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Research

The gymnosperm ortholog of the angiosperm central cell-specification gene *CKI1* provides an essential clue to endosperm origin

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Summary

- A defining feature of angiosperms is double fertilization involving the female gametophyte central cell and formation of a nutrient-storing tissue called endosperm. The route for the evolutionary origin of endosperm from a gymnosperm ancestor, particularly the molecular steps involved, has remained elusive.
- Recently, the histidine kinase gene *Cytokinin-Independent 1* (*CKI1*), an activator of cytokinin signaling, was described as a key to specification of the endosperm precursor central cell in *Arabidopsis*. Here, we have investigated the function and expression of a putative ortholog of *CKI1* in the gymnosperm *Ginkgo biloba*.
- We demonstrate that *Ginkgo CKI1* can partially rescue an *Arabidopsis cki1* mutant and promote weak activation of the cytokinin signaling pathway in the *Arabidopsis* embryo sac, but does not confer central cell specification. *Ginkgo CKI1* is expressed in both male and female gametophytes of *Ginkgo*. In the latter, it is expressed in the ventral canal cell, which is sister to the egg cell in the archegonium. As in *Arabidopsis*, *Ginkgo CKI1* is not expressed in the egg cell.
- The similarities in expression patterns of *CKI1* in *Ginkgo* and *Arabidopsis* female gametophytes suggest that extant gymnosperms possess an essential component of the molecular machinery required for angiosperm endosperm development, and provide new insights into endosperm origin from a gymnospermous ancestor.

Introduction

The process of double fertilization that gives rise to a diploid embryo and typically a triploid endosperm has long been considered as a defining feature of angiosperms. The evolutionary origin of the endosperm has been a major unsolved question in the emergence of flowering plants from an unknown ancestral gymnosperm (Friedman, 2001). Over the past century, two main theories have been proposed for this origin (Friedman, 1992, 2001; Baroux *et al.*, 2002). In one theory, the endosperm is hypothesized to have evolved from a second supernumerary 'altruistic' embryo within the fertilized female gametophyte (FG) that acquired a different fate to become nutritive tissue for the first embryo (Sargant, 1900). This is supported by the formation of diploid endosperm in the early-diverging lineages Nymphaeales and Austrobaileyales (Friedman & Bachelier, 2013). This scenario posits endosperm homology with the sporophytic phase of the life cycle (Friedman, 2001; Rudall & Bateman, 2007). In the alternate theory, the endosperm is hypothesized to have evolved by co-opting the nutritive function of the large FG of gymnosperms (endosperm homologous to the FG), which became dependent upon fertilization by a second sperm cell for its subsequent development (Coulter, 1911); i.e., homology with the gametophytic phase of the plant life cycle (discussed in Friedman & Floyd, 2001; Baroux *et al.*, 2002). To date, molecular evidence in support of or providing mechanistic feasibility for either theory has been lacking because gymnosperms are large, slow-reproducing plants with limited genetics and transgenic capabilities, and virtually no information is available about identity, expression, or function of their FG genes.

Recently, we reported the gene *Cytokinin-Independent 1* (*CKI1*), a constitutive activator of cytokinin signaling, to be the key determinant of the angiosperm central cell, the precursor of the endosperm (Yuan *et al.*, 2016). CKI1 does not bind cytokinin, but similar to other histidine kinases acting in cytokinin signaling (Kieber & Schaller, 2014), CKI1 contains an N-terminus transmembrane domain, a histidine kinase domain, an ATPase domain, and a C-terminus receiver domain. The

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receiver domain is responsible for the recognition of CKI1 downstream signaling partners, the *Arabidopsis thaliana* histidine phosphotransfer proteins (AHPs). In *Arabidopsis*, CKI1 specifically interacts with the AHP2, AHP3, and AHP5 proteins to activate the cytokinin signaling pathway (Pekárová *et al.*, 2011). These downstream AHPs are required for *CKI1* function in the embryo sac (Deng *et al.*, 2010; Liu *et al.*, 2017). *CKI1* is both necessary and sufficient for central cell specification, and subsequently for endosperm formation, either with or without fertilization (Yuan *et al.*, 2016). In a null *cki1* mutant, the central cell acquires egg cell fate, whereas ectopic expression of *CKI1* can confer central cell fate to an egg cell or to the accessory synergid cells. *CKI1* is not present in the sequenced genomes of nonseed plants, but appears to be present in both angiosperms and gymnosperms, despite the absence of endosperm in the latter (Yuan *et al.*, 2016). Here, we have further characterized the *CKI1* ortholog from a gymnosperm, *Ginkgo biloba*.

Ginkgo FG development is comparable to that of most gymnosperms. After formation of a single functional megaspore deep within the nucellus, the FG undergoes a period of free nuclear divisions. Coenocytic development continues until the gametophyte contains several thousand free nuclei, followed by cellularization and formation of typically two archegonia at the micropylar end (Friedman and Goliber, 1986). This ancient species has retained its reproductive structures essentially unchanged for over *c.* 170 Myr (Zhou & Zheng, 2003). In addition, in contrast with conifers like *Pinus* and *Picea*, the ovules of *Ginkgo* are not protected by scales and their FGs can be more easily accessed. The characterization of *Ginkgo CKI1* gene expression and function in this study provides new molecular insights into the role played by an ancestral *CKI1* gene in the origin of the endosperm.

Materials and Methods

Plant materials and growth conditions

A. thaliana wild-types (WTs) Wassilewskija (Ws), Columbia-0 (Col-0), and Landsberg erecta were used in this study. The *cki1-9* (Col-0) and the *cki1-5* (Ws) mutants were obtained from the ABRC Stock Center. Plants were grown in a controlled chamber with a 16 h : 8 h, light : dark cycle at 22°C at 60% relative humidity.

G. biloba ovules were collected at different successive developmental stages from a female tree in Davis, California (USA): 03/24, 03/31, 04/07, 04/14, 04/21, 05/12, 05/15, 06/01, 07/05, 07/12, and 07/26 (month/day).

Anatomy and microscopy

For whole-mount clearing observations, samples were prepared as described previously (Pagnussat *et al.*, 2007, 2009). For fluorescence microscopy, individual *Arabidopsis* ovules were dissected from the pistils/siliques and then mounted with distilled water (Panoli *et al.*, 2015). Samples were observed under a Zeiss Axioplan imaging 2 microscope (Axioskop 2 plus) (Zeiss, Oberkochem, Germany). Images were captured with ZEN (LSM 710) or AxioVision (Axioskop 2 plus) software. Images were processed by Adobe Photoshop and Adobe Fireworks software.

Cloning of *GbCKI1* from *Ginkgo biloba*

The available genome sequence of *CKI1*-like genes from *Amborella trichopoda* and *Picea abies* (Norway spruce) were obtained from online genome resources (Amborella Genome Project, 2013; Nystedt *et al.*, 2013) (http://www.amborella.org/; http://congenie.org/start). Sequence alignments were carried out between *Amborella* and *Picea* using the ClustalW2 website, and a 500 bp region containing highly conserved short nucleotides (20–25 bp) was identified. One forward primer (PaCKI1-Mid-F) and two reverse primers (PaCKI1-Mid-R1 and PaCKI1-Mid-R2) were designed against the Norway spruce *CKI1* sequence because spruce is also a gymnosperm (primers shown in Supporting Information Table S1). Nested PCR reactions were carried out using *Ginkgo* genome DNA as a template. A unique band *c.* 450 bp was obtained and sequenced. Full-length complementary DNA (cDNA) was then obtained by SMARTer RACE cDNA amplification techniques (Clontech; primers, see Table S1).

Constructs for complementation and ectopic expression

pAtCKI1::GbCKI1: first *Arabidopsis CKI1* promoter was amplified and ligated into *pCAMBIA1300-NOST* to generate *pAtCKI1-NOST*. Then *Ginkgo CKI1* gene coding DNA sequence (CDS) was amplified by primer pair GBCKI1-Kpn-F and GBCKI1-Kpn-R and inserted into *pAtCKI1-NOST* by *Kpn*I to obtain *pAtCKI1::GbCKI1* (primers, see Table S1).

pAtCKI1::HbCKI1: GbCKI1 N-terminal region was amplified by primers GBCKI1-Kpn-F and GbCKI1-InR. And *AtCKI1* receiver domain was amplified by primers AtCKI1Rec-F and AtCKI1CDS-KpnR. These two fragments served as templates for further overlapping PCR with primer pair GBCKI1-Kpn-F and AtCKI1CDS-KpnR to obtain hybrid *CKI1* fragment. Eventually the hybrid *CKI1* fragment was inserted into *pAtCKI1-NOST* by *Kpn*I to obtain *pAtCKI1::HbCKI1* (primers, see Table S1).

pES1::GbCKI1: *GbCKI1* CDS was amplified by primers GbCKI1-SacI-F and GbCKI1-Pst-R (Table S1), and ligated into the vector pCAMBIA1300-pES1-NOST to generate *pES1::GbCKI1*. Sequences were further confirmed by sequencing. After transformation into plants homozygous for either the pTCSn::NLS-3XeGFP or the pDD22::H2B-eGFP marker, BASTA was used to screen for positive transgenic plants.

Individual binary expression vectors were transformed into *Agrobacterium tumefaciens* AGL1 strain, and then used to transform *Arabidopsis* WT plants, mutant plants, or marker lines.

Quantitative real-time polymerase chain reaction

Total RNA was isolated with Qiagen RNeasy Kit using the manufacturer's recommended protocol (Qiagen, http://www.qiagen.com/). Total RNA (10 μg) was treated with the Ambion TURBO DNA-free Kit according to the manufacturer's protocol

(Ambion, https://www.thermofisher.com). RNA concentrations were determined by using a Nanodrop Spectrophotometer (NanoDrop Technologies). First-strand cDNA was synthesized from the DNase-treated RNA (1.5 μg) by using Invitrogen SuperScript II reverse transcriptase following the manufacturer's instructions. cDNA was diluted 1 : 10 with TE buffer (10 mM Tris, 1 mM EDTA (pH 8.0)), and 1 μl of each reaction solution was added to 12.5 μl of 2× SYBR® green supermix (Bio-Rad, http://www.bio-rad.com/) containing the relevant primer pairs (Table S1) according to the manufacturer's instructions. A Bio-Rad iCycler was used to monitor the real-time quantitative reverse transcription PCRs for 50 cycles. *GbActin* was used as the internal control (primers, see Table S1).

RNA *in situ* hybridization

Nonradioactive *in situ* hybridization was performed as previously described (Yang *et al.*, 1999; Chen *et al.*, 2007). *Ginkgo* ovules from different stages were fixed in 4% paraformaldehyde and 2% glutaraldehyde; after dehydration in an ethanol series, samples were embedded in paraffin. Ovules were sectioned longitudinally at 8 μm thickness. For RNA probe synthesis, a 413 bp CDS fragment of *GbCKI1* was amplified (primers, see Table S1) and cloned into pGEM-T Easy vector (Promega). Plasmids were linearized using *Nco*I (sense) and *Sal*I (antisense). Probes were synthesized in the presence of DIG-rUTP (Roche) by MEGAscript T7 and Sp6 Transcription Kit (Ambion, Thermo Fisher Scientific).

Results

Characterization of a putative gymnosperm *CKI1* ortholog from *Ginkgo biloba*

Orthologs of the *CKI1* gene can be identified in all sequenced angiosperms, which is consistent with its essential reproductive function in central cell specification. As expected, no clear orthologs of *CKI1* could be identified in sequenced genomes from nonseed plants, such as the moss *Physcomitrella* or the lycophyte *Selaginella*. However, on searching the newly released *P. abies* genome (Nystedt *et al.*, 2013), and publicly released *Pinus taeda* genome sequences, we could identify single-copy *CKI1*-like genes in both conifers. Because gymnosperms do not have an equivalent of the angiosperm central cell, the presence of *CKI1* genes in these conifers was unexpected. To understand *CKI1* evolution in seed plants, we selected the putative *CKI1* ortholog from *G. biloba* for detailed characterization (Fig. 1). A comprehensive phylogenetic analysis was carried out among all the histidine kinase family members in different species, which include CKI1 clade, AHK1–AHK5, ETR1, and ERS1 clades (Yuan *et al.*, 2016). In the CKI1 clade, we can clearly identify an angiosperm subclade, which can be subdivided into monocots, eudicots and the earliest diverging lineage angiosperm *Amborella*, and a separate gymnosperm subclade. GbCKI1 falls clearly within the gymnosperm subclade, together with the conifer subgroup of *Pinus* and Norway spruce (Yuan *et al.*, 2016).

GbCKI1 can partially rescue early abortion phenotype in *Arabidopsis cki1* mutant

Using primers based on the Norway spruce and *Amborella CKI1* sequences, we cloned a full-length cDNA, *GbCKI1*, encoding a protein closely related to *Arabidopsis CKI1* in structural features (Fig. 1a). Further sequence alignment revealed that, in the H-box of the histidine kinase domain, all CKI1 orthologs contain an HD motif at the active histidine residue, whereas the other plant histidine kinases contain an HE motif (Fig. 1b). The HD motif in GbCKI1 was used to confirm its CKI1 identity (Fig. 1b). To evaluate functional conservation, we introduced *GbCKI1* under the *AtCKI1* promoter into heterozygous *cki1-5* mutant *Arabidopsis* plants carrying egg cell and central cell double markers FGR1.0 (*pEC1.1::NLS-3XRFP; pDD22::GFP*). In *cki1-5*/+ plants, 45.6% ($n = 704$) of ovules showed WT expression pattern, and 26% of ovules did not show any marker expression, representing embryo sacs arrested at the FG4 stage, a characteristic of the *cki1-5* allele in the Ws ecotype. A further 28.4% of ovules showed ectopic egg cells and no central cell marker expression (Table 1), representing loss of central cell fate and gain of egg cell fate, which is also a characteristic phenotype found in *cki1* mutants (Yuan *et al.*, 2016). In the transgenic *pAtCKI::GbCKI1/+ cki1-5/+ Arabidopsis* plants, remarkably, we observed partial rescue of the *cki1-5* mutation, with a lower ratio of FG4 arrested embryo sacs, which corresponded to a higher ratio of mature embryo sacs with ectopic egg cells (32.1–41.5%, vs 28.4-% in *cki1-5*/+ plants; Fig. 1b,c; Table 1). These results show that *GbCKI1* can partially suppress the growth arrest phenotype of *cki1-5*, but not the central cell specification defect, possibly because of the absence of this cell type in gymnosperms. Another interesting aspect is that *GbCKI1* appears to compete with *AtCKI1*. When we transformed *pAtCKI::GbCKI1* into WT plants homozygous for FGR1.0, we noted an early abortion phenotype, detected by failure of a fraction of ovules to express either the egg cell or the central marker carried by FGR1.0 (14.9–43.3%, Table 1). Similarly, increases in ovule abortion frequency were also observed with *GbCKI1* in *cki1-5/+ FGR1.0/FGR1.0* plants.

Ectopic *GbCKI1* expression in *Arabidopsis* embryo sacs can activate the cytokinin signaling pathway but does not confer central cell fate

In order to understand why *GbCKI1* cannot fully rescue the *Arabidopsis cki1* mutant, we investigated the activation of cytokinin signaling by *GbCKI1*. We used the FG-specific promoter p*ES1* (Yu *et al.*, 2005) to drive expression of a full-length *GbCKI1* cDNA clone in all four cell types (synergid cell, egg cell, central cell, and antipodal cell). This construct was introduced into an *Arabidopsis* line homozygous for the cytokinin signaling reporter, *pTCSn::NLS-3XeGFP* (Yuan *et al.*, 2016). In WT *Arabidopsis* plants, the two-component signaling sensor (TCS) reporter is only activated in the antipodal cells and central cells (Fig. 2a,b; Table 2), consistent with *AtCKI1* expression in those cells (Yuan *et al.*, 2016). But in the ectopic *GbCKI1* expression lines, TCS signals are additionally observed in the egg cells and

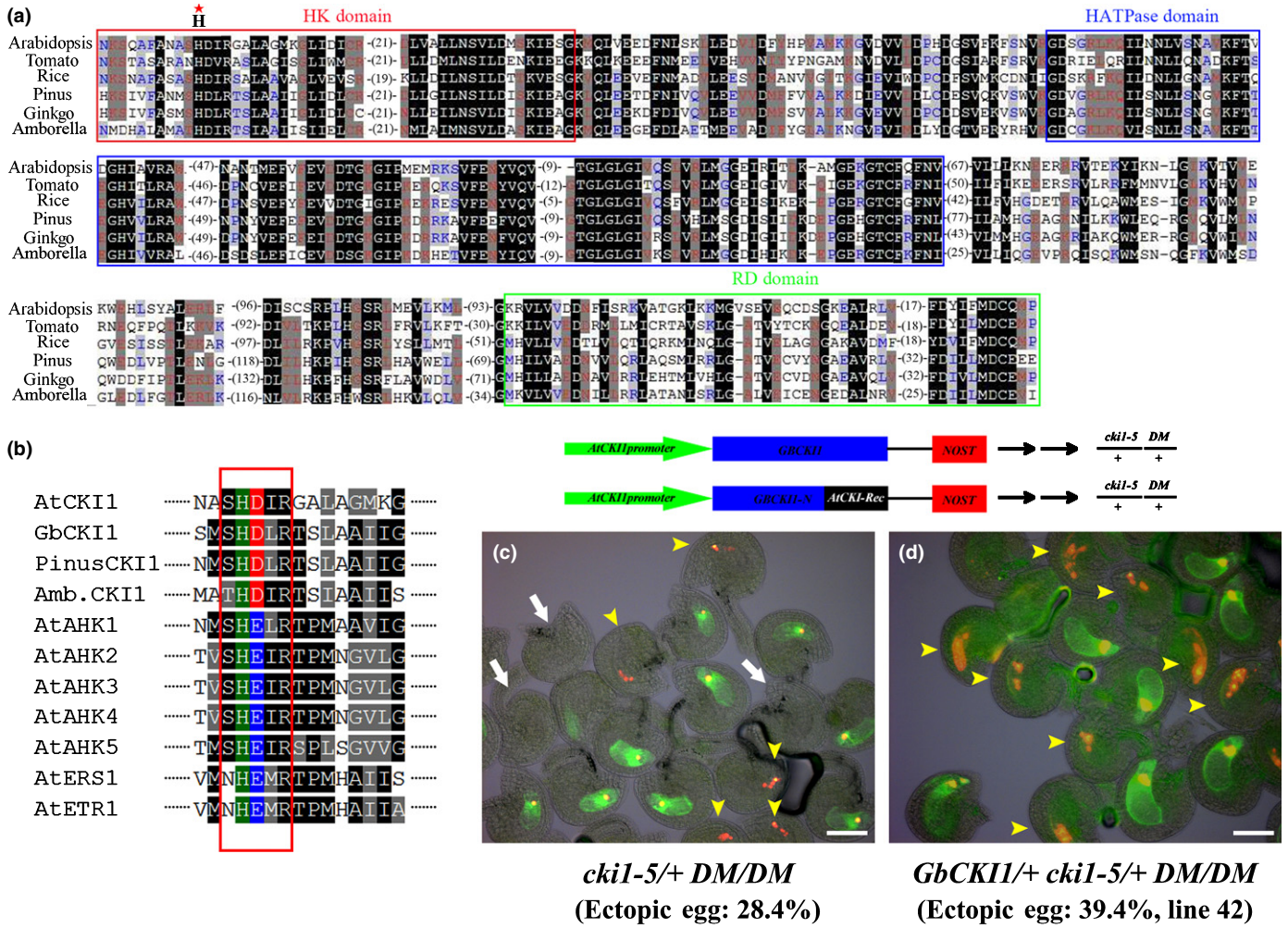

Fig. 1 CKI1 phylogeny in seed plants and characterization of *GbCKI1*. (a) Sequence alignment of CKI1 proteins from different taxa including: *Amborella*, *Oryza*, *Arabidopsis*, *Solanum*, *Pinus*, and *Ginkgo*. HK domain, HATPase domain, and RD domain (receiver domain) are highly conserved in all the species. H represents the conserved histidine (H) in the HK domain. (b) Conserved residues in the H-box of HK domains of *Arabidopsis* histidine kinases aligned with CKI1 orthologs from gymnosperms *Pinus taeda* and *Ginkgo biloba*, and the early-diverging angiosperm *Amborella*. All CKI1 proteins contain an HD signature motif, but other histidine kinases have an HE motif. (c, d) *GbCKI1* and *GbCKI1N-AtRec* can partially rescue early aborted ovules in *Arabidopsis cki1-5* mutant. The assay is complicated by sensitivity of female gametophytes (FGs) to *CKI1* dosage; extra copies of native *pAtCKI::AtCKI1* cause variable FG abortion, an effect that is also observed with *pAtCKI::GbCKI1* and *pAtCKI::GbCKI1N-AtRec* expression in wild-type plants. Expression of egg cell (EC) and central cell double markers (FGR1.0, *pEC1.1::NLS-3XRFP; pDD22::GFP*) in *cki1-5* mutant is shown in three categories of ovules observed: (i) ovules with wild-type pattern; (ii) ovules with ectopic EC marker (c, d, yellow arrowheads); (iii) ovules with no marker expression due to the early abortions (c, white arrows). After transformation with *pAtCKI1::GbCKI1*, the early aborted ovules in *cki1-5* mutant can be partially rescued, indicated by increasing fractions of ovules with ectopic EC marker expression (d, yellow arrowheads; Table 1). *pAtCKI::GbCKI1N-AtRec* is discussed separately. Bars, 25 μm.

synergid cells as well (Fig. 2c–h; 35.5–38.5%, Table 2). However, the TCS reporter signals intensity in egg cells and synergid cells were relatively weak compared to those observed in the central cells and antipodal cells (Figs 2c–h, S1). We have shown previously that if *AtCKI1* is ectopically expressed in *Arabidopsis* under the same FG promoter, the strength of TCS reporter expression in the egg cells and synergid cells is similar to that observed in the central cells and antipodal cells (Yuan *et al.*, 2016). These observations suggest that *GbCKI1* might be less efficient than the endogenous *AtCKI1* in activating cytokinin signaling in the *Arabidopsis* embryo sac. Previously, we have found that ectopic *AtCKI1* expression is fully penetrant in terms of frequency of ectopic TCS vs WT signals (Yuan *et al.*, 2016). *GbCKI1* shows a frequency of WT signals (61.5–64.9%; Table 2) that is higher than the 50% expected for full penetrance, again suggesting a lower efficiency of activating cytokinin signaling for *GbCKI1* compared with *AtCKI1*.

In *Arabidopsis*, ectopic *AtCKI1* expression resulted in cell-fate switching of synergid cells and egg cells to central cells (Yuan *et al.*, 2016). To determine whether ectopic *GbCKI1* expression, despite weaker activation of cytokinin signaling, could still result in change of cell fates, we checked expression of the central-cell-specific marker DD22 in the *pES1::GbCKI1* lines ectopically expressing *GbCKI1* in the FG. We could observe 5.5–7.8% ectopic DD22 marker expression either in synergid cells or egg cells in our transgenic lines, but never in both cell types at the

Table 1 Expression of FGR1.0 (egg cell and central cell) double marker in embryo sacs of *cki1-5* mutant complemented by *pAtCKI1::GbCKI1*

Genotype	Line	WT pattern (%)	Ectopic EC marker (%)	Signal negative (early aborted) (%)	*n*	*P* value
cki1-5/+; FGR1.0/FGR1.0		45.6	28.4	26.0	704	
pAtCKI1::GbCKI1/+; cki1-5/+; FGR1.0/FGR1.0	82	34.2	43.3	22.5	573	2.803×10^{-15}
	42	41.7	39.4	18.8	398	1.022×10^{-6}
	27	33.1	36.8	30.2	242	0.0039
	16	36.4	35.0	28.6	472	0.0016
	19	23.2	34.8	42.0	500	0.0015
	28	45.4	32.1	22.5	240	0.2057
pAtCKI1::GbCKI1/+; FGR1.0/FGR1.0	6	56.7		43.3	240	
	9	85.1		14.9	308	
	77	80.9		18.7	267	

WT, wild-type; EC, egg cell.

FGR1.0 (*pEC1.1::NLS-3XRFP; pDD22::GFP*). The marker labels EC nucleus and the whole central cell (CC) in WT *Arabidopsis* ovules. Red box highlights the expression ratios of ectopic EC marker expression in the original *cki1-5* mutant and in the *cki1-5* mutant complemented by *pAtCKI1::GbCKI1*. *Arabidopsis* female gametophytes exhibit sensitivity to *GbCKI1* expression under the *AtCKI1* promoter. When the *pAtCKI::GbCKI1* construct was introduced into WT plants homozygous for the FGR1.0 marker, varying frequencies of early arrest and abortion were observed, as the different transformants failed to express FGR1.0 in 14.9–43.3% of the ovules (lines 6, 9, and 77 in this table). When the *pAtCKI::GbCKI1* construct was introduced into *cki1-5/+; FGR1.0/FGR1.0* plants, some of the aborted ovules might be due to *GbCKI1* expression. This is seen by the decrease of ovules showing WT expression pattern in the *cki1-5/+* transformants (23.2–45.4%), relative to the *cki1-5/+* plants without the transgene (45.6%). In *cki1-5/+; FGR1.0/FGR1.0* plants, 29.8% embryo sacs are predicted to be carrying both the *cki1-5* mutation and the marker due to early arrest and abortion of 20.2% of the embryo sacs. The percentage of embryo sacs expressing ectopic EC marker is slightly less, 28.4%. χ^2 test is calculated based on an expectation of 28.4% ectopic EC marker expression in *pAtCKI1::GbCKI1/+; cki1-5/+; FGR1.0/FGR1.0* plants (the null hypothesis is that there is no rescue of the *cki1-5* early abortion phenotype).

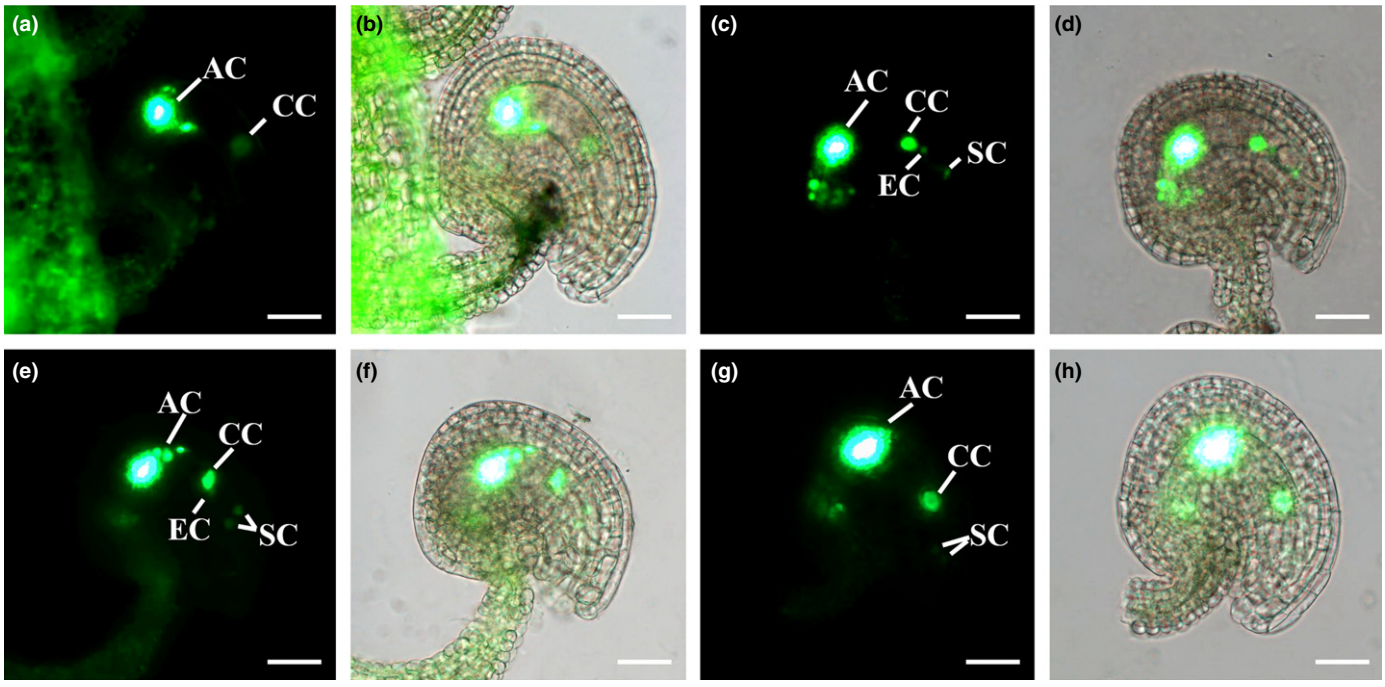

Fig. 2 *GbCKI1* activates cytokinin signaling pathway in *Arabidopsis*. (a, b) Ovules expressing cytokinin signaling marker *pTCSn::NLS-3XeGFP*. Signals are detected in antipodal cells (ACs) and central cells (CCs) inside the wild-type embryo sac. Two-component signaling sensor new (TCSn)-green fluorescent protein (GFP) signals can be detected in ACs and CCs (a), and bright field and GFP channel are merged in (b). (c–h) Ectopic TCSn-GFP signals were observed in transgenic lines *pES1::GbCKI1*. GFP signals were presented in egg cells (ECs) and synergid cell (SCs) in (c) and (e), while in the case of (g) the signals can only be observed in SCs. (d), (f), and (h) are the merged images of bright field and GFP channel. Bars, 30 μm.

same time (Fig. S2; Table S2). In addition to the low ratios of ectopic DD22 marker expression, the signal strength is also quite weak (Fig. S2), indicating that even in these cases the egg cells and synergid cells are not fully transformed into central cells. Another indication of an incomplete transformation to central cells is that the size of these ectopic DD22-expressing cells

Table 2 Expression of cytokinin signaling marker two-component signaling sensor new (TCSn) in the embryo sacs of *pES1::GbCKI1*/+ plants

Genotype	Line	Wild-type pattern (%)	Ectopic signals	*n*	*P* value
pTCSn::NLS-3XeGFP/pTCSn::NLS-3XeGFP		100.0	0	112	
pES1::GbCKI1/+; pTCSn::NLS-3XeGFP/pTCSn::NLS-3XeGFP	1	61.5	0.385	109	5.7493×10^{-5}
	2	64.9	0.351	97	0.0006
	3	64.7	0.353	102	0.0004

χ^2 test is calculated based on an expectation of 100% wild-type (WT) pattern of marker expression in *pES1::GbCKI1 Arabidopsis* plants (the null hypothesis is that there is no difference of marker expression in WT and *pES1::GbCKI1* transgenic plants).

remains unchanged from the normal egg cells and synergid cells. We have previously shown that when the egg cells and synergid cells are fully transformed into central cells they also exhibit larger sizes (Yuan *et al.*, 2016). Finally, in the ectopic *pES1::GbCKI1* lines, we never observed the formation of white seeds containing dual endosperms without embryos, which is observed with ectopic *AtCKI1* expression (Yuan *et al.*, 2016). In summary, we have shown that *GbCKI1* can partially activate the cytokinin signaling pathway in the *Arabidopsis* FG, and can confer some central cell attributes manifested by ectopic expression of the central cell marker DD22. However, *GbCKI1* cannot transform egg cells or synergids into central cells, likely related to its inability to fully activate cytokinin signaling in *Arabidopsis* FGs.

The *Arabidopsis* CKI1 receiver domain does not confer central cell specification functions to GbCKI1

In *Arabidopsis*, the CKI1 receiver domain specifically interacts with downstream effectors of cytokinin signaling AHP2, AHP3, and AHP5 (Pekárová *et al.*, 2011). It is possible that inability of *GbCKI1* to specify central cell fate is due to poor interactions with these downstream effectors. Therefore, we replaced the GbCKI1 receiver domain with AtCKI1 receiver domain, to generate a gene *pAtCKI1::GbCKI1N-AtRec* encoding the hybrid CKI1 protein under the *Arabidopsis CKI1* promoter (Table 3). The hybrid gene was transformed into *cki1-5/CKI1* mutant plants homozygous for the double marker FGR1.0. In the T0 generation, plants genotyped as *pAtCKI1::HbCKI1/+ cki1-5/+* were subjected to phenotypic analysis. We observed that similar to GbCKI1, the hybrid CKI1 protein is able to rescue early abortion phenotype by increasing the ectopic egg cell ratio in the transgenic plants (35.5–43.6% vs 28.4% in *cki1-5* mutant, Table 3). However, the central cell specification defect was not rescued due to the fact that normal central cell pattern was not recovered compared with *cki1-5* mutant (Table 3). Similarly, the ratio of early aborted ovules with no marker signals is also increased (23.0–39.0% vs 26.0% in *cki1-5* mutant, Table 3), indicating the hybrid protein can also compete with native AtCKI1 protein. This was confirmed when we introduced hybrid *CKI1* into plants homozygous for FGR1.0 double marker (aborted ovules can increase to 47.9% vs 26.0% in *cki1-5* mutant, Table 3). We conclude that the partial functionality of GbCKI1 cannot be enhanced using the *Arabidopsis* AtCKI1 receiver domain, and that other domains of the CKI1 kinase must be required for complete functionality.

GbCKI1 is expressed gametophytically in *Ginkgo biloba*

We next investigated the expression of *GbCKI1* in sporophytic and gametophytic tissues of *Ginkgo* using quantitative PCR (qPCR; Fig. 3). We collected samples of young and mature leaves from male and female trees, young male cones before pollen shedding, male cones with mature pollen, and ovules at different stages of development. We separated sporophytic parts and gametophytic parts from late ovules, where this is possible, and eventually the archegonial region and nonarchegonium region of the ovules at the fully mature stage (Fig. 3a). The results showed that *GbCKI1* expression was not detected in any sporophytic tissue tested, but only restricted to the gametophytic tissue throughout development, with the highest expression observed in the isolated archegonial region of the mature FG (Fig. 3b). The expression specificity is similar to *Arabidopsis CKI1*, which is also highest in the female reproductive tissue (Deng *et al.*, 2010), except that *GbCKI1* also shows some expression in male gametophyte before or at the pollination stage (Figs 3b, 4j,k). Male gametophyte expression of *CKI1* is not observed in *Arabidopsis*, suggesting that this expression has been lost in angiosperms. The qPCR results were verified by RNA *in situ* hybridization on reproductive tissues (Fig. 4).

Samples from three time points were chosen to perform the *in situ* experiments. In ovules from 21 April, the FG is still at the stage of free nuclei, although partial cellularization has already begun at its periphery (Fig. 4a–c). By RNA *in situ* hybridization, the first notable result is that *GbCKI1* expresses exclusively in the FG. In Fig. 3(b), labeled free nuclei of the central region can be clearly observed. A similar expression pattern was observed with the ovules collected 3 wk later (Fig. 4d–f, sampled on 12 May). Owing to rapid enlargement of the FG, the large central vacuole, and relatively thin cytoplasm, at this stage the whole FG is completely separated from the sporophytic tissue after sample fixation (Fig. 4d–f). The FG is at a later free-nucleate stage, and *GbCKI1* is highly expressed in the gametophytic noncellularized nuclei (Fig. 3e). In the ovules from a later stage, cellularization is nearly complete (Fig. 4g–i, sampled on 1 June), and only a few hundred nuclei remain uncellularized in the center of the FG. *GbCKI1* RNA signals disappeared from cellularized nuclei and are only present in the free nuclei area, which leads to the conclusion that *GbCKI1* is highly associated with centrally localized nuclei before cellularization. Consistent with previous qPCR results, we observed *GbCKI1* RNA presence in pollen grains near or at the time of shedding (Fig. 4j–l). In summary, *GBCKI1* is highly

Table 3 Expression of FGR1.0 (egg cell and central cell) double marker in embryo sacs of *cki1-5* mutant complemented by *pAtCKI1::HbCKI1*

Genotype	Line	WT pattern (%)	Ectopic EC marker (%)	Signal negative (early aborted) (%)	*n*	*P* value
cki1-5/+; FGR1.0/FGR1.0		45.6	28.4	26.0	704	
pAtCKI1::HbCKI1/+; cki1-5/+; FGR1.0/FGR1.0	53	25.4	36.8	37.8	209	0.0068
	58	33.9	43.1	23.0	239	4.6953×10^{-7}
	63	33.2	35.5	31.4	220	0.0203
	71	24.8	36.2	39.0	246	0.0068
	72	28.8	40.6	30.7	313	1.7819×10^{-6}
pAtCKI1::HbCKI1/+; FGR1.0/FGR1.0	55	87.9		12.1	257	
	61	52.1		47.9	315	
	64	88.4		11.6	346	

WT, wild-type; EC, egg cell.
χ^2 test is calculated based on an expectation of 28.4% ectopic EC marker expression in *pAtCKI1::HbCKI1/+; cki1-5/+; FGR1.0/FGR1.0 Arabidopsis* plants (the null hypothesis is that there is no rescue of the *cki1-5* early abortion phenotype). Red box highlights the expression ratios of ectopic EC marker expression in the original *cki1-5* mutant and the *cki1-5* mutant complemented by *pAtCKI1::HbCKI1*.

expressed in the free uncellularized nuclei in early FG development, and meanwhile it is also expressed in the pollen grains. The pattern indicates that *GbCKI1* might be functional during early development of both male gametophytes and FGs in *Ginkgo*.

Ginkgo CKI1 is expressed in the developing female gametophyte archegonia and excluded from the egg cell nucleus at maturity

In *Ginkgo*, an archegonium initial cell divides periclinally to yield a small neck initial cell toward the surface of the FG that will produce four neck cells, and a larger cell to the interior called the 'central cell'. Before fertilization, this 'central cell' undergoes an asymmetric mitotic division to form a small ventral canal (VC) cell and a much larger egg cell nucleus. So at maturity, one archegonium possesses one egg cell nucleus, one VC cell with a nucleus, and four neck cells at the opening to the archegonial chamber (the physical space between the surface of the FG and the nucellus).

Detailed analysis of *GbCKI1* expression in *Ginkgo* archegonia was carried out by RNA *in situ* hybridization. Following the division of the archegonial initial, *GbCKI1* shows strong expression in both gymnosperm central cell (GCC) nucleus and neck initial cell (Fig. 5a). Weak *GbCKI1* expression was observed in the nuclei surrounding the archegonium, reflecting a general association of *CKI1* expression with dividing FG cells (Fig. 4). Later, as the GCC becomes enlarged, *GbCKI1* RNA signals are visible in the GCC nucleus and in the surrounding cytoplasm (Fig. 4c). *GbCK1* continues to be expressed in the secondary neck mother cells. Surrounding expression is increasingly confined to the archegonium periphery ('archegonial jacket') (Fig. 5c). Finally, following the asymmetric division of the GCC to form the egg cell nucleus and VC cell, *GbCKI1* RNA signals were not detected in the egg cell nucleus (Fig. 5d–i), which is diffuse but can be clearly visualized by counterstaining with 4′,6-diamidino-2-phenylindole (Fig. 5f,i). At this final stage of FG development, *GbCKI1* RNA signals are clearly present in the VC cell, which is sister to the egg cell (Fig. 5d,e), and slightly weaker in the neck cell nuclei (Fig. 5d,g).

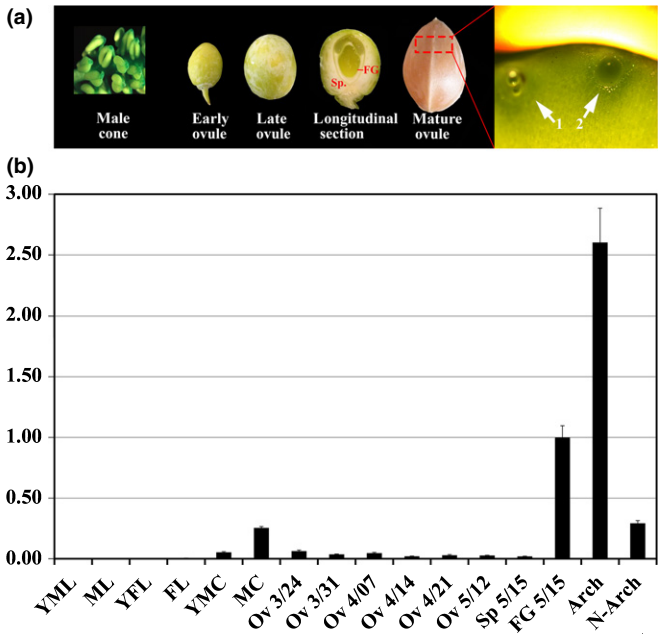

Fig. 3 *GbCKI1* has highest expression in the archegonial region of *Ginkgo* female gametophyte. (a) Samples used for quantitative PCR analysis in this research. *Ginkgo* female gametophytes (FG) and sporophytic tissues (Sp.) are not separable in early ovules (from date 3/24 to 5/12 (date format: month/day)), while separation later became possible after longitudinal dissection. Arrows indicate two archegonia inside a mature ovule (fleshy sarcotesta removed). (b) Quantitative PCR analysis of *GbCKI1* expression in different tissues and different developmental stages. Error bars indicate + SD. YML, young male leaf; ML, male leaf; YFL, young female leaf; FL, female leaf; YMC, young male cone; MC, male cone; Ov, ovule; FG, female gametophyte; Sp, sporophytic tissue; Arch, archegonial region; N-Arch, nonarchegonium region.

Discussion

The phylogeny of plant histidine kinases shows that the origin of *CKI1* can be traced to the origin of the gymnosperms (Yuan *et al.*, 2016). Owing to the difficulties of mutant studies and

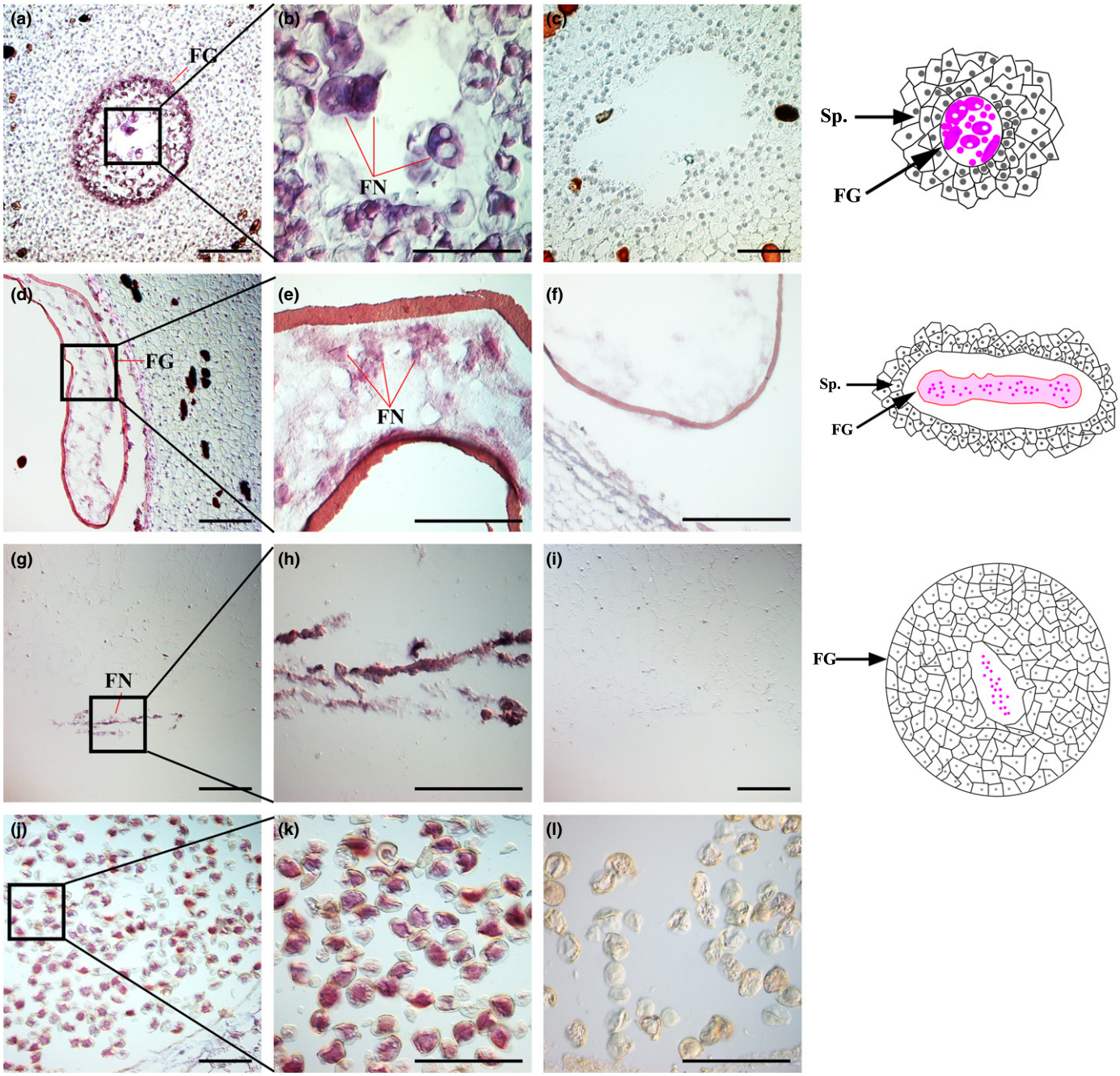

Fig. 4 *In situ* localization of *GbCKI1* mRNA during *Ginkgo* gametophyte development. (a–i) *In situ* RNA expression of *GbCKI1* in *Ginkgo* female gametophytes from different developmental stages. (b, e, h, k) are higher magnification views of the insets in (a, d, g, j). Sense probe control from same developmental stages are showing in (c, f, i). (a–c) Ovules were collected on 21 April, and female gametophyte was at free nuclear (coenocytic) stage. *GbCKI1* specifically expresses in the female gametophyte (a, b). (d–f) Ovules were collected on 12 May, and female gametophytes are still at rapidly dividing free nuclear stage. *GbCKI1* is exclusively detected in free-nucleate female gametophytes (d, e). Owing to rapid enlargement of the female gametophyte and relatively thin cytoplasm at this stage, the whole female gametophyte is extremely delicate to preserve during the fixation process and becomes completely separated from the sporophytic tissue. (g–i) Ovules were collected on 1 June, and female gametophyte at late free-nucleate stage when only a few hundred nuclei in the center of female gametophytes remain uncellularized whereas peripheral region is cellularized. *GbCKI1* is exclusively detected in the uncellularized nuclei (g, h). (j–l) *In situ* RNA expression of *GbCKI1* in *Ginkgo* male cone near pollen-shedding stage. *GbCKI* mRNA is detected in the pollen grains (j, k). (k) High-magnification view of (j); (l) the sense probe control. FG, female gametophyte; FN, free nuclei; Sp., sporophytic tissue; FG, female gametophyte. Bars, 200 μm.

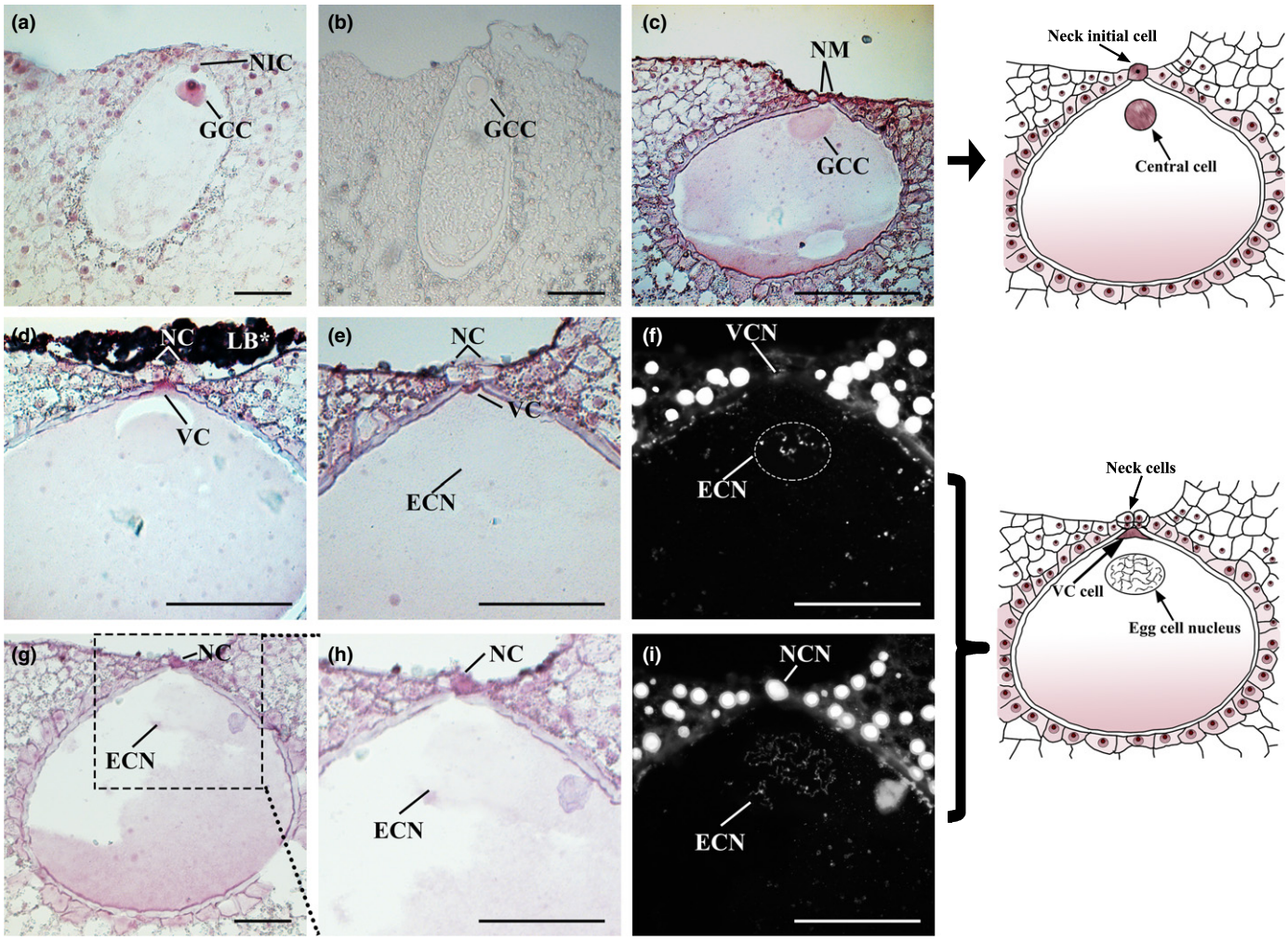

Fig. 5 *In situ* localization of *GbCKI1* mRNA during archegonium development. (a–c) Two stages of *Ginkgo* archegonia after formation of gymnosperm central cell (GCC): earlier stage (sampled on 7/5 (date format: month/day), a) and later stage (sampled on 7/12, c). *GbCKI1* mRNA was detected in GCC (a, c), neck initial cell (NIC) (a, slightly displaced during sectioning) and neck mother (NM) cells (c). (b) Median longitudinal section of female gametophyte (FG) from the same stage as in (a) hybridized with sense RNA probe control. (d–i) Mature *Ginkgo* archegonia (sampled on 7/26). No signals were detected in egg cell nucleus (ECN); strong signals can be detected in ventral canal (VC) cell (d, e), and weaker signals can be observed in neck cell nuclei (NCNs) (d, g). (e, h) Two adjacent serial longitudinal sections of the same FG; (h) is enlargement of boxed area in (g). (f, i) The same sections as in (e, h) counterstained with 4′,6-diamidino-2-phenylindole. Diffuse ECN (f, i, only partially visible in image f, circled), ventral canal cell nucleus (VCN) (f) and one NCN are visible under UV fluorescence (i). NC, neck cell; LB, lipid bodies and noncellular debris, possibly tannins, sometimes collect on the surface of the FG (white asterisk). Bars, 200 μm.

transformation experiments in long-lived and massive organisms, any conclusions about the biological function of *CKI1* in gymnosperms are necessarily indirect. Nonetheless, inferences can be drawn from comparative developmental studies of equivalent stages of the life cycle. To provide insights into the evolution of *CKI1* expression and function, we characterized *GbCKI1*, a putative *CKI1* ortholog from *Ginkgo*, an early diverging gymnosperm lineage and a genus considered as 'living fossil', having survived more or less unchanged from *c.* 170 Ma (Zhou & Zheng, 2003).

We show here that *GbCKI1* can partially rescue the embryo sac arrest phenotype of the *cki1-5* mutant in the *Arabidopsis* ecotype Ws, but not the misspecification of central cells as egg cells (Fig. 1b,c; Table 1). However, in WT *Arabidopsis*, significant ovule abortion was also observed due to CKI1 expression (Table 1). CKI1 has previously been shown to form homodimers (Hejatko *et al.*, 2009). It is possible that introduction of GbCKI1 into *Arabidopsis* results in the formation of nonproductive dimers with AtCKI1, and thereby causes a partial dominant-negative phenotype. We show here also that *GbCKI1* expression promotes activation of the cytokinin signaling pathway in the *Arabidopsis* embryo sac, but not to the same extent as *AtCKI1*, and that ectopic *GbCKI1* does not confer central cell specification to the egg cells or synergid cells (Figs 2, S1, S2; Tables 2, S2). Thus, while *GbCKI1* has some of the *AtCKI1* functionality in terms of cytokinin signaling, it lacks the full capability of the *AtCKI1* kinase in terms of cell specification. A domain swap with the receiver domain of *AtCKI1* did not result in higher frequencies of central cell specification, suggesting that the reduced function of GbCKI1 might reside in one of the other conserved domains (Table 3). Finally, we examined the expression pattern of

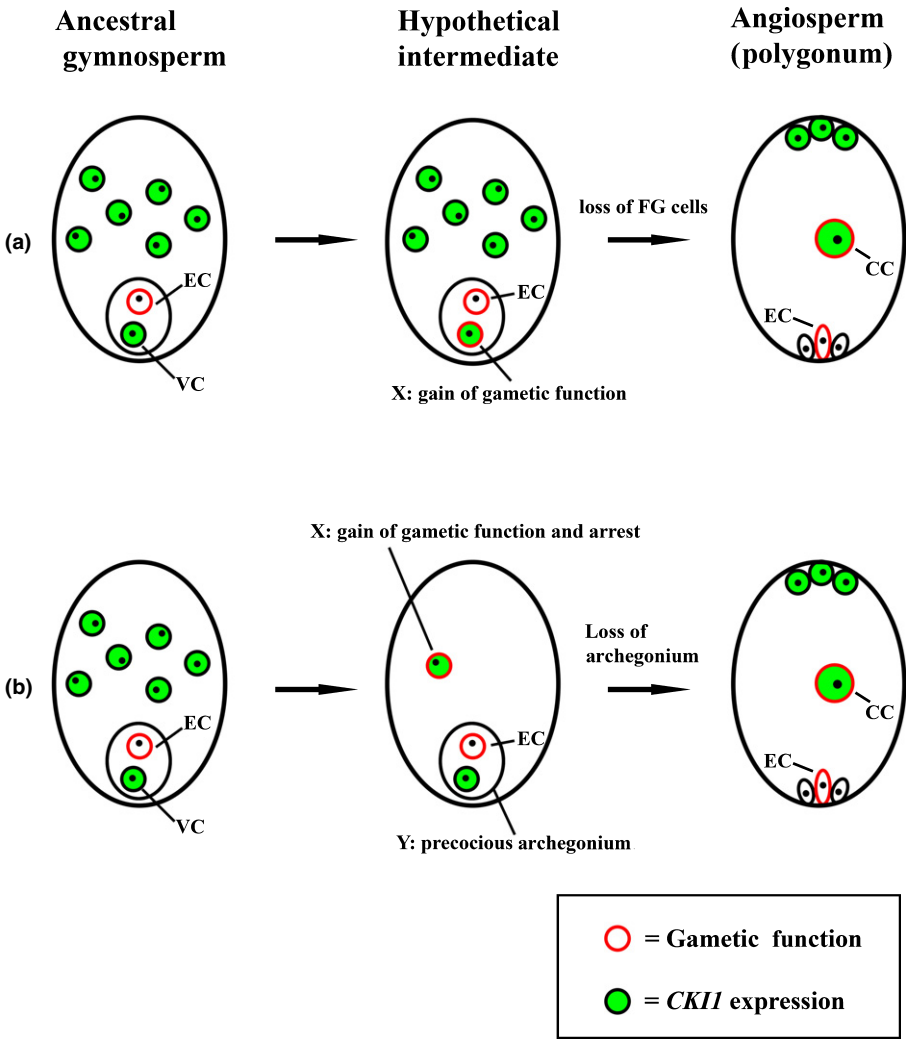

Fig. 6 Schematic diagram of possible evolutionary routes incorporating CKI1 expression and function for the two hypotheses for endosperm origin. (a) Route corresponding to 'altruistic embryo' theory, with two major steps for the hypothetical intermediate: (1) fixation of gametic capability in an archegonial cell expressing *CKI1* (one candidate being the ancestral ventral canal cell, sister to the egg cell), followed by (2) loss of female gametophyte (FG) cells outside the archegonium. (b) Route corresponding to the hypothesis that the endosperm is a homolog of the gymnosperm FG with three major steps: (1) progenic formation of an archegonium (FG still with few nuclei or cells); (2) early arrest of the nutritive FG cell lineage expressing *CKI1*, and simultaneous gain of gametic capability by this arrested cell; (3) loss of archegonium while retaining the egg cell (EC). VC, ventral canal cell; CC, central cell.

GbCKI1 and found that it is restricted to the gametophytic phase of the life cycle (Fig. 3). In FG development, *GbCKI1* is expressed initially in the free nuclear FG, becomes largely restricted to the archegonium, and disappears from the egg cell nucleus during maturation (Fig. 4). This pattern is strongly reminiscent of what has been observed in *Arabidopsis*, where *CKI1* expression is initially throughout all cells before FG4 stage and becomes more restricted later, with eventual exclusion from the egg apparatus (Yuan *et al.*, 2016), and suggests a common ancestral requirement for absence of *CKI1* expression in the egg cell.

Diplospermy, the release of two sperms at fertilization, is a synapomorphic trait in seed plants (Norstog *et al.*, 2004; Rudall & Bateman, 2007). Indeed, a second sperm nucleus is generally available for fertilization in gymnosperms, and occasional examples of 'double fertilization' have been described, notably in *Ephedra* and *Gnetum* (Friedman, 1990, 1991; Carmichael & Friedman, 1996). Interestingly, the second fertilization in *Ephedra* involves the VC cell nucleus, which is sister to the egg cell nucleus, and at least in *Ginkgo* the equivalent cell expresses *CKI1*. In *Arabidopsis,* the central cell both contains a sister nucleus of the egg cell and expresses *CKI1*, and could thus be homologous to the archegonial VC cell as was first suggested by Friedman (1998). However, FG cells other than the VC cells also express *CKI1* in *Ginkgo* before maturation of its FG, raising broader possibilities for the evolutionary relationship of *Ginkgo* FG cells to endosperm precursor cells.

Evolutionary implications for endosperm origin from gymnospermous ancestors

The evolution of the endosperm as a fertilization-dependent nutritive tissue for the embryo marks a significant step in flowering plant evolution; it is associated with the drastic reduction in size of the FG, and consequently the ovules, which in turn may have facilitated the accommodation of multiple ovules within a carpel. Gymnosperms typically invest resources in nutritive tissues irrespective of successful fertilization and embryo development, producing large FGs that can consist of thousands of cells. Both endosperm origin theories (sporophytic or unfertilized gametophytic tissue homology) are based on developmental anatomical observations and require several assumptions as yet unsupported by molecular

data; and, conspicuously, the numbers of evolutionary steps needed are undefined. A parsimonious route to endosperm evolution as a diversion from the typical product of gamete fusion, to form an 'altruistic proembryo' (Sargant, 1900) is suggested by findings from this and our previous study (Yuan *et al.*, 2016). First, ectopic endosperm formation in *Arabidopsis* arises from expression of a single gene, *CKI1*, that can solely control the post-fertilization fate of gametophytic cells (Yuan *et al.*, 2016). Second, gymnosperms already possess *CKI1* orthologous genes expressed in the FG, especially in the archegonium (Fig. 6). Therefore, a mutation leading to ectopic expression of the *CKI1* gene in a potential gametic cell of an ancestral gymnosperm and followed by a second fertilization event involving that cell and the 'extra' sperm cell could have been sufficient to switch the post-fertilization fate from embryo to nourishing tissue (i.e. endosperm) in the earliest angiosperms or an immediate ancestor. Given the demonstrated potential for second fertilization in the gymnosperms (Friedman, 1998), subsequent selection for such an event in an ancestral gymnosperm would then have led to the evolution of a distinct endosperm in angiosperm descendants (Fig. 6a). The expression and function of *CKI1* as a key endosperm precursor determinant can also be incorporated into the FG origin hypothesis (Fig. 6b). In this context, it is interesting to note that a much reduced megagametophyte with significant post-fertilization growth and development can be found in *Gnetum* (Friedman & Carmichael, 1998), raising the possibility that *Gnetum* represents a progression along this route. While all Gnetales share the loss of flagella in sperms with conifers and angiosperms, *Gnetum* and its sister taxon *Welwitschia mirabilis* are the only ones to have also lost their archegonia (Friedman, 2015). These two taxa share the same evolutionary trend toward reduced FG, storing nutrients mostly after fertilization, yet they do not have a biparental storage tissue, an endosperm. Molecular phylogeny studies do not support a direct shared ancestral relationship of Gnetales to the angiosperms (Friedman & Floyd, 2001; Maia *et al.*, 2014), but it remains possible that the types of innovations observed in *Gnetum* and *Welwitschia* also occurred independently in a separate lineage that is directly ancestral to angiosperms. In summary, both routes to endosperm evolution would require the concurrent gain of gametic function by a *CKI1*-expressing FG cell (Fig. 6b). What this study suggests is that the *CKI1* gene had already evolved in gymnosperms to perform an undetermined function in the gymnosperm FG, which likely involved hormone-independent activation of the cytokinin signaling pathway. This original *CKI1* function in the gymnosperm FG must have been subsequently co-opted during angiosperm evolution to specify a fertilization-dependent endosperm precursor cell (angiosperm central cell).

Major evolutionary steps, such as evolution of language ability in primates or multicellularity in fungi, have been proposed to result from mutations in single regulatory genes, rather than changes involving large numbers of genes (Konopka *et al.*, 2009; Oud *et al.*, 2013). Despite the considerable evolutionary distance between angiosperms and *G. biloba*, and the lack of information on the intermediary steps, it is plausible that changes in a small number of genes, including *CKI1*, formed the basis for the major evolutionary steps leading to the flowering plant FG. The identification and characterization of more gymnosperm genes, particularly orthologs of angiosperm genes with FG functions, could provide additional insights into this question.

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Author contributions

V.S. conceived the study. L.Y. and V.S. designed the overall experimental scheme. L.Y., Z.L. and X.S. designed and executed the experiments. J.J. provided *Ginkgo* material. L.Y., Z.L., V.S. and J.J. wrote the manuscript. L.Y. and Z.L. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Quantification of GFP fluorescent intensity of TCS reporter in WT and *GbCKI1* expressing embryo sacs.

Fig. S2 *GbCKI1* can activate central cell specific marker DD22 expression at low ratio.

Table S1 Primers used in this research

Table S2 Expression of central cell specific marker DD22 in the embryo sacs of *pES1::GbCKI1*/+ plants

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