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GhCOR27, Which Encodes a Cold-related Gene, Is Involved in Cotton Tolerance to Cold Stress

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Abstract: The growth and development of crop plants are greatly affected by various abiotic stresses such as cold stress. Cold stress tolerance is triggered by a sensing mechanism, leading to signaling transduction in plants. Especially in the model species *Arabidopsis* (*Arabidopsis thaliana*) it was found that cold-related genes play important roles via cold stress. Cotton is thermophilic and sensitive to cold temperature during its development and growth. However, the mechanism of about cotton how to sense the cold response remains elusive. Here, we isolated a gene from cotton seedling cDNA, which phylogenetic analysis clustered it into the Cold-Related gene member and the protein sequences showed that its closest homolog is *Arabidopsis AtCOR27*, so we defined it as *GhCOR27*. Furthermore, the expression pattern analysis via real-time PCR used peculiar *GhCOR27* primers demonstrated that *GhCOR27* mRNA accumulated abundance was gradually induced at low temperature. In addition, the cotton seedlings of silenced-*GhCOR27* through virus-induced gene silencing (VIGS) exhibited a more severe chilling injury phenotype at low temperature. These results suggested that *GhCOR27* may play a role in response to cotton cold stress response.

Keywords: *Gossypium hirsutum*, Cold Stress, Cold-related Gene (COR), Virus-induced Gene Silencing (VIGS)

1. Introduction

Plant suffers various biotic and abiotic factors in its life cycle which affect plant growth and development. Abiotic factors such as drought, temperature and salt can cause irreversible injury to plant leading to poor growth, the loss of yield and even leading to death. For many annual plants such as maize, cotton and rice which are sensitive to temperature, cold stress becomes the primary environmental factor affecting the growth, development and yield [1]. Also, the degree of damages depends on the period of exposure to cold stress and the ability of adaption to cold stress [2]. In order to survive under cold condition, over a long evolutionary period, plants develop the ability to sense low temperature and the mechanisms of stress tolerance [3].

The evolved adaption to cold stress called cold acclimation, is associated with a complex process involved in multiple signaling pathways including biochemical and physiological alterations such as activating second messengers and other components to respond to cold tress. According to previous studies, low temperature activates second messengers Ca^{2+} to trigger plant responses under cold stress, which cellular Ca^{2+} dynamics are detected in response to cold through a aequorin-based Ca^{2+}-signaling mechanism [4] and the reduction in membrane fluidity caused by cold stress appears to be a primary event of cold perception to activate the Ca^{2+} channel in plants [5]. Molecular traits were revealed that many genes are involved in the cold acclimation. A group of genes named cold regulated genes (*COR*) genes have been characterized as down-stream functional protein in *CBF*-dependent pathway designed as the ICE1-CBF-COR cascade which were widely studied. In this system, CBFs/DREBs are rapidly induced by cold, and bind to the

promoter regions of COR genes to activate their transcription [6]. Furthermore, expression of many *COR* genes was induced by cold stress than that of *CBF* genes [7]. So far, many *COR* genes have been isolated from plant species, and reported to play roles in low temperature pathway, especially in *Arabidopsis*, the *COR* genes such as *COR6.6*, *COR15a*, *COR47*, and *COR27*, *COR28*, *COR78*, have been well characterized and acknowledged [8-11]. But, bioinformatic analysis indicated that *COR* family have lower homology in plants compared with the *CBF* family [12].

Cotton, as widely planted crop in China, often suffer from low temperature leading to loss of yield and even to death especially in spring in some districts of China such as Xinjiang. Therefore, based on biological and agricultural need, the research and understanding on the cotton cold acclimation mechanism at biochemical and molecular level will provide important help. By now, the knowledges of *COR* genes in response to cold stress in cotton remain less. Here, we isolated a gene from cotton seedling cDNA. Polygenetic analysis by amino sequence alignment indicated that it belongs to the same clade with *AtCOR27*, so we defined it as *GhCOR27*. The expression pattern analysis demonstrated that *GhCOR27* was induced and its mRNA accumulated abundance increased at low temperature. Furthermore, the cotton plants of silenced-*GhCOR27* through virus-induced gene silencing (VIGS) exhibited a more severe chilling injury phenotype at low temperature. These results suggested that *GhCOR27* may play a role in response to cotton cold stress.

2. Materials and Methods

2.1. Cotton Growth and Gene Expression Analysis

Seeds of upland cotton cv TM-1 (Gossypium hirsutum) were delinted with H_2SO_4 (98%) and sterilized in 70% ethanol for a few seconds, followed by three rinses in sterile water. Cotton plants were grown in a controlled environment chamber at 28°C, with a 12 hours photoperiod. For cold treatments, 3-week-old seedlings were transferred to 10°C, and were collected at 3, 6, 12, 24 and 48 h timepoint used for RNA extraction and real-time PCR analysis. Total RNA was isolated from leaf tissue using the Biospin Plant Total RNA Extraction Kit (Bioer Technology Co., Ltd. China) and First-strand cDNA was synthesized by TranScript-Uni One-Step gDNA Removal and cDNA synthesis SuperMix kit (TRANSGEN BIOTECH Co., Ltd. China). For qRT-PCR analysis, the gene-specific primers *GhCOR27*-F: 5-GGAGAAAAGACCAGTTATGC-3 and *GhCOR27*-R: 5-TAATGTGTGTCAGACTTCGG-3 and the primers *histon3*-F: 5-GCCAAGCGTGTCACAATTATGC-3 and *histon3*-R: 5-ACATCACATTGAACCTACCACTACC-3 were selected and checked via BLASTN search.

2.2. Phylogenetic Analysis

The neighbor-joining method was used to construct the phylogenetic tree of using MEGA v. 5.1 [www.megasoftware.net]. Amino acid sequences were analyzed using DNAMAN (DNAMAN Inc). The peptide sequences were aligned with the ClustalW program (http://www.ebi.ac.uk), and phylogenetic analysis was employed to investigate the evolutionary relationships among the aquaporins. A minimum evolution tree was generated in MEGA-X.

2.3. Construction of Vigs Vectors and Agrobacterium Mediated Vigs

Tobacco rattle virus (TRV)-based virus-induced gene silencing was performed. *GhCOR27* was amplified by polymerase chain reaction from cDNA of TM-1 leaf tissues with primers *GhCOR27*-F: 5-GAATTCCTTCTCATCAGTTTTCTATCCT-3, *GhCOR27*-R: 5-GGGGTACCGTAATGTGTGTCAGACTTCGGG-3, and inserted into the pYL156 vector with restriction enzymes *Eco*RI and *Kpn*I digestion. *GhCLA1* was cloned into the pYL156 (pTRV-RNA2) vector as control, which is used as a visible marker to monitor the efficiency of VIGS. The plasmids containing binary TRV vectors pTRV-RNA1 and pTRV-RNA2 (pYL156) vector, pYL156-*GhCOR27* and pYL156-*GhCLA1*, were transformed into *Agrobacterium tumefaciens* strain GV3101, respectively. Ten-d-old seedlings were transfected with the mixture (1:1, v/v) of *Agrobacterium* cultures (OD600=1.5) harboring pTRV1 with pTRV2 or its derivative plasmids. After completion of agro-inoculation, the seedlings were grown at 25°C under a 16 h/8 h light/dark cycle in a controlled environmental chamber. After two weeks of cultivation, the plants were inoculated for two weeks with low temperature. The experiments were performed with at least 30 plants per treatment and repeated three times.

3. Results

3.1. GhCOR27 Bioinformatic and Phylogenetic Analyses

It remains unknown how many COR family members exist in cotton and what their biological functions are. To identify the genes involved in response to low temperature in cotton, the published amino acid sequences of *AtCOR27* genes were retrieved and used as query to blast against the *G. hirsutum* unigene database (https://www.cottongen.org/). Nine genes from the *G. hirsutum* unigene database, Gh_A01G0983, Gh_A06G1853, Gh_A09G0755, Gh_D01G0071, Gh_D01G1032, Gh_D05G1942, Gh_D06G0147, Gh_D09G0756 and Gh_D12G0215 were retrieved. We performed the expression analysis under low temperature treatment for all the nine genes (data not shown) and showed Gh_A09G0755 was induced by low temperature. Then, by using online blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the deduced amino acid sequence of Gh_A09G0755 was used to blast with AtCOR27 (NP_568615.3, Arabidopsis thaliana), PvCOR27 (XP_031267232.1, *Pistacia vera*), PgCOR27 (XP_031394028.1, *Punica granatum*), TcCOR27 (EOY14453.1, *Theobroma cacao*) and GaCOR27

(KAA3476458.1, *Gossypium australe*) and exhibited high identity with GaCOR27 (89.86%), TcCOR27 (74.06%), but lower identity with PvCOR27 (52.57%), AtCOR27 (47.86%) and PgCOR27 (35.23%) (Figure 1A). Meanwhile alignment was performed using the multalin website (http://multalin.toulouse.inra.fr/multalin/) and shown these *COR* family member containing common conserved domain, so we designed Gh_A09G0755 as *GhCOR27* (Figure 1B).

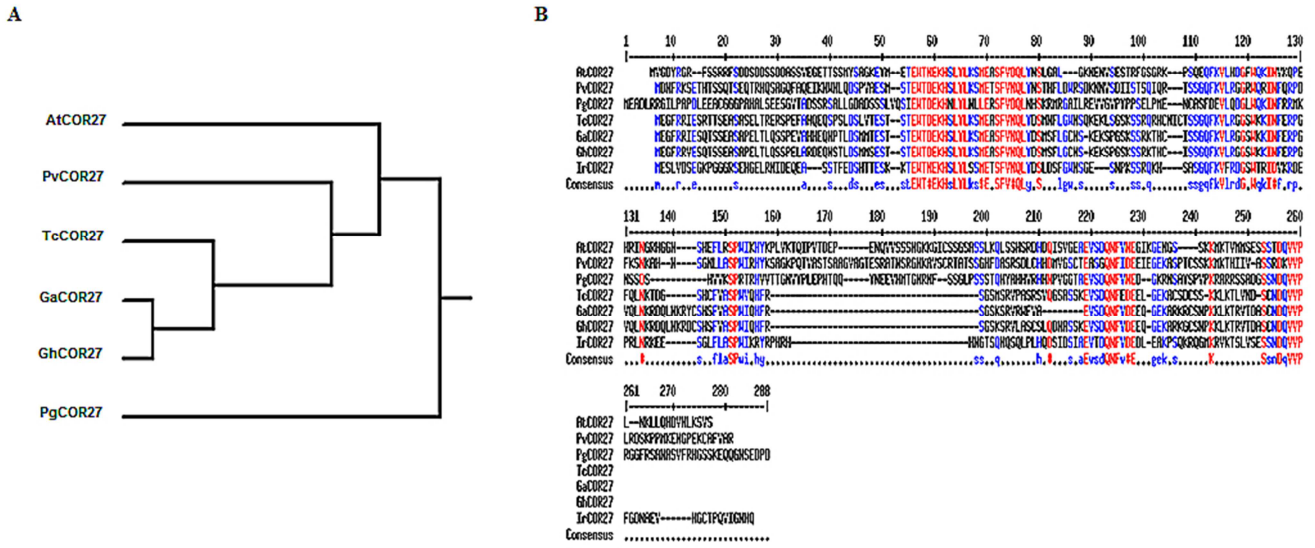

Figure 1. *A Sequence alignment and B phylogenetic analysis of cotton GhCOR27 and other species COR27 family members.*

The alignment was performed using the Multalin website (http://multalin.toulouse.inra.fr/multalin/) with a hierarchical clustering approach, and the phylogenetic tree was constructed using CLUSTALW and rooted phylogenetic tree (http://www.genome.jp/tools/clustalw/).

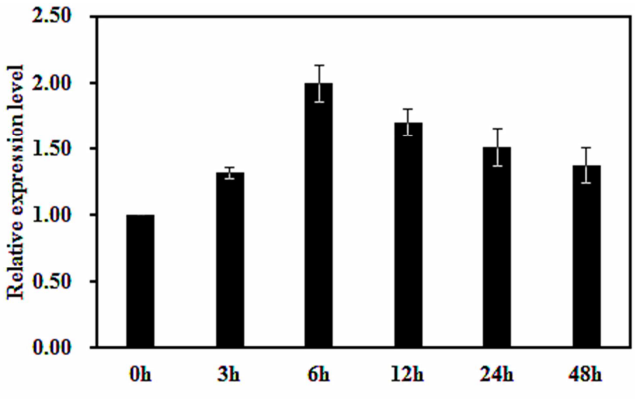

Figure 2. *Relative expression analysis of GhCOR27 in cotton under low temperature treatments.*

GhCOR27 expression patterns were analyzed in 3-week-old plants growing at 26°C after treatments at10°C for 0, 3, 6, 12, 24 and 48 h. The results are the mean value of three independent experiments. For each experiment 25-30 plants were used per treatment. Bars represent SEs, P<0.05.

3.2. The Expression of GhCOR27 Is Induced by Low Temperature

To investigate whether GhCOR27 is involved in defense low temperature stress, we checked the expressive pattern of *GhCOR27* at low temperature. the seedlings were shifted from 26°C to 10°C and then were collected at 3h, 6h, 12h, 24h, 48h time points respectively for transcriptional analysis. An induction was observed following treatment at 10°C, with the increasement of incubation time at low temperature, the transcriptional level of *GhCOR27* was gradually induced, a peak expression was showed at 6h-time point (Figure 2). Based on the result, we speculated that *GhCOR27* may function in the cold acclimation process in cotton.

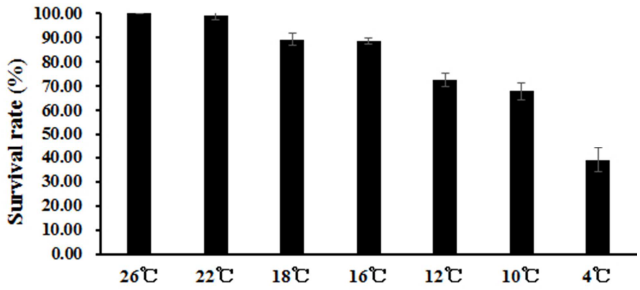

Figure 3. *Survival rates of cotton seedlings treated at different temperature.*

Plants were grown at 26°C for 3 weeks and transferred directly to 22°C, 18°C, 16°C, 12°C, 10°C and 4°C for 3d in normal photoperiod (12h-light/12h-dark cycle). And then returned to 26°C for 72 h. The survival rates were monitored. The results are the mean value of three independent experiments. For each experiment 25-30 plants were used per treatment. Bars represent SEs, P<0.05.

3.3. Assessment of Survival Following Low Temperature Treatment in Acclimated and Non-acclimated Plants

Cotton as a crop sensitive to temperature, its growth development has great chance of suffering from cold stress, Seedlings exposed to cold often produce lower survival [2]. Here, a test based on survival rate to establish proper low temperature at which phenotypic differences between seedlings with gene-overexpressing or gene-silencing and wild-type seedlings can be told clearly was performed.

3-week-old cotton seedlings growing at 26°C were shifted to 22°C, 18°C, 16°C, 12°C, 10°C and 4°C for 3 days in normal photoperiod (12-h-light/12-h-dark cycle). And the percentage of survival was investigated after transferring back to 26°C for 3d. As shown, with the decrease of temperature, seedlings exhibited increasing sensitivity to low temperature with decreased survival rates, and survival rates reached the lowest especially when seedlings transferred from 26°C to 4°C (Figure 3). The compared result indicated that temperature begin to show impact on seedlings in survival rate at 18°C and use of lower temperature at 4°C was lethal for cotton. But for the purpose of telling significant difference between VIGS plants and control for following gene function analysis under certain cold stress condition, the pre-test proved that the better cold stress treatment was at 10°C.

3.4. VIGS Gene-silencing Cotton Plants Exhibited Supersensitive to Low Temperature

Since VIGS gene-silencing technique is a rapid way for studying gene profile in plant and is wildly applied to discovery functional gene in cotton [13-15]. To further test whether *GhCOR27* was required for cotton resistance at low temperature, we adopted VIGS assay in this study. First, we infiltrated *GhCLA1* into seedling leave as a visual marker to verify the efficiency in TM-1, after 10-15 days, newly emerged true leave exhibited albino phenotype, Meanwhile decreased transcriptional level for *GhCLA1* was also recorded by RT-PCR and qPCR (Figure 4A and 4B). So, the VIGS assay was establish in this study for silencing *GhCOR27* in cotton. Then we designed the primers to amplify the conserved region of *GhCOR27* and inserted it into TRV-RNA2 vector pYL156. The *Agrobacterium* carrying the recombinant TRV vectors with either vector control or *GhCOR27* fragment were infiltrated into the cotton seedlings, seedlings infiltrated with *GhCLA1* were set as marker to assess the silencing efficiency for *GhCOR27* silencing in this study. When the marker seedings showed albino phenotype, we challenged the control and *GhCOR27*, seedlings with low temperature. The phenotype in TM-1 plants silenced with *GhCOR27* by VIGS was observed, the expression of interesting gene was analysis by both RT-PCR and qPCR (4B and 4C). Also the percentage of survival plants was scored after treatment at low temperature. As shown, plants silenced with *GhCOR27* exhibited a more severe chilling injury phenotype than plants infiltrated with the vector control (Figure 4C). The percentage of survival plants silenced with *GhCOR27* was also lower than those with control infiltration (Figure 4D). Thus, loss of function analysis suggested that *GhCOR27* may play a role in cold stress signal pathway.

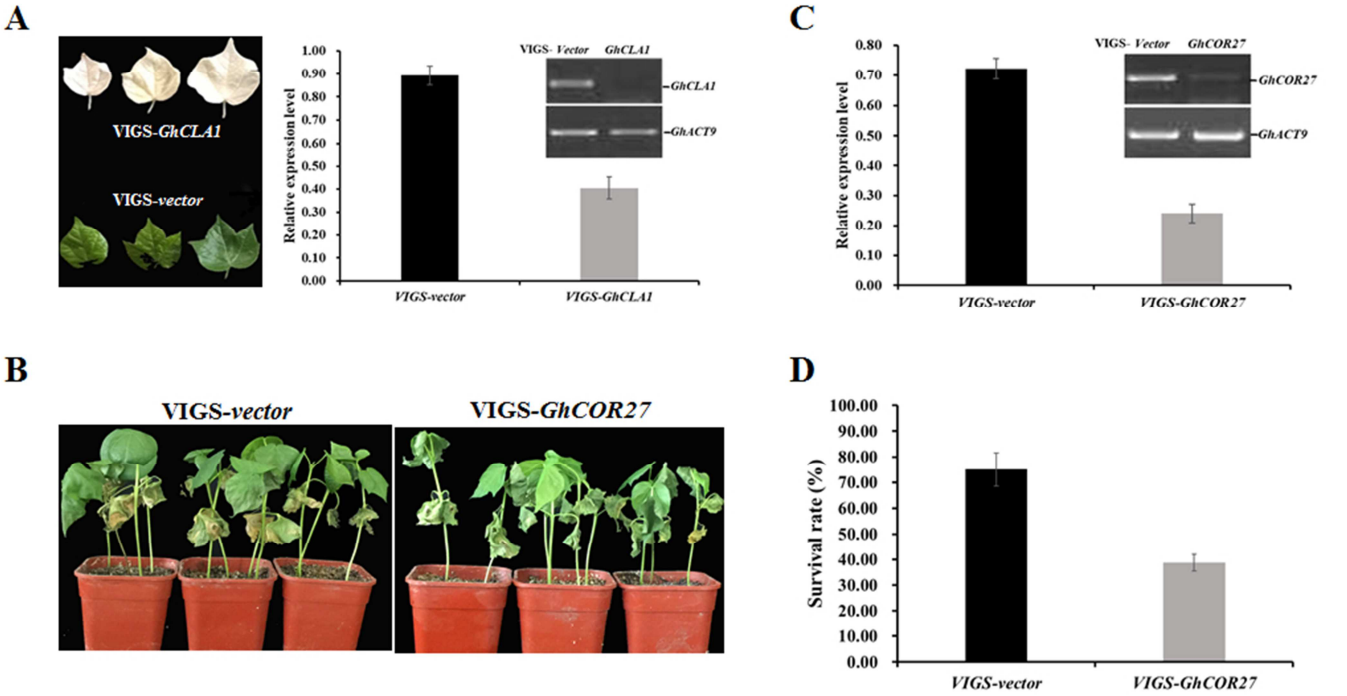

Figure 4. *Silencing of GhCOR27 enhanced plant susceptibility to cold stress.*

A. The photobleaching phenotype of *GhCLA1*-silenced cotton leaves. Agrobacterium carrying pTRV-RNA1 and pYL156-*GhCLA1* were infiltrated into two fully expanded cotyledons of 2-week-old seedlings. The right and the inset show the expression of *GhCLA1* in control and silenced plants by RT-PCR and qRT-PCR analysis. *GhACT9* was used as a control. B. Phenotype of control and silenced seedlings under low temperature condition. C. The relative expression of *GhCOR27* in control and *GhCOR27*-silenced plants, the inset show the expression of *GhCLA1* in control and silenced plants by RT-PCR. D. Survival rate of *GhCOR27*-silenced seedlings after low temperature treatment.

4. Discussion

Many *COR* genes from different plants have been described, including five groups of *COR* genes from *A. thaliana*, *CbCOR15* from *C. bungeana*, *BN115*, *BN26*, and *BN19* genes from *B. napus*, *COR14b* gene from *Hordeum vulgare*, and *CbCOR15a* from *C. bursa-pastoris*. These genes are of different expression patterns. For example, *BnCOR25* from *B. napus* transcription level significantly increased after 3 h cold treatment, and declined to low level after 6 h and enhanced again after 12 h cold treatment [16, 17]. Different expression patterns may indicate different roles of these COR genes in each species, for example in *Arabidopsis*, *COR27* and *COR28* mediate cold signaling input to circadian clock and represent a trade-off between freezing tolerance and appropriate period length of circadian rhythm [11]. Cotton (Gossypium spp.) is the most important fiber producing plant in the world and also a significant oilseed crop. Due to cotton is thermophilic and sensitive to cold temperature during its development and growth, it is important to improve our understanding of genes that directly or indirectly impact such cold traits. Based on the whole cotton genome sequence database, a number of cotton genes were identified and characterized, the understanding of gene function not only at genome-wide level, but also at individual level becomes systematic. In this study, we used the full length *AtCOR27* amino acid sequence as query to blast against the genome of *G. hirsutum* database and screened *COR* family member in the cotton genome. Total nine genes were identified from *G. hirsutum* database. Then, quantitative PCR was performed to find out which candidates were markedly up-regulated under cold stress. The expression level of *GhCOR27* increased after 3h cold treatment and then reached the peak that was 1.92 fold higher in the cold-treated seedlings than in those of the control plants, even treated after 48h, it remained a higher transcription level than non-treatment condition, which indicate that *GhCOR27* may play an important role in response to cold stress.

For further understanding of the function of *GhCOR27* under cold stress, we should have generated over-expressing or loss of function cotton plants to characterize it. As the major impediment to analyzing cotton gene function on a large scale is the laborious and time-consuming process of generating transgenic cotton. Moreover, many cotton cultivars and Gossypium species that contain important genes for cotton improvement are recalcitrant to genetic transformation. Therefore, there is an urgent need to develop a rapid method for functional analysis of cotton genes on a genomic scale. Virus-induced gene silencing (VIGS) offers such a possibility because it allows the investigation of gene functions without plant. By now, VIGS has been a reliable gene-silencing technique for studying gene characterization in plant. Furthermore, VIGS vectors have been modified to apply to different species and been already proved to work efficiently. Among these vectors, the tobacco rattle virus (TRV) vector is widely used in many plants such as tobacco [18], tomato [19], pepper [20] and petunia [21]. By this approach, genes referred to abiotic stress in cotton were identified and characterized as well [14, 15, 22]. More important, VIGS provides rapid way to discovery and study gene because when the Agrobacterium carrying the target gene is inoculated in cotton cotyledons of 2-week-old seedling, the effect of gene silencing will emerge in 2 weeks after the inoculation [20], which is especially helpful for those plants which have difficulty in obtaining transgenic plants. Silencing begins within a few weeks of inoculation and can extend throughout the developing period. VIGS can also be used to test the function of essential genes [23]. Gao found that *GhMKK2* and *GhVe1* are required for resistance to *Verticillium dahliae* in the cotton CA4002 line by VIGS method [20]. So, in this study we used the VIGS technology for the purpose of generating *GhCOR27*-silenced cotton plants. The comparison result indicated that silencing *GhCOR27* led to plant susceptibility to cold stress which the survival rate of *GhCOR27*-silenced seedlings was only at 31.09% lower than those of non-silenced seedlings at 53.27%. These results suggest that *GhCOR27* may play a role in response to cotton cold stress response and demonstrate the TRV-VIGS system can be used to rapidly identify functions of genes that play a role in cotton abiotic stress.

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