Running head: Casein kinase I regulates somatic embryogenesis

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LEAFY COTYLEDON1-CASEIN KINASE I-TCP15-PHYTOCHROME
INTERACTING FACTOR4 network regulates somatic embryogenesis by regulating auxin homeostasis

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One sentence summary: A novel gene network regulates auxin homeostasis, thereby affecting cell proliferation and the transition from non-embryogenic callus to somatic embryos during somatic embryogenesis.
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ABSTRACT

Somatic embryogenesis (SE) is an efficient tool for the propagation of plant species and is also a useful model for studying the regulatory networks in embryo development. However, the regulatory networks underlying the transition from non-embryogenic callus to somatic embryos during SE remain poorly understood. Here, we describe a *Gossypium hirsutum* CASEIN KINASE I gene, *GhCKI*, which is a novel key regulatory factor that strongly affects SE. Overexpressing *GhCKI* halted the formation of embryoids and plant regeneration due to a block in the transition from non-embryogenic callus to somatic embryos. In contrast, defective *GhCKI* in plants facilitated SE. To better understand the mechanism by which *GhCKI* regulates SE, the regulatory network was analyzed. A direct upstream negative regulator protein, *Gossypium hirsutum* LEAFY COTYLEDON1 (GhLEC1), was identified to be targeted to a cis-element, CTTTTC, in the promoter of *GhCKI*. Moreover, GhCKI interacted with and phosphorylated GhTCP15 by coordinately regulating the expression of *Gossypium hirsutum* PHYTOCHROME INTERACTING FACTOR4 (*PIF4*), finally disrupting auxin homeostasis, which led to increased cell proliferation and aborted somatic embryo formation in *GhCKI*-overexpressing somatic cells. Our results demonstrate a complex process of SE that is negatively regulated by *GhCKI* through a complex regulatory network.
INTRODUCTION

Somatic embryogenesis (SE) is a process in which somatic cells are reprogrammed to generate a complete new embryo in response to external stimuli, without the fusion of gametes. The process of SE resembles zygotic embryogenesis, and developmental and regulatory mechanisms of SE could provide an accessible reference for studying the earliest developmental events of the zygotic embryo in the life cycle of higher plants (Mordhorst et al., 1997). In addition, SE is a key step in the realization of genetic transformation, somatic hybridization, and somaclonal variation screening in most plants, especially cash crops (Zeng et al., 2006). Cotton, as a key fiber crop, requires a highly successful regeneration procedure from somatic cells in order to perform genetic manipulation. Although we have conducted research on SE in cotton by expression profile analysis and demonstrated that transcriptional regulation in SE is a complex process with the interaction of multiple molecules (Zeng et al., 2006; Yang et al., 2012), the detailed functions of key transcriptional regulators and the mechanisms underlying the different developmental processes are less well understood, and only a few genes that regulate SE in cotton have been identified (Hu et al., 2011).

SE comprises two styles: direct SE and indirect SE, in which embryos are formed from explant tissues without or with a callus phase, respectively. During SE in cotton, non-embryogenic callus (NEC) and embryogenic callus (EC) are present (Yang et al., 2012), indicating that SE occurs in an indirect style, which consists of four different phases: dedifferentiation from explants, cell division and proliferation, transition from NEC to EC, and transition from EC to somatic embryos (Yang et al., 2012; Filonova et al., 2000).

During the past three decades, numerous transcription factors, kinases, and types of hormones involved in SE in different species have been identified. *Arabidopsis thaliana* PLANT GROWTH ACTIVATOR (*PGA6*) belonging to the homeodomain transcription factor family and identical to WUSCHEL (*WUS*) plays a critical role in SE. Gain and loss-of-function *pga6* mutants can promote and compromise the vegetative-to-embryogenic transition, respectively (Zuo et al., 2002). LEAFY
*COTYLEDON1* (*LEC1*), which encodes a HAP3 subunit of the CCAAT box-binding factor complex, has been shown to be necessary for somatic embryogenesis. Mutations in *LEC1* cause defective embryo maturation, and thus, *LEC1* participates mainly in promoting embryonic cell differentiation during late SE developmental stages (Lotan et al., 1998). *Arabidopsis LEC2, FUSCA3*, and *ABA INSENSITIVE3*-encoding B3 domain transcription factors are essential for several features of somatic embryo development (Braybrook et al., 2006; Stone et al., 2008).

*AGAMOUS-LIKE15* is a MADS-domain transcription factor that is primarily expressed during embryogenesis. There are no significant differences between *agl15* and wild type in terms of SE, but *agl15/agl18* double mutants display a compromised ability to produce somatic embryos (Heck et al., 1995; Zheng et al., 2009).

Other than transcription factors, kinases have been shown to be involved in SE. Kinases play vital roles in signal transduction pathways, regulating downstream signaling proteins via phosphorylation. The most famous type of kinase involved in SE is *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*). Five putative *SERK* genes in *Arabidopsis* have been characterized, and the overexpression of *AtSERK1* strengthened the capacity of suspension cells to undergo SE. In addition, expression analysis of three alfalfa *SERK* genes revealed that these genes are expressed in early stage embryogenic tissues but not in non-embryogenic tissues or in the mature embryo (Becraft, 2002; Hecht et al., 2001; Nolan et al., 2003). These results suggest that *SERKs* play a conserved role in mediating SE in plants.

In addition to transcription factors and kinases, hormone homeostasis and stress also have been proposed to be key plant growth factors in promoting the dedifferentiation of explants or initiating an embryogenic pathway. Auxin is an important growth regulator for embryo induction (Yang et al., 2012), but cytokinin, abscisic acid, ethylene, jasmonates, and brassinosteroids also play roles in initiating SE (Sagare et al., 2000; Depuydt and Hardtke, 2011). Interestingly, all of the above mentioned genes involved in SE are related to auxin, among which *LEC1* is known...
to upregulate the auxin biosynthesis gene \textit{YUCCA10} (\textit{YUC10}) by binding to its promoter (Junker et al., 2012). Overexpression of \textit{LEC1} could substitute for exogenous auxin and induce SE (Lotan et al., 1998). \textit{LEC2} was found to bind directly to the regulatory regions of \textit{YUC4} and to activate the auxin signaling pathway in somatic cells to promote the formation of embryonic cells (Stone et al., 2008). In addition, the expression of \textit{SERK1} is also responsive to auxin treatment (Nolan et al., 2003; Gazzarrini et al., 2004).

Casein kinase I (CKI) is a highly conserved serine/threonine protein kinase which has been implicated in cell proliferation, apoptosis, tumorigenesis, and development in mammals (Gross and Anderson, 1998; Peters et al., 1999; Price, 2006). In yeasts and plants, CKI is involved in regulating sugar signaling (Moriya and Johnston, 2004), root development (Liu et al., 2003), and rice flowering time (Dai and Xue, 2010). In our previous study, we showed that both transgenic overexpression of \textit{GhCKI} in \textit{Arabidopsis} or the induction of high expression of \textit{GhCKI} by high temperature (HT) in early stage anthers in HT-sensitive cotton line caused male sterility. We have speculated that increased \textit{GhCKI} expression inhibited the activities of starch synthases, disrupted the homeostasis of glucose, IAA, and ABA; and caused anther abortion (Min et al., 2013; Min et al., 2014). Additionally, \textit{GhCKI} was found to be expressed strongly in NEC and globular embryos, while expression was low in embryonic callus, and overexpressing \textit{GhCKI} led to a failure of SE and a very low levels of transgenic plant production (Min et al., 2013); we therefore considered \textit{GhCKI} as a potential regulator of SE. To understand how \textit{GhCKI} alters cell fate during the transition from NEC to somatic embryos, molecular and genetic experiments were performed. Based on the analysis of upstream and downstream regulatory networks involving \textit{GhCKI}, we found that a novel gene network (\textit{LEC1-CKI-TCP15-PIF4}) regulates auxin homeostasis to affect the fate of callus cells during SE.

**RESULTS**

**Kinetics of cotton somatic embryogenesis (SE) in response to \textit{GhCKI}**
Our previous study showed that overexpressing GhCKI led to a failure of SE and a very low level of transgenic plant regeneration (Min et al., 2013). To further investigate the role of GhCKI in SE, we transformed cotton with gene constructs for both the overexpression (35S::GhCKI) and a variable region RNAi (35S::iGhCKIv), with 35S::GUS serving as a negative control (Figure S1A-D). Explants transformed with 35S::GUS and 35S::iGhCKIv underwent SE, producing embryonic callus and somatic embryos, and most explants of 35S::iGhCKIv showed more evidence of SE than 35S::GUS explants (Figure 1A and 1B). On the other hand, 35S::GhCKI explants failed to undergo SE after induction for 90 d (Figure 1C). We obtained only 2 transgenic cotton lines carrying the 35S::GhCKI following more than 20 transformation experiments using more than 4,000 explants. The 2 lines showed little increase in GhCKI expression and exhibited male sterility (Min et al., 2013). However, 45 and 83 lines carrying the 35S::iGhCKIv and 35S::GUS were isolated from 400 explants, respectively. To confirm the SE phenotypes caused by 35S::iGhCKIv, hypocotyl sections from 5 day-old seedlings of T3 plants of 35S::iGhCKIv-34 showing the lowest expression (Figure S2A) and 35S::GUS transformants (with GUS staining) were cultured for callus induction. As shown in Figure S2B-2D, more than 95% (94/100, 97/100, 95/100) of the explants from 35S::iGhCKIv-34 transgenic plants produced embryonic callus and somatic embryos at 90 d (Figure S2C, S2D), while approximately 50% (45/100, 49/100, 54/100) of the explants from 35S::GUS produced somatic embryos (Figure S2B, S2D). These results suggest that GhCKI plays a role in inhibiting SE.

To search for potential reason of GhCKI inhibited SE, changes in explants transformed with 35S::GUS, 35S::iGhCKIv, and 35S::GhCKI were determined at 25 d, 40 d, 60 d, and 90 d based on histological analysis (Figure 1D-O). Little change was observed in the cross sections of explants transformed with three type constructs at 25 d (Figure 1D-F), 40 d (Figure 1G-I), 60 d (Figure 1J-L), and 90 d (Figure 1M-O) under bright light. To detect the programmed cell death of explants transformed with three type constructs, cross sections of explants were stained by
aniline blue and observed under UV light (Figure 1a-l). No significant difference in
the strength of fluorescent signal of aniline blue under UV light was observed at 25
d. However, obvious differences were observed in transformants for longer
induction (Figure 1d-l). Compared to 35S::GUS (Figure 1d, g), the fluorescent
signals were enhanced and inhibited in 35S:iGhCKIv and 35S:GhCKI explants after
induction for 40 d (Figure 1e, f) and 60 d (Figure 1h, i) respectively. In addition,
more regularly shaped and content rich cells with strong aniline blue staining were
found in 35S::GUS and 35S:iGhCKIv 90-d induction callus (Figure M, N, j, k),
whereas 35S:GhCKI callus contained irregular and content less cells with weak
aniline blue staining (Figure O, l). These results suggest that GhCKI plays a role in
inhibiting programmed cell death during SE.

Expression pattern of GhCKI during SE
To uncover the basis of the differential SE of 35S:GhCKI and 35S::GUS (containing
natural GhCKI expression), a more detailed expression pattern analysis was
performed by comparing the activities of a cloned fragment of the native GhCKI
promoter and 35S. Hypocotyl sections of 5 day-old seedlings of 35S::GUS and
ProGhCKI::GUS T3 transgenic cotton plants were employed to induce SE in vitro,
and changes in GUS staining during SE were determined for explants that were
cultured for 0 d, 3 d, 7 d, 25 d, 40 d, 60 d, and 90 d (Figure S3A and S3B). The
results revealed a large accumulation of GUS protein throughout the entire SE
process in 35S::GUS transgenic explants. However, lower accumulations of GUS
protein were detected in ProGhCKI::GUS, especially in 25-d, 40-d, 60-d, and 90-d
explants. This GhCKI expression pattern was confirmed by quantitative RT-PCR
(qRT-PCR) in wild type explants during SE (Figure S3C). Somatic embryos usually
begin to form when explants are cultured for 40 d in cotton (Yang et al., 2012). Thus,
we considered overexpression of GhCKI might block the possibly necessary
decrease in expression of GhCKI from 25 d to 90 d, to block SE progression. The
decreased expression of GhCKI in the 25 d to 90 d cultured explants suggests there
may exist an suppressor binding site upstreaming GhCKI, and this was further
investigated.

Functional analysis of promoter fragments of GhCKI during SE in Arabidopsis

As a first step to understand the potential upstream mechanism regulating GhCKI during SE, four distinct regions based on the location of known cis-elements were cloned, with a view to analyze the functions of the GhCKI promoter (ProGhCKI) (Figure 2A). The regions from -1 to -168 bp, -169 to -478 bp, -479 to -733 bp, and -734 to -1002 bp of the ProGhCKI were designated as regions A to D, respectively. Region A contains two elements, one from -72 to -64 bp (TTAGGGT TT) and another from -94 to -88 bp (ACGTGA), which were identified as telo-boxes required for the activation of gene expression in root primordia and root hairs, respectively. A soybean embryo factor (SEF) binding site from -150 to -155 bp (TGGGTT) was also found in region A. Region B contains a CGGTTG motif that serves as a core recognition site for all MYB proteins (Urao et al., 1993) and a P-box (AACCTAAC) for MYB26 protein binding. Region C contains a core sequence of the sulfur-responsive element (SURE) containing the auxin response factor binding sequence and three SEF binding sites. Region D contains two HDZIP2ATATHB2 (TAATGATTA), which were found to interact with ATHB-2 (Ohgishi et al., 2001), a variant of the CArG motif (CAAACAAGG) for the MADS-domain protein AGL15 (Tang and Perry, 2003), and two CPBCSPOR elements for cytokinin-enhanced protein binding.

To identify the core regulatory region responsible for SE regulation in GhCKI promoter, three progressive 5′ deletions of ProGhCKI together with the whole sequence were fused with GUS (Figure 2B) and used to transform Arabidopsis. The expression patterns were determined in cultures induced from T3 seeds germinated on Arabidopsis seed somatic embryogenesis (ASSE) medium (see Experimental Procedures), as shown in Figure 2C. Region A alone (ΔBCD) showed weak GUS staining in the 7-d, 14-d, 21-d, and 30-d cultures (Figure 2C). Promoters lacking the C and D region (ΔCD) could not drive GUS expression in any of the culture stages. Strong staining for ProGhCKI::GUS and ΔD::GUS in the meristem and vascular
tissues was observed in 7-d and 14-d cultures. At 21 d and 40 d, the cultures from ProGhCKI::GUS and ΔD::GUS showed strong GUS activity. Higher GUS activities were detected at all stages of the cultured explants transformed with ProGhCKI::GUS compared with other deletion constructs.

Based on these results, we speculate that the cis-elements in region B may provide the binding site for repressors of GhCKI during SE and that regions C and D may contain cis-elements for the binding of activators of GhCKI affecting SE (Figure 2C). However, either ΔD::GUS or ProGhCKI::GUS showed significantly impaired GUS activity in the 30-d cultures (Figure 2C) when embryogenic callus and somatic embryos were generated at this stage (Lotan et al., 1998). Considering that the GUS activity of ProGhCKI::GUS transgenic tissue was significantly lower than in 35S::GUS explants at 25 d, 40 d, 60 d, and 90 d post induction (Figure S3A, S3B), we speculate that the repressors of GhCKI may bind to region B, then suppress the transcription of GhCKI at 30-d Arabidopsis cultures. In addition, we hypothesize that potential repressors of GhCKI might be highly expressed in embryogenic callus and somatic embryos under normal conditions.

The transcription of GhCKI is putatively suppressed by GhLEC1 via binding to the cis-element CTTTTC in region B of ProGhCKI

To search for potential upstream repressors of GhCKI during SE, the B region of the ProGhCKI sequence was used to screen an Arabidopsis transcription factor library using the yeast one-hybrid (Y1H) system (Ou et al., 2011). Among the transcription factors found to bind region B of ProGhCKI in yeast, AtLEC1, necessary for embryonic cell differentiation (Lotan et al., 1998), was a candidate. Therefore, the homologous protein of AtLEC1 in cotton, GhLEC1, was identified. The full-length open reading frame of GhLEC1 consists of 651 nucleotides encoding a peptide of 216 amino acid (aa) residues with a predicted molecular mass of 24 kDa (Figure S4A). GhLEC1 was found to bind the full ProGhCKI and the region B of ProGhCKI in one-to-one Y1H experiments (Figure 3A).

To determine the exact functional binding site for GhLEC1 in region B, six
distinct regions (B1 to B6) progressing from the 5′ to 3′ direction in region B were synthesized (Figure S4B). Next, we incubated his-tagged recombinant GhLEC1 (Figure S4C) with the B1 to B6 double-stranded DNA oligonucleotide probes respectively to evaluate binding using in vitro electrophoretic mobility shift assays (EMSA); two shifted bands indicative of GhLEC1 binding to B3 were detected.

Next, region B3 was deleted progressively in the 3′ to 5′ direction to design four probes (B3-1 to B3-4) for EMSA (Figure 3B). In each lane, two mobility shifts, dig-tagged B3-1 and B3-2, were observed in the polyacrylamide gels, and B3-2 showed a slightly reduced intensity of the shifted bands compared with B3-1. In contrast, only one weak band was found in B3-3, and no interaction was detected between GhLEC1 and B3-4 (Figure 3C). These results indicated that CTTTTC (ML1) is a putative binding site for GhLEC1.

To further confirm this possibility, competition experiments were performed. As shown in Figure 3D, the binding of GhLEC1 to B3 could be effectively competed by adding excessive amounts of unlabeled B3 probe (10×, 50×). Furthermore, unlabeled B3 probes containing one and two ML1-mutated (TGGGGT) sequences could be partially and completely competed for binding of GhLEC1 to the B3 fragment of the GhCKI promoter (Figure 3D). Parallel experiments indicated that GhLEC1 was unable to bind labeled B3 probes containing two ML1-mutated regions (Figure 3D). In addition, ProGhCKI containing two ML1 mutations (mProGhCKI) was also cloned and mated with GhLEC1 in yeast. No visible clones were observed (Figure 3E). These results indicate that the ML1 core sequence of ProGhCKI is required for GhLEC1 binding.

Moreover, the interaction between the GhLEC1 and ProGhCKI was quantified using a dual-luciferase reporter (DLR) system in cotton protoplasts. Compared with negative controls, GhLEC1 strongly downregulated the activity of the LUC reporter in response to ProGhCKI, whereas this activity was not changed in mProGhCKI compared with the controls (Figure 3F). These results show that GhLEC1 putatively suppresses the transcription of GhCKI via binding to the ML1 sequences of the B region of ProGhCKI in vitro.
GhCKI interacts with and phosphorylates GhTCP15 in vitro

To understand the regulatory network of which GhCKI is a component during SE, full-length GhCKI was used to screen two yeast two-hybrid (Y2H) libraries: one Arabidopsis transcription factor library, and the other a cotton embryogenic callus library. Sixty-four and 28 candidate GhCKI-interacting proteins were identified in the two libraries, respectively. Among these proteins, we identified a class I TCP transcription factor (AtTCP15) from the Arabidopsis transcription factor library and a protein from an upland cotton embryogenic callus cDNA library that was completely homologous to GbTCP (Figure S5, Hao et al., 2012). GbTCP has the greatest similarity to AtTCP15, and therefore, the protein was also named GhTCP15.

Two recent studies have demonstrated that AtTCP15 is involved in the regulation of cell division, proliferation, and differentiation (Kieffer et al., 2011; Li et al., 2012). Thus, the open reading frame of GhTCP15, encoding 344 aa with a calculated molecular weight of 37.6 kDa, was cloned and mated to GhCKI. The result showed that GhCKI interacted with GhTCP15 (Figure 4A), and the result was further verified by ß-galactosidase and bimolecular fluorescence complementation (BiFC) assays using cotton protoplasts from embryonic callus (Figure 4A and 4B).

Because GhCKI interacted with GhTCP15, and the aa sequence of GhTCP15 was found to contain several consensus CKI phosphorylation sites (Figure S6), it was necessary to determine whether GhCKI phosphorylates GhTCP15. Therefore, GhCKI and GhTCP15 proteins were synthesized using high-yield wheat germ cell-free and prokaryotic protein expression systems, respectively (Figure 4C and 4D). The GhCKI and GhTCP15 proteins were subjected to in vitro phosphorylation assays and visualized by SDS-PAGE containing Phos-tag and MnCl₂. As shown in Figure 4E, GhTCP15 was phosphorylated by GhCKI, and the kinase activity of GhCKI was disrupted by Ic261 (a CKI-specific inhibitor). The impaired density of the phosphorylation band of GhTCP15 corresponded to the appearance of lambda phosphatase, which suggested that GhCKI represents a typical CKI and is able to use GhTCP15 as a substrate for phosphorylation in vitro.
**GhLEC1, GhCKI, and GhTCP15 affect callus proliferation**

Through Y1H and Y2H experiments, *GhLEC1* and *GhTCP15* were identified as regulatory genes upstream and downstream of *GhCKI*. To further confirm the effects of *GhCKI* on SE, we employed *Arabidopsis* overexpressing *GhCKI* (OE25-6, with moderate *GhCKI* expression, showing normal male fertility similar to WT; OE15-7, with high *GhCKI* expression, showing male sterility) that were previously generated (Min et al., 2013). In addition, because a multiple sequence alignment of the conserved domain of CKI, *AtCKL1* and *AtCKL2* showed a high degree of nucleotide sequences conservation with *GhCKI* (Figure S7A), we also transformed *Arabidopsis* plants using an RNAi vector containing the conserved region of *GhCKI* (35S:iGhCKIc) (Figure S1B). Among 16 independent transformants, two iGhCKIc lines (CDi-1 and CDi-6) contained only one copy and showed significantly downregulated expression of *AtCKL1* and *AtCKL2* were used for further study (Figure S7B and S7C). In addition, *Arabidopsis* were transformed with an *GhLEC1* overexpression construct to produce lines Lov6-29 and Lov7-11 (Figure S8A and S8B). The *lec1-1* mutant (CS8101) was obtained from ABRC. It was found that the expression of *AtCKL1* and *AtCKL2* was down-regulated by overexpressing *GhLEC1* but up-regulated in *lec1-1* mutants (Figure S8C), suggesting that LEC1 acts as a repressor of the transcription of *GhCKI*.

To analyse the role of *GhTCP15* further, a homozygous T3 line with a high expression level of *GhTCP15* (OETCP6-9, Hao et al., 2012), and the tcp14-4/tcp15-3 double mutant (tcp14/tcp15) (*AtTCP14* and *AtTCP15* are two closely related genes, Kieffer et al., 2011) were collected. Then Col-WT, OE25-6, OE15-7, CDi-1, Lov6-29, lec1-1, OETCP6-9 and tcp14/tcp15 (Table 1) seeds of *Arabidopsis* were cultured on ASSE medium to conduct a holistic analysis of the effect on callus formation and growth. After induction for 21 d, OE15-7, OETCP6-9, and lec1-1 explants showed a greater callus fresh and dry weight than controls (Figure 5A-5C). Lov6-29, CDi-1 and tcp14/tcp15 produced a smaller fresh and dry weight of callus compared with Col-WT and OE25-6 (Figure 5A-5C). Moreover,
for longer induction up to 30 d, the trends for callus fresh and dry weight for the
eight genotypes were similar with those of 21 d (Figure S9). These results indicated
that *GhLEC1*, *GhCKI*, and *GhTCP15* influence callus mass during SE.

Changes in organ size and mass rely on alterations in cell size or number, or
both (Mizukami et al., 2000). In general, the cell numbers in the developing organ
depend essentially on cell proliferation (Mizukami et al., 2000). To analyze the
mechanism by which *GhCKI* affected callus mass, we compared the callus mass
ratio in Col-WT, *OE25-6*, *OE15-7*, *CDi-1*, *Lov6-29*, *lec1-1*, *OETCP6-9*, and
tcp14/tcp15 at two time points (21 d/0 d, 30 d/21 d) (Figure S9). The callus
proliferation rates of *OE15-7* and *OETCP6-9* were significantly higher, and those of
*Lov6-29* and *CDi-1* significantly lower, than those of Col-WT and *OE25-6*.
However, no significant differences in proliferation rate were seen between
tcp14/tcp15 and *lec1-1* and Col-WT or *OE25-6* (Figure S9). We also used the
tetrazolium compound

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-te
trazolium, inner salt; MTS] and an electronic coupling agent (phenazine ethosulfate)
to test proliferation at the biochemical level. Protoplasts (2×10^5) from 21 d-old
callus from each line were prepared for MTS assays, then the optical density (OD)
value at 490 nm was determined every 0.5 h for 3 h using a microplate reader. After
0.5 h, *OE15-7* showed significantly higher OD values than the other lines. From 1 h
to 3 h, the OD values of eight genotype lines in MTS assays (Figure 5D) showed the
same trends as callus proliferation rate analysis (Figure S9). In addition, the
expression of some cyclin-dependent protein kinase genes (*CYCA3;2*, *CYCD3;2,
*CYCD3;3*) associated with cell proliferation (Vandepoele et al., 2002; Dewitte et al.,
2007) was markedly increased in *OE15-7* and *OETCP6-9* and significantly
decreased in *Lov6-29* and *CDi-1* at 21 d and 30 d cultures, compared to *OE25-6* and
Col-WT. However, the expression of three cyclins was slightly changed in *lec1-1*,
except *CYCA3;2* and *CYCD3;2* which were significantly increased in *lec1-1* 30 d
cultures. In tcp14/tcp15, the expression of three cyclins was slightly increased,
except the expression of *CYCD3;3* was significantly decreased in 21 d (Figure 5E).
These results were consistent with the MTS assays (Figure 5D). Taken together, 
GhLEC1, GhCKI, and GhTCP15 may affect callus mass by regulating cell 
proliferation.

**GhLEC1-GhCKI-GhTCP15 regulates cell proliferation by regulating auxin biosynthesis**

It is well established that different cellular auxin signaling cascades are translated 
into different cellular responses, including cell expansion, cell proliferation, and cell differentiation (Benjamins and Scheres, 2008; Vanneste and Friml, 2009; Ishida et al., 2010). A high concentration of auxin has often been shown to function as an efficient initiator of callus formation and proliferation during SE, when the explants are exposed at an early stage (Leyser, 2002). In our previous study, GhCKI was found to be involved in the auxin signaling pathway (Min et al., 2014). To investigate the effects of GhCKI on auxin metabolism, the IAA contents were determined for the 21 d-induced Arabidopsis explants (Figure 6A). The OE15-7, OETCP6-9, and lec1-1 explants showed highest IAA contents; Col-WT, OE25-6, and tcp14/tcp15 showed medium IAA contents; CDi-1 and Lov6-29 showed lowest IAA contents. The IAA contents were reflected in the expression of auxin biosynthesis-related genes (AtYUC2, AtYUC5, AtYUC8) (Figure 6B). IAA content and the expression of IAA biosynthesis genes were consistent with the trends of callus mass and callus proliferation rate of the eight genotypes lines. These results indicated that GhLEC1, GhCKI, and GhTCP15 may affect cell proliferation by influencing the homeostasis of endogenous IAA through regulating the expression level of auxin biosynthesis genes.

**GhCKI might regulate auxin biosynthesis by regulating the transcription of GhPIF4 via phosphorylating GhTCP15**

GhCKI and auxin were implicated in the SE process, as suggested by the increased levels of IAA detected in 21 d callus of Arabidopsis GhCKI overexpression line (OE15-7) (Figure 6A) and in cotton explants derived from 35S::GUS, 35S::GhCKI,
and 35S:iGhCKIv (Figure 6C). Compared to 35S::GUS, the 35S::GhCKI explants showed significantly higher levels of IAA at 25 d, 60 d, and 90 d, and 35S:iGhCKIv showed significantly lower level of IAA at 60 d. The IAA contents also were reflected in the expression of auxin biosynthesis-related genes (GhYUC2 and GhYUC5) in cotton explants (Figure 6D). Then, the hypocotyls of the homozygous 35S:iGhCKIv lines were directly induced on medium lacking exogenous auxin, with WT-YZ1 and 35S::GUS serving as controls. As shown in Figure 6E, WT-YZ1, 35S::GUS, and 35S:iGhCKIv-55 (the expression of GhCKI was slightly decreased, Figure S2A) showed significantly increases in callus mass accompanied by the appearance of somatic embryos. However, the inhibition of callus induction in 35S:iGhCKIv-28 and -34 cotton explants leading to SE abortion was observed. These results further support a link between GhCKI and IAA during SE.

It is well established PHYTOCHROME-INTERACTING FACTOR GENES (PIFs) directly regulate multiple auxin biosynthesis genes by binding to G-box sequences in Arabidopsis genes promoters (Franklin et al., 2011). To further characterize a possible link between GhCKI and IAA, the expression of three cotton PIFs was measured by RT-PCR (Figure 6D), and the results showed that GhPIF4, GhPIF5, and GhPIF6 were expressed to higher levels in 35S::GhCKI cultures but to a reduced extent in 35S:iGhCKIv cultures compared to 35S::GUS, and this is consistent with the changes in the expression of GhYUC2 and GhYUC5 (Figure 6D). We therefore speculate that GhCKI may regulate auxin signaling during SE via GhPIFs regulation of YUC genes, although no direct interaction between GhCKI and GhPIFs was found.

Interestingly, there is an interaction between GhCKI and GhTCP15 (Figure 4), to examine whether the GhTCP15 phosphorylated by GhCKI affected DNA binding to GhPIF promoters, we searched the class I TCP binding sequence GTGGGNCC and the class I and II TCP common binding element site II (TGGGCC/T) (Tremousaygue et al., 2003) in the region 1.8 Kb upstream of GhPIF4, GhPIF5, and GhPIF6. The typical class I TCP binding sequence GTGGGNCC (Tremousaygue et al., 2003) appeared in the GhPIF4 promoter (proPIF4) from -935 to -942 bp. To
investigate the nature of the protein complex binding the promoter, a purified
his-tagged GhTCP15 was used to perform EMSA analysis after incubation of the
proteins with GhCKI and ATP. In the absence of GhCKI and ATP, GhTCP15 only
weakly bound to proPIF4 to form a complex (Figure 6F, lane 2). Incubation of
GhTCP15 with GhCKI and ATP prior to EMSA resulted in a strong increase in the
DNA-binding activity of GhTCP15 (Figure 6F, lane 3). However, after the addition
of 10× and 100× unlabeled proPIF4 to compete the binding, or addition of the CKI
inhibitor Ic261, the binding activities were impaired (Figure 6F, lane 4, 5, and 6).
The DNA-binding activity was also impaired when the GhTCP15 protein was
pre-treated with GhCKI and ATP and then incubated with lambda phosphatase prior
to EMSA (Figure 6F, lane 7). In contrast, incubation of GhTCP15 with both GhCKI
and ATP using the proPIF4 mutant (mproPIF4) as the competitor resulted in no
detectable differences in binding activity (Figure 6F, lane 8 and 9). The results
implied that GhCKI phosphorylates GhTCP15 to regulate the transcription of
GhPIF4, and thereby may regulate auxin biosynthesis to affect cell proliferation.

**GhLEC1, GhCKI, and GhTCP15 affect cell differentiation**

GhLEC1, GhCKI, and GhTCP15 may affect cell proliferation by regulating auxin
biosynthesis, but whether GhLEC1, GhCKI, and GhTCP15 affects the production of
competent embryogenic cells and subsequent formation of embryogenic callus and
somatic embryos requires further investigation. Somatic embryos can develop from
embryogenic callus derived from competent embryogenic cells. Genes known to
induce SE when overexpressed include PLANT GROWTH ACTIVATOR 6
(PGA6/WUS) and BABY BOOM (BBM), which play predominant roles in
maintaining embryonic cell identity (Boutilier et al., 2002; Zuo et al., 2002; Hecht
et al., 2001). Therefore, the expression levels of PGA6/WUS and BBM were
examined at 21 d in callus derived from Col-WT, OE25-6, OE15-7, CDi-1, Lov6-29,
lec1-1, OETCP6-9, and tcp14/tcp15.

No significant differences in the expression of PGA6/WUS and BBM were
observed in Col-WT, OE25-6, OE15-7, lec1-1, OETCP6-9, and tcp14/tcp15 callus.
However, in CDi-1 and Lov6-29 tissues, the expression of PGA6 and BBM genes was slightly increased compared to Col-WT (Figure S10A). The results indicate that AtLEC1 mutant (lec1-1) and overexpression of GhCKI and GhTCP15 did not affect the production of competent embryogenic cells from NEC. In order to check whether GhLEC1, GhCKI, and GhTCP15 expression was associated with the formation of embryogenic callus and somatic embryos, the expression levels of AtLEC1, AtFUS3, and AtABI3 in 30-d callus of eight genotypes lines were analysed by qRT-PCR (Figure S10B). AtLEC1 was expressed at low levels in OE15-7, OETCP6-9, and tcp14/tcp15, but undetectable in lec1-1. AtFUS3 and AtABI3 genes were highly expressed in 30 d-old callus of CDi-1 and Lov6-29, expressed at intermediate levels in Col-WT, OE25-6, lec1-1, and tcp14/tcp15, but expressed at low levels in OE15-7 and OETCP6-9 (Figure S10B).

The 30 d-old callus represents the stage in which somatic embryos are formed (Lotan et al., 1998), as illustrated by transmission electron microscopy (TEM). The cultures of OE15-7, OETCP6-9, and lec1-1 consisted of abnormal cells with a large central vacuole, thin cytoplasm, and few organelles; the other cultures consisted of cells with identical diameters and dense cytoplasms, small vacuoles, rich, starchy grains, and large nuclei (Figure S11). In plants, non-embryogenic cells are characterized by a large central vacuole and thin cytoplasm, while embryogenic callus cells typically have dense cytoplasm, small vacuoles and large nuclei. In addition, the mitochondria in OE15-7, OETCP6-9, and lec1-1 cultured cells showed a complete outer membrane and no internal vacuolization while cells of Col-WT, OE25-6, CDi-1, Lov6-29, and tcp14/15 displayed internal vacuolization in the mitochondrias. These observations suggest that overexpression of GhLEC1 may promote somatic embryo formation, while overexpression of GhCKI and GhTCP15 suppress somatic embryo formation by promoting cell proliferation and decreasing the competence for embryogenic cell differentiation.

**Discussion**

**GhCKI is a negative regulator of somatic embryo formation**
The Y1H system revealed GhLEC1 binds to the promoter of GhCKI. LEC1 is required for the normal development of both early and late embryo morphogenetic events and efficiently promotes embryonic cell differentiation from vegetative cells; the lec1-1 mutation causes defective embryo maturation (Lotan et al., 1998). However, a credible target binding site for LEC1 has not been identified previously. LEC1, which encodes a HAP3 subunit of the CCAAT, is presumed to interact with the other HAP subunits to form a complex that binds a CCAAT-box (Lotan et al., 1998). However, no consensus CCAAT DNA sequence was found in the promoter of GhCKI. In addition, LEC1 belongs to the NF-YB family, and many studies have demonstrated that some NF-YB members (such as LEC2, ABI3, and FUS3) can bind to the RY/Sph motif ([A/C]TGCATG) (Braybrook et al., 2006), whereas no typical RY/Sph motif was found within ProGhCKI. Based on the expression profile analysis of the 5′ deleted ProGhCKI, we found that region B was sufficient for GhLEC1 binding. EMSA assays were performed between GhLEC1 and the 5′ deletions of region B in ProGhCKI, and the results suggested that B3 is critical for the GhLEC1 binding. Further EMSA assays demonstrated that ML1 (CTTTTC) is the specific recognition site for GhLEC1, which is the first LEC1 binding site to be identified.

Previous studies have found genes that are regulated by LEC2 through microarray analysis (Braybrook et al., 2006), and AGL15 was determined to be a direct target gene based on the ChIP-chip approach and Affymetrix tiling arrays (Zheng et al., 2009). However, no reports have described direct target genes of LEC1. Confirmation of the ML1 motif provided evidence for the identification of potential targets that are directly regulated by LEC1. We and others have shown that ectopic overexpression of LEC1 promoted cellular processes by enhancing embryo induction in vegetative cells, suggesting the LEC1 gene is necessary and sufficient for the induction of somatic embryo formation. As a direct target of GhLEC1, GhCKI showed an opposite expression pattern compared with GhLEC1, as confirmed by EMSA and LUC assays that GhLEC1 directly suppressed the expression of GhCKI. Because CDi-1 showed phenotypes similar to GhLEC1
overexpression lines (Figure 5) and overexpression of \textit{GhCKI} in cotton and
\textit{Arabidopsis} inhibited somatic embryo formation, it is possible that GhCKI plays a
role distinct from that of GhLEC1 in SE and acts as a negative regulator of somatic
embryo formation.

Both \textit{GhCKI} and \textit{GhTCP15} are key genes in cellular proliferation

We have described the interaction of GhCKI with GhTCP15 and their similar
eexpression patterns during SE. Overexpression of \textit{GhTCP15} resulted in a phenotype
similar to that observed for the overexpression of \textit{GhCKI} (Figure 5). TCP proteins
are plant-specific transcription factors with important roles in multiple plant
developmental events and are associated with cell proliferation (Kieffer et al., 2011;
Li et al., 2005). Li et al. (2005) reported that class I and II TCP factors function in
an opposite manner in plant cell growth and proliferation, with class II TCP factors
inhibiting but class I factors promoting cell proliferation. GhTCP15, a homolog of
AtTCP15, is a class I TCP protein. Therefore, we propose that GhTCP15 and its
interactive protein GhCKI are both critical for cell proliferation because they may
regulate the expression of cell cycle-associated genes. Class I TCP proteins regulate
the expression of the \textit{CYCB1;1} gene in the G2/M phase of the cell cycle and of
proliferation cellular nuclear antigen 2 during the G1/S transition to control the
balance between cell proliferation and endoreduplication (Boudolf et al., 2004).
Class II TCP proteins reduce the expression of histones and cyclins (cell cycle
marker genes) to negatively regulate cell growth (Gaudin et al., 2000). Interestingly,
\textit{CYCP3;1}, \textit{CYCP3;2}, and one cyclin \textit{A} gene were all upregulated in \textit{Arabidopsis} root
or cotton fibers by overexpressing \textit{GbTCP} (\textit{GhTCP15}; Hao et al., 2012), and the
binding site for TCP protein was often found together with a telo box in the
promoters of cell cycle-related genes (Tremousaygue et al., 2003). In our Y1H
assays, AtTCP4 potentially regulated \textit{GhCKI} (data not shown), and the telo box was
also found in the promoter of \textit{GhCKI} (Figure 3). GhCKI is a member of the CKI
family, and studies in mammals have demonstrated that CKI is involved in circadian
rhythms, DNA repair pathways, and cell cycle progression, among others (Peters et
al., 1999; Dhillon and Hoekstra, 1994; Behrend et al., 2000). Considering these findings together, we propose that GhCKI might participate in or regulate cell cycle progression. Thus, it is possible that the overexpression of GhCKI or GhTCP15 accelerates the cell cycle and ultimately causes excessive callus proliferation.

In addition to the excessive callus proliferation observed both in GhCKI- and GhTCP15-overexpressing Arabidopsis, more branches, more flowers, and shorter siliques with few seeds were also observed in the GhTCP15 highly overexpressing lines (Hao et al., 2012), which were similar to those observed in OE15-7 with high expression of GhCKI (Min et al., 2013). These data suggest that both GhCKI and GhTCP15 cooperatively regulate multiple developmental processes.

**GhCKI coordinates cell proliferation and differentiation by altering auxin homeostasis**

We found that GhCKI positively regulates the callus mass, with a smaller mass in the RNAi line CDi-1 and a larger mass in the overexpresser OE15-7. Increase in callus mass is determined primarily by cell proliferation rather than water uptake, shown by the increasing dry weight (Figure 5C). This finding suggests that GhCKI positively regulates cell proliferation. Somatic embryo formation is regulated by the coordination of cellular proliferation and differentiation, and plant hormones are involved in regulating these processes. Auxin, ethylene, ABA, GA, brassinosteroids, and cytokinin have been reported to participate in callus formation, development, and SE processes (Depuydt and Hardtke, 2011; Goren et al., 1979). Of these, auxin and cytokinin are the most studied hormones in SE; the balance between these two types of hormones determines cell proliferation and differentiation (Skoog and Miller, 1957).

Interestingly, LEC1 upregulated the auxin biosynthesis gene YUC10 (Junker et al., 2012). In our study, GhLEC1 directly but negatively regulated GhCKI, which involved in the auxin (Min et al., 2014) biosynthetic pathways, indicating that GhLEC1 and GhCKI have essential functions in auxin homeostasis during SE. AtTCP15 was involved in the alteration in auxin homeostasis (Kieffer et al., 2011).
Furthermore, a high concentration of auxin has often been shown to function as an efficient initiator of callus formation and proliferation during SE, when the explants are exposed at an early stage (Leyser, 2002). But a high concentration of auxin inhibited the callus differentiation and somatic embryo formation (Filonova et al., 2000). Thus, we propose that auxin homeostasis play roles in the balance of cell proliferation and cell differentiation. Overexpression of \textit{GhCKI} or \textit{GhTCP15} disrupted auxin homeostasis (Figure 6A and 6C), which altered the transition from cell proliferation to cell differentiation, resulting in the arrest of somatic embryo formation.

**Conclusion**

Based on an integration of the relationships between different morphologies and biochemical changes during SE in the \textit{Arabidopsis} and cotton lines, we speculate that \textit{GhCKI} plays a positive role in explant dedifferentiation, NEC formation and cell proliferation by phosphorylating \textit{GhTCP15}. Phosphorylated \textit{GhTCP15} has an enhanced binding to the promoter of \textit{GhPIF4} to regulate the transcription of \textit{GhPIF4}, thereby regulating auxin biosynthesis at an early stage of SE. During later stages of SE, \textit{GhLEC1} expression was initiated, repressing the transcription of \textit{GhCKI} via binding to the cis-element CTTTTC in the promoter of \textit{GhCKI}. This might reduce \textit{GhTCP15} phosphorylation activity, decrease the transcription of \textit{GhPIF4} and auxin biosynthesis genes, to promote the transition from cell proliferation to cell differentiation and somatic embryo formation (Figure 7).

**EXPERIMENTAL PROCEDURES**

**Plant materials and culture media**

Seeds of YZ1 (\textit{G. hirsutum}), \textit{Arabidopsis} transgenic plants [Col-0], and the mutant lines (\textit{lec1-1}, \textit{Arabidopsis} [Ws]); \textit{tcp14/15}, \textit{Arabidopsis} [Col-0]) were sterilized and washed as previously described (Hu et al., 2011; Zhang et al., 2006). Cotton seeds were germinated on 1/2 MS medium supplemented with 1.5% (m/v) glucose, and solidified with 0.25% (m/v) phytagel (Sigma, USA) at 28°C in the dark for 7 days.
The hypocotyls of the seedling were cut into 0.5-0.8 cm sections and used as explants for callus induction or transformation. Callus induction was carried out on basic MSB medium (MS and B5 vitamins) containing 3% (m/v) glucose, 0.25% (m/v) phytagel, 0.1 mg/L 2,4-D, and 0.1 mg/L kinetin for 25 d. Then explants were subcultured on MSB medium supplemented with 3% (m/v) glucose, 0.25% (m/v) phytagel, 0.5 mg/L indolebutyric acid, and 0.1 mg/L kinetin for induction of embryogenic callus or for sampling. Embryogenic callus were used for protoplast isolation and cultured on MSB medium containing 3% (m/v) glucose, 0.25% (m/v) phytagel, 0.5 mg/L indolebutyric acid and 0.15 mg/L kinetin. The sterilized Arabidopsis seeds were cultured on ASSE medium [basic MSB medium containing 2.0 mg/L 2,4-D, 1.0 mg/L 6-BA, 0.3 g/L casein acid hydrolysate, 0.5 g/L glutamine, 0.5 g/L proline, 3% sucrose, and 0.25% phytagel (pH=5.8)] at 28°C in the dark.

**Vector construction and transformation**

The 1002-bp upstream sequence of the *GhCKI* start codon (*ProGhCKI*) was cloned (Min et al., 2013), and the putative cis-elements were predicted using the PLACE database (Higo et al., 1999). *ProGhCKI* and its three progressive 5′ deletions were identified and inserted into Gateway vector pGWB433 (Nakagawa et al., 2007) and fused to *GUS* by BP and LR recombination (Invitrogen) to obtain vectors ΔBCD::GUS, ΔCD::GUS, ΔD::GUS, and *ProGhCKI*:GUS. The isolation of *GhCKI* and *GhTCP15* cDNAs and the construction of their overexpression vectors were performed as previously described (Min et al., 2013; Hao et al., 2012). An empty pCAMBIA2300S vector (35S::GUS) served as a control. The full-length *GhLEC1* ORF was obtained from RNA extracted from cotton EC as a template, and the encoded protein was found to be 53% homologous to the AtLEC1 protein using Clustalx 1.83. The *GhLEC1* overexpression vector was constructed with pK2GW7 by BP and LR reactions using primers Lov-F/R. Two RNAi constructs carrying *GhCKI* conserved and variable regions (35S:iGhCKIc and 35S:iGhCKIv) were constructed using pHellsgate 4 through BP recombination reactions. The *GhCKI*, *GhTCP15*, *GhLEC1* overexpression constructs, ΔBCD::GUS, ΔCD::GUS, and...
$\Delta D::GUS$ constructs, and $\text{ProGhCKI}::GUS$ and $35S::i\text{GhCKIc}$ constructs were used to transform Arabidopsis (Col-0) by the floral dip method (Zhang et al., 2006). $\text{GhCKI}$ overexpression and $35S::i\text{GhCKIv}$, $\text{ProGhCKI}::GUS$, and $35S::GUS$ constructs were introduced into cotton (YZ1) by transforming hypocotyl sections via Agrobacterium tumefaciens (Jin et al., 2005). The primers used in this study are listed in Table S1.

**GUS activity and histochemical and microscopic analyses**

The histochemical localization of GUS activity in $\text{ProGhCKI}::GUS$, $35S::GUS$, $\Delta BCD::GUS$, $\Delta CD::GUS$, and $\Delta D::GUS$ cotton and Arabidopsis transformants was performed as described by Min et al. (2013). To evaluate the potential production of embryogenic callus, cotton explants that were transformed with $35S::GUS$, $35S::\text{GhCKI}$, and $35S::i\text{GhCKIv}$, callus of transgenic Arabidopsis plants and mutant lines from different culture times were imaged using a Nikon D40 camera. The cotton explants were sectioned into 8-μm-thick slices with a microtome, stained with 0.1% aniline blue, and observed according to Min et al. (2014). Electronic microscopic analyses (TEM) of the callus were conducted as previously described (Min et al., 2013).

**Yeast one-hybrid assay**

To search for potential upstream regulators of \text{GhCKI} during SE, the $\text{ProGhCKI}$ sequence was amplified using the primer pair $\text{ProGhCKI-Y1H-F/R}$ with adapters containing $\text{EcoRI-XbaI}$ digestion sites and cloned into the pHisi-1 vector to generate pHisi-1-$\text{ProGhCKI}$. The vectors were linearized with XhoI and then transferred into YM4271 yeast strains to screen an Arabidopsis transcription factor library (Ou et al., 2011). Positive clones were selected on SD-Trp-His medium with different concentrations of 3-AT for 3-5 d at 30°C. To characterize the interaction between \text{GhLEC1} and the promoter regions of \text{GhCKI} in yeast, the full-length $\text{GhLEC1}$ cDNA was cloned into a pDEST22 vector using the Gateway LR reaction (Invitrogen) and then transferred into the Y187 yeast strain. The different regions of
ProGhCKI and mProGhCKI were amplified using specific primer pairs (Table S1) to generate the pHisi-1-ProGhCKI A, B, C, D, and pHisi-1-mProGhCKI vectors as described above. A mating-based Y1H assay was conducted according to the protocol described by Ou et al. (2011). The primers used in the Y1H assay are listed in Table S1.

Electrophoretic mobility shift assays
GhLEC1 and GhTCP15 were cloned into a pET-28a vector for expression induction in *Escherichia coli* BL21 strain with 1 mM IPTG (isopropyl-1-thio-b-D-galactopyranoside) for 4 h at 18°C. His-tagged GhLEC1 and GhTCP15 were extracted and purified using the MagneHis™ protein purification kit (Promega). The promoter regions of *GhCKI* and *GhPIF4* were synthesized and used as probes. Probe labeling, DNA-protein binding reactions, and probe shift detection were performed according to the user manual of a 2nd Generation DIG gel shift kit (Roche) as described by Li et al. (2014). The primers and oligonucleotides used in the EMSA assay are listed in Table S1.

Dual-luciferase reporter assays
The dual-luciferase reporter assays were performed as described previously (Hao et al., 2010). The fragment of *GhLEC1* was obtained by PCR using the primer pair GhLEC1-GAL4BD-F/R (Table S1). The PCR product was then ligated into the 35S-pBDGAL4 vector using the In-fusion HD Cloning Kit (Clontech) to obtain plasmid 35S-GhLEC1 as an effector; the None and 35S-pBDGAL4 vectors served as negative controls. The *ProGhCKI* sequence containing two CTTTTC core sequences was amplified using the primer pair ProGhCKI-LUC-F/R with adapters containing *HindIII-SalI* digestion sites utilizing *ProGhCKI::GUS* vector as a template, and the relevant *mProGhCKI* sequence containing two CTTTTC-mutated versions was obtained by overlap extension PCR. The two fragments were inserted into GAL4-LUC to generate *ProGhCKI*-GAL4-LUC and *mProGhCKI*-GAL4-LUC as reporter constructs, with *AtUbiquitin3*-Renilla-LUC as an internal control. To
analyze the interaction between GhLEC1 and ProGhCKI, protoplasts were isolated from cotton ECs according to Yang et al. (2008), and effector, reporter, and internal controls (6 μg, 6 μg, and 0.5 μg, respectively) were co-transformed into protoplasts using PEG4000. The transformed protoplasts were cultured at 28°C in the dark for 16 h, and firefly and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and the Multimode Plate Reader (PerkinElmer).

**Yeast two-hybrid assay**

The bait construct (pDEST32-GhCKI) was prepared as previously described (Min et al., 2013). An Arabidopsis transcription factor library was kindly provided by L. J. Qu, Peking University, and a cotton SE cDNA library was prepared from ECs using Matchmaker Library Construction and Screening Kits (Clontech). The two libraries were screened using pDEST32-GhCKI by yeast mating. To confirm the interaction between GhCKI and GhTCP15, the full-length CDS of GhTCP15 was amplified using the primer pair GhTCP15-pDEST22-F/R and cloned into pDEST22 via the Gateway LR reaction (Invitrogen) to generate pDEST22-GhTCP15. Next, pDEST22-GhTCP15 was introduced into yeast strain Y187, which was mated with the AH109 yeast strain containing pDEST32-GhCKI and selected on SD-Leu-Trp, SD-Leu-Trp-His, or SD-Leu-Trp-His+X-α-gal.

**BiFC assays**

BiFC assays were performed as described (Waadt et al., 2008). For generation of the BiFC constructs, the VenusC:GhCKI was constructed (Min et al., 2013), and the ORF of GhTCP15 was amplified with primer pair GhTCP15-VN-F/R (Table S1) and cloned at the SalI site of pVYNE(R) by In-fusion enzyme to obtain the VenusN:GhTCP15. Protoplasts were prepared from cotton ECs and the following procedures were performed as our previous description (Min et al., 2013).

**Cell-free protein expression**
Cell-free protein expression was performed using the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega). To generate the protein expression vector, the ORF of *GhCKI* was amplified using the primer pair *GhCKI*-CF-F/R (Table S1) and cloned into the *Sgf*I-*Pme*I sites in pF3KWG (BYDV) Flexi vector, resulting in pF3KWG-*GhCKI*. The mixture of pF3KWG-*GhCKI* and TNT SP6 High-Yield Wheat Germ Master was incubated at 25°C for 2 h for translation. Mixtures containing no DNA and Luciferase SP6 Control DNA served as negative and positive control reactions, respectively. The translation products were detected by SDS-PAGE followed by blotting with conserved or specific *GhCKI* antibodies.

**In vitro phosphorylation and dephosphorylation assays**

*In vitro* kinase assays for *GhCKI* were performed according to the manufacturer’s instructions using the Casein Kinase I Assay Kit (Sigma) with minor modifications. Cell-free-expressed *GhCKI* as the kinase and *Escherichia coli*-induced and expressed *GhTCP15* as the substrate protein were mixed with 1× kinase assay buffer (40 mM HEPES, pH 7.5, 130 mM KCl, 10 mM MgCl₂, 0.01 mM ATP, 5 mM DTT, 5 mM β-glycerophosphate, and 0.2 mM sodium orthovanadate; Sigma) and 0.1 mM ATP and ddH₂O or inhibitor Ic261 in a total volume of 24 μL. The reactions were incubated at 37°C for 15 min and then stopped by boiling for 5 min. For the dephosphorylation assays, the products of *GhTCP15* phosphorylation by *GhCKI* were incubated with 1× dephosphorylation assay buffer (50 mM, HEPES, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% Brij35, and 1 mM MnCl₂) and 40 units LambdaPPase at 30°C for 1 h. The phosphorylation and dephosphorylation assay products were separated by electrophoresis using Phos-tag™ SDS-PAGE (8% acrylamide gels, 0.375 M Tris, 0.1% SDS, 0.1 mM MnCl₂, 0.05 mM Phos-tag™ AAL-107), and the gels were stained, destained, and visualized.

**RT-PCR and qRT-PCR**

To confirm if the transformants are obtained, RT-PCR and qRT-PCR analysis were performed as described in a previous study (Min *et al.*, 2014). *GhUB7* and *AtACT7*
were used as internal controls for cotton and *Arabidopsis*, respectively. The primers used in the study are listed in Table S1.

**IAA determination**

For determination of endogenous IAA concentrations, samples were homogenized in 1 ml of 80% cold methanol and shaken at 4°C overnight in the dark. Further extraction was performed as described previously (Liu et al., 2012), and the extracts dissolution, filtration, storage, and quantifications of endogenous IAA were according to our previous report (Min et al., 2014).

**Callus proliferation and MTS assays**

Seeds from eight *Arabidopsis* lines, including Col-WT, *OE25-6, OE15-7, CDi-1, Lov6-29, lecl-1, OETCP6-9*, and *tcp14/15* were cultured on ASSE medium; for each line, more than six replicates were assessed. After culturing for 7 d, 14 d, 21 d, and 30 d, the cultures were harvested and weighed. The average weight of each line from five cultures was used to calculate the proliferation rate.

The MTS assay was also used to measure cell proliferation using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega). In the MTS assays, the protoplast preparation and culture conditions were performed as previously described (Hu et al., 2011), and the number of protoplasts was counted with a hemacytometer under light microscopy. Protoplasts (2×10⁵) were cultured in 100 μL W5 medium, and 20 μL CellTiter 96 Aqueous One Solution reagent was added to quantify MTS at 23°C based on four replicates. The OD values at 490 nm at different time points were determined using a Multimode Plate Reader (PerkinElmer).

**Statistics**

Each graphical data point represents the results of multiple independent experiments (*n* ≥ 3), and the means ± s.e.m are shown. The *p* values for physiological parameters (fresh weight, dry weight, cell proliferation rate, and LUC activity) were evaluated...
based on the shortest significant ranges (SSR; \( p \) values <0.05) post hoc analysis, and values that did not share a common letter were considered statistically significant. Statistically significant results for the content of IAA and qRT-PCR were determined using the two-tailed unpaired Student’s \( t \)-test, and \( p \) values <0.05 were considered statistically significant.

Accession numbers

The accession numbers in this article were shown in Table S1.

SUPPLEMENTAL DATA

Figure S1. Constructs used for cotton and Arabidopsis transformation.

Figure S2. Comparison of embryogenic callus (EC) formation in the control and \( GhCKI \) variable region RNAi transformants.

Figure S3. Expression pattern of \( GhCKI \) during SE.

Figure S4. Isolation and expression of \( GhLEC1 \).

Figure S5. Sequence alignment between \( GbTCP \) and \( GhTCP15 \).

Figure S6. Predicted phosphorylation sites by CKI in \( GhTCP15 \).

Figure S7. Downregulation expression of \( GhCKI \) homologous genes in Arabidopsis by RNAi.

Figure S8. Isolation of ectopically overexpressed \( GhLEC1 \) Arabidopsis plants.

Figure S9. Effects of \( GhLEC1 \), \( GhCKI \), and \( GhTCP15 \) on callus mass during SE.

Figure S10: \( GhLEC1-GhCKI-GhTCP15 \) governs the switch from cell proliferation to cell differentiation by regulating the homeostasis of auxin.

Figure S11. TEM analyses of cultures after induction for 30 d in WT-Col, \( OE25-6 \), \( OE15-7 \), \( CDi-1 \), \( Lov6-29 \), \( lec1-1 \), \( OETCP6-9 \), and \( tcp14/15 \).

Table S1. Oligonucleotides used in this study.

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AUTHOR CONTRIBUTIONS

X.Z. and L.M. designed the experiment. L.M., Q.H., Y.L., J.X., and Y.M. performed the experiments. L.M., X.Z., L.Z., and X.Y. analyzed the data. L.M. and X.Z. wrote the article.

FIGURE LEGENDS

Figure 1: Schematic representation and histological observation of explants at different induction stages for 35S::GUS, 35S:iGhCKIv, and 35S:GhCKI transformants.

(A-C) Transformants from 35S::GUS (A), 35S:iGhCKIv (B), and 35S:GhCKI (C) induced for 90 d. No embryogenic callus were observed in the GhCKI-overexpressed explants (35S:GhCKI), but most explants of 35S:iGhCKIv showed high quality embryogenic callus (red arrows), better than the control explants (35S::GUS).

(D-O) Cross sections of explants from 35S::GUS (D, G, J, M), 35S:iGhCKIv (E, H, K, N), and 35S:GhCKI (F, I, L, O) induced for 25 d (D, E, F), 40 d (G, H, I), 60 d (J, K, L), and 90 d (M, N, O) under bright light condition. No significant differences were observed in the explants transformed with three type constructs at 25 d, 40 d, 60 d, and 90 d under bright light.

(a-l) Cross sections of explants from 35S::GUS, 35S:iGhCKIv, and 35S:GhCKI were stained with aniline blue to detect cell growth and death, as shown by bright blue fluorescence under ultraviolet light condition. Explants of 35S::GUS (a, d, g, j), 35S:iGhCKIv (b, e, h, k), and 35S:GhCKI (c, f, i, l) were induced for 25 d (a, b, c), 40 d (d, e, f), 60 d (g, h, i), and 90 d (j, k, l). No significant differences were observed in the density of blue fluorescence in explants at 25 d. However, explants of 35S:iGhCKIv and 35S:GhCKI at 40 d, 60 d, and 90 d showed strong and weak blue
fluorescence respectively, compared with 35S::GUS (control). UV, ultraviolet light; Bars in (A-C), 1 cm. Bars in (D-O, a-l), 100 μm.

**Figure 2:** Diagram showing the deletion of the GhCKI promoter (ProGhCKI) and expression patterns of GUS driven by full and truncated ProGhCKI sequence in transgenic Arabidopsis plants.

(A) Structure of the ProGhCKI sequence. Regions A, B, C, and D ranged from -1 to -168 bp, -169 to -478 bp, -479 to -733 bp, and -734 to -1002 bp of ProGhCKI, respectively. Partially predicted cis-regulatory elements in different regions of ProGhCKI are labeled.

(B and C) Deletion of the GhCKI promoter fused to GUS protein (B) and comparison of GUS expression in transformed Arabidopsis among different constructs (C). The Δbcd, Δcd, and Δd indicate regions A, A+B, and A+B+C, respectively. The 7 d, 14 d, 21 d, and 30 d indicate different culture times. Bars, 2 mm.

**Figure 3:** Expression of GhCKI was directly suppressed by GhLEC1 via binding to the cis-element CTTTTC upstream of GhCKI

(A) Y1H assays showing the binding of GhLEC1 to region B in the promoter of GhCKI. Regions A, B, C, and D ranged from -1 to -168 bp, -169 to -478 bp, -479 to -733 bp, and -734 to -1002 bp of ProGhCKI, respectively. Empty pDEST22 vector was used as a negative control. The growth of transformants on SD-Trp-His+8 mM 3-AT medium showed that GhLEC1 was able to bind to the GhCKI promoter.

(B) Truncated B3 region of the ProGhCKI sequences and different mutated versions, as indicated in the text. The underlined ML1 and mML1 are the CTTTTC core sequence and mutated CTTTTC (TGGGGT) sequence, respectively.

(C) and (D) EMSA analysis of GhLEC1 protein interacting with dig-labeled oligonucleotide, as shown in (B). (C) The binding status between GhLEC1 and different B3 regions of ProGhCKI deleted progressively in the 3' to 5' direction. (D) GhLEC1 binding to the B3 region of ProGhCKI was confirmed by competition
experiments via ML1 core sequence mutation. The figure below shows no binding between GhLEC1 and B3 with two ML1 mutations. Black arrows in C and D indicate shifted bands. FP, free probe.

(E) GhLEC1 failed to bind ProGhCKI when two ML1s were mutated (mProGhCKI) in the Y1H assays. Empty pDEST22 vector served as a negative control.

SD-Trp-His+8 mM 3-AT medium was used to check the binding.

(F) Transcriptional suppression of GhLEC1 on the promoter of GhCKI in vivo. (a) Effectors prepared for cotton EC protoplast transient assays. 35S, promoter of cauliflower mosaic virus; Ω, translation enhancer; Nos, nopaline synthase; GAL4BD, GAL4 DNA-binding domain. (b) Schematic representation of the reporters and GhLEC1 suppression of GhCKI transcription as revealed by the relative LUC activity. The effectors (None, GAL4BD, and GhLEC1) and reporters (ProGhCKI:LUC and mProGhCKI:LUC) were co-transformed. GhLEC1 displayed high and no repression activity for the transcription of wild-type and mutated GhCKI promoters, respectively. None and GAL4BD served as negative controls.

Figure 4: GhCKI interacted with and phosphorylated GhTCP15.

(A) GhCKI interacts with GhTCP15 in Y2H assays. The empty pDEST22 and pDEST32 were used as negative controls. Blue colonies on SD-Trp-Leu-His+X-α-gal medium indicated positive interactions.

(B) BiFC assay showing the interaction of GhCKI with GhTCP15 in vivo. The yellow fluorescence indicates a positive interaction. The expression of GhCKI or GhTCP15 alone (GhCKI-VYNE or VYCE-GhTCP15) in cotton protoplasts was assessed as a negative control. YFP, yellow fluorescence protein.

(C) GhCKI protein expressed via cell-free protein expression assays were detected by western blot. CF-None indicates mixture containing no DNA as a negative control reaction. pF3KWG-GhCKI indicates cell-free expression mixture with expressed GhCKI. Sp and Cp indicate antibodies prepared using the specific and conserved domain sequences, respectively, of the GhCKI protein as probes. Black arrow indicates hybridization signals.
Induced expression of His-tagged GhTCP15 was verified by western blot. The molecular weight of expressed GhTCP15 consisted with the calculated molecular weight (37.6 kDa).

Phosphorylation of GhTCP15 by GhCKI was verified by in vitro phosphorylation assays using Phos-tag SDS-PAGE. Ic261, CKI inhibitor; LambdaPPase, Lambda protein phosphatase. Red arrows indicate positive phosphorylation.

Figure 5: Ectopic expression of GhLEC1, GhCKI, and GhTCP15 in Arabidopsis affects the callus proliferation.

(A) Callus growth of WT-Col, OE25-6, OE15-7, CDi-1, Lov6-29, lec1-1, OETCP6-9, and tcp14/15 at 21 d. Callus in red cycles are highlighted on the right side of the culture plate. Bars, 1 cm in the culture plate; 1 mm in the highlighted pictures.

(B) and (C) Determination of the fresh weight (B) and dry weight (C) of WT-Col, OE25-6, OE15-7, CDi-1, Lov6-29, lec1-1, OETCP6-9, and tcp14/15 cultures cultured for 21 d. The fresh and dry weights were calculated as grams of callus from five explants.

(D) MTS assays. Protoplasts (2×10^5) were prepared from cultures of WT-Col, OE25-6, OE15-7, CDi-1, Lov6-29, lec1-1, OETCP6-9, and tcp14/15 after 21 d of culturing and then cultured in 100 μl W5 with MTS for 0.5 h to 3 h for detecting OD values.

(E) Expression levels for cell proliferation related genes (CYCA3;2, CYCD3;2, CYCD3;3) were validated by qRT-PCR in cultures of WT-Col, OE25-6, OE15-7, CDi-1, Lov6-29, lec1-1, OETCP6-9, and tcp14/15 after induction for 21 d and 30 d. CYCA3;2, Cyclin-dependent protein kinase 3;2; CYCD3;2, CYCLIN D3;2 CYCD3;3, CYCLIN D3;3.

The data in (B, C, D) represent the means ± s.e.m of biologically independent experiments (n ≥ 3). Values in (B, C) not sharing a common letter were considered statistically significant (SSR; p<0.05). Asterisks in (D) indicate statistically significant differences between different lines and wild-type (*; P<0.05, **; P<0.01,
Figure 6: GhCKI involves in auxin biosynthesis by regulating the transcription of GhPIF4 through phosphorylating GhTCP15.

(A) IAA contents of WT-Col, OE25-6, OE15-7, CDi-1, Lov6-29, lec1-1, OETCP6-9, and tcp14/15 in 21-d cultures. The data represent the means ± s.e.m of biologically independent experiments (n ≥ 3). Values not sharing a common letter were considered statistically significant (SSR; p<0.05).

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(C) Determination of IAA content in 35S::GUS, 35S:GhCKI, and 35S:iGhCKIv cultures at different culture time points of transgenic cotton explants. The data represent the means ± s.e.m of three biologically independent experiments. Red asterisks indicate statistically significant differences between 35S:GhCKI and 35S::GUS. Black asterisks indicate statistically significant differences between 35S:iGhCKIv and 35S::GUS (*; P<0.05, **; P<0.01).

(D) RT-PCR showing the expression of PIFs and auxin biosynthesis genes in 35S::GUS, 35S:GhCKI, and 35S:iGhCKIv transgenic cotton explants. YUC, YUCCA; PIFs, phytochrome-interacting factor genes. GhUB7 (Cotton Ubiquitin 7) was used as a control. The 7 d-90 d indicate explants transformed with different constructs were cultured on callus induction medium without antibiotics for 2 d (co-culture), followed by transfer to induction medium containing antibiotics and cultured for 7 d-90 d.

(E) Homozygous 35S:iGhCKIv transgenic lines were cultured on medium without applying exogenous auxin. WT-YZ1 and 35S::GUS act as controls showed significantly increases in callus mass accompanied by the appearance of somatic
embryos. However, the inhibition of callus induction in 35S:iGhCKIv-28 and -34 cotton explants led to SE abortion were observed. Callus in red circles are highlighted in the right-hand panels. Bars, 1 cm.

(F) EMSA assay demonstrating the effects of GhCKI on the binding of GhTCP15 to labeled GhTP4 probe carrying a GTGGGACC core element in the promoter of GhPIF4. Lanes 2-6: untreated GhTCP15 (lane 2) or GhTCP15 pre-incubated with GhCKI and ATP (lane 3), 10× or 100× unlabeled GhTP4 added to lane 3 reaction solution (lanes 4 and 5), and the CKI inhibitor Ic261 added to lane 3 (lane 6). To examine the effect of dephosphorylation, the mixture in lane 3 was incubated with LambdaPPase (lane 7). Additionally, 10× or 100× unlabeled mproPIF4 was added to lane 3 (lanes 8 and 9, respectively). Ic261, the CKI inhibitor; LambdaPPase, Lambda protein phosphatase; 10× or 100×, 10× or 100× unlabeled proPIF4; m10× or m100×, m10× or m100× unlabeled mproPIF4; 32P-GhTCP15-proPIF4 and GhTCP15-proPIF4 indicate phosphorylated GhTCP15 and GhTCP15 binding to labelled proPIF4, respectively. FP, free probe.

Figure 7: Schematic diagram illustrating how GhCKI cooperates the upstream GhLEC1 and the downstream GhTCP15 to regulate SE by modulating the auxin signaling pathway via GhPIF4. Without GhLEC1 expression, GhCKI expression is enhanced, GhCKI kinases phosphorylate GhTCP15 at an early stage of SE (explant dedifferentiation, NEC formation and cell proliferation), and increased binding of phosphorylated GhTCP15 to the promoter of GhPIF4, which promotes the expression of auxin biosynthesis genes. When the expression of GhLEC1 is initiated in the late stage of SE, the expression of GhCKI is inhibited, leading to reduced phosphorylation of GhTCP15. Thus, the transcription of GhPIF4 and auxin biosynthesis genes are impaired, leading to a decrease in the accumulation of auxin, which promotes the transition from NEC to EC, thereby facilitating somatic embryo formation. TFs, transcription factors; CKI, CASEIN KINASE I; LEC, LEAFY COTYLEDON; +P, phosphorylation; PIFs, PHYTOCHROME-INTERACTING FACTOR GENES; TSS, transcriptional start site; NEC, non-embryogenic callus; EC, embryogenic callus; SE,
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<th>Code</th>
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<td><strong>OE25-6</strong></td>
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<td><em>AtLEC1</em> T-DNA insertion mutant (CS8101). High <em>GbTCP/GhTCP15</em> (GhTCP15 protein completely homologous to GbTCP) expression in Arabidopsis (Hao et al., 2012).</td>
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