Plant Communications
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CellPress
Partner Journal

Pollen self-elimination CRISPR–Cas genome editing prevents transgenic pollen dispersal in maize

Dear Editor,

Genome editing with clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated nuclease (Cas)-mediated technologies have revolutionized basic plant science and crop genetic improvement (Chen et al., 2019). Stable genetic transformation of CRISPR–*Cas* cassette(s) is the main approach to genome editing *in planta*. In many sexually reproducing plants, a major concern is the dispersal of genetically modified elements through pollen (Devos et al., 2005). Maize (*Zea mays* L.), a typical outcrossing crop species, can produce as many as two to five million pollen grains per plant (Goss, 1968) and has a recommended isolation distance of 200 m due to wind dispersal (Ma et al., 2004) or even >3 km due to foraging by insects like honey bees (Danner et al., 2014). A previously reported strategy using suicide transgenes effectively killed immature embryos and pollen harboring a *Cas9* transgene produced by T_0 plants and produced transgene-free edited T_1 plants (He et al., 2018). Especially for vegetatively propagated plants, this technology solves the problem of removing transgenic components, as it is not feasible to remove them through meiotic recombination and segregation. However, genome editing has a number of useful applications for which the *Cas* transgene needs to be retained in the plants, including RNA-guided Cas9 as an *in vivo* desired-target mutator (Li et al., 2017) and haploid induction-coupled editing (Kelliher et al., 2019; Wang et al., 2019) through the paternal haploid using a *cenh3*-null mutant as the female gametophyte (Ravi and Chan, 2010). In this correspondence, we present *PSEC*, which prevents pollen transgene dispersal from plants that harbor a T-DNA containing a pollen suicide cassette next to specific single guide RNA and Cas cassettes. At the same time, *PSEC* can still be inherited through the female gamete to the next generation and also retains CRISPR–Cas gene editing activity. Through sexual crossing, it acts in *trans* to induce efficient target mutations in the parental genome of crosses for breeding applications.

To generate a programmed *PSEC*, we introduced a male gametophyte inactivation gene, the maize alpha-amylase gene *ZmAA1*, driven by the pollen-specific promoter (*Polygalacturonase 47*, *ZmPG47*) used in our previous study (Qi et al., 2020) into a T-DNA that also holds the CRISPR–Cas9 cassette (Figure 1A). The pollen derived from these *PSEC* plants was not viable when the *PSEC* transgene was present, but the transgene was inherited to the next generation through the female gametophyte (Figure 1B). *In vivo* Cas editing activity was retained to generate new allelic target mutations when crossed with the lines (Figure 1C). In this study, we designed *PSEC* to target genes encoding three growth-regulating factors, *ZmGRF1*, *ZmGRF5*, and *ZmGRF6,* and obtain single and/or multiple mutants (Figure 1A).

We performed Agrobacterium (*Agrobacterium tumefaciens*)-mediated stable transformation of immature embryos from the maize inbred line ZC01 with *PSEC*, as described previously (Li et al., 2017), resulting in the isolation of 25 independent T_0 transformants. After a preliminary assessment of the target mutations, we selected five transformants for characterization of *PSEC* copy numbers via digital droplet PCR (Figure 1D). Plants 3-1 and 24-1 harbored a single copy of the *PSEC* transgene (Figure 1D); we thus chose transformant 3-1 for further characterization.

Plant 3-1 grew and flowered like the wild type (WT), with normal stamens and anthers. After KI/I_2 staining (Figure 1E), stamens from plant 3-1 were lighter than WT stamens, with nearly half of 3-1 pollen grains lacking purple staining. These observations were consistent with our previous study in which we produced sterile male flowers in maize with the same *PG47pro:ZmAA1* transgene (Qi et al., 2020).

Viable pollen represented half of all plant 3-1 pollen, as demonstrated by a chi-squared test ($\chi^2 < \chi^2_{0.05,1}$; Figure 1F), thus conforming to the expected segregation ratio for a single copy of *PSEC*. To confirm the presence/absence of *PSEC*, we carefully collected around 100 stained pollen and 100 unstained pollen from plant 3-1 under a stereomicroscope for genomic DNA extraction and *Cas9* PCR amplification, with three replicates. All purple pollen lacked *PSEC*, and all unstained pollen contained the transgene (Figure 1G).

We genotyped self-crossing progeny of 3-1, 3-4, and 3-7 to identify target mutations by Sanger sequencing. We identified all possible and homozygous single-, double-, and triple-mutant combinations at *ZmGRF1*, *ZmGRF5*, and *ZmGRF6* in Cas9-negative plants (Supplemental Table 2). This complete set of *Zmgrf1*, *Zmgrf5*, and *Zmgrf6* mutants can be used for both gene functional studies and breeding (Figure 1H). All mutants were shorter than the WT (Figure 1H and Supplemental Figure 1). Three of the mutations in 3-1-16, 3-1-56, and 3-1-156 may not produce knockouts and may represent weak alleles instead (Supplemental Table 2). We further genotyped and characterized the offspring of the confirmed knockout mutations above, as we

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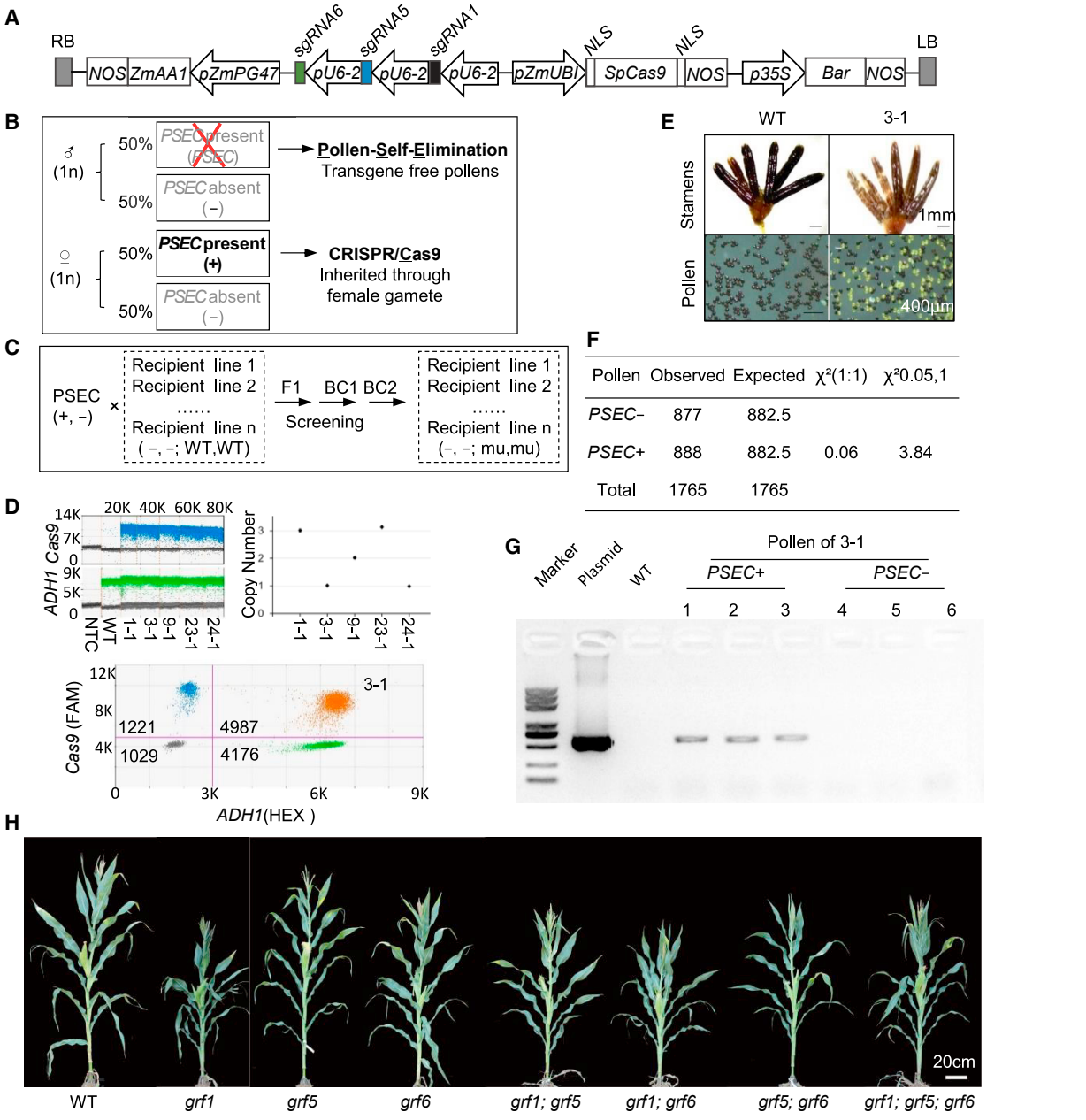

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Pollen recipient × donor	Seeds of F1 combination			χ^2 (1:1)	*PESC* generated mutations in F1 [a]		
	PESC+	*PESC*–	Expected ratio		*ZmGRF1*	*ZmGRF5*	*ZmGRF6*
JD96M × 3-2	0	272	0	NA	0% (0/40)	0% (0/40)	0% (0/39)
JD96F × 3-3	0	357	0	NA	0% (0/39)	0% (0/37)	0% (0/40)
B73 × 3-6	0	272	0	NA	0% (0/40)	0% (0/40)	0% (0/38)
3-2 × JD96M	159	181	1:1	1.30	17.5% (7/40)	40% (16/40)	77.5% (31/40)
3-3 × JD96F	147	136	1:1	0.35	51.3% (20/39)	50% (20/40)	95% (38/40)
3-6 × B73	151	138	1:1	0.50	45% (18/40)	35% (14/40)	75% (30/40)

Note: "a" indicates the ratio of *PSEC*-generated mutation in F1 (mutant/total).

Figure 1. Pollen self-elimination CRISPR–Cas9 (*PSEC*) provides transgene-free pollen and retains targeted mutations *in vivo*.
(A) Schematic diagram of the *PSEC* vector. *pPG47*, pollen-specific promoter; sgRNA1, sgRNA5, and sgRNA6, sgRNAs designed to target *ZmGRF1*, *ZmGRF5*, and *ZmGRF6*, respectively; *ZmAA*, α-amylase gene.

(legend continued on next page)

noticed high mutant activity for the retained *PSEC*, as discussed below.

To investigate how the *PSEC* transgene can be spread and inherited, we used T_1 plants as pollen donors or receptors in crosses with the three maize inbred lines JD96M, JD96F, and B73 (Figure 1I). When using plants 3-2, 3-3, and 3-6 as the pollen donors, we genotyped 272, 357, and 272 F_1 seeds produced from JD96M × 3-2, JD96F × 3-3, and B73 × 3-6 crosses by PCR for *Cas9*. None of the seeds harbored the *Cas9* gene, indicating that *PSEC* is not spread or inherited through 3-2, 3-3, or 3-6 pollen. By contrast, we detected *PSEC* in about half of all F_1 seeds produced from the 3-2 × JD96M, 3-3 × JD96F, and 3-6 × B73 crosses. These data were consistent with our expectation that *PSEC* can be spread and inherited only through the female gametophyte.

To test whether the inherited *PSEC* transgene showed efficient targeted mutation activity, we genotyped 40 F_1 seeds from each of the above crosses using plant 3-3, 3-3, 3-4 as the female parent. We identified plants with the desired homologous/bi-allelic mutations at each target site of *ZmGRF1*, *ZmGRF5*, and/or *ZmGRF6* in 17.5%–95% of all F_1 seeds (Figure 1I). These data indicate that the desired mutations can be efficiently produced through crossing with *PSEC* as the maternal parent.

In conclusion, we successfully developed a programmed *PSEC* system with a pollen-specific energy depletion cassette in which the pollen is eliminated, not the cassette, when *PSEC* is present in haploid pollen. *PSEC* can be inherited through the female gametophyte and perform Cas9-mediated genome editing activity across generations. This system could greatly alleviate serious concerns about the widespread diffusion of genetically modified elements into natural and agricultural environments via outcrossing. This technology should be applicable to other CRISPR–Cas systems and outcrossing plant species other than maize.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

FUNDING

This research was supported by the National Key Research and Development Program of China (2020YFE0202300), the Henan Provincial Department of Agriculture and Rural Affairs (grant #2022010201), the earmarked fund for CARS-Maize (CARS-02-06), and the Hainan Yazhou Bay Seed Laboratory (#B21HJ0215).

AUTHOR CONTRIBUTIONS

C.X., X.L., Q.Y., and H.W. conceived and designed the experiments. H.W., Q.Y., J.Z., C.L., H.F., and X.Z. conducted the experiments. C.X., H.W., and X.Q. wrote and revised the manuscript.

ACKNOWLEDGMENTS

No conflict of interest is declared.

Received: April 10, 2023
Revised: June 6, 2023
Accepted: June 6, 2023
Published: June 10, 2023

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https://doi.org/10.1016/j.xplc.2023.100637

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(B) Schematic representation of the PSEC system. +, *PSEC* present; –, *PSEC* absent; ♀, female gametophyte; ♂, male gametophyte; 1n, haploid cell.
(C) *PSEC* can be inherited through the female gametophyte and retain *in vivo* editing activity. mu, mutation of the target gene.
(D) Digital droplet PCR identification of single-copy *PSEC* plants. Endogenous *ZmADH1* was used as the internal reference. Top left panel, amplitudes of the targets and reference; top right panel, calculated *PSEC* copy numbers; bottom panel, typical droplet numbers with *Cas9*, *ZmADH1*, and *Cas9*/*ZmADH1* amplitudes and blank amplitudes (bottom left quadrant) of plant 3-1 plotted in four quadrants.
(E) Stamens and mature pollen of *PSEC* plant 3-1 and its wild type after KI/I_2 staining.
(F) Chi-squared test of the segregation ratio of pollen with or without *PSEC*.
(G) PCR validation of pollen from plant 3-1 with *PSEC* present or absent as indicated by KI/I_2 staining. Each sample originated from 100 purple or unstained pollen. The *PSEC* plasmid was used as the positive control.
(H) Phenotypes of representative mutants at the flowering stage. Scale bar, 20 cm.
(I) Genotyping results of F_1 progeny from crosses between self-cross of 3-2, 3-3, and 3-6 used as the pollen donor or receptor.

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