

Gibberellin mediates spermidine-induced salt tolerance and the expression of *GT-3b* in cucumber

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Abstract

Background Transcription factor GT-3b binds to the GT-1 element in the promoter of S-adenosylmethionine synthase (SAMs) gene, the key gene of spermidine (Spd) biosynthesis, to regulate its expression, thereby increasing the salt tolerance of cucumber plants. Furthermore, exogenous Spd increases the transcript level and protein abundance of SAMs, which promotes the accumulation of endogenous polyamine. However, whether Spd regulation of SAMs depending on GT-3b is largely unknown. Here, we investigated the potential mechanism of Spd in regulating of GT-3b in cucumber seedlings under salt stress.

Results Exogenous Spd significantly increased the tolerance to salt stress in cucumber, while its effects were compromised when application of methylglyoxal bis-guanylhydrazone (MGBG), an inhibitor of Spd biosynthesis. GT-3b was significantly induced by Spd under salt stress. The promoter sequence of GT-3b was predicted that there contained cis-acting regulatory elements response to phytohormones, such as gibberellin (GA), salicylic acid (SA) and methyl jasmonate (MeJA). Foliar spray of GA 3, SA and MeJA could induce the expression of GT-3b. Interestingly, exogenous Spd dramatically induced the expression of genes related to GA biosynthesis, increased the activity of gibberellin oxidase, and promoted the accumulation of GA 3, but decreased in MGBG-treated plants. Furthermore, application of GA 3 increased the expression of GT-3b and salt tolerance, while blocked when treatment with paclobutrazol (PAZ), the GA biosynthesis inhibitor. In addition, Spd-induced salt tolerance was compromised in PAZ-treated plants.

Conclusions Our results suggested that GA mediated Spd-induced salt tolerance and the expression of GT-3b in cucumber. These results provide new perspective for our understanding the molecular mechanism of Spd in regulation of salt tolerance in plants.

Background

Transcription factors are key components that generate specific stress responses. They manipulate the transcriptional expression of a variety of stress-tolerant genes and control a wide range of downstream metabolism pathways. Many transcription factors, such as NAC [1], MYB [2], ERF [3], WRKY [4] and zinc finger proteins [5], are involved in salt stress tolerance in plants [6]. The trihelix transcription factor is a unique transcription factor in plants and plays a key role in the process of plant stress signal transmission [7]. It can enhance the stress tolerance of plants by binding to cis-acting elements in the promoter region of stress-regulating genes and by regulating the expression of stress-related genes [7]. The trihelix family is divided into five subfamilies, GT-1, GT-2, GT γ , SH4 and SIP1, based on the number of DNA binding domains and the conserved amino acids in the GT protein domain [7]. Northern blotting indicates that the expression level of the GT-1 protein RML1 is significantly upregulated in rice seedling leaves infected with rice blast [8]. It was found that GT-4 interacts with TEM2 to regulate the salt response gene *Cor15A* to improve salt tolerance [9]. The GT-3b transcription factor is a trihelix transcription factor induced by NaCl and pathogens [10], which can interact with the GT-1 cis-element to promote the transcriptional expression of downstream genes and plays an important role in the salt

tolerance of plants. Our previous work found that the *GT-3b* is induced by salt stress and binds to the *GT-1* element in the promoter of *S*-adenosylmethionine synthase gene (*CsSAMs*) to trigger its expression in cucumber [11], thereby improving the salt tolerance of cucumber plants.

SAMs plays an important role in salt stress, and studies have confirmed that salt stress induces the expression of *SAMs* [11, 12]. The expression of *SAMs1* and *SAMs3* genes is obviously induced by salt stress in tomato roots and can be maintained at a high level for a long period of time [13]. Plants overexpressing *SAMs* gene are more tolerant to salt and oxidative stress in comparison to wild-type plants [14]. Furthermore, proteomic analysis found that *SAMs* is upregulated stably during salt stress and is considered as marker protein for the salt stress response in chloroplasts [15]. Similarly, an increase in the expression of *SAMs* protein in wheat was observed using two-dimensional gel electrophoresis under salt stress [16]. Overexpression of *SAMs* from *Pyropia tenera* in *E. coli* significantly enhanced high salt tolerance [17].

SAMs catalyzes the reaction of ATP and L-methionine to generate *S*-adenosylmethionine (SAM), which is the main methyl donor in organisms and the precursor for the biosynthesis of ethylene and polyamine (PA). The interaction between the *SAMs* gene and the amino transferase gene in apples affects plant growth by promoting the accumulation of PA in plants [18]. Overexpression of *SAMs* gene activates the PA metabolic pathway, increases the level of PA, and confers salt tolerance to plants. It has been reported that salt tolerance of transgenic plants overexpressing *SAMs2* is significantly improved, and the contents of spermidine (Spd) and spermine (Spm) in transgenic tobacco is significantly higher than those in wild-type tobacco [19]. In addition, overexpression of *SAMs1* gene promotes the synthesis and accumulation of Spm and Spd, activates stress-related genes, enhances the expression of the antioxidant system, thereby increasing salt tolerance in tomato plants [20]. Similarly, overexpression of *MfSAMs1* promotes the synthesis and oxidation of PAs, leading to improving antioxidant protection against H₂O₂ [21, 22]. As a stress-responsive protein, the expression of SAM is significantly inhibited by high concentrations of salts, while exogenous PA reverses the inhibitory effects of salt stress on *SAMs* at various levels, including enzymatic activity, gene and protein levels. Li [23] analyzed the effects of exogenous Spd on protein expression in cucumber leaves under salt stress and found that Spd increased the abundance of *SAMs* and other proteins related to antioxidant defense and detoxification. In addition, exogenous Spd can upregulate the expression of *SAMs* under salt stress and provide more methyl donors that can participate in the defense response by promoting the synthesis of SAM, and it may promote lignification in the vascular tissue of plants [12] and regulate water transport and ion-selective absorption under salt stress. However, whether Spd regulation the expression of *SAMs* is rely on *GT-3b* in still unclear.

In this study, we analyzed the mechanism of Spd regulation of *GT-3b* in response to salt stress in cucumber. The results showed that under salt stress, Spd upregulated the expression of *GT-3b* by increasing the level of endogenous GA in cucumber plants, which promoted the expression of *GT-3b*, resulting in enhanced the salt tolerance of cucumber plants. Our results provide new insight for understanding of the molecular mechanism of Spd enhancing tolerance to salt in plants.

Results

Spd is crucial for salt tolerance in cucumber

The growth of cucumber seedlings subjected to salt stress was significantly inhibited compared with the control (CK) plants (Fig. 1a). The plant height, shoot fresh weight, root fresh weight, shoot dry weight and root dry weight decreased by 39.63%, 32.15%, 46.59%, 19.89% and 31.25%, respectively, in comparison to CK plants and the above indexes in plants subjected to salt stress (S) with spraying of Spd (Spd+S) were 33.33%, 25.79%, 24.95%, 7.80%, and 18.18% higher than that in S-treated plants (Fig. 1b). The adverse effects-induced by salt stress were more significant in the plants treated with salt and methylglyoxal bis-guanylhydrazine (MGBG), an inhibitor of Spd biosynthesis, but was alleviated after spraying Spd (Fig. 1). Furthermore, the contents of malondialdehyde (MDA) and proline in cucumber leaves significantly increased under salt stress (Additional file 1: Figure S1). Foliar application of MGBG further promoted the accumulation of MDA and proline, while application of Spd significantly decreased the MDA and proline contents (Additional file 1: Figure S1). The results indicated that Spd could alleviate the inhibition of cucumber seedling growth by salt stress and reduce the degree of damage in plants.

Spd induced the expression of *GT-3b* under salt stress

It has been demonstrated that salt stress induced the expression of *GT-3b*, which mediates the biosynthesis of PAs to regulate salt tolerance [11]. To detect whether *GT-3b* is involved in Spd-induced the tolerance to salt, we used qPCR to analyze the expression patterns of *GT-3b* gene. As shown in Fig. 2, the expression level of *GT-3b* was upregulated under salt stress compared with the CK. The increased level of *GT-3b* in Spd+S plants was more great and reached to the peak at 12 h, while application of MGBG compromised the induction of *GT-3b* (Fig. 2). These results suggested that *GT-3b* was a salt stress response gene and was regulated by Spd.

Analysis the expression patterns of *GT-3b* in response to phytohormones

To further investigate the role of *GT-3b* in Spd-induced salt stress tolerance, we analyzed its promoter and the expression patterns in response to different phytohormones. To identify the cis-acting element in *GT-3b*, the 1278 bp sequence of the *GT-3b* promoter was isolated from genomic DNA, and the cis-acting regulatory elements were identified using the PlantCARE and PLACE online databases [24, 25].

Bioinformatics analysis revealed that the putative cis-acting regulatory elements that regulate stress and defense gene expression in different plant species were existed in this region (Additional file 2: Figure S2 and Additional file 3: Table S1). The phytohormone response elements were involved in these cis-acting elements, including the TGACG and CGTCA motifs, GARE motif and the TCA element, which are response to methyl jasmonate (MeJA), gibberellin (GA) and salicylic acid (SA), respectively. To verify whether *GT-3b* can be respond to these phytohormones, GA₃, MeJA and SA were sprayed to the leaves of cucumber seedlings to analyze the expression patterns of *GT-3b*. The results showed that GA₃, MeJA and SA upregulated the expression of *GT-3b* (Fig. 3), indicating that the transcription level of *GT-3b* is regulated by GA, MeJA, and SA.

To further determine the role of this 3 kind of phytohormones on the regulation of *GT-3b*, we replaced the *CaMV 35S* promoter in pBI121 that contained the GUS reporter gene with the *GT-3b* promoter (Fig. 4a) and constructed four promoter expression vectors of different lengths based on the position of each element (Fig. 4b). The tobacco leaves infiltrated with *Agrobacterium tumefaciens* harboring *GT-3b* promoter fragments were treated with GA₃, SA, and MeJA, which was followed by GUS histochemical staining and fluorometric assay analyses. After spraying GA₃ with the P1 vector containing the GARE motif, the staining was significantly deeper and the GUS activity was higher than other treatments (Fig. 4c,d), indicating that GA could activate *GT-3b* to participate in the stress defense response. Similarly, the GUS activity of the P2 fragment containing the CGTCA motif and the P3 fragment containing the TCA element was consistent with the staining as showed significantly increased the GUS activity treated with MeJA or SA (Fig. 4c,d). These results indicated that the GARE motif, CGTCA motif and TCA element in the *GT-3b* promoter may be response to exogenous GA, MeJA, and SA, which was consistent with the finding by qPCR that GA, MeJA and SA could upregulate the expression of *GT-3b*.

Endogenous GA₃ content and gibberellin oxidase activity in cucumber leaves

The above results showed that Spd could regulate the expression level of *GT-3b*, and the transcription level of *GT-3b* was also regulated by GA, MeJA, and SA. Therefore, we speculate that Spd regulates the expression of *GT-3b* by affecting GA, MeJA, and SA. To verify this hypothesis, we studied the effect of exogenous Spd treatment on the contents of endogenous GA₃, SA and MeJA in cucumber leaves (Fig. 5). The results showed that the content of GA₃ in leaves after Spd treatment was approximately 3.97-fold higher than that in CK plants (Fig. 5a). In contrast, the GA₃ content in MGBG treatment plants was significantly lower than that in CK plants (Fig. 5a). Unlike GA₃, the content of endogenous SA induced by Spd was not significantly different from that of the CK, while treated with MGBG significantly increased the content of SA (Fig. 5b). However, the content of endogenous MeJA in the cucumber leaves was too low to detect. On the basis of the effect of Spd on endogenous phytohormone contents, we speculate that the induction of the expression of *GT-3b* by Spd is most closely related to GA₃.

To test whether Spd was involved in regulation of GA, we examined the effects of Spd on the expression of key genes in the GA anabolic pathways. The results showed that exogenous Spd upregulated the expression of *CPS1*, *KS1*, *KO1*, *KAO1*, *KAO2*, *GA20ox1*, *GA20ox2*, *GA20ox3*, *GA20ox4* and *GA20ox5*, which are associated with GA biosynthesis (Fig. 6). The expression of *GA3ox3*, *GA3ox4* and *GA3ox5* was significantly upregulated in Spd-treated plants (Fig. 6), which promoted the conversion of GA₉ and GA₂₀ to GA₄, GA₁ and GA₃. Moreover, Spd upregulated the expression of *GA2ox1*, *GA2ox2*, *GA2ox3*, *GA2ox4* and *GA2ox7* (related to GA metabolism), which promoted the conversion of GA₁ and GA₄ to inactive gibberellin, while plants treated with the inhibitor of MGBG exhibited the opposite effects on the expression of GA anabolic pathways (Fig. 6). Furthermore, qPCR analysis showed that the GA receptor gene *GID1* was significantly up-regulated under Spd treatment (Fig. 6).

Interestingly, Spd enhanced the activity of GA3-oxidase, GA20-oxidase and GA2-oxidase, while MGBG had the opposite effect (Fig. 7). These results suggested that exogenous Spd could promote the biosynthesis of GA through regulation the gene expression and enzyme activity of GA anabolic pathways.

To determine the role of Spd and GA on the regulation of *GT-3b*, we analyzed the effects of Spd and GA on the expression of *GT-3b* using qPCR. As shown in Fig. 8a, the transcript level of *GT-3b* increased by 6.81-fold in Spd-treated plants, but decreased by 25.73% in MGBG-treated plants, in comparison to CK plants. In addition, GA₃ upregulated the expression of *GT-3b*, while application of paclobutrazol (PAZ), an inhibitor of GA biosynthesis, significantly inhibited the expression of *GT-3b* (Fig. 8b). Therefore, Spd promoted the accumulation of GA in plants and GA could induce the expression of *GT-3b*, suggesting that the regulation of *GT-3b* by Spd might mediate by GA.

Involvement of GA in Spd-induced salt stress tolerance

To investigate whether GA mediated Spd-induced salt tolerance, we first analyzed the effect of Spd on the content of GA₃ under salt stress. The content of GA₃ was significantly increased both in S and Spd+S plants, but the increased level was more profound in Spd+S plants (Fig. 9). In contrast, the content of GA₃ in MGBG+S and Spd+MGBG+S plants was dramatically lower than that in Spd+S plants, indicating that inhibition endogenous Spd compromised the induction of GA₃ under salt stress.

To further determine the role of GA₃ in Spd-mediated salt tolerance, we analyzed the effect of GA₃, PAZ and Spd on the growth of cucumber seedlings under NaCl stress. The results showed that the growth of cucumber seedlings subjected to salt stress was significantly inhibited and the inhibition was alleviated after spraying GA₃ (Fig. 10). However, the adverse effects of salt stress were more serious when plants treatment with PAZ (Fig. 10). The growth parameters in plants treatment with PAZ and Spd were slight higher than the single PAZ-treated plants under salt stress (Fig. 10). Therefore, these results indicated that GA mediated Spd-induced salt tolerance in cucumber seedlings.

Discussion

A large number of studies have reported that exogenous Spd can improve the tolerance of plants to salt stress [26-28]. Exogenous Spd could enhance the accumulation of proline and antioxidant enzymes to increase tolerance to salt stress [26-28]. Spd reduces the content of MDA, increases the content of chlorophyll, and regulates PSII activity in wheat seedlings under salt stress [29]. Spd inhibits stearic acid, linoleic acid and linolenic acid in salt-stressed plants, and reduces the total free fatty acids and the contents of JA and SA to improve salt stress tolerance [30]. In addition, exogenous Spd significantly increases the contents of free Spd and Spm [29]. It is found that MGBG enhances the adverse effects of salt stress [31]. In this study, the effects of exogenous Spd and MGBG on the growth and physiological indicators of resistance in cucumber seedlings under salt stress were analyzed, and the results were in accord with the results of previous studies. Wang et al. [32] described the molecular mechanism of exogenous Spd on improving high-temperature tolerance based on the expression of *miRNA* in cucumber.

Sang et al. [33] used proteomics studies to analyze the effects of Spd on alleviating high-temperature stress in tomato. The molecular mechanism of Spd on enhancing high-temperature tolerance has been studied via transcriptomics and proteomics. However, the molecular mechanism of Spd on improving salt tolerance in plants is poorly understood. Here, we found that Spd increased salt tolerance through promoted the accumulation of GA, which further induced the expression of *GT-3b* in cucumber.

Spd induces the expression of salt-responsive gene *GT-3b*

GT-3b is a salt stress protein induced by salt stress and pathogens [10]. We found that Spd significantly increased the expression level of *GT-3b* gene (Fig. 2). A study found that the AtGT-3b protein specifically binds to the promoter of *SCaM-4* gene to confer NaCl responsiveness [10]. Our previous study found that the GT-3b transcription factor specifically binds to the salt response element GT-1 in the promoter of *CsSAMs* to regulate its expression [11]. Studies have shown that in *Lycoris* [34], wheat [15] and tomato [12], transcription of the *SAMs* gene is elevated during salt stress. Exogenous PAs can enhance the metabolism and accumulation of endogenous PAs [35, 36]. Wu et al. [37] indicated that exogenous Spd can induce endogenous PAs production under salt stress. In the present study, we found that Spd induced the expression of *GT-3b* under salt stress (Fig. 2). Thus, exogenous Spd may upregulate the expression of *SAMs* under salt stress via GT-3b. The accumulation of SAMs promotes the synthesis of PAs [38], which is beneficial for the enhancement of the tolerance of plants to salt stress [39, 40].

Endogenous GA regulates the transcription level of *GT-3b*

The predicted stress-related cis-elements may be necessary for the regulation of stress-induced gene expression [1]. In this study, we found that the 1278 bp promoter fragment of *GT-3b* contained the TGACG and CGTCA motifs associated with MeJA signaling, the GARE motif involved in responses to GA and the TCA element involved in SA responses [41]. Interestingly, GA, SA, and MeJA can increase the degree of GUS staining and enhance GUS activity (Fig. 4), indicating that the GA, SA, and MeJA binding motifs in the *GT-3b* promoter might play an important role in its response to exogenous GA, SA and MeJA.

It has been reported that transcription factors are regulated by multiple phytohormones. Some NAC transcription factors are affected by auxin [42], ethylene [43], abscisic acid [44], GA [45], MeJA [46], SA [47] and cytokinin [48]. These phytohormones participate in signal transduction. The phytohormone-related motifs may play a vital role in regulating the expression of *GT-3b* when bound by the corresponding phytohormones [49]. In this study, we treated cucumber seedlings with GA₃, SA and MeJA and found that the relative expression level of *GT-3b* gene was upregulated (Fig. 3). Furthermore, GA₃ could rapidly induce the expression of the *GT-3b* gene, indicating that GA, SA and MeJA can regulate the transcription level of *GT-3b*. By measuring the contents of endogenous phytohormones, we found that only the content of GA₃ increased after Spd treatment (Fig. 5). qPCR analysis found that GA₃ can upregulate the expression level of *GT-3b*. These results indicate that Spd regulates *GT-3b* expression and is most closely related to GA.

Spd promotes the accumulation of endogenous GA under salt stress

It has been reported that Spd increases the concentration of GA in white clover under drought conditions [50] and regulates the changes in endogenous PA and phytohormones to reduce the structural and functional destruction of photosynthetic organs [51]. Similarly, we found that exogenous Spd significantly increased the content of endogenous GA₃ under salt stress, upregulated the expression of genes related to GA biosynthesis, enhanced the activity of GA3-oxidase and GA20-oxidation, and decreased the activity of GA2-oxidase associated with GA degradation (Fig. 6,7,9). Taken together, our results indicated that Spd may regulate the GT-3b transcription factor by enhancing the accumulation of GA.

Conclusions

This study confirmed that Spd had a positive effect on the alleviation of damage caused by salt stress in cucumber seedlings. Spd enhanced the accumulation of endogenous GA by upregulating the expression of key genes involved in GA biosynthesis and enhancing the activity of gibberellin oxidase, which thereby induced the expression of *GT-3b* transcription factors. The enhanced expression of the *GT-3b* transcription factor was beneficial for the transcriptional expression of *CsSAM*, which in turn promoted the accumulation of endogenous PAs that contributed to the tolerance of plants to stress. These results will help us to understand the molecular mechanism of Spd alleviation salt-stress in cucumber seedlings, which is important for guiding breeding that aims to increase cucumber salt tolerance.

Methods

Plant materials and growth conditions

The cucumber seeds (*Cucumis sativus* L. cv Jinchun No. 2), a commercial variety, were purchased from the Tianjin Kernel Cucumber Research Institute (Tianjin, China) and placed on moistened filter paper in the dark for 30 h at 28 °C for germination. Then, the germinated seeds were sown in seedling pots (10 cm × 7 cm × 8 cm) filled with organic substrate (vinegar, vermiculite, and peat at a volume ratio of 2:1:2) and cultured in the light incubator (RXZ-500D, Ningbo, China) at Nanjing Agricultural University, where the environmental conditions consisted of a 26 ± 2 °C / 17 ± 2 °C day/night, a photoperiod of 12 h/12 h (day/night), and a relative humidity of 60–75%. The tobacco seeds (*Nicotiana benthamiana*) were directly sown in seedling pots (5 cm × 3 cm × 4 cm) containing an organic substrate and cultured in light incubator (RXZ-500D, Ningbo, China) under the same environmental conditions. When the third leaves of the cucumber seedlings were fully expanded, the following treatment was carried out.

Salt stress and Spd treatment

To investigate the effect of Spd on the alleviation of salt stress injury in cucumber and the expression of *GT-3b*, cucumber seedlings were divided into two groups; one group was subjected to salt stress via irrigation with 50 ml 100 mM NaCl solution, and the other group was irrigated with the same amount of water. At the same time, the leaves of the salt-treated seedlings were sprayed with deionized water, 1 mM

Spd, and 1 mM MGBG solution on both sides, and the control plants were sprayed with the same amount of deionized water. The seedlings were sprayed at 5 pm every day for 7 d, and the experiment consisted of 5 treatments: plants sprayed with deionized water as control plants (CK), plants sprayed with deionized water on salt-stressed plants (S), plants sprayed with Spd on salt-stressed plants (Spd+S), plants sprayed with MGBG on salt-stressed plants (MGBG+S), and plants sprayed with Spd and MGBG on salt-stressed plants (Spd+MGBG+S).

To investigate the role of GA on the expression level of *GT-3b*, 1 mM GA₃ and 1 mM PAZ were sprayed on the cucumber leaves. To understand the effect of Spd on the content of endogenous GA, SA, and MeJA, we sprayed 1 mM of Spd and 1 mM of MGBG on cucumber seedling leaves under normal growth conditions. To investigate the role of GA in Spd-induced salt tolerance, the other 5 treatments were performed: plants sprayed with deionized water (CK), plants sprayed with deionized water on salt-stressed plants (S), plants sprayed with GA₃ on salt-stressed plants (S+GA₃), plants sprayed with PAZ on salt-stressed plants (S+PAZ), and plants sprayed with PAZ and Spd on salt-stressed plants (S+PAZ+Spd). There were 3 replicates per treatment and 10 seedlings per replicate. The samples were collected at the indicated time, frozen in liquid nitrogen and stored at -80 °C for further analysis. After treatment for 7 d, the growth parameters were measured by the method of Wang et al. [32] and Tang et al. [52].

Phytohormone treatment

To clarify the characteristics of the response of *GT-3b* to phytohormones, 100 μM GA₃, SA and MeJA solution were sprayed on both sides of cucumber leaves. The same amount of deionized water was sprayed as a control. To verify whether the *GT-3b* promoter responds to phytohormones, the tobacco plants at the 5-leaf stage were injected with an *Agrobacterium tumefaciens* promoter solution and sprayed with 100 μM GA₃, SA and MeJA, respectively. After treatment for 1 d, tobacco leaves were collected for GUS chemical tissue staining and GUS fluorescence quantitative analysis.

Promoter cloning and sequence analysis

The *GT-3b* promoter sequence was obtained from the NCBI database (GenBank No: NC_026657.1). The 1278 bp sequence upstream of the start codon (ATG) of *GT-3b* was cloned from cucumber genomic DNA for further analysis. The online search tools PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) were used to predict the cis-acting regulatory elements.

Plasmid construction and transient tobacco transformation

On the basis of the putative cis-acting elements responsible for phytohormones, four different sequences of varying lengths from the *GT-3b* promoter fragments were amplified using PCR with the specific primers (Additional file 4: Table S2) and inserted into the pBI121 vector, and the plasmids were named P1, P2, P3 and P4, respectively. All constructs were introduced into the *Agrobacterium tumefaciens* strain EHA105

using electroporation after sequencing. The activated *Agrobacterium tumefaciens* was injected in the tobacco leaves. The activated *Agrobacterium tumefaciens* with the pBI121 vector was used as a positive control, and the infiltration medium was used as a negative control.

Histochemical staining and fluorometric analysis of the GUS enzyme

The injected tobacco leaves were stained in a staining solution (25 mg mL⁻¹ X-gluc, 50 mM sodium phosphate, pH 7.0, 10 mM Na₂EDTA, pH 8.0, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferricyanide) overnight at 37 °C to detect the activity of different promoter fragments according to the method described by Jefferson et al. [53]. After staining, the tobacco leaves were bleached three times with 75 % (v/v) ethanol in a water bath at 95 °C to remove the chlorophyll. Then, we photographed the tobacco leaves with a Leica DM2500 fluorescence microscope. The fluorometric GUS assays were measured according to the method described by Wang et al. [11].

Total RNA extraction and qPCR analysis

Total RNA was extracted from the leaves of the cucumber seedlings using the RNA Simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using the PrimeScriptTM 1st Strand cDNA Synthesis Kit (Takara, Japan). Real-time quantitative PCR (qPCR) was performed according to the instructions of the SYBR Premix ExTaqTM II (Tli RNaseH Plus) Kit (Takara, Japan). The amplification was performed using the StepOnePlusTM Real-time PCR System (Applied Biosystems, USA). The cucumber *actin* gene was used as an internal reference to normalize the qPCR data. The primer sequences are shown in Additional file 4: Table S2. The gene expression fold analysis was performed using the 2^{-ΔΔCt} method [54].

Quantification of endogenous GA₃, SA and MeJA

For determination of the contents of GA₃, SA and MeJA, approximately 1.0 g of leaves was ground in liquid nitrogen and extracted three times with 4 ml precooled 80% (v/v) chromatographic methanol. The homogenate was placed on ice for 12 h in the dark and then centrifuged at 10,000 *g* for 15 min at 4 °C, and the supernatant was stored in a refrigerator at 4 °C in the dark. The supernatant was added to 1.0 g PVPP and then shaken in a shaker at 4 °C for 1 h in the dark. After centrifugation at 10,000 *g* for 15 min at 4 °C, the supernatant was passed through a C18 extraction cartridge (Waters, Ireland). The filtered liquid was stored in a 50 ml centrifuge tube and dried in a vacuum for 3 d until there was no liquid at the bottom. Then, we added 1 ml precooled chromatographic methanol to the centrifuge tube under low light conditions to completely dissolve the phytohormone as much as possible. The solution was filtered into a brown sample vial with a 0.45 μm organic ultrafiltration membrane, and the sample was stored at 4 °C in the dark. The high-performance liquid chromatography (HPLC) instrument used in the experiment was the Waters 1525 system (UPLC H-Class, USA). GA₃, SA and MeJA standards were purchased from Sigma, and chromatographic methanol was obtained from Tedia.

Measurement of GA oxidase activity

For enzymatic activity assays, 0.2 g of the cucumber leaves were ground in phosphate buffer (pH 7.4) and the homogenates were centrifuged at 4 °C 4,000 *g* for 15 min. The GA3, GA2 and GA20 oxidase activity were measured with the plant GA3-oxidase, GA2-oxidase and GA20-oxidase ELISA Assay Kit (Sino Best Biology Technology, China) according to the manufacturer's instructions.

Determination of the malondialdehyde and proline contents

To determine the content of malondialdehyde (MDA), 0.2 g of cucumber leaves were ground in 0.1% trichloroacetic acid (TCA), and the homogenates were centrifuged at 6,000 *g* for 10 min. Then, 2 mL of 0.6% thiobarbituric acid (TBA) was added into the 2 mL supernatant. The mixture was heated in a water bath at 100 °C for 10 min and then recentrifuged at 6,000 *g* for 10 min after cooling. The absorbance of the supernatant was read at 532 nm and 600 nm, and the MDA concentration was calculated as previously described [55].

To determine the content of proline, 0.2 g of cucumber leaves were ground and placed in a 15 ml centrifuge tube, to which was added 5 ml 3% sulfosalicylic acid. The mixture was heated in a water bath at 100 °C for 10 min and then recentrifuged at 3,000 *g* for 10 min after cooling. Then, 1 ml of supernatant, 1 ml water, 1 ml of glacial acetic acid and 2 ml of ninhydrin acid solution was added into a 15 ml centrifuge tube and heated in a boiling water bath for 60 min. When the mixture was cooled, we added 4 ml toluene into the centrifuge tube and vortexed for 30 s. The absorbance of the supernatant was read at 520 nm, and the proline concentration was calculated using the method described by Vicente et al. [56].

Statistical analysis

All of the data are presented as the mean \pm SE. Significant differences ($P < 0.05$) between treatments were determined using Duncan's multiple range tests in SPSS statistical software (IBM SPSS statistics 20).

Abbreviations

GA: Gibberellin; GA 2-oxidase: Gibberellin 2-oxidase; GA 20-oxidase: Gibberellin 20-oxidase; GA 3-oxidase: Gibberellin 3 β -hydroxylase; GUS: β -Glucuronidase; HPLC: High-performance liquid chromatography; MDA: Malondialdehyde; MeJA: Methyl jasmonate; MGBG: Methylglyoxal bis-guanylhydrazone; PA: polyamine; PAZ: Paclobutrazol; SA: salicylic acid; SAM: *S*-adenosylmethionine; SAMs: *S*-adenosylmethionine synthase; Spd: Spermidine; Spm: Spermine

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JS designed the experiment. XWG, YW, WKL, LK and XYS performed experiments. XWG and WY wrote the manuscript. SRG analyzed the data. All authors reviewed, revised and approved the manuscript.

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Additional Files

Additional file 1: Figure S1. Effects of NaCl stress on MDA and proline contents.

Additional file 2: Figure S2. Nucleotide sequence of the promoter region of the *GT-3b* gene.

Additional file 3: Table S1. Putative cis elements in the promoter of *CsGT-3b*.

Additional file 4: Table S2. Sequence of primers used in this study.

Figures

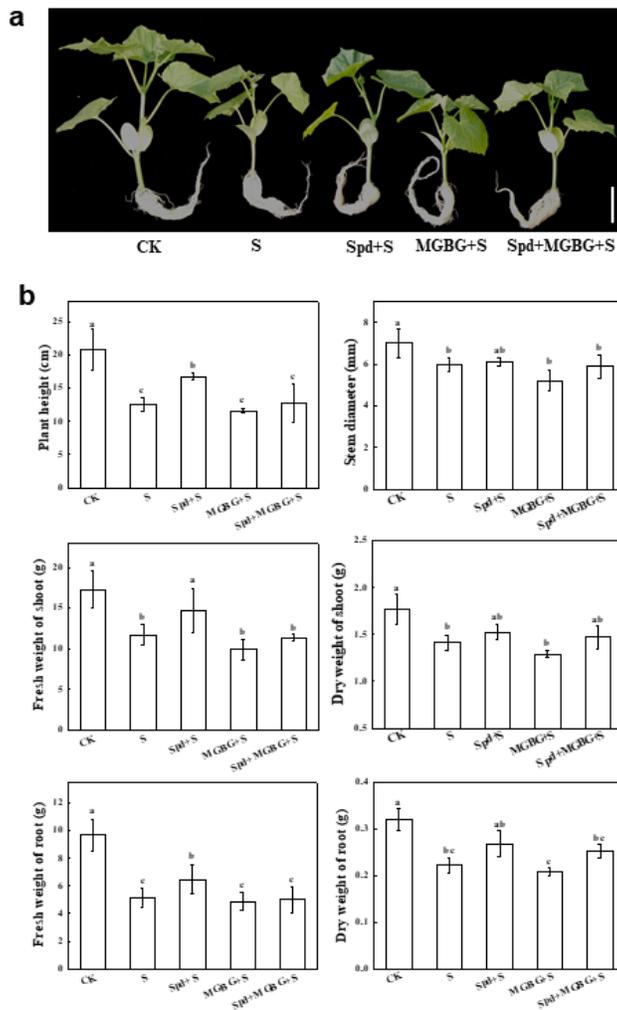


Figure 1

Effects of spermidine (Spd) on cucumber seedlings under NaCl stress. a The phenotype of cucumber seedlings. Bars: 5 cm. b The growth parameters of cucumber seedlings. Each value is the mean \pm SE ($n=3$). Different letters indicate significant differences between treatments ($P<0.05$). Three independent experiments were performed with similar results. CK: deionized water + deionized water; S: deionized

water + 100 mM NaCl; Spd + S:1 mM Spd + 100 mM NaCl; MGBG + S:1 mM MGBG + 100 mM NaCl; Spd + MGBG + S: 1 mM Spd + 1mM MGBG + 100 mM NaCl.

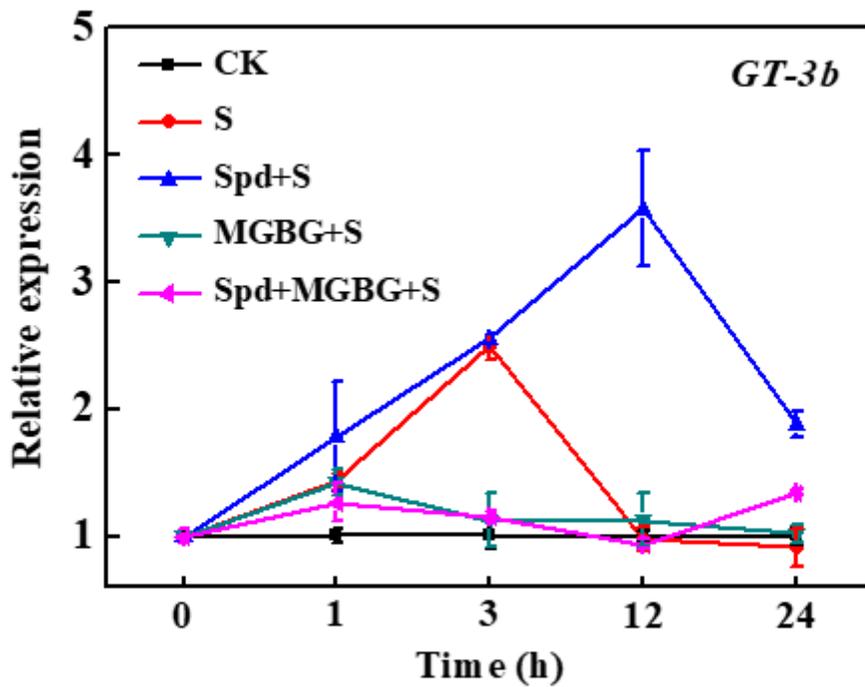


Figure 2

Effects of spermidine (Spd) on the expression of *GT-3b* under salt stress. Each value is the mean \pm SE (n=3). Three independent experiments were performed with similar results. CK: deionized water + deionized water; S: deionized water + 100 mM NaCl; Spd + S:1 mM Spd + 100 mM NaCl; MGBG + S:1 mM MGBG + 100 mM NaCl; Spd + MGBG + S: 1 mM Spd + 1mM MGBG + 100 mM NaCl.

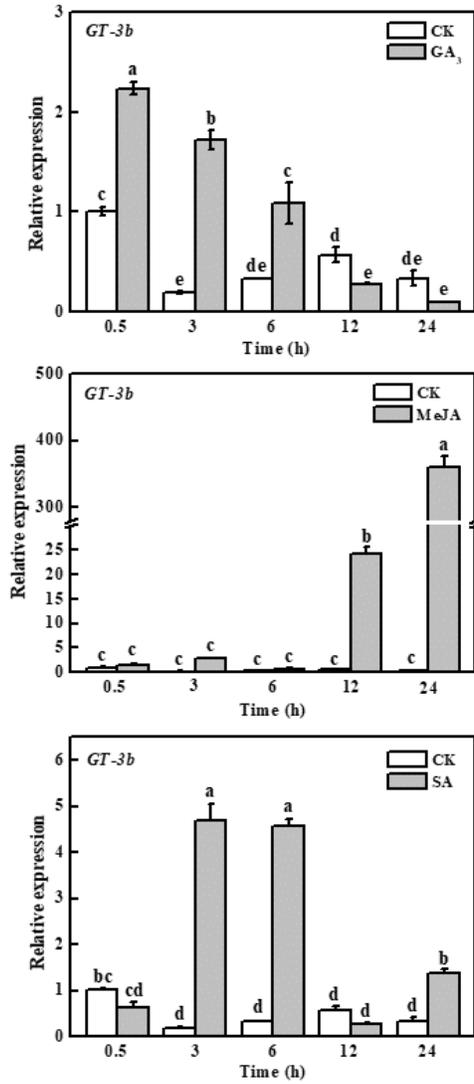


Figure 3

Real-time quantitative PCR analysis of GT-3b expression in leaf tissues of cucumber plants treated with GA₃ (100 μM), MeJA (100 μM), SA (100 μM). Each value is the mean ± SE (n=3). Different letters indicate significant differences at P<0.05 according to Duncan's multiple range test. Three independent experiments were performed with similar results.

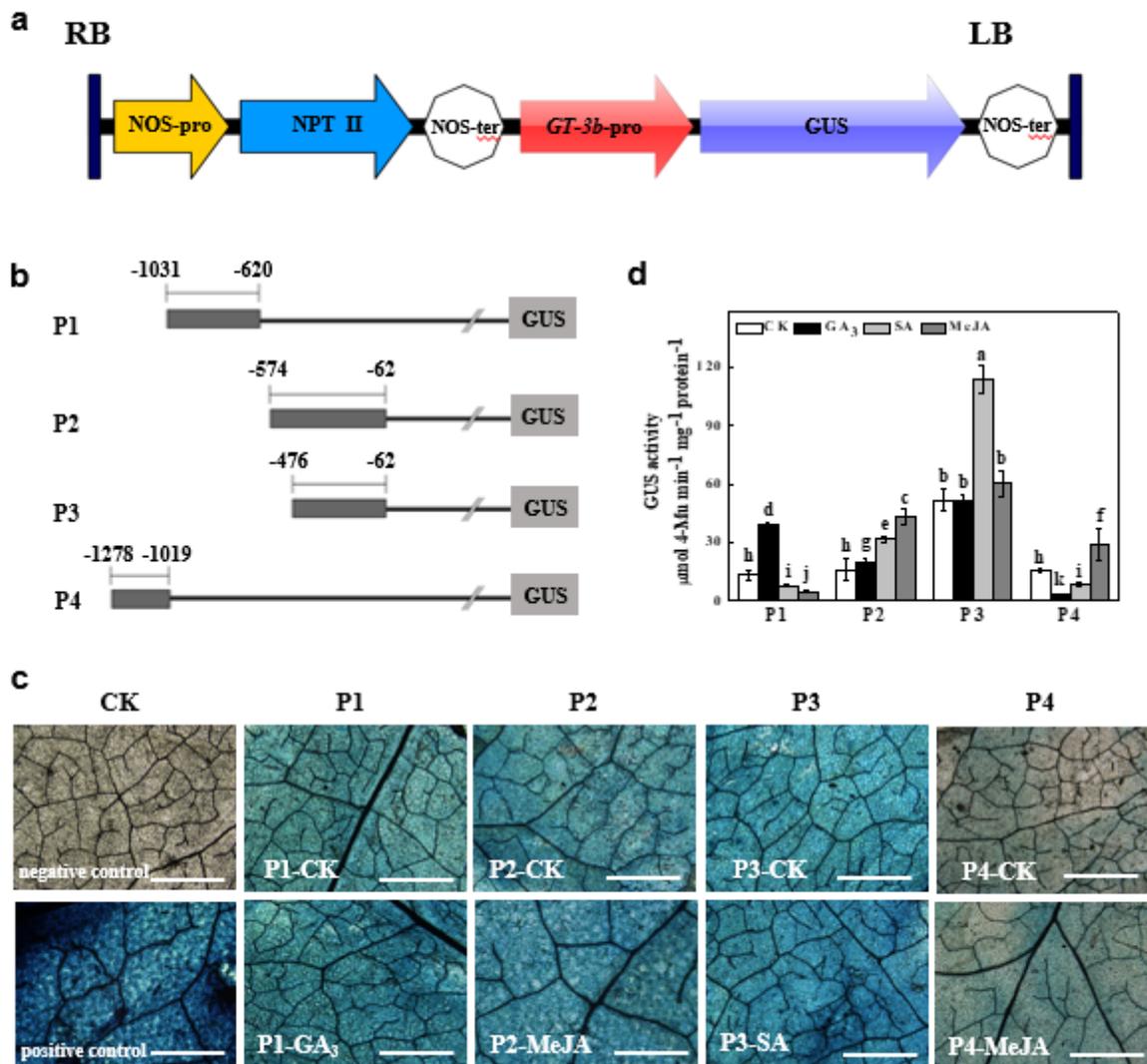


Figure 4

GUS transient expression in tobacco leaves. a Schematic representation of GT-3b promoter::GUS vector constructs. Nos-ter, nopaline synthase terminator; NPT II, neomycin phosphotransferase II gene; LB and RB, left and right T-DNA borders. b Schematic representation of the four different deletion GT-3b promoter constructs used to assay GUS activity in tobacco leaves. These constructs are based on the pBI121 vector. c Histochemical staining of four constructs under GA₃, MeJA and SA treatments. Bars: 200 µm. d GUS activity deriving from transient transformation of different GT-3b promoter constructs under phytohormones treatment. The data are presented as the mean ± SE (n=3). Different lowercase letters above the bars indicate significant differences at P<0.05. Three independent experiments were performed with similar results.

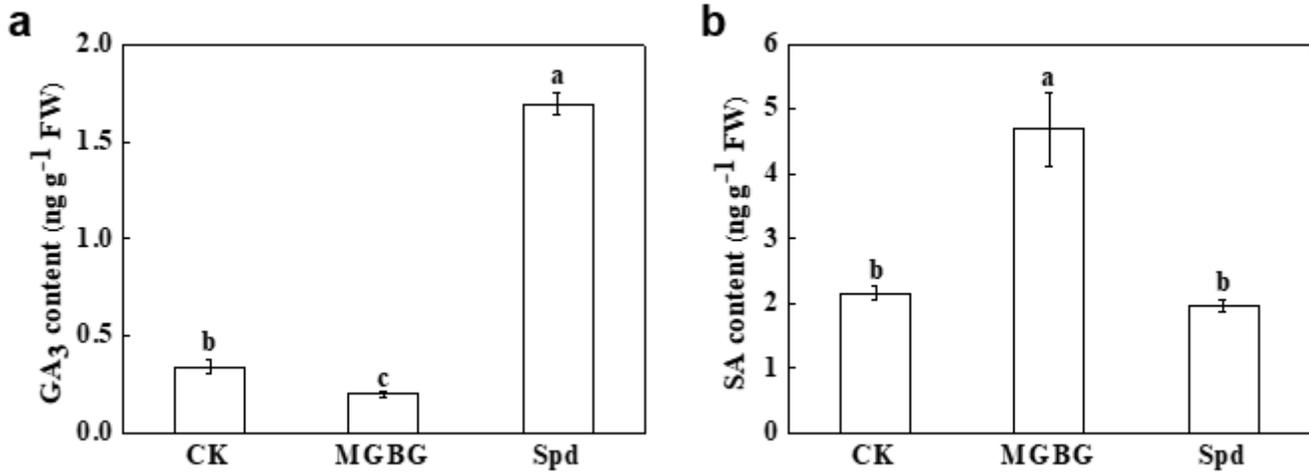


Figure 5

Effects of spermidine on endogenous GA3 and SA contents. a GA3 content. b SA content. Phytohormone contents were measured after 7 d. The data are presented as the mean \pm SE (n=3). Different lowercase letters above the bars indicate significant differences at $P < 0.05$. Three independent experiments were performed with similar results.

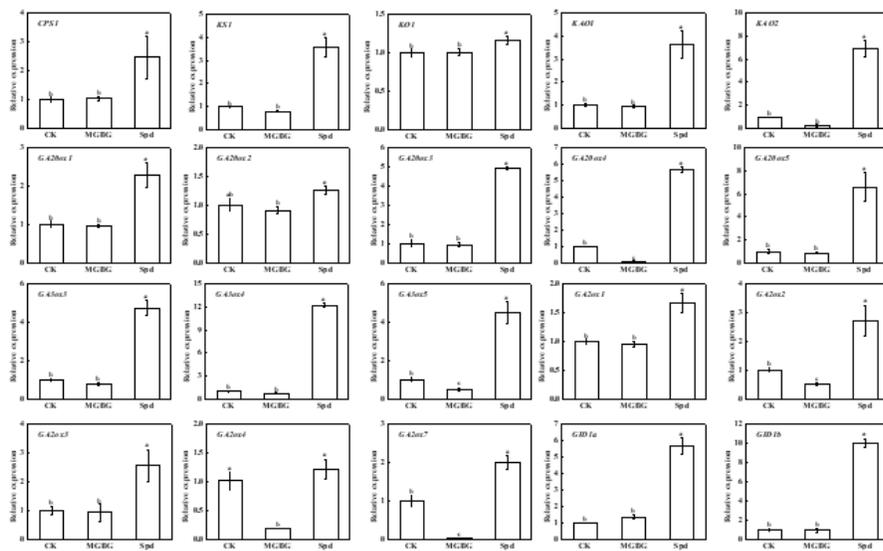


Figure 6

Effects of spermidine on the expression of gibberellin-related genes. The cucumber plants were treated with 1 mM Spd, 1 mM MGBG, and deionized water. Gibberellin-related gene expression was measured after 3 h. The data are presented as the mean \pm SE (n=3). Different lowercase letters above the bars indicate significant differences at P<0.05. Three independent experiments were performed with similar results.

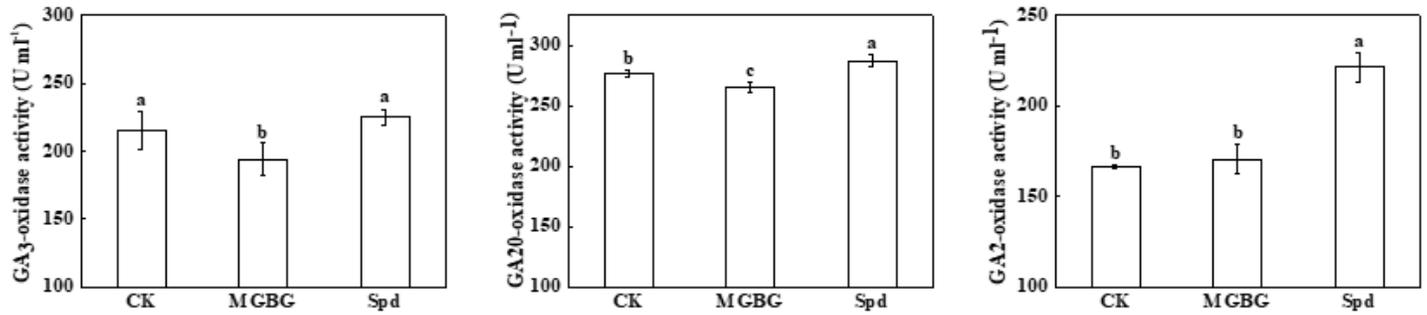


Figure 7

Effects of spermidine on the GA-oxidase activity. The cucumber plants were treated with 1 mM Spd, 1 mM MGBG, and deionized water. GA-oxidase activity measured after 1 d. The data are presented as the mean \pm SE (n=3). Different lowercase letters above the bars indicate significant differences at $P < 0.05$. Three independent experiments were performed with similar results.

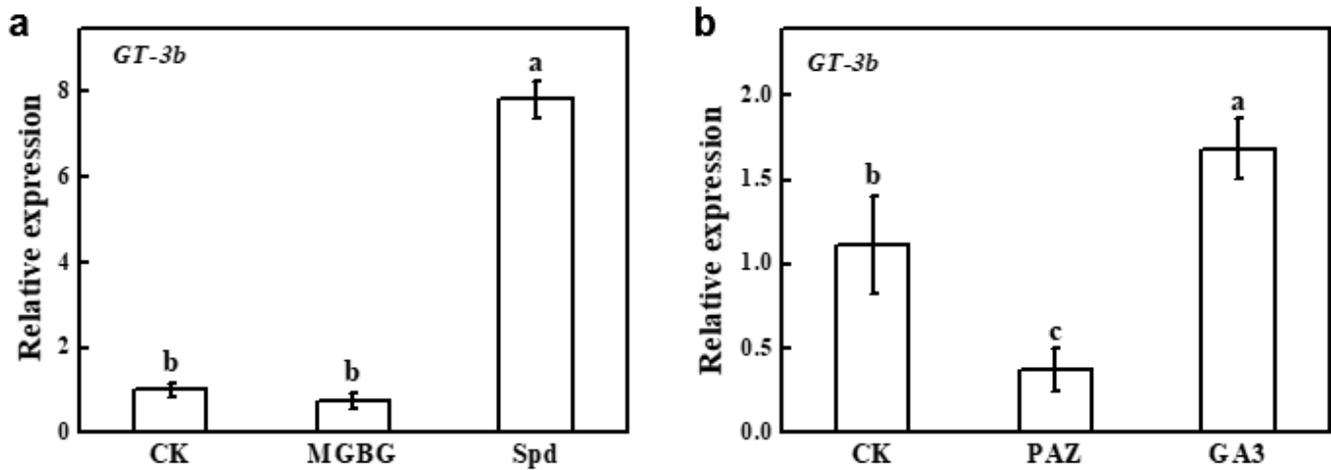


Figure 8

Effects of spermidine and gibberellin on the expression of GT-3b. a Effects of spermidine on the expression of GT-3b. b Effects of gibberellin on the expression of GT-3b. The data are presented as the mean \pm SE (n=3). Different lowercase letters above the bars indicate significant differences at $P < 0.05$. Three independent experiments were performed with similar results.

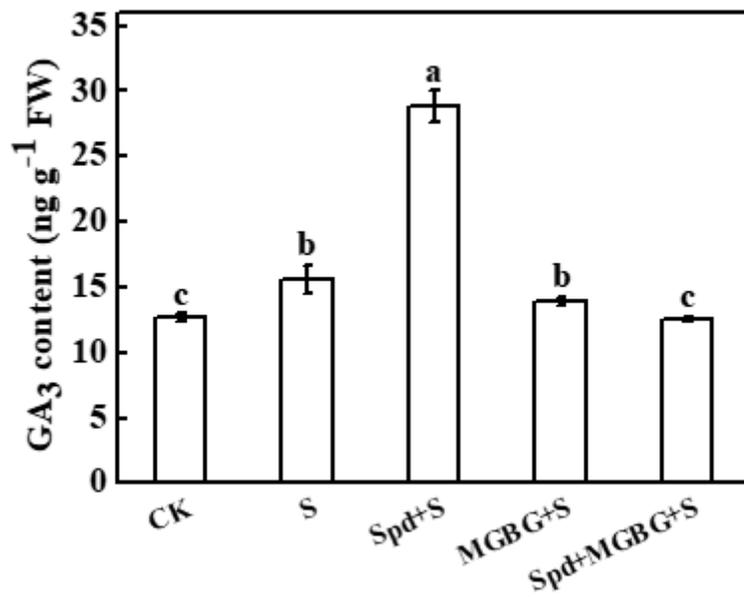


Figure 9

Effects of spermidine on the content of GA₃ under salt stress. Phytohormone contents were measured at 7 d. The data are presented as the mean \pm SE (n=3). Different lowercase letters above the bars indicate significant differences at $P < 0.05$. Three independent experiments were performed with similar results.

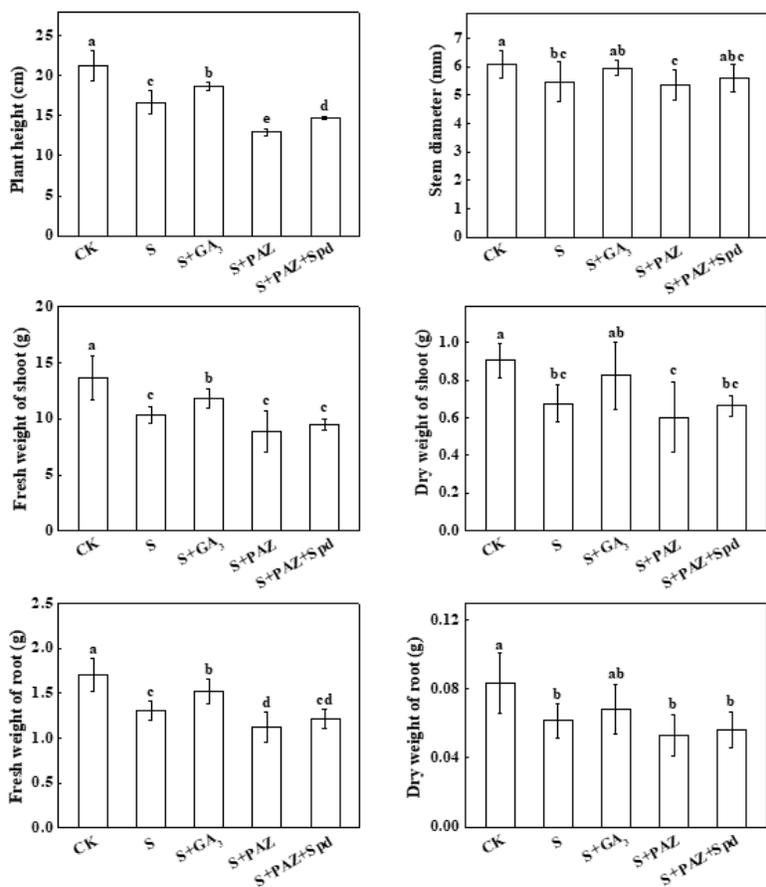


Figure 10

Gibberellin mediated spermidine (Spd)-induced salt tolerance. The growth parameters of cucumber seedlings were measured after treatment for 7 d. Each value is the mean \pm SE (n=6). Different letters indicate significant differences between treatments (P < 0.05). Three independent experiments were performed with similar results. CK: deionized water + deionized water; S: deionized water + 100 mM NaCl;

S + GA3: 1 mM GA3 + 100 mM NaCl; S + PAZ: 1 mM PAZ + 100 mM NaCl; S + PAZ + Spd: 1 mM PAZ + 1 mM Spd + 100 mM NaCl.

Supplementary Files

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