LACHESIS-dependent egg-cell signaling regulates the development of female gametophytic cells

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SUMMARY
In contrast to animals, plant germ cells are formed along with accessory cells in specialized haploid generations, termed gametophytes. The female gametophyte of flowering plants consists of four different cell types, which exert distinct functions in the reproductive process. For successful fertilization, the development of the four cell types has to be tightly coordinated; however, the underlying mechanisms are not yet understood. We have previously isolated the lachesis (lis) mutant, which forms supernumerary gametes at the expense of adjacent accessory cells. LIS codes for the Arabidopsis homolog of the pre-mRNA splicing factor PRP4 and shows a dynamic expression pattern in the maturing female gametophyte. Here, we used LIS as a molecular tool to study cell-cell communication in the female gametophyte. We show that reducing LIS transcript amounts specifically in the egg cell, affects the development of all female gametophytic cells, indicating that cell differentiation in the female gametophyte is orchestrated by the egg cell. Among the defects observed is the failure of homotypic nuclei fusion in the central cell and, as a consequence, a block in endosperm formation. LIS-mediated egg cell signaling, thus, provides a safeguard mechanism that prevents the formation of nurturing tissue in the absence of a functional egg cell.

KEY WORDS: Cell-cell communication, Egg cell, Female gametophyte

INTRODUCTION
Plant germ cells develop in specialized haploid structures, termed gametophytes. The female gametophyte of flowering plants comprises two gametes, the egg and the central cell. Both cells are fertilized by one of two sperm cells delivered by the pollen tube. The fertilized egg cell subsequently develops into an embryo, whereas the central cell generates the endosperm, which nurtures the developing embryo. This double-fertilization process distinguishes the reproductive mode of angiosperms from that of other plants and is considered to be one of the key traits responsible for the success of flowering plants (Friedman, 2006). Importantly, it ensures that the formation of nurturing tissue and hence the allocation of resources is only initiated upon sperm cell delivery. The formation of endosperm is of high metabolic cost to the plant and probably affects other life history constraints, suggesting that double fertilization is of high adaptive value. Apart from the two gametic cells, the female gametophyte forms two types of accessory cells. Two synergids lie at the entrance point of the pollen tube and are required for both the attraction of the pollen tube and the reception of the sperm cells (Higashiyama et al., 2001; Huck et al., 2003; Rotman et al., 2003; Amien et al., 2010). The function of the three antipodal cells at the opposite pole of the female gametophyte is less clear; however, they might have a nourishing function that is achieved by transferring nutrients from the surrounding sporophyte to the female gametophyte (Raghavan, 1997). The development of the distinct cell types has to be closely coordinated to ensure reproductive success and there is some evidence for cell-cell communication in the female gametophyte: plants with reduced mitochondrial activity in the central cell exhibit an increased antipodal lifespan, indicating that programmed cell death of antipodal cells is under regulatory control by the central cell (Kägi et al., 2010). Moreover, it has been shown that synergids, egg cells and central cells are symplastically connected in the early eight-nucleate female gametophytes of Torenia fournieri (Han et al., 2000). In maize, small peptides have been identified that, owing to their size, have the potential to move between cells (Dresselhaus, 2006). A further hint for communication between single female gametophytic cells comes from the analysis of the lachesis (lis) mutant (Groß-Hardt et al., 2007). lis mutants form supernumerary egg cells, resulting from misspecification of the synergids and central cell, which express morphological and molecular attributes of the egg cell. Furthermore, antipodal cells adopt a central cell fate in lis mutants, demonstrating that all cells in the female gametophyte can differentiate into gametic cells. lis is defective in the Arabidopsis homologue of the yeast pre-mRNA splicing factor PRP4 (Groß-Hardt et al., 2007), and promoter-reporter analysis has suggested that cell specification in wild-type female gametophytes correlates with a strong upregulation of LIS expression in gametic cells. By contrast, LIS expression appears to be downregulated in accessory cells of mature female gametophytes, which has led to the hypothesis that the gametic potential of accessory cells is repressed by lateral inhibition from the gametic cells (Groß-Hardt et al., 2007). Whereas LIS appears to be involved in the maintenance of cell fates, the plant hormone auxin has been implicated in the establishment of the distinct female gametophytic cells. Pagnussat et al. have studied the effects of altered auxin synthesis and response and analyzed the expression of the auxin response regulator DR5 (Pagnussat et al., 2009). The presented data are consistent with a model whereby a micropylar-chalazal auxin gradient determines the different cell fates in the female gametophyte.
Here, we have made use of *LIS* as a molecular tool to study cell-cell communication in the female gametophyte. We show that reducing *LIS* transcript amounts specifically in the egg cell affects synergids, central cell and antipodals, suggesting that the egg cell acts as a signaling center for the development of all female gametophytic cells. Moreover, reducing *LIS* transcript levels in the egg cell interferes with homodiploidization of the central cell and, consequently, endosperm formation. *LIS*-mediated egg cell signaling thus reveals a safeguard mechanism, which prohibits the formation of nurturing tissue in the absence of a functional egg cell.

**MATERIALS AND METHODS**

**Plant material and growth conditions** Plants were grown on soil in growth chambers under long-day conditions (16 hours light/8 hours dark) at 18°C.

**Molecular cloning** The promoters *DD2*, *DD45*, *EC1*, *MEA*, *HSFa2* and *35S* used in front of 2xGFP, *LIS* and/or *lis* (RNAi) were amplified (see supplementary material Table S3) and cloned into the pGEM-T vector (Promega). The *DD45*, *MEA* and *EC1* promoters were cloned into the binary vector pGIIbar-LIS::2xGFP_LIS (Moll et al., 2008) in replacement of the *LIS* promoter. To generate *DD45::NLS_3xGFP* and *DD2::NLS_3xGFP*, the respective promoters were introduced into pGIIbar-NLS_3xGFP (Takada and Jürgens, 2007). For the *lis* (RNAi) construct, a 500 bp fragment was amplified from *LIS* cDNA (Groß-Hardt et al., 2007) with primers P-ASs and P-ASas containing BamHI and PacI restriction sites, and with primers P-Ss and P-Pac containing MluI and NotI restriction sites and cloned into pGEM-T (Promega). The two fragments were subsequently introduced into pGIIbar-EC1 containing an additional MCS-adaptor between the PacI and SpeI restriction sites (see supplementary material Table S3). Between the two *lis* (RNAi) fragments, a Fad2 intron of vector pEC1-1f2 (S.S., unpublished) was inserted using BamHI/MluI restriction sites. Subsequently, the *DD2*, *DD45*, *MEA*, *HSFa2* and *35S* promoters were introduced in front of the *lis* (RNAi) construct. For generating pGIIbar-EC1::NLS_3xdsRed::tNOS, the *EC1* promoter and the transcription termination sequence *tNOS* were amplified with primers RV3s/RV3as containing EcoRI/PstI restriction sites and RV4s/RV4as containing HindIII/EcoRI restriction sites and cloned into pGEM-T vector. Subsequently, the *EC1* promoter and *tNOS* were introduced into pGIIbar. The NLS_3xdsRed was excised from ER126 (T. Laux, unpublished) using the BamHI restriction site and introduced into pGIIbar containing EC1 and *tNOS*.

**mRNA isolation and semi-quantitative PCR** mRNA was isolated from leaves, buds and flowers with Dynabeads mRNA Purification Kit (Invitrogen). For-PCR and first-strand synthesis, the Revert Aid H-Minus First Strand cDNA Synthesis Kit from Fermentas was used with Oligo(dT)-primers. For semi-quantitative PCR equal amounts of cDNA were used. The cDNA fragment of *cLIS* was amplified using P-LISs and P-LIS2as, and *cACTIN* was amplified using P-AC Ts and P-AC T as see supplementary material Table S3).

**Histology and microscopy** For analysis of mature female gametophytes, the oldest closed flower bud of a given inflorescence was emasculated and harvested 2 or 3 days later. Whole-mount clearing was performed as described previously (Groß-Hardt et al., 2007). Cleared whole mounts were visualized using a Zeiss Axiolab Microscope (Zeiss, Oberkochen, Germany). GFP- and dsRED-expressing specimens were observed using a Leica DMIF6000B.

**RESULTS AND DISCUSSION**

In the female gametophyte, *LIS* is non-mobile and preferentially accumulates in gametic cells. We have previously shown that the *LIS* expression pattern in maturing female gametophytes appears to be dynamic: a nuclear localized β-glucuronidase (GUS) reporter, fused to the *LIS* promoter, confers expression to all cells of young eight-nucleate gametophytes. However, shortly after cellularization, expression is hardly detectable in accessory cells and is upregulated in gametic cells (Groß-Hardt et al., 2007). To address the question of whether the transcriptional dynamics were reflected at the protein level, we tagged the *LIS* cDNA N-terminally with two copies of the green fluorescent protein (2xGFP). We have previously shown that the chimeric LIS protein complements the *lis* mutant, indicating that the GFP did not interfere with *LIS* protein function (Moll et al., 2008).

In mature female gametophytes of wild-type plants containing the *LIS::2xGFP_LIS* construct, the fluorescence signal was detected in all female gametophytic cells, with the strongest expression in the egg cell and the central cell, indicating that the upregulation of *LIS* promoter activity in gametic cells translates into increased protein amounts (Fig. 1A). The fact that the GFP-tagged LIS protein was detected in both accessory cell types suggests that the transcriptional downregulation of *LIS* in these cells is masked by the stability of the protein. Alternatively, we could not rule out the possibility that the LIS protein is mobile. We, therefore, expressed 2xGFP_LIS specifically in the egg cell and in the central cell using the *DD45* and the *MEA* promoters, respectively (Groß-Hardt et al., 2007; Steffen et al., 2007). The expression profile of *DD45* was originally described for the Columbia accession and by analyzing a *DD45::NLS_3xGFP* reporter construct, we could confirm the egg cell-specific expression profile in Landsberg *erecta* plants (Fig. 1B).

Importantly, in *DD45::2xGFP_LIS* and *MEA::2xGFP_LIS* plants, we detected the fluorophore specifically in the egg cell and the central cell, respectively (Fig. 1C,D). This indicates that the GFP-tagged LIS protein is not mobile in the time window covered by the respective two promoters and suggests that the presence of 2xGFP_LIS in the accessory cells is due to protein stability.

**Fig. 1.** The LIS protein is immobile and preferentially accumulates in gametic cells. (A) *LIS::2xGFP_LIS* is detected ubiquitously with strongest expression in the egg and central cell. (B) In Landsberg *erecta* DD45::NLS_3xGFP plants, the fluorescence signal is observed only in the egg cell. (C,D) In *DD45::2xGFP_LIS* plants (C), GFP is specifically detected in the egg cell (n=208) and in *MEA::2xGFP_LIS* plants (D), GFP is specifically detected in the central cell (n=276). White arrowhead, synergid nucleus; black arrowheads, egg cell nucleus; black asterisk, central cell nucleus; white asterisk, antipodal nucleus. Scale bar: 25 μm.
The upregulation of LIS in gametic cells correlates temporally with the establishment of the distinct cell types, and we next asked whether enhanced LIS expression in gametic cells was of functional relevance for the development of female gametophytic cells. In order to reduce LIS transcript amounts specifically, we made use of a RNA interference (RNAi) approach.

**Constitutive lis (RNAi) lines exhibit reduced LIS mRNA levels and growth retardation**

To analyze whether a lis (RNAi) construct could effectively reduce LIS transcript levels, we expressed the construct ubiquitously in sporophytic tissue using the 35S promoter. Of 12 independent 35S::lis (RNAi)/− lines, six showed a dwarf-like phenotype (see supplementary material Fig. S1A). Semi-quantitative PCR of three independent lines revealed that the respective plants contained substantially reduced LIS transcript amounts, suggesting that 35S::lis (RNAi)/− plants are lis hypomorphs (see supplementary material Fig. S1B). We had previously shown that homozygous lis plants are lethal (Groß-Hardt et al., 2007) and the severe growth retardation in 35S::lis (RNAi)/− plants is in line with an essential role of LIS in plant development.

**Reduced LIS expression in the egg cell compromises the development of all female gametophytic cells**

To modulate LIS transcript dynamics in maturing egg cells, we next expressed the RNAi construct from the DD45 promoter, which is specifically active in the egg cell and early embryos (Steffen et al., 2007). Of 19 independent transformants, seven lines exhibited a reduced fertility (see Table S1A in the supplementary material). Of these seven lines, four showed morphological abnormalities in the female gametophyte strongly resembling lis mutants (Fig. 2A,C,E,F; Fig. 4A). We consistently observed a failure of polar nuclei fusion in the central cell. Additionally, synergid nuclei size was increased and comparable with egg cell nuclei size in three lines. In four lines, degeneration of antipodals was impaired and in some cases we observed that the antipodals had protruded towards the central cell (Fig. 4F). As in lis mutants, the morphological defects in DD45::lis (RNAi)/− correlated with reduced fertility, albeit to a lower extent (see supplementary material Table S1A). A hallmark of the lis mutant defect is ectopic expression of the egg cell marker ET1119 in central cell and synergids. However, in DD45::lis (RNAi)/− plants, we did not observe ectopic expression of this reporter (data not shown), and expression of a second egg cell marker line, EC1::NLS_3xdsRED, was also comparable between wild-type and DD45::lis (RNAi)/− plants (see supplementary material Fig. S3). This molecular discrepancy might be accounted for by temporal differences in LIS transcript depletion, which in lis initiates with the onset of megagametogenesis and in DD45::lis (RNAi) only after cellularization.

lis mutant female gametophytes become infrequently fertilized and the resulting seeds contain undeveloped endosperm, which is a likely consequence of central cell mis-specification (Groß-Hardt et al., 2007) (Fig. 2B,D). Notably, this defect was also observed in DD45::lis (RNAi)/− plants, indicating that the central cell is functionally impaired (Fig. 2G). At first glance, these results suggest that defects in the egg cell impair the development of other female gametophytic cells. However, it has been established that RNAi can spread between cells (Baulcombe, 2004) and we could therefore not exclude that the broad spatial range of symptoms observed in DD45::lis (RNAi) was due to systemic downregulation
of LIS in the entire female gametophyte. To test whether DD45::lis (RNAi) specifically reduced LIS transcript amounts in the egg cell, we introduced a GFP-tagged LIS cDNA under the control of the EC1 promoter (Ingouff et al., 2009) into the DD45::lis (RNAi)/– T2-4 line. EC1 confers expression specifically to the egg cell in Columbia accessions (Ingouff et al., 2009). However, in Landsberg erecta plants, we occasionally detected an additional moderate expression in synergids and a very weak signal in the central cell.

To assess the effects of reduced LIS transcript in the cells adjacent to the egg cell, we therefore targeted the RNAi construct to the synergids, central cell and antipodal cells, using the cell-specific promoters DD2 (Steffen et al., 2007), MEA (Groll-Hardt et al., 2007) and HSFa2 (Kägi et al., 2010), respectively. Fortunately, not only the promoter of MEA but also the one of DD2 is additionally active after fertilization in the endosperm allowing direct assessment of the functionality of both constructs (Fig. 4 B,C) (Köhler et al., 2003; Steffen et al., 2007). Of 15 independent MEA::lis (RNAi)/– transformants, six showed defects after fertilization (see supplementary material Table S1B). These plants exhibited various degrees of reduced endosperm formation and the seeds eventually aborted. Two lines arrested at the megaspore stage resulting in sterile ovules. As we never detected MEA expression at this stage (data not shown), the defect is likely to be due to nonspecific defects caused by the T-DNA insertion. Importantly, female gametophyte development was indistinguishable from wild type in the remaining lines. Experiments using the DD2 promoter yielded similar results. Of 18 transgenic lines analyzed, 15 exhibited an increased number of aborted seeds (see supplementary material Table S2A), which could be traced back to severe defects in endosperm development. Two of the DD2::lis (RNAi)/– lines revealed defects in the maturing female gametophyte. However, by introducing EC1::2xGFP_LIS as a reporter for LIS transcript amounts, we could show that in line 0 LIS mRNA levels appeared to be not reduced, whereas in line C LIS mRNA levels were additionally reduced in the egg cell (see supplementary material Fig. S4). These results suggest that the defects are not caused by a synergid-specific reduction of LIS transcripts.

Finally, we expressed lis (RNAi) under control of the antipodal promoter HSFa2 (Fig. 4D, supplementary material Table S2B). None of the 20 independent transformants analyzed exhibited a defect in the mature female gametophyte. Together, our data indicate that a moderate reduction of LIS transcript amounts in the synergids, central cell and antipodal cells is tolerated, whereas reduction of LIS transcript in the egg cell affects all female gametophytic cells. This indicates that cell differentiation in the female gametophyte is orchestrated by the egg cell through a LIS-dependent lateral inhibition signal (Fig. 4E).

Together, we have shown that DD45::lis (RNAi) targets LIS specifically in the egg cell. Surprisingly, downregulating LIS in the egg cell only affects the development of the entire female gametophyte with terminal consequences: the resulting female gametophyte does not only show severe morphological abnormalities but additionally reduced fertility. These results imply that egg cell integrity is required for completion of female gametophyte development. Among the defects observed in DD45::lis (RNAi) gametophytes is the failure of polar nuclei fusion, indicating that this process, which is a prerequisite for
endosperm formation, is possible only in the presence of a functional egg cell. The *LIS* dependent cell-cell communication thus suggests a safeguard mechanism, whereby the formation of endosperm, which is of high metabolic cost, is only enabled in the presence of a fertilizable egg cell.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.075234/-/DC1

**References**


**Fig. 4.** *DD45::lis (RNAi)* affects the development of all female gametophytic cells. (A) Morphological characterization of four *DD45::lis (RNAi)/–* lines. (B, C) Expression of DD2::NLS_3xGFP (B) and of MEA::NLS_GUS (C) in the four-nucleate endosperm. (D) Expression of HSFa2::NLS_GUS in antipodals. (E) Model of LIS function. pn, polar nuclei; syn, synergids; ec, egg cell; ap antipodals; white arrowheads, endosperm nucleus; white asterisk, antipodal cells. Scale bar: 25 μm.