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SHORT COMMUNICATION



Arabidopsis CKI1 mediated two-component signaling in the specification of female gametophyte

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ABSTRACT

Cytokinin independent 1 (CKI1) is a histidine kinase involved in the two-component signaling pathway and acts as a master regulator of central cell specification *via* CKI1-mediated two-component signaling. In this study, the dynamic distribution of two-component system (TCS) signals was primarily investigated during *Arabidopsis* embryo sac development. TCS signals were stably detected in female gametophytes cells from the megaspore stage all through to the mature embryo sac stage. CKI1 acts as the primary activator of the TCS signaling pathway in embryo sacs. Accordingly, focusing on CKI1, two alternate models are proposed for female gametophyte cell fate specification. In the first model, CKI1 co-determines the central cell fate in combination with a hypothetical X factor at the micropylar pole, and in the alternate model, CKI1 alone determines the central cell fate.

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Introduction

Two-component system (TCS)-mediated phosphorylation is one of the primary mechanisms in the regulation of signal transduction in plant cells. Kamberov et al. first identified two-component signaling elements in *Escherichia coli* when they investigated the nitrogen regulatory protein system.¹ In plants, the basic two-component scheme is a complicated multistep phosphorylation system that employs hybrid histidine kinase (HK) comprising HK and receiver domains, a histidine-containing phosphotransfer protein, and a separate response regulator (RR).² The activation of TCS is dependent on the upstream hybrid HK, which is capable of autophosphorylating itself on a conserved histidine residue and transferring phosphate to downstream regulators. In *Arabidopsis*, seven hybrid HK proteins, including three cytokinin receptor proteins (i.e., AHK2, AHK3, and AHK4), AHK1, CKI1, AHK5, and an ethylene receptor (i.e., ETR1) can act as upstream activators of TCS.^{2–7}

CYTOKININ INDEPENDENT 1 (*CKI1*) was identified as an HK gene, whose ectopic expression induces a typical cytokinin response in the absence of exogenous cytokinin.⁸ CKI1 contains two transmembrane domains in the N-terminal region and mainly localizes in the endoplasmic reticulum and plasma membrane.^{9–11} CKI1 acts upstream of AHPs to activate the two-component signaling pathway.^{12–15} *CKI1* has loss-of-function mutants in the form of female gametophytic lethal mutations but no homozygous mutants, indicating that *CKI1* is required for megagametogenesis.^{9,13,16,17} In our previous study, we introduced female gametophytic cell type-specific single and double markers into a *cki1-9/+* mutant. We observed that central and antipodal cell fates were completely lost and transformed into egg or synergid cell fates. On the other hand, the ectopic expression of

CKI1 in the micropylar domain transformed the egg and synergid cell fates into a central cell fate. These findings indicated that *CKI1* is a master regulator of central cell specification.^{10,18}

The female gametophyte (embryo sac) is critical to many steps of the angiosperm reproductive processes, including pollen tube guidance, fertilization, seed initiation after fertilization, and maternal control of seed development after fertilization.¹⁹ Female gametophyte development, as one of the most important developmental events in the plant life cycle, is important for fundamental research in plant reproduction and agricultural breeding. Thus far, functional research on plant TCS mainly focuses on stress adaption and vegetative growth. Meanwhile, research on reproductive development, particularly female gametophyte development and cell fate specification remains limited. In this study, we aim to supplement current knowledge on CKI1-mediated TCS signaling in *Arabidopsis* female gametophyte specification to provide insights into this elaborate process.

Dynamic distribution of TCS signals during arabidopsis embryo sac development

Similar to the synthetic auxin-responsive reporter *DR5*, TCS, which contains the type-B RR binding motifs and a minimal 35S promoter, is often used as a universal cytokinin-responsive reporter and HK-activated two-component signaling reporter.²⁰ Bencivenga et al.²¹ detected TCS signals in embryo sacs with the *TCSpro::GFP* line but only detected signals in the basal part of the nucellus and in the funiculus starting from stage 2–III. They also found that the GFP signal was drastically reduced and hardly visible at

stage 3-V except in the funiculus, where its expression was stable. Notably, this version of TCS-mediated reporter expression is considerably weak in the embryo sac.^{13,16,17,21} GFP expression progressively decreases with increasing generations.¹³ To overcome these barriers, a superior version TCS new (*TCSn*), with optimized response elements was developed;^{22,23} this version exhibited robust and high sensitivity to cytokinin and two-component signaling. Furthermore, *TCSn::GFP* expression was detected in female gametophytes until the mature (FG7) stage, where some GFP signals were reported at the micropylar end.²² The *TCSn* reporter has also been utilized in rice.²⁴

To determine the precise stage and location of TCS signals during embryo sac development, we utilized a nuclear localized GFP reporter *TCSnpro::NLS-3XeGFP* in combination with a *AKVpro::NLS-Mcherry* reporter that labels all the nuclei in the embryo sac.¹⁰ As shown in Figure 1, TCS signals were stably detected from the FG1 stage to the mature embryo sac (FG7) stage in plants carrying both the *TCSnpro::NLS-3XeGFP* and *AKVpro::NLS-Mcherry* reporters. The colocalization of GFP and RFP signals in the female gametophytic nuclei suggests that TCS signaling is active throughout the female gametophyte in the early stages of development (Figure 1 A-D). At the later stages, beginning with polar nuclei migration and cellularization, TCS signals were weaker in the egg cell nucleus and two synergid nuclei relative to the polar nuclei and antipodal nuclei (Figure 1 E). During the progression from fusion of the two polar nuclei fusion to maturity of the embryo sac, the TCS signals disappeared entirely from the egg cell and synergid cell, but remained strong in the antipodal cells and the central cell (Figure 1 F-G). Intense TCS signals were also detected in the sporophytic tissues at the chalazal end, especially at the earlier stages (Figure 1). Since there is no *CKI1* expression in these sporophytic tissues, these signals might arise from the AHK cytokinin receptors as they can activate TCS in response to cytokinin. Consistent with this interpretation, genetic studies using AHK cytokinin receptor mutants have shown that functional AHKs are required in the sporophyte for female gametophyte fertility.^{25,26}

Our previous research showed that in the *cki1-9* loss-of-function mutant, TCS signals were absent in the mature female gametophyte, whereas TCS signals in the sporophytic tissues at the chalazal end were unaffected, suggesting that CKI1 was the primary activator of the TCS signaling pathway in the embryo sac.¹⁰ However, the localization of CKI1 protein was found to be restricted to the chalazal domain from the FG4 stage.¹⁰ This polarized distribution of CKI1 differs from the TCS signals reported here, which are found in all nuclei until the final stages of development. This discrepancy might be due to the movement of the targets of CKI1 kinase, the AHP proteins, within the syncytial embryo sac. In the previous study, the CKI1-GFP fusion protein included the ER localization signal of CKI1, so that during translation the protein would remain localized to the ER associated with the nucleus in which it was transcribed, and not free to localize to other nuclei within the coenocyte, thereby preserving the polarized CKI1 expression.¹⁰ In contrast, the AHP proteins have been shown to shuttle between the cytoplasm

and the nucleus.^{27,28} It is possible that CKI1 in the chalazal domain phosphorylates cytoplasmic AHPs that can then enter any of the nuclei within the coenocyte to activate TCS signaling. However, after cellularization such movement of AHPs would be restricted by the cell membranes, and the TCS signals would then colocalize with CKI1 expression, i.e., present only in the antipodal cells and the central cell, as shown in Figure 1F-G.

Models for cell fate specification in embryo sacs

Cell fate specification is an elaborate process involving numerous genes and pathways. In our previous work, we already showed that CKI1 acts as an upstream regulator within TCS pathway network in female gametophyte development process, particularly in central cell and antipodal cell specification.^{10,15} Here, focusing on CKI1, we proposed two models for cell fate specification in *Arabidopsis* embryo sacs.

Model 1: CKI1 and an X factor in the micropylar end codetermine the central cell fate

In this model (Figure 2 A), an X factor located in the micropylar end is indispensable for egg and synergid cell fates. CKI1, the Y factor, is located in the chalazal end and is necessary for antipodal and central cell fates. In wild-type embryo sacs, the Y factor limits the spread of the X factor from the micropylar pole to the chalazal pole, thereby ensuring the polarity between both factors. At the FG5 stage, one nucleus with the Y factor from the chalazal pole and the other nucleus with the X factor from the micropylar pole centrally migrate, thereby initializing the fusion process. Consequently, after cellularization (FG6 stage), the fused cell acquires the Y and X factors to confer the central cell fate. The X factor alone specifies the egg cell fate, but the synergid cell fate is codetermined by the X factor, and an additional W factor. The W factor exists only in synergids and polar nuclei and is activated from the beginning of the cell fate specification stage. Antipodal cells fate is specified by Y factor alone, without the X factor from the micropylar pole. In the *cki1/+* loss-of-function mutants, the X factor is relieved of the influence of the Y factor and spreads to the chalazal end; thus, three antipodal cells and two unfused polar nuclei acquire the X factor, which primarily confers egg cell fate. The W factor in the polar nuclei, together with the X factor, could alter the cell fate in polar nuclei, thereby switching from the egg cell fate to the synergid cell fate, which could explain the spatiotemporal dynamic expression of egg and synergid cell markers in the *cki1-9/+* mutant.¹⁰ Synergid cells in the micropylar end could maintain their cell fates because of the presence of the X and W factors. However, when *CKI1* is ectopically overexpressed (CKI1-OX) in the micropylar end, the Y factor is introduced and the polarity is disturbed. Thus, three gametophytic cells in the micropylar end acquire both the Y and X factors, thereby leading to the specification of the central cell fate. Antipodal cells in the chalazal end could maintain its cell fate because of the presence of the Y factor (CKI1). This model does not require an additional factor from the chalazal pole ("Z", see below) for antipodal specification; however, it does not exclude the possibility that such an additional factor might also be involved.

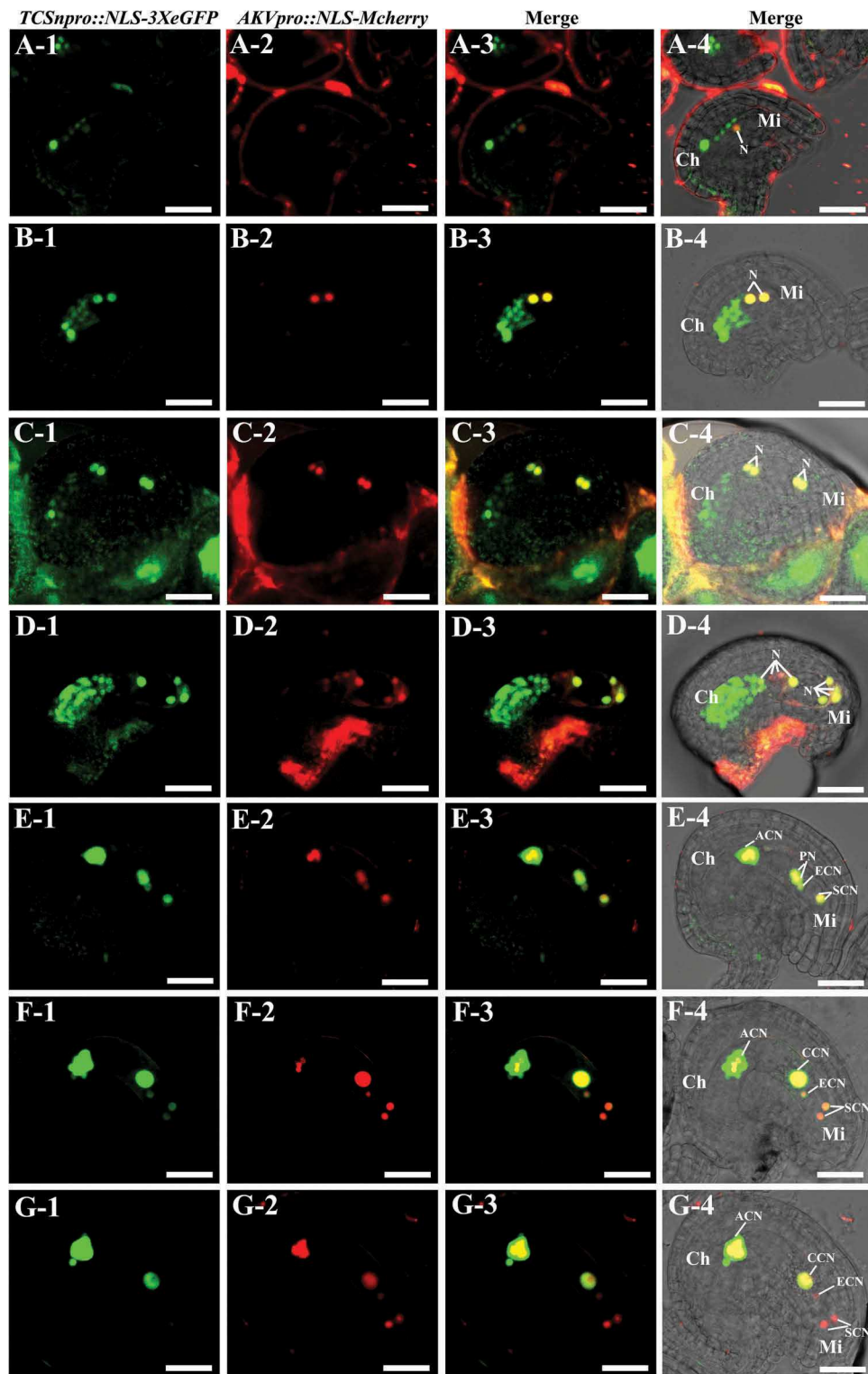


Figure 1. Dynamic distribution of TCS signals during *Arabidopsis* embryo sac development. (A) TCS signals in the FG1 stage, where the nucleus shows TCS signals. (B) TCS signals in the FG2–FG3 stages, where both nuclei show TCS signals. (C) TCS signals in the FG4 stage, where all of the four nuclei show TCS signals. (D) TCS signals in the FG5 stage, where all of the eight nuclei show TCS signals. (E) TCS signals in the FG6 stage, where the two polar nuclei fuse and all of the eight nuclei show TCS signals, whereas sporophytic TCS signals in the chalazal end become hardly detectable. (F) and (G) TCS signals in the FG7 stage. Ch, chalazal end; Mi, micropylar end; N, nucleus; CCN, central cell nucleus; PN, polar nuclei; SCN, synergid cell nuclei; ECN, egg cell nucleus; ACN, antipodal cell nuclei. Scale bars = 20 μ m.

Model 2: CKI1 alone determines the central cell fate

In this model (Figure 2 B), CKI1 (Y factor) alone determines central cell fate, and an unknown X factor determines egg cell fate. The synergid cell fate is codetermined by the X and W factors. Antipodal cell fate is codetermined by the Y and Z

factors. The Z factor may exist in antipodal cells or may originate from the surrounding sporophytic tissues at the chalazal pole. In wild-type embryo sacs, the Y factor could still limit the spread of the X factor from the micropylar pole to the chalazal pole to ensure the polarity of the two factors. After polar nuclei

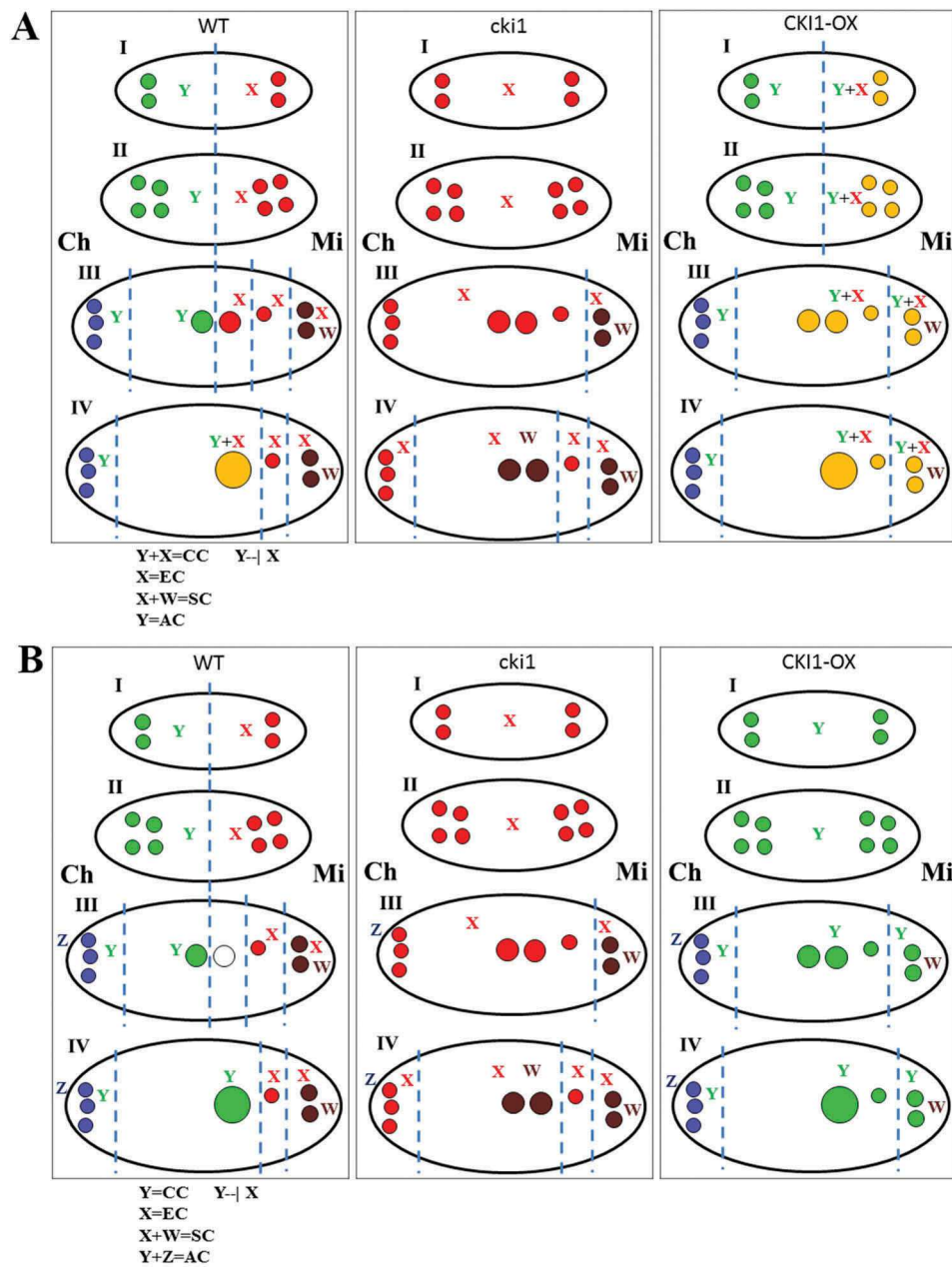


Figure 2. Models for cell fate specification in *Arabidopsis* embryo sacs. (A) Model 1: CKI1 in the chalazal end and X factor in the micropylar end codetermine central cell fate. $Y + X = CC$, where Y and X factors codetermine central cell fate; $Y = AC$, where Y factor alone determines antipodal cell fate; $X = EC$, where X factor alone determines egg cell fate; $X + W = SC$, where X and W factors codetermine synergid cell fate; $Y \dashv X$, where Y factor suppresses X factor. See text for details. (B) Model 2: CKI1 in the chalazal alone determines the central cell fate. X, Y, Z, and W are the female gametophytic cell fate determinant factors; CKI1 is the Y factor. $Y = CC$, where Y factor alone determines central cell fate; $X = EC$, where X factor alone determines egg cell fate; $X + W = SC$, where X and W factors codetermine synergid cell fate; $Y + Z = AC$, where Y and Z factors codetermine antipodal cell fate; $Y \dashv X$, where Y factor suppresses X factor from the chalazal domain. Ch, chalazal pole; Mi, micropylar pole. I, II, III, and IV represent the embryo sac development stages. The antipodal cell nucleus is marked with a blue circle; the central cell nucleus is separately marked with a yellow circle in Model 1 and a green circle in Model 2; the egg cell nucleus is marked with a red circle; the synergid cell nucleus is marked with a purple circle.

fusion and cellularization (FG5–FG6), the fused cell acquires the Y factor from one nucleus, which centrally migrates from the chalazal pole, and confers the central cell fate. In the *cki1/+* loss-of-function mutants, the mechanism of the cell fate switch is identical to that described in Model 1. However, in *CKI1-OX* embryo sacs, three gametophytic cells in the micropylar end acquire the Y factor; thus, these cells are transformed into central cells. Antipodal cell fate is unaltered because of the presence of the Y and Z factors in the chalazal end.

Apart from the Y factor CKI1, little is known about other factors, which are hypothesized to be cell fate determination-related proteins or plant growth regulators. Studies of genetic evidence of egg cell-related genes, such as *LACHESIS* (*LIS*),^{29,30} *CLOTHO* (*CLO*), *ATROPOS* (*ATO*),³¹ and *ZmEAL1*,³² as well as laser ablation to deplete the FG of a functional egg cell^{33,34} have indicated an important role of the egg cell in the differentiation of synergids, central cell, and antipodal cells.³⁵ Further research showed that the overexpression of *RKD1* and *RKD2* in

ovule sporophytic tissues could induce egg cell-like structures with egg cell-specific markers.^{33,36} Moreover, interference of the single-copy *RKD* gene in liverwort *Marchantia* exhibited defects in egg cell development.^{37,38} These results indicate the roles of *RKDs* in egg cell differentiation. However, no female gametophytic defects were observed in the *rkd1* and *rkd2* single and double mutants. Moreover, *RKD1* and *RKD2* were expressed only from the mature egg cell stage, thereby indicating that both are unlikely to be the egg cell fate determinant.

Auxin or its downstream targets could be a candidate X factor, as proposed by Pagnussat et al.³⁹ who demonstrated that micropylar cell fates (egg cell and synergids) could be altered by manipulation of auxin signaling. Furthermore, Panoli et al.⁴⁰ investigated the expression patterns of *AUX1* with *AUX1pro::AUX1-GFP* and determined that gametophytic *AUX1* was first detected in the micropylar end from the FG4 stage. This polarized localization of *AUX1* at the micropylar pole contrasts with localization of CKI1 (the Y factor) at the chalazal pole. However, no female gametophytic defects were observed in *aux1* mutants, possibly due to gene function redundancy with other members of the *AUX* gene family. Therefore, further research is needed to confirm whether auxin, *AUX1* or downstream participants in auxin signaling such as ARFs⁴¹ might fill the role of the proposed X factor.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

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