SI Appendix Materials and Methods

Plant growth and selection

Arabidopsis seeds were sown on propagation plugs and stratified for three days at 4 °C. Plants were grown about ~35 days (22°C, 16 h light/8 h dark) before emasculation.

Transgenic plants were selected on MS medium (4.3 g MS salts (Duchefa), 0.5 g MES, 0.8 % agar, pH 5.8 (KOH)) supplemented with either 10 mg/l gluphosinate-ammonium or 50 mg/l kanamycin.

Sample preparation

After around 35 days of growth all stage 12c flowers (1) on the main stem were emasculated and harvested after 1 to 7 days. Harvested pistils were fixed onto double sided tape and dissected with a hypodermic needle (0,45 x 12 mm) under a dissection microscope. For the morphological analysis of wild-type and mutant ovules the placenta with attached ovules were fixed overnight in a solution of 4 % (v/v) glutaraldehyde and 12,5 mM sodium cacodylate (pH 6.9) at 4 °C (1). After fixation, the tissues were transferred to a clearing solution (chloralhydrate : water : glycerol, 8:2:1) for further dissection and then mounted onto glass slides in the same medium. The samples were left to clear for at least two days before viewing.

GUS staining of pistils was performed as described (2), followed by mounting in the chloralhydrate-based clearing solution.

For examining living tissues, the placenta and the ovules were removed from the ovarium and placed in a 5 % (v/v) glycerol solution for further dissection. The tissues were then mounted in the 5 % (v/v) glycerol solution and observed under the microscope within the hour.

Microscopy and image processing

DIC imaging was done with an Olympus BX51 DIC microscope (PLN 40X objective). Fluorescence imaging was done with a confocal laser scanning microscope (LSM710, Zeiss). All 8-bit images (1024 x 1024) were collected through the Zen software (Zeiss). A water-immersed C-Apochromat 40x/1,20 W Korr M27 objective was used. Settings: GFP, excitation 488 nm, emission 500 -580 nm; Chlorophyll a, emission > 650 nm; Fixed samples, excitation 488 nm, emission 500 – 600 nm. Histogram adjustments were made in Fiji (ImageJ version 1.51s) (3, 4). All images were exported as TIFF files and assembled into figures in Inkscape (version 0.92.2) (https://inkscape.org).

Analysis of phenotypical data

The analysis of all counting data was done in RStudio (https://www.rstudio.com, R version 4.0.3). The counting data was first imported into RStudio and summarized per genotype and/or timepoint. The summarization encompassed pooling the data from same-age pistils and collapsing the data into a binary format: Ovules/cells were scored to be either fully intact or in a stage of decay. Ovules/synergids that died of unnatural causes (aborted category), were malformed or could not be viewed properly (unsure category) were not included in the analysis. The counting data was then analyzed by means of chi-squared tests or Fisher' s exact tests (to account for low expected values). The null hypothesis assuming no difference in the occurrence of intact and degenerated ovules/cells within a contrast. The results were plotted using the *ggplot2* package for R (5).

Gateway cloning of reporter and rescue constructs

For making the different constructs the promoters of *ACT11*, *DD45*, *DD27*, *DD33, NAP*, *SHYG* and *ORE1* were PCR amplified from Col-0 DNA with primers outfitted with *attB4* and *attB1R* sites or XhoI and BamHI recognition sites (all primers listed in Supplementary Table 1). The amplicons were subsequently recombined with pDONRP4-P1R (BP reaction, Invitrogen) or ligated into pENTR-L4-R1 after digestion of the amplicon and vector backbone with BamHI and XhoI. To generate the transactivation constructs (*pACT11>>HTA6-GFP* and *pDD33>>HTA6-GFP*) the cloning proceeded as described previously (6). For the other reporter constructs the desired segments (tdTomato or NLS-GFP-GUS in pDONR221) were combined by recombinational cloning with the destination vector pK7m24GW,3 or pB7m24GW,3 (7).

For the rescue construct the coding sequence of *NAP* was cloned from cDNA (Col-0) and cloned into pDONR221. The entry clone was subsequently used in an LR reaction together with pEN-R2-GFP*-L3, pEN-L4-pNAP-R1 (described previously) and the destination vector pB7m34GW,0 (7) to produce the desired expression vector (*pNAP::NAP-GFP*). Vectors were transferred to *Agrobacterium tumefaciens* (GV3101) by electroporation. *Arabidopsis* plants were then transformed by floral dip (8). All lines were followed up until homozygous, single insertion T3 lines were obtained.

CRISPR vector construction and establishment of triple mutant *nap shyg nac* lines

Two efficient protospacer sequences per gene targeting the coding exons of *NAP/ANAC029*, *SHYG-1/ANAC047* and *ORE1/ANAC092* were selected using the Geneious Prime 2019 software (Supplemental Table 1). Protospacer sequences with overhangs (Supplementary Table 1) were cloned into BbsI-HF (New England Biolabs) linearized Golden Gate entry vectors with unarmed gRNA modules (Decaestecker et al. 2019) via Gibson assembly using 2× NEBuilder Hifi DNA Assembly Mix (New England Biolabs). The gRNA armed Golden Gate entry vectors were verified via Sanger sequencing. To generate the Cas9 gRNA expressing

vector, the Golden Gate entry vectors were assembled in the destination vector PcUBIP-Cas9-mTagBFP2-G7T-A-ccdB-CmR-G_pFASTRK (https://gatewayvectors.vib.be) via Golden Gate cloning (9). The expression vector was verified via restriction digestion and Sanger sequencing, performed by Eurofins Scientific using the Mix2Seq service. Vectors were transferred to *Agrobacterium tumefaciens* (GV3101) by electroporation. Plant vectors were transformed in *Agrobacterium tumefaciens* (strain GV3101) by electroporation. Transformation into *Arabidopsis* Col-0 plants was performed via the floral-dip method (8). T1 transgenic seeds were selected under a Leica M165FC fluorescence stereomicroscope and transferred to soil. Seeds of plants showing an ovule longevity phenotype were collected and T2 plants were genotyped using genotyping primers flanking both gRNA target sites of each gene (all primers listed in Supplementary Table 1). FAST negative (Cas9 free) T2 seeds of lines showing indels in all three genes of interest were selected and genotyped in T3. Homozygous frameshift mutations in all three genes were confirmed in the T4 generation for the selected line which was used for phenotypic analysis.

Genotyping of CRISPR mutants

To extract DNA leaf discs were collected, frozen and ground. 50µl extraction buffer (0.1M Tris-HCL pH 9.5; 0.25M KCl; 0.01M EDTA) was added. The samples were incubated at 95°C for 10 min and cooled on ice for 5 min. 50µl 3 % BSA was added and the mixtures were vortexed and centrifuged at 13,200 rpm for 1 min. Supernatants were collected and subjected to PCR analysis. 1 µl of DNA extraction was used as the template in a 20µl PCR reaction.

PCR was performed using the ALLin™ Red Taq Mastermix, 2X (highQu) according to the manufacturer's instructions. PCR product was column purified using GeneJET PCR Purification Kit (Thermo Scientific) and sent for Sanger sequencing.

RNA extraction and sequencing

Unpollinated pistils of emasculated wild-type flowers were dissected as described previously; for each time point the ovules of over 75 pistils (approximately 4000 ovules) were collected on dry ice with the help of a vacuum-driven micro-aspirator (10). The whole time series was sampled in three independent batches (four time points in each round, 2 to 5 DAE). After subsequent tissue grinding, RNA was extracted with the Spectrum Plant Total RNA kit (Sigma). RNA quantity and quality was assessed by spectrophotometric analysis (NanoDrop ND-2000, Thermo) and on-chip electrophoresis (RNA 6000 Nano Kit, Agilent) (Supplementary Data 4). Because the RNA quality of the last time point (5 DAE) was never satisfactory (RIN < 7), the sample was not included for the RNA sequencing. Samples were sent to the VIB Nucleomics Core Facility (https://nucleomicscore.sites.vib.be/en) for sequencing on the Illumina HiSeq platform, 50bp paired-end reads sequenced on a single lane.

RNA sequencing data analysis

BAM files were imported into the Galaxy Workflow Environment (11). After quality control and trimming, the reads were mapped to the reference genome using an *Arabidopsis thaliana* annotation file (TAIR10, GFF3 file) available from Ensembl (plants.ensembl.org/). After mapping the read counts were summarized into a TXT file. To infer differential expression the raw read counts were imported into RStudio (https://www.rstudio.com/, RStudio Team, 2016) for statistical analysis (R version 4.0.3). Statistical analysis was performed with the R packages *limma* and *edgeR* for the analysis of digital gene expression (12-14). To filter raw read counts a CPM-based cut-off value was calculated based on a read count of 10 reads for a given library. Genes were omitted if none of the samples exceeded the cut-off value. The raw read counts were subsequently normalized by the trimmed mean of M-values method (TMM). After normalization a general linearized model was fitted to the counting data with the age in DAE and batch (block effect) as factors. Differential expression was tested by doing pairwise comparisons between different time points. The p values were adjusted according to the Benjamini-Hochberg method (15). To narrow down the list of DE genes the list was filtered for FDR < 0.05, $|Log_2FC| > 1.6$. Gene lists from other datasets were also imported and marked in the final output file. For creating the heatmaps the R package *pheatmap* was used. For the identification of TFs we downloaded a list of TFs from PlantTFDB 3.0 (http://planttfdb.cbi.pku.edu.cn) (16).

Gene ontology enrichment analysis

1410 DEG's (2 vs 4 DAE) were fed into the PANTHER 17.0 tool (geneontology.org) and compared to all *Arabidopsis thaliana* genes (27430) as reference list. The analysis type was PANTHER Overrepresentation Test (Released 20220712) and the annotation version was GO Ontology database DOI: 10.5281/zenodo.6799722 (Released 2022-07-01). As annotation data set, "GO biological process complete" was selected. The analysis used a Fisher's Exact test with a correction based on the False Discovery Rate (FDR). Only results with an FDR < 0.05 are reported.

Quantitative PCR

RNA was extracted from unpollinated ovaria (i.e. pistil minus abscission zone, stigma and style) at 2, 4 and 6 DAE using the Spectrum Plant Total RNA kit (Sigma). Next, 500 ng RNA was reverse transcribed with the iScript cDNA synthesis kit (BioRad). The cDNA was diluted 1:20 in RNase-free water and mixed with LightCycler 480 SYBR Green I Master Mix (Roche) and 0.5 µM primer mix (Supplementary Table 1). The qPCR program consisted of 45 amplification cycles at an annealing temperature of 60 °C.

Stigma grafting based semi-*in-vitro* fertilization

In short, pistils of different senescence stages (2-7 DAE) were removed from the plant with a razor blade, and their stigmas were removed by cutting just below the style. Then, these "stock" pistils were placed horizontally into prepared trenches cut in a plate containing pollen germination medium as described (17) supplemented with 0.8 % agar (No.4 – Plant Tissue Culture Grade, Neogen). The pedicels of the stock pistils were inserted into the medium to safeguard water and solute supply to the severed pistil. Next, young flowers (2 DAE) were pollinated by pollen carrying a *pRPS5A::NLS-GFP* reporter construct. The pollinated stigmas were cut just below the style, and placed as a "scion" on the decapitated "stock" pistils on the agar plate. Shortly placing the cut scion stigmas onto the surface of the germination medium provided a liquid film of pollen germination medium on the cut face that was sufficient to provide adherence of the scion stigma to the stock pistil. Pollen tubes germinated on the scion stigma and crossed the graft junction to invade the stock pistil and were able to fertilize up to 50 % of the ovules in a young stock (2 DAE). Fertilization was detected at 3 days after pollination and grafting by extracting the ovules from their pistils and analyzing them by CLSM. Fertilization rates were quantified based on the presence of proliferating endosperm expressing a *pRPS5A::NLS-GFP* reporter introduced by the pollen.

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