Carpel-specific down-regulation of \textit{GhCKXs} in cotton significantly enhances seed and fiber yield

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Abstract

Cytokinin is considered to be an important driver of seed yield. To increase the yield of cotton while avoiding the negative consequences caused by constitutive overproduction of cytokinin, we down-regulated specifically the carpel genes for cytokinin oxidase/dehydrogenase (CKX), a key negative regulator of cytokinin levels, in transgenic cotton. The carpel-specific down-regulation of \textit{CKXs} significantly enhanced cytokinin levels in the carpels. The elevated cytokinin promoted the expression of carpel- and ovule-development-associated genes, \textit{GhSTK2}, \textit{GhAG1}, and \textit{GhSHP}, boosting ovule formation and thus producing more seeds in the ovary. Field experiments showed that the carpel-specific increase of cytokinin significantly increased both seed yield and fiber yield of cotton, without resulting in detrimental phenotypes. Our study details the regulatory mechanism of cytokinin signaling for seed development, and provides an effective and feasible strategy for yield improvement of seed crops.

Keywords: AG subfamily gene, carpel-specific, cotton yield, cytokinin, cytokinin oxidase, ovule initiation.

Introduction

Cotton is a major source of natural fibers for the global textile industry. Cotton seeds contain about 23% protein and 21% oil, and therefore are also an important source of foodstuff, feed, and edible oil (Zhang et al., 2002; Sunilkumar et al., 2006; Chen et al., 2007; Ma et al., 2016). A cotton boll contains approximately 30 seeds, and approximately 25% of the ovular epidermal cells of each ovule are able to differentiate into the commercially important lint fibers (Kim and Triplett, 2001; Mansoor and Paterson, 2012). Hence, the seed size and seed number of cotton determine the yield of fibers and seeds. However, seed size is negatively correlated with seed number due to the limitation of space and nutrition, and enhancing fiber abundance usually results in smaller seeds (J. Zhang et al., 2005; M. Zhang et al., 2011).

Generally, a seed is derived from a fertilized ovule, and thus, ovule development is critical to seed yield. The number of ovules per ovary depends on the ovule identity and ovule primordia initiation, which are controlled by an array of genes (Franks et al., 2002; Favaro et al., 2003; Sridhar et al., 2006; Azhakanandam et al., 2008; Jiang et al., 2020), hormone signals (Higuchi et al., 2004; Bartrina et al., 2011; Bencivenga et al., 2012; Zu et al., 2022), and environmental factors (Meyer, 1966; Reddy et al., 1991; Ritchie et al., 2007; Sita et al., 2017; Jiang et al., 2019). Ovules originally arise from carpel (ovary) tissue as new meristematic formation, and the cell fate in proliferating ovule primordia is specified by particular ovule identity factors, such as MADS box AG subfamily members Seedstick (STK),
Cytokinin promotes cotton ovule initiation

Shatterproof (SHP1/2), and Agamous (AG) (Favaro et al., 2003; Pinyopich et al., 2003; Brambilla et al., 2007; Zu et al., 2022). It was reported that ectopic expression of their genes can promote the formation of carpels and ovules (Favaro et al., 2003; Pinyopich et al., 2003; Xu et al., 2004; Guo et al., 2007; Liu et al., 2009, 2010; de Moura et al., 2017; Nardeli et al., 2018).

Cytokinins (CKs) are an important group of phytohormones that regulate the proliferation and differentiation of plant cells (Ioio et al., 2008) and control many developmental and physiological processes in plants, including leaf senescence (Gan and Amasino, 1995; Hönig et al., 2018), organ formation (Lohar et al., 2004; Zhao et al., 2009), nutrient uptake and allocation (Séguela et al., 2008; Gu et al., 2018), as well as biotic and abiotic stress (Siemens et al., 2006; Rivero et al., 2007; Choi et al., 2010; Peleg et al., 2011; Cortleven et al., 2019). A noteworthy role of cytokinins is to regulate seed yield (Sawan et al., 2000; Ashkari et al., 2005; Barrtrina et al., 2011).

The signal transduction pathway of cytokinins has been well investigated in Arabidopsis. The signaling pathway is initiated by binding of cytokinin to Arabidopsis histidine kinase receptors (AHNs), following phosphoryl group transport via Arabidopsis histidine proteins (AHPs) to Arabidopsis response regulators (ARRs). The B-type ARRs that contain conserved GARP DNA binding and activation domains can bind to a short 5′-(A/G) GAT(T/C)-3′ core DNA sequence to activate the transcription of downstream genes (Sakai et al., 2000, 2001; Mason et al., 2004, 2005; Argyros et al., 2008; Werner and Schmülling, 2009; Argueso et al., 2010; Zubo and Schaller, 2020). Among ARR-activated genes, members of the AG subfamily, such as STK, AG1, and SHP, have been known to be involved in ovule initiation (Barrtrina et al., 2011; Zu et al., 2022). However, details about cytokinin signaling pathway in carpel development need to be investigated.

Cytokinin levels are regulated through biosynthesis, activation, degradation, and conjugation of the bioactive molecules (Sakakibara, 2006; Jameson and Song, 2016). Cytokinin oxidase/dehydrogenase (CKX), which catalyses the irreversible degradation of the cytokinins by oxidative side chain cleavage, is a crucial regulator controlling endogenous cytokinin levels in the plant kingdom (Jones and Schreiber, 1997; Schmülling et al., 2003; Kowalska et al., 2010). Down-regulation of CKX genes could significantly increase cytokinin levels in transgenic plants and contribute to the enhancement of seed (grain) yield (Ashikari et al., 2005; Zalewski et al., 2012; Yeh et al., 2015; Holubová et al., 2018). However, constitutive down-regulation of CKXs in plants usually causes cytokinin overproduction phenotypes, including dwarfism, sterility, and root growth inhibition (Zalewski et al., 2012; Gao et al., 2014; Zhao et al., 2015; Gasparis et al., 2019). Thus, the key to regulating CKXs for seed yield improvement is to express the genes at the right time in the right place (Daskalova et al., 2007; Werner et al., 2010; Gao et al., 2014; Ramireddy et al., 2018).

In this study, we used a chimeric carpel- and stamen-specific promoter (Busch et al., 1999; Deyholos and Sieburth, 2000), proAGIP, to down-regulate a functional cotton CKX gene (proAGIP::GhCKX3b-RNAi) in cotton carpels. We showed that the carpel-specific down-regulation of GhCKXs could enhance CK levels at the position where the ovule initiates in the carpel, without causing abnormal growth phenotypes. Cytokinin-activated GhARR1, GhARR2a, and GhARR11 in turn promoted the expression of GhSTK2, GhAG1, and GhSHP by binding their promoter sequences, thus boosting carpel development and ovule formation. As a result, seed and fiber yield of proAGIP::GhCKX3b-RNAi cotton was significantly increased compared with that of the non-transgenic control. Our results indicate the biotechnological potential of manipulation of CKs in cotton carpels for the concurrent improvement of seed and fiber yield.

Materials and methods

Plasmid construction and plant materials

The construction of GhCKX3b-RNAi (previously named GhCKX-RNAi) and the genotype of pro35S::GhCKX-RNAi transgenic cotton have been described previously (Zeng et al., 2012; Zhao et al., 2015). To construct the vectors used for genetic transformation, the 1653 bp sequence of the 3′ end of the second intron of the AtAG gene was amplified from the gDNA of Arabidopsis and fused with a 46 bp minimal CaMV35S promoter to create a functional proAGIP promoter, as previously described (Fang et al., 1989; Deyholos and Sieburth, 2000). For the proAGIP::GhCKX3b-RNAi construct, the proAGIP promoter was linked with the GhCKX3b-RNAi fragment and assembled with HindIII and EcoRI-linearized p5 vector (Luo et al., 2007). proAGIP::GUS was constructed by replacing the CaMV35S promoter of the pBl121 vector with the proAGIP::GhCKX3b-RNAi fragment, the coding regions of GhARR1, GhARR2a, GhARR2b, GhARR11, and GhARR12 were amplified from carrot cDNA of upland cotton ‘Jimin 14’ (with or without stop codon). Similarly, Yellow Fluorescent Protein (YFP) was amplified from plasmid pLGN-pro35S::GhPIN3a::YFP (Zeng et al., 2019) (with or without stop codon). For the pro35S::GhARR1, pro35S::GhARR2a, pro35S::GhARR2b, pro35S::GhARR11, and pro35S::GhARR12 constructs, the coding regions of GhARR1, GhARR2a, GhARR2b, GhARR11, and GhARR12 (with stop codon), respectively, were assembled with SpeI and SalI-linearized p5 vector (Zeng et al., 2019). For the pro35S::GhARR1::YFP, pro35S::GhARR2a::YFP, and pro35S::GhARR2b::YFP constructs, the coding regions of GhARR1, GhARR2a, and GhARR2b (without stop codon), respectively, were fused to the 5′ terminal region of YFP (with stop codon) and assembled with SpeI and EcoRI-linearized p5 vector. For the pro35S::YFP::GhARR11 and pro35S::YFP::GhARR12 constructs, the coding regions of GhARR11 and GhARR12 (with stop codon), respectively, were fused to the 3′ terminal region of YFP (without stop codon) and assembled with SpeI and EcoRI-linearized p5 vector.
RNA extraction and real-time quantitative PCR
Total RNA was extracted using the EASY spin plant RNA extraction kit (Aidlab Biotech, China). Approximately 1 µg RNA was transcribed into first-strand cDNA using the NovoScript Plus All-in-one First Strand cDNA Synthesis SuperMix (gDNA Purge, Novoprotein, China). The real-time quantitative PCR (RT-qPCR) assays were performed on a CFX Connect Real-Time System (Bio-Rad Laboratories) with 2×NovoStart SYBR qPCR SuperMix plus (Novoprotein, China).

Sequence retrieval, phylogenetic analysis, and sequence alignment
The amino acid sequences of AtCKXs and B-type AtARRs were obtained from the Arabidopsis genome databases (https://www.Arabidopsis.org/, accessed on 30 December 2019). The GhCKX and B-type GhARR homologs were identified using the BLASTP tool with default parameters in the CottonFGD database (https://cottonfgd.org/, accessed on 30 December 2019) using AtCKX and B-type AtARR sequences, respectively. The cutoff values were 0 for the sequence retrieval of GhCKXs and B-type GhARRs.

In situ hybridization
The linearized DNA template of the gene-specific GhCKX3b probe was amplified directly from a vector carrying the coding sequence of GhCKX3b. In situ hybridization of GhCKX3b mRNA was performed following the method described in Zhang et al. (2017). The sections incubated with the sense RNA probe served as the negative control. Images were captured on a microscope (CKX41, Olympus, Japan).

Immunohistochemical localization of cytokinins
Immunolocalization of cytokinins was performed following the method described by Zhang et al. (2017) with some modifications. Briefly, sections (10 µm) of cotton bud at the pinhead square stage were incubated with antibody (against trans-zeatin riboside (iZR) and trans-zeatin (iZ), Agrisera, Sweden) and then the signal was detected by using DyLight 550-labeled secondary antibody (Abcam, UK), and visualized on a laser-scanning confocal microscope (SP8, Leica, Germany). Sections incubated without the primary antibody served as the negative control.

Transient expression
Four-week-old leaves of Nicotiana benthamiana were used for transient expression. Agrobacterium tumefaciens strain GV3101 containing a plant expression vector was cultured overnight at 28 °C to OD600 of 1.0. The pelleted cells were resuspended and diluted with infiltration buffer (Chen et al., 2021) to OD600 of 0.01–0.05. The infiltrated leaves were used for analysis 3 d later.

Microscopic observations
GUS-stained samples were observed using a stereo-microscope imaging system (SteREO Discovery V20, Zeiss, Germany). Fiber initiation was observed on an S-3400N scanning electron microscope (Hitachi, Japan) as described previously by Zeng et al. (2019). The protein subcellular localization and cytokinin immunolocalization was observed on a laser-scanning confocal microscope (SP8, Leica, Germany). The fluorescence signal was detected by an HyD detector under a ×40 oil immersion objective lens. The imaging condition was set up manually: DyLight 550 (excitation: 552 nm, emission: 560–600 nm) and YFP (excitation: 514 nm, emission: 520–560 nm). The intensity of fluorescence signal was quantified using software Leica Application Suite X.

Histochemical staining and quantification of β-glucuronidase activity
Histochemical staining of β-glucuronidase (GUS) was performed as previously described by Jefferson et al. (1987). Briefly, detached or hand-sectioned tissues were immediately immersed in the staining solution (Zeng et al., 2019) and then placed in the dark at 37 °C for 12 h. The stained samples were bleached and fixed in 75% ethanol before photographing. Images were captured on a stereo-microscope imaging system (SteREO Discovery V20, Zeiss, Germany). Fluorometric assays of GUS activity in vegetative and reproductive organs of pro35S::GUS transgenic cotton were performed as described by Hou et al. (2008). Each sample was ground in liquid nitrogen. Protein estimation was performed using the method of Bradford (1976). GUS activity was calculated as pmol 4-methylumbelliferone (4-MU) per minute per microgram protein and each test was represented by three biological replicates.
Gold by the LiAc-mediated method. The pGADT7 prey vector was transformed into Y1H Gold cells harboring the Bait-pAbAi. Transformants were screened in the synthetic dextrose medium (SDM) containing 100 or 200 ng ml$^{-1}$ aureobasidin A (AbA) and lacking Ura and Leu (SDM/+AbA/~Leu). The yeast growth was captured after 5 d inoculation at 30 °C.

**Dual-luciferase reporter assay**

A dual-luciferase reporter assay was performed as previously described by Hellens et al. (2005) and Yan et al. (2018). Briefly, the promoter fragments were amplified and ligated into the pGreenII 0800-LUC vector to produce firefly luciferase (LUC) reporters, and the coding regions of GhARRs were amplified and ligated into the pLGN vector to produce effectors. The reporters and effectors were co-infiltrated into 4-week-old leaves of N. benthamiana as described in ‘Transient expression’. The effector expressing pro35S::YFP served as internal control. After 3 d of growth at 25 °C, 10 μM tZ was infiltrated into the leaves 5 h before sampling. The leaves were infiltrated with the same volume of dimethyl sulfoxide (DMSO) as the negative control (0 μM tZ). The Dual-Glo Luciferase Assay System (Promega, USA) was employed to measure the LUC activity, which was calculated based on the ratio of LUC/Renilla luciferase (REN). Gene specific primers are listed in Supplementary Table S1.

**Statistical analysis**

Statistical analysis was performed with Student’s $t$-test or one-way ANOVA followed by Tukey multiple comparisons test ($P<0.05$). Each experiment comprised at least three replicates. The intensity of fluorescence signal was calculated using Leica Application Suite X software. Standard errors and standard deviations were calculated using Microsoft Excel (2016) and IBM SPSS Statistics (version 19).

**Results**

**GhCKX3b is preferentially expressed in carpels**

By detecting GUS activity, we estimated cytokinin activities in vegetative and reproductive organs of cotton expressing the cytokinin signaling reporter proTCS::GUS (Müller and Sheen, 2008; Zeng et al., 2019). The maximum GUS activity was discernible in the flower bud at the pinhead square stage (approximately −21 DPA) when the ovule formation begins (Fig. 1A, B), suggesting that a high activity of cytokinins is required for ovule morphogenesis.

**Fig. 1.** Cytokinin activities and GhCKX3b transcription levels in different cotton tissues. (A, B) The endogenous cytokinin activities indicated by GUS activity (A) and GUS staining (B) of the proTCS::GUS reporter system in cotton tissues. GUS activities were measured by methylumbelliferyl glucuronide assay in proTCS::GUS transgenic cotton roots (10 d after sowing; DAS), stems (the third internode from the apex at 110 DAS cotton plants), leaves (the third leaf from the apex), flower buds (approximately −21 DPA), petals, stamens, pistils, and ovules at anthesis. Wild-type flower buds were used as the negative control. Error bars indicate standard deviation (SD) of three biological repeats. Buds at approximately −21 DPA were used for GUS staining. (C, D) The GhCKX3b transcription levels detected by RT-qPCR (C) and mRNA in situ hybridization (D) in wild-type roots (10 DAS), stems (the third internode from the apex at 110 DAS cotton plants), leaves (the third leaf from the apex), carpels (ovaries), bracts, sepals petals, and stamens at approximately −21 DPA. GhHis3 and GhUbiquitin served as internal control. Error bars indicate SD of three replicates. Bud sections of 10 μm at approximately −21 DPA were used for in situ hybridization with gene-specific probe GhCKX3b. The sections incubated with sense RNA probe served as the negative control. The lower panels in (D) show the enlarged image of carpels (ovaries). Scale bars=500 μm. Car, carpel; Op, ovule primordia; Pe, petal; Sep, sepal; Sta, stamen.
Fig. 2. Molecular and phenotypic identification of proAGIP::GhCKX3b-RNAi transgenic cotton. (A) GhCKX3b transcription levels in cotton carpels (ovaries) at the pinhead square stage (approximately −21 DPA) of T₀ proAGIP::GhCKX3b-RNAi transgenic lines and wild type. GhHis3 and GhUbiquitin served as internal control. Error bars indicate SD of three replicates. (B) Southern blot analysis of T₁ proAGIP::GhCKX3b-RNAi transgenic lines ACR1 and ACR5. Red arrows indicate the positive hybridization signal. (C) Phenotypes of 120 DAS cotton plants grown in the field. Control, non-transgenic segregated line of proAGIP::GhCKX3b-RNAi transgenic cotton; 35SCR13 and 35SCR17, lines #13 and #17 of pro35S::GhCKX3b-RNAi transgenic cotton; scale bar=10 cm. (D) Fiber initiation on 0 DPA ovules; scale bar=50 μm. (E) The phenotype of 30 DPA cotton bolls; scale bar=1 cm.
To identify cotton CKXs, which are the key negative regulators of cytokinins in plants (Schmülling et al., 2003), we screened the genomic database of Gossypium hirsutum (https://cottonrgd.org/). Twenty-seven GhCKX homologous genes to Arabidopsis AtCKXs (https://www.arabidopsis.org/) were identified (Supplementary Fig. S1). Among them, GhCKX3b, GhCKX3c, GhCKX5a, and GhCKX6b were expressed preferentially in the carpel and stamen (Fig. 1C; Supplementary Fig. S2). Our previous study demonstrated that GhCKX3b is a functional cytokinin oxidase (previously named GhCKX; Zeng et al., 2012; Zhao et al., 2015). In situ mRNA hybridization confirmed a strong signal of GhCKX3b transcript in the carpel at the place where ovules formed (Fig. 1D).

**Specific down-regulation of GhCKX3b increases CK levels in carpel**

Down-regulation of CKXs could significantly increase cytokinin levels in transgenic plants (Ashikari et al., 2005; Zalewski et al., 2012; Yeh et al., 2015; Holubová et al., 2018). However, constitutional down-regulation of CKXs in cotton usually resulted in cytokinin overproduction phenotypes, including dwarfism, sterility, and shortened root (Fig. 2C; Zhao et al., 2015).

To avoid the side effects on plant growth from the global overproduction of cytokinins, while increasing the seed number through cytokinin manipulation, we decided to increase the cytokinin content spatiotemporally in carpels. To this end, we used *proAGIP*, a chimeric carpel- and stamen-specific promoter (Busch et al., 1999; Deyholos and Sieburth, 2000), to direct the transcription of a GhCKX3b-RNAi sequence (Supplementary Fig. S3). The GUS pattern in *proAGIP::GUS* transgenic tobacco (*Nicotiana tabacum*) confirmed the carpel-specificity of *proAGIP* promoter (Supplementary Fig. S4; Deyholos and Sieburth, 2000; Wang et al., 2008).

No discernible phenotypic alteration in plant growth and development was observed in transgenic cotton holding *proAGIP::GhCKX3b-RNAi* (ACR) (Fig. 2C–E). RT-qPCR showed that the transcriptional level of *GhCKX3b* in the carpel of transgenic lines was obviously reduced. Two transgenic lines, ACR1 and ACR5, in which *GhCKX3b* was largely down-regulated, were selected for further study (Figs 2A, B, 3).

Then, we performed LC-MS/MS to detect the content of six primarily active cytokinins, namely tZ, tZR, N6-isopentenyladenine, N6-isopentenyladenosine, dihydrozeatin, and dihydrozeatin riboside, in carpels of cotton. Besides a non-transgenic negative control, a transgenic *pro35S::GhCKX* RNAi line, 35SCR6, which showed a moderate increase of cytokinins with normal growth, and had the best yield performance among the *pro35S::GhCKX-RNAi* cottons (Zhao et al., 2015), was used as a positive control. In carpels of ACR1 and ACR5 transgenic cotton, the total cytokinins were 29.53 ± 1.46 ng g⁻¹ and 27.46 ± 1.63 ng g⁻¹, respectively, significantly higher than in the 35SCR6 line (23.46 ± 1.81 ng g⁻¹) and the non-transgenic control (15.05 ± 1.73 ng g⁻¹) (Table 1), indicating a carpel-specific increase of cytokinins by *proAGIP::GhCKX3b-RNAi*. Immunolocalization assays supported that tZ and tZR signals, which made up the majority (85.0%) of the active CKs (Table 1), mainly appeared at the position where the ovule initiated, and the signals from ACR1 and ACR5 were visibly stronger than those in 35SCR6 and the control (Fig. 4).

**Table 1.** Active CK content in carpels during cotton ovule initiation (ng g⁻¹ FW)

| Line     | IZ   | tZR  | iP   | iPR  | DZ   | DZR  | Total CKs |
|----------|------|------|------|------|------|------|-----------|
| Control  | 5.17 | 7.63 | 0.47 | 1.50 | 0.19 | 0.09 | 15.05     |
| ACR1     | 10.04| 15.62| 0.90 | 2.51 | 0.28 | 0.19 | 29.53     |
| ACR5     | 10.68| 14.23| 0.56 | 1.53 | 0.28 | 0.17 | 27.46     |
| 35SCR6   | 8.57 | 12.20| 0.76 | 1.58 | 0.21 | 0.14 | 23.46     |

Sampled carpels (ovaries) from buds at the pinhead square stage (approximately −21 DPA). Total CKs, sum of contents of IZ (trans-zeatin), tZ (tZ riboside), iP (N6-isopentenyladenine), iPR (N6-isopentenyladenosine), DZ (dihydrozeatin), and DZR (DZ riboside). Data are presented as means ±SD (n=3). Within each column, means that are followed different letters are significantly different at P<0.05 by one-way ANOVA with a Tukey multiple comparisons test. FW, fresh weight; Control, non-transgenic segregated line of *proAGIP::GhCKX3b-RNAi* transgenic cotton; ACR, *proAGIP::GhCKX3b-RNAi* transgenic cotton; 35SCR6, line #6 of *pro35S::GhCKX3b-RNAi* transgenic cotton.
Increase of cytokinin promotes the expression of ovule initiation-related genes

It has been reported that the expression of genes of the AG subfamily, such as those for Seedstick (STK), Shatterproof (SHP), and Agamous (AG), was able to promote the formation of carpels and ovules (Favaro et al., 2003; Pinyopich et al., 2003; Xu et al., 2004; Guo et al., 2007; Liu et al., 2009, 2010; de Moura et al., 2017; Nardeli et al., 2018). In line with previous reports, AG subfamily genes, including *GhSTK1*, *GhSTK2*, *GhSHP*, and *GhAG1* were up-regulated in the carpel of the ACR1 and ACR5 lines (Fig. 5A; Supplementary Table S5). To confirm these results, we treated 0 DPA wild-type ovules with 50 μM tZ, an active cytokinin, for 12 h. The exposure resulted in a noticeable increase in the expression of AG subfamily genes (Fig. 5B), confirming the promotive effect of the cytokinin on the expression of these genes.

Cytokinins regulate the expression of downstream signal transduction genes by activating B-type ARR genes (Hwang and Sheen, 2001; Meng et al., 2017). To identify B-type ARR genes that are involved in cotton carpel development, we conducted a comparative phylogenetic analysis using data from Arabidopsis (https://www.arabidopsis.org/) and Gossypium hirsutum (https://cottonfgd.org/) (D’Agostino et al., 2000; Sakai et al., 2000; Mason et al., 2004, 2005; Zubo and Schaller, 2020). Five carpel expressive GhARRs, i.e. GhARR1, GhARR2a, GhARR2b, GhARR11, and GhARR12, which predominantly localize to the nuclei of *N. benthamiana* pavement cells, were identified (Supplementary Fig. S5). Dual-luciferase reporter and Y1H assays indicated that GhARR1 could bind directly to the promoter sequences of the AG subfamily genes *GhSTK2* and *GhAG1*, GhARR2a to the promoter of *GhAG1*, and GhARR12 to the promoter of *GhSHP* (Fig. 5E; Supplementary Fig. S6). This binding could activate the transcription of these AG subfamily genes (Fig. 5C–D), thereby promoting the expression of AG downstream genes.
Fig. 5. Increase of cytokinin promoted the expression of ovule initiation-related genes. (A) Comparison of the relative expression levels of AG subfamily genes in T2 ACR1 and ACR5 transgenic lines with those of the T2 35SCR6 and the non-transgenic control. Total RNAs were extracted from cotton carpels at the pinhead square stage (approximately −21 DPA). The relative transcription levels were determined by RT-qPCR. GhHis3 and GhUbiquitin served as internal controls. Error bars indicate SD of three replicates. Control, non-transgenic segregated line of proAGIP::GhCKX3b-RNAi transgenic cotton; ACR, proAGIP::GhCKX3b-RNAi transgenic cotton; 35SCR6, line #6 of pro35S::GhCKX3b-RNAi transgenic cotton. (B) The relative transcription
levels of AG subfamily genes in tZ-treated ovules and the control. Total RNA was extracted from 0 DPA ovules treated with 50 μM tZ or the same volume of DMSO for the negative control, for 12 h. (C) Schematic representation of the constructs used in the dual-luciferase assay. (D) The promoters of AG subfamily genes involved in ovule initiation were activated under the expression of GhARRs and treatment with 10 μM tZ via a dual-luciferase assay. The effector expressing pro35S::YFP served as internal control. The leaves were infiltrated with 10 μM tZ or the same volume of DMSO for the negative control (0 μM tZ). The transactivation activity of AG subfamily gene promoters by GhARRs was calculated based on the ratio LUC/REN. Error bars indicate SD of three biological replicates. Asterisks represent significant difference (versus internal control, or 0 μM tZ). The transactivation activity of AG subfamily gene promoters by GhARRs was calculated based on the ratio LUC/REN. Error bars indicate SD of three biological replicates. Asterisks represent significant difference (versus internal control, or 0 μM tZ).

Discussion

The promotive effect of cytokinins on seed yield was first observed by exogenous application of cytokinins in various crops (Dyer et al., 1987; Atkins and Pigeaire, 1993; Sawan et al., 2000; Zuniga-Mayo et al., 2018), followed by genetic expression of cytokinin biosynthetic genes, such as the gene for isopentenyltransferase, to endogenously increase the cytokinin levels in transgenic plants (Ma et al., 2008; Atkins et al., 2011; Peleg et al., 2011). CKX, which inactivates cytokinin irreversibly in plant cells, has been identified as a key negative regulator of cytokinin content in monocots and dicots (Jones and Schreiber, 1997; Schmülling et al., 2003; Kowalska et al., 2010; Zeng et al., 2012; Zhao et al., 2015; Ogonowska et al., 2019). Accumulating evidence has demonstrated that CKX is a key regulator for seed number, and down-regulation of CKXs is an effective strategy for yield improvement of seed crops (Zalewski et al., 2010; Bartrina et al., 2011; Li et al., 2013; Yeh et al., 2015; Schwarz et al., 2020). In our previous study, we generated cytokinin-enhanced transgenic cotton in which a CKX gene was constitutively down-regulated. We found that some transgenic cottons displayed cytokinin overproduction-related aberrations, including dwarf growth, shortened root, and sterility. Only a moderate increase of cytokinin could show a positive effect on cotton yield (Zhao et al., 2015). In this study, we revealed that GhCKX3b, GhCKX3c, GhCKX5a, and GhCKX6b were expressed preferentially in the carpel and stamen (Fig. 1C; Supplementary Fig. S2), suggesting a possible function of these genes in carpel development. We thus designed a GhCKX3b-RNAi sequence, which could interfere with the expression of GhCKX3b, GhCKX3c, GhCKX5a, and GhCKX6b (Figs 3, 5A; Supplementary Fig. S3; Supplementary Table S5). The cytokinin content in the carpels of transgenic proAGIP::GhCKX3b-RNAi cotton lines was significantly higher not only over the wild-type control but also over the positive control pro35S::GhCKX3b-RNAi carpels (Table 1; Fig. 4). Compared with pro35S::GhCKX3b-RNAi cotton, of which some exhibited abnormal phenotypes, all proAGIP::GhCKX3b-RNAi cotton lines grew well without any

Carpel-specific down-regulation of GhCKXs significantly increases seed and fiber yield

By counting, we found that the number of ovules per locule (0 DPA) of ACR1 and ACR5 was significantly higher than that of the non-transgenic control as well as the constitutively down-regulated 35SCR6 line (Fig. 6; Supplementary Fig. S7). The average number of ovules per locule of the ACR1 and ACR5 lines was 9.6 ± 0.5 and 9.3 ± 0.4, respectively, increasing to 21.5% and 17.7%, respectively, over that of the non-transgenic control (7.9 ± 0.6) and 11.6% and 8.1%, respectively, increasing to 21.5% and 17.7%, respectively, over that of the non-transgenic control (7.9 ± 0.6), and 11.6% and 8.1%, respectively, increasing to 21.5% and 17.7%, respectively, over that of the non-transgenic control as well as the constitutively down-regulated 35SCR6 line (8.6 ± 0.4). The seed number per boll of ACR1 and ACR5 lines was 9.6 ± 0.5 and 9.3 ± 0.4, respectively, increasing to 35SCR6 line (8.6 ± 0.4). The seed number per boll of ACR1 and ACR5 lines was 9.6 ± 0.5 and 9.3 ± 0.4, respectively, increasing to 35SCR6 line (8.6 ± 0.4). The seed number per boll of ACR1 and ACR5 lines was 9.6 ± 0.5 and 9.3 ± 0.4, respectively, increasing to 35SCR6 line (8.6 ± 0.4). The seed number per boll of ACR1 and ACR5 lines was 9.6 ± 0.5 and 9.3 ± 0.4, respectively, increasing to 35SCR6 line (8.6 ± 0.4).

For ACR1 locules, the majority (59.2%) contained 10 or 11 ovules in each locule. In contrast, for the non-transgenic cotton, 4.3% of locules had 10 ovules, and none had 11 ovules. For the 35SCR6 cotton, 4.3% of locules had 10 ovules, and none had 11 ovules. The increase of ovules per locule of ACR cotton was also constant during the flower stage of cotton development (Supplementary Fig. S8).

More ovules per locule usually means more seeds per boll. To assess the agronomic performance of the proAGIP::GhCKX3b-RNAi transgenic cotton, we conducted field trials at the experimental farm located in Chongqing, China (29°49′N, 106°24′E) in 2018 and 2019. The consecutive two-year field experiments showed a significant increase of seeds per boll in ACR lines (Table 2). The seed number per boll of ACR1 and ACR5 was 31.1 ± 0.7 and 29.8 ± 0.6, respectively, significantly higher than that of 35SCR6 (26.1 ± 0.9) and the control (24.8 ± 0.2). Consequently, the seed yield of ACR1 and ACR5 increased 22.4% and 26.5%, respectively, as compared with that of the control. The increased seeds resulted in a significant increase of lint yield. The lint yield of ACR1 and ACR5 was increased by 32.3% and 25.8%, respectively, compared with the control. The seed yield of the ACR1 line was lower (but not statistically significant) than that of 35SCR6 cotton, due to a slight decrease in seed size (indicated by seed index, the weight in grams of 100 seeds). Nevertheless, the lint yield of the ACR1 line was still significantly higher than that of the 35SCR6 line (Table 2), with the advantage of more seeds per boll. The fiber quality, fiber length, and fiber strength of ACR1 were lower than those of the control; but the values of ACR5 were not significantly different from those of the control (Supplementary Table S6; Supplementary Fig. S9).
cytokinin overproduction-related aberrations. More importantly, the fiber yield of the two transgenic proAGIP::GhCKX3b-RNAi cotton lines was significantly higher than that of both wild-type control and the pro35S::GhCKX3b-RNAi line (Table 2), indicating an advantage of the tissue-specific regulation strategy over the constitutive one.
Cotton plants were grown at the experimental farm located in Chongqing, China (29°49′11″N, 106°24′43″E). The experiment was designed as a randomized comparative trial with three replicates. Plots were arranged randomly with an area of 18 m² (4 m x 4.5 m). Results are presented as means ±SD (n=3). Within each column, means that are followed by different letters are significantly different at <0.05 by one-way ANOVA with a Tukey multiple comparisons test. Control, non-transgenic segregated line of proAG1P::GhCKX3b-RNAi transgenic cotton; ACR, proAGIP::GhCKX3b-RNAi transgenic cotton; 35SCR6, line #6 of pro3SS::GhCKX3b-RNAi transgenic cotton.

**Fig. 7.** A model showing the promotive effect of carpel-specific enhancement of cytokinins (CKs) on cotton yield. The carpel-specific down-regulation of GhCKXs results in an increase of CK levels at the place where ovules initiate. The increased cytokinin content stimulates the activation of B-type ARR (Hwang and Sheen, 2001; Meng et al., 2017), which in turn promotes the transcription of AG subfamily genes (e.g. GhAG1, GhAG1, and GhSHP) by binding the upstream region of the genes. The up-regulation of these AG subfamily genes boosts carpel differentiation and ovule formation (Favaro et al., 2003; Pinyopich et al., 2003; Xu et al., 2004; Guo et al., 2007; Liu et al., 2009, 2010; de Moura et al., 2017; Nardeli et al., 2018). This promotion of ovule formation results in production of more seeds in a boll, thus increasing the yield of both seeds and fibers of cotton.

Cytokinins regulate growth and development of plants through the cytokinin–ARR signaling pathway (Hwang and Sheen, 2001; Meng et al., 2017). Among ARR–activated genes, AG subfamily genes STK2, AG1, and SHP are positive regulators of ovule identity and initiation (Favaro et al., 2003; Pinyopich et al., 2003; Xu et al., 2004; Guo et al., 2007; Liu et al., 2009, 2010; de Moura et al., 2017; Nardeli et al., 2018). Our transcriptome and RT-qPCR data showed that the expression of AG subfamily genes was obviously increased in the ACR transgenic cotton carpels at the place where ovules formed (Fig. 5A; Supplementary Table S5). Our Y1H and dual-luciferase assays further revealed that GhARR1, GhARR2a, and GhARR11 could directly bind to the upstream sequences of GhAG1 and GhSTK2, GhAG1, and GhSHP, respectively, and in turn activate the transcription of these AG subfamily genes (Fig. 5C–E; Supplementary Fig. S6). The enhanced expression of these AG subfamily genes could in turn promote carpel development and ovule formation, thus increasing the yield of seeds. The mechanism for increasing cotton yield by the carpel-specific enhancement of cytokinins is summarized in Fig. 7.

Fiber length, strength, and fineness are the three major traits determining the quality and economic value of cotton (Han et al., 2013; Long et al., 2018). It has been reported that a high concentration of kinetin (>5 µM), a type of cytokinin, inhibits fiber elongation, whereas a low concentration (<0.5 µM) stimulates fiber elongation (Beasley and Ting, 1974; Yu et al., 2000b). In addition, fiber elongation was inhibited in transgenic cotton expressing cytokinin biosynthesis isopen-tenytransferase gene, ipt, under the control of seed-specific promoter Ph/P (Yu et al., 2000a). Our previous study demonstrated that constitutive overexpression of GhCKX-RNAi had little negative effect on fiber quality, such as length, strength, and fineness (Zhao et al., 2015). In this current study, we found that the fiber length and fiber strength of the ACR.1 transgenic line was significantly decreased, whereas
there was no significant alteration in ACR.5. We noticed that the seed number per boll of ACR.1 (31.1 ± 0.7) was higher than that of ACR.5 (29.8 ± 0.6), while its seed size (indicated by the seed index) declined (Table 2). One explanation for the deterioration in quality of transgenic ACR.1 fibers is that more seeds per boll means a lower nutritional share, which may reduce the quality of the fibers. The impact of manipulation of cytokinins on fiber quality awaits further investigation.

Taken together, our data indicate that without any hindrance of plant development, carpel-specific up-regulation of endogenous cytokinins by down-regulation of CKX is a feasible and effective strategy for seed yield improvement, not only for cotton but perhaps also for other dicotyledons, such as canola and soybean.

**Supplementary data**

The following supplementary data are available at *JXB* online.

- Fig. S1. Phylogenetic analysis of CKX proteins.
- Fig. S2. Transcription levels of *GhCKXs* in different wild-type upland cotton tissues.
- Fig. S3. Sequence alignment of partial *GhCKXs* which were preferentially expressed in the carpel.
- Fig. S4. The activity pattern of *proAGIP* in *Nicotiana tabacum*.
- Fig. S5. Expression patterns and nuclear localization of GhARRs.
- Fig. S6. Diagram of ARR binding elements in the AG sub-family gene promoters.
- Fig. S7. Comparison of ovule number per 0 DPA locule or boll between T0 *proAGIP*: *GhCKX3b*-RNAi transgenic cottons and wild type.
- Fig. S8. Comparison of ovule number per 0 DPA locule or boll between T2 *proAGIP*: *GhCKX3b*-RNAi transgenic cotton and the non-transgenic segregated line at different growth temperatures.
- Fig. S9. Phenotypes of *proAGIP*: *GhCKX3b*-RNAi transgenic cotton bolls and seeds.
- Table S1. Primer and fragment information for plasmid construction.
- Table S2. Primers pairs used for RT-qPCR assay.
- Table S3. MIQE checklist.
- Table S4. Analysis parameters for CKs using LC-MS/MS.
- Table S5. Transcriptomic analysis of transcripts that were significantly changed between *proAGIP*: *GhCKX3b*-RNAi transgenic cotton and the non-transgenic segregated line.
- Table S6. Comparison of mature fiber qualities of *proAGIP*: *GhCKX3b*-RNAi transgenic cottons and the non-transgenic segregated line in field trial.

**Author contributions**

YP and JZ conceived the experiments and wrote the manuscript; JZ, XY, WB, YC, JZ, XD, RL, FW, HR, JZ, BD, HL, MZ, XL, LH, and YX performed the experiments and analysed the data; YP, JZ, YX, XY, and MZ contributed to the interpretation of the results. All authors reviewed and approved the manuscript.

**Conflict of interest**

The authors declare no conflicts of interest.

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**Data availability**

The original contributions presented in the study are included in the article and its supplementary data, and further enquiries can be directed to the corresponding author.

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