Communication

Hidden pitfalls of using onion pollen in molecular research

Majd Mardini [1,‡], Aleksey Ermolaev [1,2,‡] and Ludmila Khrustaleva [1,3,4,*]

[1] Center of Molecular Biotechnology, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy (RGAU-MTAA), 49, Timiryazevskaya Str., 127550 Moscow, Russia; mr.majdmardini@gmail.com
[2] Laboratory of Applied Genomics and Crop Breeding, All-Russia Research Institute of Agricultural Biotechnology, Timiryazevskaya str. 42, Moscow 127550, Russia, All-Russia Research Institute of Agricultural Biotechnology, Moscow, Russia; ermol-2012@yandex.ru
[3] Department of Botany, Breeding and Seed Production of Garden Plants, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy (RGAU-MTAA), 49, Timiryazevskaya Str., 127550 Moscow, Russia; ludmila.khrustaleva19@gmail.com
[4] Plant Cell Engineering Laboratory, All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskay 42 Str., 127550 Moscow, Russia
* Correspondence: ludmila.khrustaleva19@gmail.com or khrustaleva@rgau-msha.ru
‡ These authors contributed equally to this work.

Abstract: There is little information on the use of pollen in molecular research, despite the increased interest to genome editing by pollen mediated transformation. PCR is useful tool as an express-method to evaluate editing results before pollination. A direct PCR protocol for pollen suspension has been adapted without the need for DNA pre-extraction. We showed that pollenkitt is a limiting factor for successful PCR on pollen. A simple pre-washing step of pollen suspension was able to eliminate the pollenkitt and enormously affect the PCR results. All currently existing methods of delivery of the editing system to pollen are carried out in a wet medium. Our pollenkitt study helped us develop a simple and effective pollination method using wetted onion pollen grains.

Keywords: onion; PCR inhibition; pollenkitt; wetted pollen; pollination

1. Introduction

For many years, pollen has been an appealing subject in wide spectrum of studies, such as paleontology [1–3], forensics [4], vaccine development [5,6], allergology [7] and many others. Furthermore, pollen research is also considered a major player in the field of plant breeding and crops improvement. The fact that a pollen grain consists of 2-3 haploid cells gives a unique chance to conduct fundamental studies like DNA genotyping and population genetics [8–14]. Another promising line of research is the use of pollen as a natural carrier of exogenous DNA for genome editing [15–23]. Although many articles have been published on the study of pollen, there is scarce information on the use of pollen in molecular research.

PCR is the most used instrument in molecular biology for analysis of DNA variations. In the case of genetic manipulation of pollen, PCR might be used as an express-method to evaluate results before pollination. Since the genetic material inside pollen grains is well protected by the rigid wall, DNA extraction is considered a critical step for successful PCR. This step is usually conducted either by mechanical crushing or enzymatic lysis or a combination of the two [10,12,18]. However, some observations suggest that DNA might be passively released from pollen grains due to the high temperature during PCR amplification [24], or in some certain species, pollen grains simply burst upon contact with water solution [25]. Another issue is the possible inhibition of PCR due to the natural presence of certain substances in pollen grains. A pre-washing step before PCR is obviously required in many protocols [3], however several studies claim that constituents of pollen grains have no effect on PCR activity [12]. Nevertheless, PCR on pollen might be a species-specific procedure. In addition, there is a marked lack of research on commercially important crops, highlighting the need to adapt protocols for new species.

Pollen-mediated genetic manipulation is a revolutionary approach in crops improvement. This promising technique gives the ability to readily obtain transformed plants in the

 doi:10.20944/preprints202212.0429.v1

form of seeds, which means bypassing the laborious steps of tissue culture and regeneration. Since the mid-1970s, scientists have been suggesting a wide range of methods to solve this problem, including free uptake of DNA [16], particle bombardment [19,22], electroporation of pollen tubes [17,18], pollen magnetofection [21] and uptake of nanoassemblies [23]. Regardless of the many distinctive variations among these methods, all of them require a *wet medium* in order to facilitate the main procedure. However, in most available protocols, little attention has been paid to the impact of wet mediums on pollen viability and subsequent pollination.

In this paper we are presenting an essential tool box of technical procedures and observations for molecular studies on onion (*Allium cepa* L.) pollen. A direct PCR protocol for pollen suspension has been adapted without the need for DNA pre-extraction. We showed that a limiting factor for successful PCR on pollen suspension is the outer layer of lipids known as pollenkitt. A simple pre-washing step of pollen suspension was able to eliminate the pollenkitt and enormously affect the PCR results.

Additionally, phase-contrast and dark-field microscopy were used to determine the dynamics of pollenkitt release from pollen grains upon contact with a liquid medium. It was clear that pollenkitt plays a major role in increasing the surface area of the pollen grain, hence affecting floating and adhesion behavior. Our investigation on pollenkitt helped us to develop a simple and efficient method for pollination of onion flowers using wetted pollen grains. All the observations and methods in this paper will be particularly useful for future research on gene editing and manipulation of onion pollen.

2. Results

During experimental studies on genetic manipulation of onion pollen we encountered a number of hidden pitfalls due to the lack of a basic methodology for evaluating results and post-experimental application. In order to address the lack of practical knowledge, we investigated the factors that influence the polymerase chain reaction (PCR) on pollen grains, the viability of pollen, and its further use for pollination and seed setting.

2.1. Direct PCR on pollen suspension of onion

A gene encoding Lachrymatory Factor Synthase (LFS) was used as a target for PCR on pollen. This is a single-locus gene encoding enzyme involved in the synthesis of the volatile sulfur compounds released during wounding [26,27]. In the first attempt of PCR using fresh intact pollen as a template we faced a complete inhibition of amplification. During early stages of our experiment we believed that the inaccessibility of pollen DNA could be the reason for unsuccessful PCR. Most of the available protocols for PCR on pollen require DNA extraction [10,12,18,28]. Accordingly, we tried different techniques of DNA extraction through mechanical damaging of the pollen wall: vortexing, grinding with pestel, steel beads or glass powder. None of these techniques showed any positive PCR results (data not shown). Finally, a simple washing procedure using distilled water was able to restore normal amplification (Figure 1). We concluded that an unknown factor/substance coating the surface of the pollen grain might be responsible for PCR inhibition.

In all angiosperms, the common material for coating pollen grains is the sticky tapetal tissue secretion, also known as pollenkitt [29]. In addition to a wide range of functions [30], pollen serves as a binding agent in entomophilous plants [31]. According to Dobson [32], pollenkitt is a hydrophobic mixture of materials composed mainly of saturated and unsaturated lipids. Lipids are known to be major inhibitors of PCR [33].

During washing procedure of the pollen suspension, we noticed that the discarded supernatant always had a cloudy appearance. Since pollenkitt is a hydrophobic substance, we assumed that the cloudiness is probably due to the insoluble debris of the pollenkitt, which released into water during washing (vortexing and centrifusion). This was confirmed using dark field microscopy (Figure 2a,b).

Upon repeated washing of the suspension, pollenkitt residues were removed together with the supernatant until their concentration became negligible for PCR inhibition (Fig-

 doi:10.20944/preprints202212.0429.v1

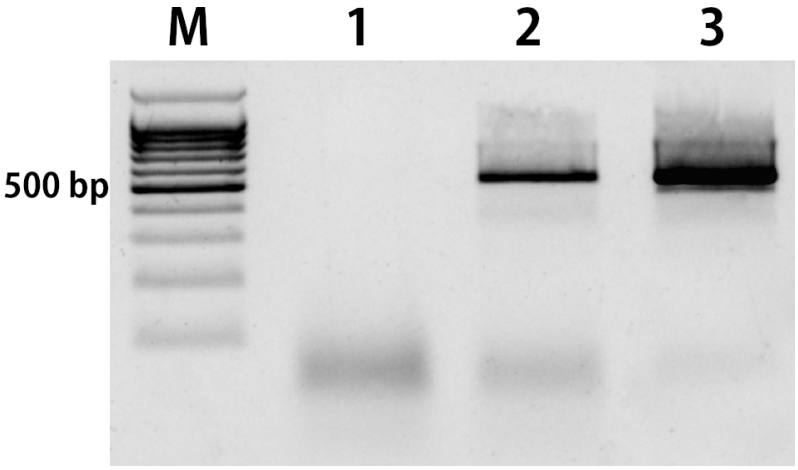

Figure 1. PCR amplification of the gene encoding LFS in onion: M — DNA Ladder 100bp+; lane 1 — mature pollen without washing; lane 2 — mature pollen after 10-times washing in ddH_2O; lane 3 — genomic DNA of *A. cepa*.

ure 2c). For onion pollen, 10 times by vortexing in 500 μL of distilled water for 10 sec and centrifuging for 5 sec using minispin centrifuge (750×g) was optimal.

In order to verify the relationship between PCR inhibition and pollenkitt, we conducted a series of PCR with different dilutions of pollenkitt added to genomic DNA of *A. cepa* as an amplification template and the LFS gene as a target (see Material and Methods). Electrophoresis showed a clear correlation between pollenkitt concentration and the intensity of the PCR band (Figure 3).

2.2. Hand pollination with wetted pollen grains

All currently existing methods of delivery of the editing system to pollen are carried out in a wet medium. One of the main objectives of our study was to verify whether it is possible to pollinate onion flowers using wetted pollen grains. Since hydration is an inevitable step in every technique of pollen genetic manipulation, it is crucial to verify such matter. *In vitro* experiments of pollen tube growing we found that onion pollen grains cannot grow after dehydration. Thus, drying the suspension was excluded. The necessity of the use of wet pollen led us to conduct detailed studies of the dynamics of pollen wetting using microscopy.

Imaging under a stereo microscope showed a rapid movement of the pollen clump immediately upon contact with liquid. This rapid movement was mainly caused by the momentary swelling of the pollen grains (Video S1). Despite the vigorous movement, it

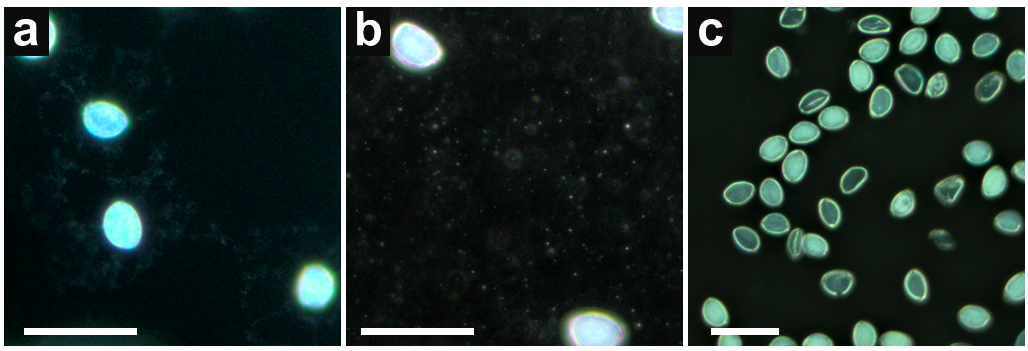

Figure 2. The dark field microscopy of pollen suspension. **(a)** pollen before washing — pollenkitt appears as a bright material around each pollen grain; **(b)** supernatant after vortexing and centrifusion — pollenkitt is no longer accumulated around each pollen grains, but rather scattered into fine granular particles; **(c)** pollen suspension after washing. Bars represent 50 μm.

 doi:10.20944/preprints202212.0429.v1

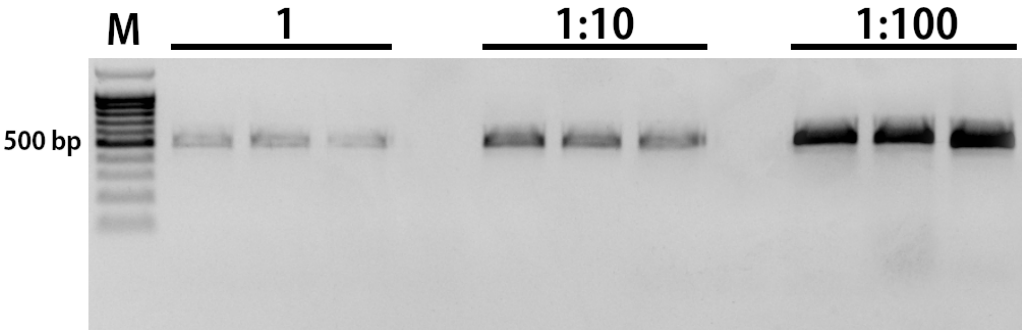

Figure 3. Inhibition of PCR upon addition of aqueous dilutions of pollenkitt to *A. cepa* genomic DNA as an amplification template and the LFS gene as a target. PCR experiment was repeated three times with each dilution. Dilution 1 represents the equivalent amount of pollenkitt eluated from 10 μg of pollen.

was noticeable that the neighboring pollen grains could not come into direct contact with each other, as if they were surrounded by an invisible structure (Video S2). In less than a minute, the rapid movement stopped, and all the pollen grains that were previously in one clump floated steadily on the surface of the droplet. Using phase-contrast microscopy a light-dense structure surrounding the floating pollen grains was observed (Figure 4a). We suggest that this structure is in fact the pollenkitt released from the surface of pollen grains upon contact with water. Within 4-5 minutes after contact with the liquid surface, adjacent fragments of pollenkitt began to join together, forming an interwoven "blanket" of pollenkitt components (Figure 4b).

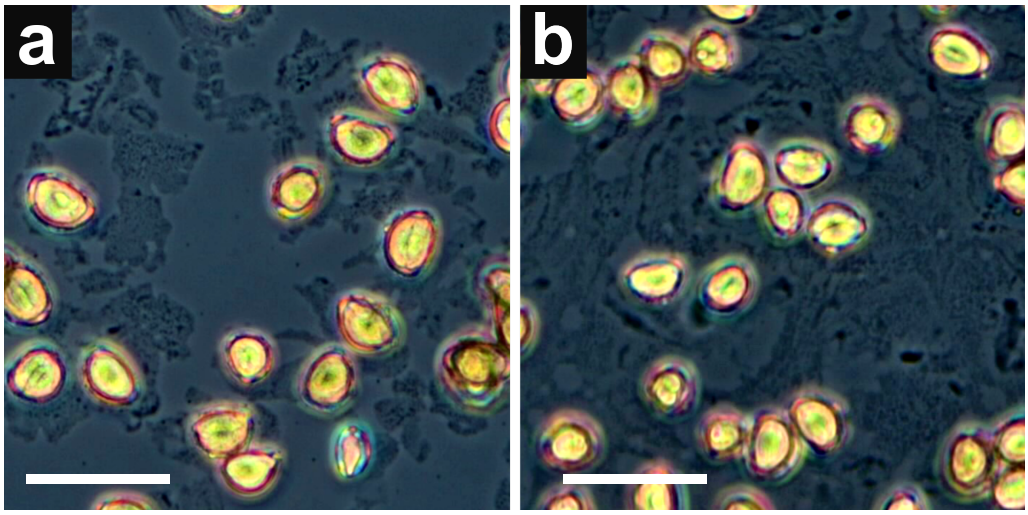

Figure 4. The phase-contrast microscopy of the pollenkitt in *A. cepa*: **(a)** pollen grains immediately after placing on a droplet of distilled water; pollenkitt appears as fragments released from the surface of pollen grains. **(b)** the same pollen grains after 5 minutes; pollenkitt appears as a "blanket" that keeps pollen grains on the surface of the droplet. Bars represent 50 μm.

This "blanket" obviously affects the floating and adhesion properties of pollen grains. Based on this observation the optimal technique of transferring the wet pollen grains onto the stigma was developed. Using an inoculation loop, a lump of the blanket was easily collected from a liquid surface by the hollow center of the loop which enabled us to easily apply an even layer of wetted pollen grains to the stigmatic surface (Video S3).

Two different liquid mediums were tested: (1) 12% (w/v) sucrose water solution and (2) pollen growing medium according to Brewbaker and Kwack [34] as following: 0.1 g/L H_3BO_3, 0.3 g/L $Ca(NO_3)_2 \times 4H_2O$, 0.3 g/L $MgSO_4 \times 7H_2O$, 0.1 g/L KNO_3 dissolved in 12% sucrose. There were two positive controls: (3) open pollination with insects and (4) hand

 doi:10.20944/preprints202212.0429.v1

pollination with fresh intact pollen grains using a brush. A negative control (5) was also used to test castration and inflorescence isolation.

The seed set percentage using pollen growing medium was the lowest — 35.7%, while open pollination by insects was the highest — 93.5% (Table 1). The percentage of set seeds in the experiment with 12% sucrose water solution was almost the same as in the positive control of intact pollen grains (63.5% and 65.5%, respectively). This result means that 12% sucrose water solution and the method of applying pollen using a loop are the most optimal for further work related to pollen genetic manipulations.

Table 1. The effect of different pollination methods on seed setting in the *A. cepa* umbels.

Treatment	Number of flowers in umbel	Number flowers that set seeds, (%)
Wet pollination with pollen growing medium	56	20 (35.7)
Wet pollination with 12% sucrose solution	52	33 (63.5)
Manual pollination with un-wetted pollen grains	61	40 (65.5)
Open pollination by insects	62	58 (93.5)
Negative control	0	0 (0)

In summary, our results demonstrate a feasible technique of using wetted pollen grains to pollinate onion flowers without affecting viability and seed setting. Minimizing influence of technical procedures is always favorable, especially in experiments as genetic manipulation. Therefore, engaging our wet pollination technique, and PCR using pollen DNA as a template might be very beneficial in future experiments based on genetic manipulation of onion pollen aimed to producing seeds with edited genome.

3. Materials and Methods

3.1. Plant materials and pollen collection

Bulbs of onion (*Allium cepa* L.) cultivar "Myachkovsky 300" were kindly provided by Federal Scientific Vegetable Center. By the end of April 2022 vernalized onion bulbs were planted within 40 cm distance in open field. Since flowering within one umbel is not synchronized, pollen was collected once or twice a day for 3-4 days. The pollen was used immediately or stored in a small Petri dish at –20 °C for further use.

3.2. PCR on pollen suspension

Using a sensitive analytical balance, 0.5 mg of pollen was weighed inside a 1.5 mL microtube. A simple washing technique was performed by vortexing in 500 μL of distilled water for 10 sec and centrifuged for 5 sec using minispin centrifuge (750×g). After discarding the supernatant, fresh distilled water was added and the same procedure was repeated for 10 rounds. To ensure that no precipitated pollen grains are lost during washing, only 450 μL of the supernatant was carefully discarded between rounds. As a result, after discarding the last supernatant, 50 μL of a suspension of pollen grains with a concentration of 10 $\mu g/\mu L$ is obtained.

PCR was performed using 10 μg of mature intact pollen (1 μl of the pre-washed suspension), or 50 ng of *A. cepa* genomic DNA as a positive control. 25 μL of 1× PCR mix contained 2.5 μL of 10× Taq Turbo buffer (25 mM $MgCl_2$, pH = 8.3) (Evrogen, Moscow), 0.2 mM of each dNTP (Evrogen, Moscow), 0.2 μM both forward and reverse primers and 5 U of Taq-polymerase (Evrogen, Moscow). Primers (F: TCAAGCACTGCAAACCTCTTC; R: ATGGAGCTAAATCCTGGTGCA) were designed for amplification of ORF (Open Reading Frame) of the LFS gene (GenBank: AB089203.1). Touchdown PCR was performed

 doi:10.20944/preprints202212.0429.v1

in order to amplify desirable amplicons without non-specific PCR-product. Cycling was performed as follows: 10 minutes of initial denaturation at 95 °C, 10 cycles (touchdown stage) — 30 seconds of denaturation at 95 °C, 30 seconds of annealing (from 67 °C to 62 °C, decrement at 0.5 °C/cycle), 45 seconds of elongation at 72 °C, 30 cycles (standard PCR) - 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 62 °C, 45 seconds of elongation at 72 °C, 10 minutes of final elongation at 72 °C. PCR product were visualized in agarose gel (2% of agarose in 0.5× TBE buffer, 0.5 μg/mL of ethidium bromide, 3 V/cm).

3.3. Evaluating the effect of pollenkitt on PCR

To test our hypothesis of pollenkitt inhibition of PCR, we conducted a series of PCR reactions with different dilutions of pollenkitt, using genomic DNA of *A. cepa* as a template and the LFS gene as an amplification target. PCR mix, cycling conditions and gel electrophoresis were performed as in the previous section (positive control). Pollenkitt was extracted from pollen grains as the following: in 1.5 mL tube, 1 mg pollen was thoroughly vortexed with 100 μL ddH_2O for 30 sec then centrifuged for 5 sec using minispin centrifuge (750×g). Since pollen grains are much heavier than the pollenkitt debris, they are precipitated first, and the latter remain in the supernatant. 1 μL of the pollen supernatant was immediately added to the PCR reaction either as a 1:10 or 1:100 aqueous dilution.

3.4. Microscopy of the onion pollenkitt

For microscope preparation, a large droplet of distilled water (100 μL) was placed on a glass slide. Using a microspatula or dissecting needle, clumps of pollen (~1 mg) were carefully placed on a drop. Microscopy was performed using Zeiss SterREO LUMAR.V12 and Zeiss Axiolab 5.

3.5. Wet manual pollination of onion flowers

Two different liquid mediums were tested in wet pollination experiments: (1) 12% (w/v) sucrose water solution and (2) pollen growing medium according to Brewbaker and Kwack [34] as following: 0.1 g/L H_3BO_3, 0.3 g/L $Ca(NO_3)_2 \times 4H_2O$, 0.3 g/L $MgSO_4 \times 7H_2O$, 0.1 g/L KNO_3 dissolved in 12% sucrose. The pollen clump was added to 100 μL of liquid medium placed on a glass slide.The floating cover of pollen was easily transferred to the stigma using a small bacterial inoculation loop (Video S3).

About 70-75% of the flowers in selected umbels have been removed to make subsequent steps more convenient.Umbels were proofed against flying insects using isolation cages made of 4-layered medical gauze. Castration was conducted on a daily basis. Due to the non-synchronous flowering, the umbrellas were constantly checked to immediately remove the immature anthers from the newly opened flowers. The flowering period for a single umbel (the time between the opening of the first flower until the drying of the last flower) lasted for 8-10 days on average.

Author Contributions: M.M. — invistigation, validation, visualization, writing - original draft, methodology; A.E. — invistigation, validation, writing - original draft, methodology, L.K. — conceptualization, funding acquisition, project administration, supervision, writing - original draft, writing - review & editing.

Funding: This research was funded by the Russian Foundation for Basic Research, grant number 20-016-00065.

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Acknowledgments: We thanks to Alexander Voronkov, K.A. Timiryazev Institute of Plant Physiology RAS, for practival advice on pollen collection.

Conflicts of Interest: The authors declare no conflict of interest.

 doi:10.20944/preprints202212.0429.v1

References

1. Paffetti, D.; Vettori, C.; Caramelli, D.; Vernesi, C.; Lari, M.; Paganelli, A.; Paule, L.; Giannini, R. Unexpected presence of Fagus orientalis complex in Italy as inferred from 45,000-year-old DNA pollen samples from Venice lagoon. *BMC Evolutionary Biology* **2007**, *7*, 1–13.
2. Parducci, L.; Suyama, Y.; Lascoux, M.; Bennett, K.D. Ancient DNA from pollen: a genetic record of population history in Scots pine. *Molecular Ecology* **2005**, *14*, 2873–2882.
3. Suyama, Y.; Kawamuro, K.; Kinoshita, I.; Yoshimura, K.; Tsumura, Y.; Takahara, H. DNA sequence from a fossil pollen of Abies spp. from Pleistocene peat. *Genes & genetic systems* **1996**, *71*, 145–149.
4. Alotaibi, S.S.; Sayed, S.M.; Alosaimi, M.; Alharthi, R.; Banjar, A.; Abdulqader, N.; Alhamed, R. Pollen molecular biology: Applications in the forensic palynology and future prospects: A review. *Saudi journal of biological sciences* **2020**, *27*, 1185–1190.
5. Lale, S.V.; Gill, H.S. Pollen grains as a novel microcarrier for oral delivery of proteins. *International journal of pharmaceutics* **2018**, *552*, 352–359.
6. Atwe, S.U.; Ma, Y.; Gill, H.S. Pollen grains for oral vaccination. *Journal of Controlled Release* **2014**, *194*, 45–52.
7. Ravindra, K.; Goyal, A.; Mor, S. Pollen allergy: Developing multi-sectorial strategies for its prevention and control in lower and middle-income countries. *International Journal of Hygiene and Environmental Health* **2022**, *242*, 113951.
8. Ziegenhagen, B.; Schauerte, M.; Kormutak, A.; Scholz, F. Plastid DNA polymorphism of megagametophytes and pollen in two Abies species. *Silvae genetica* **1996**, *45*, 355–358.
9. Aziz, A.N.; Seabrook, J.E.; Tai, G.C. Amplification of RAPD markers from single pollen grains of diploid (2N= 2X= 24) potato. *American journal of potato research* **1999**, *76*, 179–182.
10. Matsuki, Y.; Isagi, Y.; Suyama, Y. The determination of multiple microsatellite genotypes and DNA sequences from a single pollen grain. *Molecular Ecology Notes* **2007**, *7*, 194–198.
11. Aziz, A.; Sauve, R. Genetic mapping of Echinacea purpurea via individual pollen DNA fingerprinting. *Molecular Breeding* **2008**, *21*, 227–232.
12. Chen, P.H.; Pan, Y.B.; Chen, R.K. High-throughput procedure for single pollen grain collection and polymerase chain reaction in plants. *Journal of Integrative Plant Biology* **2008**, *50*, 375–383.
13. Mase, N.; Sawamura, Y.; Yamamoto, T.; Takada, N.; Nishio, S.; Saito, T.; Iketani, H. Direct genotyping of single pollen grains of a self-compatible mutant of Japanese pear (Pyrus pyrifolia) revealed inheritance of a duplicated chromosomal segment containing a second S-haplotype. *Euphytica* **2014**, *200*, 297–304.
14. Matsunaga, S.; Schütze, K.; Donnison, I.S.; Grant, S.R.; Kuroiwa, T.; Kawano, S. Single pollen typing combined with laser-mediated manipulation. *The Plant Journal* **1999**, *20*, 371–378.
15. Hess, D.; Gresshoff, P.M.; Fielitz, U.; Gleiss, D. Uptake of protein and bacteriophage into swelling and germinating pollen of Petunia hybrida. *Zeitschrift für Pflanzenphysiologie* **1975**, *74*, 371–376.
16. Ohta, Y. High-efficiency genetic transformation of maize by a mixture of pollen and exogenous DNA. *Proceedings of the National Academy of Sciences* **1986**, *83*, 715–719.
17. Booy, G.; Krens, F.; Huizing, H. Attempted pollen-mediated transformation of maize. *Journal of plant physiology* **1989**, *135*, 319–324.
18. Abdul-Baki, A.A.; Saunders, J.A.; Matthews, B.F.; Pittarelli, G.W. DNA uptake during electroporation of germinating pollen grains. *Plant Science* **1990**, *70*, 181–190.
19. Twell, D.; Klein, T.M.; McCormick, S. Transformation of pollen by particle bombardment. In *Plant tissue culture manual*; Springer, 1991; pp. 631–644.
20. Schreiber, D.N.; Dresselhaus, T. In vitro pollen germination and transient transformation ofZea mays and other plant species. *Plant Molecular Biology Reporter* **2003**, *21*, 31–41.
21. Zhao, X.; Meng, Z.; Wang, Y.; Chen, W.; Sun, C.; Cui, B.; Cui, J.; Yu, M.; Zeng, Z.; Guo, S.; et al. Pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers. *Nature plants* **2017**, *3*, 956–964.
22. Nagahara, S.; Higashiyama, T.; Mizuta, Y. Detection of a biolistic delivery of fluorescent markers and CRISPR/Cas9 to the pollen tube. *Plant reproduction* **2021**, *34*, 191–205.
23. Gogoi, N.; Kanwal, M.; Norman, M.; Downs, J.; Ahmad, N.; Mago, R.; Bariana, H.; Muellner, M.; Bansal, U.; Jones, B.J. Wheat pollen uptake of CRISPR/Cas9 RNP-PDMAEMA nanoassemblies results in targeted loss of gene function in progeny. *bioRxiv* **2022**.
24. Levin, I.; Gilboa, N. Direct PCR using tomato pollen grain suspensions. *BioTechniques* **1997**, *23*, 986–990.
25. Petersen, G.; Johansen, B.; Seberg, O. PCR and sequencing from a single pollen grain. *Plant Molecular Biology* **1996**, *31*, 189–191.
26. Imai, S.; Tsuge, N.; Tomotake, M.; Nagatome, Y.; Sawada, H.; Nagata, T.; Kumagai, H. An onion enzyme that makes the eyes water. *Nature* **2002**, *419*, 685–685.
27. Masamura, N.; McCallum, J.; Khrustaleva, L.; Kenel, F.; Pither-Joyce, M.; Shono, J.; Suzuki, G.; Mukai, Y.; Yamauchi, N.; Shigyo, M. Chromosomal organization and sequence diversity of genes encoding lachrymatory factor synthase in Allium cepa L. *G3: Genes | Genomes | Genetics* **2012**, *2*, 643–651.
28. Suyama, Y. Procedure for single-pollen genotyping. In *Single-pollen genotyping*; Springer, 2011; pp. 7–15.
29. Heslop-Harrison, J. Tapetal origin of pollen-coat substances in Lilium. *New Phytologist* **1968**, *67*, 779–786.
30. Pacini, E.; Hesse, M. Pollenkitt–its composition, forms and functions. *Flora-Morphology, Distribution, Functional Ecology of Plants* **2005**, *200*, 399–415.

31. Hesse, M. Pollenkitt development and composition in Tilia platyphyllos (Tiliaceae) analysed by conventional. *The Tapetum: Cytology, Function, Biochemistry and Evolution* **2012**, *7*, 39.
32. Dobson, H.E. Survey of pollen and pollenkitt lipids–chemical cues to flower visitors? *American journal of botany* **1988**, *75*, 170–182.
33. Wilson, I.G. Inhibition and facilitation of nucleic acid amplification. *Applied and environmental microbiology* **1997**, *63*, 3741–3751.
34. Brewbaker, J.L.; Kwack, B.H. The essential role of calcium ion in pollen germination and pollen tube growth. *American journal of botany* **1963**, *50*, 859–865.