Improved salt tolerance and delayed leaf senescence in transgenic cotton expressing the *Agrobacterium IPT* gene

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Abstract

The manipulation of cytokinin contents *via Agrobacterium*-mediated transformation is an efficient tool for delaying leaf senescence and improving the resistance to environmental stresses. In the present study, cotton transformants harbouring the *Agrobacterium tumefaciens isopentenyl transferase* (*IPT*) gene under the control of the promoter of *Gossypium hirsutum* cysteine proteinase (*Ghcysp*) were generated. PCR and Southern blot analysis indicated that the foreign DNA fragment was successfully integrated into the cotton genome. The chlorophyll and cytokinin contents, and ROS-scavenging enzymatic activities were significantly increased in transgenic cotton lines, which resulted in a significant delay in leaf senescence. The growth characteristics of transgenic cotton lines resembled the non-transgenic lines except delaying premature senescence and the lint yield and fiber quality of transgenic lines were improved. In addition, the transgenic lines had higher biomasses, *IPT* transcripts, and endogenous cytokinin contents compared with those of non-transgenic lines under 200 mM NaCl stress.

Additional key words: *Gossypium hirsutum*, isopentenyl transferase**,** cytokinins, NaCl, senescence-specific promoter.

Introduction

Leaf senescence is not simply a degenerative process but plays an important role in the recycling of nutrients from old, non-functional leaves to young productive leaves and developing seeds. While most genes are inactivated during leaf senescence, distinct sets of genes are activated (referred to as senescence-associated genes, SAGs; Gan and Amasino 1997, Liu *et al.* 2008). Among SAGs, SAG12 is found to encode a cysteine endopeptidase in *Arabidopsis*. The promoter of this gene, P_{SAG12}, has been isolated and exploited in strategies to control and modify plant senescence. The isopentenyl transferase gene (*IPT*) encodes a rate-limiting enzyme in the cytokinin biosynthetic pathway (Barry *et al.* 1984). When *IPT* is combined with a senescence-inducible promoter, the expression of *IPT* is governed by the promoter (Nguyen *et al.* 2010). This fusion directs *IPT* to express in leaves only at the onset of senescence. Senescence is therefore delayed due to the increased contents of cytokinins. In turn, high cytokinin content leads to the down-regulation of senescence-specific promoter that prevents cytokinin from too high accumulation (Gan and Amasino 1995). In modified tobacco with a fusion between *IPT* and *SAG12* promoter, senescence was significantly delayed and plants showed 40 % increase in biomass and a 52 % increase in seed yield (Jordi *et al.* 2000). To date, introduction of the *IPT* gene, linked to senescence-associated SAG promoters, has been reported in many plant species, such as rice (Lin *et al.* 2002), *Medicago sativa* (Calderini *et al.* 2007), *Petunia hybrida* (Casanova *et al.* 2005). In these transformed plants, the activation of

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Abbreviations: CTAB - cetyltrimethyl-ammonium bromide; DAE - days after emergence; DAP - days after planting; iP - isopentenyladenine; iPA - isopentenyladenosine; IPT - isopentenyl transferase; LTN - leaves of the tenth node on main stem; ROS - reactive oxygen species; Z - zeatin; ZR - zeatin-riboside.
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IPT transcription is triggered mainly in senescing leaves, and plants develop normally.

The onset and progression of leaf senescence are regulated by both endogenous and environmental factors and their interactions (Munné-Bosch and Alegre 2004). Salinity reduces plant productivity firstly by reducing plant growth due to osmotic stress. In turn, leaf senescence is mostly induced by the toxicity when excessive salt is accumulated in transpiring leaves (Munns 2002, Pic *et al.* 2002). Much evidence suggests that the both processes are related to hormonal signals generated in response to salt stress. For example, the total cytokinin content progressively decreased due to salinity stress in tomato plants (Ghanem *et al.* 2008). It seems that the plant salinity tolerance could be enhanced by delaying the salt-induced senescence of leaves. Therefore, it is plausible to assume that the introduction of P_{SAG}*::IPT* construct is capable of not only delaying leaf senescence but also enhancing salt stress tolerance.

Premature senescence is one of the most limiting factors to cotton productivity and fiber quality. Previous study has found that *cysteine proteinase* (*Ghcysp*) is a cotton senescence-enhanced protease from the same cysteine endopeptidase family as *SAG12* (Shen *et al.* 2004). We report here the isolation of *Ghcysp* promoter region and its exploitation in cotton.

Materials and methods

Promoter isolation and sequencing: The 5' upstream region of *Ghcysp* gene was isolated by adaptor PCR using a cloning kit (*TaKaRa*, Dalian, China). Briefly, 0.5 μg of genomic DNA of *Gossypium hirsutum* L. cv. Liaomian 9 digested with *BamHI* was ligated to the dephosphorylated *BamHI* adaptor. The ligated products were amplified in the first round of PCR using the 5'-adaptor primer C1, 5'-GTACATATTGTCGTT AGAACGCGTAATACGACTCA-3', and the *Ghcysp* 3' specific primer S1, 5'-TGATAGAACCAGAGC AACGAAGAG-3'. The second round of PCR was performed using the first PCR product as template with the 5'-adaptor primer C2, 5'-CGTTAGAACGCGTAA TACGACTCACTATAGGGAGA-3', and the second 3' specific primer S2, 5'-GGGTTTTGAAGTGGG GTTTTATAG-3'. The PCR product was cloned into the *pGEM-T* vector (*Promega*, Madison, USA) and sequenced. The *PLACE* database and *PlantCARE* were used for promoter nucleotide sequence analysis.

Vector construction: *Agrobacterium tumefaciens IPT* cDNA fragment (*GenBank* accession No. X14410) was amplified from *A. tumefaciens* (C58) using primer sequences: 5'-GTCTAGATTCAACATATCGCAAGA CCG-3' (containing the *XbaI* site) and 5'-GGA GCTCCACAACAAAGAACGAACATC-3' (containing the *SacI* site) and cloned into the *pGEM-T* vector to produce *pT-IPT*. The *GUS* gene of pBI121 was replaced with *IPT* at the *XbaI* and *SacI* restriction sites and produced the construct *pB-IPT*.

According to the sequence, the 5' upstream region of the *Ghcysp* gene was amplified by PCR from total genomic DNA of *Liaomian 9*. The PCR primer pairs were 5' *Ghcysp* (5'-GAAGCTT*TTGCGTCGTCTGGTA TAATCCTTG*-3') and 3' *Ghcysp* (5'-GTCTAGA *GGGTTTTGAAGTGGGGTTTTATAG*-3'). The italic letters in the primer sequence corresponded to the sequence of the *Ghcysp* promoter. Restriction enzyme sites *HindIII* (in the 5' *Ghcysp* primer) and *XbaI* (in the 3' *Ghcysp* primer) were underlined. The PCR amplification conditions were: 95 °C for 5 min (pre-denaturation), 95 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min; 35 cycles in total. An approximately 920 bp fragment was obtained and subsequently subcloned into the *pGEM-T* vector. The *Ghcysp* promoter fragment was inserted into the corresponding sites of the *pB-IPT* vector in place of a CaMV 35S promoter (Fig. 2*A*). The plant transformation vector containing the P_{GHCP}*::IPT* fusion gene was delivered into *A. tumefaciens* strain LAB 4404 by the freeze-thaw method (Hofgen and Willmitzer 1988).

Cotton transformation: Cotton (*Gossypium hirsutum* L., cv. Zhongmian 10) seeds were cultured *in vitro*. Hypocotyl segments (5 to 6 mm in length) excised from 5 to 7-d-old seedlings were used for transformation. They were used directly or precultured for 2 d on hormone-free Murashige and Skoog (MS) medium with B5 vitamins. The explants were infected with *A. tumefaciens* LBA4404 containing the *pBGI* in the bacterial suspension for about 20 min, and blotted dry on a sterile filter paper to remove excess *A. tumefaciens*. The transformants were cultured and selected using the method described by Sunilkumar and Rathore (2001). Selected well-grown shoots with true leaves without roots were cut-off and grafting on wild-type cotton rootstocks growing in greenhouse. Forty five batches of transformation experiments were carried out (40 explants each) to confirm the reproducibility of protocol.

PCR, Southern blot and Northern blot: Total genomic DNA was extracted from frozen cotton leaves using cetyltrimethyl-ammonium bromide (CTAB) method. PCR was performed with the *ipt*-forward (5'-GCG TCCAATGCTGTCCTCAACT-3')/*ipt*-reverse (5'-TGG TGGTCCTTCAAACGCTTCG-3') pair of primers. The conditions were as follows: 35 cycles of 94 °C for 1 min, 46 °C for 1 min, and 72 °C for 1 min 30 s.

About 20 μg of genomic DNA was digested with *XbaI* and *SacI*, and resolved by electrophoresis in 0.8 %

agarose gel and transferred onto a nylon membrane (*Amersham*, USA). A [^{32}P]-labeled probe of an *XbaI* and *SacI IPT* gene fragment was prepared using *Megaprime* DNA labeling system (*Amersham*) according to the manufacturer's instructions. The hybridized membrane was washed with 0.2× SSC (pH 7.2) and 0.1 % (m/v) sodiumdodecyl sulphate (SDS) at 60 °C. The hybridization signals were detected by exposing them onto film (*RX-U*, *Fuji*, Tokyo, Japan).

Total RNA was extracted from frozen samples using the RNeasy plant mini kit (*Qiagen*, Fremont, CA, USA) according to the manufacturer's instructions. Total RNA (30 μg) was run in 1.5 % formaldehyde-agarose gels, and blotted onto nylon membranes (*Amersham*). The filter was probed with the [^{32}P]-labeled *XbaI* and *SacI IPT* gene fragment prepared as for the Southern blot. The membrane was hybridized with the probe in 50 % (m/v) formamide, 5× SSC, 50 mM sodium phosphate (pH 7.0), 0.1 % (m/v) SDS, 50 μg cm^{-3} salmon sperm DNA, and 1 × Denhardt's at 45 °C for 12 h. The blots were washed at 65 °C and exposed to X-ray film under an intensifying screen at -80 °C. RNA samples for each experiment were analyzed in at least three independent blots.

Real-time PCR analysis: Fresh tissues were used for total RNA isolation, and cDNA was synthesized from 5 μg of RNA using the *SuperScript* first-strand synthesis system for RT-PCR (*Invitrogen*, Carlsbad, CA, USA) with random hexamer primers according to the manufacturer's instructions. Primers for *IPT* gene and predicted amplicon sizes were as follows: forward, 5'-CGTCCAATGCTGTCCTCAAC-3' and reverse, 5'-GCTTGCTTGGCTGTAATGAT-3' (120 bp). Cotton 18S rRNA (Accession number U42827) was used for RNA normalization. The primers used were: forward primer 5′-CCATAAACGATGCCGACCAG-3′ and reverse primer: 5′-AGCCTTGCGACCATACTC-3′. The *SuperScript™ III platinum®* two-step qRT-PCR kit with *SYBR® Green* (*Invitrogen*) was used for detecting the expression levels of the genes. Quantitative RT-PCR reactions contained the first strand cDNA of each tissue as a template, specific primers and qPCR core kit for *SYBR Green* (*Eurogentec*, Leige, Belgium) in a final volume of 0.025 cm^3. Amplifications were performed for 40 cycles, consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, and an initial preheating at 95 °C for 15 min. All reactions were done in triplicate. The threshold cycle (C_t) values of the triplicate PCRs were averaged. The relative cotton *IPT* gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). For calibration, the *IPT* gene expression in the leaves of 55-d-old transgenic plants was used.

Field test and analysis of leaf senescence, yield, and fiber quality: Leaves of the tenth node on the main stem, LTN (counting after the cotyledons) in different days after emergence were used for assessing chlorophylls, *IPT* expression, cytokinins, and antioxidant enzyme activities. All samples were collected and immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

Number of bolls per plant was counted on September 15. On September 20, 50 bolls in the central position of the plants were harvested in each plot for the measurements of boll mass, seed and lint yield and fiber quality using a model *900-A* (*HIV*, Charlotte, NC, USA).

Salt stress responses: To assess the ability of seeds to germinate under salt stress conditions, 50 seeds from each transgenic line and its corresponding non-transgenic line were placed on germination paper moistened with 250 mM NaCl in separate Petri dishes. Dishes were sealed with *Parafilm* and placed in the dark at 28 °C. Seven days after imbibition, the germination rate was determined.

Then the seedlings were grown in pots containing aerated nutrient solution. At the five leaf stage, six seedlings were exposed to salinity by adding NaCl to the growth medium in 50 mM increments every 12 h, until the final concentration of 200 mM was reached. The leaves were collected and immediately frozen in liquid N_2 for subsequent assays. Uniform seedlings were harvested for biomass determination after drying in an oven at 80 °C to a constant mass.

Determination of chlorophyll content: Chlorophyll was extracted by homogenizing 500 mg (fresh mass) leaf tissue in liquid nitrogen and resuspending in 5 cm^3 of cold 80 % acetone. After overnight incubation at -20 °C, the extracts were cleared by centrifugation and measured at 645 and 663 nm using a *SPAD-502* chlorophyll meter (*Minolta*, Osaka, Japan).

Analysis of cytokinins: The extraction and purification of isopentenyl-adenine and isopentenyl adenosine (iP and iPA) and zeatin and zeatin-riboside (Z and ZR) in leaves were done essentially as described by Dong *et al.* (2008). The cytokinin contents were determined by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (*Phytodetek*, USA.) following the protocol provided by the manufacturer. The sum of free bases, ribosides, nucleotides and 9N-glucosides of the corresponding cytokinins was measured. For simplicity, the endogenous cytokinins were identified as Z+ZR and iP+iPA, and shown as total content.

Statistical analysis: All experiments were performed three times with four samples. The statistical significance of differences in the means between the transgenic lines and their corresponding non-transgenic lines was determined using Duncan's multiple range test at $P = 0.05$ or $P < 0.01$.

Fig. 1. Nucleotide sequences of the 5' flanking region (*GenBank* accession number GQ919043) of the cotton (*G. hirsutum*) cysteine proteinase (*Ghcysp*) gene. The putative transcription site is designated ATG as +1. The *Ghcysp* coding region starting from an ATG is shown in *bold* letters. The TATA box, CAAT box are indicated and *underlined*. All putative *cis*-acting elements are *boxed*, including GT-1 box (salt responsive element), CRT (cytokinin-regulated transcription factor), MYB (MYB binding site), RWS (responsive to water stress), CAT-box (response to cold stress), ARW (activation of response to wounding), ABRE (ABA-responsive element), CDPB (cytokinin-dependent protein binding), JARE (senescence and jasmonate-responsive element), EAG (embryogenesis-abundant genes) and ERE (ethylene-responsive element).

Results

Isolation of *Ghcysp* promoter: The putative 5' regulatory region of *Ghcysp* gene was carried out to define putative *cis*-elements in the *Ghcysp* sequence using the software programs *PLACE* and *PlantCARE*. A number of potential regulatory motifs corresponding to known *cis*-elements were identified (Fig. 1). The A of translation initiation codon (ATG) is defined as +1. A putative TATA box and a CAAT box, which serve as basal promoter elements for the transcription of eukaryotic genes, were identified 100 to 107 bp and 151 to 158 bp upstream from the translation start site, respectively. An ABRE-like sequences, as a *cis*-acting element in response to drought and high-salinity stresses (Narusaka *et al.* 2003, Nakashima *et al.* 2006), was located at positions -612 to -606 (sequence ACGTGGC). ERE (ethylene responsive element) has been identified in promoter regions of senescence-responsive plant genes as *cis*-acting elements involved in dehydration stress, dark-induced senescence, and jasmonate-induced senescence (Tapia *et al.* 2005). Two ERE-like sequences were observed at positions -59 to -52 (sequence ACTTCAAA) and -162 to -157 (sequence GCCGCC), respectively. In addition, a sequence that matches a novel *cis*-element exhibiting cytokinin-dependent protein binding (CDPB**)** was located at positions -569 to -564 (sequence TATTAG). MYB recognition sites have been identified in promoter regions of stress-responsive plant genes as *cis*-acting elements involved in ABA-responsive signal transduction (Abe *et al.* 2003). In the 5′-flanking region of the *Ghcysp* gene, a plant MYB-like sequence were observed at positions -697 to -692 (sequence TAACTG). Taken together, the 1000-bp promoter region contains a number of putative senescence-specific, cytokinin-responsive, or stress-induced regulatory motifs, implying that the *Ghcysp* promoter may be under a complex regulation.

Transformation, regeneration, and selection of transgenic P_{GHCP}::*IPT* lines: Our preliminary results, using a P_{GHCP}::*GUS* chimera, showed that *Ghcysp* expression gradually increased in senescence leaves. About 1 800 hypocotyl explants were infected with the *A. tumefaciens* strain LBA4404 containing the *pBGI*, a binary vector carrying the P_{GHCP}::*IPT* construct (Fig. 2*A*). Using this transformation protocol, 96 independent regenerated shoots were obtained. Well-grown shoots were cut-off and grafted to wild-type rootstocks. More than 85 % of the grafted shoots gave rise to plantlets. About 50 putative transgenic plants were allowed to flower, and backcrossed to parental cv. Zhongmiansuo 10. The resulting T_1 seeds were heterozygous and segregated 1:1 for the presence:absence of the transgene in single-copy transformation events. Segregants that

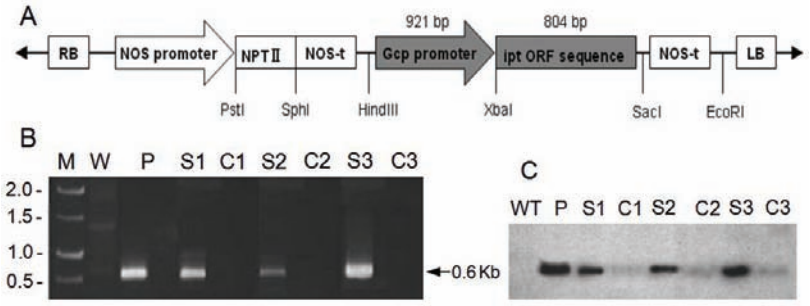

Fig. 2. Molecular characterization of plants of the transgenic cotton lines. A schematic representation of the binary vector containing *Ghcysp* promoter and *IPT* ORF sequence for cotton transformation. Restriction enzyme sites used for Southern analyses are indicated: *XbaI*, and *SacI*, respectively (*A*). DNA molecular size marker (M), wild type plants (Zhongmiansuo 10) (W), plasmid DNA containing P_{GHCP}::*IPT* as a positive control (P), three independently transgenic lines (S1, S2, and S3), and non-transgenic lines (C1, C2, and C3) as a negative control for PCR (*B*) and Southern blot analysis (*C*).

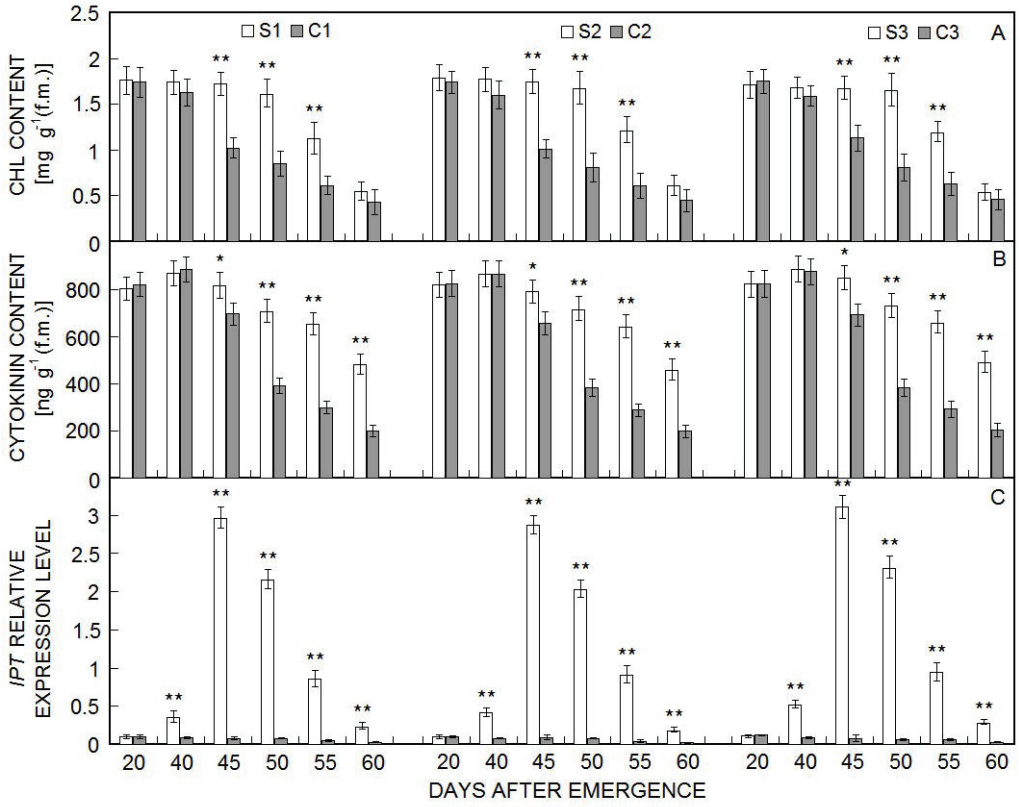

Fig. 3. Chlorophyll contents (*A*), total endogenous cytokinin (Z+ZR+iP+iPA) contents (*B*) and relative *IPT* expression levels (*C*) in the tenth node main-stem leaves of three independently transgenic lines (S1, S2, and S3) and their corresponding non-transgenic lines (C1, C2, and C3) at 20, 40, 45, 50, 55 and 60 d after emergence, respectively. Values are means ± SD, $n = 4$, * and ** show significant differences at $P < 0.05$ and $P < 0.01$.

contained the transgene were referred to as S1, S2 and S3. The corresponding non-transgenic segregants were termed C1, C2, and C3, respectively. After a rigorous four-generation selfing and kanamycin selection, we got the homozygous transgenic offspring. PCR amplifications produced a 0.60 kb fragment of the *IPT* gene from the putative transgenic lines as expected (Fig. 2*B*). Southern blot analysis (Fig. 2*C*) with *IPT* probes further confirmed the integration of the transgenes into the genome of these lines. Homozygous lines were selected through two further rounds of backcross and selfing selections, and lines giving 100 % survival were deemed homozygous, and used for further experiments.

Leaf senescence of transgenic cotton plants: Several agronomically important traits of the homozygous P_{GHCP}*::IPT* plants were evaluated under well-watered conditions in the field. The transgenic plants displayed leaf senescence later than their corresponding non-transgenic plants. The oldest leaves of non-transgenic plants turned yellow about 2 weeks earlier than the corresponding leaves of transgenic plants. LTN of non-transgenic plants reached their full expansion 20 d after emergence (20 DAE), displayed the first signs of leaf senescence 40 DAE and complete necrosis at 60 DAE. On the other hand, the transgenic plants displayed the first signs of leaf senescence at 50 to 55 DAE. The life span of the leaves from full expansion to the first signs of leaf senescence was 10 to 15 d longer than that of non-transgenic plants. However, there was no significant difference in the life span of the leaves from emergence to death between the transgenic and non-transgenic plants.

The total chlorophyll contents in both the transgenic and non-transgenic lines decreased with age from 40 to 60 DAE (Fig. 3*A*). However, the decrease was faster in the non-transgenic lines than in the transgenic lines. The decrease between 40 and 45 DAE in the non-transgenic C1 line and the transgenic S1 line was about 37.4 and 1.2 %, respectively. Similar trends are seen in comparisons of S2/C2 and S3/C3. The leaves of C1, C2 and C3 were completely senescent and contained no measurable chlorophyll at 65 DAE, while the leaves of transgenic plants (S1, S2 and S3) were still with relatively high chlorophyll contents (data not shown). These results indicated that the chlorophyll degradation in the leaves of non-transgenic plants occurred earlier when compared with those of transgenic plants at the same developmental stage.

The seedling emergence, squaring, flowering, peak flowering, peak boll-setting, and boll-opening occurred at about 8, 40, 61, 80, 95, and 110 d after planting (DAP), respectively, in both P_{GHCP}*::IPT* plants and non-transgenic plants. No significant differences of phenotype were found between the transgenic and non-transgenic plants, indicating that the expression of P_{GHCP}*::IPT* gene had no negative effect on the growth of cotton plants. Nevertheless, the cotton yield, yield components, and fiber quality were significantly improved in the transgenic lines. The seed yield of S1, S2 and S3 lines was higher by 25.4, 23.8 and 26.1 % than in corresponding non-transgenic lines, respectively. The lint yield of transgenic lines increased on average by 34.2 %. Furthermore, the fibers produced by the transgenic lines were generally more uniform, stronger and longer than those produced by the non-transgenic lines. All these improvements suggest that the expression of *IPT* gene in transgenic cotton could result in increased productivity by delaying the process of leaf senescence.

***IPT* expression and cytokinin content in the senescing leaves of transgenic plants:** Northern analysis indicated the *IPT* transcript in the senescing leaves of transgenic lines, but not in either expanding or senescing leaves of non-transgenic plants (Fig. 4). The relative expression of *IPT* gene in developing leaves was investigated by qRT-PCR. Transcript levels of *IPT* gene in expanding leaves of S1, S2, and S3 were barely detectable before 40 DAE, reached the peaks at 45 DAE, and then declined from 50 to 60 DAE (Fig. 3*C*). The *IPT* transcript levels in the senescing leaves of three transgenic lines at 45 DAE were by 746, 600 and 498 % higher than that at 40 DAE, respectively. These results suggest that the expression of *IPT* gene in transgenic plants was suppressed in young and fully matured leaves, but could be activated by a senescence development signal in early matured leaves.

Expression of *IPT* leads to increased contents of cytokinins. At 20 and 40 DAE, there was no statistical difference in the cytokinin contents between the transgenic and non-transgenic plants (Fig. 3*B*). At 45 DAE, cytokinin content increased in transgenic plants. At 50 to 60 DAE, the contents of cytokinins in transgenic plants was higher than in non-transgenic plants ($P < 0.01$) because of the dramatic decrease of cytokinins content after 50 DAE in non-transgenic plants (Fig. 3*B*). At 50 DAE, the content of cytokinins of S1, S2 and S3 was

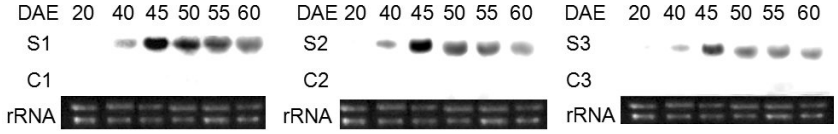

Fig. 4. Northern blot analysis of *IPT* expression in the tenth node main-stem leaves of three independent LTN (S1, S2, and S3) and their corresponding non-transgenic lines (C1, C2, and C3) at 20, 40, 45, 50, 55 and 60 d after emergence.

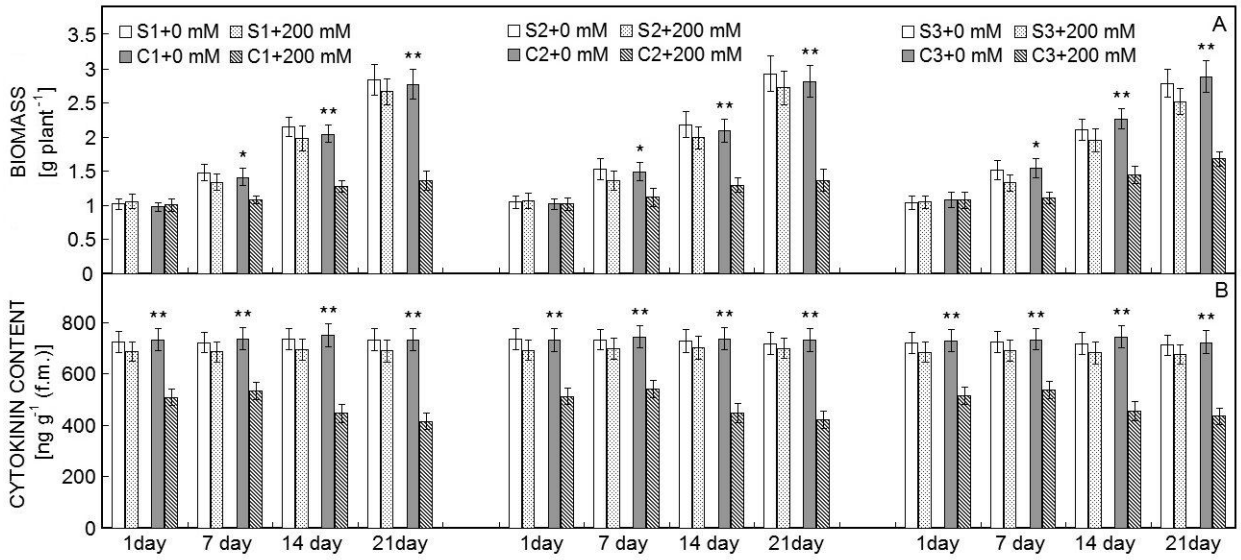

Fig. 5. The biomass (*A*) and total endogenous cytokinin (Z+ZR+iP+iPA) content (*B*) in three independent transgenic lines (S1, S2, and S3) and their corresponding non-transgenic lines (C1, C2, and C3) at 1, 7, 14 and 21 d after 200 mM NaCl treatment. Values are means ± SD; $n = 4$, * and ** show significant differences at $P < 0.05$ and $P < 0.01$.

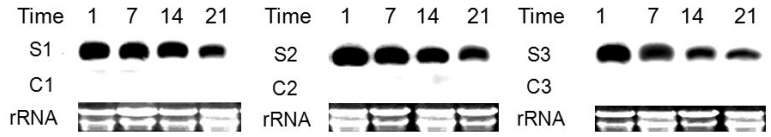

Fig. 6. Northern blot analysis of *IPT* expression in the seedlings of three independent transgenic lines (S1, S2, and S3) and their corresponding non-transgenic lines (C1, C2, and C3) at 1, 7, 14 and 21 d after 200 mM NaCl treatment.

by 79.8, 86.5 and 90.1 % higher than in corresponding non-transgenic plants (Fig. 3*B*). These results showed that the dynamic changes of cytokinins content in the leaves at 45 DAE and afterward correlated positively with the *IPT* gene expression.

Salt tolerance of the transgenic cotton plants: On the medium without NaCl, the germination percentage of all lines was around 95 %. Under 250 mM NaCl treatment, the germination percentage of the transgenic plants S1, S2, and S3 was 80.3, 78.2 and 82.1 %, respectively, whereas the germination percentage of their corresponding non-transgenic plants were 9.2, 9.6 and 10.5 %, respectively.

To assess the salt tolerance of transgenic P_{GHCP}::*IPT* cotton, seedlings at the stage of five leaves were subjected to salt stress by adding 200 mM NaCl to the nutrient solution. The average total dry mass per plant was determined after 1, 7, 14 and 21 d of salt treatment (Fig. 5*A*). During 1 - 14 d, no significant difference was observed between the transgenic and non-transgenic lines. After 21 d, the total dry mass of the transgenic lines decreased by 5.98 - 9.67 %, which was not significantly different from their non-stressed counterparts. The non-transgenic plants, on the other hand, had a significantly lower dry mass (from 41.66 to 51.24 %) than the corresponding non-stressed plants.

Under 200 mM NaCl treatment, levels of the *IPT* transcripts of S1, S2, and S3 seedlings reached the maximum within 1 d, decreased at 7 d, and remained constant at 14 and 21 d. However, the *IPT* transcript was not detected in the seedlings of C1, C2 and C3 plants under salt stress. The expression of *IPT* induced by salinity confirmed the salt responsiveness of the *GHCP* promoter (Fig. 5*B*).

Under normal growth conditions, the content of cytokinins was similar in the transgenic and non-transgenic lines (Fig. 5*B*). After 21 d of salt treatment, cytokinins content was reduced in the seedlings of all lines. The reduction in the three transgenic lines ranged from 2.78 to 5.87 %, and was not significantly different from that in their non-stressed counterparts. However, the non-transgenic lines had a drastic reduction (39.75 to 43.09 %) in cytokinins content. Thus, the cytokinins content in the three transgenic lines was significantly higher than in their corresponding non-transgenic lines.

Discussion

Senescence is presumably accompanied by a decline of cytokinins content in leaves and cytokinin supplement can delay leaf senescence. However, systemic expression of *IPT* gene or *kn1* (a homeobox gene) in transgenic plants results in extreme phenotypes (Chuck *et al.* 1996, Khan *et al.* 2010). In this paper, we reported the manipulation of cytokinins content by senescence-regulated expression of the *A. tumefaciens IPT* gene through its control by the *Ghcysp* promoter in cotton. The transgenic cotton had significantly higher cytokinin and chlorophyll contents, and ROS-scavenging enzyme activities in comparison with wild-type, which resulted in a significant delay in leaf senescence. These results were consistent with cytokinin function reported in *Arabidopsis* (Xu *et al.* 2009), *Medicago sativa* (Calderini *et al.* 2007), *Triticum aestivum* (Sykorova *et al.* 2008), and *maize* (Robson *et al.* 2004). As a result of *IPT* expression, cotton production and fiber quality were improved. In addition, we showed that the expression of *IPT* gene, driven by the *Ghcysp* promoter, contributed to the enhanced tolerance of the transgenic cotton to salt stress.

Cytokinins are known to modulate the activity of genes coding light-harvesting chlorophyll-binding proteins and to promote the reversal of senescence by stimulating the expression of genes active in the redifferentiation of senescent plastids (gerontoplasts) into chloroplasts (Synková *et al*. 1997). Much evidence suggests that cytokinins are the major leaf senescence-inhibiting hormones, since senescence is delayed after the application of cytokinins (Van Staden *et al.* 1988) or due to the overproduction of cytokinins in transgenic plants transformed with cytokinin biosynthesis-related genes (Cowan *et al.* 2005, Havlova *et al.* 2008). Leaf senescence can only be initiated when leaf cytokinins content falls below a threshold (Noodén *et al.* 1997). Maintaining cytokinin content above this threshold inhibits transcriptional regulation of senescence-related genes and prevents the onset of senescence in the entire plant (Gan and Amasino 1995). The phenotypes of P_{GHCP}*::IPT* cotton lines are consistent with the suppression of senescence initiation and senescence-associated catabolic processes by cytokinins. Our work showed that the life span of leaves from full expansion to the first signs of leaf senescence in transgenic *IPT* lines was 14 to 16 d longer than that in their corresponding non-transgenic lines. However, leaf senescence was dramatically accelerated after the first signs of leaf senescence, and the life span of the leaves from emergence to death was not significantly different between the transgenic and non-transgenic lines. Based on the fact that senescence modification took the form of delayed initiation rather than reduced rate, leaf ageing and senescence are likely to be two distinct processes.

Jordi *et al.* (2000) showed that the senescence-activated cytokinin production caused the inhibition of chlorophyll breakdown in the basal leaves of tobacco, affecting source/sink relationships and reducing nitrogen mobilization to younger and growing tissues that resulted in an inverted vertical nitrogen profile within the plant canopy. In transgenic maize, the senescence-activated cytokinin production induced a marked delay in flowering (Robson *et al.* 2004). Our work showed that the growth characteristics during vegetative period of P_{GHCP}*::IPT* cotton plants resembled those in wild-type. Both plants grew and developed in a similar time-course and no delay was observed in flowering and seed set, which seems to contradict the results for *Arabidopsis* (Xu *et al.* 2009) and maize (Robson *et al.* 2004).

Altered metabolism of ROS is one of the most characteristic traits of leaf senescence, *e.g.* in maize, the senescence of leaves was characterized with increased oxidative stress and decreased antioxidant activity (Procházková *et al.* 2001). In transgenic cotton plants, the delayed senescence might be correlated with higher activity of antioxidative enzymes, and suppression of lipid peroxidation and membrane leakage (data not shown).

Our results revealed the reduced susceptibility to salt stress by the overproduction of cytokinin in cotton. The alteration of cytokinin content in plants exposed to various stresses has been frequently reported. For instance, trans-zeatin (tZ) and trans-zeatin riboside (tZR) content rapidly decreased in the elongation zone of barley leaves within several minutes after salinity stress treatment (Fricke *et al.* 2006). The large drop of cytokinin contents in salinized tomato leaves (Ghanem *et al.* 2008) could contribute to the progression of senescence under stress. Applied cytokinins trigger phosphoenolpyruvate carboxylase and proline accumulation in *Mesembryanthemum crystallinum*, and led to enhanced tolerance to salt stress (Thomas *et al.* 1992). Transgenic plants with *IPT* gene accumulate higher contents of endogenous cytokinins and this affects plant growth, development, senescence and the resistance to abiotic (Synková *et al.* 1997, Havlova *et al.* 2008) and biotic (Pogány *et al.* 2004) stresses.

In conclusion, our results showed that the cytokinin and chlorophyll contents were increased in transgenic *IPT* cotton plants under the control of *Ghcysp* promoter, which led to the significant delay of leaf senescence. The lint yield and fiber quality of transgenic cotton were also improved. In addition, the suppression of salt-induced leaf senescence resulted in outstanding salt tolerance. This result is exciting because the expression of *IPT* gene could facilitate the development of transgenic cotton able to grow in salt soils.

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