

High-frequency biparental inheritance of plant mitochondria upon chilling stress and loss of a genome-degrading nuclease

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Mitochondria are inherited maternally in the vast majority of eukaryotes. Occasional transmission of paternal mitochondria (paternal leakage) can lead to heterochondriomy and recombination between maternal and paternal mitochondrial genomes. Despite its potential physiological and evolutionary consequences, the extent of paternal leakage and the cellular processes governing mitochondrial inheritance remain largely unknown. Here we have established a robust genetic screen to detect paternal mitochondrial inheritance in tobacco (*Nicotiana tabacum*). Our data reveal an unexpectedly high paternal transmission frequency of 0.18%, which increased markedly to 7.34% when the organellar exonuclease DPD1 was disrupted and pollen development occurred at low temperature. Notably, paternally transmitted mitochondria restored growth, development and male fertility in progeny that inherited dysfunctional mitochondria from the maternal parent. Together, our findings uncover molecular mechanisms underlying maternal mitochondrial inheritance, and highlight the potential of biparental transmission to rescue mitochondrial function and generate novel mitochondrial genotypes through recombination.

In photosynthetic eukaryotes, genetic material is stored in three compartments: the nucleus, the plastids and the mitochondria^{1–3}. Although plastid and mitochondrial genomes encode only a limited number of proteins, they are essential for key cellular processes including photosynthesis and respiration^{4–6}. Consequently, mutations in cytoplasmic genomes can have profound effects on plant growth and development. A striking example is cytoplasmic male sterility (CMS), which arises from mutations in the mitochondrial genome that lead to the failure to produce functional pollen⁷. As cytoplasmic genomes are maternally inherited in most angiosperms, traits associated with cytoplasmic

genomes are typically passed down across generations exclusively through the maternal lineage^{8,9}.

The uniparental inheritance of cytoplasmic genomes stands in stark contrast to the biparental transmission of nuclear DNA¹⁰. Although numerous cytological studies support the predominance of maternal inheritance, accumulating evidence suggests that this maternal bias is not absolute. Rare instances of paternal plastid transmission have been reported in species traditionally considered to follow strict maternal inheritance, including *Arabidopsis thaliana*¹¹, *Brassica napus*¹² and *Nicotiana tabacum* (tobacco)¹³. These findings

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indicate that exceptional cases of paternal leakage can be identified when (1) highly sensitive methods for paternal plastids detection are employed and (2) sufficiently large sample sizes are analysed. Leveraging such sensitive, large-scale screening strategies, we have recently identified genetic and environmental factors that govern plastid inheritance in tobacco¹⁴.

In contrast to the progress made with uncovering the mechanisms underlying plastid inheritance, the cellular processes governing mitochondrial inheritance remain largely elusive. A prevailing hypothesis posits that the abundance of mitochondrial DNA in male gametes plays a crucial role in shaping mitochondrial inheritance¹⁵. In *Arabidopsis*, mitochondria are regularly present in sperm cells and can be transmitted to the egg cell upon fertilization¹⁶. However, during pollen maturation, mitochondrial DNA is actively degraded by nucleases, thus preventing its paternal transmission^{17–20}. Although some nucleases involved in mitochondrial DNA degradation have been identified^{21,22}, no instances of paternal mitochondrial transmission could be detected when the corresponding mutants were used as pollen donors. Consequently, the genes that regulate mitochondrial inheritance remain unknown and our understanding of paternal mitochondrial leakage is limited.

This knowledge gap is largely due to the lack of sensitive assays for the detection of paternal transmission events. In particular, the inability to express selectable marker genes from the mitochondrial genome has hindered the development of robust experimental systems for the study of mitochondrial inheritance. Although molecular techniques such as restriction fragment length polymorphism (RFLP) and derived cleaved amplified polymorphic sequence (dCAPS) have been used to specifically detect paternal mitochondria^{22–24}, these approaches are labour intensive and lack the throughput required for large-scale screening.

Recent advances in mitochondrial genome engineering have opened up new possibilities to address this major bottleneck^{25–28}. Using TALEN-mediated targeted mutagenesis, we have generated tobacco mitochondrial mutants that display distinct developmental phenotypes²⁹. By leveraging these phenotypic differences between mutant and wild-type plants, we set out to establish an experimental strategy to detect paternal mitochondrial transmission events. Leveraging this strategy, we report here the identification of factors that control the maternal inheritance of mitochondria. We demonstrate that manipulation of these factors changes the mode of mitochondrial inheritance, thus modulating phenotype and physiology of the progeny. Importantly, our findings show that paternal mitochondria, once transmitted, can enter the germline and become stably inherited across generations, suggesting an impact on the evolution of mitochondrial genomes and speciation processes in plants.

Results

An experimental system to detect paternal mitochondrial transmission

The robust detection of paternal leakage in mitochondrial inheritance requires a high-throughput screening strategy. To identify a screenable phenotypic marker, we evaluated mitochondrial mutants that could be used as maternal parent in crosses with a paternal parent that harbours wild-type mitochondria. Any paternally transmitted mitochondria would then restore a wild-type-like phenotype in the progeny, which ideally, would be distinguishable from the mutant phenotype early upon seed germination. The mitochondrial *Δnad9* mutant met these criteria. In this mutant, the *nad9* gene has been deleted from the mitochondrial genome (Extended Data Fig. 1), leading to disrupted complex I biogenesis and a range of macroscopic phenotypes, including impaired growth and development, and male sterility²⁹. These *Δnad9*-associated defects are stably passed on to the progeny through the maternal lineage. Most importantly, the *Δnad9* progeny exhibits strongly delayed seed germination and seedling

growth. While wild-type seedlings typically produce true leaves after 14 days, *Δnad9* seeds germinate at a slower rate, and at day 14, only the cotyledons have emerged (Extended Data Fig. 2). This clear phenotypic difference provides a robust system for distinguishing the two mitochondrial genotypes (wild-type versus *Δnad9*). We reasoned that after crossing *Δnad9* mutants with paternal plants carrying wild-type mitochondria, progeny harbouring paternal (wild-type) mitochondria would germinate and grow faster than the progeny that harbours only maternal (*Δnad9*) mitochondria. Such a phenotypic restoration would provide an easily screenable readout of paternal mitochondrial leakage events (Fig. 1a).

To test this strategy, we fertilized emasculated *Δnad9* mutant flowers with pollen from chilling-stressed *Δdpd1* (*defective in pollen organelle DNA degradation 1*) mutants carrying wild-type mitochondria. We used the chilling-stressed *Δdpd1* mutant as pollen donor, because we recently showed that the combination of low temperature and the *Δdpd1* allele significantly promotes paternal plastid transmission¹⁴. The *DPD1* gene encodes an organellar exonuclease, whose knockout exhibits defective organellar DNA degradation in pollen^{14,22}. We hypothesized that similar conditions would also promote paternal mitochondrial transmission and maximize the likelihood of detecting paternal leakage events. In an initial experiment (Experiment 1, see Methods), a total of 1,000 seeds from the *Δnad9* × *Δdpd1* 10 °C cross were sown, and their germination was monitored by daily inspection. We identified 5 F₁ seedlings that germinated at a speed comparable to the wild type (Fig. 1b). When these 5 seedlings had already developed their cotyledons, the rest of the progeny only showed radicle emergence (Fig. 1b). We considered these 5 seedlings as potentially derived from paternal mitochondrial leakage events, and they will hereafter be referred to as PMt plants. To further assess their growth phenotypes, we transferred the PMt seedlings to Magenta boxes. While the PMt-3 plant showed retarded growth and abnormal leaf morphology, closely resembling the characteristic *Δnad9* phenotype²⁹, the other four PMt plants displayed growth and development similar to wild-type plants (Extended Data Fig. 3).

To test whether the phenotypic restoration in PMt plants was due to the transmission of paternal mitochondria that provide *nad9* expression, RNA was extracted from their leaf and root tissues, followed by polymerase chain reactions with reverse transcription (RT-PCR) with *nad9*-specific primers to assess *nad9* expression. As expected, *nad9* transcripts were completely absent from the *Δnad9* mutant, but could be readily detected in both leaf and root tissues of PMt-1, -2, -4 and -5 plants. Notably, for PMt-3, *nad9* transcripts were detected only in roots (Fig. 1c and Extended Data Fig. 4). The absence of *nad9* transcripts from leaf tissues probably explains the leaf developmental defects observed in this line. However, presence of wild-type mitochondria in the roots of PMt-3 suggests that *nad9* expression in the root is sufficient to rescue the germination phenotype of the *nad9* mutant. Taken together, the presence of *nad9* transcripts in all PMt plants provides strong evidence for paternal mitochondrial transmission being the cause of the observed restoration of wild-type-like seed germination.

To exclude the possibility that the PMt plants originated from contamination by wild-type seeds, genotyping experiments were conducted to verify both the nuclear and mitochondrial genotypes of the paternal leakage lines. Since the paternal parent was homozygous for the *Δdpd1* allele in the nuclear genome, genuine PMt plants are expected to be heterozygous for *Δdpd1*. In addition, despite paternal leakage, PMt plants should also inherit *Δnad9* mitochondria from the maternal plants. Genotyping experiments confirmed the presence of the nuclear *Δdpd1* and the mitochondrial *Δnad9* loci in all PMt plants (Fig. 1d,e). Together, these results ultimately confirm that the PMt plants represent bona fide cases of paternal transmission and demonstrate that our experimental strategy is suitable to detect paternal mitochondrial leakage events.

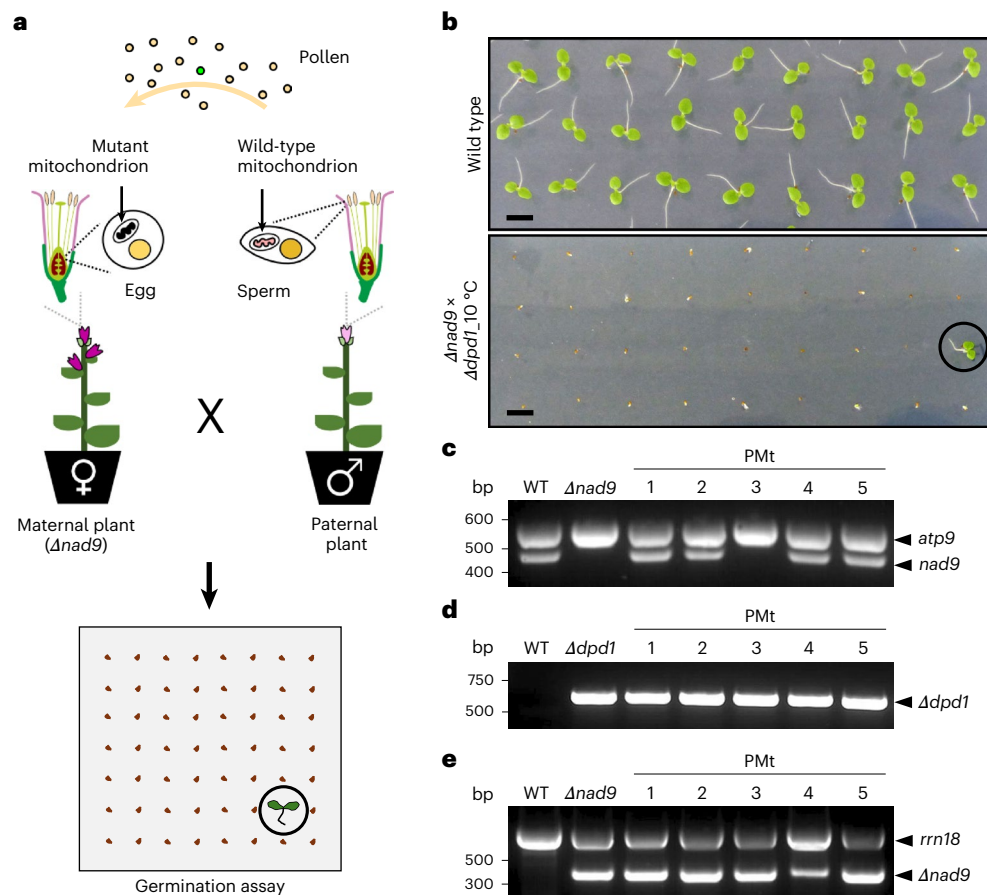


Fig. 1 | Detection of paternal mitochondrial transmission in tobacco by a phenotypic screen. **a**, Experimental design for the detection of paternal mitochondrial transmission events. The deletion of *nad9* from the mitochondrial genome impairs mitochondrial function, leading to delayed seed germination. Upon maternal inheritance, the progeny of $\Delta nad9$ mother plants inherit only mutated mitochondrial genomes, thus exhibiting the delayed germination phenotype. This phenotype provides a robust assay for progeny that has inherited functional paternal mitochondria, which restore full respiratory activity and thus wild-type-like germination (encircled seedling).

b, Identification of paternal mitochondrial leakage events by germination assays. Pictures were captured on day 10 after sowing. Top: wild-type seeds. Bottom: seeds from crosses using a $\Delta nad9$ plant as the maternal parent and a $\Delta dpd1$ plant grown at 10 °C ($\Delta dpd1_{10\text{ }^{\circ}\text{C}}$) as the pollen donor (Experiment 1). Open circle: a seedling displaying exceptionally fast germination, potentially carrying paternal

mitochondria (PMt plantlet). Scale bars, 1 cm. **c**, Detection of *nad9* transcripts by RT-PCR. RNA was extracted from leaf tissues of wild-type (WT), $\Delta nad9$ and 5 PMt plants exhibiting exceptionally fast germination. *atp9* and *nad9* transcripts were co-amplified using gene-specific primers. *atp9* served as an internal reference and was detected in all samples. *nad9* transcripts were absent from the $\Delta nad9$ mutant, but detected in PMt-1, -2, -4 and -5 plants. RT-PCR followed by gel electrophoresis were performed once per sample. **d,e**, Verification of PMt plants by nuclear and mitochondrial genotyping. Genotypes were analysed by PCR using primers specific to the *dpd1* (**d**) and *nad9* (**e**) deletions. Both deletions were detected in all PMt samples, confirming that the identified PMt plants represent progeny derived from the $\Delta nad9 \times \Delta dpd1_{10\text{ }^{\circ}\text{C}}$ cross. Simultaneous amplification of the mitochondrial genomic locus encoding the 18S rRNA (*rrn18*) served as an internal control. PCRs followed by gel electrophoresis were performed once per sample.

Vegetative segregation of parental mitochondrial genomes

The detection of the $\Delta nad9$ locus in the PMt plants (that had been phenotypically rescued by wild-type mitochondria) indicates the coexistence of maternal and paternal mitochondrial genotypes in the tissues examined, a state known as heterochondriomy. To investigate whether plant growth and development is influenced by the relative abundances of the two parental mitochondrial genomes, we transferred PMt plants to soil and cultivated them under standard greenhouse conditions.

We observed three distinct phenotypes in the PMt plants. The first type, exemplified by the PMt-3 plant, exhibited the $\Delta nad9$ phenotype characterized by rolled-up leaf margins and curled flower corolla (Fig. 2a). These morphological abnormalities were expected, given the lack of detectable *nad9* transcripts in the shoot tissues (Fig. 1c). The second type included PMt-1, -2 and -4, all of which showed wild-type-like growth rates, leaf shape and flower morphology (Fig. 2a), suggesting that *nad9* expression originating from paternally inherited mitochondria provided sufficient mitochondrial activity to sustain normal growth and development. The third category, represented by

PMt-5, exhibited a chimaeric phenotype. Although *nad9* transcripts were detected in its leaf tissues, the PMt-5 plant produced a mix of wild-type-like and $\Delta nad9$ -like leaves. Notably, the $\Delta nad9$ phenotypes became less pronounced as the plant developed, and all emerging flowers resembled those of the wild type (Fig. 2a). This finding suggests a shift in mitochondrial genome ratios over time, with paternally derived *nad9* expression eventually reaching levels that restored mitochondrial function to an extent that allowed normal development of reproductive organs.

Paternal mitochondrial transmission reverses cytoplasmic male sterility

Deletion of the *nad9* gene from the mitochondrial genome causes CMS in tobacco²⁹. In line with this known phenotypic consequence, we observed a severe reduction in pollen production in the PMt-3 plant that lacked *nad9* expression in its shoot tissues. Strikingly, all other PMt plants showed abundant pollen deposition in the anthers (Fig. 2a), suggesting restoration of male fertility. To assess pollen viability,

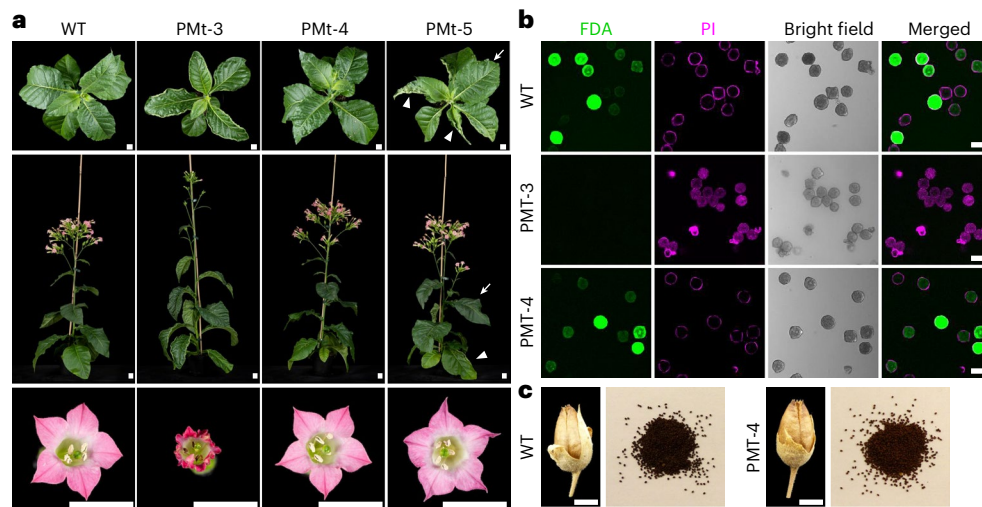


Fig. 2 | Restoration of male fertility by paternal mitochondrial inheritance.

a, Growth and developmental phenotypes of WT and PMt plants cultivated in the greenhouse. Images were captured at 55 days (top) and 75 days (middle) after sowing. Bottom: close-up views of floral morphology in WT, PMt-3, -4 and -5 plants. PMt-3 displayed a $\Delta nad9$ -like mutant phenotype, including rolled-up leaf margins, impaired flower development with curly corolla margins and vestigial anthers lacking pollen. By contrast, PMt-4 resembled the WT plant in leaf and floral morphology, and exhibited WT-like pollen production. PMt-5 exhibited signs of heterochondriomy, indicated by a mixture of $\Delta nad9$ -like (arrowheads) and WT-like leaves (arrows) at early stages (top). However, as the plant continued to grow, later-emerging leaves no longer exhibited the $\Delta nad9$ phenotype (middle), and all flowers appeared WT-like with normal pollen production (bottom). Scale bars, 2 cm. **b**, Confocal microscopy images of pollen from WT, PMt-3 and -4 plants. Pollen viability was assessed using FDA and PI staining. Both WT and PMt-4 plants produce viable pollen, indicated by FDA-derived green fluorescence observed in pollen grains. By contrast, PMt-3 pollen was non-viable, exhibiting only PI fluorescence due to the absence of cytoplasmic esterase activity required for FDA–fluorescein conversion and increased permeability to PI. Scale bars, 50 μ m. The pollen viability assay was performed twice per plant and representative images are shown. **c**, Representative seed capsules that originated from self-pollinated WT and PMt-4 flowers. Except for PMt-3, all PMt plants produced WT-like seed capsules and exhibited normal seed yields. Scale bars, 1 cm.

we performed fluorescein diacetate (FDA) and propidium iodide (PI) staining of pollen collected from wild-type and PMt plants. The vast majority of PMt-3 pollen grains were non-viable, as indicated by PI fluorescence (Fig. 2b). By contrast, pollen from wild-type and PMt-4 plants showed predominant green fluorescence from FDA staining, indicating viability (Fig. 2b). We further assessed pollen fertility through pollination assays. Pollen from PMt-1, -2, -4 and -5 plants successfully fertilized flowers upon self- or cross-pollination, resulting in the formation of seed capsules that were similar in size to those of the wild type (Fig. 2c). These findings confirmed the restored male fertility in all PMt plants except PMt-3.

The restoration of male fertility suggests that the paternally derived *nad9* gene is present and expressed in reproductive tissues. Hence, it can be expected that the *nad9*-containing genomes readily enter the female germline and are transmitted to the progeny via maternal inheritance. To investigate whether the paternally derived *nad9* genes are inherited to the subsequent F_2 generation, we collected individual seed capsules from self-pollinated PMt-4 plants and harvested their seeds separately. Germination assays were performed with seeds from three capsules. While seeds from capsules 1 and 2 showed wild-type-like germination and seedling growth, those of capsule 3 exhibited mixed phenotypes: while most seedlings germinated normally, a subset showed delayed germination and resembled the $\Delta nad9$ mutant (Extended Data Fig. 5a). We reasoned that the germ cells that had given rise to capsule 3 contained a mixture of wild-type and $\Delta nad9$ mitochondrial genomes. Genotyping experiments revealed that the $\Delta nad9$ genome was undetectable in seedlings from capsules 1 and 2, but was present in some seedlings from capsule 3 (Extended Data Fig. 5b). These results indicate that, in PMt plants, the mitochondrial genomes received from both parents continue to segregate during development, and either a single genotype or both genotypes can be incorporated into the germline and transmitted to the next generation.

Unbiased detection of paternal mitochondrial transmission

Our data described above indicated that the relative abundances of parental mitochondrial genomes and their segregation in different cell lineages (somatic versus germline cells) and tissues (root versus shoot) determine the phenotypic outcome of biparental mitochondrial inheritance. We, therefore, reasoned that our phenotype-based screening system, which relies on the detection of restored seed germination time, is inherently influenced by the segregation and proliferation of transmitted paternal mitochondria during embryogenesis. If one assumes that phenotypic restoration requires the presence of a substantial number of paternal mitochondria in the embryo, the sensitivity of our screen may be limited in that only those paternal leakage events are identified that involve a substantial level of paternal mitochondrial transmission. Consequently, the phenotypic screen may underestimate the true frequency of paternal leakage. To address this potential limitation, we set out to develop a more sensitive approach that allows detection of paternal mitochondria also in progeny that do not exhibit phenotypic restoration.

The nuclear genome of tobacco harbours abundant mitochondrial DNA sequences (NUMTs) that originate from intracellular DNA transfer. Some of these NUMTs are very similar in sequence to the mitochondrial *nad9* gene (which comes from paternally inherited mitochondria in our screens)²⁹. For this reason, paternal leakage cannot be detected unambiguously by direct amplification of genomic DNA. We, therefore, used *nad9* expression as an indicator of paternal mitochondrial leakage. *nad9* expression can be very sensitively detected by RT–PCR assays (Fig. 1c), and *nad9* transcripts are exclusively derived from mitochondria.

To validate the relationship between *nad9* expression levels and paternal leakage, we measured the number of wild-type (paternal) *nad9* gene copies and *nad9* transcript abundance in additional PMt seedlings (Extended Data Fig. 6a) by quantitative real-time PCR (qPCR) and quantitative PCR with reverse transcription (RT–qPCR), respectively. After

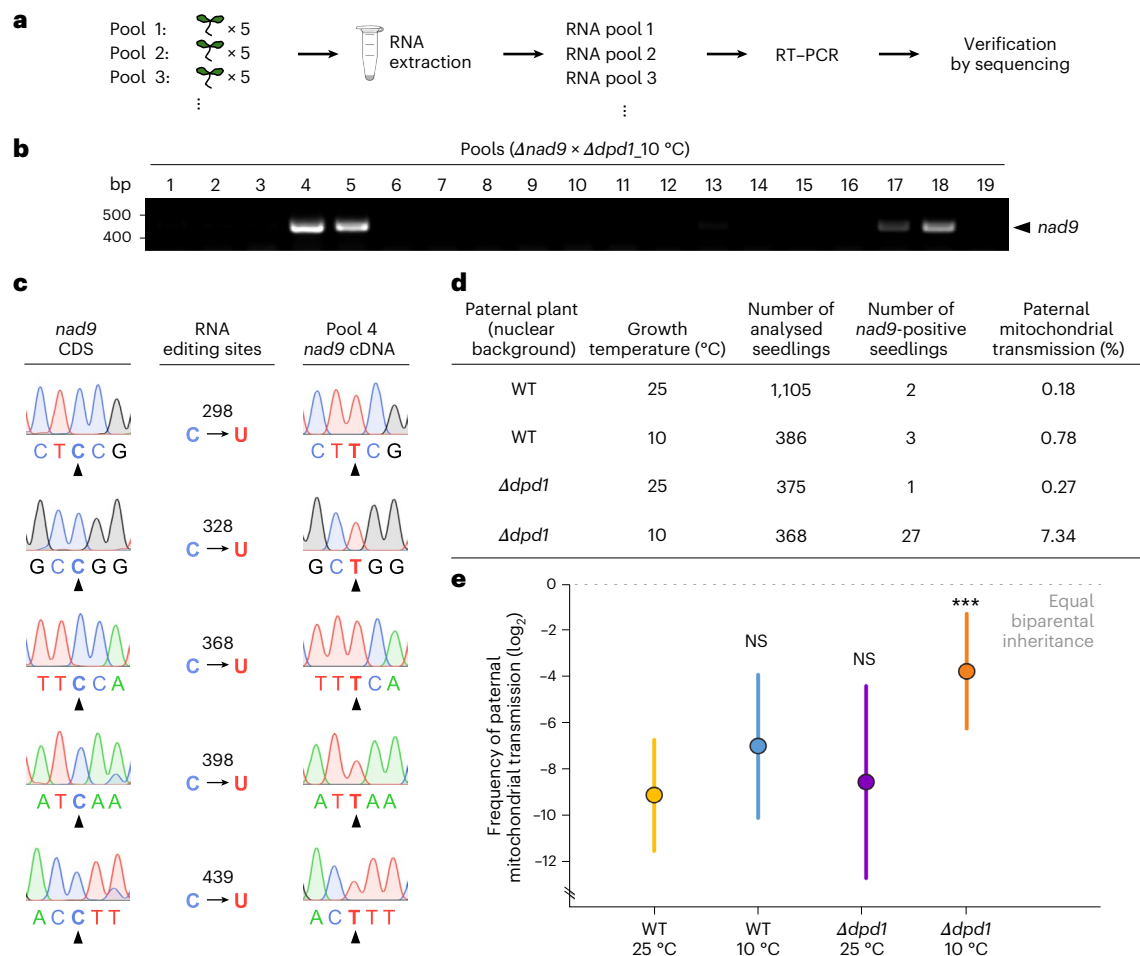


Fig. 3 | Synergistic effect of the *dpd1* mutation and low temperature during pollen development leads to enhanced paternal mitochondrial transmission.

a, Experimental design of the PCR-based screening approach for the detection of paternal mitochondrial transmission. Five progeny seedlings were randomly selected and allocated to a single pool for RNA extraction. RT-PCR was performed to assess *nad9* expression. Detected *nad9* cDNA was subsequently recovered and sequenced to validate transcript identity. **b**, Example of an RT-PCR analysis performed on RNA pools from progeny derived from $\Delta nad9 \times \Delta dpd1_{10^\circ C}$ crosses (Experiment 2). *nad9* transcripts were detected in pools 4, 5, 17 and 18. PCR followed by gel electrophoresis was performed once per sample. **c**, Sequencing data from a representative *nad9*-positive sample. Five previously published RNA editing sites (nucleotide positions 298, 328, 368, 398 and 439)^{29,30} were analysed to assess the RNA editing status of the recovered *nad9* cDNA. All five sites were fully edited (arrowheads). **d**, Quantification of the impact of chilling stress ($10^\circ C$), the *dpd1* mutation and their combination on paternal mitochondrial inheritance (Experiment 2). Four sets of crosses were analysed, with all 4 using $\Delta nad9$ as the maternal parent and the following pollen donors (nuclear background, growth temperature): (1) WT_25 $^\circ C$ ($n = 1,105$ seedlings); (2) WT_10 $^\circ C$ ($n = 386$); (3) $\Delta dpd1_{25^\circ C}$ ($n = 375$); and (4) $\Delta dpd1_{10^\circ C}$ ($n = 368$). Seedlings displaying WT-like germination were classified as exceptional and were individually processed (that is, without pooling) for *nad9* expression analysis.

The remaining progenies were pooled before RNA extraction (five seedlings per pool). Positive *nad9* amplification signals from pools were considered to derive from a single *nad9*-positive seedling. The frequency of paternal mitochondrial transmission is the number of *nad9*-positive seedlings divided by the total number of seedlings analysed. **e**, Statistical analysis of paternal mitochondrial transmission in Experiment 2. Circles represent the frequency of paternal mitochondrial transmission per group as single data points (no replicates within the experiment). Differences in paternal transmission between each group and the control condition (WT_25 $^\circ C$) were analysed through a model based on binomial distribution. Individual seedlings are defined as the units of study for the analysis, based on explicit modelling assumptions (see Methods, 'Modelling and statistics'). Differences were tested simultaneously using two-sided z-tests and corrected for multiple comparisons through the Holm method. Coloured vertical lines: 95% confidence intervals (CI95) of the measured transmission frequency. Effects are expressed as $\log_2$ fold change ($\log_2 FC$). The cross using $\Delta dpd1$ plants exposed to $10^\circ C$ as pollen donors shows a significant effect compared with the control ($\log_2 FC = 5.34$; CI95: 2.86–7.82; $z = 5.069$; $P = 1.2 \times 10^{-6}$), while no significant effects were found for WT_10 $^\circ C$ ($\log_2 FC = 2.10$; CI95: -0.99 to 5.19; $z = 1.600$; $P = 0.219$) and $\Delta dpd1_{25^\circ C}$ ($\log_2 FC = 0.55$; CI95: -3.59 to 4.71; $z = 0.317$; $P = 0.751$). $\alpha = 0.05$; not significant (NS), $P > 0.05$; *** $P < 0.001$.

quantification and exclusion of *nad9*-containing NUMTs, we found a strong positive correlation between the number of mitochondrial *nad9* gene copies and transcript levels in PMt plants ($R^2 = 0.9449$; Extended Data Fig. 6b). Notably, PMt seedlings with intermediate phenotypes (that is, with some rolled-up leaf margins, resembling the $\Delta nad9$ mutant) showed substantial levels of *nad9* transcripts (26–55% of the wild-type level), suggesting that seedlings derived from paternal leakage are indistinguishable in morphology from $\Delta nad9$ seedlings at low *nad9* copy numbers (and transcript levels).

We implemented an RT-PCR-based screening approach, in which we randomly sampled progeny, extracted RNA from whole seedlings and tested for the presence of *nad9* transcripts. To increase the number of seedlings that can be assayed while maintaining sensitivity of *nad9* transcript detection, we pooled five seedlings per sample for RNA extraction (Fig. 3a). RT-PCR on pooled RNA samples leads to the detection of paternally transmitted *nad9* transcripts (Fig. 3b, a representative RT-PCR result showing the detection of *nad9* transcripts in 4 out of 19 pools derived from the $\Delta nad9 \times \Delta dpd1_{10^\circ C}$ cross;

Experiment 2). In Experiment 1, we detected *nad9* transcripts in 8 out of 20 pools derived from the $\Delta nad9 \times \Delta dpd1$ 10 °C cross, whereas no *nad9* transcripts were detected in 20 pools of the control cross ($\Delta nad9 \times WT$ 25 °C). In mitochondria of tobacco, the *nad9* mRNA is known to undergo post-transcriptional processing by C-to-U RNA editing at multiple sites²⁸. Sequencing of the amplified RT-PCR products confirmed the presence of the edited nucleotides at the expected sites³⁰, thus unambiguously demonstrating that *nad9* was expressed and its mRNA correctly edited inside mitochondria (Fig. 3c). Notably, none of these *nad9*-positive RNA pools contained seedlings showing phenotypic restoration. These findings demonstrate that the PCR-based screening approach, coupled with validation by complementary DNA (cDNA) sequencing, can reliably and highly sensitively identify paternal leakage events, including events that would go undetected by phenotype-based screening.

The exonuclease DPD1 and the temperature during pollen development control mitochondrial inheritance

Leveraging our sensitive PCR-based approach, we next wanted to quantify the effects of low temperature and the $\Delta dpd1$ allele on mitochondrial inheritance. To this end, we performed sets of crosses with WT and $\Delta dpd1$ plants grown at either 10 °C or 25 °C as pollen donors (Experiment 2; Fig. 3d). The F_1 progenies were then screened for paternal leakage using *nad9* transcript detection by RT-PCR assays. Contrary to the widely held assumption that mitochondria are strictly maternally inherited, we observed a surprisingly high paternal mitochondrial transmission frequency of 0.18% under standard conditions (that is, in the $\Delta nad9 \times WT$ 25 °C cross). The highest frequency of paternal mitochondrial inheritance, 7.34%, was observed in the $\Delta nad9 \times \Delta dpd1$ 10 °C cross. By contrast, crosses involving only low temperature (that is, $\Delta nad9 \times WT$ 10 °C) or only the *dpd1* deletion allele ($\Delta nad9 \times \Delta dpd1$ 25 °C) did not show statistically significant differences in paternal transmission frequencies compared with the $\Delta nad9 \times WT$ 25 °C cross (Fig. 3d,e). Together, these results indicate that the combination of low temperature during pollen maturation and $\Delta dpd1$ mutation is required to trigger substantial paternal mitochondrial transmission.

Cytological basis of paternal mitochondrial transmission

Given the high paternal transmission frequency observed in the $\Delta nad9 \times \Delta dpd1$ 10 °C cross, we next sought to identify the cellular processes that are affected in chilling-stressed $\Delta dpd1$ pollen. Chilling stress is known to increase plastid abundance in generative cells (GCs), thereby promoting paternal chloroplast transmission¹⁴. We therefore speculated that low temperature may likewise enhance mitochondrial abundance in GCs. In addition, we wanted to investigate the potential effects of temperature on mitochondrial integrity by analysing organelle morphology at the ultrastructural level. To this end, we examined mitochondrial distribution and morphology in pollen developed at 10 °C or 25 °C by electron tomography (ET). Three-dimensional GC reconstruction by ET allowed quantitative assessment of the mitochondrial abundance in GCs (Fig. 4a, Extended Data Fig. 7, and Supplementary Videos 1 and 2). Higher numbers of mitochondria were observed in the GCs of pollen grains that had developed at 10 °C versus those at 25 °C (Fig. 4b), suggesting that organelle entry into the GC contributes to the elevated paternal transmission frequency observed in the $\Delta nad9 \times \Delta dpd1$ 10 °C cross. We did not observe any notable differences in mitochondrial morphology in pollen developed at 10 °C versus those at 25 °C (Fig. 4a and Extended Data Fig. 7), indicating that low temperature does not appreciably affect mitochondrial integrity.

While the presence of mitochondria in GC may enable paternal leakage, the abundance of mitochondrial DNA (mtDNA) in pollen has been proposed as a critical factor governing mitochondrial inheritance^{15,31}. In *Arabidopsis*, the dually targeted organellar exonuclease DPD1 plays a key role in degrading mtDNA during pollen maturation²², thus potentially restricting paternal transmission. To

examine the effect of the *dpd1* loss-of-function allele on mtDNA abundance in tobacco pollen, we performed DAPI and MitoTracker staining experiments using in vitro-germinating $\Delta dpd1$ pollen tubes. Discrete punctate DAPI fluorescence signals, indicative of cytoplasmic DNA, were observed in $\Delta dpd1$ pollen tubes but not in wild-type pollen tubes (Fig. 4c). Importantly, we observed co-localization of the DAPI and MitoTracker fluorescence signals (Fig. 4d), indicating that mtDNA is retained in $\Delta dpd1$ pollen.

Together, these findings demonstrate that the combined effects of chilling stress and $\Delta dpd1$ mutation lead to an increased abundance of DNA-containing mitochondria in male gametes, thereby promoting paternal mitochondrial transmission.

Discussion

In this study, we report the occurrence of biparental mitochondrial inheritance in tobacco, a species previously believed to inherit its mitochondria strictly maternally. We established phenotypic and RT-PCR-based screens to detect paternal mitochondrial transmission events at high sensitivity. While conventional methods for assaying mitochondrial inheritance have exclusively relied on sequence polymorphisms^{32–36}, our phenotype-based screen identifies paternal leakage through phenotypic restoration, followed by validation through expression analysis and genotyping (Fig. 1). To further improve sensitivity, we developed a PCR-based screening method capable of identifying paternal leakage events even in progeny that do not show phenotypic restoration (Fig. 3). This approach revealed a paternal mitochondrial transmission frequency as high as 7.34% for chilling-stressed *dpd1* pollen, which is substantially higher than the 0.5% (5 PMt plants in 1,000 progenies analysed) detected through phenotype-based screening of the same treatment group. A small limitation of this approach lies in the pooling step, where a positive PCR signal from a pooled sample is attributed to a single leakage event, leading to an underestimation of the actual paternal mitochondrial transmission frequency. Correction for this effect (Methods) results in an estimated transmission frequency of 8.7%.

Applying our screening approaches, we showed that the use of chilling-stressed $\Delta dpd1$ plants as pollen donors significantly enhances paternal mitochondrial transmission. This finding suggests that the inheritance of both mitochondria and plastids is influenced by a common set of genetic and environmental factors¹⁴. However, our findings also reveal differences in the mechanisms controlling the inheritance of the two DNA-containing organelles. For example, neither low temperature nor $\Delta dpd1$ mutation alone were sufficient to markedly elevate the paternal transmission frequency of mitochondria (Fig. 3e). This observation stands in stark contrast to what was found previously for plastids, where low temperature and $\Delta dpd1$ mutation, on their own, caused a 150-fold and 120-fold increase in paternal transmission, respectively¹⁴. The differences between plastid and mitochondrial inheritance observed here are consistent with previous work showing that the inheritance of the two DNA-containing organelles involves distinct mechanisms. For example, in the seed plant cucumber, the mitochondrial genome is inherited paternally, while the chloroplast genome is inherited maternally³².

Chilling stress has been shown to impair the organelle exclusion mechanism in pollen mitosis I—a key barrier that prevents paternal plastid inheritance¹⁴. Interference with this mechanism leads to increased inclusion of plastids in the GC¹⁴. Our electron tomographic analyses also revealed a higher abundance of mitochondria in the GCs of chilling-stressed pollen than in those developing at 25 °C (Fig. 4b). However, it is worth noting that the exclusion mechanism appears to be inherently less effective for mitochondria than for plastids, as mitochondria are regularly observed in GCs and sperm cells of tobacco^{37,38}. In *Arabidopsis*, paternal mitochondria have been seen to enter the egg cell upon fertilization¹⁶. Interestingly, despite their regular presence in gametes, paternal mitochondrial transmission is considered to be

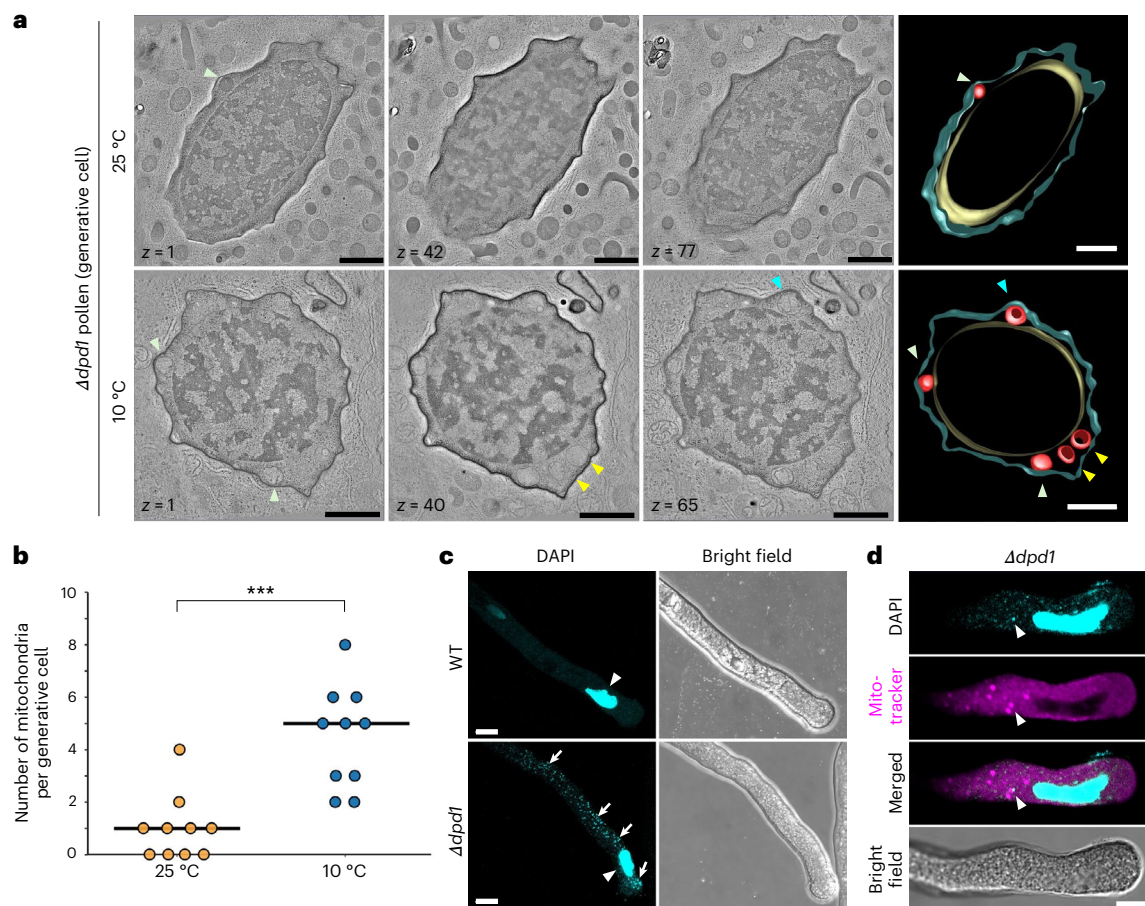


Fig. 4 | The impact of low temperature and *dpd1* mutation on mitochondria in male gametes. **a**, Identification of mitochondria in GCs (residing within the cytosol of the vegetative cell) of $\Delta dpd1$ pollen developed at 25 °C or 10 °C by electron tomography. Mitochondria were identified on the basis of their characteristic morphology. Serial sections were collected for tomographic analysis. The reconstructed tomograms were used to generate 3D models of the GC with enclosed mitochondria (right). Coloured arrowheads indicate individual mitochondria corresponding to those identified in the respective section layers. Scale bars, 1 μ m. For a schematic depiction of a pollen grain with vegetative and generative cell, see Extended Data Fig. 8. **b**, Number of mitochondria observed in the GC of pollen developed at 25 °C and 10 °C ($n = 10$ electron tomograms per group). Data points from individual electron tomograms are represented as circles. The difference in means between groups was tested using a *t*-test for unpaired samples (two-tailed and assuming unequal variances). The effect of low temperature on the abundance of GC mitochondria is significant (difference

in means = 3.5; CI95: 1.94–5.06; $t = 4.7678$; d.f. = 15.272; $P = 2.374 \times 10^{-4}$). $\alpha = 0.05$; *** $P < 0.001$. Horizontal lines, median. **c**, Confocal microscopy images of wild-type (top) and $\Delta dpd1$ (bottom) pollen tubes. In vitro-growing pollen tubes were stained with DAPI to visualize nuclear and cytoplasmic DNA. Z-stack images were captured and overlaid to provide an overview of the cytoplasmic DNA abundance in pollen tubes. DAPI signals from the generative cell nucleus were observed in both WT and $\Delta dpd1$ pollen tubes (arrowheads), while cytoplasmic DNA was observed exclusively in the $\Delta dpd1$ mutant (arrows). Scale bars, 10 μ m. Pollen staining followed by microscopy was performed once per genotype and a representative image is shown. **d**, Confocal microscopy images of a $\Delta dpd1$ pollen tube stained with DAPI and MitoTracker. The co-localization of DAPI and MitoTracker signals (arrowheads) suggests retention of mitochondrial DNA in the $\Delta dpd1$ mutant. Pollen staining followed by microscopy was performed once and a representative image is shown. Scale bar, 5 μ m.

very rare in these species^{22,29}. The prevailing view is that the mere presence of mitochondria in male gametes is insufficient to confer paternal transmission of the mitochondrial genome. Instead, mitochondrial inheritance is primarily governed by mechanisms that regulate mtDNA abundance in male gametes^{15,39–41}. Notably, a recent study identified an endonuclease, MTII, which is expressed in the fertilized egg and selectively degrades maternal mtDNA, thereby causing predominantly paternal mitochondrial inheritance in cucumber⁴². These findings highlight the critical role of mtDNA abundance in determining inheritance patterns and may explain why an increase in mitochondrial abundance in the GC alone is insufficient to substantially promote paternal transmission (Fig. 3d,e).

In *Arabidopsis*, DPDI has been shown to mediate the degradation of both plastid and mitochondrial DNA in maturing pollen²². In line with this, we observed the persistent presence of mtDNA in mature pollen of $\Delta dpd1$ tobacco plants (Fig. 4d). Intriguingly, other nucleases have also been implicated in mtDNA degradation during male gametogenesis²¹.

It therefore seems plausible that multiple nucleases act redundantly to mediate mtDNA degradation, and this redundancy may limit the impact of $\Delta dpd1$ mutation on mitochondrial inheritance (Fig. 3d,e). By contrast, in chilling-stressed $\Delta dpd1$ pollen, the enzymatic activity of all mtDNA-degrading nucleases may be reduced, thus enhancing the mtDNA retention phenotype associated with $\Delta dpd1$ mutation. Together with the chilling-induced increase in mitochondrial abundance, the synergistic effect of low temperature and the $\Delta dpd1$ allele probably leads to a higher abundance of DNA-containing mitochondria in the GC, accounting for the markedly elevated paternal transmission frequency (Fig. 3d,e).

Unexpectedly, we observed a relatively high paternal mitochondrial transmission frequency of 0.18% under standard conditions, when using greenhouse-grown wild-type plants as pollen donors (Fig. 3d). This frequency is substantially higher than the reported paternal plastid leakage frequency of 0.0016% (ref. 14). Moreover, the combined effect of low temperature and the $\Delta dpd1$ mutation causes an even

higher paternal leakage for mitochondria (7.34%) than for plastids (2–3%)¹⁴ under the same conditions. This could be explained by two factors. First, as discussed above, the organelle exclusion mechanism that effectively prevents paternal plastid transmission appears to be less efficient for mitochondria. Second, we screened for transmission of the paternal *nad9* gene, which complements the *nad9* defect in the maternal genome. If the paternally transmitted *nad9* confers a selective advantage already in the zygote, the paternal mitochondria could preferentially proliferate during embryogenesis. Given that, in the zygote, the paternal organelles are largely outnumbered by the maternal ones^{15,43}, a selective advantage of the transmitted paternal mitochondria could reduce the probability of their loss due to random segregation and sorting out. Previous attempts to detect paternal mitochondrial transmission in *Arabidopsis* and tobacco have been mostly unsuccessful^{22,24,44,45}, possibly due to the inability to specifically select for paternal mitochondria. Investigating the inheritance of neutral mitochondrial genome variants would offer valuable insight into the possible role of selection in the early maintenance of paternally transmitted mitochondria. Regardless, our findings challenge the widely held belief that paternal leakage is rare and suggest that paternal mitochondrial transmission occurs more frequently than previously assumed.

The occurrence of paternal mitochondrial leakage has profound implications for both plant physiology and evolutionary biology. Consistent with previously reported transgenerational effects of mitochondrial leakage⁴⁶, our findings show that the inherited paternal mitochondria affect progeny growth and development across generations (Extended Data Fig. 5). Importantly, by promoting paternal mitochondrial transmission, we successfully restored male fertility, offering a novel strategy to manipulate CMS, a trait of great relevance in crop breeding⁴⁷. CMS typically results from mitochondrial mutations, and the associated male-sterile phenotype cannot be reversed through crosses with wild-type plants due to maternal inheritance⁴⁸. Paternal mitochondrial transmission offers a novel possibility to restore fertility, which does not even require previous identification of the causal CMS loci or the corresponding nuclear fertility-restorer genes.

From an evolutionary perspective, an intriguing consequence of paternal leakage is the potential recombination between parental mitochondrial genomes upon mitochondrial fusion^{49–51}. Genome recombination can introduce genetic diversity and help counteract Muller's ratchet^{52,53} especially in taxa harbouring mitochondrial genomes with exceptionally high mutation rates (for example, *Silene*⁵⁴ and *Plantago*⁵⁵). Interestingly, paternal mitochondrial leakage occurs in *Silene vulgaris* at rates between 0.2% and 10% (ref. 56). Paternal leakage also has important implications for phylogenetic studies in that it can obscure lineage relationships and thus lead to misinterpretations in phylogenetic analyses^{57,58}.

In summary, we have shown that mitochondrial inheritance is shaped by both environmental and genetic factors. The targeted interference with key cellular processes during male gametogenesis can lead to the breakdown of maternal inheritance, thus opening up new possibilities to employ biparental inheritance as a powerful tool in crop breeding.

Methods

Plant material and growth conditions

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were used for all experiments. The mitochondrial mutant *Δnad9* (*Δnad9-29* line²⁹) was used as the maternal parent in all crossing experiments. The *Δnad9* mutant is homochondriomic, that is, lacks any residual copies of the wild-type mitochondrial genome²⁹. Transplastomic plants (harbouring *gfp* and *aadA* expression cassettes in their plastid genome¹⁴) with either a *dpd1* knockout (*Δdpd1*) or WT nuclear background were used as paternal parents. The *DPD1* exonuclease is encoded by the *DPD1S* and *DPD1T* loci in the tobacco nuclear genome. The *Δdpd1* mutant

used in this study is a doubly homozygous derivative of the previously generated mutant¹⁴, and is homozygous for the $\Delta 1/+1$ allele in *DPD1S* and homozygous for a 1.8 kb deletion in *DPD1T*. In the context of genotyping, detection of the *Δdpd1* deletion always refers to the 1.8 kb deletion allele in *DPD1T*.

Seeds were germinated on agar-solidified Murashige and Skoog (MS) medium⁵⁹ (0.68% w/v) supplemented with 3% (w/v) sucrose, and the pH adjusted to 5.8. To monitor early plant development, seedlings were transferred to Magenta boxes and cultivated under a 16 h light (25 °C, 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 8 h dark (20 °C) cycle. For continued vegetative and reproductive growth, plants were transferred to soil and grown in a greenhouse under standard conditions (~300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light intensity) with a 16 h light/8 h dark photoperiod (day/night temperatures: -25 °C/-20 °C).

Chilling stress experiments were performed in controlled environment chambers maintained at 10 °C (~150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light intensity, with a 16 h light/8 h dark photoperiod). Paternal plants were first cultivated under standard greenhouse conditions and then transferred to the 10 °C chamber at the onset of flowering. To generate chilling-stressed pollen, the plants were kept at 10 °C throughout the flowering period.

Crosses and screens for paternal mitochondrial transmission

Pollen from *Δdpd1* or wild-type plants grown in standard greenhouse conditions or at 10 °C was collected and used for manual pollination. Greenhouse-grown *Δnad9* plants were used as the maternal parent. Parental plants were raised and subsequently crossed in two independent experiments: Experiment 1, a small-scale exploratory experiment that included two sets of crosses (*Δnad9* × WT_{25 °C} and *Δnad9* × *Δdpd1*_{10 °C}), and Experiment 2, a large-scale experiment designed to (1) replicate the findings of Experiment 1 for the two crosses performed and (2) explore the effects of individual factors by including two additional sets of crosses (*Δnad9* × WT_{10 °C} and *Δnad9* × *Δdpd1*_{25 °C}). Seeds resulting from these four sets of crosses were harvested and screened for paternal mitochondrial inheritance. To prevent self-pollination, all flowers used for crosses were emasculated, including the flowers of the *Δnad9* mutant (to make sure that, even if there was low-level leakiness of the CMS trait, there is no risk of self-pollination).

To assess paternal mitochondrial transmission, the progenies were germinated on MS medium solidified with 0.68% (w/v) agar and supplemented with 3% (w/v) sucrose (pH 5.8), at a density of 100 seedlings per 120 mm square plate (10 seeds per row, 10 rows per plate). Plates were incubated in controlled environment chambers under a 16 h light (25 °C, 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 8 h dark (20 °C) cycle. Germination and early seedling development were monitored daily by visual inspection. Paternal leakage was detected using two approaches: phenotype-based screening of the seedlings (by evaluating germination and growth rates) and PCR-based molecular screening (by randomly selecting seedlings for RNA extraction followed by RT-PCR analysis).

RNA extraction and RT-PCR for paternal mitochondrial transmission screens

Leaf and root tissues were harvested from WT, *Δnad9* and PMt plants for RNA extraction using the NucleoSpin RNA Plant kit (Macherey-Nagel), following manufacturer protocol except that the DNase digestion step was deliberately omitted. Reverse transcription was carried out using SuperScript III reverse transcriptase (Invitrogen) with oligonucleotides oJF113 and oJF1144 as primers (Extended Data Table 1), which bind to the 3' UTRs of *nad9* and *atp9*, respectively. The synthesized cDNA was purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and used as template for PCR with primers P1 (binding to the *nad9* CDS), oJF1145 (binding to the *atp9* 5' UTR) and P2 (an adapter-specific primer binding to the 5' terminal sequence of both

oligonucleotides oJF1113 and oJF1144, thus excluding amplification of residual DNA present in the RNA preparations; Extended Data Table 1). The expected PCR product sizes were 465 bp for *nad9* and 568 bp for *atp9*. PCR products were resolved and visualized via electrophoresis in 2% agarose gels. Recovered PCR products were analysed by Sanger sequencing to determine the RNA editing pattern of the *nad9* cDNA. PCR reactions were performed using GoTaq DNA polymerase (Promega) according to manufacturer instructions. All oligonucleotides used in this work were generated by chemical synthesis and acquired from a commercial provider (Eurofins).

Measurements of *nad9* gene copy numbers and relative transcript abundances by qPCR and RT-qPCR

Wild-type, $\Delta nad9$ and PMt samples derived from flash-frozen whole seedlings were divided for extraction (in parallel) of genomic DNA and RNA. Total genomic DNA was isolated following a cetyltrimethylammonium bromide (CTAB)-based protocol⁶⁰. Total RNA was isolated using the NucleoSpin RNA Plant kit (Macherey-Nagel) following manufacturer instructions, including the recommended DNase treatment step. Synthesis of cDNA was carried out with the SuperScript III Reverse Transcriptase kit (Invitrogen) following manufacturer instructions, using 250 ng total RNA and 2.5 μ M random hexamer primer in a reaction volume of 40 μ l. Negative control reactions (from which the reverse transcriptase was omitted) were done in parallel and included in downstream measurements. All measurements were carried out in technical triplicates using the LightCycler 480 Real-Time PCR System and LightCycler SYBR Green reaction mixtures (Roche) in reaction volumes of 5 μ l. Purified genomic DNA (5 ng) or 1/200 of the cDNA reaction was used as template. All oligonucleotide primers target regions within single exons so that amplicons derived from genomic DNA or cDNA templates are of identical size. Transcript levels and relative gene copy numbers of *nad9* were calculated relative to those of the nuclear reference genes *L25* (ref. 61) or *TUBB2*, using an efficiency-adjusted ΔC_q method⁶².

Genotyping experiments

DNA was isolated from leaf tissues of WT, $\Delta nad9$ and PMt plants, and used as template for PCR reactions. Primer pairs (1) P3–P4, (2) oJF883–oJF884 and (3) oEG327–oEG332 (Extended Data Table 1) were employed to detect $\Delta nad9$ mitochondrial genomes, the mitochondrial 18S rRNA gene *rrn18* (as an internal control) and the $\Delta dpd1$ allele in the nuclear genome, respectively. PCR reactions were performed using DreamTaq DNA polymerase (Thermo Fisher) according to manufacturer instructions.

Pollen viability assay

To assess pollen viability, anthers were detached from WT and PMt flowers at anthesis, and collected in a 5 ml Eppendorf tube. Pollen grains were collected by vortexing with pollen harvesting buffer (100 mM Na_3PO_4 (pH 7.0) and 1 mM Na_2EDTA (pH 8.0)), followed by centrifugation at $1,000 \times g$ for 5 min. The pollen pellet was then resuspended and stained in pollen viability solution (100 mM Na_3PO_4 (pH 7.0), 1 mM Na_2EDTA (pH 8.0), 10 μ M PI (Abcam) and 20 μ g ml⁻¹ FDA (Sigma)) at room temperature for 30 min. The stained samples were imaged by confocal laser-scanning microscopy (TCS SP8, Leica). FDA was excited with a 488 nm argon laser and the emission signal was detected at 500–550 nm. PI was excited with a 561 nm argon laser and its emission signal was detected at 600–650 nm.

Electron tomography

Pollen samples for electron microscopic analysis were processed through high-pressure freezing, freeze substitution, resin embedding and ultramicrotomy as previously described⁶³. Pollen was collected from $\Delta dpd1$ plants grown under standard (22–25 °C) and low-temperature (10 °C) conditions. The pollen samples were placed

in 3 mm planchettes for high-pressure freezing, followed by freeze substitution at –80 °C for 24 h, with gradual warming to room temperature over 36 h. After rinsing three times with acetone to remove any excess osmium tetroxide, pollen samples were subsequently embedded in Embed-812 resin (Electron Microscopy Sciences, 14120). Thin sections (90 nm thick) were prepared from sample blocks of each stage and examined with a Hitachi 7400 TEM operated at 80 kV. Serial thick sections (300 nm thick) were collected for tomography. Tilt series were collected from 60° to –60° (1.5° intervals) with a 200 kV Tecnai F20 intermediate voltage electron microscope (FEI). For dual-axis tomography analysis, tilt series around two orthogonal axes were acquired from each section using the microscope's tomography programme. Tomograms were reconstructed as described⁶⁴. Tomograms were collected using TOMOGRAPHY v.4.0.3.4538 and reconstructed using Etomo v.5.0.0.10. Tomogram segmentation was performed using IMOD v.5.0.2. For three-dimensional (3D) modelling of generative cells and their enclosed mitochondria, contours were manually traced using the '3dmod' software package. The generative cell membrane, the nucleus and the mitochondria were outlined in every 8th slice, with manual corrections applied as needed. Final 3D models were generated using the 'imodmesh' command to mesh the corrected contours.

DAPI and MitoTracker staining and confocal laser-scanning microscopy

To visualize mitochondrial DNA, DAPI and MitoTracker staining was performed with in vitro-germinating pollen tubes. Mature pollen grains were collected from either WT or $\Delta dpd1$ plants and suspended in pollen germination buffer (1.6 mM boric acid, 3.0 mM calcium nitrate, 1.0 mM potassium nitrate, 0.8 mM magnesium sulfate, 10 % sucrose, pH 7.4), followed by an incubation at room temperature for 2–3 h to allow pollen tube germination. Pollen tubes were stained with 4,6-diamidino-2-phenylindole (DAPI) solution (100 mM Na_3PO_4 (pH 7.0), 0.1% (v/v) Triton X-100, 1 mM Na_2EDTA (pH 8.0) and 1 μ g ml⁻¹ DAPI) and 100 nM MitoTracker Red CMXRos (Invitrogen, M7512) for 30 min at room temperature. DAPI and MitoTracker fluorescence signals were imaged by confocal laser-scanning microscopy (TCS SP8, Leica), using (1) a 405 nm laser diode for excitation and a 430–495 nm filter for detection of DAPI fluorescence emission, and (2) a 561 nm laser for excitation and 585–615 nm for detection of MitoTracker fluorescence emission.

Modelling and statistics

All statistical tests and assembly of statistical models were done using R v.4.3.3 (<https://www.R-project.org/>). The detection of the *nad9* transcript in pools and individual samples by RT-PCR was used as a proxy for the transmission of paternal mitochondria. Modelling for analysis of Experiment 2 was performed by considering individual seedlings as the unit of study based on the following assumptions: (1) paternal transmission of mitochondria is the result of a binomially distributed process (that is, a seedling being *nad9*-positive is one out of two possible outcomes and occurs at a set probability that defines the binomial process), (2) transmission events observed in individual seedlings are independent of each other and (3) for all samples derived from pooled seedlings, the detected *nad9* transcripts originated from a single seedling in the pool. Paternal mitochondrial transmission was then calculated as the percentage of *nad9*-positive seedlings.

The variation in paternal mitochondrial transmission in response to different combinations of nuclear genotype ($\Delta dpd1$ or wild-type) and temperature during pollen development (25/20 °C or 10 °C) was analysed through a binomial model constructed with the maximum likelihood method in R. The model was assembled using the glm() function, using 'log' as the link function. Parameters in the model represent the difference between a given group (a combination of genotype and temperature) and the control condition (wild type at 25 °C). Hypothesis testing of the differences between these groups was performed using the multcomp v.1.4-25 package. Null hypotheses were stated using the

glht() function and tested simultaneously with z-tests using the cftest() function. To account for multiple comparisons, *P* values were corrected through the Holm method. Simultaneous 95% confidence intervals were obtained using the confint() function. The following packages were used to provide adequate code functionality: ggplot2 v.3.5.1, ggsignif v.0.6.4, rstudioapi v.0.16.0, MASS v.7.3-60.0.1 and dplyr v.1.1.4.

A corrected estimate of the paternal transmission frequency of mitochondria in Experiment 2 (that accounts for multiple transmission events in a single pool) was obtained for the *Δdpd1* 10 °C group using Monte Carlo simulations in R. For simplicity and only for this calculation, we considered all *nad9*-positive samples obtained experimentally for this group (7 from individual seedlings and 20 from pools) as pool derived and assumed that the frequency of paternal mitochondrial inheritance (that is, the number of individual *nad9*-positive seedlings among the total) follows a binomial distribution. After stipulating a set of possible transmission frequencies, a fixed pool size (5 seedlings) and a fixed number of samples (74 pools), we simulated the random allocation of *nad9*-positive seedlings to pools (10,000 simulations). For each given transmission frequency, we calculated the average of *nad9*-positive pools (samples) obtained across all simulations. The corrected transmission frequency estimate (8.7%) is the value that produced an average of *nad9*-positive samples equal to the experimental value (27).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Data supporting the findings of this work are available within the paper, its extended data figures and tables, and Supplementary Videos. NCBI entry [NC_006581.1](#) was used as the reference mitochondrial genome for tobacco. Source data are provided with this paper.

Code availability

The R code used for statistical tests and the model is available via Zenodo at <https://doi.org/10.5281/zenodo.18684523> (ref. 65).

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Author contributions

K.P.C. and R.B. designed the research. E.G.-D., Z.L., W.W., D.K. and K.P.C. performed the experiments. E.G.-D., Z.L., J.F., D.K., W.W., L.J., K.P.C. and R.B. contributed to data analysis and interpretation. K.P.C. wrote the paper draft, R.B. revised it, and E.G.-D., J.F. and Z.L. provided additional input.

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Competing interests

The authors declare no competing interests.

Additional information

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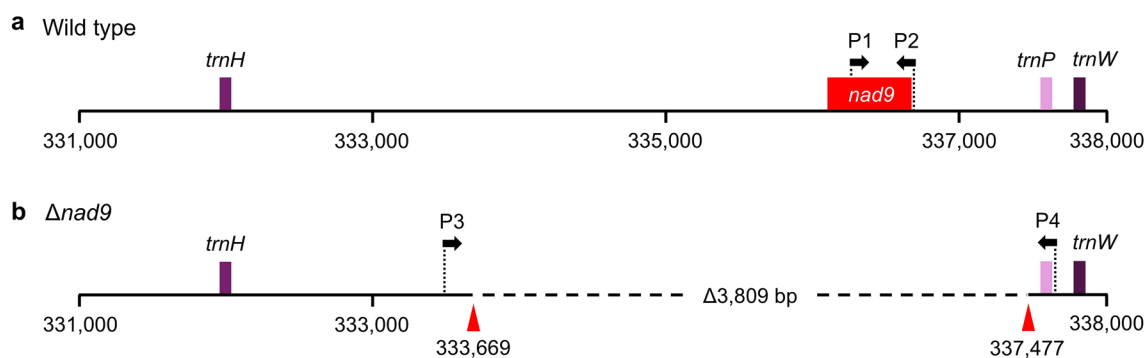
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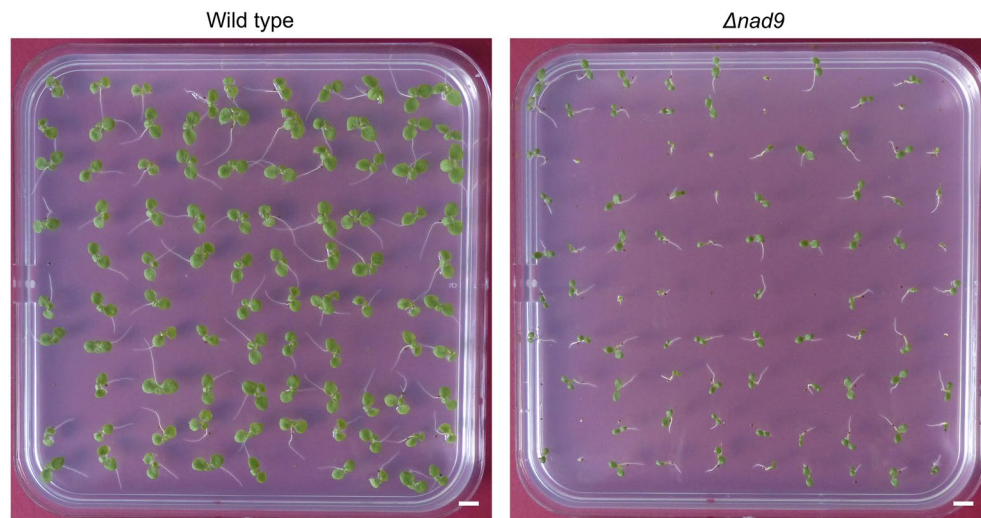
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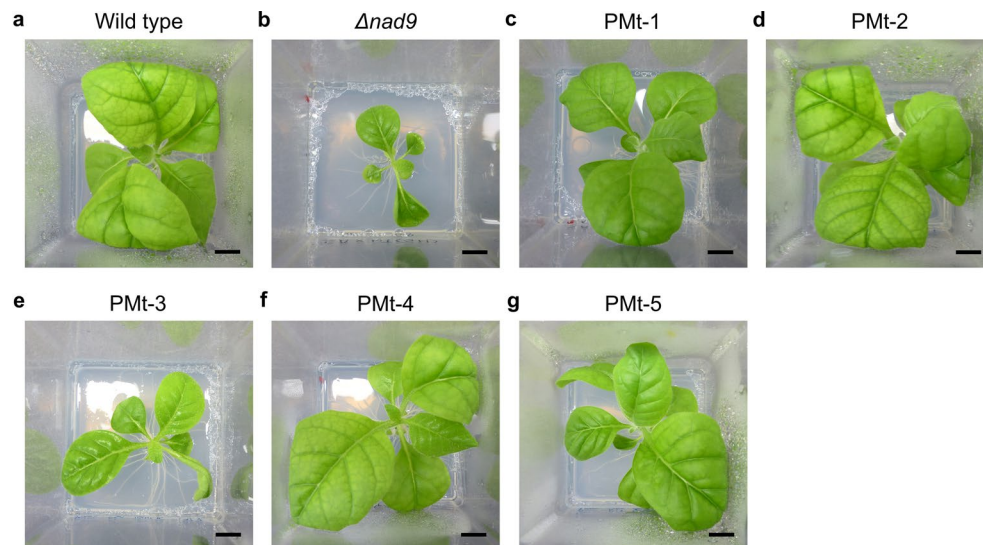
Extended Data Fig. 1 | Schematic representation of the wild-type and the $\Delta nad9$ mitochondrial genomes. The region from nucleotide position 331,000 to 338,000 (based on the *Nicotiana tabacum* reference genome; [NC_006581](#)) is shown. **a**, Map of the *nad9* region in the wild-type mitochondrial genome. The *nad9* coding sequence is located between nucleotide positions 336,111 and 336,683. The *nad9*-specific primers P1 and P2 were used to detect *nad9*

transcripts. **b**, Map of the corresponding genomic region in the mitochondrial genome of the $\Delta nad9$ mutant. A 3,809-nucleotide segment of the mitochondrial genome has been deleted (indicated by red arrowheads), leading to the complete loss of the *nad9* gene. To detect the deletion, primers P3 and P4 were used that result in amplification of a short 325 bp fragment.



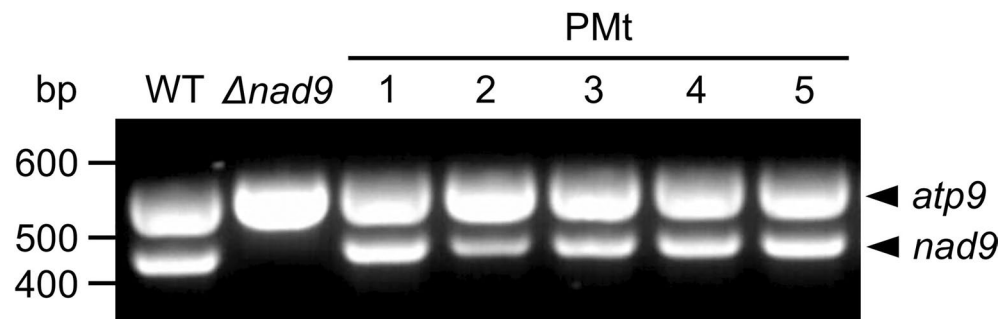
Extended Data Fig. 2 | The $\Delta nad9$ mutant exhibits delayed seed germination and retarded seedling growth. Surface-sterilized wild-type and $\Delta nad9$ seeds were sown on MS medium supplemented with 3% sucrose, stratified for two

days, and then transferred to a growth chamber. Each plate contains 100 seeds. Pictures of wild-type and $\Delta nad9$ seedlings were taken on day 14 after sowing. Scale bars, 1 cm.

**Extended Data Fig. 3 | Vegetative growth phenotypes of PMt plants.**

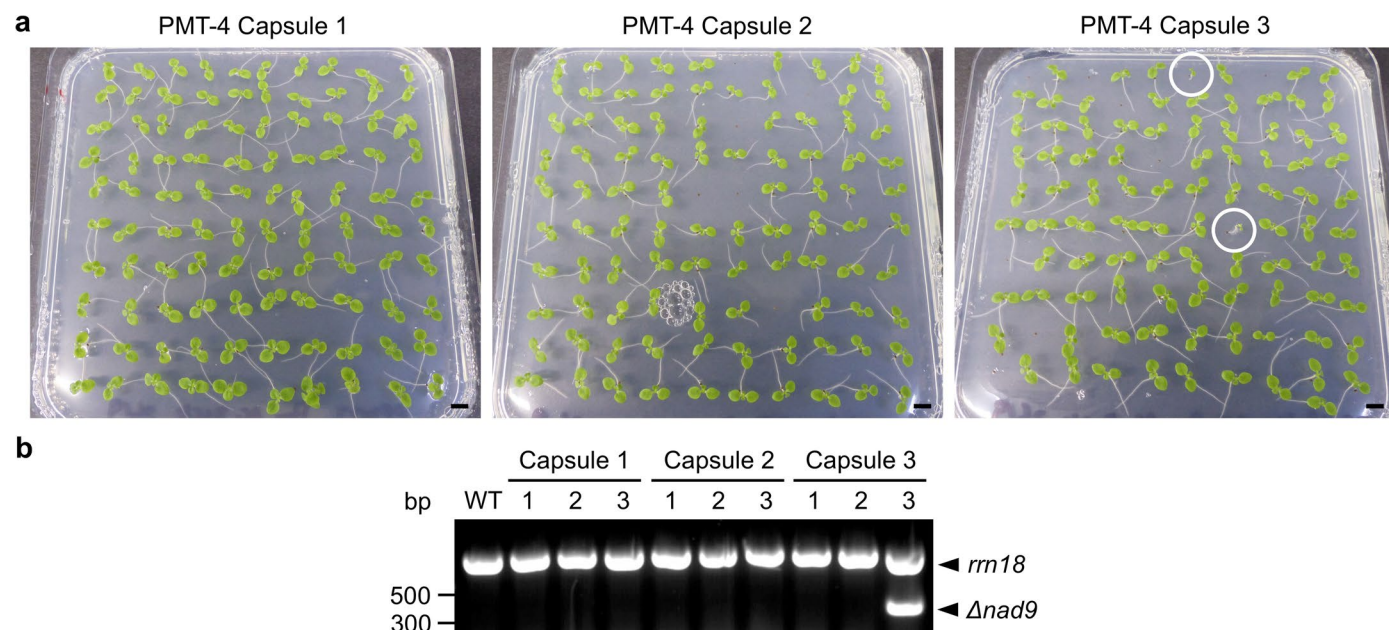
a–g, Seedlings were transferred from germination plates to Magenta boxes. Pictures were captured on day 40 after sowing. Note the stunted growth exhibited by the $\Delta nad9$ mutant (**b**). While PMt-1, PMt-2, PMt-4, and PMt-5 (**c,d,f,g**)

display a growth phenotype similar to wild-type plants, the PMt-3 plant (**e**) shows a $\Delta nad9$ -like phenotype, consistent with the absence of *nad9* transcripts from its leaf tissues (see Fig. 1c). Scale bars, 1 cm.



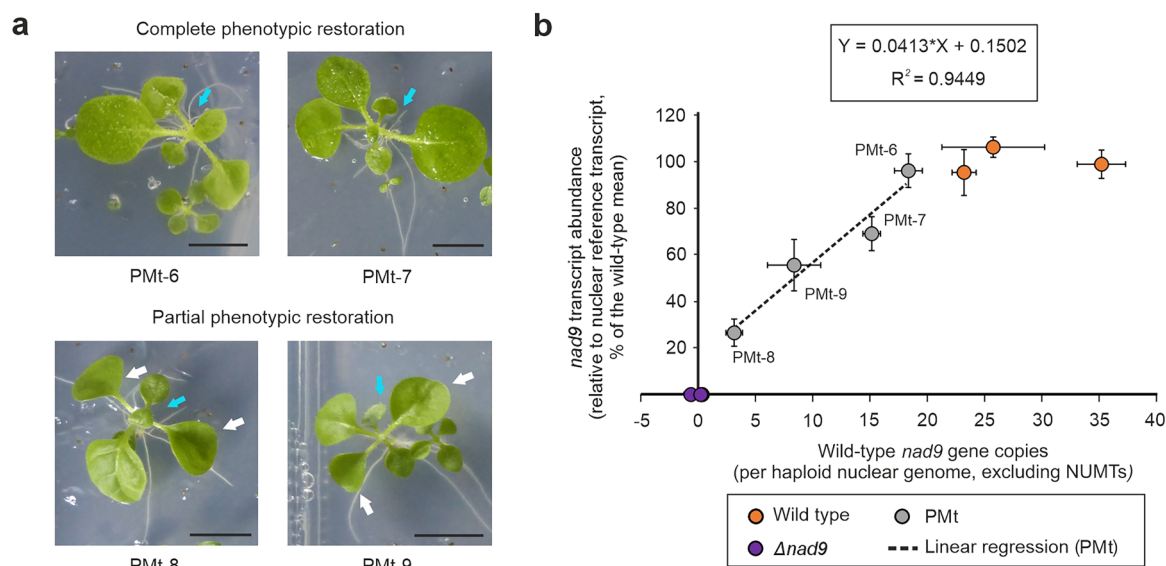
Extended Data Fig. 4 | Presence of *nad9* transcripts in root tissues of PMt plants. RNA was extracted from the root tissues of wild-type, $\Delta nad9$, and PMt plants. *atp9* and *nad9* transcripts were co-amplified using gene-specific primers

(see Methods), with *atp9* serving as an internal control. *nad9* transcripts are detected in all samples except for the $\Delta nad9$ mutant. RT-PCR followed by gel electrophoresis were performed once per sample.



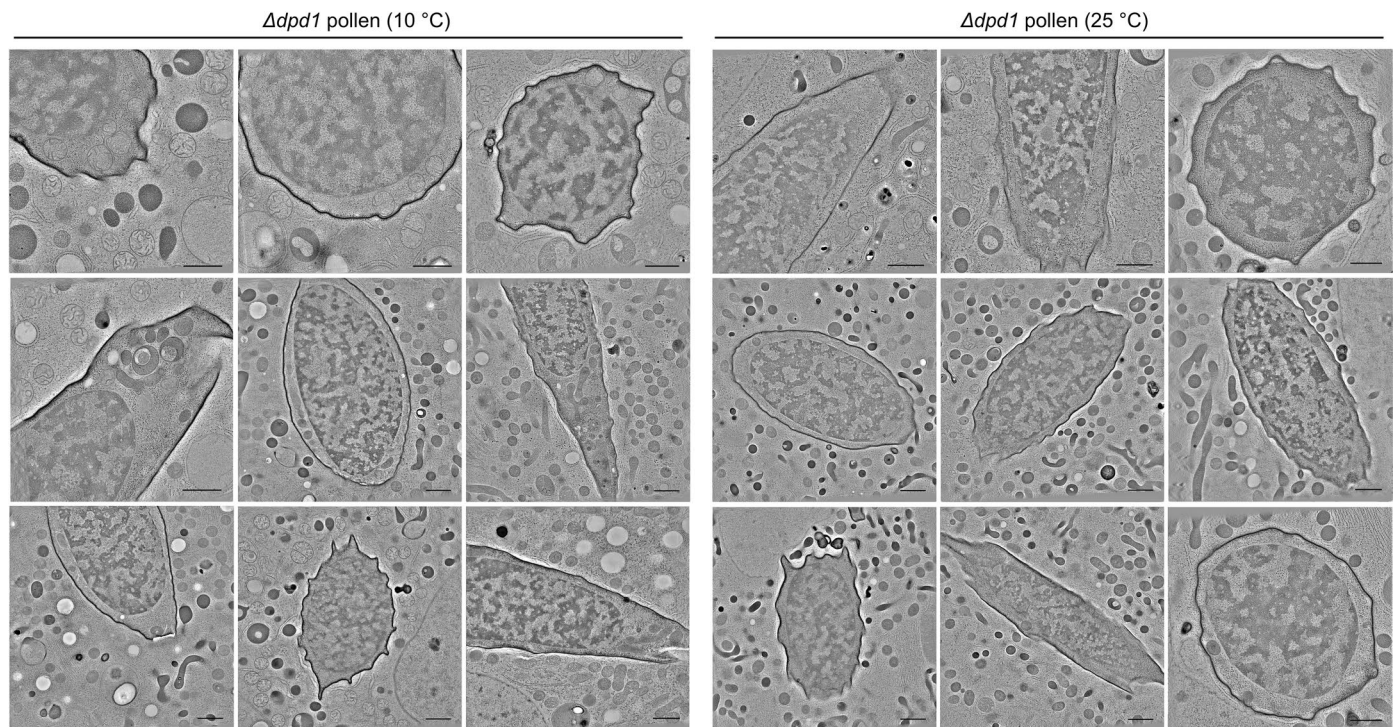
Extended Data Fig. 5 | Transmission of paternal mitochondria into the next generation. **a**, F_2 seedlings derived from individual capsules of the self-pollinated PMt-4 plant growing in MS medium. Pictures were taken on day 14 after sowing. While the majority of seedlings exhibit a WT-like germination and growth rate, some seedlings of capsule 3 show delayed germination and growth (indicated by white circles), suggesting the persistence of Δ *nad9* mitochondrial genomes in the progeny of this capsule. Scale bars, 1 cm. **b**, PCR amplification of Δ *nad9* mitochondrial genomes in the F_2 generation. Five seedlings were collected at

random and grouped into pools, with three pools (1–3) generated per seed capsule. DNA was extracted from each pool, and PCR was performed using primers designed to amplify the *nad9* deletion locus (see Extended Data Fig. 1B). The *nad9* deletion locus was detected in Pool 3 of Capsule 3, indicating that both paternal and maternal mitochondria are retained in the germ cells of the PMt-4 plant and can be transmitted to the next generation. Amplification of the mitochondrial genomic locus encoding the 18S rRNA (*rrn18*) served as an internal control.



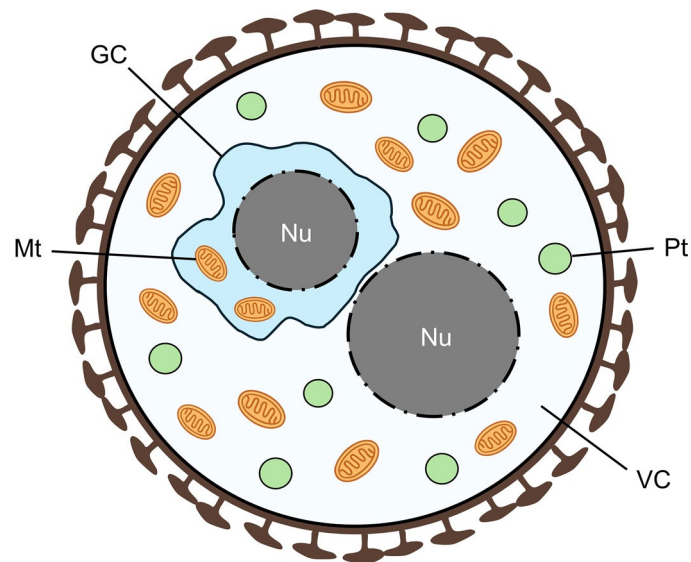
Extended Data Fig. 6 | Correlation between phenotypic restoration, *nad9* copy number and transcript abundance in PMt plants. a, Phenotypes of PMt seedlings (cyan arrows) 28 days after sowing. Plants with partial phenotypic restoration display features reminiscent of the Δ *nad9* mutant, such as the typical rolled-up leaf margins (white arrows). Scale bars, 1 cm. **b**, Relationship between relative *nad9* transcript abundance and the number of wild-type *nad9* gene copies (of paternal origin in PMt plants), as quantified by RT-qPCR and qPCR, respectively. Each circle corresponds to measurements from a whole seedling (biological replicate), and represents mean values from three independent quantification experiments, each using a different combination of *nad9* amplicon and reference gene: (i) *nad9* amplicon A / *L25*, (ii) *nad9* amplicon B

/ *L25*, and (iii) *nad9* amplicon A / *TUBB2* (Supplementary Table 1). Within one quantification experiment, all measurements were done in triplicate in the same 384-well PCR plate. *nad9* transcript abundance was determined relative to the transcript abundance of a nuclear reference gene, and in parallel, preliminary *nad9* copy numbers (that is, including *nad9*-containing NUMTs) were calculated in relation to gene copies of the same reference gene. NUMTs were quantified in the Δ *nad9* mutants [14.5 ± 0.4 ; mean $\pm$ standard error of the mean (SEM) across experiments] and subtracted from the preliminary values to yield the wild-type mitochondrial *nad9* copy numbers per haploid nuclear genome. Error bars: $\pm$ SEM of relative transcript abundance (vertical) or gene copy number (horizontal) across experiments.



Extended Data Fig. 7 | Electron tomography of generative cells of $\Delta dpd1$ pollen developed at 10 °C or 25 °C. Sections of pollen samples were collected for tomographic analysis. Tomograms were reconstructed from tilt series

covering a significant volume of the GCs to quantify mitochondrial abundance. Pollen harvest, followed by sample preparation and electron microscopy, was performed once. Scale bars, 1 μ m.



Extended Data Fig. 8 | Schematic depiction of a pollen grain showing the generative cell (GC) embedded within the vegetative cell (VC). The nuclei (Nu) of the two cells are indicated. Plastids (Pt) and mitochondria (Mt) are largely excluded from the GC, but two mitochondria are shown here within the GC to illustrate the paternal leakage reported in this work (cf. Figure 4a).

Extended Data Table 1 | Synthetic oligonucleotides used in this study

Primer	Sequence (5' to 3')	Purpose
P1	GAGTTGATTATCCCTCTCGA	Detection of <i>nad9</i> transcripts
P2	ATGCCGGACATCTTAAGGAG	Adapter sequence binding to the 5' terminal sequence of both oligonucleotides oJF1113 and oJF1144
P3	GAATGAATATCGGACCCTACT	Genotyping of <i>nad9</i> deletion
P4	AATGGATCCCTCGTTACTGC	Genotyping of <i>nad9</i> deletion
oJF883	GCTAAAGGTAGCTTGTCTCG	Amplification of <i>rm18</i>
oJF884	CACTCATCGTTTACGGCATG	Amplification of <i>rm18</i>
oEG327	GAAGCATTAGCTCCAAGGTTC	Genotyping of <i>dpd1</i> deletion
oEG332	GCAAAGATCAATGCCAAGGC	Genotyping of <i>dpd1</i> deletion
oJF1113	ATGCCGGACATCTTAAGGAGAATAGCATTTCTATTGATTGTCC	For <i>nad9</i> cDNA synthesis
oJF1144	ATGCCGGACATCTTAAGGAGAAATGGGACCGCGAAGACAAC	For <i>atp9</i> cDNA synthesis
oJF1145	CCTATGCTTTGCATGAACATCTC	Detection of <i>atp9</i> transcripts
oJF1194	CATCAATCATCCGGATCTACGC	qPCR amplification of <i>nad9</i> (amplicon A)
oJF1195	CTTGGGTCATCTCAATGGGTTC	qPCR amplification of <i>nad9</i> (amplicon A)
oJF1383	GATGATCCAGAGAAACGTGTGG	qPCR amplification of <i>nad9</i> (amplicon B)
oJF1384	CCTATTGATTTGTCCCCTGGAC	qPCR amplification of <i>nad9</i> (amplicon B)
oEG566	GTTGCCAAGGCTGTCAAGTCAG	qPCR amplification of <i>L25</i>
oEG567	CCTTCCAGGTGCACTAATACGAG	qPCR amplification of <i>L25</i>
oJF1385	ATCAGCCACAATGTCTGGTGTC	qPCR amplification of <i>TUBB2</i>
oJF1386	AAGCTCTGTACTGCTGAGAACC	qPCR amplification of <i>TUBB2</i>

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Data collection	Tomograms were collected using TOMOGRAPHY v. 4.0.3.4538, and reconstructed by Etomo v 5.0.0 10. Tomogram segmentation was done by IMOD v 5.0.2.
Data analysis	Statistical tests and model assembly were performed in R v 4.3.3, using the following packages: ggplot2 v. 3.5.1, ggsignif v. 0.6.4, rstudioapi v. 0.16.0, MASS v. 7.3-60.0.1, dplyr v. 1.1.4, multcomp v.1.4-25. The R code is deposited under https://github.com/egonzalezduran/modelsmitoinheritance

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Data supporting the findings of this work are available within the paper, its Extended Data Figures and Tables, and in Supplementary Videos. Source data are provided with this paper. NCBI entry NC_006581.1 was used as the reference mitochondrial genome for tobacco.

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Life sciences study design

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Sample size	Sample sizes for the phenotype-based screen was meant to detect transmission of paternal mitochondria at levels comparable to the plastid paternal transmission frequencies described for $\Delta\text{d}p\text{d}1_10^\circ\text{C}$ in Chung et al. 2023, Nat. Plants 9, 68–80. In the PCR-based screens, Experiment 1 suggested there was a large difference in paternal transmission frequencies of mitochondria between the $\Delta\text{d}p\text{d}1_10^\circ\text{C}$ and the $\text{WT_}25^\circ\text{C}$ groups. As a consequence, in Experiment 2 screen we focused our increased sampling depth on the $\text{WT_}25^\circ\text{C}$ group: this was done to ensure that, in the case we did not find any inheritance events in this group, we would still be able to provide an informative estimate for the paternal mitochondrial transmission frequency of <i>N. tabacum</i> at standard growth conditions ($<0.33\%$ at 1,100 seedlings with 95% confidence, as reported in the output of a two-sided binomial test in R, using the <code>binom.test()</code> function for zero successes in 1,100 trials): in the experiment, this sampling depth led to the detection of two events in Experiment 2. For the remaining groups, sample sizes were chosen after considering possible effect sizes by calculations of Cohen's <i>w</i> (Cohen, J. Statistical Power Analysis for the Behavioral Sciences, Routledge, 2013). The selected sample size ($n \geq 350$) was calculated to be sufficient to detect differences from $\text{WT_}25^\circ\text{C}$ with effect size $w = 0.122$ at power = 0.8, with $\text{df} = 1$ and $\alpha = 0.0166$ (i.e., $0.05/3$, a Bonferroni-corrected value accounting for the three pair-wise comparisons between $\text{WT_}25^\circ\text{C}$ and each of the other groups): this was calculated using the <code>pwr.chisq.test()</code> function in R's <code>pwr</code> package v.1.3-0; https://cran.r-project.org/web/packages/pwr/). These sample sizes are appropriate to find effects that are smaller than those found between $\text{WT_}25^\circ\text{C}$ and the other groups in the paternal plastid inheritance study ($w = 0.49 - 7.92$). <i>w</i> values were calculated in R through the <code>ES.w1()</code> function in the <code>pwr</code> package.
Data exclusions	No data were excluded
Replication	Two independent crossing and screening experiments (1 and 2) were conducted: Experiment 2 successfully replicated the findings of Experiment 1. Quantification of transcript levels and gene copy number were done simultaneously in one 384-well qPCR plate. All measurements were realized in technical triplicates and with at least 3 seedlings (biological replicates) per group. Every quantification experiment included, in the same plate, dilution series for construction of standard curves and calculation of primer efficiencies. Quantification was replicated successfully in three independent experiments, using different amplicons for <i>nad9</i> and/or different reference genes.

Randomization

Plants were assigned randomly to treatment groups. For crosses, Δ ad9 plants were used as maternal parents, where each plant received pollen from multiple paternal parents from a single genotype-temperature combination: the pairing between pollen donor groups and each maternal parent was done at random. For pooling and RNA extraction, in Experiment 1 seedlings were collected at random. In Experiment 2, seedlings with early germination (before day 13 after sowing) were classified as exceptional and processed individually (i.e., without pooling), whereas the remaining seedlings were collected and allocated to pools at random. For electron tomography studies, pollen was randomly harvested from individual plants and placed in planchettes for high-pressure freezing to prepare multiple sample blocks. Thin sections were prepared from random sample blocks and observed.

Blinding

Data collection and analysis was not blinded. There were no measurements or treatments that could significantly benefit from a blind design.

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☐	☒ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Plants

Seed stocks

Tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were used for all experiments. The mitochondrial mutant Δ ad9 (Δ ad9-29 line) was generated by our group and was described in Forner et al. (2023), Nat. Plants 9, 1818–1831. (<https://doi.org/10.1038/s41477-023-01538-2>). Transplastomic plants with either a *dpd1* knockout (Δ *dpd1*) or wild-type (WT) nuclear background were generated by our group and described in Chung et al. (2023), Nat. Plants 9, 68–80. (<https://doi.org/10.1038/s41477-022-01323-7>).

Novel plant genotypes

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