The fastest and easiest way to transform genes to the pollen of a pear (Rosaceae) variety

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Method Article

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Abstract

Background To date, the success rate of using pollen as a transgenic vector in agriculture has been very low, especially for fruit trees of the Rosaceae family.

Results We selected a widely cultivated pear variety, 'Wonhuwang'(*Pyrus pyrifolia* Nakai. cv. Wonhuwang), which can be used to successfully transform genes into mature pollen within 4 min under negative pressure (- 80 Kpa). The pollen morphology of this pear variety is irregular, but its viability is not reduced. Pollen containing the green fluorescent protein (GFP) gene can complete pollination and fertilization, and the GFP gene is expressed in embryos. The greatest advantage of this method is that it does not require fresh pollen, and it can be used to verify gene function.

Conclusion This method is the simplest and fastest among reported transgenic methods for transforming genes into pollen.

Introduction

Great progress has been made in applying various transgenic plant technologies in crop species, including large-scale commercialization 1. Regardless of trends in transgenic technology development, transformation protocols should evolve toward simplicity, ease of operation and convenience of breeding 2,3. However, most techniques developed based on *Agrobacterium*-mediated and direct DNA transfer require time-consuming, labor-intensive, and expensive procedures such as long-term in vitro culture, creating the conditions necessary for regenerating protoplasts or explants 4, and the use of expensive instruments such as particle guns 5. Pollen grains, which are ideal vectors for introducing foreign genes into the germline, are abundant and easy to manipulate 6,7. However, the success rate of using pollen as a vector to generate transgenic plants is very low, especially for trees 8, mainly because two wall layers are formed during pollen development 9,10, preventing foreign genes from entering the pollen 11. This is the first and most difficult step when using pollen as a vector for transgenic plants. Therefore, researchers have developed 12 methods to introduce foreign genes into the pollen of crop species 6. Systems for generating transgenic plants have been more recently developed for tree species than for crop species, and efficient transformation systems for many species have not yet been successfully established. Moreover, the complexity and diversity of tree genomes make generating transgenic plants more difficult in tree species. The establishment of a tree transgene system for tree breeding is urgently needed 8. In this study, from a large number of pear varieties, we successfully selected the widely cultivated 'Wonhuwang' pear, into whose pollen foreign genes can be easily introduced.

Materials and methods

Materials

Pollen collection

Blooming pear flowers were harvested, and pollen was collected. The pollen was then preserved at -20 °C.

Methods

Pollen germination—a test of pollen viability

Pollen grains were germinated in medium containing 0.55 mmol/l $Ca(NO_3)_2$, 1.6 mmol/l H_3BO_3, 1.6 mmol/l $MgSO_4$, 1 mmol/l KNO_3, 440 mmol/l sucrose and 5 mmol/l 5,2-(N-morpholino) ethane sulfonic acid hydrate (MES), and the pH was adjusted to 6.0 with Tris.

Monitoring of pollen tube growth in the style

After rinsing with water, fixed styles of the 'Qiuyue' variety were cleared in KOH (5%) at room temperature until most tissues became transparent. They were then rinsed in water and stained for more than 1 h with aniline blue (0.1% in 1/30 mol/L K_3PO_4). Pollen tube growth in the style tissue was examined under a fluorescence microscope (EVOS Auto 2, Thermo Fisher, USA).

Arabidopsis pollen tubes

Arabidopsis (*Arabidopsis thaliana*) plants were cultivated at light intensities ranging from 120-150 μmol m^{-2} s^{-1} over a 12-h daily light period at a temperature of 22 ± 2 °C.

Newly opened *Arabidopsis thaliana* flowers were picked, and pollen grains were dusted onto the medium. The pollen was cultured for 6 h at 25 °C on medium supplemented with 18% (w/v) sucrose, 0.01% (w/v) H_3BO_3, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, and 1 mM $MgSO_4$ at pH 7.0 (KOH).

Electron microscopy of maize pollen

After spraying with gold, the pollen grains were observed under a scanning electron microscope (ZEISS Merlin Compact, Germany) at 3.0 kV.

Measurement of the total anthocyanin content in the pollen tube

Total anthocyanins were extracted using a methanol-HCl method and measured as described by Lee and Wicker (1991) 12.

Quantitative real-time RT-PCR to determine gene expression

To estimate mRNA expression levels, qRT—PCR analysis was performed. Total RNA was extracted from pollen and styles with TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized from 2 μg of total RNA using an Omniscript RT kit (Qiagen). The primers for MdGH3.1 were F: CTCGGATATGGCCAAACACG and R: AGAAGGCTTGCACATAGGGT. Gene expression data were normalized to actin gene levels and are expressed as ratios to the relevant control (assigned a value of 1).

Statistical analyses

The data are expressed as the means ± SEs and were analyzed using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences between the experimental groups were analyzed using Student's t test.

Results

Introduction of the GFP gene into pollen by negative pressure infiltration

To determine whether foreign genes can be transformed into pollen, we used a negative pressure infiltration method for 4 min to insert the GFP gene into pollen grains and then cultured the pollen for 2-3 h, after which GFP expression in pollen tubes was assessed (Figure 1A). As a control, the pollen tube of normally cultured ‘Wonhuwang’ pollen had no fluorescence, and only the pollen grains exhibited fluorescence (Figure 1B), corresponding to self-fluorescence. After the empty vector was introduced, the pollen tube still exhibited no fluorescence (Figure 1C). However, the pollen tube of pollen transformed with the GFP gene showed obvious fluorescence (Figure 1D). We selected another pear variety (*Pyrus pyrifolia* ‘Qiuyue’) and treated it in the same manner, and we observed no fluorescence in the pollen tube (Figure 1E). The pollen tubes of *Arabidopsis thaliana* also did not fluoresce when treated via the same method (Figure 1F). Moreover, negative pressure infiltration had no significant effect on the growth of pollen tubes or the pollen germination rate (Supplementary Figure S1A and B). These results suggested that genes can be introduced into ‘Wonhuwang’ pollen by negative pressure infiltration and can be expressed in the pollen tube.

Scanning electron microscopy (SEM) was used to observe the pear pollen. The normal dried pollen grains of ‘Qiuyue’ were prolate elliptic and tricolpate monads (Figure 2A). The polar diameter range was 32–48 μm, and the equatorial diameter range was 14–25 μm (Supplementary Figure S2). In contrast, the pollen of ‘Wonhuwang’ significantly differed from that of ‘Qiuyue’ (Figure 2B). Many of the pollen grains showed irregular morphology, but this did not affect the viability of the pollen (Figure 2B). There was no significant difference between the germination rate and the length of the pollen tubes of ‘Wonhuwang’ pollen and the pollen of the other variety in vitro (Supplementary Figure S1). ‘Wonhuwang’ pollen was used to pollinate the stigma of the ‘Qiuyue’ pear, and the pollen tube grew to the bottom of the style (Figure 2C). In the case of self-pollinating ‘Wonhuwang’, the pollen tube reached only one-third of the length of the stigma (Figure 2D), indicating that ‘Wonhuwang’ pear has typical gametophytic self-incompatibility. This finding also further suggested that the irregular morphology of ‘Wonhuwang’ pollen does not decrease its viability.

GFP gene-transformed ‘Wonhuwang’ pollen pollinated the style of ‘Qiuyue’ pear

Unpollinated stigmas (Figure 3A and B), stigmas with pollen not transformed with the GFP gene (control) (Figure 3C and D) and stigmas with pollen transformed with the empty vector (Figure 3E and F) exhibited no fluorescence. However, after pollination with pollen harboring the GFP gene, there was obvious

fluorescence in the stigma (Figure 3G and H). After 15 d of pollination, the young fruit was cut to observe the embryo. The fluorescence of the control embryo was very weak (self-fluorescence) (Figure 3I and J), while the embryo transformed with the GFP gene exhibited obvious fluorescence (Figure 3K and L). These results suggested that the pollen containing the GFP gene can complete pollination and fertilization.

Verification of gene overexpression

The transcription factor MdMYB1 can directly regulate the accumulation of anthocyanins in apple (*Malus domestica*) fruit cells 13. We transformed a plasmid containing the MdMYB1 gene linked to a reporter gene, which can also be expressed in pollen tubes, into ‘Wonhuwang’ pollen via negative pressure infiltration (Figure 4A). After 3 h of cultivation, the anthocyanin content of the transgenic pollen significantly increased (Figure 4B).

When we studied metabolic physiological disorders in apple fruits, the auxin response gene MdGH3.1 was significantly overexpressed at the site of metabolic disorder 14. This gene can inhibit auxin synthesis and cause plant dwarfing 15,16. We transformed this apple gene into ‘Wonhuwang’ pollen via negative pressure infiltration. The gene was highly expressed in the pollen tube of ‘Wonhuwang’ (Figure 5A) and inhibited pollen tube growth (Figure 5B). When auxin was added to the culture medium, pollen tube growth was restored (Figure 5C). These results suggested
that ‘Wonhuwang’ pollen can be used for gene function verification.

Discussion

The existing method of introducing foreign genes into pollen, the first step of generating transgenic plants with pollen as a vector, is not suitable for fruit trees of the Rosaceae family. When vacuum infiltration is used, fresh buds are needed to allow foreign genes to enter pollen. At this time, the pollen is immature, and the whole plant must be under vacuum conditions during the procedure 17. The ultrasonic introduction of foreign genes requires fresh pollen and decreases pollen viability. After pollination, maize pollen produced by ultrasonication can only produce an average of 0.5-2 seeds per ear 18. Nanomagnetic technology provides a new method of introducing foreign genes into fresh maize pollen 18 and cotton pollen 11, though with very controversial results 19. *Agrobacterium*-mediated transformation is one of the most commonly used methods, but its efficiency is very low, especially for fruit tree pollen. Particle gun bombardment requires fresh pollen and expensive equipment. For Rosaceae fruit trees such as temperate apple and pear fruit trees, the annual flowering period is approximately 7 d. The above methods of introducing foreign genes into pollen are difficult in practice.

At present, gene function verification in fruit trees involves the transfer of genes into herbaceous plants, such as Arabidopsis, tobacco, or tomato. These plants differ greatly from Rosaceae fruit trees in terms of their biological characteristics and have a long life cycle. The pollen tubes of pear grow very quickly and begin to germinate after 30 min. Pollen of this variety and the transgenic method presented here can be used for rapid verification of the gene function of fruit trees.

A better prospect for this approach is its application to fruit tree breeding. Since the most appreciated pear varieties have a high degree of heterozygosity and since crossbreeding leads to considerable progeny variation, using this traditional method to select new genotypes is time-consuming and expensive 20. The S genotype of ‘Wonhuwang’ is S3St, which can be pollinated with any pear variety that does not contain both the S3 and St genotypes. Pollen can quickly transfer the target gene into pear seeds, possibly overcoming some of the limitations of traditional breeding to improve selection efficiency.

Conclusion

In summary, the negative pressure infiltration method can be used to quickly transform foreign genes into ‘Wonhuwang’ pollen and express them. In addition, the plasmid can enter the pollen of this variety without negative pressure infiltration, but the gene expression level was lower than that under negative pressure infiltration (Supplementary Figure S3 and Fig. 4C).

Declarations

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Author contributions

Q.H.Y. conceived and designed the study. S.Q.R., W.L.R. and Z.Z.Y. performed the experiments. Q.H.Y. and Z.Y.G. analyzed the data. Q.H.Y. wrote the manuscript text and prepared Figures 1-5. All authors reviewed the manuscript.

The authors declare that they have no conflicts of interest regarding the content of this article.

Ethical approval

Ethics approval was not required for this research.

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Figures

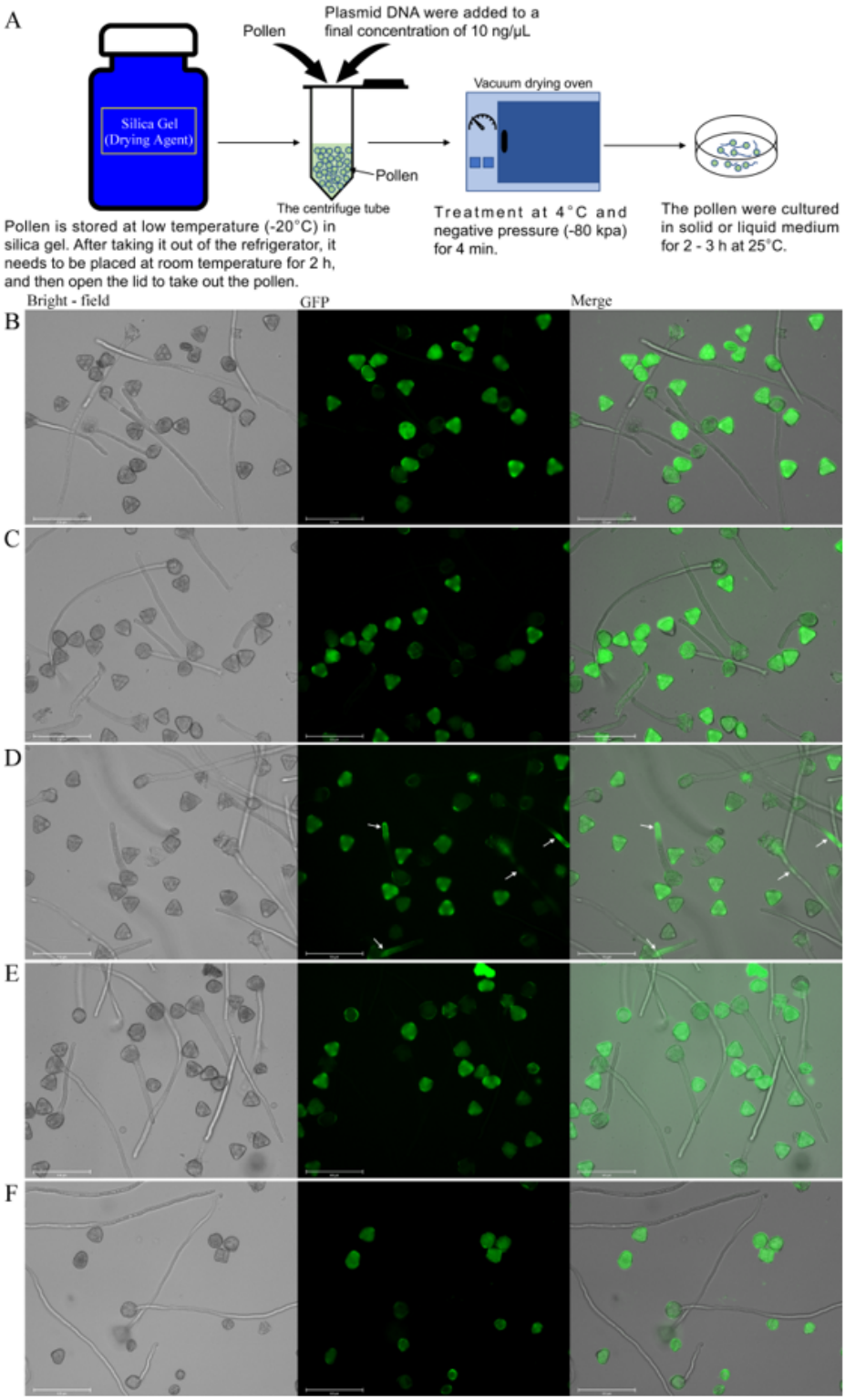

Figure 1

Verification of transgenic pollen transformed with the GFP gene. Pollen culture was performed for 3 h at 25 °C. (A) The process of transforming genes into pollen by negative pressure infiltration. (B) No transgenic ‘Wonhuwang’ pollen (control). (C) The empty vector was transformed into ‘Wonhuwang’ pollen. (D) The plasmid containing the GFP gene was transformed into ‘Wonhuwang’ pollen. The white arrow points to where the fluorescent protein-encoding gene is expressed. (E) The plasmid containing the

GFP gene was transformed into the pollen of the pear cultivar ‘Qiuyue’. (F) The plasmid containing the GFP gene was transformed into the pollen of *Arabidopsis thaliana*.

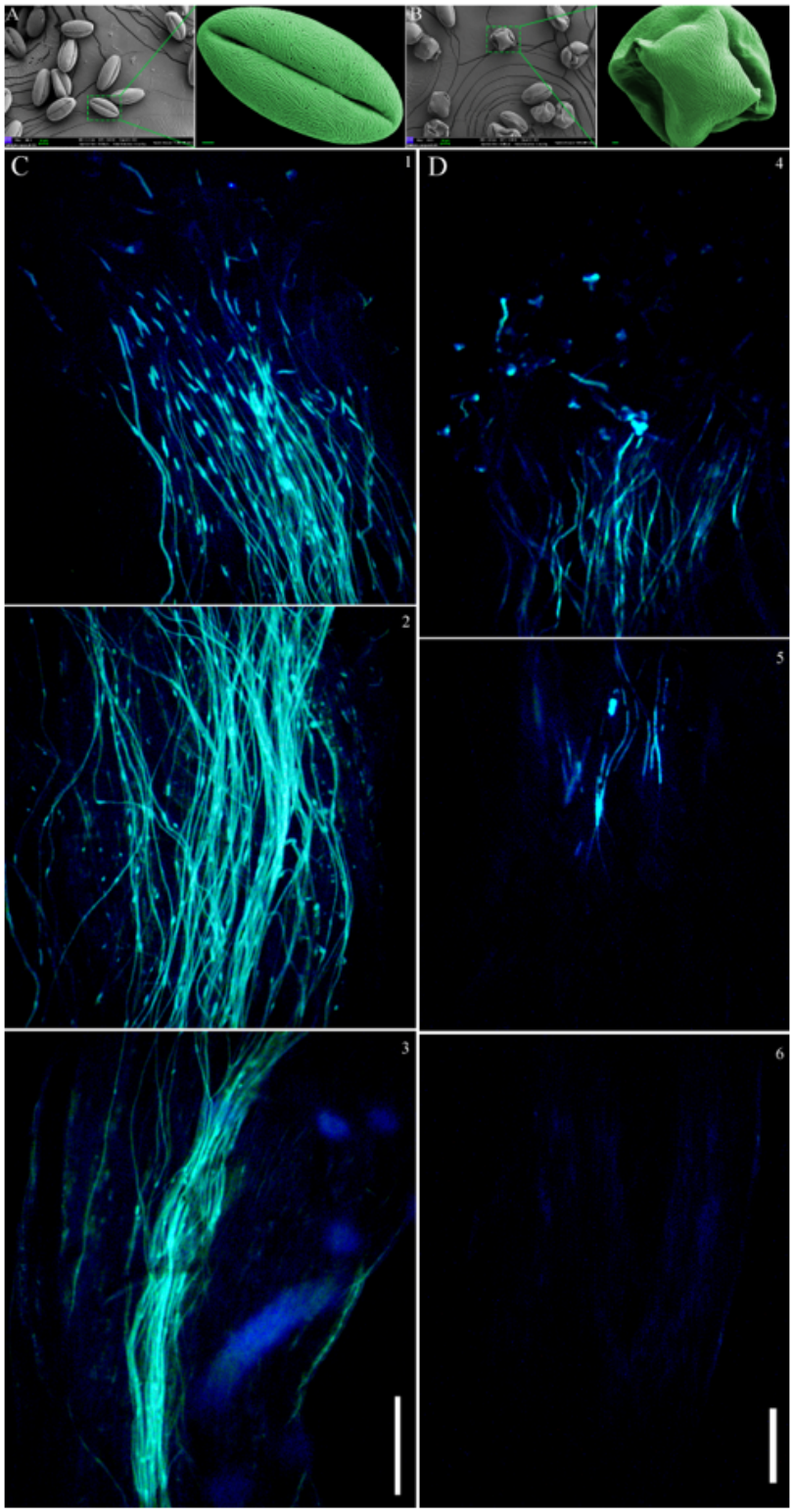

Figure 2

Scanning electron microscopy (SEM) of pollen and in vivo pollination. (A) SEM images of the dried pollen of the ‘Qiuyue’ cultivar. The scale bar is 2 μm in A. (B) SEM images of the dried pollen of the cultivar

‘Wonhuwang’. The scale bar in B is 1 μm. (C) A pollinated style stained with aniline blue. The growth of the ‘Wonhuwang’ pollen tube in the ‘Qiuyue’ pear style for 72 h (compatible pollination). 1. Upper style of ‘Qiuyue’, pollinated with ‘Wonhuwang’ pollen; 2. middle style of ‘Qiuyue’, pollinated with ‘Wonhuwang’ pollen; 3. lower style of ‘Qiuyue’, pollinated with ‘Wonhuwang’ pollen. (D) Growth of the ‘Wonhuwang’ pollen tube in the ‘Wonhuwang’ pear style after 72 h (incompatible pollination). 4. Upper style with self-pollination; 5. middle style with self-pollination; 6. lower style with self-pollination. The scale bar is 0.8 mm in (C and D).

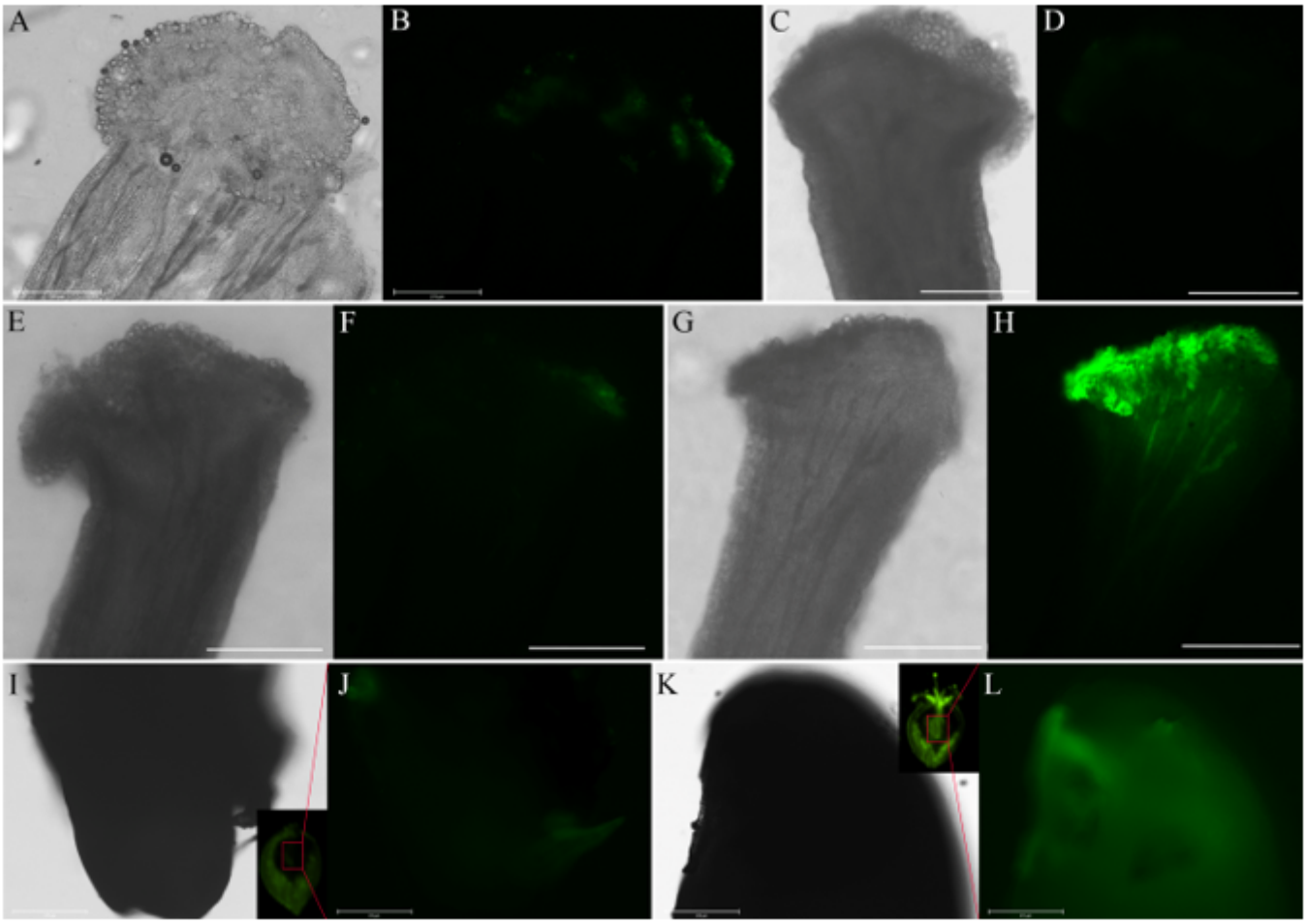

Figure 3

‘Wonhuwang’ pollen transformed with the GFP gene pollinates the stigma of ‘Qiuyue’. (A - B) The stigma of ‘Qiuyue’ without pollination. (C - D) Nontransgenic ‘Wonhuwang’ pollen was used to pollinate the stigma of ‘Qiuyue’ for 30 h. (E - F) ‘Wonhuwang’ pollen transformed with the empty vector was used to pollinate the stigma of ‘Qiuyue’ for 30 h. (G - H) The stigma of ‘Qiuyue’ pear was pollinated with ‘Wonhuwang’ pollen transformed with the GFP gene for 30 h. The scale bar represents 275 μm. (I – J) Control: The stigma of ‘Qiuyue’ pear was pollinated with ‘Wonhuwang’ pollen without the GFP gene for 15 d. The inner image in (I) shows the cut young fruit, and the innermost seed cavity was observed by a stereo fluorescence microscope. (I) Bright field image obtained with a fluorescence microscope. (J) The GFP channel was observed with a fluorescence microscope. (K – L) The stigma of ‘Qiuyue’ pear was

pollinated with ‘Wonhuwang’ pollen transformed with the GFP gene for 15 d. The inset image in (K) shows the cut young fruit, and the innermost seed cavity was observed by stereo fluorescence. (K) Bright field image obtained with a fluorescence microscope. (L) The GFP channel was observed with a fluorescence microscope.

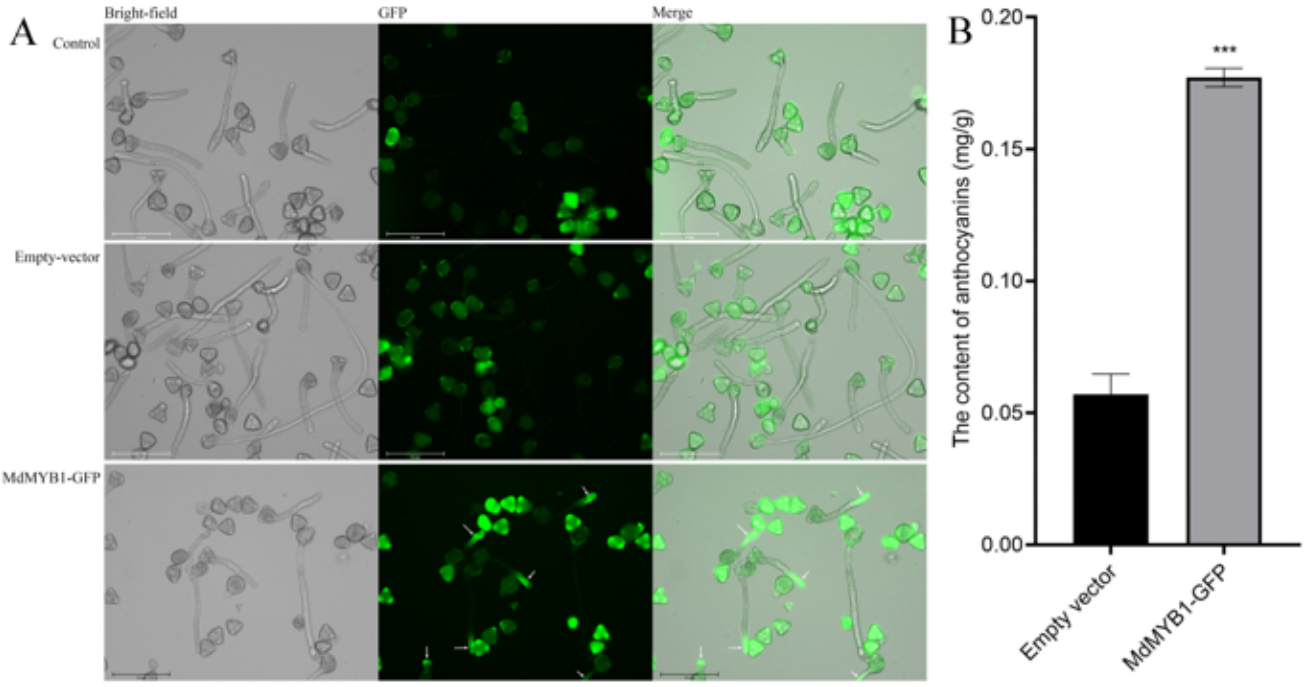

Figure 4

The MdMYB1-GFP gene was transformed into ‘Wonhuwang’ pollen, which was subsequently cultured on solid medium for 3 h. (A) No transgenic ‘Wonhuwang’ pollen (control). The empty vector was transformed into ‘Wonhuwang’ pollen (empty vector). The plasmid containing the MdMYB1-GFP gene was then transformed into ‘Wonhuwang’ pollen (MdMYB1-GFP). The white arrow points to where the fluorescent protein-encoding gene is expressed. (B) The content of anthocyanins in pollen tubes. *** indicates significant differences at $P < 0.001$. The data are presented as the means ± standard errors.

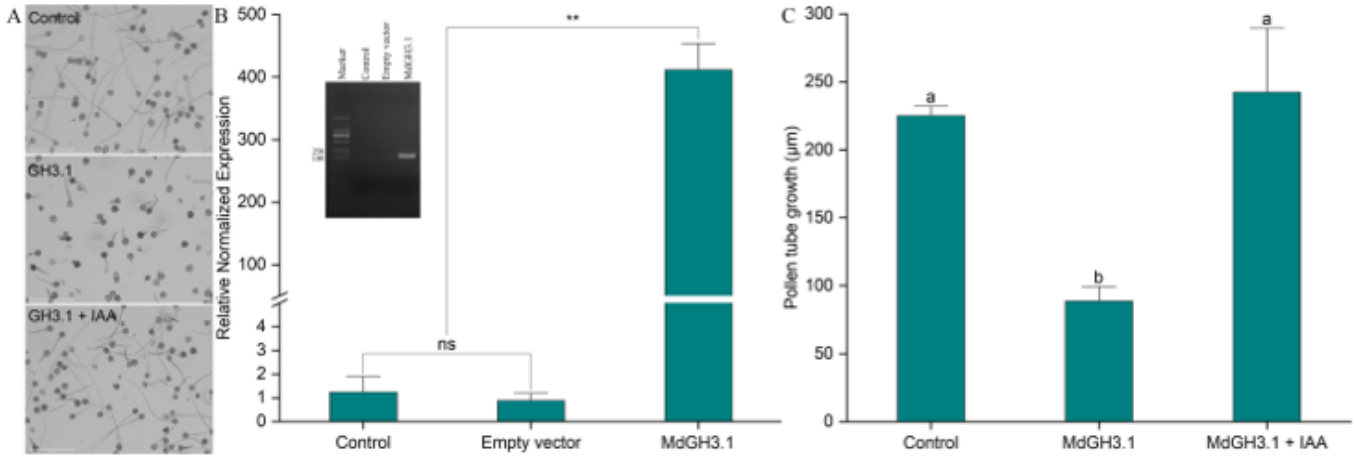

Figure 5

The MdGH3.1 gene of apple was transformed into ‘Wonhuwang’ pollen for functional verification. (A) Pollen culture in vitro for 3 h. Pollen that was not transformed was used as a control. The MdGH3.1 gene was transformed into ‘Wonhuwang’ pollen via negative pressure infiltration, after which the pollen was subsequently cultured on medium (GH3.1). ‘Wonhuwang’ pollen was cultured on medium supplemented with 6 μmol/L IAA after the pollen was transformed with the MdGH3.1 gene (GH3.1 + IAA). (B) Quantitative measurement of MdGH3.1 expression in pollen tubes. The inner image shows the DNA gel electrophoresis bands after quantitative PCR. ns: No significant difference. **P < 0.01. (C) Statistical analysis of pollen tube length. The data are presented as the means ± standard errors. Different letters indicate significant differences (P < 0.05).

Supplementary Files

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