42.22% to 8.89%, respectively. After the cold-stress treatment, the CG methylation of the repeats in the *ACO3* promoter did not change, while the CNG and CHH methylation of the repeats in the *ACO3* promoter decreased significantly, from 65.89% to 20% and from 42.22% to 8.16%, respectively. After salt-stress treatment, the CG methylation of the repeats in the *ACO3* promoter did not change significantly, while the CNG and CHH methylation of the repeats in the *ACO3* promoter decreased significantly, from 65.89% to 21.43% and from 42.22% to 9.19%, respectively (Figure 3B).

DNA methylation of the *GSTF14* promoter was analyzed next. After drought-stress treatment, the CG, CNG, and CHH methylation of the repeats in the *GSTF14* promoter decreased from 90.30% to 75.49%, from 64.04% to 48.61%, and from 20.78% to 8.72%, respectively. After cold-stress treatment, the CG methylation of the repeats in the *GSTF14* promoter decreased nonsignificantly, from 90.30% to 76.52%, while the CNG and CHH methylation decreased significantly, from 64.04% to 51.46% and from 20.78% to 9.63%, respectively. After salt-stress treatment, the CG methylation of the repeats in the *GSTF14* promoter decreased nonsignificantly, from 90.30% to 78.56% while the CNG and CHH methylation decreased significantly, from 60.45% to 52.75% and from 20.78% to 8.65%, respectively (Figure 3C). Our results revealed that drought, cold, and salt stresses could induce DNA demethylation of the repeats in the gene promoters and increase the expression of these defense and stress resistance genes. Moreover, under drought, cold, and salt stresses, the pattern of DNA methylation variation of the *ACD6* and *GSTF14* promoters was different from that of the *ACO3* promoter.

2.4 Role of *ROS1* in the regulation of the SA defense 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 detect the expression of related genes in plants mutated at key functional elements of the RdDM pathway. The results showed that the expression of *ACD6* and *GSTF14* clearly increased in the mutant *ago4* and DNA methyltransferase mutants *met1* and *drm1/2*, with ecotypes Col-0 and Landsberg *erecta* (Ler) as controls (Figure 4A). RT-qPCR results further confirmed that *ACD6*, *GSTF14*, and *ACO3* were upregulated in the *ago4* mutant (Figure 4B), indicating that RdDM has an important role in maintaining the low transcription levels of *ACD6*, *GSTF14*, and *ACO3* in wild-type plants; however, the *ago4*, *met1*, and *drm1/2* mutants showed increased transcript levels for those genes.

To determine whether *ROS1* plays a role in the responses of these genes to abiotic stress, we performed cold-stress treatment on *ros1* mutants and compared the expression of the *ACD6* gene between the cold stress-treated *ros1* mutants (*ros1*+cold) and the cold stress-treated Col-0 (Col-0+cold). The results showed that when Col-0 was used as the control, the expression of *ACD6* in the cold stress-treated Col-0 plants significantly increased. However, when the cold stress-treated Col-0 plants were used as a control, the increase in *ACD6* expression in the cold stress-treated *ros1* mutants and *ros1 dml2 dml3 (rdd)* mutants was significantly inhibited (Figure 4C). *ROS1* plays an important role in the activation of defense

and stress resistance genes in response to abiotic stress, and this finding was confirmed by the expression of another gene, *GSTF14*. When the cold stress-treated Col-0 plants were used as the control, the increase in *GSTF14* expression was inhibited in the cold stress-treated *ros1* mutants, but the increase in *ACO3* expression was not affected in the cold stress-treated *ros1* mutants (Figure 4C). The results further showed that the expression levels of these genes were lower in the *ros1* and *rdd* mutants, when the Col-0 plants were used as the control (Figure 4D).

Sequencing analysis confirmed that the DNA methylation levels of the repeats in the *ACD6* promoter in cold stress-treated Col-0 plants were significantly reduced, including the CG, CNG and CHH sites, while the DNA methylation levels of the repeats in the *ACD6* promoter in cold stress-treated *ros1* mutants were not significantly decreased (Figure 4E). The results further demonstrated that the DNA methylation at CNG and CHH sites in the *ACO3* promoter in cold stress-treated Col-0 plants was significantly decreased, while the DNA methylation at CNG and CHH sites in the *ACO3* promoter in cold stress-treated *ros1* mutants was not significantly reduced (Figure 4F). Moreover, the DNA methylation levels of the repeats in the *ACD6* and *ACO3* promoters in *ros1* mutants were elevated (Figure 4E, F). Our results revealed that the activation of the expression of the regulator *ACD6*, the stress resistance gene *GSTF14* and *ACO3* by abiotic stresses was related to ROS1-mediated DNA demethylation.

3. Discussion

In recent years, scientists have begun to pay attention to the important role of hormones in the regulation of plant growth and development and resistance to abiotic stresses. 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* [31]. For example, the 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 [32]. We know less about the role of the SA defense pathway in the response of plants to abiotic stresses and the related molecular mechanisms.

This study investigated the role of the SA pathway and related defense genes in the response of plants to abiotic stresses. The results showed that drought, cold and salt stresses induced the expression of the upstream regulator *ACD6* of the SA pathway, the stress resistance gene *GSTF14*, and the ABA pathway-related gene *ACO3* in *Arabidopsis* plants (Figure 1E, F). The gain-of-function mutant of *ACD6*, *acd6-1*, can increase the expression of the genes *ACD6-1*, *EDS1*, *PAD4*, and *NPR1* and induce an increase in SA accumulation [24-29]. Therefore, we hypothesized that the increase in *ACD6* expression would further activate the expression of defense genes *NPR1* and *PR5* (Figure 1G) in the SA pathway.

Under the same stress conditions, different genes differ in the levels and patterns of DNA methylation (Figure 3), suggesting complex molecular mechanisms regulate the expression of these genes. Sequencing results confirmed that the increase in the expression of *ACD6*, *GSTF14*, and *ACO3* was related to the reduction in DNA methylation levels of the promoters of these genes. The CG, CNG, and CHH

methylation in the *ACD6* promoter decreased to varying degrees, and the CG methylation decreased significantly (Figure 3A). However, the CG methylation of the repeats in the *ACO3* promoter barely changed, but their CHG and CHH methylation significantly decreased (Figure 3B). ROS1-mediated DNA demethylation can act on the three DNA methylation sites, CG, CHG, and CHH [33]. DNA methylation sequencing of *ros1* mutants has revealed that ROS1 generally targets genes that contain CG, CNG, and CNN methylation in transposable elements and repeats but does not target genes that contain only CG methylation [34]. We speculate that ROS1-mediated DNA demethylation could play an important role in the transcriptional activation of the upstream regulator *ACD6* of the SA pathway and in that of the stress resistance gene *GSTF14* and *ACO3*. Our results reveal that abiotic stresses (cold stress, drought, and salt stress) induced DNA demethylation of the *ACD6*, *ACO3*, and *GSTF14* promoters and transcriptionally activated the expression of defense and stress resistance genes, thereby enhancing the adaptability of plants to abiotic stresses.

Further studies revealed that the expression of the regulator *ACD6* of the SA pathway and stress resistance gene *GSTF14* in the mutants *ago4*, *drm1/2*, and *met1* was higher than in Col-0 (Figure 4A). RT-qPCR results confirmed that *ACD6*, *ACO3*, and *GSTF14* in the mutant *ago4* were upregulated (Figure 4B), indicating that the RdDM pathway has an important role in maintaining the low transcription levels of *ACD6*, *GSTF14*, and *ACO3* in wild-type plants. DNA methylation can be removed by DNA glycosylases/lyases in *Arabidopsis*, in which ROS1 can negatively regulate the RdDM pathway [7, 8]. Our results further reveal that ROS1 also plays an important role in the responses of the defense genes in the SA pathway and stress resistance genes to abiotic stresses. When the Col-0 plants were used as the control, the upregulation of *ACD6* and *GSTF14* was significant in Col-0 plants treated with cold stress for 24 h (Figure 4C). When the cold stress-treated Col-0 plants were used as the control, the increase in the expression of *ACD6* and *GSTF14* in *ros1* mutants treated with cold stress for 24 h was significantly inhibited (Figure 4C). Furthermore, after 24 h of the cold-stress treatment of Col-0, DNA methylation levels in the repeats in the *ACD6* promoter were significantly reduced, while DNA methylation levels in the repeats in the *ACD6* promoter in cold stress-treated *ros1* mutants were not significantly reduced (Figure 4E). These results further confirm that ROS1-mediated DNA demethylation played an important role in the transcriptional activation of the upstream regulator *ACD6* of the SA pathway and the stress resistance gene *GSTF14* in response to cold stress.

Our study reveals that plant defense genes in the SA pathway are involved in response to various abiotic stresses. Epigenetic regulation, such as DNA demethylation, plays an important role in this process. For example, ROS1-mediated DNA demethylation is known as active demethylation. Due to the complexity of the dynamic regulation of DNA methylation, the molecular mechanisms by which plants adapt to various adverse environmental factors and the ways different signaling pathways interact still require in-depth study.

4. Materials And Methods

4.1. Plant growth and abiotic stress treatments

Arabidopsis thaliana ecotype Columbia (Col-0) and the mutant plants were used for this work. The *ros1* and *ros1dml2dml3* mutant seeds were provided by Chengguo Duan, Shanghai Center for Plant Stress Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (CAS). The *ago4*, *met1*, *drm1/2* and *cmt3* mutant seeds were provided by Prof. Xiaofeng Cao, 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 before they were transplanted to soil.

Arabidopsis thaliana Col-0 plants were treated with abiotic stresses, such as cold stress (4 °C, 24 h), salt stress (150 mM NaCl, 3 days), and drought stress (not watered, 7 days). The significant experimental details were as follows.

Cold stress	Salt stress	Drought stress
4°C	150 mM NaCl	not watered
24 hours	3 days	7 days

4.2. RT-sqPCR, RT-qPCR and northern blotting analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocols. The total RNA was subsequently used for RT-sqPCR, RT-qPCR, and RNA gel blotting analysis. For RT-sqPCR, total RNA was extracted from the treated plants and subsequently used for reverse transcription and semiquantitative PCR. For RT-qPCR, the complementary DNA synthesis was performed using the 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 analysis, 10 mg of total RNA was extracted from the treated plants and separated on 1% agarose-formaldehyde gels, transferred to Hybond-Np membranes, and hybridized as described previously [4]. *ACD6* (AT4G14400) and *GSTF14* (AT1G49860) probe primer pairs were as follows: F (*ACD6*), 5'-TCTCCCTGGTGAAGATGTCG-3' and R (*ACD6*), 5'-TTACCGATGCAACAAGAGCC-3'; F (*GSTF14*), 5'-AGGCGAGTCTCCTTACTTGG-3' and R (*GSTF14*), 5'-TTATAGGCAAACGA CGCTGC-3'; F (*ACO3*), 5'-ACGAGTCAATCACCAAGGGT-3' and R (*ACO3*), 5'-GAAGTCCT TACGGTCAACGC-3'.

4.3. 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'-AAGTTTATTGATGAAAGGAG-3' and R (*ACD6*), 5'-CTTACTT (G/A) TCTTCATCAA-3'; F (*GSTF14*), 5'-TTTGAAAGTTGGTGTATT AAA-3' and R (*GSTF14*), 5'-CCCATACCTATCATATTTTCAT-3'; F (*ACO3*), 5'-GTAATATT AGTAAAGATGTGT-3' and R (*ACO3*), 5'-CACTACTTTTCATTATACTCTTT-3'. PCR cycles include

95°C 30s 55°C 30s 50°C 30s 62°C 2min repeat 40 cycles. The cytosine methylation analysis was performed as described previously [35].

Declarations

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 *ros1dml2dml3* mutant seeds and Prof. Xiaofeng Cao, Institute of Genetics and Developmental Biology, CAS, for *ago4-1*, *met1*, *drm1/2* and *cmt3* mutant seeds. This work was supported by the Key Laboratory of Jilin Province for Plant Resources Science and Green Production, China. 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).

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Figures

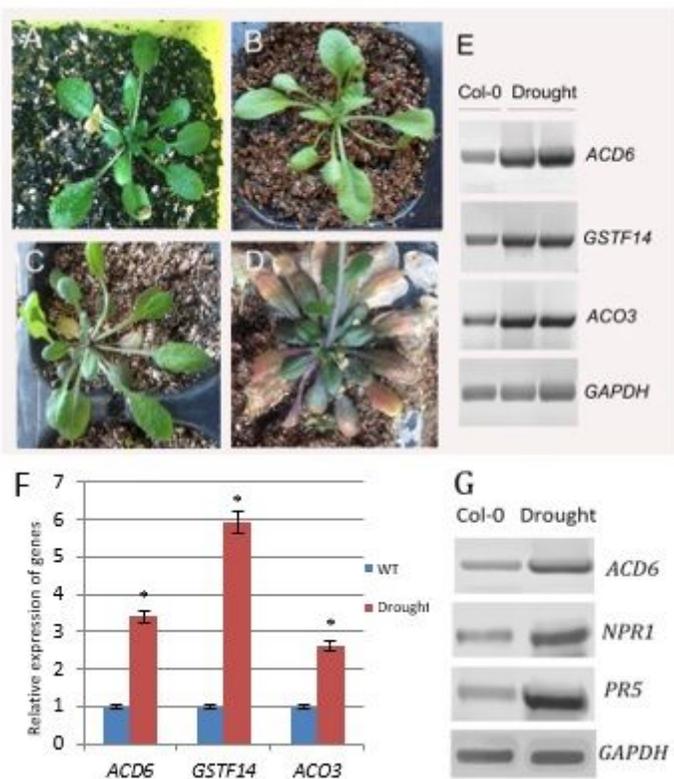


Figure 1

Detection and analyses of the expression of defense genes in Arabidopsis plants treated with drought stress (A) The untreated Arabidopsis Col-0 plants. (B, C) The leaves of Arabidopsis plants treated with drought stress turned slightly yellow and shrunk during days 5-7. (D) Anthocyanin accumulation in the leaves of Arabidopsis plants treated with drought stress clearly increased, and the leaves turned severely yellow and withered on day 14. (E) Transcript levels of related genes in Arabidopsis plants treated with drought stress were analyzed by sqPCR; untreated Col-0 plants served as controls. (F) Transcript levels of related genes in Arabidopsis plants treated with drought stress were analyzed by qPCR. The statistical analysis was performed; asterisks indicate statistically significant differences compared with control plants ($P < 0.05$). (G) Transcript levels of defense genes in Arabidopsis plants treated with drought stress were analyzed by sqPCR.

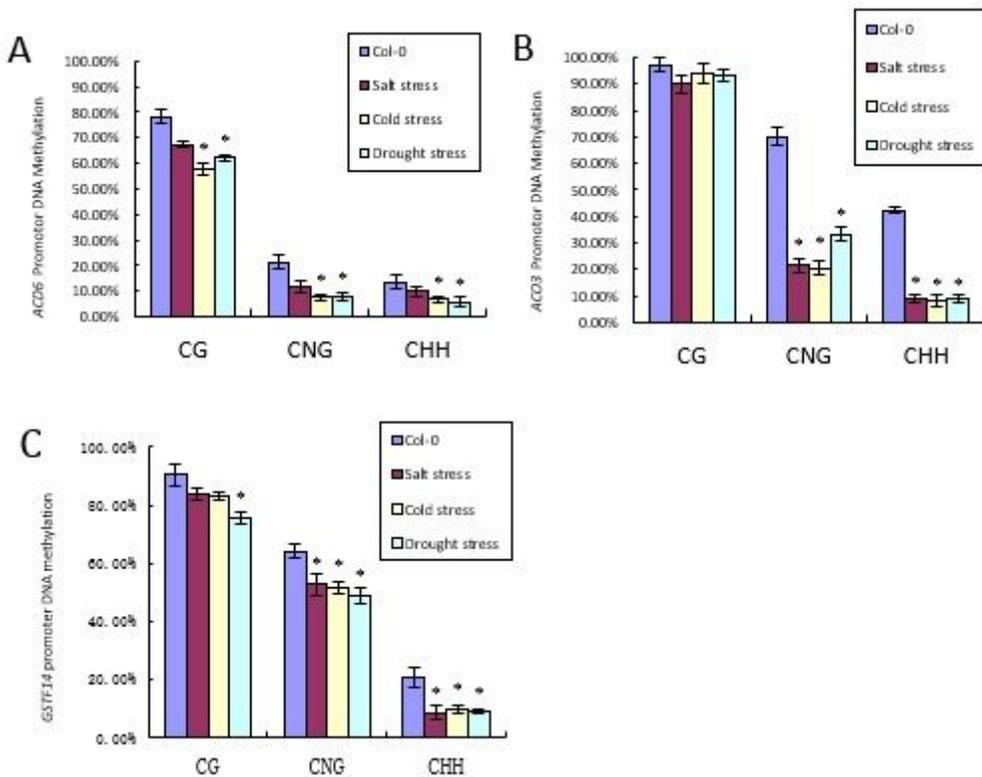


Figure 2

Detection and analyses of the expression of defense genes and stress resistance genes in Arabidopsis (A, B) The defense genes and ACO3 transcript levels in Arabidopsis plants treated with cold and salt stress were analyzed by sqPCR; untreated Col-0 plants served as controls. (C, D) ACD6, GSTF14, and ACO3 transcript levels in Arabidopsis plants treated with cold and salt stress were analyzed by qPCR; untreated Col-0 plants served as controls. The statistical analysis was performed; asterisks indicate statistically significant differences compared with control plants ($P < 0.05$).

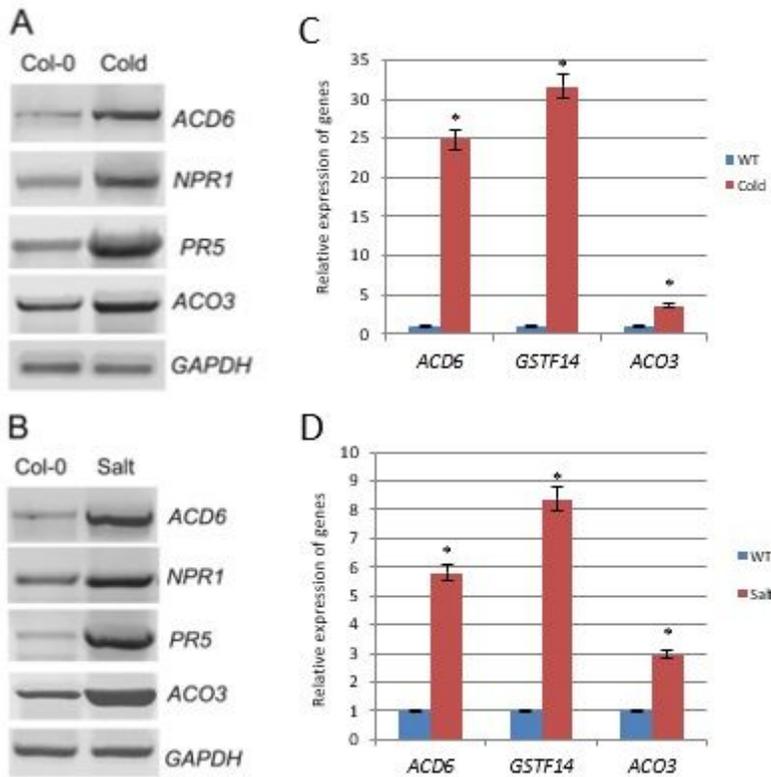


Figure 3

Analyses of DNA methylation of the promoters in plants treated with different stresses (A) Percentage of DNA methylation in the repeat regions of the ACD6 promoter in plants treated with different stresses and untreated Col-0 plants. (B) Percentage of DNA methylation in the repeat regions of the ACO3 promoter in plants treated with different stresses and untreated Col-0 plants. (C) Percentage of DNA methylation in the repeat regions of the GSTF14 promoter in plants treated with different stresses and untreated Col-0 plants. Fifteen individual clones of each genotype were used for sequencing, and the original data are shown in supplement Data S1. The statistical analysis was performed using OriginPro 8 (<http://www.originlab.com>). Values are means \pm SEM, and asterisks indicate statistically significant differences compared with control plants (one-way analysis of variance, $P < 0.05$).

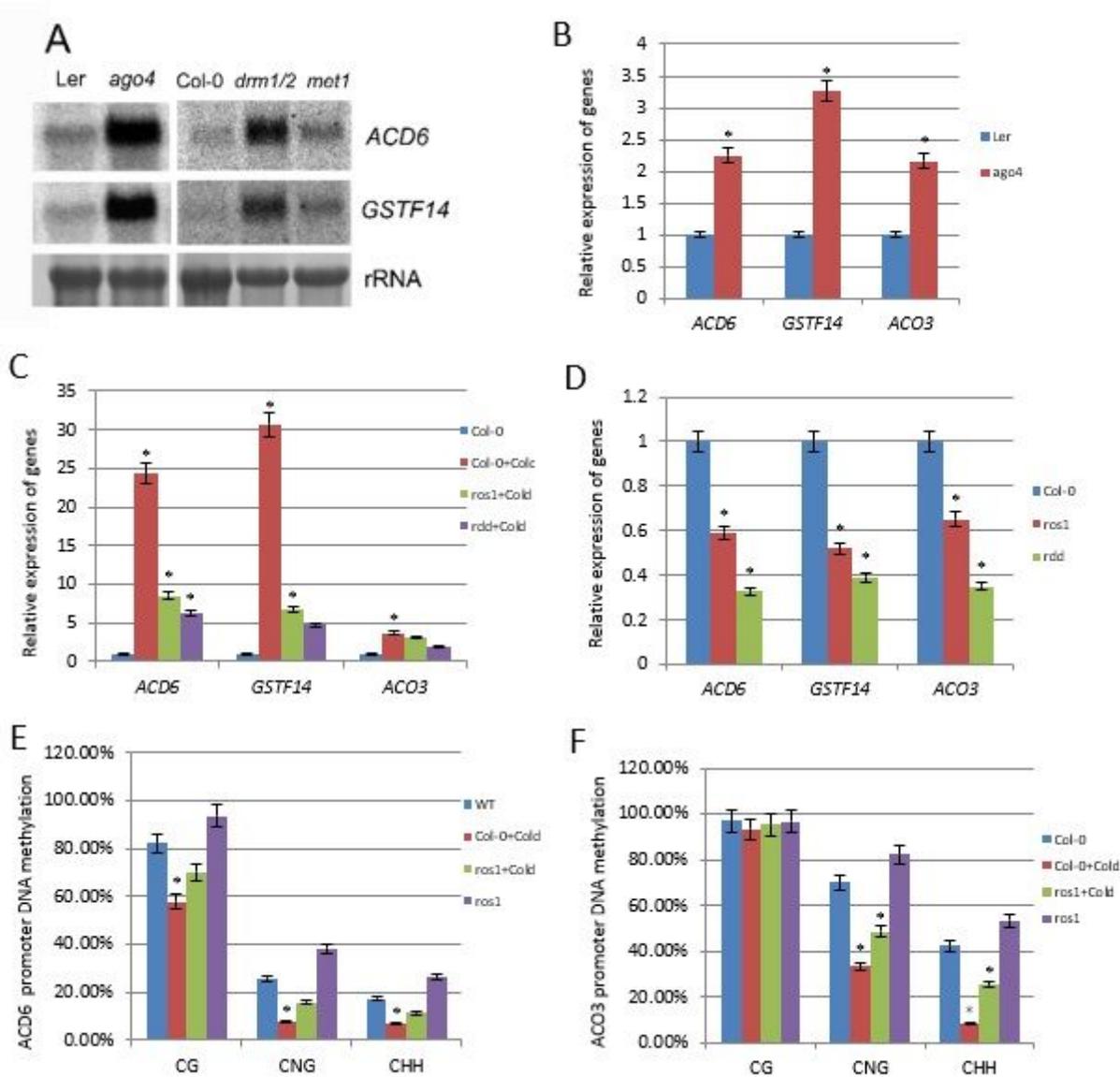


Figure 4

Analyses of DNA methylation and the expression levels of genes (A) Analyses of the expression levels of ACD6 and GSTF14 in the mutants *ago4*, *met1*, and *drm1/2* by northern blotting; wild-type Col-0 and Ler ecotype served as background controls for the mutant genotypes. (B) Analyses of the expression levels of ACD6, ACO3, and GSTF14 by RT-qPCR in DNA methylation mutant plants *ago4*, with wild-type Ler ecotype as background control for the mutant genotypes. (C) The related genes were detected in the untreated Col-0, the Col-0 treated with cold stress, *ros1*, and *ros1 dml2 dml3* (*rdd*) mutant plants treated with cold stress by RT-qPCR. (D) The related genes were detected in the untreated Col-0 and *ros1* plants by RT-qPCR. (E) Analyses of DNA methylation in the repeat regions of the ACD6 promoter in Col-0, *ros1* mutants, and Col-0 plants and *ros1* plants treated with cold stress. (F) Analyses of DNA methylation in the repeat regions of the ACO3 promoter in Col-0, *ros1* mutants, the Col-0 plants, and *ros1* plants treated with cold stress. The statistical analysis was performed; asterisks indicate statistically significant differences compared with control plants ($P < 0.05$).

Supplementary Files

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