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Decreased active cytokinin levels inhibited the cytokinin signaling pathways to increase the lateral root number in mepiquat chloride-treated cotton (*Gossypium hirsutum* L.) seedlings

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Abstract

Background Mepiquat chloride (MC) is a widely used plant growth regulator in cotton (*Gossypium hirsutum* L.). It regulates endogenous hormone content and crosstalk to control plant height and promote lateral root (LR) development. However, the roles of cytokinins (CTKs) in the MC-induced increase in LR number in cotton seedlings remain unclear. Therefore, in this study, whole-genome transcriptome analysis was performed to elucidate the molecular mechanisms, CTK transformation, and CTK signaling pathway response to MC in cotton roots.

Results In the present study, MC reduced the contents of the active CTK *trans*-zeatin (tZ) and N⁶-isopentenyladenine (iP) but increased the levels of the nucleoside CTK *trans*-zeatin riboside (tZR) and N⁶-isopentenyladenine riboside (iPR). RNA-seq data showed that the CTK biosynthesis genes *GhIPTs* and active CTK catabolism genes *GhCKXs* were obviously upregulated after MC treatment. The CTK-activating enzyme gene *GhLOGs* was repressed compared with the control. Furthermore, MC inhibited the expression of *GhAHK4* and *GhARR2/12*, which are involved in the CTK signaling pathway, and activated the IAA-IAA14-ARF7/19 signaling module. Meanwhile, MC increased the expression levels of genes involved in sucrose synthesis, the cell cycle, cell division, and cell wall biosynthesis pathways. Silencing the *GhCKX* family separately decreased the LR number and active indole-3-acetic acid (IAA) level. The expression levels of *GhPIN1*, *GhARF7*, *GhARF19*, *GhLBD16*, *GhLBD18*, *GhLBD29*, and *GhLBD33* were downregulated, but *GhARR2/12* and *GhIAA14* were upregulated. The total content of active CTKs was noticeably increased. The results of silencing the *GhLOGs* family were opposite to those of silencing *GhCKXs*. Silencing *GhARR12* could upregulate *GhPIN1* expression and increase LR number. In addition, the silenced *GhCKXs*, *GhLOGs*, and *GhARR12* were less responsive to MC-induced LR growth than the control.

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Conclusion These results suggested that MC treatment could upregulate CTK-nucleoside biosynthesis and CTK metabolism genes to decrease active CTK levels, promoting crosstalk between CTKs and auxin signaling pathways to enhance LR initiation.

Keywords Auxin, Cytokinins, *Gossypium hirsutum* L., Mepiquat chloride, RNA-seq, Seed soak *Agroinoculation*-Virus-induced gene silencing (VIGS)

Introduction

Mepiquat chloride (MC; N,N-dimethylpiperidinium chloride) is a synthetic plant growth regulator that has been used worldwide in cotton production (Reddy et al. 1996). MC efficiently reduced plant height and vegetative growth, increased the number of lateral roots (LRs), and improved resistance to low temperatures and drought (Zhao et al. 2000; Kerby 1985; York 1983; Duan et al. 2002; Xu et al. 1992; Wang et al. 2014).

MC could significantly increase endogenous indole-3-acetic acid (IAA) levels in cotton seedling roots, advance the development of lateral root primordia (LRPs) earlier than 2 days, and promote the number and length of the total LRs (Chen 2017; Chen et al. 2018). Furthermore, the contents of nucleoside cytokinins (CTKs) were significantly increased while the levels of active free base CTKs were significantly decreased in MC-treated cotton seedling roots (Gou et al. 2010; Zhang et al. 2022). LR growth is regulated antagonistically by the plant hormones auxin and CTKs (Malamy et al. 1997; Woodward et al. 2005; Casimiro et al. 2001; Laplace et al. 2008). Hence, we presumed that the number of LRs increased by MC may be related to the changes in IAA and free base CTK levels.

Auxin regulates LR growth by targeting the degradation of auxin/indole-3-acetic acid (Aux/IAA) proteins such as SOLITARY-ROOT (SLR)/IAA14 (Fukaki et al. 2002), which can derepress the transcription factors AUXIN RESPONSE FACTOR7 (ARF7) and AUXIN RESPONSE FACTOR19 (ARF19) (Fukaki et al. 2005). Subsequently, ARF7 and ARF19 activate downstream genes *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (*LBD16/ASL18*) and *LBD29/ASL16* expression to mediate LR patterning (Okushima et al. 2007).

CTKs mainly inhibit LR growth (Jing and Strader 2019). The reduced N⁶-isopentenyladenine (iP) and *trans*-zeatin (tZ) contents could increase the number of LRs and adventitious roots (Werner et al. 2001, 2003). The changes in different types of CTKs are regulated by a variety of enzymes involved in the CTK metabolism pathway. The first and rate-limiting enzyme is adenylate isopentenyl-transferase (IPT), which catalyzes dimethylallyl diphosphate (DMAPP) N-prenylation to produce iP riboside 5'-triphosphate (iPRTP), iP riboside 5'-diphosphate (iPRDP), or iP riboside 5'-monophosphate (iPRMP) in higher plants (Takei et al. 2001). Another enzyme is

cytochrome P450 monooxygenase (CYP735A), which utilizes iP-nucleotides to produce tZ-nucleotides (Takei et al. 2004). Furthermore, CTK nucleoside 5'-monophosphate phosphoribohydrolase (LOG), a CK-activating enzyme, participates in the final step of bioactive CTK synthesis and directly catalyzes CTK nucleotides to active nucleobases (Kurakawa et al. 2007; Kamada-Nobusada et al. 2009). CTK degradation is regulated by cytokinin oxidase/dehydrogenase (CKX) enzymes, which irreversibly degrade CTKs to produce adenine and unsaturated aldehyde 3-methyl-2-butenal (Johansson et al. 2002; Galuszka et al. 2000). Previous studies showed that MC treatment could significantly upregulate the expression levels of the auxin response factors *GhARF7* and *GhARF19* and the IAA amidohydrolase (IAHs) genes *GhILR1*, *GhILL6b*, *GhIAR3a*, and *GhIAR3b* (Chen et al. 2018). Nevertheless, how the genes of CTK synthesis and metabolism pathways are involved in the transformation of different types is not clear in the process of the MC-induced increase in LR number in cotton seedlings.

Virus-induced gene silencing (VIGS) is one of the simplest and most straightforward methods for inducing transient gene knockdown to rapidly verify functional target genes (Schachtsiek et al. 2019). In cotton, an *Agrobacterium*-mediated VIGS assay using tobacco rattle virus (TRV) vectors has been utilized in young seedlings and roots to effectively repress gene expression levels (Senthil-Kumar et al. 2014; Zhang et al. 2018).

This research aimed to study the role of CTKs in the MC-induced increase in LR number in cotton seedlings. Therefore, the expression patterns of genes encoding CTK metabolism during LR growth were determined by transcriptome sequencing. Subsequently, *GhLOG* genes, *GhCKX* genes, and *GhARR12* were silenced in early seedlings to verify the RNA-seq results.

Materials and methods

Plant materials and treatment for RNA-seq

Our previous results showed that the promoting effect of MC on LRP and LR numbers was greatest at 400 mg·L⁻¹ by evaluating these effects in cotton treated with 0–800 mg·L⁻¹ MC (Chen 2017). Hence, 400 mg·L⁻¹ MC solution was utilized for soaking cotton seeds in this study. The cotton seeds (cv. Xinkang 4) were soaked in sterile H₂O (control treatment) and 400 mg·L⁻¹ MC

solution, respectively, at 30 °C for 12 h. The MC standard (purity, 97.0%) was purchased from Hebei Guoxin Non-ong Biotechnology Co., Ltd. (Hejian, Hebei, China). Then, the seeds were incubated in rolls of germination paper (25 cm wide, 38 cm long, catalog number OP1015, Hoffman Manufacturing, Inc., Albany, OR, USA) at 25 °C and 70% relative humidity. The roots of seeds on germination paper were harvested at 12 h, 24 h, and 48 h, immediately weighed, frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA-seq analysis.

Sample extraction, purification, and quantification for IAA and CTKs

The extraction methods of IAA and CTKs were the same. Briefly, the frozen roots were homogenized to a powder in liquid nitrogen and weighed. Then, a 0.2 g (fresh weight) sample was added to 4 mL of 80% methanol for extraction at 4 °C overnight. The slurry was centrifuged at 11 610 g for 15 min at 4 °C; subsequently, the root residue was re-extracted with 1 mL of 80% methanol for 2 h under the same conditions. The supernatants were combined and evaporated under a stream of N₂, and the residue was redissolved in 1 mL of phosphate-buffered saline (PBS) buffer.

The active CTKs tZ and iP and the nucleoside CTKs *trans*-zeatin-riboside (tZR) and N⁶-isopentenyladenine riboside (iPR) were purified and quantified by immunoaffinity columns (IAC, prepared in our laboratory) combined with ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC–ESI–MS/MS, Waters, Milford, MA, USA) (Zhang et al. 2022). The IAA concentration in cotton roots was detected by an ic-ELISA kit produced in our laboratory.

RNA extraction, quantitative real-time polymerase chain reaction (qRT-PCR) analysis, cDNA library construction, and Illumina sequencing

Total RNA was extracted from the roots with a Plant RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Subsequently, 2 µg of total RNA was treated with DNase I and used for cDNA synthesis with oligo (dT) primers and reverse transcriptase (Promega, USA).

qRT-PCR was carried out on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA); the reaction volume was 15 µL and contained 1.2 µL of diluted cDNA, 0.3 µL of ROX reference dye, 0.3 µL of each forward primer and reverse primer (each at 10 µmolL^{−1}), and 7.5 µL of SYBR Premier Ex Taq mix (Takara, Japan), with ddH₂O added to 15 µL. The *GhActin9* gene was used as an internal control. PCR amplification was performed using two-step cycling conditions of 95 °C for 30 s, followed by 40 cycles of 95 °C

for 5 s and 60 °C for 35 s. The primers used are listed in Supplementary Table 1. The levels of each gene transcript were calculated relative to those in corresponding untreated controls. Fold changes in RNA transcripts were calculated by the 2^{−ΔΔCt} method (Livak et al. 2001), and the values reported represent the average of three independent trials.

After qRT-PCR analysis, high-quality RNA samples were used for cDNA library preparation and sequencing. Six libraries were constructed and sequenced by using the Illumina HiSeq 2500 platform according to the Illumina standard procedure in Majorbio Bio-pharm Technology (Shanghai, China).

Data processing and de novo transcriptome assembly

The data were analyzed on the free online Majorbio Cloud Platform (<http://www.majorbio.com>). Raw reads were cleaned by removing adapter reads, ambiguous reads ('N' > 10%), low-quality sequences (Q20 < 20%), and unknown sequences of reads. Sequences with a length less than 50 bp were discarded after removing adaptors. Clean reads were then de novo assembled using SeqPrep (<https://github.com/jstjohn/SeqPrep>) with default parameters.

Differentially expressed gene (DEG) analysis, enrichment, and annotation analyses

The fragments per kilobase per million reads (FPKM) method was used to calculate the expression of each gene. The DESeq2 package was used to obtain the “base mean” value for identifying differentially expressed genes (DEGs). A false discovery rate (FDR) ≤ 0.05 and an absolute value of log₂ ratio ≥ 0.263 were set as the thresholds for defining the gene expression difference between the two samples as significant.

Gene ontology (GO) enrichment analysis of DEGs was performed by Goatools software. Fisher's exact test was used, and terms with an adjusted *P* value (*P* adjusted) < 0.01 and FDR < 0.05 were considered significantly enriched (Young et al. 2010). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was carried out for the genes/transcripts in the gene concentration using R software, and the calculation principle was the same as that of GO enrichment analysis. The KEGG pathways with *P* adjusted < 0.05 were regarded as significantly enriched.

Seed soak Agrobacterium VIGS (SSA-VIGS) procedure

To construct the pTRV-VIGS vectors, we mainly followed the method of Zhang et al. (2018). Briefly, pTRV2 was linearized with *EcoRI* and *KpnI*. Then, cDNA fragments of cotton *GhLOG3* (311 bp), *GhLOG7* (306 bp), *GhLOG8* (328 bp), *GhCKX1* (327 bp), *GhCKX3* (300 bp), *GhCKX5*

(336 bp), *GhCKX6* (319 bp), *GhCKX7* (318 bp), and *type-B ARABIDOPSIS RESPONSE REGULATOR12* (*GhARR12*, 322 bp) were cloned into linearized vector to construct recombinant vectors pTRV-GhLOG3, pTRV-GhLOG7, pTRV-GhLOG8, pTRV-GhCKX1, pTRV-GhCKX3, pTRV-GhCKX5, pTRV-GhCKX6, pTRV-GhCKX7, and pTRV-ARR12, respectively. All related primers are shown in Supplementary Table 1. Other vectors, pTRV-RNA1, pTRV-GhCLA1, and pTRV-GFP, were a gift from Engineering Research Center of Plant Growth Regulator, China Agricultural University. These recombinant vectors were transformed into *Agrobacterium tumefaciens* strain GV3101, cultivated, and resuspended as described by Zhang et al. (2018). The cells were incubated at 24 °C for 3 h after the OD_{600 nm} was diluted to 1.3 (Mu 2016). Subsequently, *Agrobacterium* cultures of pTRV2 recombinant vectors with pTRV1 were separately mixed in a 1:1 volume ratio.

Due to LR initiation being advanced to 2 d in MC-treated seedlings (Chen et al. 2018), CTK-related genes were silenced by soaking seeds in *Agroinoculation*. Hence, before LRP initiation, *Agrobacterium*-treated seedlings should be soaked in MC solution to verify the effect of MC. The cotton seed soak *Agroinoculation* procedure was performed as described by Zhang et al. (2018). The seeds were surface-sterilized and washed and then soaked in sterile ddH₂O at 30 °C for 12 h. After peeling the seed coats, the naked seeds were dipped into *Agrobacterium* suspensions at 20 °C for 90 min. Subsequently, these seeds were cultured in Murashige and Skoog (MS) liquid medium with 200 µM adenine sulfate (AS) at 24 °C in the dark for 2 d. To sift the soaking time and MC solution concentration of root soaking, two-day-old seedlings were soaked in 0, 10, 20, 40, and 80 mg·L⁻¹ MC solution for 6, 12, and 20 h, respectively. Then, the roots were washed with ddH₂O and cultured in Hoagland nutrient solution under a 14 h photoperiod, a 24/20 °C day/night temperature cycle, a 550 µmol·m⁻²·s⁻¹ light intensity, and 60% relative humidity. The number of

LRs was measured at 5 d after MC treatment. Under the optimal conditions of root soaking, twenty-four cotton seedling roots were treated in MC solution and ddH₂O and cultured as described above. Gene silencing efficiency was estimated by detecting the relative expression levels of *GhCKX1* at different times.

Statistic analysis

The data were analyzed by SPSS 25 (IBM, Armonk, NY, USA). The least significant difference (LSD) tests were used for mean comparisons and differences were considered significant at $P < 0.05$.

Results

MC increased nucleoside form CTK levels and decreased free base CTKs in cotton roots

To evaluate the effect of CTKs on LR growth under MC treatment, the content of CTKs was measured by IAC–UPLC–ESI–MS/MS at 12, 24, and 48 h after treatment. Compared with the CK treatment, MC reduced the contents of tZ and iP by 2.60%–32.26% and 12.50%–26.79%, respectively, while it increased the tZR and iPR levels by 10.61%–38.04% and 4.69%–51.26%, respectively (Table 1). In the sampling period, MC did not affect the primary root length and weight of seedlings (Table S2). These data revealed that MC may reduce free base CTKs to increase the LR number.

Sources of variability in RNA-seq data and functional annotation of the transcripts

To elucidate the molecular mechanisms of different types of CTK transformation under MC treatment, RNA-seq analysis of cotton roots at 12, 24, and 48 h after MC treatment was carried out. Six independent libraries were created with Illumina Hi-Seq sequencing results. After the quality control, 135.42 Gb of clean reads were obtained. Each sample reached more than 7.08 Gb of clean reads, and the percentage of Q30 bases exceeded 95.92%. A total of 81 091 unigenes were identified, and

Table 1 Contents of CTKs detected in cotton seedling roots

Sampling period	Treatment	Content/(ng·g ⁻¹ FW)			
		tZR	iPR	tZ	iP
12 h	CK	4.89 ± 0.01	1.19 ± 0.08	0.77 ± 0.02**	0.48 ± 0.01*
	MC	6.75 ± 0.02**	1.80 ± 0.17**	0.75 ± 0.01	0.42 ± 0.03
24 h	CK	1.79 ± 0.04	1.08 ± 0.11	0.62 ± 0.01	0.64 ± 0.01*
	MC	1.98 ± 0.08*	1.44 ± 0.16**	0.42 ± 0.03	0.51 ± 0.03
48 h	CK	0.17 ± 0.01	0.64 ± 0.02	0.19 ± 0.01*	0.56 ± 0.01**
	MC	0.22 ± 0.01**	0.67 ± 0.02	0.17 ± 0.01	0.41 ± 0.02

All measurements were performed in triplicate. * and ** indicates the level of significance at $P < 0.05$ and $P < 0.01$, respectively

approximately 73.0% of the identified transcripts had mapped reads between 200 and 1 800. Fifty-six percent of transcripts had more than 1 000 reads, and 26% had more than 1 800 mapped reads (Fig. S1). The unigenes were annotated by BLAST searches against public databases. In total, 70 158 unigenes were annotated, including 66 017 annotated in the cluster of orthologous groups of proteins (COG), 53 245 in GO, 28 908 in KEGG, 57 538 in Pfam, 54 769 in Swiss-Prot, and 70 119 in Non-Redundant Protein Sequence Database (NR) (Table S3).

Differential gene expression analysis

The total number of clean reads for all identified transcripts was used for differential expression analysis in DESeq with an FDR (P value) ≤ 0.05 and $|\log_2(\text{fold change})| \geq 0.263$. Then, we found 7 805 DEGs in roots by MC treatment. Compared with the control samples, 2 351, 1 435, and 4 019 DEGs were identified at 12, 24, and 48 h, respectively. Among these DEGs, 1 269, 812, and 2 037 were upregulated, and 1 082, 623, and 1 982 were downregulated. However, only four genes were activated at all three time points, and two genes were repressed (Fig. 1). To prove the reliability of the RNA-seq results, five CTK synthesis genes, *GhIPT2* (*Gh_A09G0887*), *GhIPT3* (*Gh_D11G1264*), *GhLOG1* (*Gh_A05G0902*), *GhLOG7* (*Gh_A05G0315*), and *GhLOG8* (*Gh_A05G0315*), were selected for real-time qRT-PCR by using the RNA pools used for RNA sequencing. The expression patterns of these genes were generally consistent with the RNA-seq data (Fig. S2), confirming the reliability of the transcriptomic data obtained by the RNA-seq analysis.

Annotation and enrichment analysis of the DEGs

To understand the DEGs in MC-treated roots, a total of 4 118 upregulated and 3 687 downregulated DEGs at three time points showed a similar classification pattern based on GO annotation analysis. The DEGs were classified into biological processes, cellular components, and molecular functions (Fig. S3A). Furthermore, the results of KEGG annotation analysis showed that the DEGs were mainly classified into metabolism, genetic and environmental information processing, cellular processes, and organismal systems (Fig. S3B-C).

Subsequently, GO and KEGG functional enrichment analyses were performed to identify the biological process and category of DEGs. GO enrichment analysis showed that the DEGs were significantly enriched in various biological processes. These genes were mainly involved in the cellular protein modification process (602), protein modification process (602), macromolecule modification (625), cell wall organization or biogenesis (100), external encapsulating structure organization (93), and cell wall organization (90) (Fig. 2A). Moreover, the KEGG pathway enrichment analysis revealed that these DEGs mainly clustered in ether lipid metabolism, glycerophospholipid metabolism, zeatin biosynthesis, and RNA transport (Fig. 2B).

DEGs involved in CTKs and auxin synthesis and metabolism

To understand the transformation of different types of CTK contents, the genes involved in the biosynthesis and metabolism of CTKs were analyzed and identified. The main CTK synthesis enzymes include IPT, CYP735A, and LOG. In comparison with the control, the expression

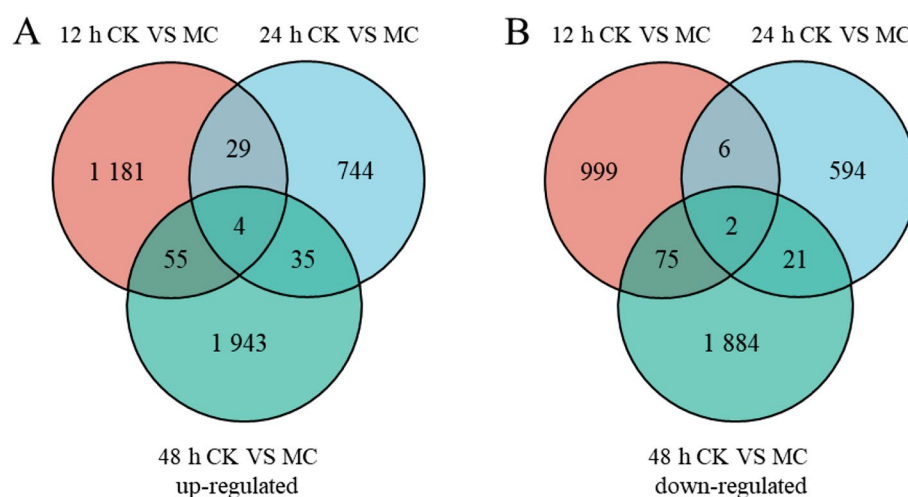


Fig. 1 Venn diagram of the dynamic transcriptome changed at different time points after treatment of cotton seedling root. **A** significantly up-regulated genes. **B** significantly down-regulated genes

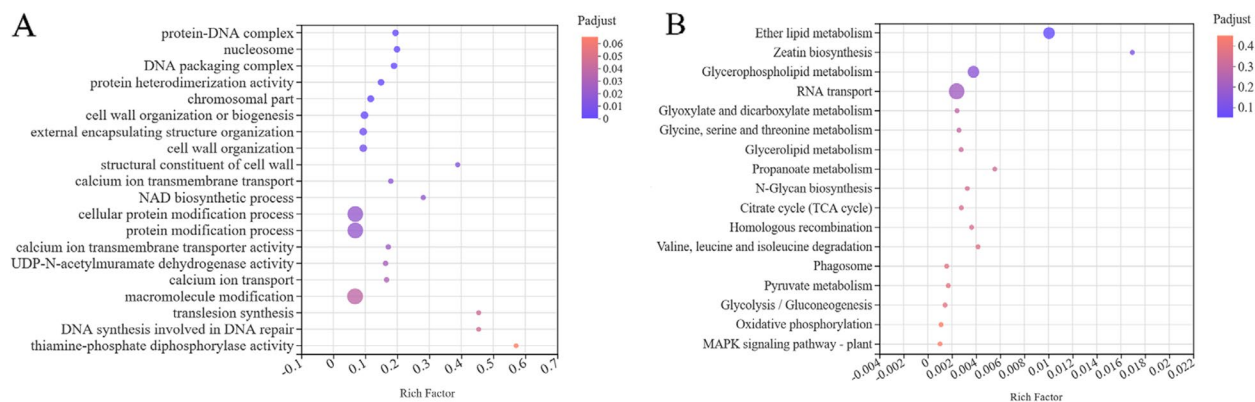


Fig. 2 GO enrichment analysis of all the DEGs and KEGG enrichment during sampling time. **A** GO enrichment of the DEGs at three time points. **B** KEGG enrichment of the DEGs at two and three sampling time points. The vertical axis represents GO term, and the horizontal axis represents Rich factor [the ratio of the number of genes/sample number enriched in the GO term to the number of the annotation gene/background number]. The larger the Rich factor, the greater the degree of enrichment. The size of the dot indicates the number of genes/transcripts in this GO term, and the color of the dots corresponds to a different FDR range

of *GhIPT2* (*Gh_A09G0887*), *GhIPT3* (*Gh_D08G0730*), *GhIPT5* (*Gh_D10G1519*), and *GhIPT9* (*Gh_D05G3327*) was upregulated during the sampling time. However, the expression of *GhLOGs* was repressed at three time points after MC treatment (Fig. 3A-B). Therefore, the increased content of nucleoside CTKs may be related to the upregulated expression of *GhIPTs* in MC-treated seedlings.

The content of active CTKs was also related to metabolism and glycosylation. The expression of *GhCKX1* (*Gh_A09G1128*), *GhCKX5* (*Gh_D01G0972*), and *GhCKX7* (*Gh_D13G2023*) was upregulated (Fig. 3A-B), and *GhCKX3* and *GhCKX6* were also increased at 24 h (data not shown). CTKs combine with glucosides to form O-glucosides and N-glucosides to adjust the homeostasis of hormones (Hou et al. 2004). The expression levels of *UGT76C2* (*Gh_A05G3018*) were increased at 12 and 24 h, whereas *UGT73C1s* were upregulated at 24 and 48 h (Fig. 3A-B). These findings indicated that the decrease in active iP and tZ contents is mainly due to the down-regulated expression levels of the CTK synthesis gene *GhLOGs* and increased CTK metabolism (Fig. 3A-B).

Considering that several stages of LR growth are regulated by auxin, auxin synthesis and metabolism-related genes have also been investigated. IAA, the main type of auxin, is synthesized by parallel pathways. Tryptophan aminotransferase (TAA1), TAA1-related proteins (TARs), and flavin-containing monooxygenases (YUC) participate in the two-step IPA pathway, which is the main plant IAA biosynthetic pathway (Livak et al. 2001). RNA-seq analysis demonstrated that *GhTAA1* (*Gh_A03G1194*, *Gh_D02G1629*) and *GhTAR2* (*Gh_D09G1630*, *Gh_A09G1558*) were repressed, while *GhYUCs* were significantly upregulated in cotton seedling roots. In contrast, the genes *GhILLs* encoding the

IAA-amino acid hydrolase ILR1-like to catalyze IAA-amino acid conjugates to free IAA were slightly activated (Fig. 4A-B). Moreover, IAA inactivation-related genes, such as those encoding IAA-amido synthase GRETCHEN HAGEN 3 (GH3) and encoding 2-oxoglutarate-dependent Fe(II) dioxygenase DIOXYGENASE FOR AUXIN OXIDATION (DAO), were also repressed (Fig. 4A-B). These results showed that MC increased the expression levels of genes encoding key rate-limiting enzymes *GhYUCs* of IAA synthesis and IAA-amino acid conjugate hydrolyses but reduced the expression levels of IAA inactivation genes.

DEGs involved in LR growth

Auxin signaling pathways, such as solitary-root (SLR)/IAA14 and auxin response factor ARF7/19, are involved in LR initiation. The results showed that the expression of *GhIAA14* was suppressed while *GhARF19* was upregulated (Fig. 4A, C). The CTK receptors AHK3 and CRE1/AHK4 and type-B response regulators in the CTK signaling pathway could influence LR growth by antagonizing auxin signaling. MC activated *GhAHK3* and *GhARR1* and repressed *GhAHK4*, *GhARR2*, and *GhARR12* (Fig. 3A, C). LR initiation requires an auxin gradient that is mediated by the auxin efflux carrier PIN-FORMED (PIN). MC increased the expression of *GhPINs* (Fig. 4A) and *GNOM*, which encodes ADP RIBOSYLATION FACTOR-GUANIDINE EXCHANGE FACTOR (ARF-GEF) to regulate the trafficking of PIN proteins (Fig. 5A). Furthermore, maintaining xylem pole pericycle cells in a mitosis-competent state, the nuclear protein ABERRANT LR FORMATION4 (ALF4)-related gene, and MYB transcription factor MYB77 interacted with ARF7 to regulate LR formation were all upregulated (Fig. 5A). The gene encoding PICKLE

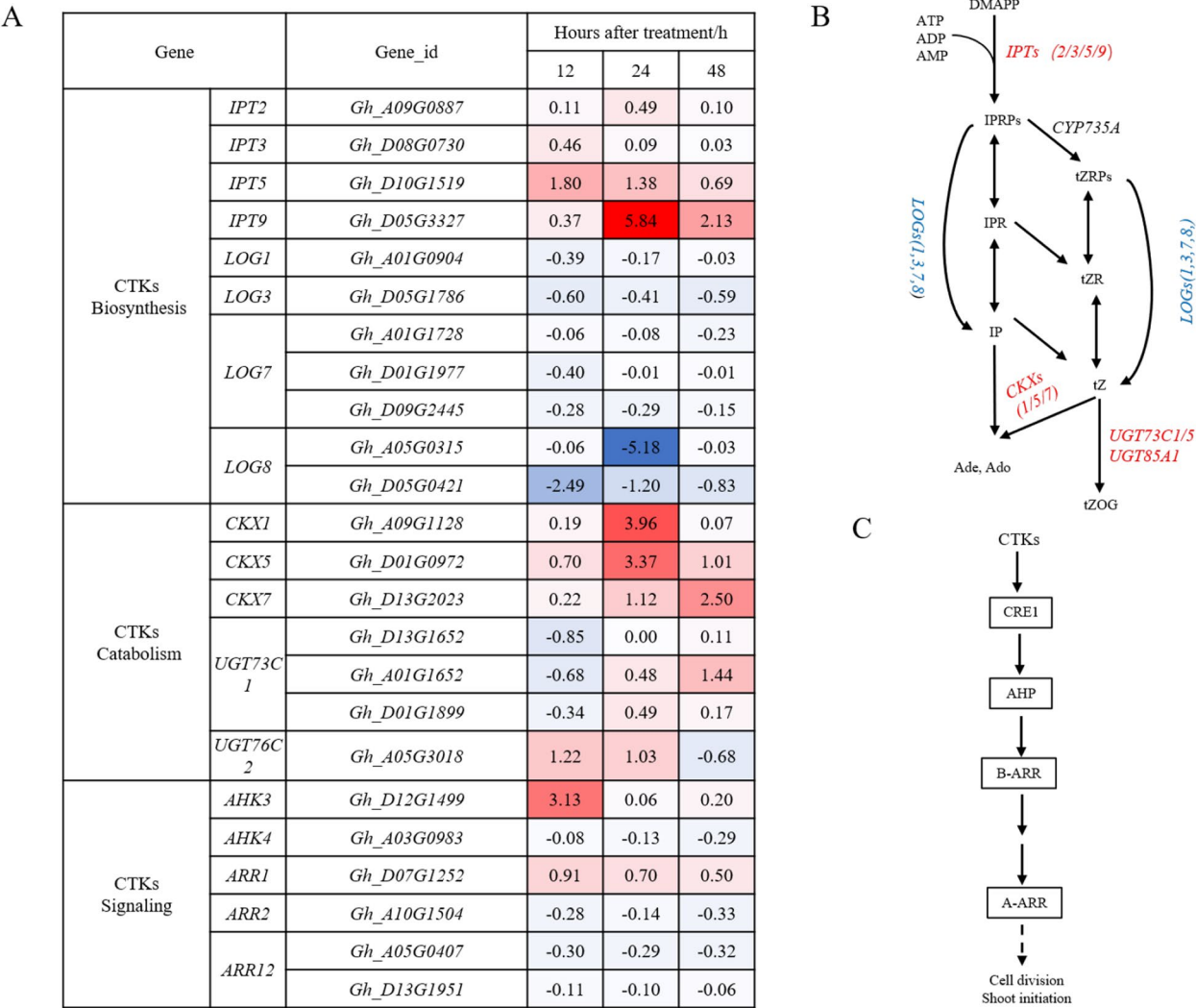


Fig. 3 Expression pattern of CTKs related genes during three sampling times. **A** The heatmap of related genes involved in CTKs. **B** CTKs biosynthetic and catabolism. **C** signal transduction pathway. DMAPP, dimethylallyl diphosphate; IPR, isopentenyladenine Riboside; iP, isopentenyladenine; tZR, trans-zeatin riboside; tZ, trans-zeatin; IPT, adenosine phosphate-isopentenyltransferase; CYP735A, cytochrome P450 monooxygenases; LOG, cytochrome P450 monooxygenases; CKX, CK oxidase; UGT85A1, UGT73C5 and UGT73C1, O-glucosyltransferases. The genes up- or down-regulated were represented in red or blue, respectively. The number in each cell is the log₂ value of fold change (log₂(fold change))

(PKL), a chromatin-remodeling factor that can repress ARF7/19 activity to inhibit LR initiation, was downregulated (Fig. 5A). Putative APETALA2/Ethylene-Responsive Element Binding Protein (AP2/EREBP) transcription factor and LBD16, LBD18, and LBD29, as targets of ARF7/19 mediated LR primordium development and LR initiation, respectively, were upregulated by MC treatment (Fig. 5A).

MC treatment promoted the cell cycle, cell division, cell wall biosynthesis, and sucrose synthesis

The cell cycle and cell division are the headstones for LR initiation. The genes involved in cell division and the cell cycle were analyzed. The results revealed that the

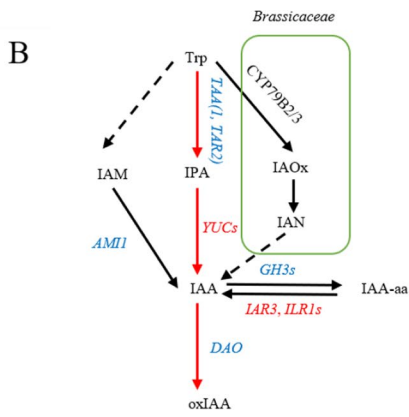
expression of *GhCDKBs* was upregulated at 24 h and 48 h compared with the control (Fig. 5B). The expression of the cyclin genes *GhCYCBs* was increased, and *GhCYCAs* and genes in the D-type cyclin (*CYCD*) gene family were upregulated at 24 h and 48 h after MC treatment. *GhE2Fa* and *GhE2Fb*, as transcriptional activators of the cell cycle, were upregulated by MC treatment. Unsurprisingly, *GhCDKs* interactor/inhibitor of CDKs (CKIs) and Kip-related proteins (KRP6s) were repressed after MC treatment (Fig. 5B).

Cell elongation and cell division require modification of cell wall rigidity. MC activated the genes related to cell-wall biosynthesis, including cellulose synthase (CESA),

A

Gene		Gene_id	Hours after treatment/h		
			12	24	48
IAA Biosynthesis	TAA1	<i>Gh_A03G1194</i>	-0.37	-0.22	-0.67
		<i>Gh_D02G1629</i>	0.01	0.05	-0.01
	TAR2	<i>Gh_A09G1558</i>	-0.12	-0.25	-0.17
		<i>Gh_D09G1630</i>	-0.38	-0.19	-0.14
	YUC	<i>Gh_A06G0538</i>	0.92	1.37	1.85
		<i>Gh_D06G0596</i>	-0.03	0.67	2.44
		<i>Gh_D07G0235</i>	0.35	0.05	1.59
		<i>Gh_Sca010895G01</i>	0.06	0.43	0.22
	AMI1	<i>Gh_D08G1285</i>	0.26	1.60	-0.20
		<i>Gh_A11G2395</i>	0.04	-3.07	-1.56
	ILL3	<i>Gh_D11G2710</i>	-0.16	-0.25	-0.80
		<i>Gh_D04G0924</i>	0.01	0.03	0.01
	ILL4	<i>Gh_Sca006015G02</i>	0.06	0.04	0.04
		<i>Gh_A06G0614</i>	0.15	1.48	0.02
IAA Catabolism	DAO	<i>Gh_A10G0550</i>	0.00	0.63	0.00
		<i>Gh_D06G0411</i>	0.14	0.32	0.06
	GH3s	<i>Gh_A06G0381</i>	0.12	0.41	0.26
		<i>Gh_A07G1653</i>	-1.17	-0.37	0.06
		<i>Gh_D07G1858</i>	-0.31	-3.50	-0.02
		<i>Gh_A03G1429</i>	-0.73	-0.63	-0.03
		<i>Gh_D02G2045</i>	-0.55	-0.08	-0.36
		<i>Gh_A13G0392</i>	-0.31	-0.13	-0.42
		<i>Gh_D08G1987</i>	-0.39	-0.05	-0.37
		<i>Gh_D11G1989</i>	-0.11	-0.07	-0.29
IAA Signaling	ARF19	<i>Gh_A06G0710</i>	0.81	0.76	2.89
		<i>Gh_A07G2353</i>	0.17	0.03	0.20
		<i>Gh_D04G0067</i>	1.11	0.38	1.56
	LAA14	<i>Gh_A02G0341</i>	-0.13	-0.07	-2.21
		<i>Gh_Sca006585G01</i>	-0.14	-0.14	-0.05
Auxin efflux carrier	PIN1	<i>Gh_A12G2431</i>	0.12	0.11	0.03
		<i>Gh_D10G1392</i>	0.28	0.00	1.12
		<i>Gh_D12G2556</i>	0.03	0.13	0.11
	PIN2	<i>Gh_A05G0014</i>	0.31	0.54	0.04
		<i>Gh_A01G1254</i>	0.01	0.42	0.06
	PIN3	<i>Gh_A13G0107</i>	0.79	0.11	0.10
		<i>Gh_D01G1471</i>	0.22	0.29	0.72
	PIN6	<i>Gh_A10G1348</i>	0.08	0.31	0.06
		<i>Gh_D02G0734</i>	0.44	0.55	0.14
		<i>Gh_D10G1122</i>	0.07	0.01	0.19

B



C

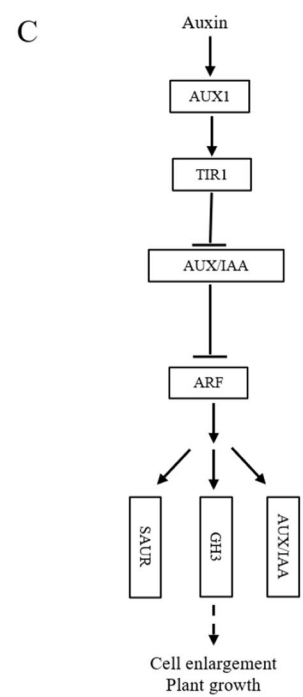


Fig. 4 Expression pattern of IAA related genes during three sampling times. **A** The heatmap of related genes involved in IAA. **B** IAA biosynthetic and catabolism. **C** signal transduction pathway. Trp, tryptophan; IPA, indole-3-pyruvic acid; TAA, tryptophan aminotransferase; TAR2, TAA-related 2; YUC, YUCCA. ILLs, IAA-amino acid hydrolase ILR1-like. DAO, DIOXYGENASE FOR AUXIN OXIDATION; GH3, GRETCHEN HAGEN3; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; IAOx, indole-3-acetaldoxime; oxIAA, 2-oxindole-3-acetic acid; PIN, PIN-FORMED; ARF, auxin response factor. The genes up- or down-regulated were represented in red or blue, respectively. The number in each cell is the log₂ value of fold change (log₂(fold change))

cellulose synthase-like (CSL), and glucan synthase-like (GSL), and participated in the synthesis of pectin and hemicelluloses, two UDP-glucose dehydrogenases (UGD) (Fig. 5C). The results showed that the expression levels of all genes mentioned above was upregulated at three sampling times.

SPS and SPP are mainly genes involved in the last two steps of sucrose synthesis. MC could increase *GhSPS1Fs*, *GhSPS2Fs*, and *GhSPP2* expression levels and upregulate *GhSPS3F* expression at 12 h (Fig. 5D). These results

showed that MC treatment could induce the cell cycle, division, cell-wall biosynthesis, and sucrose synthesis to promote LR initiation (Fig. 5D).

Seed soak Agroinoculation VIGS silenced *GhLOGs*, *GhCKXs*, and *GhARR12*

To verify the hypothesis that MC treatment could increase *GhCKX* expression levels and downregulate *GhLOGs* to decrease active CTK levels while promote crosstalk between the CTKs and auxin signaling

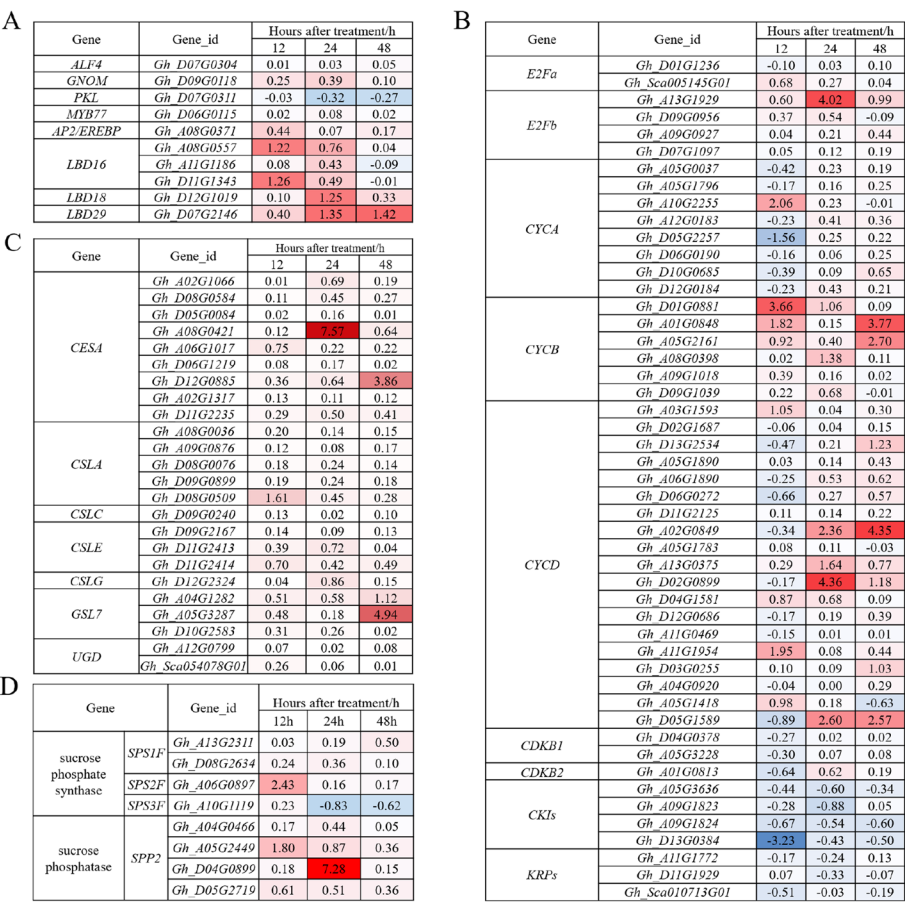


Fig. 5 Expression pattern of LR growth, cell cycle, cell division, cell wall biosynthesis, and sucrose synthesis genes. **A** Expression of genes involved in LR growth. **B** cell cycle and division. **C** cell wall biosynthesis. **D** sucrose synthesis. ALF4, ABERRANT LATERAL ROOT FORMATION4; PKL, PICKLE; AP2/EREBP, putative APETALA2/Ethylene-Responsive Element Binding Protein; LBD, LATERALORGAN Boundaries-domain. GNOM, guanine nucleotide exchange factor; MYB77, MYB transcription factor; PKL, PICKLE; AP2/EREBP, putative APETALA2/Ethylene-Responsive Element Binding Protein; LBD, LATERALORGAN Boundaries-domain. E2F, E2F transcription factor; CYCA, A-type cyclin; CYCB, B-type cyclin; CYCD, D-type cyclin; CDBK, B1-type CDKs; CKIs, interactor/inhibitor of CDKs; KRPs, Kip-related proteins; CESA, cellulose synthase; CSL, cellulose synthase-like; GSL, glucan synthase-like; UGD, UDP-glucose dehydrogenases; SPS, sucrose phosphate synthase; SPP, sucrose phosphatase. The genes up- or down-regulated were represented in red or blue, respectively. The number in each cell is the log2 value of fold change (log₂(fold change))

pathways to enhance the LR number of cotton seedling roots, the genes *GhLOG3*, *GhLOG7*, *GhLOG8*, *GhCKX1*, *GhCKX3*, *GhCKX5*, *GhCKX6*, *GhCKX7*, and *GhARR12* were silenced in early cotton seedlings.

Optimizations the method of soaking root

The effects of varying MC concentrations (0–40 mg·L⁻¹) on the number of cotton seedling LR were dose dependent (Table 2, Fig. S4). Treatment with prolonged soaking time (6–12 h) enhanced the number of LR. However, soaking roots in 80 mg·L⁻¹ MC solution or soaking roots for 20 h significantly decreased the number of LR. When roots were soaked in 10–80 mg·L⁻¹ MC solution for 12 h, compared with the CK, the number of LR increased by 15.5%, 14.3%, 16.4%, and 3.7% in 5-day-old

cotton seedlings after treatment. The effect was most pronounced at a concentration of 40 mg·L⁻¹ for 12 h.

GhCKX1 was silenced in roots by SSA-VIGS

The *CKX1* gene is mainly expressed in close proximity to the vascular cylinder at the site of growing LR. Hence,

Table 2 LR number at 7 d after MC soaking root treatment

MC Treatment/ (mg·L ⁻¹)	6 h	12 h	20 h
0	73.0 ± 2.0c	80.5 ± 1.5b	66.7 ± 1.2c
10	78.0 ± 0.0bc	93.0 ± 0.0a	83.0 ± 2.4b
20	78.5 ± 1.5b	92.0 ± 0.8a	85.0 ± 0.0ab
40	87.5 ± 2.5a	93.7 ± 2.9a	87.5 ± 1.5a
80	75.7 ± 3.3bc	83.5 ± 1.5b	80.5 ± 0.5b

Different letters indicate a significant difference at *P* < 0.05 between different treatments at the same treatment times

GhCKX1 was selected to confirm whether gene silencing can be induced in roots by using the SSA-VIGS method. pTRV:*GhCKX1* recombinant vectors were constructed (Fig. 6A) and then inoculated into cotton seeds to silence *GhCKX1* expression. The LR number of pTRV:*GhCKX1* seedlings was reduced by 41.0% and 45.5% at 15 and 22 days post inoculation (dpi), respectively, compared with that of pTRV:*GhGFP* seedlings (Fig. 6B, Fig. S5). Then, the relative expression levels of *GhCKX1* in roots at different dpi were measured to ascertain the start silence time. As shown in Fig. 6C, the expression level of *GhCKX1* was reduced from 2 to 22 dpi in pTRV:*GhCKX1* roots, and the maximum decreased 91.0% at 2 dpi. This result showed that SSA-VIGS can silence genes in seedling roots as early as 2 dpi.

Silencing of *GhCKX1/3/5/6/7* by SSA-VIGS decreased the number of cotton seedling LR

In the present RNA-seq results, the expression of *GhCKX1*, *GhCKX3*, *GhCKX5*, *GhCKX6*, and *GhCKX7* was upregulated after MC treatment. To determine whether MC treatment increased the LR number of cotton seedlings by upregulating *GhCKX* expression, the genes *GhCKX1/3/5/6/7* were separately silenced and then treated by soaking roots in 40 mg·L⁻¹ MC solution for 12 h. The seedling roots were harvested at 22 dpi. The pTRV:*GhCKXs* seedling LR numbers of the

control and MC treatment were significantly decreased by 4.44%–43.33% compared with the pTRV:*GhGFP*. In the pTRV:*GhGFP* seedlings, the LR numbers increased by 62.78% after MC treatment (Fig. 7A), while in the pTRV:*GhCKXs* seedlings, the LR numbers after MC treatment were increased by 7.50%–19.37% compared with that of the CK treatment (Fig. 7A, Fig. S6). In the pTRV:*GhCKXs* roots, the expression levels of *GhCKX1*, *GhCKX3*, *GhCKX5*, *GhCKX6*, and *GhCKX7* were markedly reduced by 15.3%–76.36% (Fig. S7A–E). Compared with the pTRV:*GhGFP*, the relative transcript abundances of *GhARR2*, *GhARR12*, and *GhIAA14* were dramatically upregulated by 42.80%–275.46%, 6.43%–153.96%, and 12.82%–70.84%, respectively, and those of *GhPIN1*, *GhARF7*, *GhARF19*, *GhLBD16*, *GhLBD18*, *GhLBD29*, and *GhLBD33* were downregulated by 8.91%–63.01%, 6.61%–49.92%, 18.61%–47.64%, 5.24%–47.37%, 19.67%–5.12%, 39.82%–67.05%, and 5.96%–59.28%, respectively (Fig. 7B–K). The expression levels of the aforementioned genes in both MC-treated pTRV:*GhCKXs* seedlings and the control were consistent with expectations.

The active tZ, iP, and active IAA contents of control and MC-treated pTRV:*GhGFP* and pTRV:*GhCKXs* roots were quantified. Compared with the pTRV:*GhGFP*-CK, the tZ contents of pTRV:*GhCKX5* and pTRV:*GhCKX7* were significantly increased by 69.23%–92.31%, the

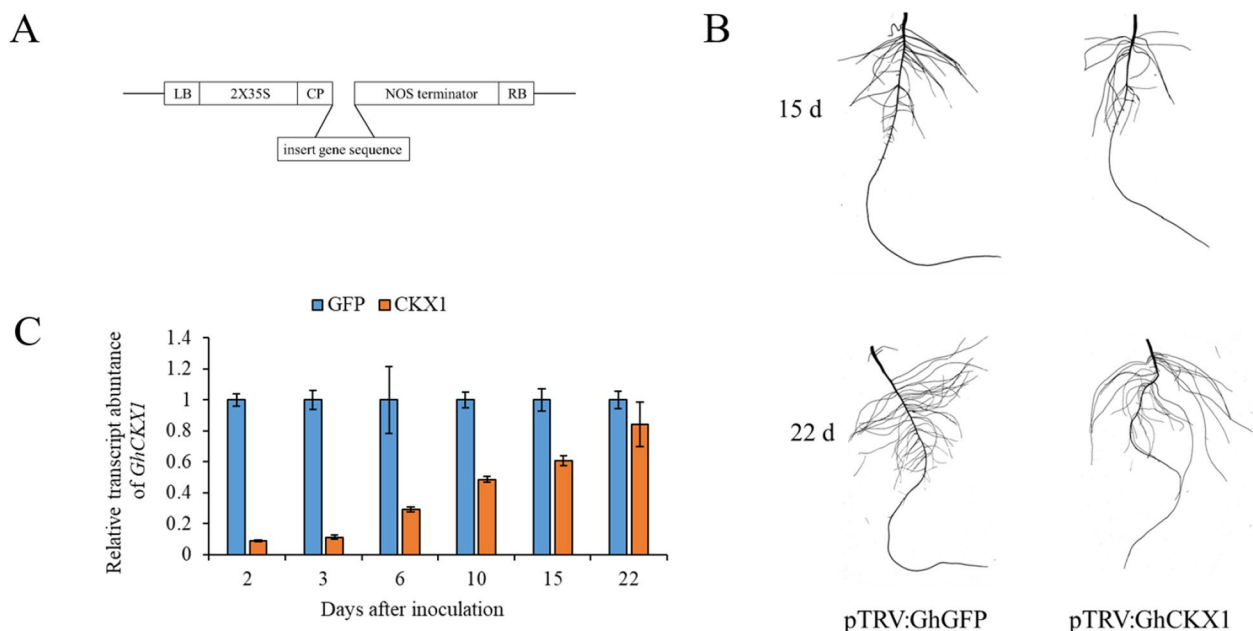


Fig. 6 Silencing of *GhCKX1* by SSA-VIGS method in cotton seedling roots. **A** Schematic representation of TRV2 vectors used for VIGS. **B** The root phenotypes of cotton seedlings inoculated with pTRV:*GhGFP* and pTRV:*GhCKX1* at 15 dpi and 22 dpi. **C** The relative expression levels of *GhCKX1* in pTRV:*GhGFP* and pTRV:*GhCKX1* seedling roots at different time points after inoculation. *GhActin9* was used as an internal control. Each bar value represents mean $\pm$ SD ($n=3$)

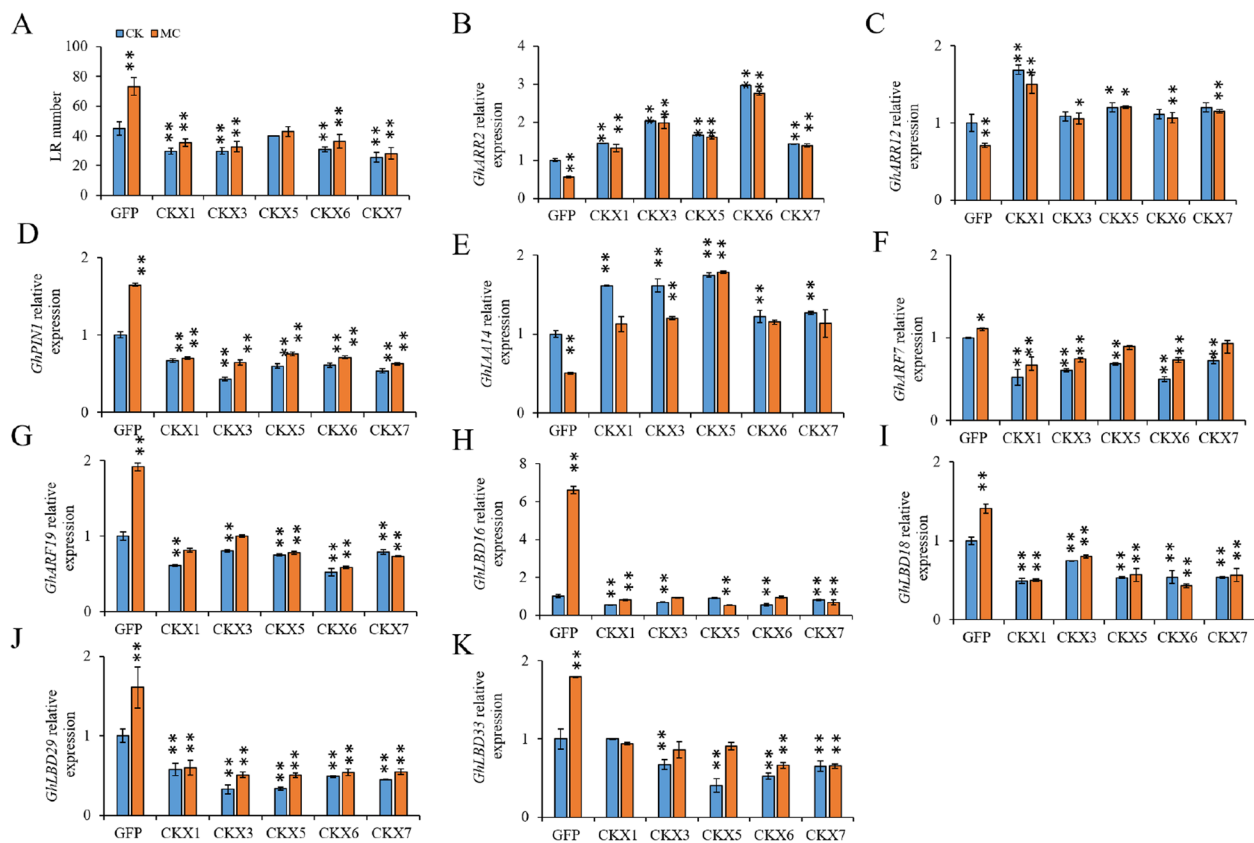


Fig. 7 Silencing of *GhCKXs* by SSA-VIGS method in cotton seedling roots. **A** The LR number of pTRV:GhGFP and pTRV:GhCKXs seedling with or without MC treatment. **B–K** The relative expression levels of *GhARR2* (**B**), *GhARR12* (**C**), *GhPIN1* (**D**), *GhIAA14* (**E**), *GhARF7* (**F**), *GhARF19* (**G**), *GhLBD16* (**H**), *GhLBD18* (**I**), *GhLBD29* (**J**), *GhLBD33* (**K**) in pTRV:GhGFP and pTRV:GhCKXs seedling roots at 22 dpi, respectively. GFP, CKX1, CKX3, CKX5, CKX6, CKX7 represent pTRV:GhCKX1, pTRV:GhCKX3, pTRV:GhCKX5 and pTRV:GhCKX6, pTRV:GhCKX7, respectively. *GhActin9* was used as an internal control. Each bar value represents mean $\pm$ SD ($n=3$). * and ** indicate a significant difference at $P < 0.05$ and $P < 0.01$, respectively

iP and total active CTK levels greatly increased, and the IAA contents decreased by 7.42%–80.96%. In MC-treated pTRV:GhGFP seedlings, tZR, iPR, and IAA were increased by 14.29%, 73.91%, and 55.21%, while tZ and iP contents were reduced by 15.38% and 57.89%, respectively, in contrast to control seedlings (Table 3). These results were consistent with previous results. Taken together, these data suggest that *GhCKXs* might play an important role in LR formation in cotton seedlings in response to MC treatment.

Silencing of *GhLOG3/7/8* and *GhARR12* by SSA-VIGS increased the number of cotton seedling LRs

Compared with the pTRV:GhGFP control seedlings, the number of LRs in pTRV:GhLOG3/7/8 and pTRV:GhARR12 was remarkably increased by 10.00%–57.14% (Fig. 8A and Fig. S8). In the presence of MC, the number of LRs in

pTRV:GhLOGs and pTRV:GhARR12 was enhanced by 5.72%–17.37% compared with that in the control seedlings (Fig. 8A, Fig. S8). The relative transcript abundances of *GhLOG3*, *GhLOG7*, *GhLOG8*, *GhARR2*, *GhARR12*, and *GhIAA14* were severely downregulated by 93.76% and 80.72%, 90.07% and 89.23%, 45.32% and 31.55%, 73.02%–93.15%, 17.20%–92.68%, and 33.14%–51.40%, respectively, and the expression level of *GhPIN1*, *GhARF7*, *GhARF19*, *GhLBD16*, *GhLBD18*, *GhLBD29*, and *GhLBD33* was significantly upregulated in MC-treated pTRV:GhLOGs roots, respectively (Fig. 8B–K, Fig. S7F–I). Compared with the pTRV:GhGFP-CK, tZ and iP levels were decreased by 23.08%–61.54% and 21.05%–94.74%, respectively, and the active IAA contents were observably enhanced by 8.25%–90.91% in pTRV:GhLOGs seedlings (Table 3). These results suggest that reduced *GhLOGs* and *GhARR12* abundance in MC-treated seedlings could increase LR number.

Table 3 Contents of CTKs and IAA detected in SSA-VIGS cotton seedling roots

Treatment	Content/(ng·g ⁻¹ FW)				
	Nucleosides forms		Free base forms		Active forms
	tZR	iPR	tZ	iP	IAA
pTRV:GhGFP-CK	0.98±0.02	0.46±0.01	0.13±0.00	0.19±0.00	122.06±5.20
pTRV:GhGFP-MC	1.12±0.01*	0.80±0.01**	0.11±0.00	0.08±0.00**	189.45±1.69**
pTRV:GhLOG3-CK	1.02±0.00*	0.65±0.02**	0.08±0.01**	0.15±0.00**	132.22±0.54*
pTRV:GhLOG3-MC	1.00±0.00*	1.07±0.00**	0.10±0.00	0.12±0.00**	132.13±0.00*
pTRV:GhLOG7-CK	0.88±0.02*	0.75±0.00**	0.08±0.00**	0.08±0.00**	138.18±0.00*
pTRV:GhLOG7-MC	1.04±0.00	0.83±0.00**	0.05±0.00**	0.14±0.00**	168.97±12.32**
pTRV:GhLOG8-CK	1.02±0.05	0.80±0.00**	0.06±0.01**	0.01±0.02**	158.72±10.30**
pTRV:GhLOG8-MC	1.04±0.01	0.71±0.00**	0.05±0.00**	0.10±0.02**	233.02±9.00**
pTRV:GhCKX1-CK	0.61±0.01**	0.56±0.01*	0.10±0.01	0.30±0.00**	75.59±10.21**
pTRV:GhCKX1-MC	0.56±0.00**	0.61±0.01**	0.10±0.00	0.34±0.00**	82.18±7.55**
pTRV:GhCKX3-CK	0.57±0.01**	0.49±0.01	0.11±0.00	0.24±0.00**	117.29±13.27
pTRV:GhCKX3-MC	0.36±0.00**	0.46±0.01	0.11±0.00	0.23±0.00*	122.90±0.95
pTRV:GhCKX5-CK	0.60±0.00**	0.61±0.01**	0.23±0.00**	0.21±0.00*	111.32±8.34
pTRV:GhCKX5-MC	0.75±0.01**	0.61±0.01**	0.22±0.00**	0.19±0.00	68.24±7.42**
pTRV:GhCKX6-CK	1.11±0.00*	0.69±0.00**	0.14±0.00	0.27±0.00**	102.74±1.64*
pTRV:GhCKX6-MC	1.28±0.01**	0.71±0.00**	0.14±0.00	0.25±0.00**	113.00±9.06
pTRV:GhCKX7-CK	1.16±0.01*	0.64±0.00**	0.25±0.00**	0.29±0.00**	38.74±1.95**
pTRV:GhCKX7-MC	0.68±0.01**	0.81±0.02**	0.22±0.00**	0.32±0.00**	23.24±4.15**

Data represent mean ± SD (n=3). * and ** indicate a significant difference between pTRV:GhGFP-CK and other treatment at $P<0.05$ and $P<0.01$, respectively

Discussion

Promoting LR formation by MC treatment could increase the resistance of cotton seedlings (Duan et al. 2002; Xu and Taylor 1992). Auxin and CTK are key regulators of LR growth, and decreasing the biologically active CTKs was beneficial to promote LR emergence (Jing et al. 2019). MC could significantly enhance nucleoside form CTK levels and reduce active free base form CTKs in the roots. RNA-seq results showed that MC upregulated *GhIPTs* expression levels, which may cause the accumulation of nucleosides from CTKs. CTK catabolic genes (*GhCKXs*) and glycosyltransferase genes were activated, and the CTK-activating gene *GhLOGs* were repressed after MC treatment, which may lead to the levels of active free base CTKs being significantly decreased (Fig. 3A). After silencing *GhCKX* genes in early cotton seedling roots, the active free base CTK contents increased, and the LR number noticeably decreased (Fig. 7A, Fig. S6, Table 3). The results of silencing *GhLOGs* and *GhARR12* were opposite to those of silencing *GhCKXs* (Fig. 8A, Fig. S8, Table 3). This phenomenon was also observed in other plants. *CKX* gene transgenic hairy roots of *Lotus japonicus* significantly increased the number of LRs (Lohar et al. 2004). *AtCKX1* and *AtCKX2* transgenic tobacco and overexpression of *AtCKX1* and *AtCKX2* in

Arabidopsis significantly reduced active free base CTK contents and increased the number of LRs and adventitious roots (Werner et al. 2001, 2003). RNA interference of *MtLOG1* in *Medicago truncatula*, the LR density and number were higher than those of the control, and the primary root length was shortened (Mortier et al. 2014). Similarly, with exogenously applied iPR in an *Arabidopsis* triple mutant (*log3 log4 log7*), the LRs still emerged (Kuroha et al. 2009). Hence, increasing the expression levels of *CKXs* and decreasing the expression levels of *LOGs* to reduce the content of active CTKs probably enhance the number of LRs. However, a reduced response to MC-induced LR growth was observed in silenced *GhCKXs*, *GhLOGs*, and *GhARR12* cotton seedlings; the LR number of MC-treated pTRV:GhCKXs and pTRV:GhLOGs seedlings increased by 5.72%–19.37% compared with the control, which may be related to the fact that the *LOG* and *CKX* genes redundantly regulate LR formation (Kuroha et al. 2009; Werner et al. 2003).

Auxin accumulated in the position that will form LR primordia. PIN proteins mediate auxin efflux to establish an auxin gradient that is necessary for LR growth (Benková et al. 2003). CTK antagonizes auxin through the *AHK3/ARR1* signaling pathway and activates the auxin response inhibitor SHY2/IAA3 (SHY2), which represses *PIN1/3/7* expression (Dello Ioio et al. 2008). Furthermore,

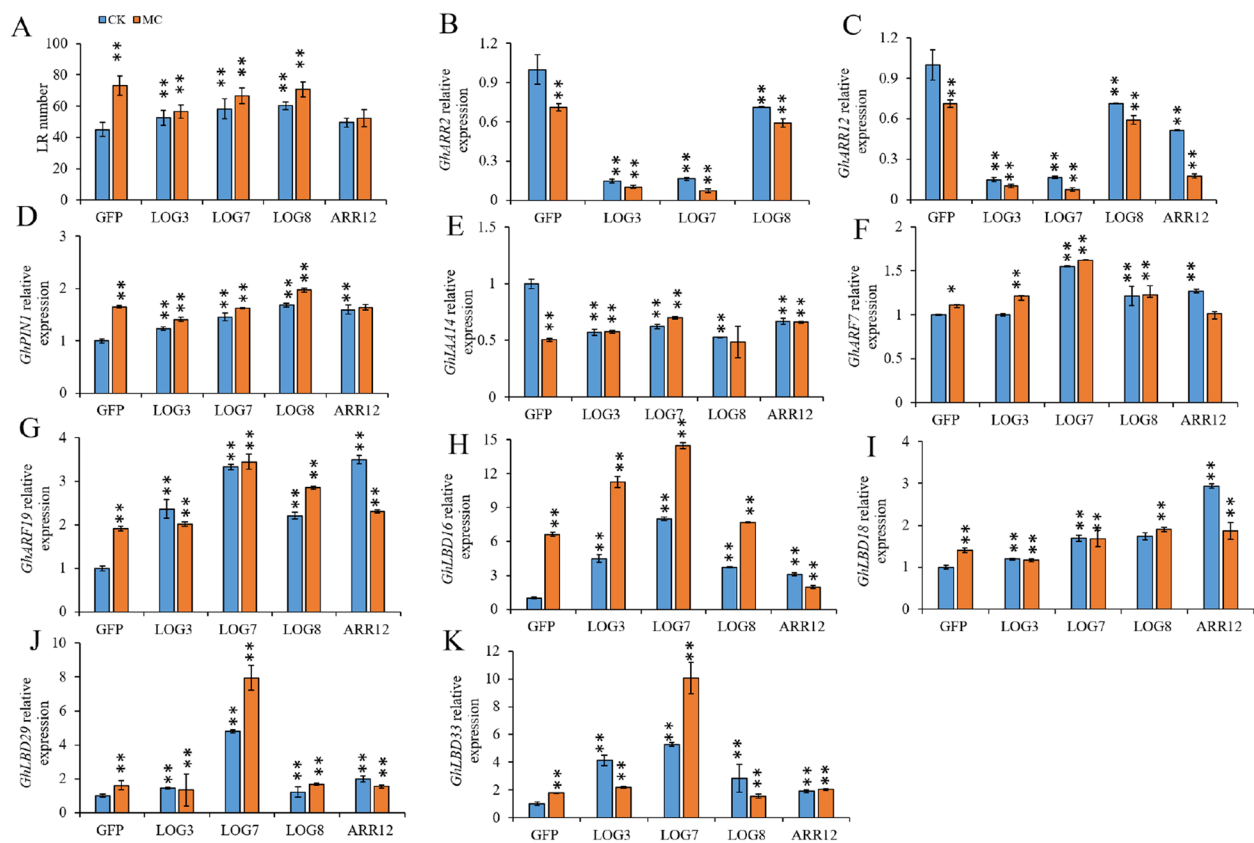


Fig. 8 Silencing of *GhLOGs* and *GhARR12* by SSA-VIGS method in cotton seedling roots. **A** The LR number of pTRV:GhGFP, pTRV:LOGs, and pTRV:ARR12 seedling with or without MC treatment. **B–K** The relative expression levels of *GhARR2* (**B**), *GhARR12* (**C**), *GhPIN1* (**D**), *GhIAA14* (**E**), *GhARF7* (**F**), *GhARF19* (**G**), *GhLBD16* (**H**), *GhLBD18* (**I**), *GhLBD29* (**J**), *GhLBD33* (**K**) in pTRV:GhGFP, pTRV:GhLOGs and pTRV:ARR12 seedling roots at 22 dpi, respectively. GFP, LOG3, LOG7, LOG8, ARR12 represent pTRV:GhLOG3, pTRV:GhLOG7, pTRV:GhLOG8 and pTRV:GhARR12, respectively. *GhActin9* was used as an internal control. Each bar value represents mean $\pm$ SD ($n = 3$). * and ** indicate a significant difference at $P < 0.05$ and $P < 0.01$, respectively

through AHK4/ARR2 and ARR12, CTK promoted PIN1 lytic degradation or regulated vacuole endocytic PIN1 to mediate LR formation (Marhavý et al. 2011). MC upregulated the expression levels of *GhAHK3* and *GhARR1* and repressed the genes *GhAHK4*, *GhARR2*, and *GhARR12*, which indirectly activated the expression of *GhPINs* to promote auxin gradient establishment (Figs. 3A, 4A, 9). Silencing *GhCKX* genes in early cotton seedling roots increased the total content of active free base CTKs, dramatically upregulated the expression levels of *GhARR2* and *GhARR12* and downregulated the expression levels of *GhPIN1* (Fig. 7B–D). The data for silencing *GhLOGs* and *GhARR12* were contrary to those for silencing *GhCKXs* (Fig. 8B–D). These results indicated that the change in CTK content could affect *PIN* gene expression. Next, auxin regulated LR initiation through the SLR/IAA14-ARF7/19 signaling pathway. However, in the absence or low levels of auxin, SLR/IAA14 combined with ARF7/19 to form a heterodimerization that suppressed auxin signal pathway-regulated gene expression.

Luckily, a high auxin level was able to remove this inhibition (Fukaki et al. 2002, 2005). In current results, MC could increase the active IAA content by inducing the expression of IAA biosynthesis genes *GhYUCs* and IAA amidohydrolase genes and downregulating the expression of IAA-amido synthase *GhGH3s* and dioxygenase *GhDAOs* (Fig. 4A) (Lunn et al. 1990). ARF7 and ARF19, as transcriptional activators in the auxin signaling pathway, activate *LBD16*, *LBD18*, *LBD29*, and *LBD33* and then induce *CycD* expression to mediate LR formation (Okushima et al. 2007, 2005; Lee et al. 2009; Berckmans et al. 2011; Feng et al. 2012; Hirota et al. 2007). Furthermore, *LBD18/LBD33* can activate the transcriptional activators E2Fa and E2Fb, which induce cell division by promoting G2-to-M transitions (Magyar et al. 2005; Casanova-Sáez et al. 2019; Berckmans et al. 2011). Our results showed that the expression level of *GhIAA14* was suppressed and that of *GhARF19*, *GhLBD16*, *GhLBD18*, *GhLBD29*, *GhE2Fa*, and *GhE2Fb* was upregulated after MC treatment (Figs. 4A, 5A–B, 9). Silencing *GhLOGs* and

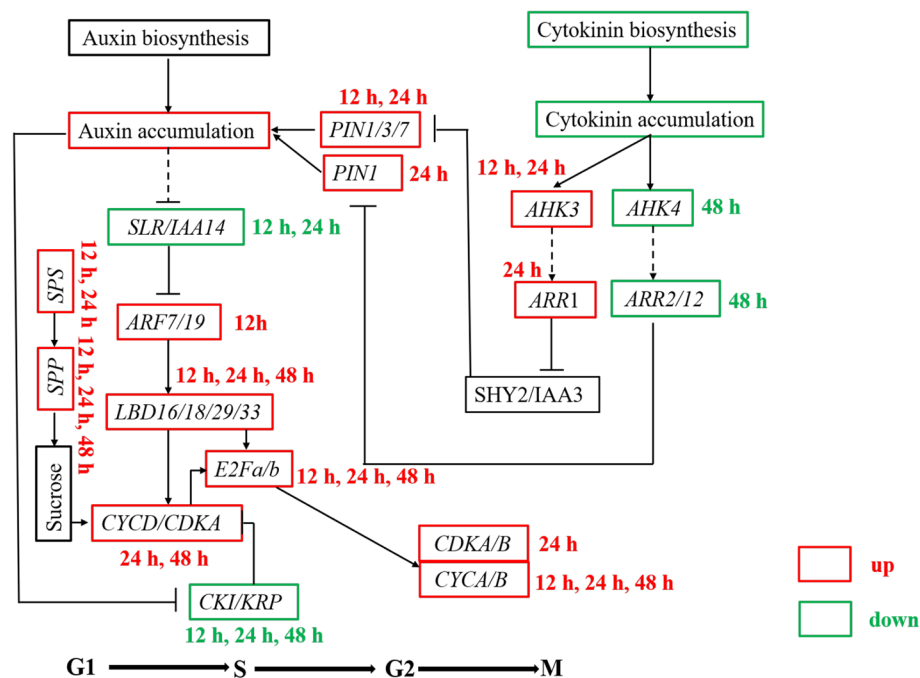


Fig. 9 A model of MC promoted LR formation in cotton. MC decreased CTKs content, repressed *GhAHK4*, *GhARR2*, *GhARR12* expressed and up-regulated *GhAHK3*, *GhARR1* to promoted *GhPINs* expression. MC increased auxin concentration to suppressed *GhLAA14* and *CKI/KRP* and activated *GhARF19*, *GhLBD16s* which could activate *GhE2Fa/b* to promoted asymmetric cell division. Moreover, auxin increased the expression of *CYCD/CDKA*, *CDKB*, *CYCA/B* to promoted both G1/S and G2/M transitions. MC also could up-regulated *SPS*, *SPP* to increase the level of sucrose to promoted *CYCD* family expression

GhARR12 genes in early cotton seedling roots showed similar results with RNA-seq (Fig. 8E-K). The data for *GhCKXs* silencing were contrary to the RNA-seq results (Fig. 7E-K). These data indicated that MC repressed the CTK signaling pathway, activated the expression levels of *PIN* genes to establish an auxin gradient, and then activated the expression of auxin signaling pathway-related genes, causing cell division to promote LR initiation.

Xylem pole pericycle cells (XPP cells) are activated by auxin for asymmetric cell division at the start of LR initiation (Péret et al. 2009). Auxin primed XPP cells to divide by inducing the genes *CDKB* and cyclin genes *CYCB1* and *CYCB2* at the G2/M phase for the cell cycle (Himanen et al. 2002, 2004; Tank et al. 2011). The CDK inhibitors CKIs (De Veylder et al. 2001) and KPP2 (Himanen et al. 2002) prevent LR initiation by inactivating CDKA/cyclin D complexes to block the G1/S transition (Wang et al. 1998) and are repressed by auxin. Overexpression of *KPP2* could reduce LR number and inactivate pericycle cells (De Veylder et al. 2001). MC treatment upregulated *GhCDKBs* and *GhCYCBs* and suppressed *GhCKIs* and *GhKRP6s* expression. *CYCD* acts at the G1/S-phase transition to accelerate cell division, and it might be a key limiting factor in LR formation because it is expressed in the overall process

(Riou-Khamlichi et al. 1999; Francis et al. 2001). The *GhCycD* family was also mainly upregulated at 24 h and 48 h (Figs. 5B, 9). The culture medium containing sucrose could promote cell division (Soni et al. 1995), MC increased the expression levels of *GhSPS* and *GhSPP* (Figs. 5D, 9), which may increase sucrose accumulation. Several studies have reported that sucrose can promote cell division by inducing *CycD* at the G0/G1 phase (Francis et al. 2001). Modification of cell wall rigidity is necessary for cell elongation, division, and organ growth (Kutschera 1990). It has also been reported that induction of cell wall remodeling enzymes could promote cell separation prior to the emergence of LR (Swarup et al. 2008; Carpita 2011). Cell wall biosynthesis genes were all activated by MC treatment (Fig. 5C). Therefore, promoting the cell cycle, cell division, cell-wall biosynthesis, and sucrose synthesis are prerequisites for enhancing LR formation (Fig. 9).

Conclusion

In this study, the nucleoside CTK contents (tZR and iPR) increased, while the levels of free bases tZ and iP significantly decreased in MC-treated seedling roots. MC upregulated the expression levels of *GhIPTs*, which encoded the rate-limiting enzyme for CTK synthesis, and improved the

accumulation of nucleoside CTKs. Moreover, the endogenous free base CTKs in seedlings under MC treatment were decreased by downregulating *GhLOGs* and upregulating *GhCKXs* and glycosyltransferase genes. Furthermore, MC treatment repressed *GhAHK4* and *GhARR2/12* of CTK signaling to activate auxin efflux carrier *PIN* expression and then promoted auxin accumulation, which activated the IAA-IAA14-ARF7/19 signal model to promote LR initiation. Meanwhile, MC increased the expression of genes involved in sucrose synthesis, the cell cycle, cell division, and cell wall biosynthesis pathways. Silencing *GhCKX* genes in early cotton seedling roots noticeably decreased the LR number, *GhPIN1* expression was repressed, the active free base CTK contents were increased, and active IAA levels were reduced. The results of silencing *GhLOGs* and *GhARR12* were opposite to those of silencing *GhCKXs*. Meanwhile, the response to MC-induced LR growth was reduced in silenced *GhCKXs*, *GhLOGs*, and *GhARR12* cotton seedlings. These results showed that MC could promote the metabolism of CTKs, especially the degradation of free base CTKs, increase the content of IAA, and then promote auxin signaling while attenuating CTK signaling to enhance LR initiation.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions

Wang BM and Chen XJ conceived the study. Zhang M was responsible for transcriptome data analysis, hormone determination and article writing. Zhang JQ was responsible for VIGS experiment and article writing. Chen YJ was responsible for cotton cultivation and root scanning experiment. Wu YX, Shi Y, and Xing HY are responsible for sample collection, sample processing and RNA extraction. The first draft of the manuscript was written by Zhang M and Zhang JQ. All authors read and approved the final manuscript.

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Data availability

All the data relevant to the present study are included in the article. Any further details related to the experiments conducted can be made available by requesting the corresponding author.

Declarations

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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