LACHESIS Restricts Gametic Cell Fate in the Female Gametophyte of Arabidopsis

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In flowering plants, the egg and sperm cells form within haploid gametophytes. The female gametophyte of Arabidopsis consists of two gametic cells, the egg cell and the central cell, which are flanked by five accessory cells. Both gametic and accessory cells are vital for fertilization; however, the mechanisms that underlie the formation of accessory versus gametic cell fate are unknown. In a screen for regulators of egg cell fate, we isolated the lachesis (lis) mutant which forms supernumerary egg cells. In lis mutants, accessory cells differentiate gametic cell fate, indicating that LIS is involved in a mechanism that prevents accessory cells from adopting gametic cell fate. The temporal and spatial pattern of LIS expression suggests that this mechanism is generated in gametic cells. LIS is homologous to the yeast splicing factor PRP4, indicating that components of the splice apparatus participate in cell fate decisions.

Introduction

The formation of gametes is a key step in the lifecycle of any sexually reproducing organism. In flowering plants, the egg and sperm cells develop within haploid gametophytes (Figure 1). The female gametophyte of Arabidopsis originates from a single haploid spore (Figure 1A) through three nuclear division cycles. The resulting syncytium of eight nuclei (Figure 1B) cellularizes and differentiates four distinct cell fates [1–4] (Figure 1C and 1D). Both the egg and central cells are fertilized by one sperm cell each to form the embryo and the surrounding endosperm, respectively. These gametic cells are flanked by accessory cells. Two synergids lie at the micropylar pole, the entry point of the pollen tube. Synergids are necessary for the attraction of the pollen tube and induce the subsequent release of the sperm cells [5–7]. The opposite pole is occupied by three antipodal cells that degenerate prior to fertilization and whose function is unclear [3].

Although collections of female gametophytic mutants have been reported [8,9], mechanisms that underlie the specification of gametic versus accessory cell fate are unknown. In the present study, we took advantage of an egg cell–specific marker that we isolated in a screen for enhancer detector (ET) and gene trap (GT) lines, to examine the regulation of gametic cell fate. Our results indicate that a combinatorial mechanism operates to ensure maximum likelihood that the key reproductive gametic cells are formed, while at the same time deleterious excess gametic cell formation is prevented.

Results/Discussion

In lachesis-1 Female Gametophytes, All Cells Differentiate Gametic Cell Fate

We performed a screen for ethyl methanesulfonate (EMS)-induced mutants that alter the expression of the enhancer detector line ET1119, which in wild type confers specific β-glucuronidase (GUS) expression to the egg cell (Figure 2A).

In lachesis-1 (lis-1) mutants, expression of the egg cell marker was expanded to the synergids and the central cell, suggesting that the restriction of egg cell fate to a single cell is compromised (Figure 2B and 2C). lis-1 is a loss-of-function mutation for which no homozygous plants were recovered (see below). Therefore, all analyses were performed on heterozygous plants in which only 50% of the ovules contain lis-1 mutant female gametophytes. Heterozygous lis-1/LIS plants produced fertilized seeds and aborted ovules at a 1:1 ratio (50.7%:49.3% in lis-1/LIS, n = 631; 96.6%:3.4% in wild type, n = 653; Figure 1E and 1F), consistent with a female gametophytic defect [10]. Reciprocal crosses with wild-type plants confirmed that lis-1 was rarely transmitted maternally (transmission efficiency through the female [TEf] = 8.6%, n = 347). Paternal transmission was also affected, but less severely (transmission efficiency through the male [TEM] = 59.4%, n = 367).

To determine whether lis-1 female gametophytes are indeed defective in cell specification, we examined morphological, molecular, and functional characteristics of the different cell types in lis-1 gametophytes (Figures 1, 2, and 3, respectively). Until cellularization, gametophytes were indistinguishable between lis-1/LIS and wild-type plants (unpublished data), indicating that LIS is not required for any previous step, including mitotic divisions, migration of nuclei, or cellularization. The first defects in lis-1 female gametophytes were consistently observed only after cellula-
Author Summary

The selection and specification of the egg cell determine the number of eggs produced by an animal or plant, which in turn dictates how many offspring that organism can produce. In most higher plants, the egg cell forms in a specialized structure consisting of four different cell types. Two cells, the egg cell and the central cell, are fertilized by sperm cells and develop into the embryo proper and the nutritive tissue (endosperm), respectively. These two gametic cells are flanked by accessory cells; but why do some cells become gametic while others differentiate into accessory cells? To answer this question, we looked for mutants in which this process is disturbed. In the *lachesis* mutant, accessory cells become extra egg cells. Interestingly, it seems that the misspecification of these accessory cells results from defects in the gametic cells. This suggests that accessory cells monitor the state of the gametic cells to act as a backup if required, ensuring the formation of the key reproductive cells.

**Figure 1. Accessory Cells in *lis-1* Gametophytes Morphologically Resemble Gametic Cells**

(A–D) Schematic representation of wild-type female gametophyte development. Sporophytic structures are shown in grey; gametophytic structures are colored. (A) After meiosis, the haploid functional megaspore is formed. (B) A series of three mitotic divisions results in the formation of an eight-nucleate syncytium. (C) After nuclear migration and cellularization, a seven-celled gametophyte is formed containing two synergids at the micropylar end (dark green), one egg cell (red), one central cell (orange) with two polar nuclei, and three antipodal cells at the chalazal pole (light green). (D) Prior to fertilization, the two polar nuclei fuse to form one large central cell nucleus, and the antipodal cells degenerate.

(E) Wild-type silique showing full seed set.

(F) Silique of *lis-1*/*LIS* plants containing aborted ovules (arrowheads).

(G–I) Mature wild-type gametophyte. (G) At the micropylar end, the two small synergid nuclei are detected (stars). The larger egg cell nucleus (arrowhead) is oriented towards the adjacent central cell. (H) A large central cell nucleus (arrowhead) resulting from the fusion of the two polar nuclei can be detected. (I) The antipodal cells at the chalazal end degenerate (star). As a consequence, synergid and egg cell become indistinguishable when lying in a similar position (arrowheads). (J) The synergid nuclei are enlarged and mis-polarized (star). As a consequence, synergid and egg cell become indistinguishable when lying in a similar position (arrowheads). (K) Polar nuclei are unfused (arrowheads) and occasionally ectopically cellularized (arrowheads in M). (L and M) Antipodal cells do not degenerate, but enlarge and protrude towards the center (stars). (N) Disintegration of antipodal cells and fused antipodal nuclei (star).

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sperm after fertilization, approximately one half of the *lis-1* mutant gametophytes that received a pollen tube (compare Figure 3C) failed to develop endosperm (Figure 3D–3F) although embryo formation was not affected. The failure to develop endosperm is unlikely to be related to an unfertilized central cell, because embryo formation can initiate autonomous endosperm formation in an unfertilized central cell [18]. Together with the ectopic expression of the egg cell marker, our results indicate that in *lis-1* gametophytes, the central cell differentiates egg cell attributes at the expense of central cell fate.

A further striking phenotype was observed for the antipodal cells that degenerate prior to fertilization in wild type (Figure 1I). In *lis-1* female gametophytes, the antipodal cells were often enlarged and protruded into the center (Figure 1L and 1M; Table 1). Additionally, in about one third of *lis-1* gametophytes (16.9% in *lis-1/LIS* plants), the enlarged antipodal cells eventually disintegrated their cell membranes, allowing the fusion of antipodal nuclei into one large nucleus (Figure 1N). In wild-type gametophytes, fusion of nuclei was only detected in central cells (Table 1). Consistently, we observed ectopic expression of the central cell marker pMEA::GUS in the protruding antipodal cells (Figure 2M–2O), whereas the expression of GT3733, an antipodal marker, was down-regulated (Figure 2P–2R). Thus, antipodal cells that degenerate prior to fertilization in wild-type gametophytes, the antipodal cells eventually disintegrated their cell membranes, allowing the fusion of antipodal nuclei into one large nucleus (Figure 1N). In wild-type gametophytes, fusion of nuclei was only detected in central cells (Table 1). Consistently, we observed ectopic expression of the central cell marker pMEA::GUS in the protruding antipodal cells (Figure 2M–2O), whereas the expression of GT3733, an antipodal marker, was down-regulated (Figure 2P–2R). Thus, antipodal cells that degenerate prior to fertilization in wild-type gametophytes, the antipodal cells eventually disintegrated their cell membranes, allowing the fusion of antipodal nuclei into one large nucleus (Figure 1N). In wild-type gametophytes, fusion of nuclei was only detected in central cells (Table 1). Consistently, we observed ectopic expression of the central cell marker pMEA::GUS in the protruding antipodal cells (Figure 2M–2O), whereas the expression of GT3733, an antipodal marker, was down-regulated (Figure 2P–2R).
resulting in two uninucleate cells, whereas the uninucleate antipodal cells fused, producing a binucleate cell. These findings suggest that an intracellular signaling mechanism senses the number of nuclei in a given cell, and reveal a tremendous, previously unrecognized plasticity of the female gametophyte. In summary, whereas wild-type gametophytes differentiate accessory and gametic cell types, accessory cells of lis-1 mutant gametophytes frequently adopted gametic cell fate. These observations suggest that all cells in the female gametophyte are competent to differentiate gametic cell fate and that LIS is involved in a mechanism that represses gametic cell fate in the accessory cells. Interestingly, the gametic central cell in lis-1 gametophytes additionally adopted egg cell fate, suggesting that a further, LIS-dependent mechanism suppresses egg cell fate in the central cell. Thus, the lis-1 mutant phenotype reveals two levels of cell fate regulation, one between gametic and accessory cells, and one between egg and central cell.

**Successive Recruitment of Cells as Gametic Cells in lis-1 Gametophytes**

The late initiation of cell-specific marker genes indicates that in Arabidopsis distinct cell fates are only manifested after cellularization. Studies in several multicellular systems have shown that cell specification is often preceded by the asymmetric distribution of fate determinants (for review see [14]), and an analogous mechanism could be defective in lis-1 gametophytes, resulting in an instant misspecification of accessory cells. Alternatively, the lis-1 phenotype could result from defects that occur after cellularization when distinct cell fates become manifest. We analyzed the time course of egg cell and central cell marker gene expression. Interestingly, we found that the number of ovules that ectopically expressed gametic cell fate increased over time (Figure 4A and 4B), indicating that accessory cells are not instantly misspecified as gametic cells. In line with a successive misspecification of accessory cells, we found that several morphological features that distinguish lis-1/LIS plants from wild type became more pronounced over time (Figure 4C–4G). Our results suggest that during cell specification in lis-1 mutant gametophytes, accessory cells become gradually recruited as gametic cells.

**LIS Is Homologous to the Yeast Splicing Factor PRP4**

We mapped the lis-1 mutation to the At2g41500 locus, which encodes a protein with seven WD40 repeats. The lis-1 mutation creates an in-frame stop codon after three WD40 repeats (Figure 5A). The lis-1 cDNA driven by a 2.6-kilobase (kb) upstream promoter sequence complemented the lis-1 mutant phenotype (Figure 5B and 5C), indicating that lis-1 is a loss-of-function mutation.

The LIS protein is strongly conserved among eukaryotes ([15] (Figure S1), showing an overall similarity to Homo sapiens, Caenorhabditis elegans, and Saccharomyces cerevisiae of 62%, 56%, and 54%, respectively. The yeast homolog PRP4 is associated with the U4/U6 complex of the spliceosome [16,17]. PRP4 is an essential splicing factor, and loss-of-function mutants accumulate unspliced pre-mRNA [18]. PRP4 function depends on its interaction with a second splicing factor, PRP3, through its WD40 domain, and the deletion of two WD40 repeats abolishes this interaction [19]. We thus conclude that lis-1 represents the null phenotype, which is consistent with the observation that the lis-2 T-DNA insertion allele (Figure 5A) causes a very similar phenotype (Figure S2).

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**Table 1. Morphological Analysis of lis-1/LIS Plants**

| Genotype | Synergids | Central Cell | Antipodal Cells* |
|----------|-----------|--------------|-----------------|
|          | Nuclei < Egg Cell | Polarity of Egg Cell | Polar Nuclei | Fused Polar Nuclei | Ectopic Cellularization | Protruded | Membrane Disintegrated | Nuclei Fused |
| Wild-type | 99.2 | 98.8 | 92.8 | 0.0 | 0.0 | 0.0 | 28.7 | 0.0 | 0.0 |
| lis-1/LIS | 56.9 | 78.8 | 46.9 | 11.3 | 0.0 | 2.7 | 16.9 | 3.9 |

Emasculated siliques of wild-type and lis-1/LIS plants were analyzed 2 d after emasculation (*n* = 264 for wild-type, *n* = 514 for lis-1/LIS plants).

*Siliques 3 d after emasculation (*n* = 497 for wild-type, *n* = 620 for lis-1/LIS plants).

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**Figure 4. Successive Recruitment of Cells as Gametic Cells in lis-1 Gametophytes**

(A and B) Ectopic expression of the egg cell marker ET1119 (A) and the central cell marker pMEA::GUS (B) in wild-type and lis-1/LIS plants. Dark bars represent wild-type, light bars represent lis-1/LIS plants. The *y*-axis shows the percentage of ectopic expression of total GUS-staining ovules. Ectopic expression was scored zero (0d), one (1d), and two days (2d) after emasculation. Total number of ovules counted was greater than 250. (C–G) Development of five morphological features in lis-1/LIS plants as compared to wild type, zero (0d), one (1d), and two days (2d) after emasculation. The *y*-axis shows percent deviation from wild type (data from Table S1). (C) Synergid nuclei smaller than egg cell nucleus. (D) Different polarity of synergids and egg cell with respect to position of nucleus. (E) Polar nuclei unfused. (F) Ectopic cellularization. (G) Protruded antipodal cells.

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The Arabidopsis genome contains a second LIS-related sequence, At2g05720. The deduced protein (accession no. AAD25639; Figure S1) shares an overall similarity of 70%, but is only half the size of the LIS protein and notably contains only four complete WD40 repeats (Figure S1). Hence, At2g05720 is unlikely to be functionally redundant to LIS.

**LIS Is Strongly Up-Regulated in Gametic Cells**

To determine the temporal and spatial expression pattern of the LIS gene, we performed RT-PCR and analyzed the expression of a pLIS::NLS_GUS construct containing the same LIS promoter fragment as the LIS cDNA rescue construct that had fully complemented the mutant phenotype (Figure 5B and 5C). LIS expression was detected at moderate levels in all tissues examined, with strongest expression in reproductive tissues (Figure 6A). GUS expression driven by the LIS promoter was detected at all stages of female gametophyte development (Figure 6B–6E). Intriguingly, shortly after cellularization, expression in the accessory cells is down-regulated, whereas expression in the gametic cells is strongly up-regulated (Figure 6E). This suggests that the mechanism, which prevents accessory cells from adopting gametic cell fate, is not cell-autonomous, but is generated in gametic cells, which is consistent with a lateral inhibition model (Figure 6F). We propose that the gametic cells upon differentiation generate a LIS-dependent signaling molecule that is transmitted to the adjacent accessory cells to inhibit their gametic cell competence, thereby preventing excess gametic cell formation. In this view, the lis mutant phenotype is a result of impaired lateral inhibition, i.e., the gametic cells fail to identify themselves to their neighboring cells, resulting in the recruitment of all gametophytic cells as gametic cells. The observation that not all cells are synchronously specified as gametic cells implies some initial bias and suggests that the proposed lateral inhibition operates to maintain rather than to establish different cell fates.

Although this mechanism can perpetuate a binary decision between gametic and accessory cell fate, additional factors are needed to explain the generation of four distinct cell fates.

The surprising nature of the LIS protein as a splicing factor suggests the participation of components of the splicing machinery in cell fate decisions and, potentially, the generation of a lateral inhibition signal. A possible mode of action could be that LIS is involved in splicing this very signal or, much less direct, some upstream regulator.
Conclusion
Our data suggest that a combinatorial mechanism operates to pattern the female gametophyte: The competence of all cells to differentiate gametic cell fate, together with lateral inhibition from the gametic cells, can ensure maximum likelihood that the key reproductive gametic cells are formed, while at the same time, excess gametic cell formation is prevented. Both the expression of the LIS gene and its distinct function in regulating gametic cell fate are surprising, given that LIS is the Arabidopsis homolog of yPRP4, an integral part of the U4/U6 complex. In the future, identification of LIS target(s) and functional analyses of other tissue-specific splicing factors [20] will help to clarify the mechanistic role of the spliceosome in the regulation of distinct developmental processes.

Materials and Methods
Plant material and growth conditions. Plants were grown on soil in growth chambers under long-day conditions at 18°C. Enhancer detection trap lines were generated using the system of Sundaresan and colleagues [21]. (Send requests for ET and GT lines to UG, grossnik@botinst.unizh.ch.) The lis-1 allele was isolated from ET1119 in the Landsberg erecta (Ler) accession after mutagenesis; seeds were mutagenized by incubation in 0.15% EMS for 10 h. A total of 5,200 M1 plants were screened for deviating growth chambers under long-day conditions at 18°C.

ET1119 in the Landsberg erecta (Ler) accession after mutagenesis; the likelihood that the key reproductive gametic cells are formed, while at the same time, excess gametic cell formation is prevented. Both the expression of the LIS gene and its distinct function in regulating gametic cell fate are surprising, given that LIS is the Arabidopsis homolog of yPRP4, an integral part of the U4/U6 complex. In the future, identification of LIS target(s) and functional analyses of other tissue-specific splicing factors [20] will help to clarify the mechanistic role of the spliceosome in the regulation of distinct developmental processes.

Figure S1. Alignment of LIS, Arabidopsis AAD25639, H. sapiens, C. elegans, and S. cerevisiae PRP4 Proteins
The alignment was generated using CLUSTALW 1.8 with default parameters and BoxShade 3.21. Positions of identical and similar sequences are boxed in black and grey, respectively. The seven repeats of the WD40 motif as annotated by Horowitz et al. [15] are boxed in red; c indicates C. elegans, h indicates H. sapiens, and y indicates S. cerevisiae PRP4 proteins.

Supporting Information
Figure S1. Alignment of LIS, Arabidopsis AAD25639, H. sapiens, C. elegans, and S. cerevisiae PRP4 Proteins
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Figure S2. Expression Analysis of lis-2 and Wild-Type Gametophytes and Localization of T-DNA Insert
(A) Wild-type expression pattern of ET1119. (B) Abnormal expression pattern in lis-2. (C) Frequencies of ectopic egg cell marker expression. Dark bar represents wild-type, grey bar and white bar represent lis-1/LIS and lis-2/LIS plants, respectively. The y-axis shows the percentage of the scored phenotype (13.52% in lis-2/LIS plants, deduced from 6.76% expression in lis-2/LIS, ET1119%) plants, n = 1,921. For details on wild-type and lis-1/LIS data, refer to Figure 2A–2C. (D) T-DNA insertion site and sequence of left border flanking region. Red indicates the T-DNA sequence; grey indicates the intron sequence; and black indicates the exon sequence.

Table S1. Progression of the Morphological Phenotype in Wild-Type and lis-1/LIS Plants
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Accession Numbers
The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) accession numbers for proteins discussed in this paper and the supporting information are cPRP4 (NP_492936), hPRP4 (AAC51925), LIS (AAW80862), LIS homologous sequence (AAD25639), and yPRP4 (P20053).

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Author contributions. RGH, CK, and UG conceived and designed the experiments. RGH, CK, and NB performed the experiments. JMM, RB, WBG, and UG isolated ET and GT lines. RGH, CK, NB, GJ, and UG analyzed the data. RGH, GJ, and UG contributed reagents/materials/analysis tools. RGH wrote the paper.

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Competing interests. The authors have declared that no competing interests exist.
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Note Added in Proof

While this manuscript was in press, Raab and Hoth [27] reported that LIS/AtPRP4 is repressed by abscisic acid and is required for seed development.