A female *in vivo* haploid-induction system via mutagenesis of egg cell-specific peptidases

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Abstract

Crop breeding schemes can be significantly accelerated by using (doubled) haploid plants. In vivo haploid induction has been applied in plant breeding since decades, but is still not available for all crops, genotypes and haploidization rates are very low1-3. Therefore, plant breeders are highly interested in methodological improvements, especially, in new concepts to haploidization. Here, we report a novel system for the induction of haploid plants by mutating genes encoding egg cell-specific aspartic endopeptidases (ECSs). After successful sperm-egg fusion, ECSs play a critical role to ensure male and female nucleus fusion and parental genome incorporation. The ecs1 ecs2 double mutant pollinated by wild type pollen is capable to produce haploid offspring originating from semigamous zygotes. Chromosome elimination occurs during zygote and early embryo development. In summary, we report a novel approach for haploidization and additionally provide new insights into the molecular basis of fertilization.

Full Text

Haploid induction technology has been used in crop breeding since decades. The major advantage of generating haploid plants is that a genome can be doubled in a single step to generate doubled haploid plants whose genome is genetically fixed at each locus. This avoids the time and money consuming processes to generate homozygous plants required for plant breeding and seed production, which otherwise have to be generated after many cycles of inbreeding from diploid heterozygous material[1-5]. Compared with *in vitro* methods involving, for example, culture of explants containing haploid cells like isolated microspores, anthers or ovule, whose regeneration to plants is often limited to certain crops and genotypes, respectively, *in vivo* haploid induction techniques have the potential to be more widely applied and thus could significantly improve breeding efficiency and praxis[4-5].

Currently, *in vivo* haploid induction techniques are based on three major strategies: (i) induction of parthenogenesis, (ii) application of paternal inducer lines and (iii) uniparental genome elimination. Transgenic approaches have demonstrated that ectopic expression of a zygotically expressed gene *BABY BOOM LIKE1* (*BBML1*) encoding an AP2 transcription factor that is normally activated after fertilization from the paternal genome already in the egg cell is sufficient to trigger parthenogenesis in rice[6]. This approach has been transferred to other cereal crops like maize[7] and thus indicates that a single gene can be used to generate haploid embryos in grass crops. Similarly, in the dicot dandelion, expression of *PARTHENOGENESIS* (*PAR*) encoding a zinc finger EAR-domain protein in egg cells can trigger embryo development without fertilization. Recently, it was reported that expressing *PAR* in egg cells of lettuce can also induce parthenogenesis[8]. A popular technique in maize to generate double haploids is based on paternal Stock6-derived *in vivo* haploid inducer lines. The underlying gene was recently identified by three groups and named as *ZmPLA1/MTL/NLD (Zea mays PHOSPHOLIPASE A1/MATRILINEAL/NOT LIKE DAD)* encoding a sperm cell-expressed phospholipase lacking the very C-terminal domain and thus is incapable to be located in the sperm plasma membrane. If this mutated version is expressed in sperm

cells, it could be used to trigger haploidization in maize[9-12]. The system has been successfully transferred to other cereals like *rice* and *wheat*[13,14], but the molecular mechanism of haploidization remained unclear. Similarly, mutants of *ZmDMP8/9* encoding members of the conserved DUF679 domain membrane protein family that are highly expressed in pollen were used for haploid induction, but the molecular mechanism is unclear[15,16]. Uni-parental chromosome elimination was reported in *CENH3*-mediated haploid induction protocols. Mutations in either maternal or paternal CENH3 was reported to lead to a reasonable number of haploids, for example, in *Arabidopsis*, maize, wheat and cotton[17-20]. However, there are still problems associated especially with male inducer lines: e.g. some haploid inducer lines produce abnormal pollen that fail to germinate and thus affect the seed setting rate, and paternal inducer lines are still limited to a very few crops. Thus, due to the application limits of current haploid technology, plant breeders are highly interested in methodological improvements as well as in novel principles of haploidization[21].

Here, we report the identification of a novel female haploid inducer system based on mutations in egg cell-specific genes. *ECS1* and *ECS2* encode aspartic endopeptidases that are exclusively expressed in the unfertilized egg cell of *Arabidopsis*[22]. Pollen development and pollen germination of the *ecs1 ecs2* double mutant appears normal like those from wild type (WT) plants. However, selfed *ecs1 ecs2* double mutant plants contained about 15% abnormal seeds. We distinguished two types of abnormal seeds: (i) undeveloped seeds slightly larger than unfertilized ovules and (ii) developed but small seeds (Fig. 1a, b). By using a whole-mount ovule/seed clearing technique, we found that undeveloped seeds were arrested at the zygote stage. After fertilization (fusion of egg and sperm cell) these zygotes became vacuolized and finally degenerated (Fig. 1c, d). To make sure that they were indeed zygotes, we labelled the egg cell membrane of *ecs1 ecs2* double mutants using the *DD45::GFP-LTI6b* fusion construct (Supplementary Fig. 1) and carefully calculated the egg cell and sperm cell fusion rate. We found that sperm cells carrying the nuclear marker HTR10-RFP could perfectly fuse with all egg cells (n = 481; Fig. 1e, f). However, occasionally we found an unfused male nucleus inside the egg cell cytoplasm (Fig. 1e; Supplementary Fig 2). 3D image analysis of these zygotes clearly showed a sperm nucleus in the egg cell cytoplasm confirming successful gamete fusion (Supplementary Fig. 2, Supplementary movie. 1). We next analyzed small sized seeds and found aborted embryos showing abnormal cell division planes. When WT embryos developed to the eight-celled stage, embryos from the *ecs1ecs2* double mutant divided less frequent and cell division planes appeared irregular. These abnormal embryos finally arrested at different early developmental stages (Fig. 1g, h).

Furthermore, among the offspring of selfed *ecs1ecs2* double mutants, we found plants with small flowers and short siliques. We then geminated hundreds of seeds and checked the ploidy level of seedlings by flow cytometry. Among 149 randomly selected *ecs1 ecs2* double mutant offspring plants we identified two haploid plants (Fig. 2a). At anthesis haploid plants contained small flowers and generated short siliques (Fig. 2b, c). To further confirm the haploid nature of these plants, chromosome spreads were generated from male meiocyte cells at meiosis I showing that the haploid plants contained five chromosomes compared to 10 in diploid WT plants (2n=10) (Fig. 2d).

Although we have never found a single fertilization phenotype in the double mutants as mentioned above, we tested the possibility that haploid plants originate from fertilization failure due to excessive sperm cell number. We used *tes* mutant pollen possessing more than two sperm cells[23] to pollinate *ecs1 ecs2* double mutants and also WT pistils as a control. Among 190 progeny plant derived from *ecs1 ecs2* double mutants, we obtained two haploid plants, while haploids were not detected among 154 WT offspring plants. Haploidy was first confirmed by flow-cytometry (Fig. 2e). Haploid plants contained smaller flowers and generated shorter siliques compared with WT plants (Fig. 2f). Their haploid nature was further confirmed by the chromosome spread technique. Experiments revealed that haploid plants contained only five chromosomes in their somatic cells (Fig. 2g). In short, after double checking haploid plants were obtained at a rate of 1.2%.

To explore the origin of haploids, we pollinated *ecs1 ecs2* plants expressing the egg cell membrane marker *DD45::GFP-LTI6b*[24] with pollen carrying the sperm nucleus marker HTR10-RFP. In WT plants, karyogamy (fusion of gamete nuclei) usually occurs shortly after egg cell and central cell each fuse with a sperm cell. Thereafter, zygotes start to elongate showing a typical zygote morphology (Fig. 3a). In *ecs1 ecs2* double mutants we observed semigamy (a type of fertilization in which the sperm cell fuses with the egg cell, but its nucleus does not fuse with the egg nucleus) at 24 hours after pollination (HAP) (Fig. 3b, Supplementary Fig. 3, Supplementary movie 2). Few semigamous zygotes (2/1500) divided at 24 HAP, but still kept egg cell morphology and did not show zygote elongation. The male nucleus could yet be seen after semigamous zygote division (Fig. 3c, Supplementary Fig. 4, Supplementary movie 3). These findings indicate that sperm entry is sufficient to trigger cell division. A similar phenomenon has been reported in fertilized central cells, in which sperm nuclei did not fuse with polar nuclei, but central cell division could be promoted[25] Divided semigamous zygotes may eventually generate haploid plants. Furthermore, to understand the process of haploidization, we labelled the chromosomes of *ecs1 ecs2* egg cells and zygotes by using the *DD45::CENH3-GFP* fusion construct[17] (Supplementary Fig. 5). We then crossed *ecs1 ecs2* plants expressing CENH3-GFP with pollen from WT plants. At 12 and 24 hap, we observed aneuploidy (6-8 chromosomes in mutant zygotes compared with 10 chromosomes in the control) (Fig. 3d-f, Supplemental movie 4-6). This indicates that paternal and/or maternal chromosome elimination occurs at the beginning of embryogenesis. The higher rate of obtained haploid plants (1.2%; see above) compared with divided semigamous zygotes (0.13%) suggests that haploidization also occurs *in ecs1 acs2* zygotes/early embryos when karyogamy was initially successful.

In summary, we found that in addition to its reported role in degrading the pollen tube attractor LURE1 and thus to prevent polytubey[22], ECS1 and ECS2 endopeptidases possess additional roles after gamete fusion. We found that haploid offspring of the *ecs1 ecs2* double mutants could be generated after forming semigamous zygotes and/or by chromosome elimination during early embryogenesis. These phenotypes suggest that ECSs play critical roles in fertilization to ensure successful fusion of male and female nuclei and parental chromosome incorporation. Although the detailed molecular mechanism underlying the process of nuclear fusion is still not known and further ECS1 ECS2 target proteins need to be identified, we found that *ecs1 ecs2* mutant plants can be used as female haploid inducer lines, which

adds to the tools of generating double haploid plants. Notably, 3.5% haploid progeny plants were also observed in another report studying *ecs1 ecs 2* double mutants indicating that the haploidization rate could be further increased. However, we did not observe single fertilization and parthenogenetic activation of unfertilized egg cells as suggested in that report (BioRxiv, 476184 (2022); doi: https://doi.org/10.1101/2022.01.20.476184) and think it is a misinterpretation. Currently, different *in vivo* paternal haploid inducer lines are widely used for haploidization, especially in maize, which provide powerful tool for crop breeding. Staging *ecs1 ecs2* mutations with other inducers like *dmp8 dmp9* and/or *pla1/mtl/nld* might significantly increase the haploid induction rate as well as manipulation of the post-fertilization process that is regulated by ECS1 ECS2 activity. In addition, among the haploid offspring derived from *ecs1 ecs2* mutants, we found that it is easy to classify seeds or seedling into different groups according to their morphology to enrich potential haploid plants and therefore to greatly accelerate the selection and confirmation process. Thus, the female haploid induction and selection system reported here provides a novel strategy of haploidization for breeding and will now be tested in crop plants.

Methods

Plant materials and growth conditions. T-DNA insertion lines SALK_021086 (*ecs1*) and SALK_090795 (*ecs2*) were obtained from the Arabidopsis Biological Resource Center (ABRC). *Arabidopsis thaliana* Columbia-0 (Col-0) was used as wild type (WT) control. Plants were grown in soil in a greenhouse under long-day conditions (16 h light/8 h dark) at 22 °C.

Vector construction and plant transformation. All fragments were amplified by polymerase chain reaction (PCR) using the Phanta Kit (Vazyme Biotech). Primer sequences (synthesized by Sangon Biotech) are listed in Supplementary Table 1. All constructs were generated using restriction enzymes (New England Biolabs) and One Step Cloning Kit (Vazyme Biotech). To label egg cell membrane and chromosomes, *DD45::GFP-LTI6b* and *DD45::CENH3-GFP* fusion constructs were generated[17, 24]. A DNA fragment containing the promoter and coding sequence was amplified from *A. thaliana* ecotype Col-0 genomic DNA and inserted into the P094 vector. All constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and then into Arabidopsis plants by the floral dipping method[26].

Ovule clearance. To analyze seed and embryo phenotypes, siliques at different developmental stages after fertilization were dissected with a needle and quickly cleared in Herr's solution (lactic acid:chloral hydrate:phenol:clove oil:xylene = 2:2:2:2:1, wt/wt) as described previously[27]. Images were acquired using an Olympus IX71 inverted microscope with differential interference contrast optics.

Confocal microscopy and image analysis. Ovules and early seeds were observed using a confocal microscope (Leica TCS SP8) and excited with a 488-nm laser and emitted light measured at 498–550 nm for enhanced GFP as well as excited with a 552-nm laser and emitted light measured at 600–650 nm for RFP. 3D images were generated using the LAS-X software (Leica) and 3D reconstructions were produced by Leica Aivia AI Image Analysis Software.

Flow cytometry. For flow cytometry analysis, two leaves were each harvested and chopped using a razor blade in a petri dish with 500 µl of nuclei extraction buffer (Partec CyStain). 1 ml of staining reagent (Partec CyStain UV Precise-Kit) was added and specimens were incubated at room temperature for 1 min. Samples were passed through a 40 µM nylon mesh into a 5 mL round-bottom polystyrene test tube (FALCON, 352235) and filtrates were analyzed using a Beckmann CytoFlex ploidy analyzer.

Chromosome spreads. For chromosome spreads, flower buds fixed in Carnoy's solution were washed twice with 10 mM citrate buffer (pH 4.5) and digested with 0.3% cellulose and 0.3% pectolyase in citrate buffer at 25℃ for 1 h. After being washed twice with the same buffer and by adding DAPI solution (10µg/ml DAPI in citrate buffer), digested buds were squashed with a cover slip to release PMCs and samples were analyzed using a Leica SP8 CLSM.

Declarations

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Author contributions

M.-X.S., X.Z., C.S. and T.D. designed the research plan. X.Z., C.S., S.L. and B.Z. performed the phenotype and genetics analyses. T.D. and M.-X.S. contributed to the data analysis and finalized the manuscript. All authors contributed to the data collection, presentation and manuscript writing.

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Figures

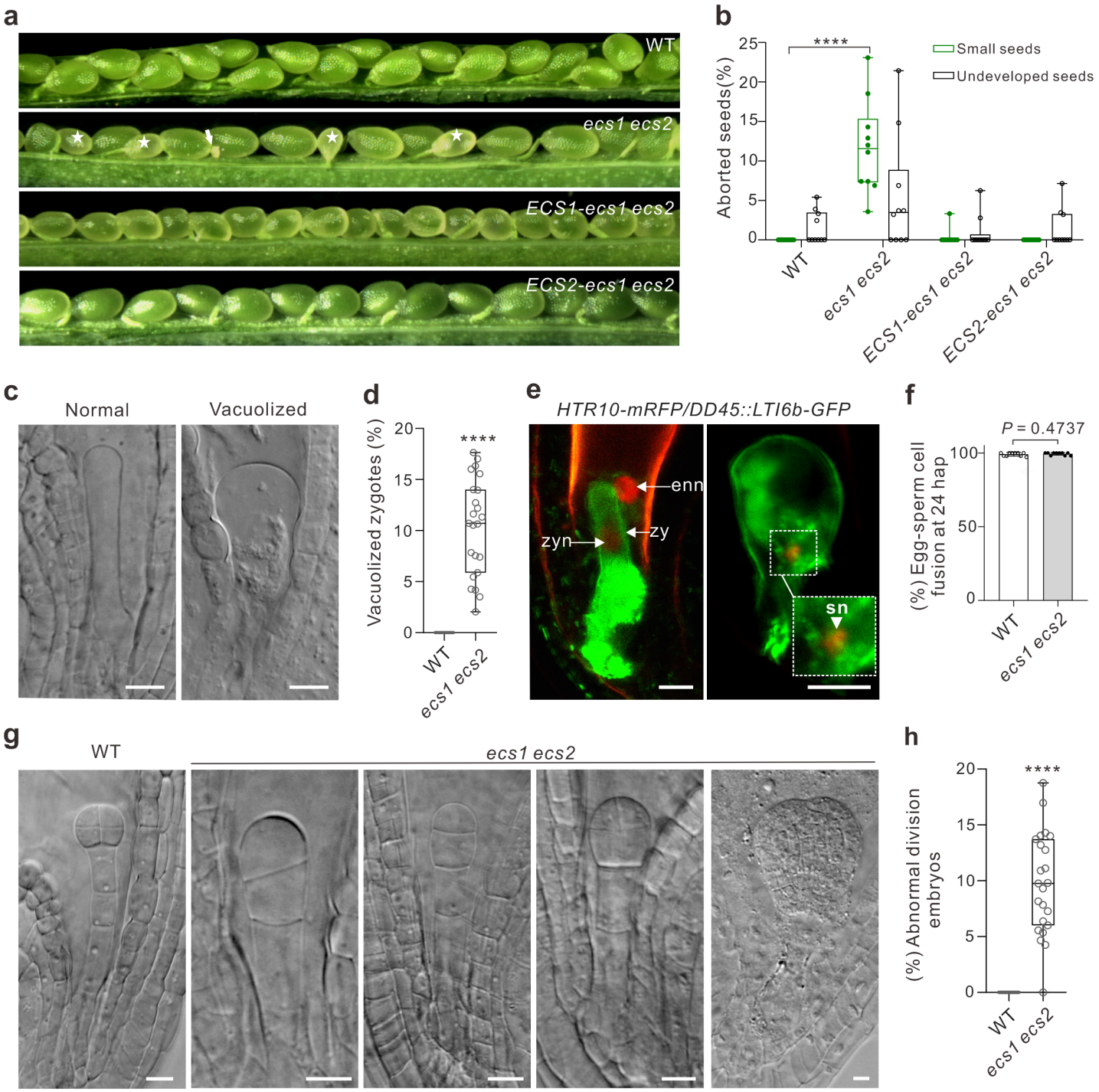

Figure 1

Mutations in *Arabidopsis ECS1/ECS2* results in multiple seed phenotypes.

a, Representative images of siliques from selfed wild type (WT) and *ecs1 ecs2* double mutants as well as complemented *ECS1-ecs1 ecs2* and *ECS1-ecs1 ecs2* lines. Asterisks, small seeds; arrow, undeveloped seed. **b,** Percentage of abnormal seeds in *ecs1 ecs2* double mutants (n = 580 for wild type (WT); 1127 for *ecs1 ecs2*; 551 for *ECS1-ecs1 ecs2*; 518 for *ECS2-ecs1 ecs2*). ****Statistical difference compared to WT (two-tailed Student's t-test; P < 0.0001). **c** Highly vacuolated zygotes at 48 hours after pollination (HAP) in

undeveloped *ecs1 ecs2* seeds. Scale bars, 10 μm. **d,** Percentage of highly vacuolated zygotes shown in (c) (n = 241 for WT; 1113 for *ecs1 ecs2*). ****Statistical difference compared to WT (two-tailed Student's t-test; P < 0.0001). **e,** A unfused sperm cell nucleus is still visible inside a fertilized egg cell at 24HAP in *ecs1 ecs2*. Sperm cell nucleus (sn) was labeled by *HTR10-RFP* (red) and egg cell membranes by *DD45::GFP-LTI6b* (green). Scale bars, 10 μm. **f,** Percentage of egg-sperm cell fusion at 24HAP (n = 465 for WT; 481 for *ecs1 ecs2*). **g,** Abnormal embryos arrested at different developmental stages in small *ecs1 ecs2* seeds. Scale bars, 10 μm. **h,** Percentage of abnormal embryonic cell divisions in *ecs1 ecs2* at 48HAP (n = 241 for WT; 1113 for *ecs1 ecs2*). ****Statistical difference compared to WT (two-tailed Student's t-test; P < 0.0001).

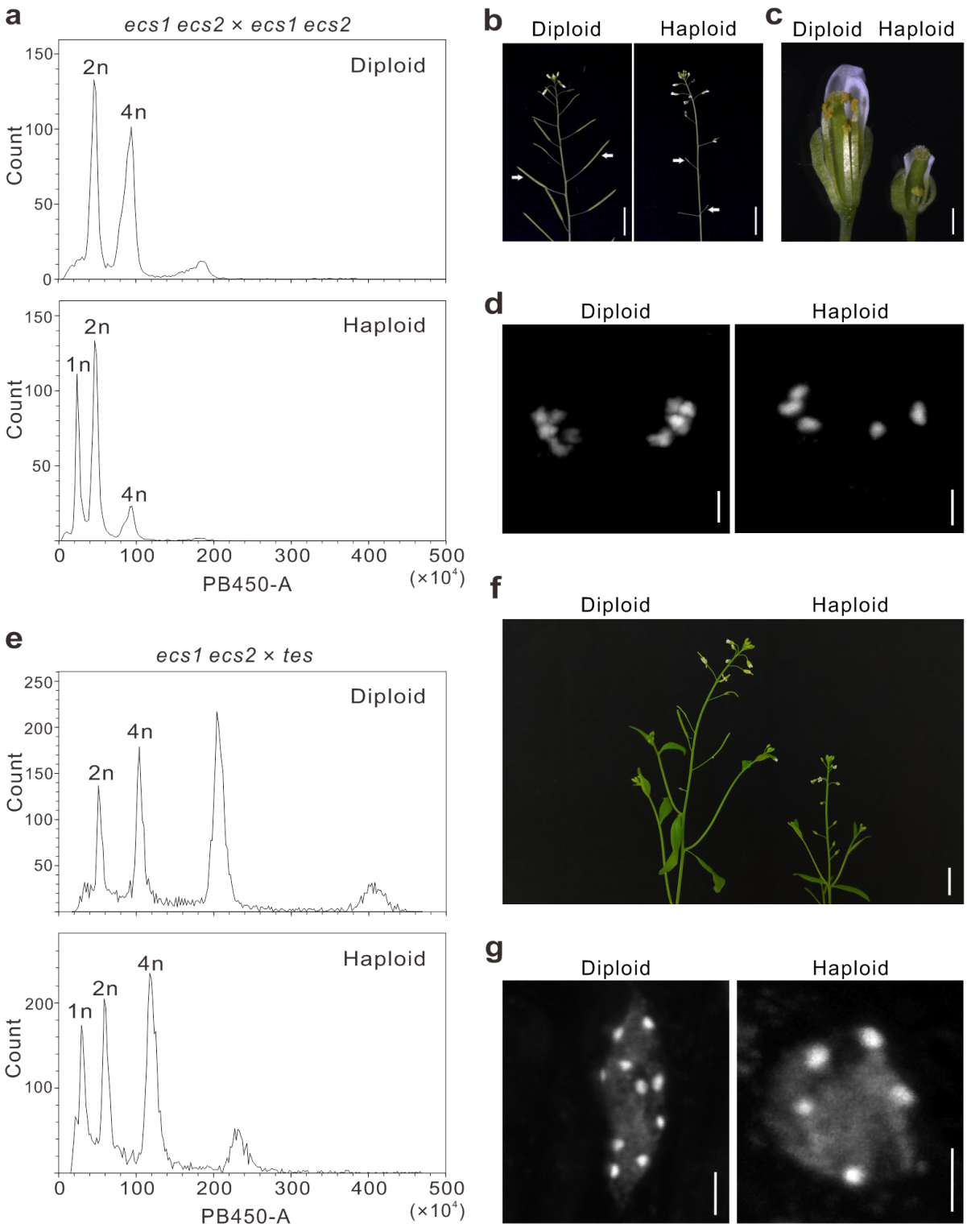

Figure 2

The *ecs1 ecs2* double mutant induces haploidization.

a, Flow cytometry of selfed *ecs1 ecs2* plants revealed diploid (2n) and haploid (1n) progeny. **b,** Haploid plants were sterile compared to diploid WT plants (here plants are shown 40 days after sowing). **c,** Haploids generate smaller flowers than diploids and were mostly male sterile. **d,** Chromosome spreads of

male meiocytes at anaphase I in diploid and haploid plants, respectively. **e,** Haploids were also observed in *ecs1 ecs2* × *tes* progenies by Flow cytometry analysis. **f,** In *ecs1 ecs2* × *tes* progenies, haploids also generate smaller flowers than diploids (30 days after sowing). **g,** Chromosome spreads from flower stalk cells in diploid (left) and haploid (right) progenies of *ecs1 ecs2* × *tes*. Scale bars, 1 cm (**b, f**), 500 μm (**c**), 2 μm (**d**) and 5 μm (**g**).

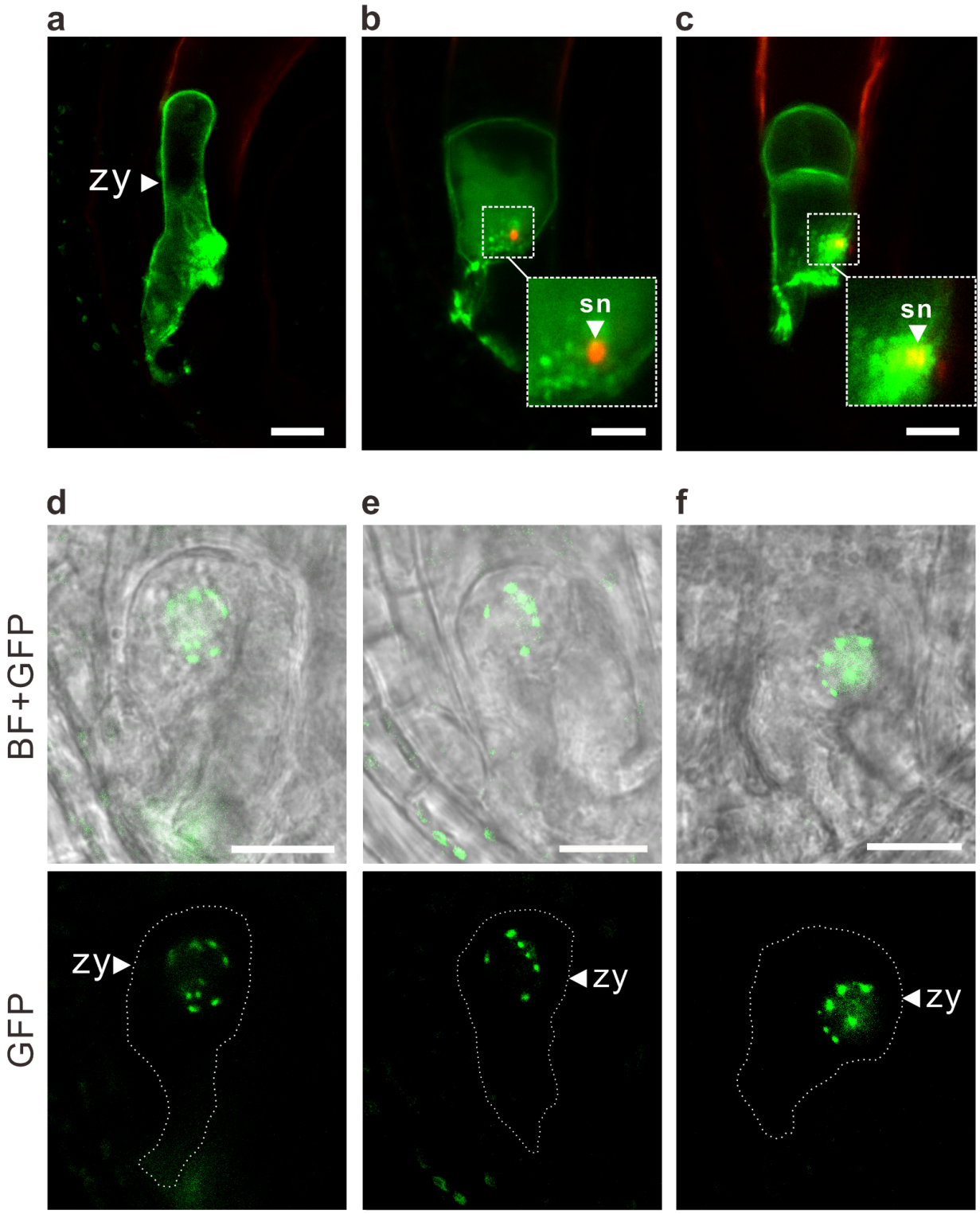

Figure 3

***ecs1 ecs2* double mutants induce haploidy after semigamy and chromosome elimination during early embryogenesis.**

a, Egg and sperm cell fusion leads to the formation of an elongated zygote 24HAP in WT ovules. **b,** In *ecs1 ecs2* double mutants, plasma membrane fusion between egg and sperm cell was completed, but karyogamy failed at 24HAP in some zygotes. SP, Sperm nucleus. **c,** Some semigamous zygotes divided asymmetrically without preceding elongation. The sperm nucleus (SN) could still be seen inside the basal cell at 24hap. **d,** *ecs1 ecs2* egg cells expressing the centromere marker *DD45::CENH3-GFP* after fusion with WT sperm. 10 chromosomes were visible in diploid zygotes at 12 hap. **e, f,** Less than 10 chromosomes (here six chromosomes in e and seven in f) were visible in early zygotes of *ecs1 esc2* derived plants at 12HAP indicating elimination of chromosomes. Scale bars, 10 µm.

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