

Fluorescent protein identity influences GESEN1 susceptibility and reveals ectopic expression from a sperm cell-specific promoter in Arabidopsis pollen

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Abstract

GESENI (GEne Silencing based on ENcoded protein's Intracellular localization) selectively silences transgenes encoding cytoplasmic proteins in *Arabidopsis thaliana* (Arabidopsis) sperm cells. To investigate the molecular basis of this phenomenon, we examined whether fluorescent protein (FP) identity influences GESENI susceptibility. Venus and Clover were completely silenced, whereas mRuby3, tdTomato, and plant codon-optimized mTFP1 escaped GESENI and were expressed in sperm cells. Unexpectedly, tdTomato and mRuby3 fluorescence was also detected in pollen vegetative cells despite expression being driven by the sperm cell-specific *HTR10* promoter. These findings establish FP identity as a key determinant of GESENI susceptibility and reveal an unexpected layer of regulation underlying sperm cell-specific transgene expression in Arabidopsis pollen.

Key Message

Fluorescent protein identity influences GESENI susceptibility, revealing an unexpected link between encoded protein properties and cell type-specific transgene regulation.

INTRODUCTION

Fluorescent proteins (FPs) have become indispensable tools for visualizing cellular structures and monitoring biological processes in living cells. As genetically encoded and minimally invasive fluorescent probes, they enable direct and specific observation of dynamic cellular events while providing quantitative information on gene expression, protein localization, and intracellular regulation at cellular and subcellular levels (Shimomura 2005; Shin et al. 2022). In *Arabidopsis thaliana* (Arabidopsis), FP-based imaging has greatly advanced our understanding of sperm cell morphology and molecular functions by enabling visualization of diverse subcellular structures. For example, FP-based imaging has enabled visualization of diverse subcellular structures, including the nucleus (*HTR10-RFP*) and centromeres (*HTR12-GFP*) (Ingouff et al. 2007), the plasma membrane (*TET9-GFP*) (Sprunck et al. 2012), the endomembrane system (*VHA-E1-GFP*), mitochondria (*mitochondrial F1-ATPase δ -subunit-GFP*) (Wudick et al. 2014), and microtubules (*GFP-TUA6*) (Park et al. 2014), among many others. Building on these advances, FP technology has been further expanded to genetically encoded fluorescent biosensors, in which fluorescent reporters are coupled with target-responsive sensing domains, enabling real-time visualization of intracellular signaling, metabolite dynamics, and other physiological processes in living cells (Frei et al. 2024). However, despite the broad utility of FP-based molecular tools, their application in Arabidopsis sperm cells has remained limited because GESENI (GEne Silencing based on ENcoded Protein's Intracellular localization) interferes with the expression and proper localization of FP-tagged proteins (Ohnishi and Kawashima 2023).

When a gene encoding a Venus-tagged cytoplasmic protein driven by an Arabidopsis sperm cell-specific promoter is introduced into plants, the expected fluorescent signal is absent in sperm cells (Fig. 1A). Consistent with this lack of fluorescence, transcripts derived from the introduced transgene are

undetectable, indicating transcriptional and/or post-transcriptional silencing. This phenomenon, designated GESENI (GEne Silencing based on ENcoded Protein's Intracellular localization), has been consistently observed across multiple independent constructs and transformants (Fig. 1B) (Ohnishi and Kawashima 2023; Ishida et al. 2026). Unlike canonical transgene silencing, which is typically associated with multicopy transgene integration and can also affect homologous endogenous genes (Napoli et al. 1990; Van Der Krol et al. 1990; Schubert et al. 2004; Zhang et al. 2015), GESENI occurs even in single-locus first-generation (T_1) transformants and selectively silences Venus-tagged transgenes without affecting their endogenous counterparts (Ohnishi and Kawashima 2023). Remarkably, when nuclear-localized (*H2B-Venus*) and cytoplasmic (*Venus*) reporters are co-transformed, only the cytoplasmic reporter is silenced, suggesting that GESENI depends on the intracellular localization of the encoded protein rather than the nucleotide sequence of the transgene. Furthermore, disruption of canonical gene-silencing pathways, including DNA methylation, H3K27me3-mediated chromatin regulation, and endogenous RNA interference, does not restore transgene expression (Ohnishi and Kawashima 2023). Collectively, these findings demonstrate that GESENI represents a previously unrecognized gene-silencing mechanism.

Remarkably, Ishida et al. (2026) recently developed a strategy to bypass GESENI, enabling the expression of transgene-encoded proteins in the cytoplasm of Arabidopsis sperm cells. To overcome GESENI, they employed two complementary approaches: transcript transfer from the vegetative cell using *ABA-HYPERSENSITIVE GERMINATION3* (*AHG3*) (Jiang et al. 2015; Sugi et al. 2024) and P2A-mediated ribosome skipping, which enables the production of two independent proteins from a single mRNA (El Amrani et al. 2004), either individually or in combination (Ishida et al. 2026). Arabidopsis plants transformed with *ProAHG3:AHG3-P2A* constructs harboring the coding sequences of the GCaMP calcium biosensor (Nakai et al. 2001), mStayGold (mSG, a highly bright and photostable dimeric green fluorescent protein) (Ando et al. 2024), or mScarlet3-H (mSc3H, a basic orange fluorescent protein) (Xiong et al. 2025), exhibited fluorescent signals in the sperm cell cytoplasm (Ishida et al. 2026). Surprisingly, successful protein expression did not require transcript transfer from the vegetative cell, as fluorescence was also detected in plants expressing *ProHTR10:AHG3-P2A-GCaMP* and *ProHTR10:SSP-P2A-GCaMP*, in which the transgenes were expressed directly in sperm cells (Ishida et al. 2026). Thus, P2A-mediated ribosome skipping alone is sufficient to bypass GESENI.

Beyond providing a practical method for FP-based analyses in sperm cells, this finding also provides important insights into the nature of GESENI. The successful expression of cytoplasmic proteins through P2A-mediated ribosome skipping demonstrates that GESENI does not directly inhibit protein translation. This distinguishes GESENI from mechanisms of intrinsic ribosome destabilization, in which a nascent polypeptide chain induces ribosome dissociation and thereby suppresses gene expression by interfering with translation (Chadani et al. 2017). These observations suggest that GESENI acts at a step before translation, despite being determined by post-translational properties of the encoded protein (Fig. 1B). How Arabidopsis sperm cells recognize intracellular localization information encoded in a transgene or its transcript during GESENI remains unknown.

To gain further insight into the molecular basis of GESENI and to facilitate the application of FP-based approaches in plant reproductive research, we investigated whether GESENI susceptibility depends on the fluorescent protein used. In our previous study (Ohnishi and Kawashima 2023), all FP-tagged constructs employed Venus, a yellow fluorescent protein derived from *Aequorea victoria* GFP through EYFP (Nagai et al. 2002). Here, we examined five additional fluorescent proteins representing diverse evolutionary origins and spectral classes: Clover, derived from *Aequorea victoria* GFP (Lam et al. 2012); mRuby3, derived from *Entacmaea quadricolor* FP611 through mRuby (Bajar et al. 2016); tdTomato, a tandem dimer derived from DsRed through dimer2 (Shaner et al. 2004; Drobizhev et al. 2011); and plant codon-optimized mTFP1 (POmTFP1), derived from *Clavularia* sp. FP484 (Ai et al. 2006; Pasin et al. 2014, 2017). Although these FPs share a conserved the β -barrel structure, they differ in their nucleotide sequences (Supplementary Fig. S1). Each FP was expressed under the control of the sperm cell-specific *ProHTR10* promoter to determine whether GESENI represents a general response to fluorescent proteins or depends on the specific FP employed.

RESULTS

We generated transgenic *Arabidopsis* lines expressing five fluorescent protein genes, *Venus*, *Clover*, *mRuby3*, *tdTomato*, and plant codon-optimized *mTFP1* (*POmTFP1*), under the control of the sperm cell-specific promoter *ProHTR10*. As controls, the same fluorescent proteins were expressed under the pollen vegetative cell-specific promoter *ProLAT52*. Independent single-locus T_1 transformants were identified, and homozygous T_2/T_3 lines were established by segregation analysis using the pAlligator/pBilligator seed fluorescence markers and confirmed by genomic PCR (Supplementary Fig. S2).

As expected, all *ProLAT52* transformants exhibited strong and stable fluorescence in the pollen vegetative cell cytoplasm (Fig. 2a). Consistent with our previous study using *Venus* (Ohnishi and Kawashima 2023), *Clover* fluorescence was completely absent from sperm cells, indicating that *Clover* is also susceptible to GESENI (Fig. 2a). In contrast, *mRuby3*, *tdTomato*, and *POmTFP1* produced detectable fluorescence in pollen grains. Notably, fluorescence from *mRuby3* and *tdTomato* was also frequently detected in pollen vegetative cells, despite expression being driven by the sperm cell-specific *ProHTR10* promoter (Figs. 2a, b). This fluorescence pattern was observed in the majority of independent T_1 transformants carrying these two fluorescent proteins (Fig. 2c; Supplementary Fig. S3).

To determine whether these fluorescence patterns were stably inherited, single-locus T_1 plants were advanced to the T_2 and T_3 generations, and pollen fluorescence was classified into four categories: sperm cell only, vegetative cell only, both cell types, or no fluorescence. In *ProHTR10:mRuby3* lines, two independent T_3 lines (#n6 and #n8) exhibited fluorescence in 86–94% of pollen grains. Among fluorescent pollen grains, most showed signals exclusively in the vegetative cell (83–89%), whereas fluorescence restricted to sperm cells (0–6%) or detected in both cell types (0–3%) was rare (Figs. 2b, d).

The two *ProHTR10:tdTomato* lines exhibited contrasting fluorescence patterns. Line #n8 showed a pattern similar to that of the *mRuby3* lines, whereas no fluorescence was detected in the T_3 generation of line #n19 (Figs. 2d, e). Interestingly, fluorescence was clearly observed in the T_1 and T_2 generations of line #n19 before disappearing in T_3 . In the T_2 generation, fluorescence was detected in 23 of 27 individuals, and nine plants, including both homozygous and heterozygous individuals, exhibited fluorescent signals in more than 40% of their pollen grains (Figs. 2d, e). In *ProHTR10:POmTFP1* lines, fluorescence was detected in 3–12% of pollen grains in both the T_2 and T_3 generations (Fig. 2d). However, because the emission spectrum of POmTFP1 partially overlapped with pollen autofluorescence, fluorescence in the vegetative cell could not be reliably quantified (Supplementary Fig. S3).

DISCUSSION

The detection of mRuby3, tdTomato, and POmTFP1 fluorescence in sperm cells demonstrates that susceptibility to GESEN1 varies among fluorescent proteins (Fig. 2). With the exception of tdTomato, which is a tandem dimer, the DNA sequences encoding these fluorescent proteins have comparable coding sequence lengths. Notably, the two fluorescent protein coding sequences comprising tdTomato are identical except for the first and last 25 bp and the linker sequence (Supplementary Fig. S1). Therefore, the distinct GESEN1 responses are unlikely to be explained simply by differences in sequence length, but may instead reflect differences in biochemical properties among fluorescent proteins, such as oligomerization tendency, folding efficiency, or maturation kinetics (Shaner et al. 2005; Nakagawa et al. 2012; Kashiwagi et al. 2019). FP tags have also been shown to affect the condensation properties of tagged proteins (Stadler et al. 2013). Furthermore, condensate dynamics can be influenced by the type of FP tag, resulting in different behaviors in vitro and in yeast cells (Fatti et al. 2025). Consistent with this notion, a recent study in yeast suggested that FP condensation is influenced by the electrostatic properties of FPs, highlighting the importance of careful FP selection in experimental studies (Lee et al. 2026). The differences in GESEN1 phenotypes observed among FP types in this study may likewise be attributable to differences in the biochemical or biophysical properties of the FPs, although this possibility remains to be experimentally tested.

An additional observation was the progressive loss of tdTomato fluorescence in the T_3 generation of line #n19, despite clear fluorescence in the corresponding T_1 and T_2 generations. This pattern differs from the previously reported characteristics of GESEN1, which occurs in single-loucs T_1 transformants and is independent of canonical transgene-silencing pathways (Ohnishi and Kawashima 2023). One possible explanation is that a conventional epigenetic silencing mechanism was superimposed on GESEN1 during successive generations. Alternatively, this phenotype may reflect a position effect associated with the transgene insertion site, as it was observed in only one independent line. At present, it remains unclear whether the progressive loss of fluorescence represents a secondary canonical transgene-silencing event or another mechanism unrelated to GESEN1. Further analyses of transgene transcription, DNA methylation, and chromatin status will be required to distinguish between these possibilities.

An even more intriguing observation was the detection of tdTomato and mRuby3 fluorescence in pollen vegetative cells (Fig. 2). This finding is particularly unexpected because *HTR10* expression is thought to be strictly restricted to sperm cells. The *HTR10* promoter is directly activated by the R2R3 MYB transcription factor DUO POLLEN1 (DUO1), a master regulator of sperm cell differentiation (Rotman et al. 2005; Borg et al. 2011). Because *DUO1* is post-transcriptionally silenced by *miR159*-mediated RNA-induced silencing complex (RISC) activity in the vegetative cell at the bicellular pollen stage, it is absent from the vegetative cell of mature tricellular pollen (Palatnik et al. 2007; Brownfield et al. 2009; Zheng et al. 2014). Consequently, *ProHTR10* is not expected to be transcriptionally active in mature vegetative cells. The vegetative cell fluorescence observed in the *ProHTR10:tdTomato* and *ProHTR10:mRuby3* lines is therefore inconsistent with the current model of *HTR10* regulation.

Assuming that *ProHTR10* is active exclusively in sperm cells, the ectopic fluorescence observed in vegetative cells raises the possibility that mRNAs synthesized in sperm cells are transported into the vegetative cell. Intercellular RNA transport from vegetative cells to sperm cells has been reported previously (Slotkin et al. 2009; Martínez et al. 2016; Wu et al. 2021), whereas transport in the opposite direction has not been demonstrated. Alternatively, the observed fluorescence could result from ectopic transcription of the transgenes in the vegetative nucleus. However, the successful expression of cytoplasmic proteins using the *HTR10* promoter and *P2A*-mediated ribosome skipping indicates that protein production can proceed in sperm cells when the corresponding mRNA is available (Ishida et al. 2026). At present, neither hypothesis explains why this phenomenon depends on both the intracellular localization of the encoded protein and the identity of the fluorescent protein. Together, these observations suggest that the mechanism underlying GESENI is more complex than previously appreciated and is not readily explained by current models of promoter specificity or intercellular RNA transport.

MATERIALS AND METHODS

Plant materials and observation

Arabidopsis thaliana plants of the Columbia-0 (Col-0) ecotype were grown under short-day conditions (8 h light at 22°C/16 h dark at 18°C) for 2–3 weeks and then transferred to continuous light conditions at 22°C. *Arabidopsis* pollen grains were isolated and observed using a Leica DM2500 LED microscope (Leica, Germany) equipped with a 40× oil-immersion objective, as described previously (Ohnishi and Kawashima 2023).

Construction and transformation

Arabidopsis constructs were generated using Multisite Gateway Technology (Invitrogen, CA, USA) and transformed as described previously (Ohnishi and Kawashima 2023). The first cassettes (P4-P1r Entry vectors) containing *ProLAT52* (Chen et al. 2009) and *mRuby3* without a stop codon (Ali et al. 2023) were

generated as described previously. The *pME-tdTomato* plasmid (Addgene plasmid #135202) was used as the middle-entry cassette for construction of tdTomato expression vectors. The *P_{Om}TFP1* sequence (Pasin et al. 2014, 2017) was synthesized de novo (GeneArt, Thermo Fisher Scientific, MA, USA) and cloned into a pENTR221 vector. Single-locus T₁ transformants and homozygous T₂/T₃ generation lines were selected based on GFP/RFP fluorescence signals in seeds carrying the pAlligator/pBilligator markers. All transformants used in this study were single-locus homozygous T₂/T₃ generation lines, except for *ProLAT52:FP* transformants, which were analyzed as heterozygous lines. The transgenes in transformants used for Fig. 1 were confirmed by PCR using genomic DNA templates (Supplementary Fig. S2). Isolated genomic DNA (20 ng/μl) was used as the template for PCR amplification with DreamTaq Green DNA Polymerase (Thermo Fisher Scientific) and primer pairs (0.1 μM each). Primer sequences are listed in Supplementary Table S1.

Declarations

Competing Interests

Y.O. is currently an employee of NTT, Inc.

Author Contribution

Y.O. performed the observation of Arabidopsis; Y.O. and T.K. designed the experiments and wrote the paper.

ACKNOWLEDGMENTS

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DATA AVAILABILITY

All data to support the conclusions of this manuscript are provided in the Figures and Supplementary Figures. Source data are provided in Supplementary Data Sets.

SUPPLEMENTARY INFORMATION (SI)

Supplementary Figure S1 Sequences of the five FPs.

Supplementary Figure S2 Conformation of transgene in *ProHTR10/ProLAT52:FP* transformants.

Supplementary Figure S3 Pollens in T₁/T₂/T₃ generations of *ProHTR10:FP* transformants.

Supplementary Table S1 List of primers used in this paper.

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Figures

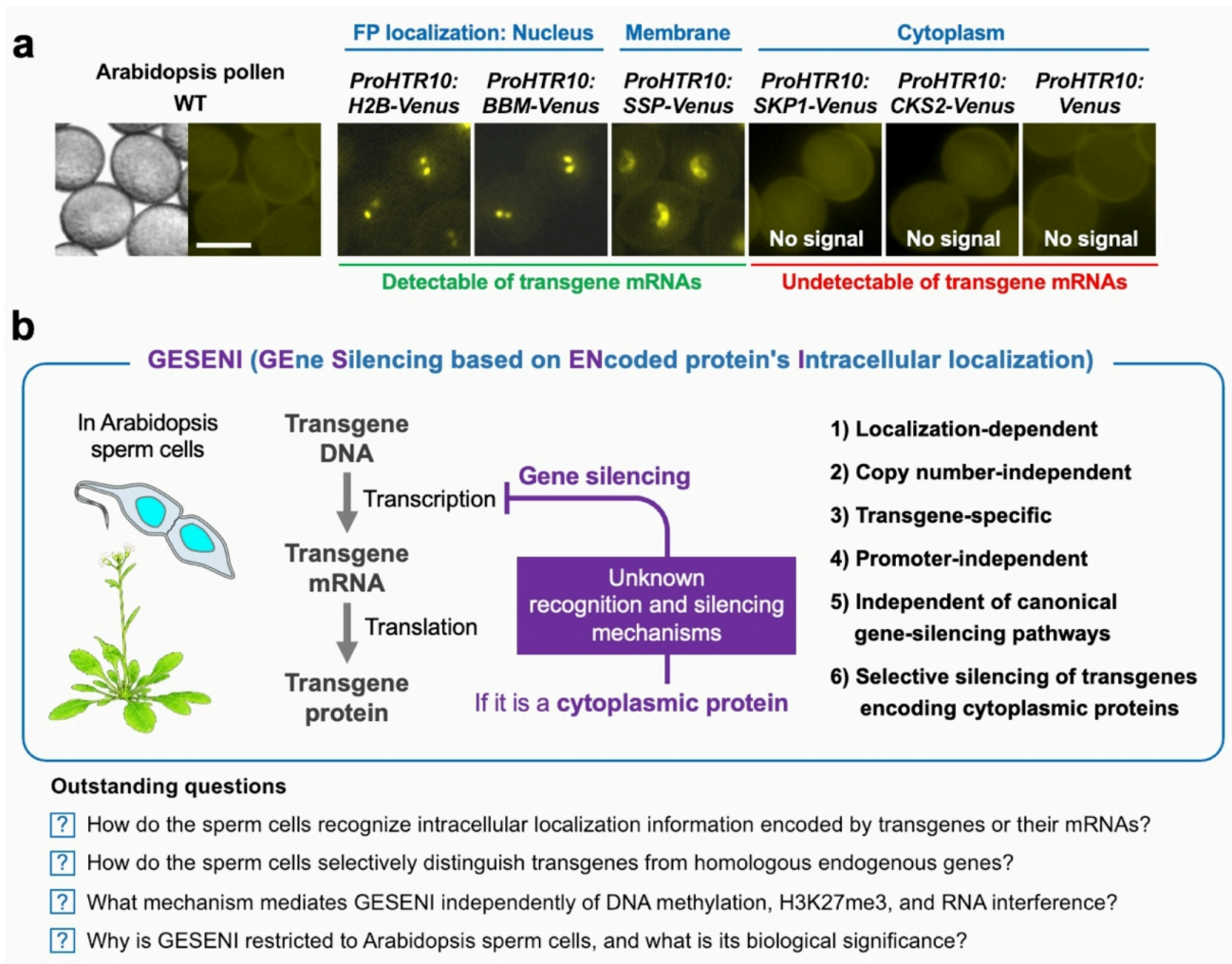


Figure 1

Unresolved questions regarding GESENI.

a, Phenotype of GESENI reported by Ohnishi and Kawashima (2023). Scale bar, 20 μ m. **b**, Summary of the known characteristics of GESENI and the remaining unresolved questions.

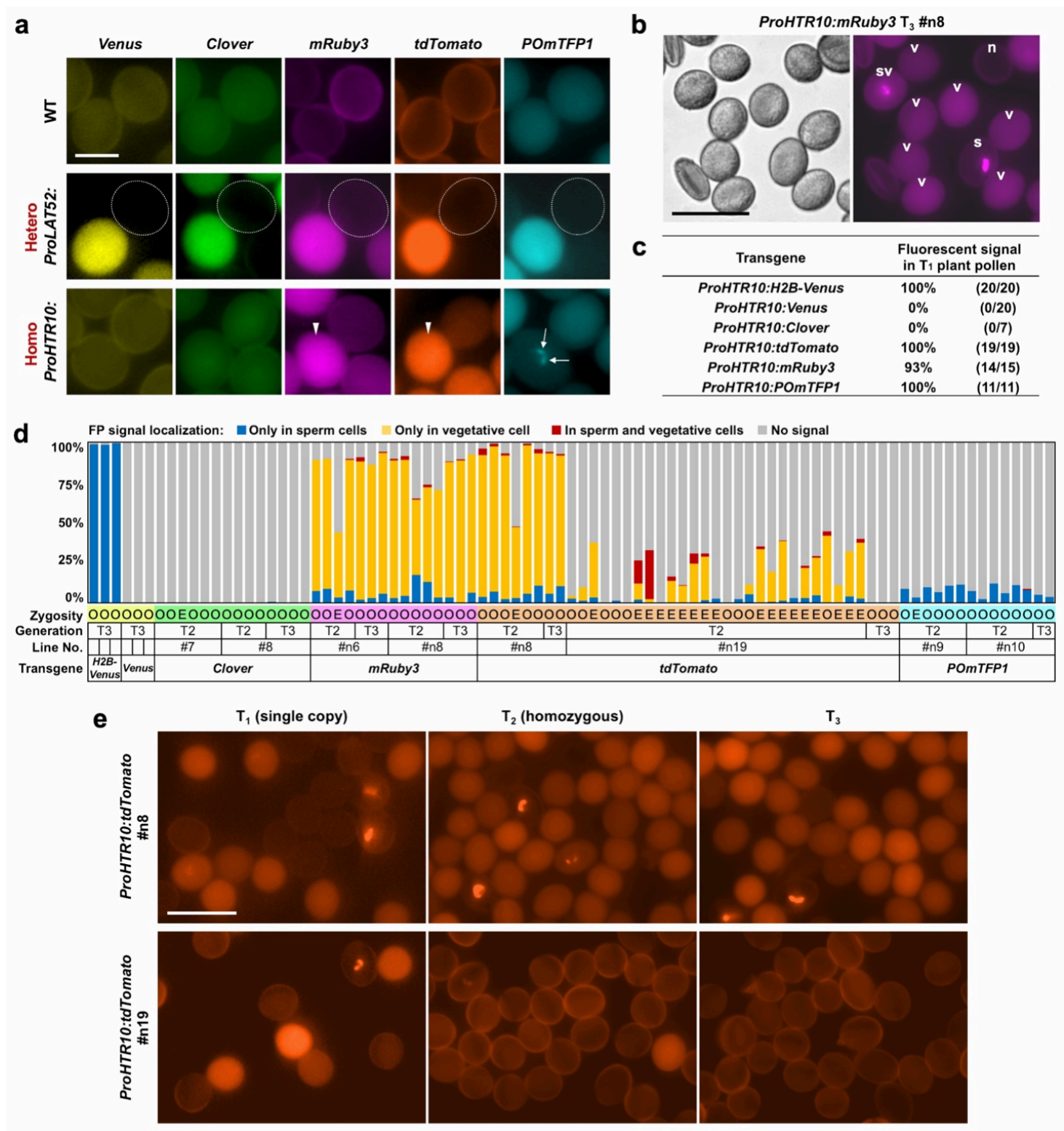


Figure 2

Fluorescent protein-dependent susceptibility to GESENI.

a, Pollen from heterozygous *ProLAT52:FP* and homozygous *ProHTR10:FP* Arabidopsis lines. T₂/T₃ pollen expressing five fluorescent protein genes, *Venus*, *Clover*, *mRuby3*, *tdTomato*, and plant codon-optimized *mTFP1* (*POMPFP1*), under the control of the sperm cell-specific promoter *ProHTR10* or the pollen

vegetative cell-specific promoter *ProLAT52*. Arrows and arrowheads indicate sperm cells and vegetative nuclei, respectively. Scale bar, 20 μ m. **b**, Representative pollen from the T_3 homozygous *ProHTR10:mRuby3* line #n8. n, no fluorescence; s, fluorescence detected only in sperm cells; v, fluorescence detected only in the vegetative cell; sv, fluorescence detected in both sperm cells and the vegetative cell. Scale bar, 50 μ m. **c**, Percentage of T_1 transformants showing the presence or absence of fluorescent signals. Transformants exhibiting fluorescence in sperm cells and/or the vegetative cell were classified as fluorescence-positive. The T_1 population included both single-locus and multi-loci transformants. **d**, Fluorescence patterns in pollen from individual single-locus T_2/T_3 lines. Bars indicate the percentages of pollen showing no fluorescence (gray), fluorescence only in sperm cells (blue), fluorescence only in the vegetative cell (yellow), or fluorescence in both sperm cells and the vegetative cell (red). A total of 118–396 pollen grains were analyzed for each line. Zygosity is indicated as homozygous (O) or heterozygous (E). Generation and line numbers are shown below each column. Data from previously reported single-locus homozygous *ProHTR10:H2B-Venus* T_3 lines (#3, #9, and #n13) and *ProHTR10:Venus* T_3 lines (#6, #n6, and #10) are included for comparison. **e**, Representative pollen from single-locus T_1 plants and their corresponding homozygous T_2 and T_3 progeny of *ProHTR10:tdTomato* lines #n8 and #n19.

Supplementary Files

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