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No evidence for transient transformation via pollen magnetofection in several monocot species

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Pollen magnetofection[1] was previously reported to deliver exogenous DNA into pollen grains both for transient transformation and for the creation of stable, genetically modified progeny. This study finds no evidence that magnetofection can accomplish transient transformation of pollen in several monocot species. Achieving stable transformation of plants remains a major hurdle except in a few species, hindering efficient progress towards both basic and applied scientific goals. Thus, the approach of pollen magnetofection, described by Zhao et al.[1], was greeted as a potentially transformative technology. In this approach, magnetic nanoparticles deliver exogenous DNA into pollen grains, which then generate genetically modified progeny incorporating that DNA into their genome. Independently, we set up trials to investigate the effectiveness of this approach in two monocot species, maize (at Oregon State University) and sorghum (at Lawrence Berkeley National Laboratory). Both species, which require specialized expertise and demanding methodologies to achieve transformation[2], are of interest as models for basic biological investigation as well as for their agricultural impact. Moreover, both species produce readily accessible pollen, raising the possibility that the pollen magnetofection approach could be easily implementable with these grasses. However, we were unable to accomplish transient transformation of pollen via magnetofection in either species, or in a third monocot, lily.

Zhao et al. focused primarily on dicot species (cotton, pepper, pumpkin and cocozelle). However, they reported some success for one monocot, lily (*Lilium brownii*), although stable transformation was not achieved. With lily, transient transformation of pollen was reported at ~90% efficiency, as detected by staining for β-glucuronidase (GUS) activity following magnetofection with a *GUS* reporter plasmid. Thus, as a prelude to attempting stable transformation in sorghum and maize, we chose to focus on testing transient transformation efficiency in pollen, as the results could be assessed rapidly (typically within a day). However, our initial experiments with sorghum indicated that *GUS* reporters would not be ideal, due to positive GUS staining in control, non-transformed pollen grains (Supplementary Fig. 1). Other published reports also indicate that GUS activity is detectable in the male gametophyte of a number of plant species[3,4], spurring our labs to use green fluorescent protein (GFP)-based reporter plasmids as an alternative. For these experiments, we chose plasmids with promoters known to be highly expressed in grass pollen (maize *Zm13*, rice *Actin1* or maize *ZmUbiquitin1*; refs. [5–7]). However, following the published protocol, 16 trials with maize pollen and 5 trials with sorghum pollen generated no indication of plasmid-induced transient expression of fluorescence (Supplementary Fig. 2 and Supplementary Table 1) (over 50,000 pollen grains screened).

To determine whether our lack of successful transient transformation was due to a grass-specific resistance to magnetofection, we turned to lily pollen as a model. Using lily pollen afforded us the ability to compare transformation efficiency via magnetofection versus biolistics, a well-established methodology[8]. The magnetofection protocol is simple enough to allow side-by-side treatment alongside the biolistic protocol, enabling the assessment of transformation efficiency in subsamples of the same population of pollen (Supplementary Methods). Briefly, pollen was collected from cut lily flowers (*Lilium* var. Santander) by vortexing anthers in pollen germination media (PGM). Biolistic microcarriers and magnetic nanoparticles were coated with the *Zm13* promoter-driven GFP reporter plasmid, and nanoparticles or microcarriers were delivered to the pollen via exposure to a magnet or biolistic bombardment, respectively. The treated pollen samples were then incubated overnight in PGM and were imaged to detect reporter expression the following day via fluorescence microscopy. Transformation efficiency was determined via blind assessment of the number of transformed pollen grains/tubes in >10 randomly chosen microscopic fields for each replicate. Over 1,000 pollen grains or tubes were screened for each experimental treatment in each of three trials (Supplementary Table 2). We followed the Zhao et al. protocol as published, with one exception: we used a PGM formulation[9] that, in our hands, gave improved pollen germination frequencies relative to that given by Zhao et al.

Transformed pollen grains and tubes were clearly recognizable by the expression of GFP fluorescence above background after biolistic bombardment with the reporter plasmid (Fig. 1). Biolistic transformation efficiency ranged from 0.4 to 1.1% in the three trials, averaging 0.7% (Table 1). As expected, no transformed pollen was found in the negative control biolistic treatments (that is, samples bombarded with microcarriers lacking plasmid DNA). However, we also observed no transformed lily pollen in the magnetofection treatments, despite screening nearly 4,000 pollen grains (Table 1). Using the Cochran–Mantel–Haenszel test to assess the repeated

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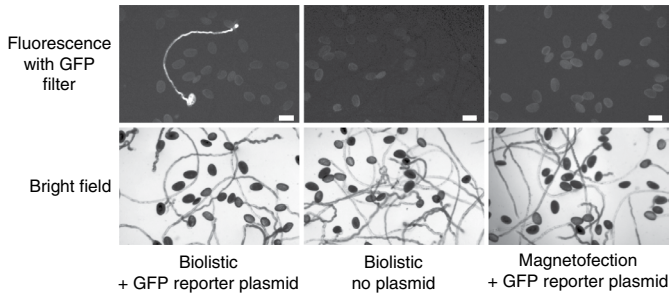

Fig. 1 | Detection of GFP fluorescence after biolistic bombardment and after magnetofection. Strong GFP fluorescence is detectable in lily pollen and pollen tubes following biolistic bombardment with the pUC19-260Zm13::GFP plasmid construct, whereas no green fluorescence is detected above background following magnetofection with the same plasmid. The experiment was repeated three times independently, with similar results (see Table 1). Scale bars, 100 μm.

Table 1 | Summary of side-by-side magnetofection and biolistic transient transformation efficiency with lily pollen, three trials

	Biolistic, with plasmid DNA	Biolistic, no DNA	Magnetofection, with plasmid DNA
Total transformants (pollen grains and pollen tubes)	33	0	0
Total pollen in imaged fields	4,651	5,559	3,731
Transformation efficiency	0.71%	0%	0%
P value, Cochran–Mantel–Haenszel test, two-sided (versus biolistic positive control)		4.09×10^{-9}	5.02×10^{-7}

trials indicates a statistically significant difference between the positive control biolistic method and both the negative control and the magnetofection protocol ($P<10^{-6}$ for both).

In conclusion, using the protocol described by Zhao et al., we were unable to reproduce any evidence of transient transformation in lily pollen via magnetofection. Although we cannot rule out transient transformation at very rare frequencies, we suspect that the report of ~90% transient expression efficiency following the magnetofection of lily pollen was due to endogenous GUS activity, rather than that from the *GUS* reporter used for assessing magnetofection success. Finally, given our lack of success in lily, sorghum and maize despite extensive trials, we also could not generate evidence to support the idea of broad applicability for pollen magnetofection across monocots. We note that we cannot address the utility of pollen magnetofection for stable transformation, as we have not tested for genomic integration of reporter DNA. However, given our results, we believe that it is important for groups working in dicot species, particularly those cited as successfully transformed (cotton, pepper and pumpkin), to provide data regarding attempts to replicate both transient and stable transformation success via pollen magnetofection.

Reporting Summary

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

Data availability

All datasets generated or analysed during the current study are included in this published article (and its Supplementary Information) or are available from the corresponding author on reasonable request.

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Author contributions

H.V.S., R.S., J.C.M., C.W. and J.E.F. conceived the experimental plan and design. R.S., C.W. and Z.V. performed the experiments and analysed the data. J.C.M., H.V.S. and J.E.F. supervised the experiments. J.E.F. wrote the initial manuscript draft, and all authors contributed to revision.

Competing interests

The authors declare no competing interests.

Additional information

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Data collection	from Methods, in Supplemental Material: Pollen in each field of view was imaged with both transmitted light (6 ms exposure time) as well as a GFP filter set (Chroma #41017 - 470/40 excitation, 495 long-pass dichroic, 525/50 emission) (800 ms exposure time) using µManager 2.0.0-gamma1 20190730 software (reference 5)
Data analysis	No software was used

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Sample size	Sample size was determined by the imaging protocol, as described in the Methods in the Supplemental Data: For each treatment, at least 10 randomly selected, non-overlapping fields of view were imaged with a 4X objective. All pollen grains in these field of view were scored blindly for GFP expression in pollen and pollen tubes. Each GFP-expressing pollen grain or pollen tube was counted as one transformation event. For each treatment, at least 1000 pollen grains were scored in this manner. The sample sizes were sufficient to detect significant differences between the positive and negative controls, and the experimental (see Table 1, Supplemental Table 2)
Data exclusions	No data were excluded from the analysis.
Replication	Reproducibility was verified by 21 initial trials, followed by three repeated side-by-side experiments as described in the main text and presented in Table 1. All trials gave the same result.
Randomization	Randomization was not a necessary part of the experimental design, as the sets of images from each experiment were randomized for blind scoring, to eliminate unconscious bias.
Blinding	This confirms that the investigator scoring images for Table 1 and Supplementary Table 2 (i.e., data analysis) was doing so blindly. In the experiments described in Supplementary Table 1, no blinding was done. In these experiments, no positive signal was ever observed - i.e., no differences between the negative control and the experimental - blinding was not deemed necessary, as it was not possible to quantitate non-existent signal.

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