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

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Abstract

DNA methylation plays an important role in the growth and development of plants and 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. In this paper, we investigated the epigenetic regulation mechanism by which an upstream regulator ACD6 in the SA pathway, ABA pathway-related gene ACO3 , and stress resistance gene GSTF14 in response to 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 transcriptional activation of ACD6 and GSTF14 was mainly dependent on ROS1-mediated DNA demethylation when Arabidopsis plants under cold stress, suggesting 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. DNA methylation directed by plant small interfering RNAs (RdDM) plays an important role in regulating gene expression, controlling the activity of transposable elements, and defending against foreign DNAs, such as of viruses (Ascencio-Ibáñez et al., 2008; Raja et al., 2008). 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 (Meister et al., 2004). The synthesized 24-nt siRNA binds to the AGO_4 protein and recruits the DNA methyltransferases DDM1/2, MET1, and CMT3 to perform *de novo* methylation and maintain methylation of the target DNA (Buchmann et al., 2009). DNA methylation can be removed by DNA glycosylases/lyases in *Arabidopsis*, and this process is known as active demethylation. Repressor of silencing 1 (ROS1) can negatively regulate the RdDM pathway (Gong et al., 2002; Yu et al., 2013; Liu et al., 2019).

Abiotic stresses mainly include drought, cold, and salt stresses, which severely threaten plant growth or crop yield (Fedoroff et al., 2010; Mirouze et al., 2011). Abiotic stresses can induce accumulation of endogenous abscisic acid (ABA), triggering ABA signal transduction to cope with adverse environmental factors (Kinoshita et al., 2014; Shinozaki et al., 2003; Zhu et al., 2016). When plants are under cold stress, ABA can regulate the expression of cold-resistant genes in plants in response to stress (Shinozaki et al., 2000; Seki et al., 2001; Maruyama et al., 2004). 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 (Chinnusamy et al., 2009). Aluminum, salt, and cold stresses induce the demethylation of the coding sequence of the NtGPDL gene in tobacco, thereby promoting the expression of this gene (Choi al., 2007). Soybean showed abnormal expression of approximately 49 transcription factors under salt stress and that the expression profiles of the MYB, b-ZIP and AP2/DREB transcription factor families were significantly correlated with the DNA

methylation of their gene sequences (Song et al., 2012). The variation of DNA methylation of four potato cultivars before and after cryopreservation indicated that the DNA methylation patterns can change in cryopreserved materials (Mirouze et al., 2011). 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 (Wildermuh et al., 2001).

Salicylic acid (SA) is an important signaling molecule in the plant defense response and can induce the expression of defense genes and acquire systemic resistance (Chen et al., 2010). 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 (Falk et al., 1999; Jirage et al.,1999; Nawrath et al., 2002; Lu et al., 2003; Cao et al., 1997; Kate et al., 1999). The molecular mechanisms underlying the induction of defense genes in the SA pathway by biotic stresses have been well studied (Yang et al., 2013; Yang et al., 2016), but the regulatory mechanism of the SA defense pathway in response to abiotic stresses remains unclear.

In this study, we determined the molecular mechanism of the upstream regulator ACD6 of the SA pathway, 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, ABA pathway-related gene *ACO3*, and 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 the increase in expression of *ACD6* and *GSTF14* mainly depended on ROS1-mediated DNA demethylation when *Arabidopsis* plants under cold stress, suggesting that ROS1 plays an important role in the response of defense genes and stress resistance genes to abiotic stresses.

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 (Yang et al., 2013; Yang et al., 2016). 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 mmol) for 1-3 days. On days 5-7, the leaves of *Arabidopsis* plants treated with drought stress turned slightly yellow and shrunk (Figure 1B, C) compared to untreated Col-0 plants (Figure 1A). On days 10-15, 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, E, F).

We extracted the total RNA from *Arabidopsis* thaliana plants on the 14th day of drought-stress treatment for comparative analysis of gene expression. The results of the reverse transcription–semi-quantitative polymerase chain reaction (RT-sqPCR) assay showed significantly increased expression levels of the regulator *ACD6* of the SA pathway, stress resistance gene *GSTF14*, and *ACO3* in the plants after drought-stress treatment compared with the untreated Col-0 plants (Figure 1G). 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 1H). 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 1I).

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 3C, 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 defense genes of the SA pathway in response to abiotic stresses, we used RNA gel blot 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 the RdDM pathway plays an important role in regulating the expression of these genes and responding to abiotic stress.

To determine whether ROS1 plays a role in the process of these genes responses to abiotic stress, we performed cold-stress treatment on *ros1* mutants and compared the expression of the *ACD6* gene in 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 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). 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, while the DNA methylation levels of the repeats in the *ACD6* promoter in cold stress-treated *ros1* mutants was not significantly decreased (Figure 4D). Our results revealed that the activation of the expression of the regulator ACD6 and stress resistance gene *GSTF14* by abiotic stresses, such as cold stress, depends mainly on ROS1-mediated DNA demethylation. Moreover, the increase in *ACD6* expression would further activate the expression of defense genes *NPR1* and *PR5*, since ACD6 is an upstream regulator in SA pathway.

To determine whether cold stress affects the expression of ROS1 or AGO4, we detected the expression levels of the *ROS1* and *AGO4* in cold stress-treated plants. The results of RT-qPCR confirmed that the expression of *ROS1* was clearly upregulated on 1 dpi, 2 dpi and 3 dpi in the cold stress-treated plants, compared with the untreated *Arabidopsis* Col-0 plants (Fig. 4E). Conversely, the expression level of *AGO4* has no obvious changes under cold stress (Fig. 4F).

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* (Seki et al., 2002). 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 (Zhu et al., 2006). 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 1G, H). 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 (Falk et al., 1999; Jirage et al.,1999; Nawrath et al., 2002; Lu et al., 2003; Cao et al., 1997; Kate et al., 1999) . Therefore, we hypothesized that the increase in *ACD6* expression would further activate the expression of defense genes *NPR1* and *PR5* (Figure 1I) 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 (Marsch-Martinez et al., 2019). 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 (Tang et al., 2016). We speculate that ROS1-mediated DNA demethylation could play a key role in the transcriptional activation of the upstream regulator *ACD6* of the SA pathway and stress resistance gene *GSTF14*. Therefore, we can better understand why *ACD6* and *GSTF14* expression increased significantly under abiotic stresses while *ACO3* gene expression did not increase significantly under abiotic stresses, especially 24 h of cold-stress treatment (Figure 2C). 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 plays a role in the expression of these genes and in response to abiotic stresses. DNA methylation can be removed by DNA glycosylases/lyases in *Arabidopsis*, in which ROS1 can negatively regulate the RdDM pathway (Gong et al., 2002; Yu et al., 2013). Our results further reveal that ROS1 plays a key role in the response of the defense and stress resistance genes in the SA pathway 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, but the increase in *ACO3* expression was not affected (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 4D). These results further confirm that ROS1-mediated DNA demethylation played a key role in the transcriptional activation of the upstream regulator *ACD6* of the SA pathway and stress resistance gene *GSTF14* and in response to cold stress, while transcriptional activation of *ACO3* was unrelated to ROS1-mediated DNA demethylation, while the expression regulation of *ACO3* mainly depended on RdDM pathway under 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, RdDM and ROS1-mediated DNA demethylation are known as passive demethylation and active demethylation, respectively. 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. The plant growth

Arabidopsis thaliana ecotype Columbia (Col-0) and the mutant plants were used for this work. 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.

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 blot analysis. For RT- sqPCR, total RNA was extracted from the inoculated plants, and subsequently used for reverse transcription and semi-quantitative PCR. For RT-qPCR, the complementary DNA synthesis was conducted 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 inoculated plants and separated on 1% agarose-formaldehyde gels, transferred to Hybond-Nþ membranes, and hybridized as described previously (Yang et al., 2016).

4.3. Bisulfite sequencing

Total DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) buffer as previously described (Chen et al., 2010) 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'-CCCATACCTATCATATTTCAT-3'; F (ACO3), 5'-GTAATATT AGTAAAGATGTGT-3' and R (ACO3), 5'- CACTACTTTCATTATACTCTTT-3'. The cytosine methylation analysis was performed as described previously (Zhang et al., 2011).

Declarations

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Figures

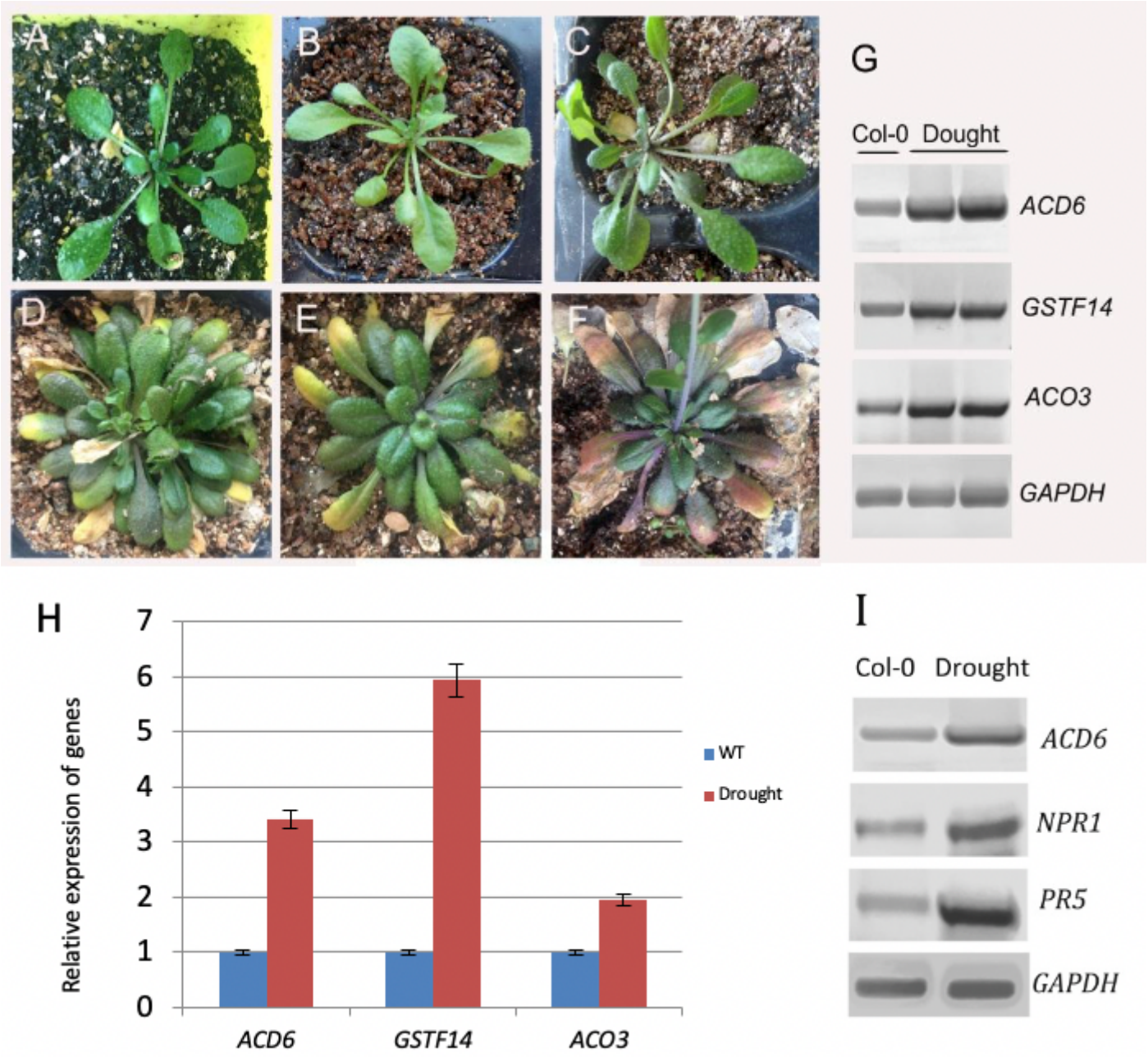

Figure 1

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 on days 5-7. (D, E, F) Anthocyanin accumulation in the leaves of Arabidopsis plants treated with drought stress clearly increased and the leaves turned severely yellow and withered on days 10-15. (G) The related genes transcript levels in Arabidopsis plants treated with drought stress were analyzed by sqPCR, untreated Col-0 plants were served as controls. (H) The related genes transcript levels in Arabidopsis plants treated with drought stress were analyzed by qPCR. (I) The defense genes transcript levels in Arabidopsis plants treated with drought stress were analyzed by sqPCR. Error bars indicate + SD (n = 3).

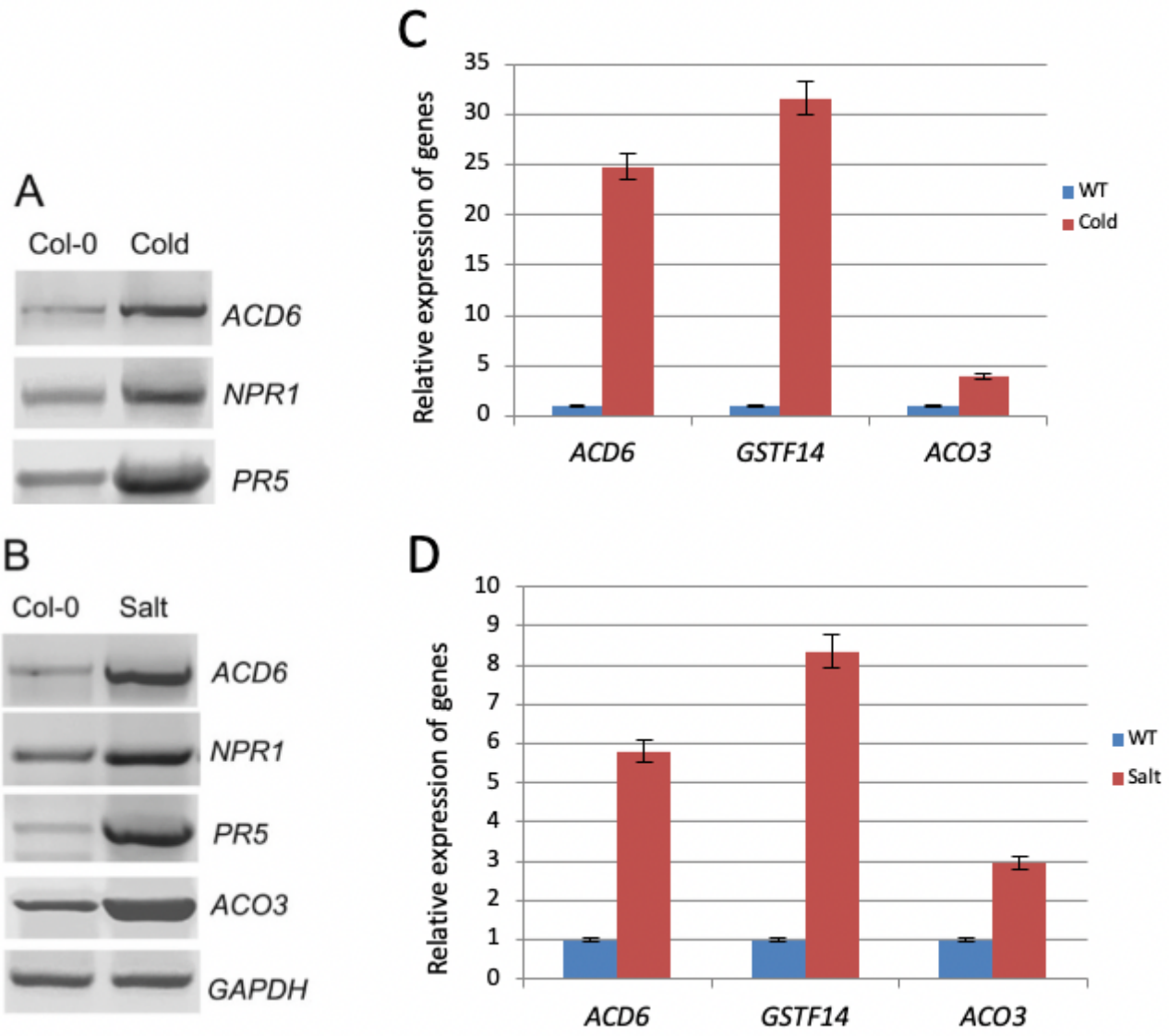

Figure 2

2 Detection and analyses of the expression of defense genes and stress resistance genes in Arabidopsis plants treated with cold or salt stress (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 were 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 were served as controls. Error bars indicate + SD (n = 3).

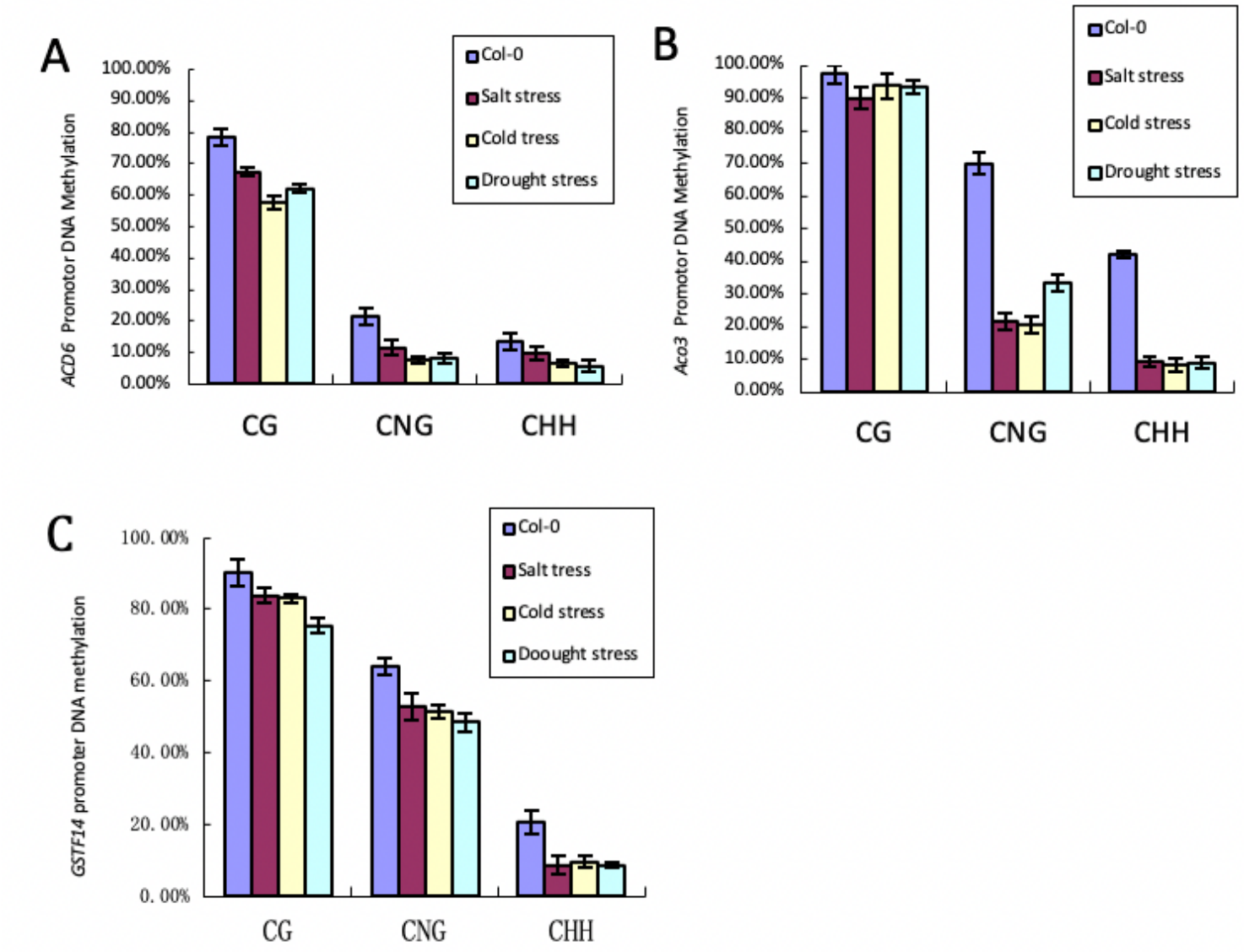

Figure 3

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 the plants treated with different stresses and untreated Col-0 plants. (B) Percentage of DNA methylation in the repeat regions of the ACO3 promoter in the plants treated with different stresses and untreated Col-0 plants. (C) Percentage of DNA methylation in the repeat regions of the GSTF14 promoter in the plants treated with different stresses and untreated Col-0 plants. DR, dispersed repeat. Fifteen individual clones of each genotype were used for sequencing. The experiments of sequencing were repeated three times and the statistical analysis was performed using OriginPro 8 (http://www.originlab.com). Error bars indicate + SD (n = 3).

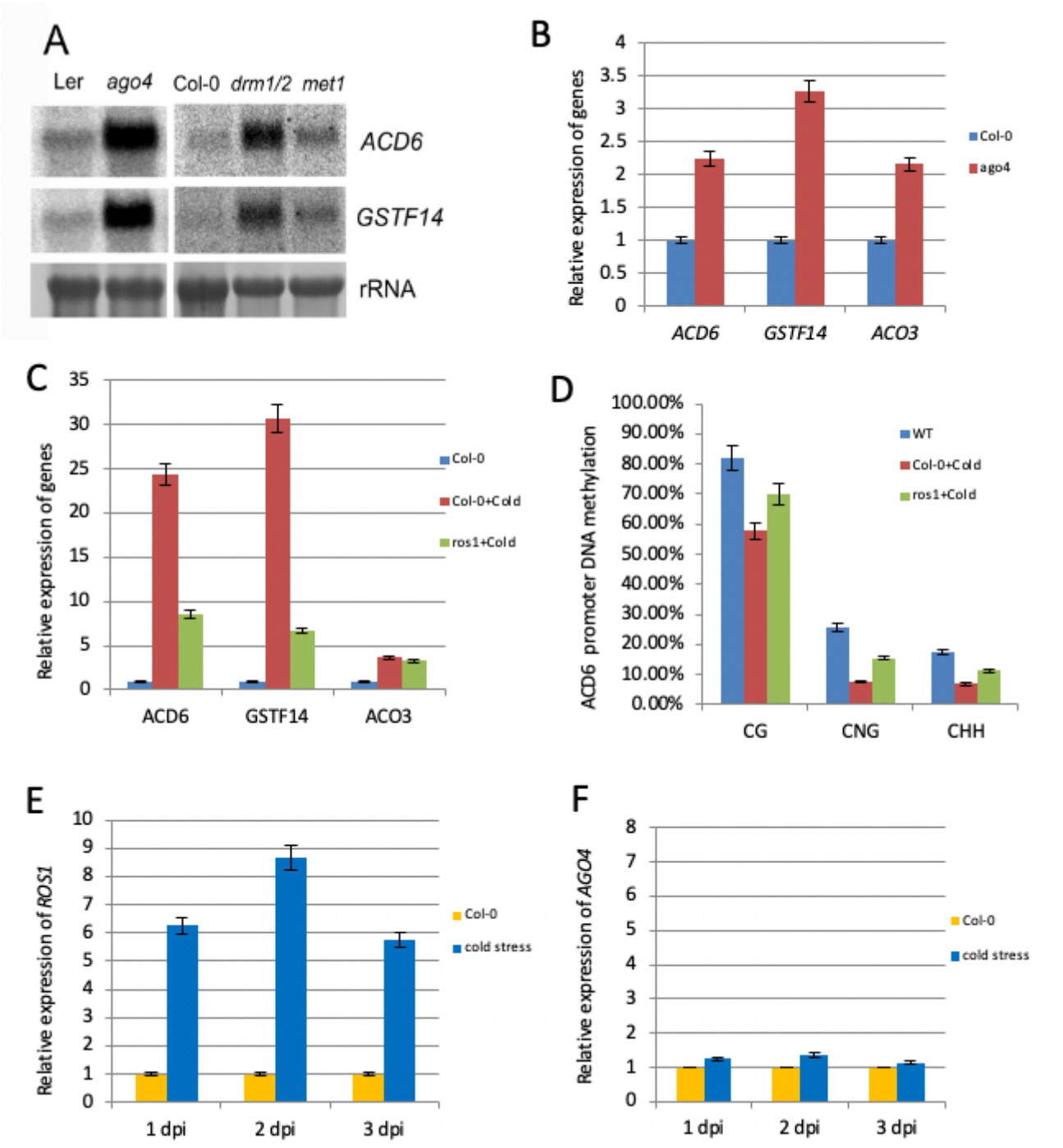

Figure 4

Analyses of DNA methylation and the expression levels of genes (A) Analyses of the expression levels of ACD6 and GSTF14 in the mutant ago4, met1 and drm1/2 by Northern blot. (B) Analyses of the expression levels of ACD6, ACO3 and GSTF14 by RT-qPCR in DNA methylation mutant plants ago4, wild-type Col-0 ecotype background control for the mutant genotypes. (C) The related genes were detected in the untreated Col-0, the Col-0 treated with cold stress and ros1 plants treated with cold stress by RT-qPCR. (D) Analyses of DNA methylation in the repeat regions of the ACD6 promoter in the plants treated with cold stress. Error bars indicate + SD (n = 3). (E, F) RT-qPCR analyses of ROS1 (E) and AGO4 (F) expression on 1 dpi, 2 dpi and 3 dpi in the cold stress-treated and untreated plants.