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An isopentyl transferase gene driven by the senescence-inducible *SAG12* promoter improves salinity stress tolerance in cotton

SHAN Yi[†], ZHAO Peng[†], LIU Zhao, LI Fangjun^{*} and TIAN Xiaoli

Abstract

Background: Soil salinity seriously affects cotton growth, leading to the reduction of yield and fiber quality. Recently, genetic engineering has become an efficient tool to increase abiotic stress tolerance in crops.

Results: In this study, isopentyl transferase (IPT), a key enzyme involved in cytokinin (CTK) biosynthesis from *Agrobacterium tumefaciens*, was selected to generate transgenic cotton via *Agrobacterium*-mediated transformation. A senescence-inducible *SAG12* promoter from *Arabidopsis* was fused with the *IPT* gene. Ectopic-expression of *SAG12::IPT* significantly promoted seed germination or seedling tolerance to salt stress. Two *IPT* transgenic lines, OE3 as a tolerant line during seed germination, and OE8 as a tolerant line at seedling stage, were selected for further physiological analysis. The data showed that ectopic-expression of *SAG12::IPT* induced the accumulation of CTKs not only in leaves and roots, but also in germinating seeds. Moreover, ectopic-expressing *IPT* increased the activity of antioxidant enzymes, which was associated with the less reactive oxygen species (ROS) accumulation compared with control plants. Also, ectopic-expression of *IPT* produced higher K^+/Na^+ ratio in cotton shoot and root.

Conclusion: The senescence-induced CTK accumulation in cotton seeds and seedlings positively regulates salt stress partially by elevating ROS scavenging capability.

Keywords: Isopentyl transferase (IPT), Cytokinin (CTK), Transgenic, Salt tolerance, Cotton

Background

Salt stress can interfere with plant growth and development. High concentration of Na^+ reduces water potential in the soil, and affects the absorption of other ions to disturb the ion homeostasis in plant cells. Furthermore, salt stress promotes accumulation of reactive oxygen species (ROS) in plants, which results in oxidative damage (Das and Roychoudhury 2014).

Cytokinins (CTKs) play an important role not only in plant growth and development, but also in response to abiotic stresses (Ha *et al.* 2012; Zwack and Rashotte 2015). Previous studies have revealed that plants either treated with exogenous CTKs or producing more endogenous CTKs have both positive and negative effects on abiotic stress tolerance (Zwack and Rashotte 2015). CTKs can directly or indirectly scavenge superoxide free

radicals (O_2^-) that induced by abiotic stresses (Leshem *et al.* 1981). Moreover, CTKs change the proportion of the membrane lipid peroxidation products and membrane fatty acids, which maintains the integrity of cell membrane, and increases the activity of antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) (Wojtania and Skrzypek 2014).

The isopentenyl transferase (*IPT*) gene from *Agrobacterium tumefaciens* encodes a rate-limiting enzyme in the adenosine monophosphate (AMP) pathway of CTK biosynthesis (Akiyoshi *et al.* 1984; Takei *et al.* 2001; Belintani *et al.* 2012), which catalyzes the first step in the *ab initio* synthesis of CTKs. With the help of *IPT*, the isomyl groups (DMAPP) are transferred to the N^6 site of AMP, which produces isopentyl adenosine-5- β -phosphate (iPMP) and isopentenyl adenosine (iPA). Then, iPMP is converted into other types of CTKs, such as zeatin riboside (ZR) (Kakimoto 2001; Takei *et al.* 2001).

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Senescence-associated (*SAG*) gene is a senescence related gene. Transgenic plants with *SAG12::IPT* have delayed leaf senescence (Yuan et al. 2002; Zakizadeh et al. 2013) and enhanced drought resistance (Rivero et al. 2007; Merewitz et al. 2010). However, estradiol-inducible overexpression of *AtIPT8* in Arabidopsis are more sensitive to salt stress due to enhanced ROS generation and decreased activities of ROS-scavenging enzymes (Wang et al. 2015).

Cotton is a worldwide cash crop as it produces fiber, seed oil, feed, and biofuel (Sunilkumar et al. 2006). In general, cotton is considered to be more drought- and salt-tolerant compared with other crops. However, salt tolerance differs greatly among cotton varieties and phenotypes (Ashraf 2002; Ohkama-Ohtsu et al. 2007; Iqbal et al. 2011). Recently, several stress-related genes were transformed into cotton to improve abiotic-stress tolerance. Overexpression of the Arabidopsis vacuolar H⁺-pyrophosphatase gene *AVP1* improves both drought and salt tolerance in cotton (Pasapula et al. 2015). Cotton transformed with the maize bZIP transcription factor *ABP9* shows resistant to drought and salt stresses (Wang et al. 2017). Overexpressing the rice NAC gene *SNAC1* improves salt and drought tolerance in cotton through enhancing root development and reducing transpiration rates (Liu et al. 2014); and overexpression of the rice SUMO E3 ligase *OsSIZ1* enhances net photosynthesis to improve drought and heat tolerance in cotton (Mishra et al. 2017). Furthermore, overexpression of *SAG12::IPT* delays leaf senescence (Liu et al. 2012a; Liu et al. 2012b; Zhao et al. 2012). Here, we found that *SAG12::IPT* transgenic cotton plants produced more CTKs and less ROS accumulation in response to salt stress. Thus, our study provided new cotton lines tolerant to salt stress via genetic engineering.

Materials and methods

Plant materials and growth conditions

Upland cotton (*Gossypium hirsutum* L.) JIHE321 was used as control plants. The plasmid *pSG529* constructed at the Richard M. Amasino Laboratory at the University of Wisconsin-Madison was donated by FU Yongcai, an associate professor at China Agricultural University. The plasmid was constructed with *IPT* gene driven by a senescence-inducible *SAG12* promoter, and possesses *NTPII* marker gene for selection. Positive *SAG12::IPT* transgenic cotton lines were obtained by *Agrobacterium*-mediated transformation with the help of the Cotton Research Institute of Shanxi Academy of Agricultural Sciences. Four transgenic cotton lines (OE3, OE6, OE7, and OE8) and JIHE321 were planted at growth room with conditions of (30 ± 2)°C day/(24 ± 2)°C night, 70~80% relative humidity, 14 h light/10 h dark, and 600 μmol·cm⁻²·s⁻¹ photosynthetically active radiation.

Salinity stress treatment

Salinity stress during germination: after being surface-sterilized by soaking in 9% H₂O₂ for 30 min, cotton seeds were soaked in 150 and 200 mmol·L⁻¹ NaCl solution for 24 h. The seeds were then wrapped with germination paper which was pre-soaked with the corresponding NaCl solution and placed in a germination box containing the corresponding NaCl solution in the dark.

Salinity stress at seedling stage: after being surface-sterilized by soaking in 9% H₂O₂ for 30 min, seeds were rinsed with tap water and germinated in sand for 4 days in the dark. After germination, seedlings at uniform growth stage were transplanted into half-strength modified Hoagland's solution containing 2.5 mmol·L⁻¹ Ca (NO₃)₂, 2.5 mmol·L⁻¹ K₂SO₄, 1 mmol·L⁻¹ MgSO₄, 500 μmol·L⁻¹ (NH₄)₂PO₄, 0.2 μmol·L⁻¹ CuSO₄, 1 μmol·L⁻¹ ZnSO₄, 100 μmol·L⁻¹ FeNaEDTA, 20 μmol·L⁻¹ H₃BO₃, 5 × 10⁻³ μmol·L⁻¹ (NH₄)₆Mo₇O₂₄, and 1 μmol·L⁻¹ MnSO₄ (Wang et al. 2012). The solutions were aerated and changed twice a week. The seedlings were treated with 220 mmol·L⁻¹ NaCl at the three-leaf stage.

Germination energy and germination rate

The number of germinated seeds was checked daily to calculate the seed germination energy and germination rate according to the following formulas.

$$\text{Germination energy} = \left(\frac{\text{Germination number at first three days}}{\text{Total number of seeds tested}} \right) \times 100\%$$

$$\text{Germination rate} = \left(\frac{\text{Germination number at first seven days}}{\text{Total number of seeds tested}} \right) \times 100\%$$

Measurement of chlorophyll content

Salt stressed cotton seedlings were used to measure chlorophyll content. The third true leaf (approximately 0.1 g) of transgenic cotton lines and the JIHE321 were collected to determine chlorophyll content following Tang et al. (2005).

Measurement of CTK content

For the salinity stress experiments performed during germination, whole germinating seeds (those with the seed coat were removed) were sampled after one, two, three, and five days of germination, and the germinating seeds sampled at the seventh day were separated into shoots and roots. For the salinity stress experiments performed at the seedling stage, the third true leaf and roots were collected after one, three, and six days of 220 mmol·L⁻¹ NaCl treatment. Endogenous zeatin (Z)/ZR and iP/iPA were determined using ELISA assay following Dong et al. (2008). The mouse monoclonal antigen

and antibodies were produced at the Center of Crop Chemical Control, China Agricultural University.

Analysis of H₂O₂ content and the activity of antioxidant enzymes in cotton leaves

The third leaf from the NaCl treated seedlings were collected at the indicated time points as described above. The H₂O₂ content was measured according to Hu et al. (2012), and POD and SOD activity were detected following Zaharieva et al. (1999) and Dhindsa et al. (1981), respectively.

Measurement of K⁺/Na⁺ ratio

After seven days of 220 mmol·L⁻¹ NaCl treatment, the third true leaf and roots were collected and dried at 80 °C, finely grounded, and digested with 1 mmol·L⁻¹ HCl at 30 °C for 24 h. The Na⁺ and K⁺ contents were determined using an atomic absorption spectrophotometer

SpectAA-50/55 (Varian, Australia). The K⁺/Na⁺ ratio was calculated by dividing K⁺ content by Na⁺ content.

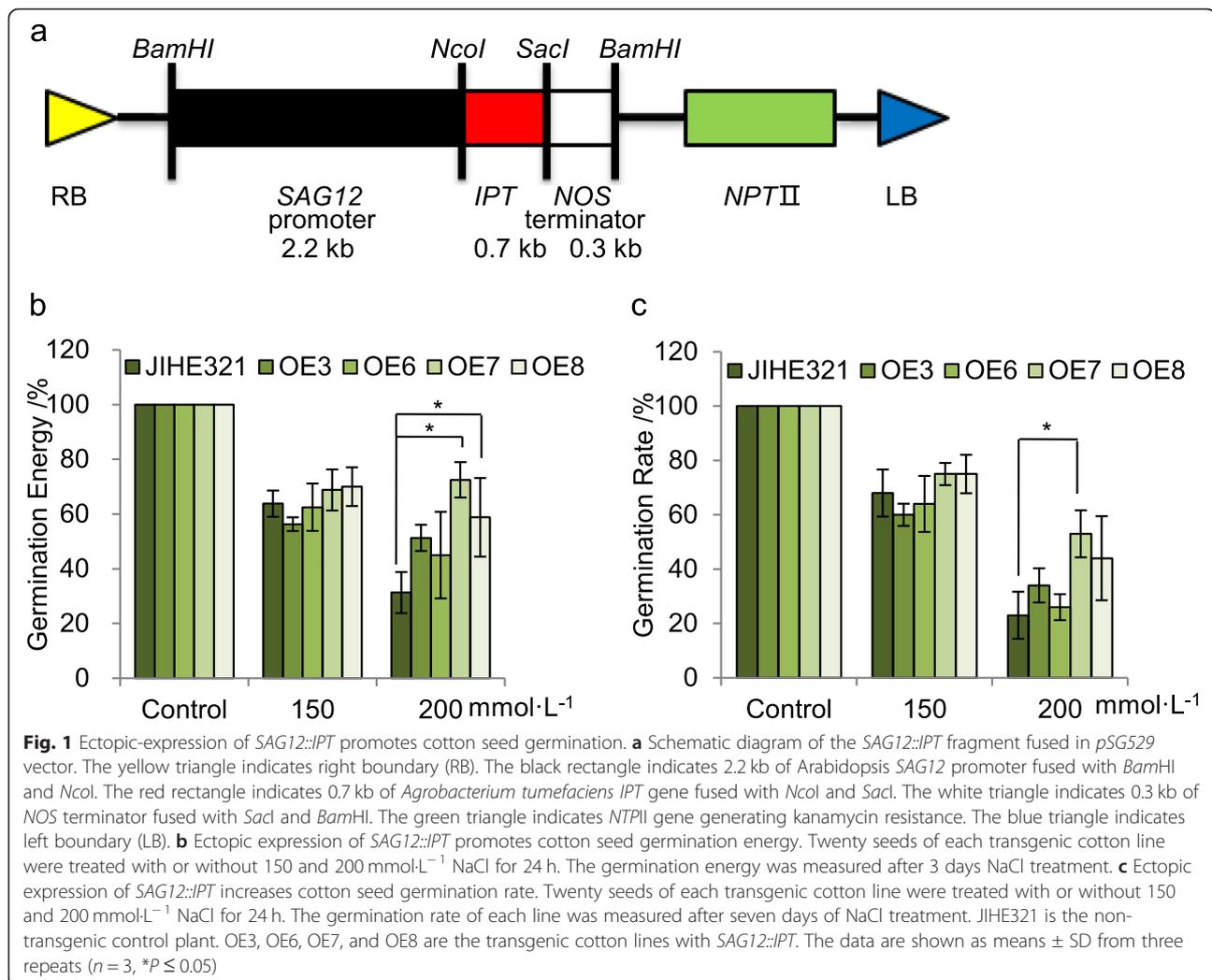
Data analysis

All experiments were repeated three times, and each treatment had at least three replicates. Data were pooled according to one representative experiment. Analysis of variance (ANOVA) was performed using the General Linear Model procedure (GLM) in the SPSS 21.0 (SPSS Inc., Chicago IL, USA). Mean values were compared using Duncan's multiple range tests at the 5% level of probability.

Results

Ectopic-expression of *SAG12::IPT* promotes cotton seed germination under salt stress

In this study, we generated transgenic cotton lines with *SAG12::IPT* vector (Fig. 1a). Among nine transgenic lines, four lines (OE3, OE6, OE7, and OE8) showed strong tolerance to salt stress, which were selected for



further investigation. We calculated the germination energy and germination rate of transgenic cotton lines whose seeds were exposed to 150 and 200 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, and found that both germination energy and germination rate decreased dramatically in a NaCl dosage dependent manner (Fig. 1). However, ectopic-expression of *SAG12::IPT* alleviated the inhibition of seed germination caused by NaCl with respect to control plants of JIHE321 (Fig. 1). Particularly, OE7 and OE8 lines showed more germination energy (Fig. 1b) and OE7 showed higher germination rate (Fig. 1c).

Ectopic-expression of *SAG12::IPT* increases salt tolerance of cotton seedlings

After being surface-sterilized by soaking in 9% H_2O_2 for 30 min, seeds were rinsed with tap water and then germinated in sand medium for 4 days in the dark. After germination, transgenic cotton lines OE3 and OE6 and the control JIHE321 were planted into half-strength modified Hoagland's solution without NaCl treatment. The seedlings at the three-leaf stage were then treated with 220 $\text{mmol}\cdot\text{L}^{-1}$ NaCl. The salt phenotype was recorded after 6 days of NaCl treatment (Fig. 2a). Approximately 0.1 g of the third leaf of transgenic cotton lines and JIHE321 were collected to determine chlorophyll content after 220 $\text{mmol}\cdot\text{L}^{-1}$ NaCl treatment for seven days (Fig. 2b). As shown in Fig. 2, ectopic-expression of *SAG12::IPT* strongly enhanced the tolerance of cotton seedlings to 220 $\text{mmol}\cdot\text{L}^{-1}$ NaCl treatment lasting for 6 days. The transgenic lines exhibited less defoliation,

greener leaves, and had more chlorophyll content compared with JIHE321 (Fig. 2a & b). Moreover, OE3 and OE6 lines showed greater tolerance than OE7 and OE8 lines.

Ectopic-expression of *SAG12::IPT* increases CTK content during cotton seed germination

We determined the CTK content in germinating seeds of OE3 line (more tolerant to salt during seed germination) and OE8 line (more tolerant to salt at seedling stage), and found that OE8 seeds showed significantly higher levels of Z/ZR compared with the control seeds after the first and second day of 220 $\text{mmol}\cdot\text{L}^{-1}$ NaCl treatment, and OE8 shoots (derived from germinating seeds) also had much more Z/ZR and iP/iPA ratios after the seventh day of NaCl treatment (Fig. 3a & b). However, the differences in Z/ZR and iP/iPA contents between the OE3 and control lines were insignificant, which is consistent with the lower salt tolerance of the OE3 line compared with the OE8 line during seed germination.

Ectopic-expression of *SAG12::IPT* increases CTK content in cotton seedlings

In leaves, OE3 and OE8 lines had more Z/ZR and iP/iPA than control plants after 3 days of 220 $\text{mmol}\cdot\text{L}^{-1}$ NaCl treatment (Fig. 4a & b). However, the differences of CTK level in roots between transgenic lines and the control did not appear until 6 days after 220 $\text{mmol}\cdot\text{L}^{-1}$ NaCl treatment (Fig. 4c & d). In particular, the OE3 line, which showed more tolerant to salt stress than the OE8

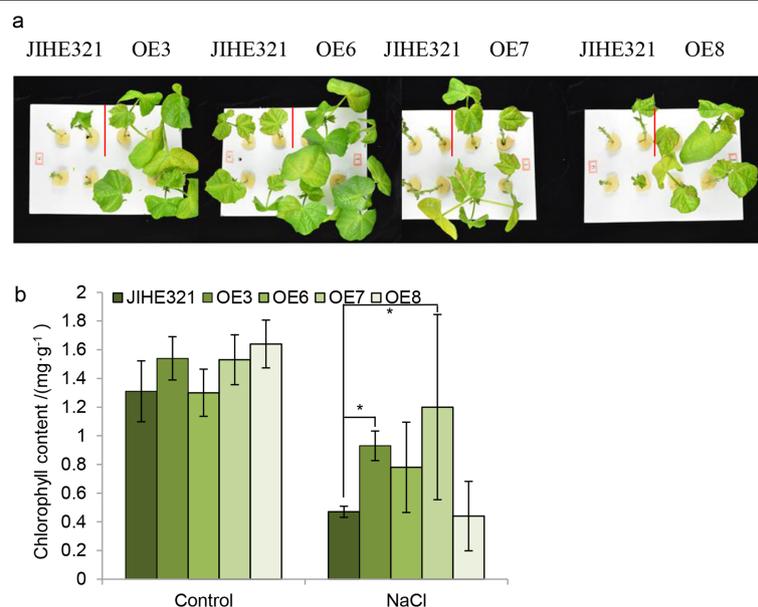


Fig. 2 Ectopic-expression of *SAG12::IPT* induces salt tolerance in cotton seedlings. **a** Ectopic expressing *SAG12::IPT* cotton plants are more tolerant to salt stress compared with JIHE321 control plants. **b** Ectopic expression of *SAG12::IPT* induces chlorophyll content in cotton leaves. The data are shown as means \pm SD from three independent repeats ($n = 3$, * $P < 0.05$)

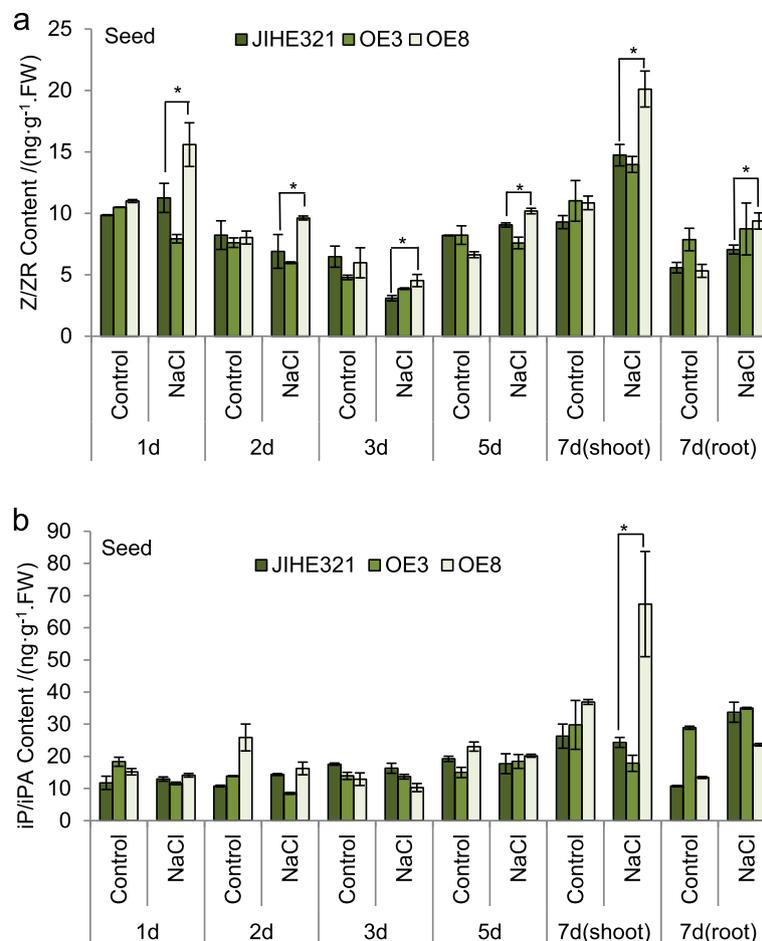


Fig. 3 Ectopic-expression of *SAG12::IPT* induces cytokinin content in cotton seeds. **a** Ectopic expression of *SAG12::IPT* induces Z/ZR-type cytokinin content in seeds of the OE3 and OE8 lines. Germinating seeds (0.5 g, without the seed coat) were sampled after one, two, three, and five days of germination; shoots and roots (0.5 g) separated from the germinating seeds with 220 mmol·L⁻¹ NaCl treatment for seven days were collected to detect the Z/ZR-type cytokinin content. **b** Ectopic expression of *SAG12::IPT* induces iP/iPA-type cytokinin content in seeds of OE3 and OE8. The samples from (a) were used for the measurement of iP/iPA-type cytokinin. The data are shown as means ± SD from three repeats ($n = 3$, * $P \leq 0.05$)

line at seedling stage, accumulated more Z/ZR and iP/iPA than the OE8 line in both leaves (after the third day of NaCl treatment) and roots (after the sixth day of NaCl treatment). Together, the data suggested that senescence-inducible ectopic-expression of *IPT* induces salt tolerance in cotton seedlings likely by promoting CTK accumulation.

Ectopic-expression of *SAG12::IPT* enhances ROS scavenging in cotton leaves under salt stress

It is known that ROS triggered by salt stress affects protein physiological function in cells. In response, cells will activate the antioxidant enzymes, like SOD and POD to protect plants from oxidative stress (Mantri et al. 2011). In this study, the relative H₂O₂ content in the third leaf of cotton seedlings was determined, and data showed that the transgenic lines OE3 and OE8 had significantly less

H₂O₂ than control plants after 3 days of 220 mmol·L⁻¹ NaCl treatment (Fig. 5a). In addition, the leaves of the OE3 line (more tolerant to salt stress than OE8 line at seedling stage) had 409%~414% and 50%~116% more SOD and POD activity, respectively, than control plants during salt stress, and the leaves of the OE8 line showed 227% and 40% more SOD and POD activity than control plants after the first and the sixth day of salt stress, respectively (Fig. 5b & c). The data indicated that maintaining high activity of ROS-scavenging system is one of the key mechanisms to protect plants from salt-induced ROS stress in ectopic-expressing *IPT* plants.

Ectopic-expression of *SAG12::IPT* maintains higher K⁺/Na⁺ ratio in leaves in response to salt stress

Maintaining the ion equilibrium of Na⁺ and K⁺ is a vital strategy to render plants tolerance of salt stress

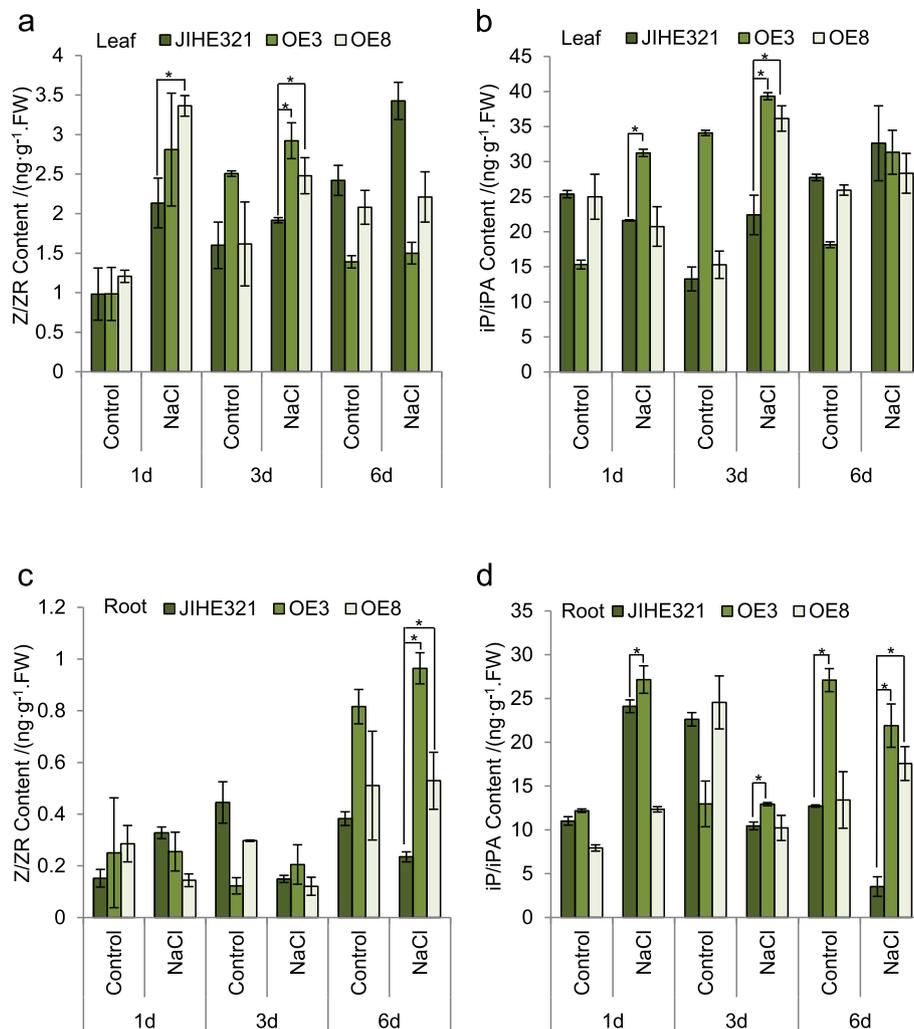


Fig. 4 Ectopic-expression of *SAG12::IPT* induces cytokinin content both in leaves and roots. **a** Ectopic expression of *SAG12::IPT* induces Z/ZR-type cytokinin content in the third true leaf of OE3 and OE8. The third true leaf (0.5 g) of transgenic lines and control cotton were collected after one, three and six days 220 mmol·L⁻¹ NaCl treatment to detect Z/ZR-type cytokinin content. **b** Ectopic expression of *SAG12::IPT* induces iP/iPA-type cytokinin content in the third true leaf of OE3 and OE8. The samples were collected as (a). **c** Ectopic expression of *SAG12::IPT* induces Z/ZR-type cytokinin content in roots of OE3 and OE8. The root samples (0.5 g) of transgenic lines and control cotton were collected after one, three and six days 220 mmol·L⁻¹ NaCl treatment to detect Z/ZR-type cytokinin content. **d** Ectopic expression of *SAG12::IPT* induces iP/iPA-type cytokinin content in roots of OE3 and OE8. The samples were collected as (c). The data are shown as means \pm SD from three repeats ($n = 3$, * $P \leq 0.05$)

(Feki et al. 2014). Compared with control plants, the OE3 line showed 79% higher K^+/Na^+ ratio in leaves after 7 days of 220 mmol·L⁻¹ NaCl treatment (Fig. 6), which may be responsible for its remarkable tolerance to salt stress. Moreover, the roots of the OE3 line, and both leaves and roots of the OE8 line had a slight, but significant higher K^+/Na^+ ratio than those of control plants (Fig. 6).

Discussion

CTKs regulate various events in plant development and responses to environmental stimuli. In the two-component CTK signaling pathway, membrane-bound

histidine kinases (HKs) perceive CTKs and cause HK auto-phosphorylation (Shi and Rashotte 2012). The signal is then transferred via histidine-containing phosphotransferase factors (AHPs), to transcription-factor-type response regulators (ARRs). Phosphorylation of B-type RRs (RRBs) activates GARP domain-containing transcription factors which positively regulate the expression of genes related to abiotic stresses (Mason et al. 2005). However, phosphorylated A-type RRs (RRAs), lacking a DNA-binding GARP domain, negatively regulate CTK signaling (Gupta and Rashotte 2012; Kieber and Schaller 2014). Moreover, HK mutants *ahk2*, *ahk3* and their double mutants are more tolerant to salt stress

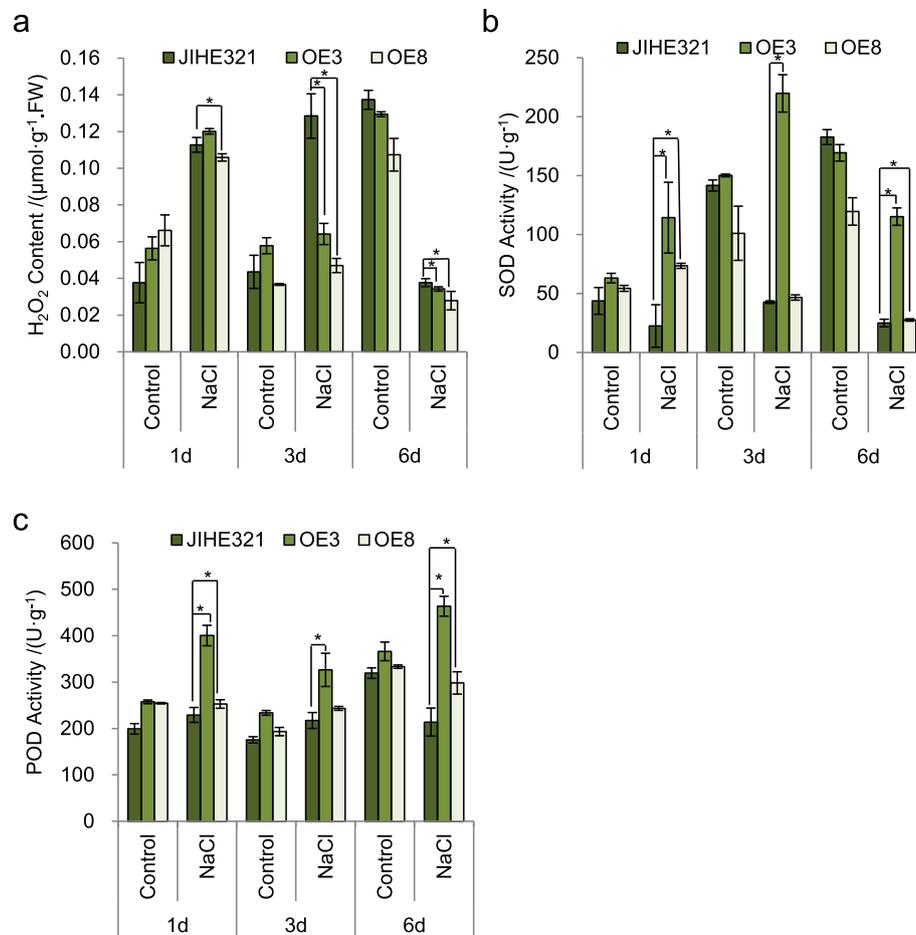


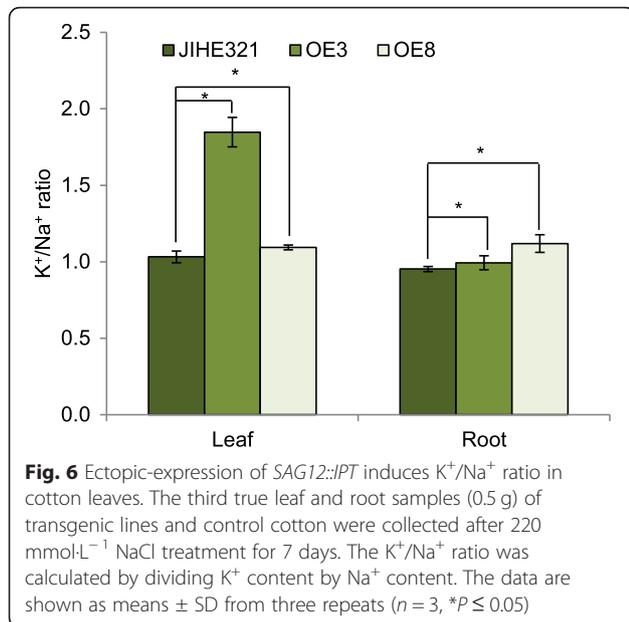
Fig. 5 Ectopic-expression of *SAG12::IPT* reduces relative H₂O₂ content, and enhances the activity of antioxidant enzymes in cotton leaves. **a** Ectopic expression of *SAG12::IPT* reduces relative H₂O₂ content in cotton leaves. The third leaf samples (0.5 g) of OE3 and OE8, and control plant were collected as Fig. 4 at indicated time points. **b** Ectopic expression of *SAG12::IPT* enhances SOD activity in the third leaf of OE3 and OE8. The samples were collected as (a). **c** Ectopic expression of *SAG12::IPT* enhances POD activity in the third leaf of OE3 and OE8. The samples were collected as (a). The data are shown as means ± SD from three repeats ($n = 3$, $*P \leq 0.05$)

(Tran et al. 2007), and RRBs double mutant *arr1arr12* also shows enhanced salt tolerance (Mason et al. 2010), indicating a negative regulation of CTK signaling in response to salt stress. This negative regulation perhaps results from the hindered root development due to higher level of CTKs (Qiu et al. 2012).

However, if driven by inducible promoters such as light-inducible *rbcS-3A*, cysteine proteinase, and salt inducible *RD29A*, overexpression of *IPT* can enhance plant salt tolerance (Thomas et al. 1995; Liu et al.; Qiu et al. 2012). Consistently, we found that ectopic-expression of *IPT* under the control of a senescence-inducible promoter *SAG12* induced endogenous CTKs and enhanced cotton salinity tolerance (Figs. 1 & 2). This could be because the temporarily increased endogenous CTKs can maintain normal cell growth under stress conditions, without affecting root growth.

Photosynthesis is one of the primary processes to be affected by salinity (Munns et al. 2006). Salt stress arises secondary oxidative stress, which can seriously affect leaf photosynthetic machinery (Ort 2001). Our study showed that overexpression of *SAG12::IPT* enhanced ROS scavenging ability (Fig. 5) and chlorophyll content (Fig. 2b) compared with JIHE321 control plants under salt stress, indicating that overexpressing *SAG12::IPT* enhanced salt tolerance likely through eliminating ROS accumulation to protect photosynthetic properties in cotton.

The reliable and stable expression of foreign genes is necessary for the successful application of genetic engineering in agriculture. However, many studies showed that the expression of the same foreign gene varies in different transgenic lines. In this study, we found that four *SAG12::IPT* transgenic cotton lines showed different responses to salt stress either during germination or



seedling stage in comparison with control plants, and the same line displayed different tolerance to salt stress at different stages. For example, the OE8 line was more tolerant to salt stress and had more CTKs than the OE3 line during germination (Figs. 1 & 3), and the OE3 line was more tolerant to salt stress and had more CTKs than the OE8 line at the seedling stage (Figs. 2 & 4). Different T-DNA insertion loci (Peach and Velten 1991; Iglesias et al. 1997) and copy number of transgenic gene (Hobbs et al. 1990; Elmayan and Vaucheret 1996) could affect the function of exogenous genes. In tobacco, transformants that have a single T-DNA insertion showed high GUS activity, while those that have multiple T-DNA insertions showed low GUS activity due to the increased methylation of the integrated T-DNA (Hobbs et al. 1990). Also, Iglesias et al. (1997) found that the GUS-positive transgenic tobacco lines was attributed to both the transcriptional enhancers located in left flanking plant DNA of the T-DNA inserts and the adjacent to telomeres of inserts (Iglesias et al. 1997). Thus, we speculate that the various salt tolerance from the *SAG12::IPT* transgenic cotton lines is the result of different insertion loci and copy number of exogenous *IPT* gene, which possibly altered the CTK biosynthesis by transcriptional or post-transcriptional regulation.

Conclusion

IPT is a key enzyme involved in CTK biosynthesis and plays an important role in response to abiotic stresses. We generated the ectopic-expressing *IPT* cotton lines under the control of a senescence-inducible *SAG12* promoter. Ectopic-expression of *SAG12::IPT* induced the

accumulation of CTKs in leaves, roots, and the germinating seeds. Furthermore, ectopic-expressing *IPT* increased the activity of antioxidant enzymes, leading to the reduction of ROS accumulation in cotton leaves. Also, ectopic-expression of *IPT* increased K⁺/Na⁺ ratio in both cotton shoot and root. Our study not only provides a blueprint on generating salt-tolerant cotton lines, but also sheds light on the functional understanding of exogenous *IPT* gene driven by an inducible promoter in response to cotton salt stress.

Abbreviations

AHPs: Histidine-containing phosphotransferase factors; AMP: Adenosine monophosphate; APX: Ascorbate peroxidase; CAT: Catalase; CTK: Cytokinin; DMAPP: Isoamyl groups; iPA: Isopentenyl adenosine; iPMP: Isopentyl adenosine-5-β-phosphate; IPT: Isopentyl transferase; POD: Peroxidase; ROS: Reactive oxygen species; SAG: Senescence-associated gene; SOD: Superoxide dismutase; ZR: Zeatin riboside

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Authors' contributions

Tian XL conceived and designed the study; Zhao P and Shan Y carried out the experiments, analyzed and interpreted the data; Shan Y, Li FJ and Tian XL prepared the manuscript. All authors read and approved the final version of manuscript.

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Availability of data and materials

No other data related to this study is available at this time.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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