A Novel Stable QTL in Chromosome A04 for Salt Tolerance at the Seed Germination Stage of Upland Cotton via a Resequencing-Based High-Density Genetic Map

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Abstract

Key message Two candidate genes *GhGASA1* and *GhADC2* playing negative roles by modulating the GA and PA signaling pathway, respectively, were identified in a major QTL for germination under salt stress.

The successful transition of a seed into a seedling is the prerequisite for plant propagation and crop yield. Germination is a vulnerable stage in a plant's life cycle which is strongly affected by environmental conditions, such as salinity. In this study, we identified a novel stable quantitative trait locus (QTL) *qRGR-A04-1* associated with relative germination rate (RGR) after treatment with salt stress based on a high-density genetic map under phytotron and filed conditions, with LOD values of 6.65-16.83 and 6.11-12.63% of phenotypic variations in all five environment tests. Two candidate genes with significantly differential expression between two parents were finally identified through RNA-seq and qRT-PCR analyses. Further functional analyses showed that *GhGASA1*- and *GhADC2*-overexpression lines were more sensitive to salt stress than wild-type in *Arabidopsis* through regulating the transcript levels of gibberellic acid (GA) and polyamine (PA) -related genes implicating in GA and PA biosynthesis with reducing the accumulation of GA and PA under salt stress, respectively. Virus-induced gene silencing analysis showed that TRV:*GASA1* and TRV:*ADC2* displayed more tolerant to salt stress by increasing the expression of GA-synthesis genes and decreasing the H_2O_2 content, respectively. Taken together, our results suggested that QTL *qRGR-A04-1* and its harbored two genes, *GhGASA1* and *GhADC2*, were promising candidates for salt tolerance improvement in cotton.

Introduction

Seed germination is a complex trait determined by both QTLs and environmental factors, such as salinity stress. Soil salinization is one of the main adverse environmental factors restricting crop growth and development (Farooq et al. 2017; Hamwieh et al. 2011), as it limits the uptake of water by the roots and is associated with the accumulation of toxic ions, and thus it influences the overall cellular physiology of the plant (Munns and Tester 2008; Roy et al. 2014). Due to climatic changes, unscientific irrigation and excessive fertilization (Han et al. 2015), salinity is becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050, which will inhibit seed germination and reduce seedling growth (Wang et al. 2003).

As the most important natural textile fiber crop in the world, cotton (*Gossypium* spp.) is also the second most inherently salt-tolerant crop, having 7.7 dS/m threshold level of that is approximately equal to 77 mM NaCl in saline soils (Huang et al. 2013). However, different cotton species even different cultivar within a species show diverse salt tolerance ability (Sun et al. 2018). High level of salt ultimately inhibited cotton growth and reduced productivity, especially when this exposure occurs at the germination or seedling stage. China is the largest producer and consumer of cotton in the world. Nowadays, saline soil becomes a key factor restricting cotton production in China. Therefore, to breed salt-tolerant cotton cultivars, it is essential to clarify the genetic mechanism of salt tolerance at the germination stage and identify the salt tolerance-related genes.

Salinity affects plants mainly focus on osmotic and ionic stress, which is affected by numerous genetic and non-genetic factors. QTL mapping based on linkage analysis and association mapping studies based on linkage disequilibrium analysis are rapid and precise alternative approach to conventional selection schemes for improving complex quantitative traits (Diouf et al. 2018; Ma et al. 2018). These analyses provided sound footing to allow researchers to link phenotypes as well as some specific regions of chromosomes. However, these strategies have limitations such as low mapping resolution and limited genetic diversity between the mapping population parents. The population structure among individuals and large extent of linkage disequilibrium can confuse the results in association mapping studies (Yano et al. 2016).

Following the completion of several draft genomes of cotton (Hu et al. 2019; Wang et al. 2018), next-generation sequencing has transformed the field of cotton breeding. In the recent past, a lot of data generated has facilitated the discovery and use of large scale of single-nucleotide polymorphisms (SNPs) in cotton (Gu et al. 2020; Ma et al. 2018; Sun et al. 2017). Accordingly, the use of SNP markers is an effective way to construct a high-quality, high-density genetic linkage map for detecting QTLs and the limitation of a low marker resolution of genetic maps previously-reported is solved. Hence, QTL mapping has become a crucial access to quantitative trait breeding, and has been largely used to agricultural fields to map an important number of traits (Luo et al. 2019; Ma et al. 2019). However, studies on identifying salt-tolerant QTLs/genes in cotton using association mapping (Cai et al. 2017; Sun et al. 2018) or even linkage mapping (Diouf et al. 2017; Oluoch et al. 2016) are limited, especially the germination-related traits under salt stress in cotton (Ashraf and Foolad 2013; Diouf et al. 2017). Thus, dissecting the genetic loci and attaining a high capacity of seed germination under salinity stress is an important objective of cotton breeding.

In the present study, a large scale recombinant inbred line (RIL) population derived from a cross between two upland cotton varieties, a high salt-tolerant cv. Nongdamian 13 (ND13) and a medium salt-tolerant cv. Nongda 601 (ND601) identified in a previous research under salt

stress (Sun et al. 2018), is used to identified QTLs related to germination rate in the greenhouse and filed condition under salt stress condition based on a high-density genetic map obtained by resequencing strategy. The QTL identified in this study could facilitate future molecular breeding programs to improve the salt tolerance in upland cotton.

Materials And Methods

Plant materials and phenotypic evaluation

A segregation population consisting of 588 F_7 RILs was used for QTL mapping (Gu et al. 2020). One of two parents was upland cotton variety ND601 with moderate salt tolerance, and the other was upland cotton variety ND13 with high salt tolerance. RIL population was developed by single-seed descent strategy.

The phenotypic evaluation for salt tolerance was performed in the phytotron under a 16/8 h light/dark cycle at a condition of 28/22°C day/night, 65% relative humidity. Seeds of the RIL population and two parents were collected from three environments, including Qingyuan Experimental Station (38°45'N, 115°29'E) of Hebei Province in 2017 (denoted E1); Sanya Experimental Station (18°21'N, 109°10'E) of Hainan Province in 2018 (denoted E2); Hejian Experimental Station (38°26'N, 116°05'E) of Hebei Province in 2019 (denoted E3). Prior to germination, the seeds were delinted with sulfuric acid solution and sterilized with sodium hypochlorite solution to minimize the danger of microbial contamination. A total of 320 healthy and full seeds were selected from each line and subdivided into four repetitions, three repetitions for salt stress and one for water control treatment. A sand culture in a germination box containing 800 g dry quartz sand and covering evenly with 250 g dry quartz sand above the seeds was used to conduct the experiment. Based on our previous results (Sun et al. 2018), 0.3% NaCl solution (m_{NaCl} / m_{sand}, approximately equal 215.6 mM NaCl), which presented a significantly differential germination rate (GR, the number of seeds germinated / total seed number used in the test) between both parents, was used to evaluate the GR of RILs after sowing seven days at seed germination stage. The relative germination rate (RGR) was calculated as follows: RGR = GR under stress treatment / control GR × 100%.

Another phenotypic measure for salt tolerance was conducted in the field condition through pot-culture method at the experimental farm of Hebei Agricultural University. Seeds of the RILs population and two parents were collected from two environments, including Sanya Experimental Station in 2018 and Hejian Experimental Station in 2019 (denoted E4 and E5, respectively). Each line with one water control and two salt stress treatments was grown in a pot (33 cm × 36 cm × 30 cm) filled with uniformly mixed substrate (dry soil: vermiculite: nutrient medium, 2:1:1) containing 0.3% NaCl under field condition. A total of 50 seeds were sown per pot with the same seeding depth. The GR of RILs was recorded after ten-days sowing, and the RGR was calculated.

The mean best linear unbiased prediction (BLUP) value for five environments under two different conditions were estimated for each line using the lme4 package in R (Poland et al. 2011). Statistical analysis of the RGR was performed using IBM SPSS Statistics SPSS 22.0.

QTL analyses

The analysis of QTL related to RGR was performed by WinQTL Cartographer 2.5 software (Wang et al. 2011). The composite interval mapping model was used to scan the genetic map and estimate the likelihood of a QTL and its corresponding effect at every 1 cM. An LOD threshold score of 2.5 was considered as the minimum necessary in order to determine that any given QTLs were significant. 99% QTL confidence interval was set as map interval. The percentage of the phenotypic variation explained (PVE) by a QTL was estimated by the coefficient of determination. The QTL nomenclature was designated as: q + trait abbreviation + chromosome number + QTL number.

Candidate genes discovery and verification

The physical region underlined by the confidence interval of the most consistent QTL was used to find candidate genes. The genes identified were searched through the CottonFGD (https://cottonfgd.org) (Zhu et al. 2017). The seeds of two parents, ND13 and ND601, from 0, 6, 12 hour (h), 1, 3, 5 and 7 day (d) during cotton seed germination process, were sampled and immediately frozen in liquid nitrogen and stored at − 80 °C for RNA extraction with an EASYspin Plant RNA Kit (Aidlab) with three replicates after treatment with salt stress. Total cDNA was synthesized with PrimeScript^{TM}RT reagent Kit (Perfect Real Time) (TaKaRa). The qRT-PCR reactions contained 10 μL SYBR® Premix Dimer Eraser™ (Perfect Real Time) (TaKaRa, China), 1.0 μL of cDNA, 0.6 μL of primer, and ddH_2O to a final volume of 20 μL. The reactions were amplified for 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s, 55 °C for 15 s and 72 °C for 15 s. All reactions were performed in three independent biological replicates, each with three technical replicates, using the Roche LightCycler96 Real-Time PCR System. *GhUBQ7* expression was used as the internal control for qRT-PCR. Relative gene expression values were calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001). The primers were listed in Table S3 in the Supplement Information.

Gene cloning and plant transformation

The full-length open reading frames of the candidate genes were amplified through PCR using cDNAs synthesized from RNA that were isolated from two parents with the corresponding SNP alleles. The amplified products were further cloned into the pGreen vector driven by the cauliflower mosaic virus 35S promoter. The resulting constructs were further transformed into *Arabidopsis thaliana* Columbia type by *Agrobacterium tumefaciens* GV3101 and selected with Basta. Two-week-old wild-type (WT) and two overexpression (OE) lines were sampled at 0 h, 3 h, 6 h and 12 h after salt treatment to analyse the gene function in *Arabidopsis*. Three weeks later, the survival rate, chlorophyll content (measured by SPAD-502, Konica Minolta, Inc., Tokyo, Japan) and fresh weight of OE and WT lines were detected after salt treatment. We used 3,3'-diaminobenzidine (DAB) staining to detect H_2O_2 with a DAB chromogenic kit (JianCheng, W026) according to the manufacturer's instructions. The primers are listed in Table S3.

For the seed germination assay, thirty seeds were surface-sterilized for 5 min in 30% NaClO, washed at least four times with sterile water, stratified at 4°C for 48 h, and plated on 1/2 Murashige & Skoog (MS) solid medium (with 1% sucrose) or 1/2 MS medium supplemented with 150 mM NaCl at 22°C under a 16 h/8 h, light/dark photoperiod for 10 days. For each germination assay, biological triplicates were performed.

Virus-induced gene silencing in cotton

The gene-specific region for candidate genes was amplified as a template and cloned into the pTRV2 vector. The resulting pTRV2 construct was co-infiltrated with pTRV1 via *A. tumefaciens* GV3101 into cotton seedlings of ND601 through syringe inoculation when the cotyledons spread (Gao and Shan 2013). The plants co-inoculated with empty pTRV2 and pTRV1 were used as controls. The cloroplastos alterados 1 gene was used as a marker to monitor the silencing efficiency. The leaves from silenced plants and the control were sampled at 0 h and 12 h after salt treatment. The primers used are listed in Table S3.

Results

Phenotypic evaluation of RILs and QTL mapping for salt tolerance

In this study, the significant difference of salt-tolerance between ND13 and ND601 were observed across five environments and the BLUP value. ND13 displayed consistently high RGR under salt stress while ND601 showed medium salt-tolerance with lower RGR (Fig. 1, Table 1). Frequency distribution graph of germination for all the population lines showed that RGR displayed continuous variation across all treatments with an absolute skewness value of less than one, predicting the presence of resistance alleles in two parents (Fig. S1-2). Significant correlations for RGR were found among the five environments (Table S1).

Table 1
Phenotypic variation of RGR in the RIL population.

Trait	Env.	Parents			RILs							
		ND13	ND601	P1–P2[a]	Minimum	Maximum	Mean	Range	SD	CV[b]	Skewness	Kurtosis
RGR	E1	0.56	0.30	0.26**	0.01	0.68	0.28	0.67	0.12	44.09%	0.55	0.07
	E2	0.73	0.43	0.30**	0.09	0.90	0.49	0.81	0.16	33.74%	0.09	−0.60
	E3	0.64	0.48	0.16**	0.24	0.83	0.59	0.59	0.09	16.06%	−0.21	0.80
	E4	0.56	0.45	0.11*	0.02	0.82	0.48	0.80	0.19	40.55%	−0.55	0.53
	E5	0.60	0.41	0.19**	0.09	0.74	0.40	0.65	0.11	28.00%	0.13	0.29
	BLUP	0.52	0.44	0.08*	0.42	0.59	0.50	0.17	0.03	6.97%	0.02	−0.04

Note: a: P1: ND13, P2: ND601. *, **, significant at $P < 0.05$ and $P < 0.01$, respectively. b: Coefficient of variation.

A high-density genetic map with 6,187 recombination bin markers based on a total of 232,946 polymorphic homozygous SNPs obtained from the two parents (ND13, 51.51Gb, 22.40×; ND601, 51.42Gb, 22.36×) and RIL lines (with average of 9.89 Gb, 4.3×) was constructed previously (Gu et al. 2020). In this study, a QTL analysis was conducted to detect markers associated with salt tolerance across five environments. A total of 11 QTLs for RGR accounting for 1.74%-12.63% of the observed PVE were identified by CIM analysis. These QTLs were located on chromosomes A01, A03, A04, A05, A07, A08, A13, D01 and D09, indicating that the salt tolerance of cotton at the germination stage was controlled by multiple genes (Table S2). Nine RGR-related QTLs were located on the At subgenome, and two were on

the Dt subgenome. Among these QTLs, one stable major QTL, *qRGR-A04-1*, was detected in all five environments with LOD values of 6.65–16.83. This QTL with 6.11–12.63% of phenotypic variation closely linked with the marker bin1322 contained the physically genetic region between the markers bin1314 and bin1329. This result suggested that the candidate gene might be located within the region between markers bin1314 and bin1329 or closer to bin1322 (Table 2).

Table 2
Information of QTL *qRGR-A04-1*.

QTL	Env.	Position (cM)	Marker interval	LOD	Additive effect	PVE%	Physical location (Mbp)
qRGR-A04-1	E1	10.11	0.0–11.2	9.26	−0.03	6.72%	82.05–85.11
	E2	5.61	4.9–9.7	10.33	−0.04	7.53%	82.05–82.81
	E3	8.11	0.0–11.9	7.94	−0.03	6.81%	81.94–85.11
	E4	12.91	0.5–17.2	6.65	−0.06	6.11%	81.14–85.11
	E5	8.61	0.0–15.3	10.81	−0.04	9.89%	81.30–85.11
	BLUP	9.11	0.0–11.4	16.83	−0.01	12.63%	81.94–85.11

GhGASA1 and GhADC2 were the candidate genes in QTL qRGR-A04-1 associated with salt tolerance

We examined the genes located in the 99% confidence intervals of the stable QTL *qRGR-A04-1* at 81.14–85.11 Mb on A04, and 349 genes were mined in this region. In order to identify the set of most robust candidate genes for salt tolerance, all the 349 genes were analyzed using “TM-1” RNA-seq expression profiles data at different time points of salt treatment 1 h, 3 h, 6 h and 12 h in leaves (http://mascotton.njau.edu.cn) (Zhang et al. 2015). And based on the SNP variation obtained from resequencing data of two parents, 21 genes were screened out. Subsequently, we performed a quantitative real-time PCR analysis to investigate whether the expression of these 21 genes in ND13 and ND601 were affected by salt stress at seed germination stage (Fig. S3). The results demonstrated that two genes (*Ghir_A04G012950* and *Ghir_A04G013460*) could be induced by salt stress. *Ghir_A04G012950* was dramatically upregulated in ND601 but not in ND13 under salt stress. In fact, the expression level of *Ghir_A04G012950* in ND601 was nearly 20-fold change compared to that in ND13 after treatment with 0.3% NaCl. *Ghir_A04G013460* was induced in ND13 at 3 d and reached the peak at 5 d under salt treatment condition. However, no expressed transcript was detected in ND601 until 5 d after salt stress. Thus, two genes, *Ghir_A04G012950* and *Ghir_A04G013460*, which were located in the QTL *qRGR-A04-1* identified in our study were predicted to be the putative candidate genes for salt tolerance at the cotton germination stage (Fig. 2A-B).

Ghir_A04G012950 has a length of 273bp and is a member of the GASA superfamily, encoding a snakin-1 protein with 90-amino-acid. We designated *Ghir_A04G012950* as *GhGASA1* which is the homologue of the gene *AT2G14900* encoding a gibberellin-regulated family protein in *Arabidopsis*. Comparing sequences of ND13 and ND601, we found a nonsynonymous SNP (A/T) in *GhGASA1*. The nonsynonymous SNP in the exon located in the GASA domain might encode a different function protein. Gene-expression test revealed that *GhGASA1* was induced by salt stress, and the expression in ND601 (T) was significantly higher than ND13 at the 5 d and 7 d during the seed germination (Fig. 2C). A previous report showed that snakin/GASA peptides participated in plant growth and development as well as in plant responses to biotic and abiotic stresses (Nahirñak et al. 2014). The expression of most of snakin/GASA genes was modulated by plant hormones and participated in hormonal signaling pathways modulating hormonal responses and levels, such as GAs. GAs is one of most important phytohormones that coordinates with a cascade of molecular signaling regulation to promote seed germination (Jiang et al. 2016). The expression levels of genes encoding biologically active GAs in ND13, such as *GhGA20ox1* and *GhGA3ox1*, were significantly higher than ND601 in germination under salt treatment. Conversely, the expression of genes encoding biologically inactive GA (*GhGA2ox1*) and DELLA protein (*GhSLR1*) in ND13 was lower than ND601 (Fig. S4).

Ghir_A04G013460, encoding an arginine decarboxylase (ADC) protein with 717 amino acid residues, is a member of the fold type III PLP-dependent enzyme family, and is a homologue of the *AtADC2* induced by salt stress through the accumulation of free putrescine (Put) in *Arabidopsis* (Urano et al. 2004). No SNP variation was found in *Ghir_A04G013460* (here named as *GhADC2*), but qRT-PCR result showed that it was induced at the 3 d by the salt stress and a significantly different expression was observed between ND13 and ND601 (Fig. 2D). Taking these together, we concluded that *GhGASA1* and *GhADC2* might be the causal genes underlying the major QTL *qRGR-A04-1* for seed germination under salt stress.

Overexpression of candidate genes depressed salt tolerance in Arabidopsis

To investigate the role of *GhGASA1* and *GhADC2* under salt stress, both genes were transferred into *Arabidopsis* to generate transgenic lines driven by the CaMV35S promoter. For gene *GhGASA1*, the transgenic lines (T3) with two haplotypes were analysis and confirmed using qRT-PCR. The expression levels of *GhGASA1* in overexpression lines (OE-T represent the ND601 genotype; OE-A represent the ND13 genotype) were remarkably higher than the WT (Fig. 3A). In *GhGASA1* transgenic *Arabidopsis*, the survival rate, fresh weight and SPAD value of OE-T lines were reduced markedly than WT and OE-A lines (Fig. 3B-E). Subsequently, germination assays were carried out using these lines on MS medium containing 0 mM and 150 mM NaCl, respectively (Fig. 3F). The germination rates of overexpressing lines (OE-T and OE-A) were similar to that of the WT under normal condition. However, when germinated on MS medium containing 150 mM NaCl, OE-T lines displayed more sensitive to NaCl than WT and OE-A lines. It is noteworthy that the OE-A *Arabidopsis* also showed a significantly different germination rate to WT (Fig. 3G), indicating that *GhGASA1* could affect the germination under salt treatment.

The GA-related genes were identified to test whether *GhGASA1* was involved in response to salt stress by altering GAs content. The results showed that expression levels of *AtGA20ox1*, *AtGA20ox3* and *AtGA3ox1* in OE-T lines were down-regulated and showed significant lower expression levels than OE-A and WT at the 6 h and 12 h after salt stress. *AtGA20ox2* in OE-T lines had significant lower gene expression level than OE-A lines at the 6 h and 12 h under salt treatment condition. *AtGA2ox2* encoding an inactive GA synthetase also had lower gene expression level in OE-T lines than WT in 12 h. Moreover, in OE-T lines, the gene *AtRGL2*, encoding a DELLA protein which was a negative regulator of the response to GA, displayed remarkable higher expression level than WT during the salt stress treatment (Fig. 3H). The results indicating that accumulation of GAs, especially the bioactive GAs, was reduced remarkably in OE-T lines which displayed more sensitive to salt treatment than the WT *Arabidopsis* (Fig. 3B). In the ABA pathway, most gene in the biosynthesis of ABA showed no significantly differential expression level (data not show). However, the *AtCYP707A1*, involving in ABA catabolism, was strongly up-regulated and significantly higher than that in WT and OE-A *Arabidopsis* (Fig. 3H). These results indicated that *GhGASA1* might play a negative role in germination through regulating the biosynthesis of GAs and promoting the breakdown of abscisic acid in response to the salt stress.

For *GhADC2*, the overexpression lines (OE-2 and OE-3) showed more sensitive to NaCl (Fig. 4A-B). The survival rate, fresh weight and SPAD value of the OE-2 and OE-3 were dramatically lower than WT (Fig. 4C-E). And the germination assays also found the germination rate for WT was higher than overexpression lines under salt treatment (Fig. 4F-G). In plants, arginine decarboxylase catalyzes the first step of polyamine (PA) biosynthesis, which is thought to play an important role for stress tolerance. Thus, we detected the expression levels of genes involved in PA biosynthesis (Fig. 4H). And the results showed that *AtSPDS1* and *AtSPDS2* synthesizing the spermidine (Spd) were induced by NaCl and the expression levels of those genes in *GhADC2*-overexpressing lines were significant decreased under salt stress, which indicating that the accumulation of Spd in WT was significantly higher than *GhADC2*-overexpressing lines. However, the transcript level of gene *AtSPMS* synthesizing the spermine (Spm) was down-regulated and displayed significantly decreased in WT plants than *GhADC2*-overexpressing lines after salt treatment. Interestingly, the transcriptions of *AtPAO1* and *AtPAO2*, functioning in polyamine catabolism, were dramatically up-regulated and showed significantly higher expression level in *GhADC2*-overexpressing lines than WT after 200 mM NaCl treatment. And *AtPAO3* showed significantly higher expression level in overexpression lines than WT even though it displayed a down-regulated trend. No significant difference was observed in *AtPAO4* between WT and *GhADC2*-overexpressing lines after salt treatment. Moreover, the expression levels of four S-adenosylmethionine decarboxylase genes (*AtSAMDC1-4*) showed no significant difference between overexpression and WT lines (data not show). With a down-regulation of PA synthesized genes and an up-regulated of catabolism genes, the accumulation of PA in *GhADC2*-overexpressing lines might display a decreasing trend. Thus, *GhADC2* may play a negative role in mediating the accumulation of PAs in response to the salt stress.

Additionally, H_2O_2 can be generated by PAO-mediated PA catabolism. It is known that H_2O_2 plays dual roles in plant responses to abiotic stresses (Liu et al. 2015). So, the transcript-level of *AtCAT* was detected in *GhADC2*-overexpressing and wild-type *Arabidopsis*. And the results showed that *GhADC2*-overexpressing lines have a significantly lower expression level than WT, indicating that a high accumulation H_2O_2 may be present in *GhADC2*-overexpressing *Arabidopsis*. Then, to determine whether *GhADC2* is involved in H_2O_2 production, *GhADC2*-overexpressing plants were stained with DAB. When they were exposed to NaCl, all seedlings accumulated H_2O_2. However, the OE lines produced and accumulated more H_2O_2 thanWT (Fig. S5). Thus, it was showed that *GhADC2* could cause excessive H_2O_2 accumulation and oxidative stress through the PA signaling pathway under salt stress.

Silencing of candidate genes enhanced cotton salt resistance

We further performed VIGS to test whether *GhGASA1* and *GhADC2* were required for cotton tolerance to salt stress. When the newly emerged leaves of plants infiltrated with TRV:*CLA1* started to display an albino phenotype (Fig. 5A), the expression of *GhGASA1* and *GhADC2* was measured by qRT-PCR to confirm the silencing of the gene, and the results showed that *GhGASA1* and *GhADC2* had been silenced (Fig. 5B). The *GhGASA1*- and *GhADC2*-silenced (TRV:*GASA1* and TRV:*ADC2*) and control (TRV:*00*) plants were subjected to salt stress at three-leaf stage (200 mM NaCl). After salt treatment, the TRV:*00* plants displayed wilting and lodging. In contrast, TRV:*GASA1* and TRV:*ADC2* seedlings displayed an increased salt tolerance (Fig. 5C-D). Subsequently, the leave of VIGS cotton plants were stained with DAB

(Fig. 5E-F), and the results showed that the accumulation of reactive oxygen species (ROS) was inhibited in the TRV:*ADC2* lines corresponding to the overexpression test, indicating that *GhADC2* can regulate ROS accumulation under salt stress conditions. TRV:*GASA1* also showed a weaker intensity of brown deposits than *TRV:00* seedlings. Moreover, the expression levels of *GhGA20ox2* and *GhGA3ox1* related to GAs biosynthesis were significantly up-regulated in TRV:*GASA1* lines, indicating that a differential accumulation of GAs synthetase transcripts in response to salt stress (Fig. S6).

All together, the functional analyses results showed that *GhGASA1* and *GhADC2* could partially explain the phenotypic variation in germination under salt stress and were the most likely candidate genes in QTL *qRGR-A04-1*.

Discussion

Good germination followed by potentially to cope up with environmental stresses has been proven to effective for yield gain. Despite having moderate salt-tolerance, cotton suffers severe yield losses to salinity stresses, largely due to being grown on saline-alkali and dry lands, especially when this exposure occurs at the germination or seedling stage. To deeply understand the salt tolerance mechanisms in cotton is a potential strategy to enhance cotton tolerance to salt stress. Whole-genome sequencing was recently shown to be a useful approach for dissecting the genetic architecture in cotton (Gu et al. 2020; Ma et al. 2018). In this work, a high-density genetic linkage map was employed to identify the QTLs for salt tolerance in cotton through phytotron and filed conditions.

To date, many QTLs for cotton salt tolerance related traits have been identified in biparental and natural populations, and the comparison of different reported QTLs is valuable to fully understand the behavior of complex traits and may support the accuracy of this map (Diouf et al. 2017; Sun et al. 2018). In the present work, seven QTLs related to RGR trait were found to share the same or overlapping confidence intervals with QTLs identified in previous QTL mapping efforts and GWASs (Table S7). One out of four novel QTLs, *qRGR-A04-1*, was found as a stable and major QTL for seed germination in cotton under salt stress and two candidate genes were identified base on a series of analyses.

Plant growth and development require complex and accurate regulation in response to different intrinsic and extrinsic stimuli. GAs and ABA are two major phytohormones that antagonistically regulate seed germination (Shu et al. 2015). Specifically, GAs promotes seed germination while ABA preserves seed dormancy. During the seed germination, high expression of GA-biosynthesis genes, such as *GA3ox1*, can promote GAs accumulation, which in turn promotes seed germination (Yamaguchi 2008). In contrast, the content of ABA would be decreased. CYP707A2, functioning in ABA catabolism, resulted in decreased ABA content in mature dry seed and a much shorter to overcome dormancy (Millar et al. 2006). A similar result with higher expression of GA-related genes and lower transcript-level of ABA-related genes was found in ND13 and ND601 germination under salt stress (Fig. S4). In addition to phytohormones, various environmental cues determine the appropriate timing for seed germination, also by mediating the ABA/GA balance. And a series of GA- and ABA-related genes were found to respond to salinity stress in plants (Zhou et al. 2020). In this study, we found a candidate gene *GhGASA1* inducing by salt stress in cotton seed germination stage (Fig. 2). *GhGASA1*-overexpression line OE-T shows more sensitive to salt stress than WT (Fig. 3). GA-biosynthesis genes in OE-T line, such as *AtGA20ox1*, *AtGA3ox1*, showed significantly lower expression levels than WT, and the transcript-level of *AtCYP707A1* was up-regulated under salt stress. When we knockdown *GhGASA1*, *TRV:GASA1* plant displayed an increased salt tolerance phenotype and the expression levels of *GhGA20ox2* and *GhGA3ox1* were significantly up-regulated under salt stress (Fig. S6). Thus, *GhGASA1* may play a negative role and involve in the regulation of GA signaling pathways of cotton germination in response to salt stress.

Polyamines, low-molecular-weight compounds, are some of the metabolites that are ubiquitously present in plants and play important roles in plant response to various environmental stresses (Kubiś et al. 2013). PAs are proposed to function in stress tolerance by maintaining membrane stability, adjusting osmotic potential, or promoting the scavenging of ROS (Liu et al. 2015). A NAC transcription factor, PtrNAC72, was found to be a repressor of putrescine biosynthesis and might negatively regulate the drought stress response via the modulation of putrescine-associated ROS homeostasis (Wu et al. 2016). In our previous study, the Spm signaling pathway contributed to resistance of the plant against *V. dahliae* in cotton (Mo et al. 2015). In the present study, a significantly higher expression levels of *AtSPDS1-2*, *AtSPMS1* and *AtPAO1-4* associated with the polyamine signaling pathway were observed in *GhADC2*-overexpression lines, showing that they were more sensitive to salt stress than WT *Arabidopsis* (Fig. 4H). Moreover, we found the expression levels of *AtPAO1* and *AtPAO2* were significantly up-regulated in *GhADC2*-overexpressing line, indicating a relative lower accumulation of Spm and Spd in *GhADC2*-overexpressing line. As a high transcript-level of *AtPAO*, H_2O_2 production is elevated, causing excessive ROS accumulation and oxidative stress, which is toxic to living cells due to lipid peroxidation and membrane damage, and can finally result in cell death (Biswas and Mano 2015). In tobacco, salinity induces cells to secrete exodus of Spd to the apoplast, where it is oxidized by PAO, thus generating abundant H_2O_2 and leading to enhanced programmed cell death (Moschou et al. 2008). Our detected results of the activities of antioxidant catalase

showed that the expression of *AtCAT* in the *GhADC2*-overexpression line was significantly lower than wild-type *Arabidopsis* under the salt stress. Moreover, the *GhADC2*-overexpression line showed a stronger intensity than that from WT in the DAB staining test (Fig. S5), indicating that high accumulation of H_2O_2 in the overexpression lines, which may enhance programmed cell death responding to salt stress. Thus, *GhADC2* may play a negative role and involve in the regulation of PA signaling pathways of cotton in response to salt stress.

Conclusion

In the present research, a consistently novel QTL was identified as *qRGR-A04-1* against salt stress based on a high-density genetic linkage map across five environments. Two candidate genes, *GhGASA1* and *GhADC2*, related to GA and PA signaling pathway were identified through tests of overexpression in *Arabidopsis* and knockdown in cotton under salt stress, which may contribute to breeding cotton varieties with high tolerance to salt stress.

Declarations

Author contribution statement ZM designed the research; QG, HK, CL, XL, ZS, ZL, WR, JY, YZ, LW, GZ, XW performed experiments; QG, XW analyzed data; QG wrote the manuscript; ZM, XW revised the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Competing financial interests The authors declare no competing financial interests.

Ethical standards The experiments were performed in compliance with the current laws of China.

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Figures

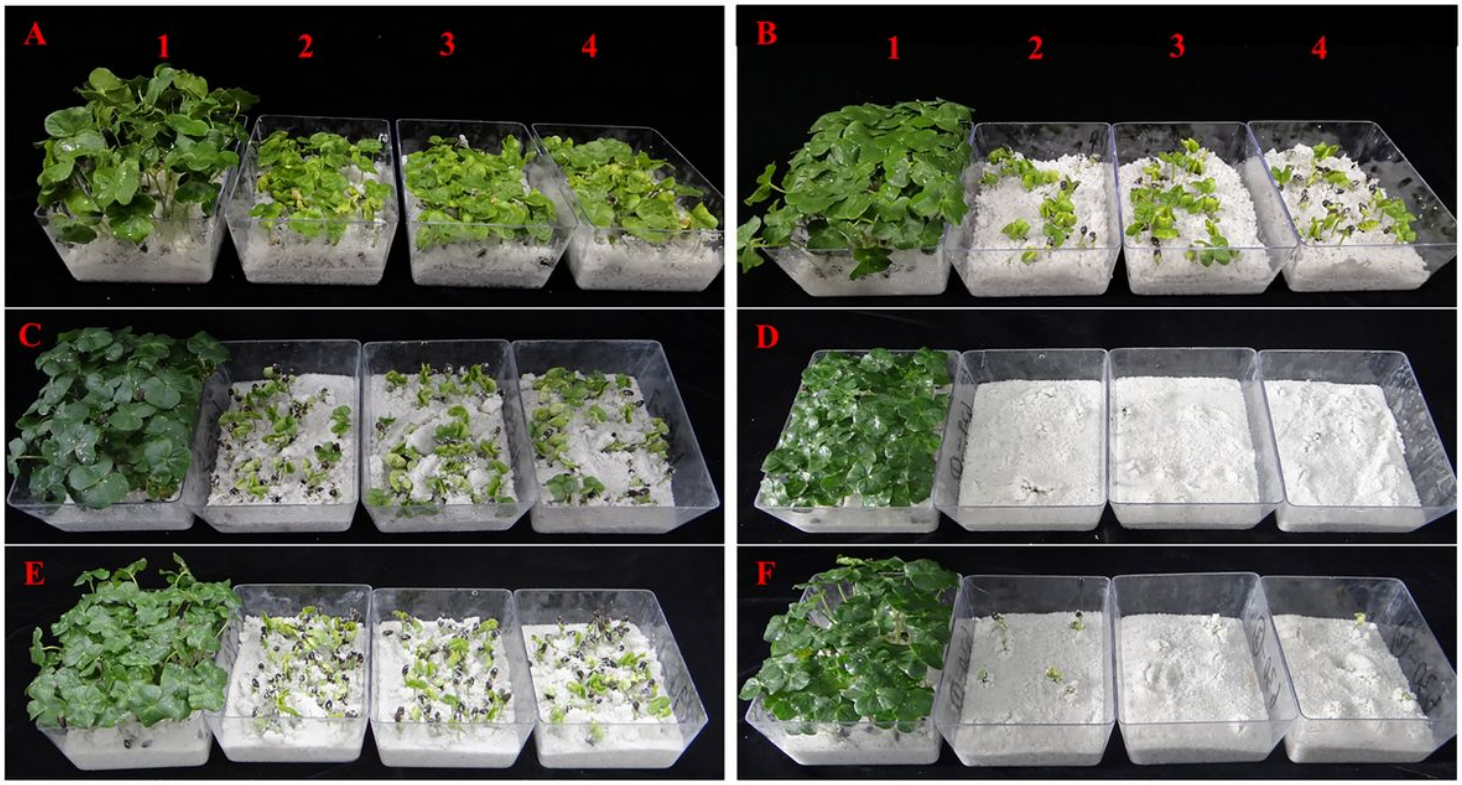

Figure 1

The salt-tolerance phenotypes of two parents and parts of RIL lines obtained in the phytotron. (A) The high salt-tolerant cv. ND13; (B) the medium salt-tolerant cv. ND601; (C, E) The high salt-tolerant lines; (D, F) The medium salt-tolerant lines. (1) Water control groups; (2-4) Salt treatment lines.

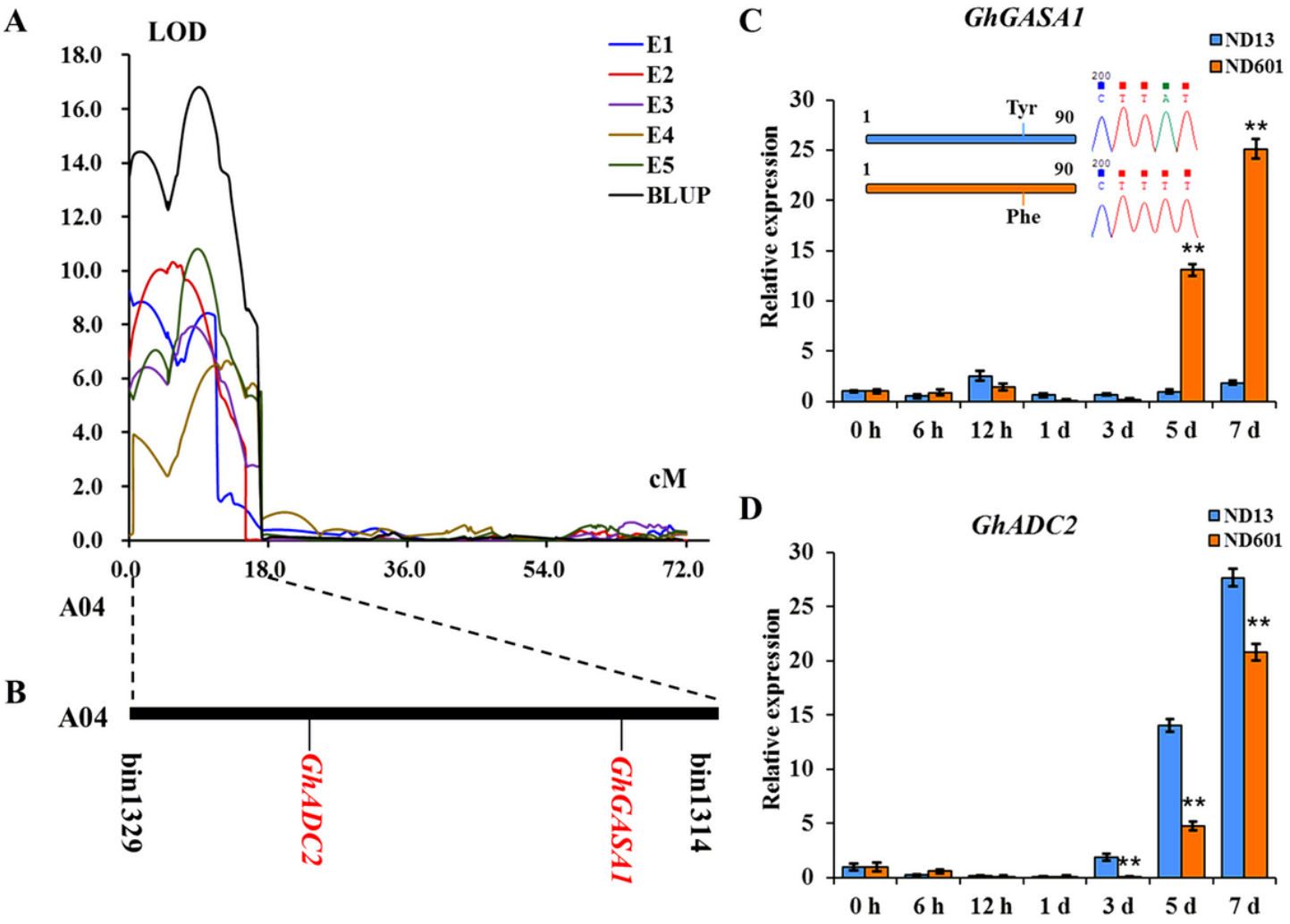

Figure 2

Identification of GhGASA1 and GhADC2 through a linkage mapping analysis. (A) qRGR-A04-1 was identified in the samples of 588 RILs collected from all five environments and BLUP value. (B) qRGR-A04-1 was mapped to the interval between markers bin1314 and bin1329 by linkage mapping and two gene GhGASA1 and GhADC2 were identified by resequencing, RNA-seq and qRT-PCR. (C-D) Expression analysis of GhGASA1 and GhADC2 after treatment with 0.3% NaCl for 0 h, 6 h, 12 h, 1 d, 3 d, 5 d and 7 d in two parents seed germination stage. The water treatment as the control to calculate the relative expression value. One nonsynonymous SNP (A/T, 203 bp) in GhGASA1 CDS region between both parents, resulting in an amino acid variation (Tyr/Phe) (** $P < 0.01$, $n = 3$).

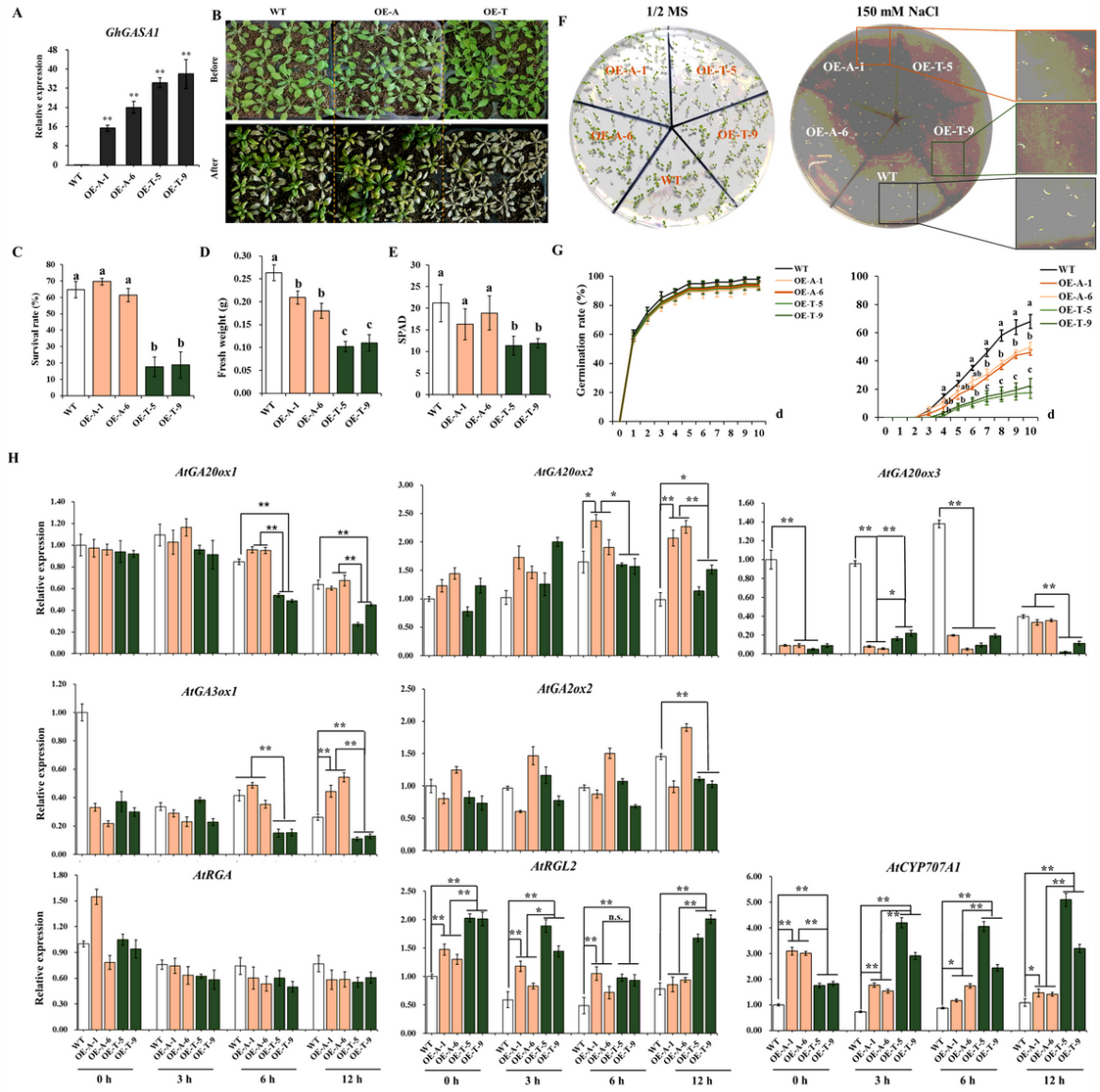

Figure 3

Performance of GhGASA1 transgenic Arabidopsis under salt stress. (A) qRT-PCR analysis of GhGASA1 for the wild-type (WT) and GhGASA1-overexpresion (OE-A and OE-T) lines (Student's t-test, ** $P < 0.01$, $n = 3$). (B) The representative phenotype of WT and OE seedlings after NaCl treatment (200 mM) for three weeks. Three independent experiments were conducted. (C-E) Survival rates, fresh weight and SPAD values of WT, OE-A and OE-T plants after NaCl treatment. Error bars indicate s.e.m. Different letters at the top of each column indicate significant differences at $P < 0.05$ ($n = 3$ biological replicates) by the Student's t-test. (F) Seed germination phenotypes. Vernalized seeds of Arabidopsis WT, OE-A and OE-T lines were transferred to MS medium or MS containing 150 mM NaCl. (G) Seed germination curves. At least 30 seeds per genotype were measured in each replicate. Error bars indicate s.e.m. Different letters at the top of each column indicate significant differences at $P < 0.05$ ($n = 3$ biological replicates) by the Student's t-test. (H) The transcript-level of GA- and ABA-related genes in GhGASA1-overexpression and wild-type Arabidopsis under salt stress. Two-week-old GhGASA1-overexpression and wild-type Arabidopsis plants were treated with 0.3% NaCl and sampled at 0 h, 3 h, 6 h and 12 h. The data are presented as the means ± s.e.m (* $P < 0.05$, or ** $P < 0.01$, $n = 3$).

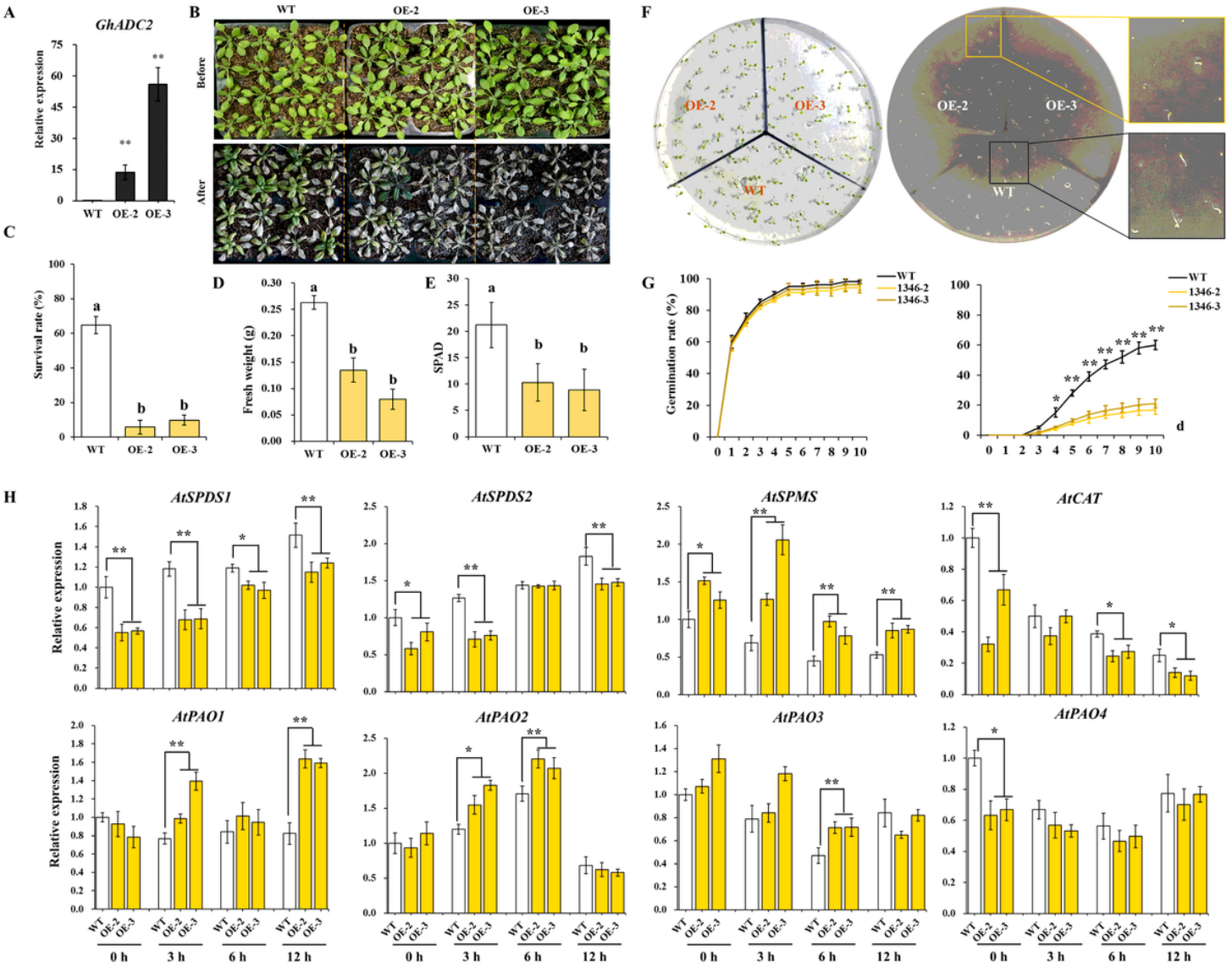

Figure 4

Performance of GhADC2 transgenic Arabidopsis under salt stress. (A) qRT-PCR analysis of GhADC2 for the WT and GhADC2-overexpresion (OE-2 and OE-3) lines (Student's t-test, ** P < 0.01, n = 3). (B) The representative phenotype of WT and OE seedlings after NaCl treatment (200 mM) for three weeks. Three independent experiments were conducted. (C-E) Survival rates, fresh weight and SPAD values of WT and GhADC2-overexpresion plants after NaCl treatment. Error bars indicate s.e.m. Different letters at the top of each column indicate significant differences at P < 0.05 (n=3 biological replicates) by the Student's t-test. (F) Seed germination phenotypes. Vernalized seeds of Arabidopsis WT and two GhADC2-overexpression (OE-2 and OE-3) lines were transferred to MS medium with or without 150 mM NaCl treatments. (G) Seed germination curves. At least 30 seeds per genotype were measured in each replicate. The data are shown as means ± s.e.m. from three independent biological repeats (* P < 0.05, **, P < 0.01). (H) The transcript-level of PA-related genes in GhADC2-overexpression and wild-type Arabidopsis under salt stress. Two-week-old GhADC2-overexpression and wild-type Arabidopsis plants were treated with 0.3% NaCl and sampled at 0 h, 3 h, 6 h and 12 h. The data are presented as the means ± s.e.m. (* P < 0.05, or ** P < 0.01, n = 3).

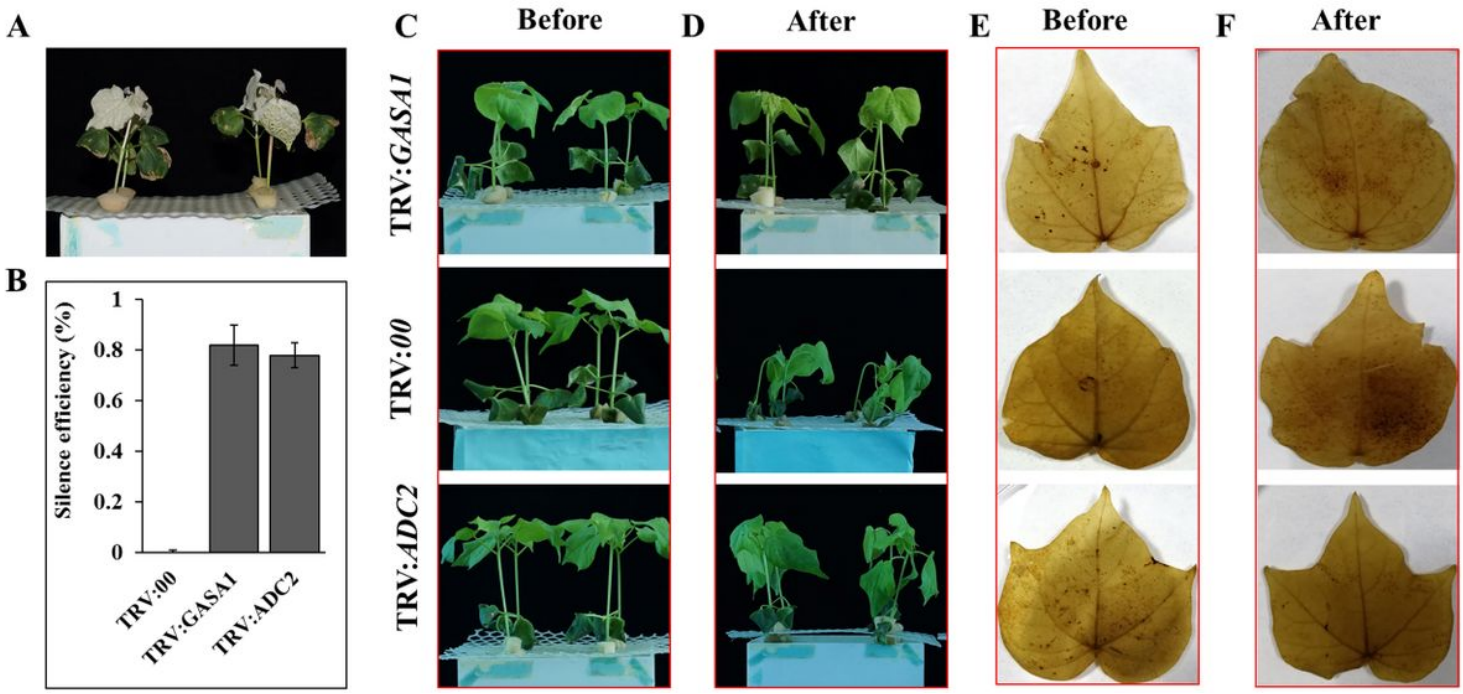

Figure 5

Silencing of GhGASA1 and GhADC2 in cotton increased the tolerance to salt stress. (A) Albino phenotype of TRV: CLA1 about two weeks after infiltration. (B) The silencing efficiency of GhGASA1 and GhADC2. Values are means ± s.e.m (n = 3 biological replicates). Error bars represent the SD of three biological replicates. (C) Phenotype of TRV:GASA1 (top), TRV:ADC2 (bottom) and the control TRV:00 (middle) plants before 200 mM NaCl treatment. (D) Phenotype of silencing cotton seedlings after salt treatment. (E-F) DAB staining of H2O2 in knockdown and control lines.

Supplementary Files

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