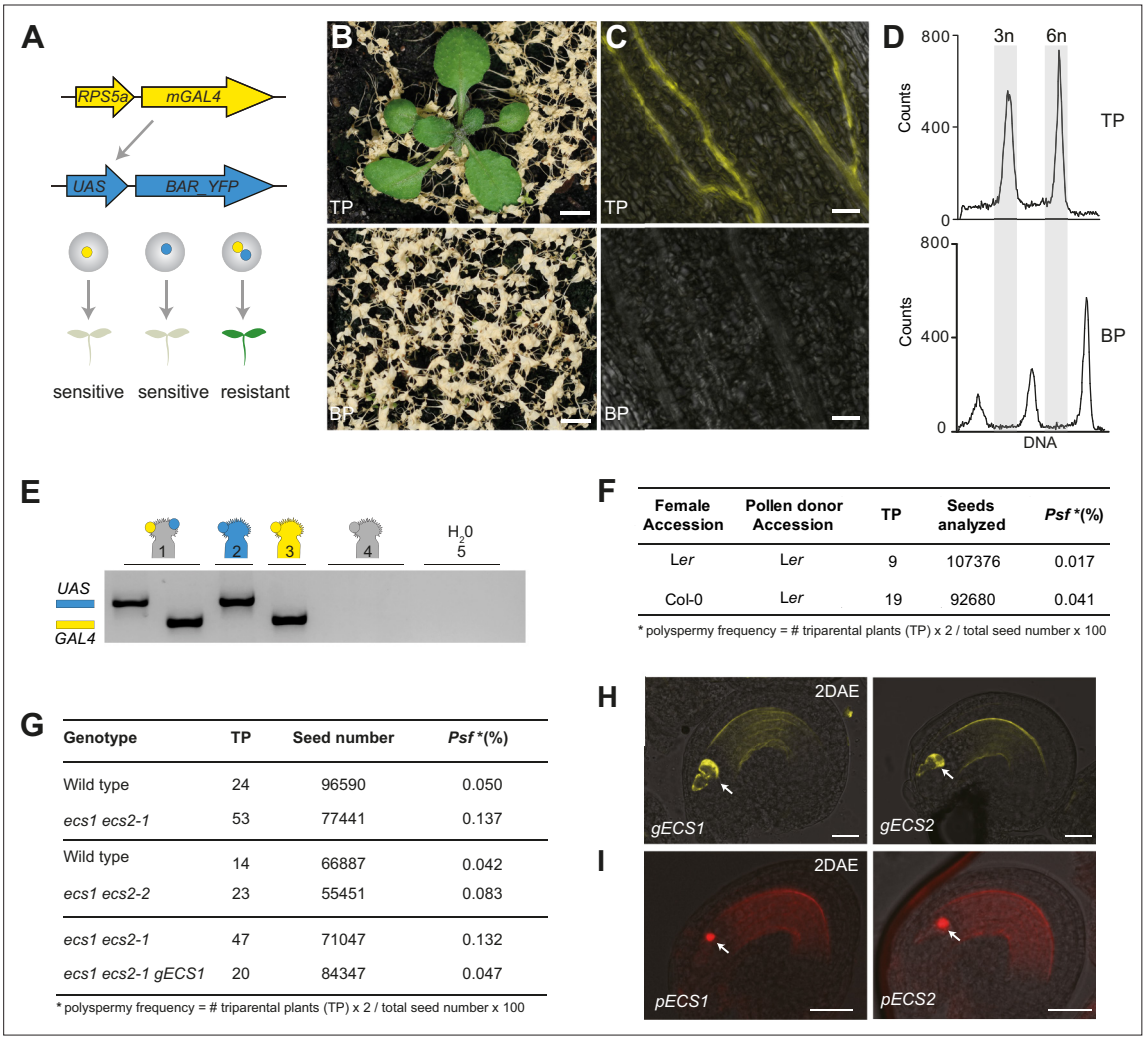
Figures and figure supplements

ECS1 and ECS2 suppress polyspermy and the formation of haploid plants by promoting double fertilization

Yanbo Mao and Thomas Nakel *et al*.



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Female Accession	Pollen donor Accession	TP	Seeds analyzed	*Psf* *(%)
Ler	*Ler*	9	107376	0.017
Col-0	*Ler*	19	92680	0.041

* polyspermy frequency = # triparental plants (TP) x 2 / total seed number x 100

G

Genotype	TP	Seed number	*Psf* *(%)
Wild type	24	96590	0.050
ecs1 ecs2-1	53	77441	0.137
Wild type	14	66887	0.042
ecs1 ecs2-2	23	55451	0.083
ecs1 ecs2-1	47	71047	0.132
ecs1 ecs2-1 gECS1	20	84347	0.047

* polyspermy frequency = # triparental plants (TP) x 2 / total seed number x 100

Figure 1. *ecs1 ecs2* double mutants segregate polyspermy-derived triparental offspring. (**A**) Illustration of the high-throughput polyspermy detection (HIPOD) assay. The two components of the yeast GAL4-UAS system are provided by distinct pollen donors (pollen donor 1, yellow and pollen donor 2, blue) such that herbicide resistance (green seedling) is only conferred if the mGAL4 transcription factor and the UAS-driven BAR_YFP protein are combined in a single egg (grey). (B–D) Herbicide treatment (**B**), YFP fluorescence (**C**), ploidy analysis (**D**) of triparental triploid plants (TP), and biparental diploid plants (BP). (**E**) PCR targeting *pUAS::BAR-YFP* (blue) and *pRPS5A::mGAL4-VP16* (yellow) in a herbicide-resistant plant recovered from HIPOD (1), *pUAS::BAR-YFP/+* (2), *pRPS5A::mGAL4-VP16/+* (3), wild-type control (4), water control (5). (**F**) Polyspermy frequency (Psf) following crosses of either wild-type *Ler* (pollen acceptor) or wild-type Col-0 (pollen acceptor) with *Ler* pollen donor 1 and *Ler* pollen donor 2. (**G**) Polyspermy frequency in *ecs1 ecs2* mutants compared to wild type and a rescue line harboring *ecs1 ecs2 pECS1::gECS1-YFP*. (**H**) ECS1-YFP and ECS2-YFP localization before fertilization in the corresponding transgenic plants of *pECS1::ECS1-YFP* and *pECS2::ECS2-YFP*. Arrow indicates egg cell. (**I**) Promoter activity analysis using *NLS-tdTomato* expression driven by a promoter fragment upstream of either *ECS1* or *ECS2*. The arrow points to the egg cell nucleus. Scale bars, 5 mm (**B**), 50 µm (**C**) and (**I**), 20 µm (**H**).

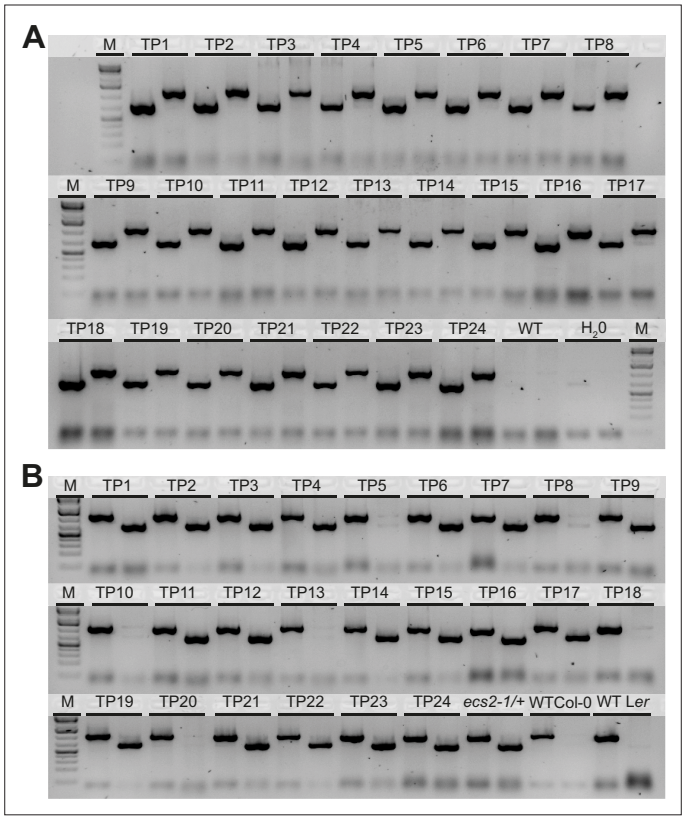


Figure 1—figure supplement 1. Triparental plants recovered from *ecs1/- ecs2-1/+* heterozygotes predominantly segregate *ecs2-1*. (**A**) PCR targeting *pRPS5A::mGAL4-VP16* (left) and *pUAS::BAR-YFP* (right) of 24 herbicide-resistant plants recovered from *ecs1/- ecs2-1/+* heterozygotes (TP1-24) subjected to HIPOD. M, 1kb plus DNA ladder. (**B**) PCR targeting wild type (left) and *ecs2-1* allele (right). M, 1kb plus DNA ladder.

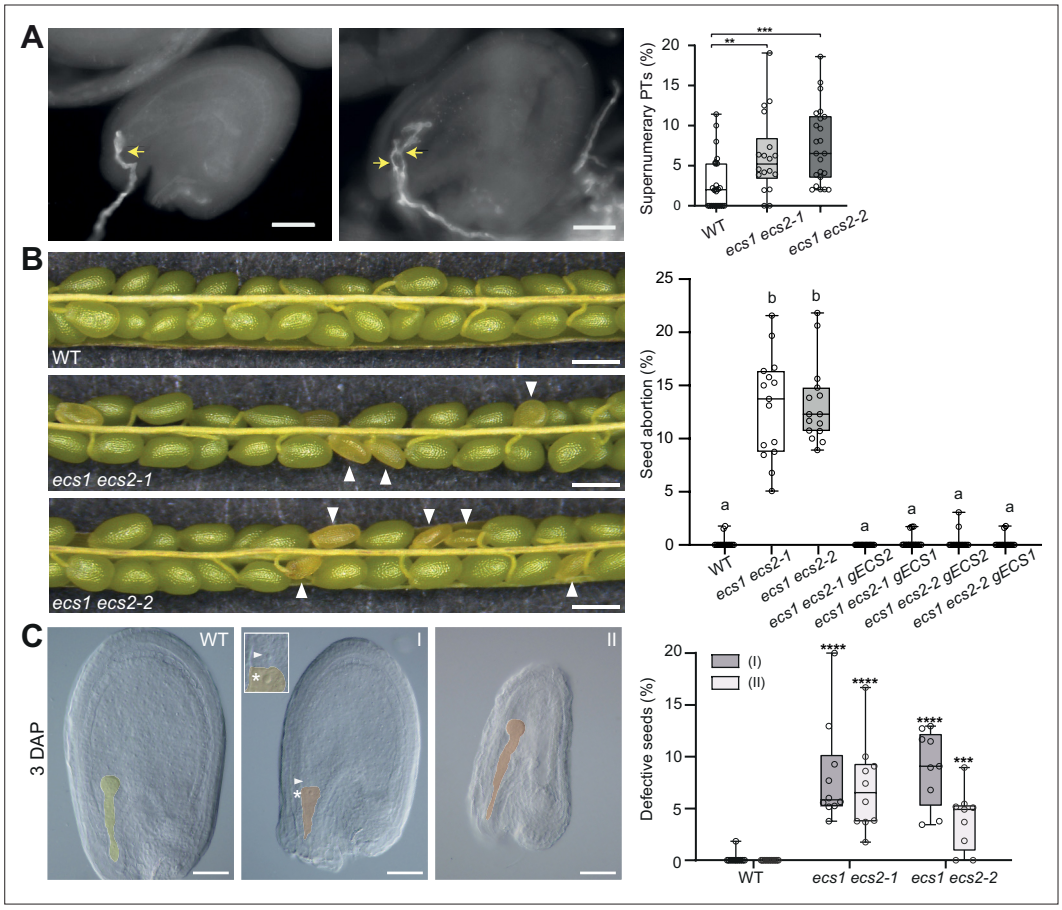
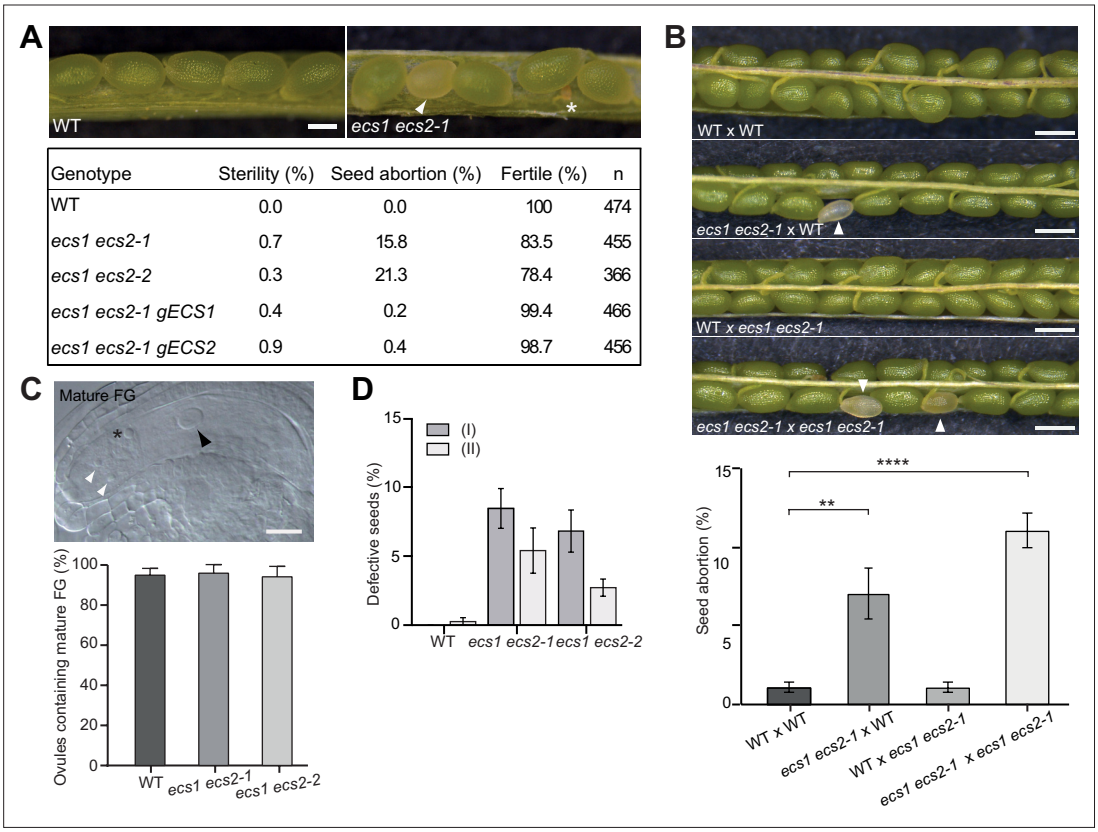


Figure 2. *ecs1 ecs2* double mutants exhibit reproductive defects. (**A**) Occurrence of polytubey in *ecs1 ecs2* mutants 20 hr after pollination (HAP) (n=1039/835/1070 for wild type/*ecs1 ecs2-1*/*ecs1 ecs2-2*, respectively). Circles represent data recovered from individual pistils. The images indicate one pollen tube (left) or two pollen tubes (right) targeting an embryo sac. Pollen tube is highlighted by a yellow arrow. (**B**) Silique of wild-type and *ecs1 ecs2* mutants with quantification of seed abortion in different genotypes (n>800 for each genotype). Circles represent data recovered from individual siliques. Arrowheads, aborted seeds. Different letters show significant difference; p<0.0001, F=106.0, by one-way ANOVA with a Tukey multiple comparison test. (**C**) Cleared whole mount and quantitative analysis of *ecs1 ecs2* mutant seed categories 3 days after pollination (DAP): In comparison to wild-type like seeds containing embryo and endosperm, *ecs1 ecs2* mutants segregate seeds without embryo (I) and seeds containing no or retarded endosperm (II) (n=655/540/522 for wild type/*ecs1 ecs2-1*/*ecs1 ecs2-2*, respectively). Circles represent data recovered from individual pistils. Embryo and unfertilized egg cells were false colored in yellow (wild type) and orange (mutant). Asterisk in image; unfertilized egg nucleus; arrowhead, endosperm nucleus. Similar results for (**B**) and (**C**) were obtained in independent experiments by a different scientist (data shown in ***Figure 2—figure supplement 1A*** and ***Figure 2-figure supplement 1D***). Data in (**A–C**) are represented in box and whisker plot, center line, median; bottom and up, 25th and 75th percentiles. Whisker, the minimum and maximum. Two-tailed Mann-Whitney comparison test between wild type and mutants. Scale bars, 50 µm (**A**) and (**C**), 500 µm (**B**). **p<0.01; ***p<0.001; ****p<0.0001.



Genotype	Sterility (%)	Seed abortion (%)	Fertile (%)	n
WT	0.0	0.0	100	474
ecs1 ecs2-1	0.7	15.8	83.5	455
ecs1 ecs2-2	0.3	21.3	78.4	366
ecs1 ecs2-1 gECS1	0.4	0.2	99.4	466
ecs1 ecs2-1 gECS2	0.9	0.4	98.7	456

Figure 2—figure supplement 1. The seed defect in *ecs1 ecs2* is of female origin. (**A**) Frequency of seed abortion in different genotypes. Upper row, representative images of the siliques of wild-type and *ecs1 ecs2-1* plants. Arrowheads indicate aborted seeds and the asterisk indicates an undeveloped seed, which are both counted as seed abortion. (**B**) Abnormal seed formation in plants resulting from crosses between different genotypes (n=614/375/382/395 for WT × WT/*ecs1 ecs2-1*× WT/ WT× *ecs1 ecs2-1*/*ecs1 ecs2-1*× *ecs1 ecs2-1*, respectively). Arrowheads, aborted seeds. Data indicates mean values ± SD; **p<0.01; ****p<0.0001 (Mann-Whitney comparison test). (**C**) Frequencies of ovules containing mature female gametophytes (FGs) (n=419/398/424 for WT/*ecs1 ecs2-1*/ecs*1 ecs2-2*, respectively). Central cell nucleus, black arrowhead; synergid (syn) nucleus, white arrowhead; egg nucleus, asterisk. Brightness was manually enhanced with Adobe Photoshop in (**C**). (**D**) Quantitative analysis of *ecs1 ecs2* mutant seed categories 3 days after pollination (DAP): In comparison to wild-type like seeds, seeds without embryo (I) and seeds containing no or retarded endosperm (II) were observed (n=356/295/366 for wild-type/*ecs1 ecs2-1*/*ecs1 ecs2-2,* respectively). Scale bar, 250 µm (**A**), 500 µm (**B**), 20 µm (**C**).

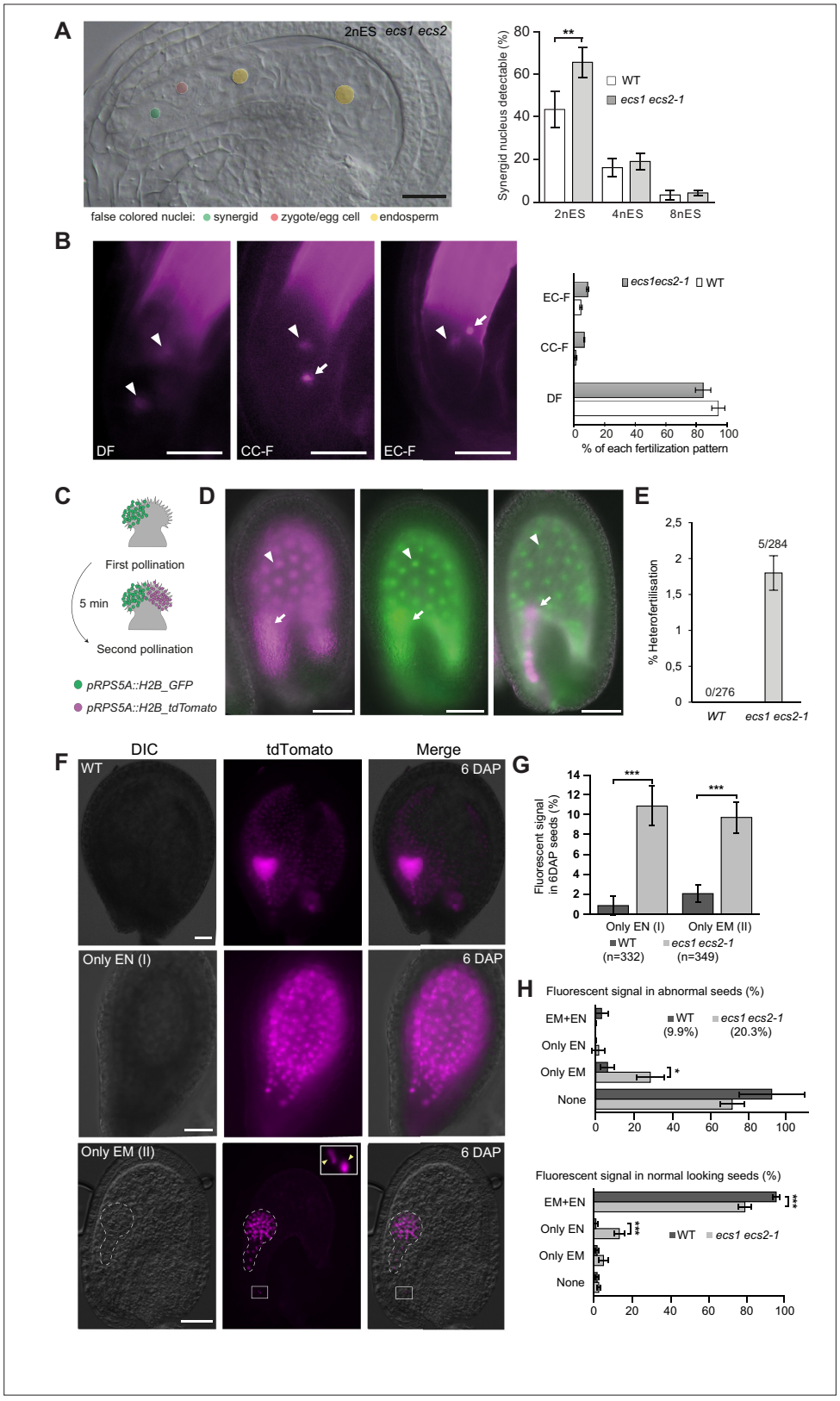


Figure 3. *ecs1 ecs2* double mutants segregate embryos that lack a paternal genome contribution. (**A**) Frequency of seeds containing a synergid nucleus at different endosperm stages (n for 2nES = 67/72, 4nES = 143/158, and 8nES = 179/141 for wild type/*ecs1 ecs2-1*, respectively). The image shows the second synergid nucleus at the two-nucleate endosperm stage. (**B**) Quantitative analyses of single fertilizations 8-10 HAP in *ecs1 ecs2* (n=90) and wild-

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type ovules (n=89) using HTR10-mRFP. Successful double fertilization (DF) is visualized by two dispersed sperm nuclei signals (arrow heads). In the middle and right panel, only one dispersed sperm cell nuclear signal (arrow heads) can be observed, while an additional sperm nuclear signal (arrows) is condensed suggesting the unfertilized state. The positions of the dispersed sperm signals correspond to the position of central cell (middle) and egg cell (right), respectively, and thus suggest different single fertilization patterns (CC-F and EC-F). (**C–E**) Analyses of seed development 3 days after dual pollination. (**C**) Diagram of the dual pollination experiment. (**D**) Successful double fertilization by a single pollen tube from either *pRPS5A::H2B_tdTomato* (left panel) or *pRPS5A::H2B_GFP* (middle panel). Heterofertilization involving the content of two genetically distinct pollen tubes is depicted in the right panel. Endosperm nuclear signal (arrow heads), embryo nuclear signal (arrows). (**E**) Percentage of heterofertilization events in wild type (n=276) and *ecs1 ecs2-1* (n=284) ovules. (**F**) Fluorescence signal in 6-day-old progenies of *ecs1ecs2-1* female plants upon paternal introduction of *pRPS5A::H2B-tdTomato*. WT: wild-type like seeds, showing paternal marker expression in both embryo and endosperm. Only EN (I): paternal marker expression only in endosperm. Only EM (II): paternal marker expression only in embryo. Dotted line; embryo, yellow arrowhead; sperm nuclei. (**G**) Percentage of 6-day-old progenies of *ecs1ecs2-1* or wild-type female plants crossed with *pRPS5A::H2B-tdTomato*, showing paternal marker expression in different fertilized tissues. (**H**) Assessment of paternally introduced marker expression in endosperm (EN) and embryo (EM) of 6-day-old siliques in *ecs1 ecs2-1* or wild type. The *ecs1 ecs2-1* seeds were divided into a normally looking and an abnormal seed fraction and analyzed individually. EM+EN; expression in both embryo and endosperm. (**G**) and (**H**), n=349/332 for wild type/*ecs1 ecs2-1*, respectively. Brightness was manually enhanced with Adobe Photoshop in (**A–C**). Data in (**A**), (**B**), (**G**), and (**H**) indicate mean ± SD. Two-tailed Mann-Whitney comparison test between wild type and mutants, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Scale bars, 20 µm (**A**), 25 µm (**B**), 75 µm (**D**), 50 µm (**F**).

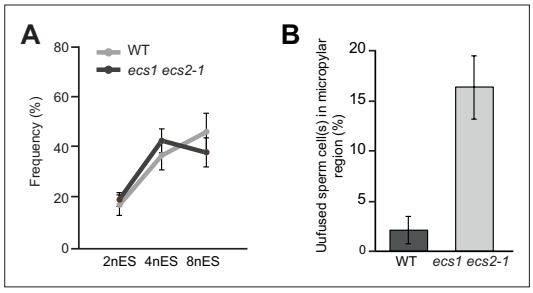

Figure 3—figure supplement 1. Early seed development in *ecs1 ecs2* mutant. (**A**) Developmental stage of wild type and *ecs1 ecs2-1* seeds 20 hr after pollination (HAP): Categories detected are two-, four- and eight-nucleate endosperm stage (2nES, 4nES, and 8nES) (n=389/371 for WT/*ecs1 ecs2-1*, respectively). No statistically significant differences detected on the basis of either Student's t-test or Mann-Whitney comparison test. (**B**) Frequency of unfused sperm cells at the micropylar end in wild-type and *ecs1 ecs2-1* ovules fertilized with pollen from *pHTR10::HTR10-mRFP* transgenic plants 1 day after pollination (DAP).

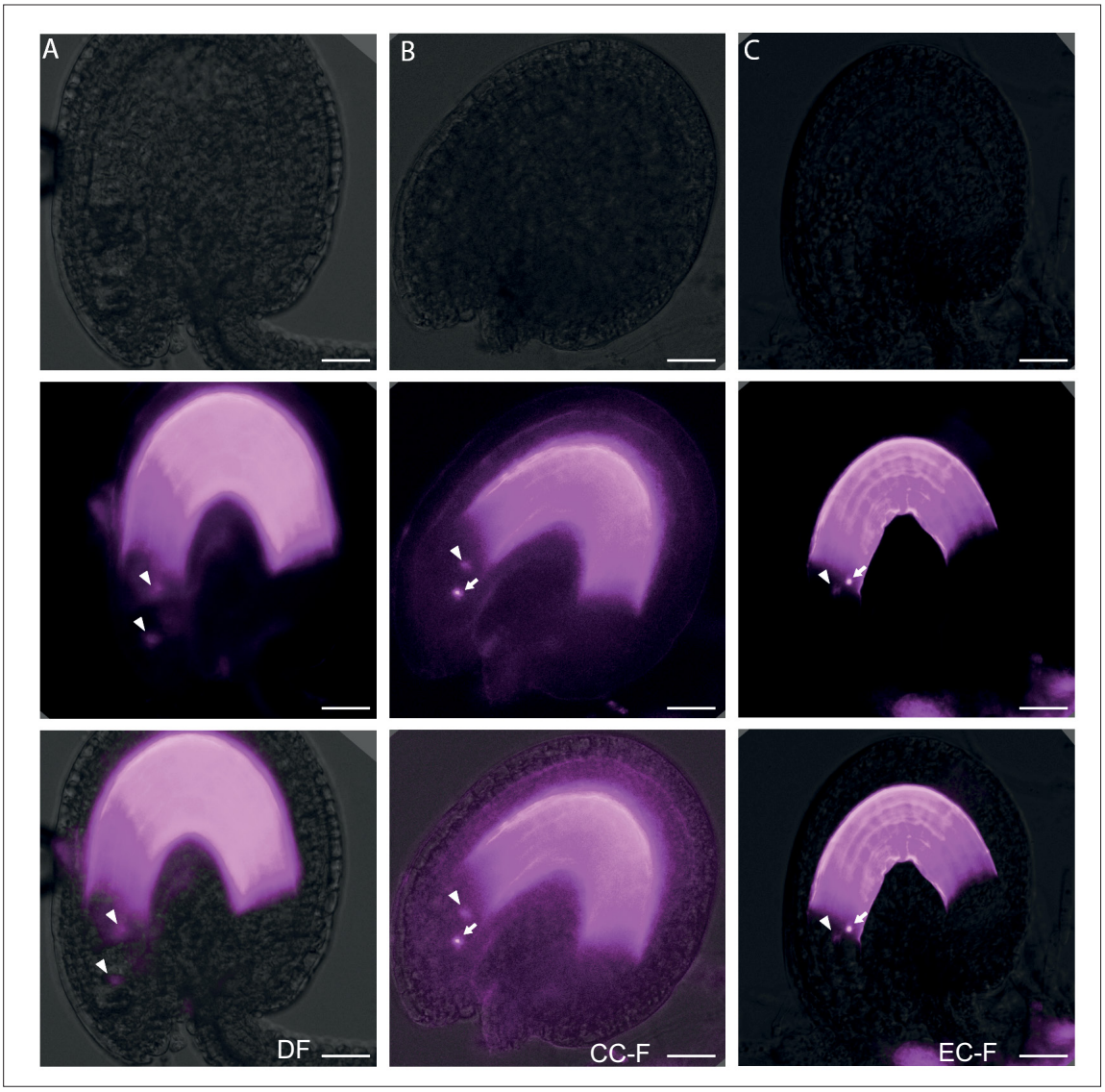

Figure 3—figure supplement 2. Detection of single fertilization events in *ecs1 ecs2* and wild-type ovules using HTR10-mRFP. (**A**) Characteristic double fertilization (DF) pattern with two dispersed sperm nuclei indicative of gamete fusion (arrow heads). (**B–C**) Ovules with only one dispersed sperm nucleus in either the position of the central cell (**B**) or egg cell (**C**). Arrow heads and arrows indicate the position of the uncondensed and condensed nucleus, respectively. Upper, differential interference contrast (DIC) channel; middle, red fluorescence; bottom, overlay. Scale bar, 25 µm. Brightness was manually enhanced with Adobe Photoshop.

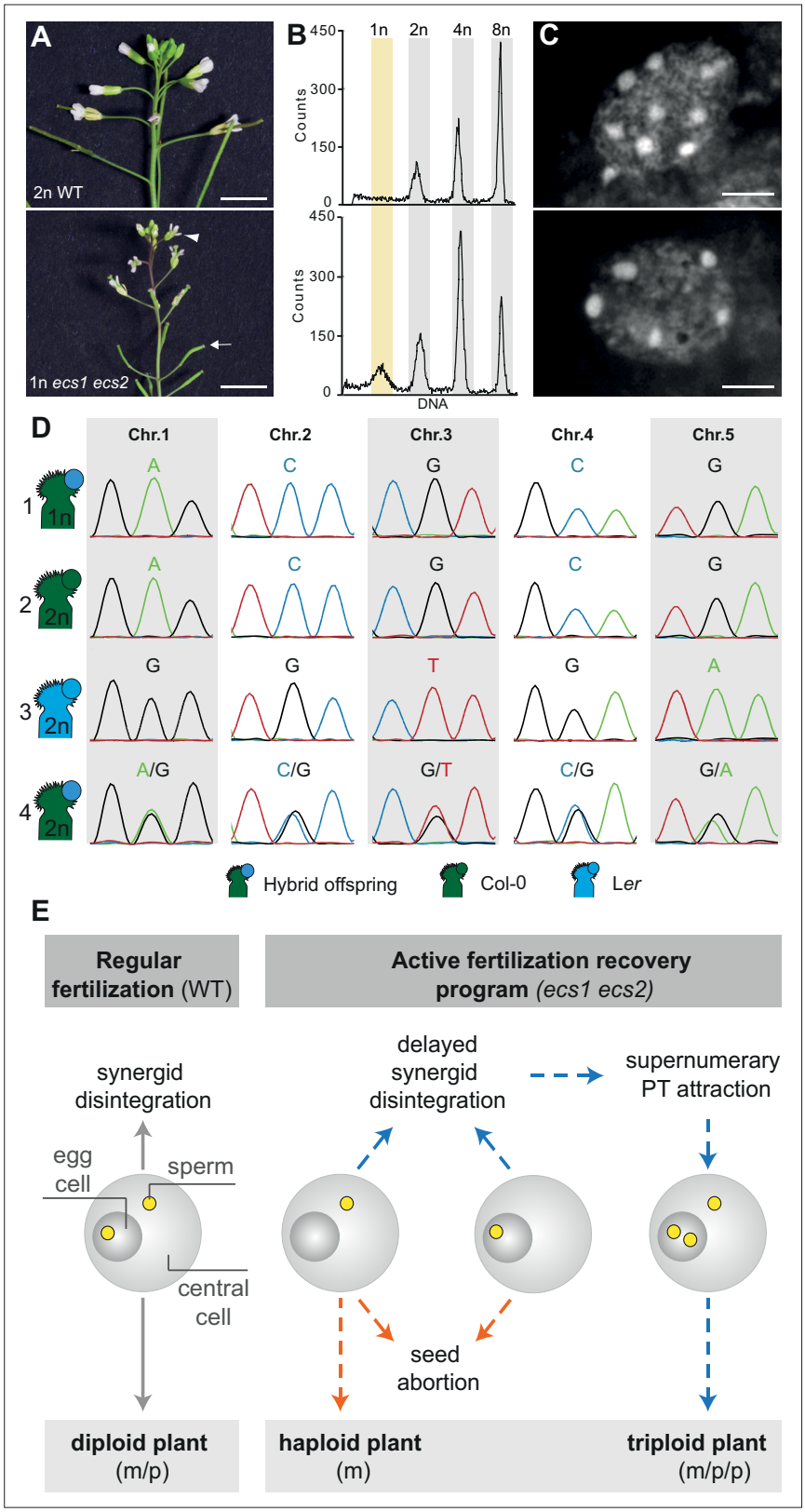

Figure 4. *ecs1 ecs2* double mutants segregate haploid offspring. (A–C) Inflorescence (**A**), flow cytometry (**B**), and DAPI stained chromosome spreads (**C**) of diploid (2n) wild-type and haploid (1n) *ecs1 ecs2* plant (n=200, see also ***Figure 4—figure supplement 1***). Brightness was manually enhanced with Adobe Photoshop in (**A**). The arrowhead and arrow point at a small flower and an undeveloped silique, respectively. (**D**) Accession-dependent

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restriction fragment length polymorphisms (RFLPs) in the hybrid progeny from a cross of *ecs1 ecs2* mutant in Col-0 background (green) with L*er* wild type pollen (blue). Scale bars, 5 mm (**A**), 2 µm (**C**). (**E**) Schematic model illustrating causes and consequences of an active fertilization recovery program in *ecs1 ecs2*. Left panel: Double fertilization of both, egg and central cell, induces synergid disintegration in wild type. Right panel: Defects in double fertilization in *ecs1 ecs2* (egg cell or central cell only) result in delayed synergid disintegration and concomitant attraction of supernumerary pollen tubes increasing the likelihood of polyspermy (blue arrows). In addition, the fertilization defect contributes to seed abortion and can infrequently result in the generation of haploid plants (orange arrows). Dotted lines indicate incomplete penetrance of the respective phenotype. m and p indicate the segregation of maternal and paternal genomes, respectively. Note that according to ***Yu et al., 2021*** the attraction of supernumerary pollen tubes is additionally controlled by ECS1/2-meditated LURE1 cleavage after fertilization.

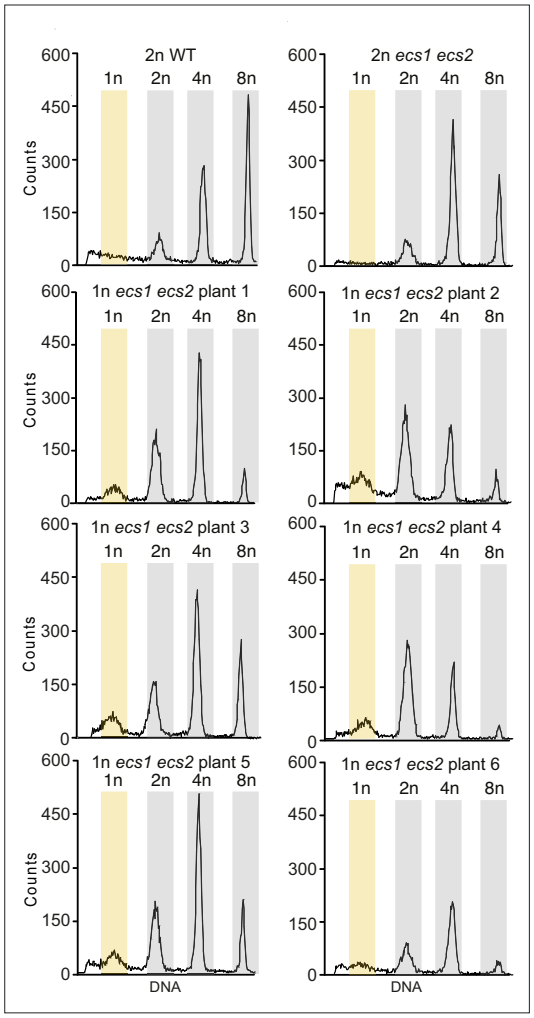

Figure 4—figure supplement 1. Ploidy analysis of *ecs1 ecs2* double mutant offspring that deviates phenotypically from wild type. Flow cytometry analysis of diploid (2n) wild-type, diploid *ecs1 ecs2*, and six haploid (1n) *ecs1 ecs2* plants. The haploid profile is highlighted by yellow color.

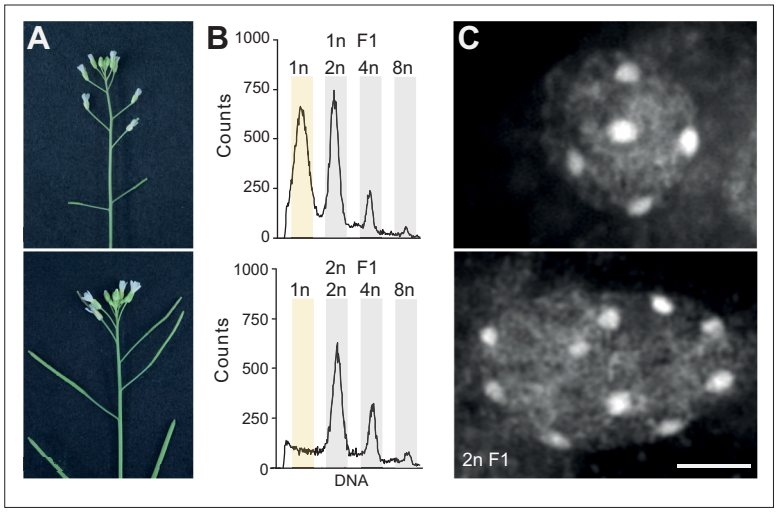

Figure 4—figure supplement 2. Inter-accession crosses between *ecs1 ecs2* (Col-0) and wild-type L*er* segregate low frequencies of haploid offspring. (**A– C**) Inflorescence (**A**), flow cytometry (**B**), and DAPI stained chromosome spreads (**C**) of haploid (1n) *ecs1 ecs2* and diploid (2n) plants derived from a cross of *ecs1 ecs2* mutant (Col-0) with wild type (L*er*).

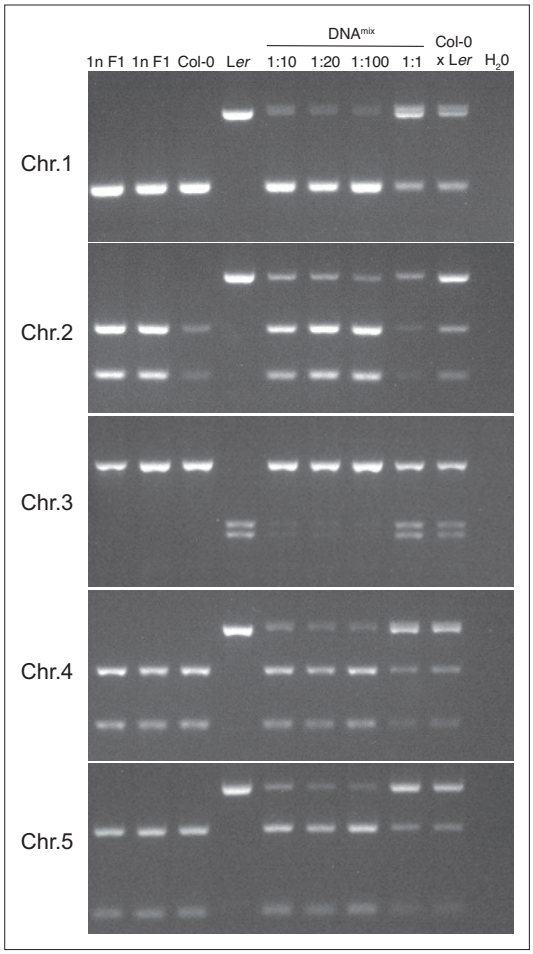

Figure 4—figure supplement 3. Haploid offspring derived from inter-accession crosses between *ecs1 ecs2* (Col-0) with wild type (L*er*) exhibit maternal genome signatures in restriction fragment length polymorphisms (RFLPs) analysis. Analysis of accession-characteristic RFLPs on different chromosomes (Chr.) in haploid offsprings (1n F1), Col-0, L*er*, different ratios of L*er*:Col-0 DNA (DNA^{mix}), cross between two different accessions (Col-0 and L*er*), H_2O.