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SUPPLEMENTARY INFORMATION

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Pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers

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Supplementary Information

Novel pollen magnetofection for plant genetic modification with magnetic nanoparticles as gene carriers

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Comparisons of pollen magnetofection and other methods

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Materials and Methods

Materials

Plasmids DNA: the pBI35SBTΔαCPTI plasmid contains *BTΔα*•*CPTI* (*BTΔα*: deletion the first α helix of *GFM Cry1Ac* gene; *CPTI*: Cowpea trypsin inhibitor gene) fusion gene under the control of a p35S promoter. The plasmid size is 16.8 Kb (see Supplementary Fig. 23).

The pBI121GUS plasmid harbors β-glucuronidase (*GUS*) gene under the control of a p35S promoter. The plasmid size is 14.6 Kb (see Supplementary Fig. 24).

The pBI35SBTCPTI plasmid contains *BT*•*CPTI* (*BT*: insecticide gene *GFM Cry1A*; *CPTI*: Cowpea trypsin inhibitor gene) fusion gene under the control of a p35S promoter. The plasmid size is 16.9 Kb (see Supplementary Fig. 25).

The pBI35SCTPBT plasmid contains *CTPBT* (*CTP*: Chloroplast target peptide; *BT*: insecticide gene *GFM Cry1A*) fusion gene under the control of a p35S promoter. The plasmid size is 15.2 Kb (see Supplementary Fig. 26).

The pBI35SMTPBT plasmid contains *MTPBT* (*MTP*: Mitochondrion target peptide; *BT*: insecticide gene *GFM Cry1A*) fusion gene under the control of a p35S promoter. The plasmid size is 15.1 Kb (see Supplementary Fig. 27).

The linearized plasmid 35S::CP4EPSPS::tNOS-EF1α::CP4EPSPS::tNOS contains two transcript boxes of *CP4-EPSPS* gene, which controlled by 35S promoter and EF1α promoter respectively. The linearized plasmid size is 7.8 Kb (see Supplementary Fig. 28).

The pBI35SGhSERK plasmid contains *GhSERK* (Cotton somatic embryogenesis receptor-like kinase) gene under the control of a p35S promoter. The plasmid size is 16.6 Kb (see Supplementary Fig. 29).

The pBIArf1SERK plasmid contains *GhSERK* (Cotton somatic embryogenesis receptor-like kinase) gene under the control of an Arf1 promoter. The plasmid size is 18.11 Kb (see Supplementary Fig. 30).

Plant materials: cotton cultivar Y18 (*G. hirsutum* L.) and SU12 (*G. hirsutum* L.), were provided by Biotechnology Research Institute, Chinese Academy of Agricultural Sciences. Pepper pollen, pumpkin pollen, cocozelle pollen and lily pollen were provided by The Institute of vegetables and Flowers, Chinese Academy of Agricultural Sciences. Polyethyleneimine modified Fe_3O_4 magnetic nanoparticles (MNPs) were purchased from Chemicell (Berlin, Germany).

Methods

PCR analysis. Genomic DNA of cotton was isolated from leaf materials of

transgenic lines using the hexadecyltrimethylammonium bromide (CTAB) method[1]. Primers used in PCR were listed as follows:

CAGGGCTCCTATGTTCTCTTGG (BT-F)

GTTGCAGTAACTGGAATGAACTCG (BT-R).

CGTCGCTCGTCGTGCGTGGCCG (CP4_ F)

ATGTATAATTGCGGGACTCTAATC (CP4_R)

AAAGATGTCGGAAGTGGTTAG (SERK_F)

CCGCCTCCACGTCAACTCACTCGAG (SERK_R)

PCR reaction mixture (50 μL) consisted of 10 ng cotton genomic DNA, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 10 nM of each primer and 2.5 units of Taq DNA polymerase (Takara, Japan). BTΔα•CPTI plasmid was used as a positive control. The PCR mixture was denatured at 95℃ for 5 minutes, amplified 35 cycles (94℃ for 30 seconds, 60℃ for 30 seconds and 72℃ for 30 seconds), and then incubated at 72℃ for 10 minutes. PCR products were analyzed by 1% (w/v) agarose gel electrophoresis.

RT PCR. Leaf total RNA of all transgenic cotton plants were extracted by the EASYspin RNA extraction kit (Yuan Ping Hao Bio Co Ltd, China. CAT. No. HF109-02). The quality and concentration of total RNA were analyzed by agarose gel electrophoresis and spectrophotometric analysis. And first-strand cDNA was synthesized by Rever Tra Ace-α kit (TOYOBO Co., Ltd. Japan. CAT. No.FSK-100). RT PCR was conducted by amplification of cDNA and gel electrophoresis analysis of PCR products. Cotton elongation factor 1α gene (EF1α) was used as RT PCR standard control. Each template cDNA was diluted according to standard control gene's amplification product until the quantity of all samples amplification product was consistent. And diluted cDNA templates were used to amplify exogenous gene. Variation of exogenous gene expression was analyzed by gel electrophoresis of the

amplification products. The RT PCR program was as follows: pre-denaturation at 94℃ for 5 min; 25 cycles of amplification (94℃ for 30 s, 58℃ for 30 s, 72℃ for 30 s); and then 5 min at 72℃. Primers used in RT PCR and real time PCR were listed as follows:

AGACCACCAAGTACTACTGCAC (EF1α_F)

CCACCAATCTTGTACACATCC (EF1α_R)

TGGGGTAATTCATCCATCTTCTC (BT_ F)

GTTGCAGTAACTGGAATGAACTCG (BT_ R)

ACGCGATAGAAAACAAAATATAG (CP4_ F)

GGCGCCCATGGCCTGCATGGC (CP4_ R)

AAAGATGTCGGAAGTGGTTAG (SERK_F)

CCGCCTCCACGTCAACTCACTCGAG (SERK_R)

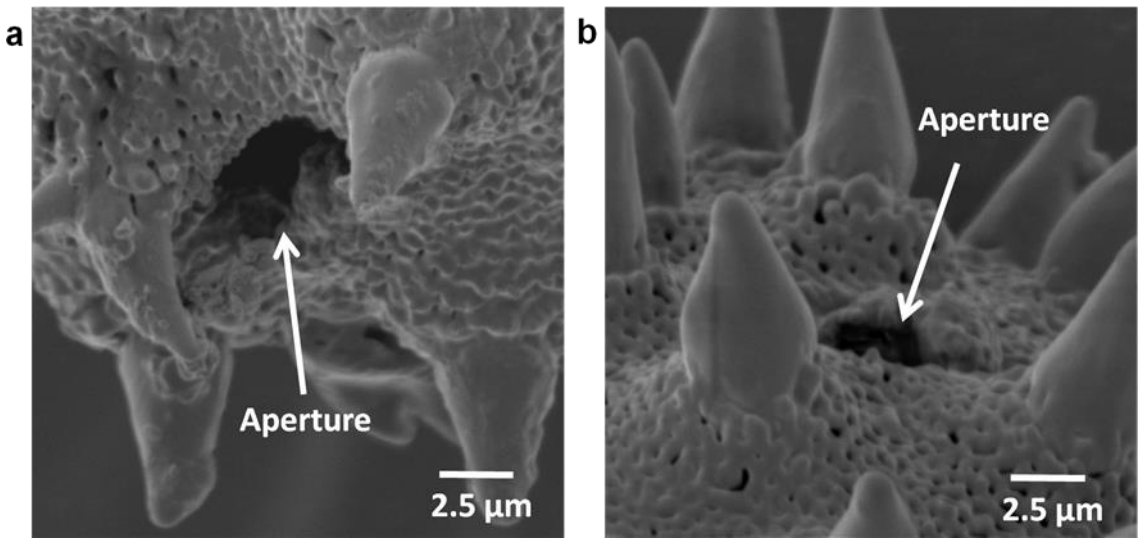
Quantitative Real Time PCR. Quantitative real time PCR was performed using SYBR® Green real time PCR Master Mix (TOYOBO Co., Ltd. Japan. CAT. No. QPK-201), according to the manufacturer's instructions. The cDNA templates for real time PCR analysis were prepared as described above. The standard control in the real time PCR was cotton elongation factor 1α gene (EF1α). The program of real time PCR was as follows: pre-denaturation at 98℃ for 30 s; amplification for 30 cycles (98℃ for 10 s, 60℃ for 30 s, 72℃ for 30 s and reading fluorescence data from plate); and then elongation for 10 min at 72℃. Amplified product melting curve analysis was performed to check the specificity of quantitative real time PCR and the temperate was set from 50℃ to 95℃ (1 s hold per 0.5℃ increase). The threshold value was 0.5, and the threshold cycles (CT) were calculated with the Chromo4 Real Time PCR System (Bio-RAD) software.

Southern blot analysis. Genomic DNA (20 μg) was digested with *Hind* III and

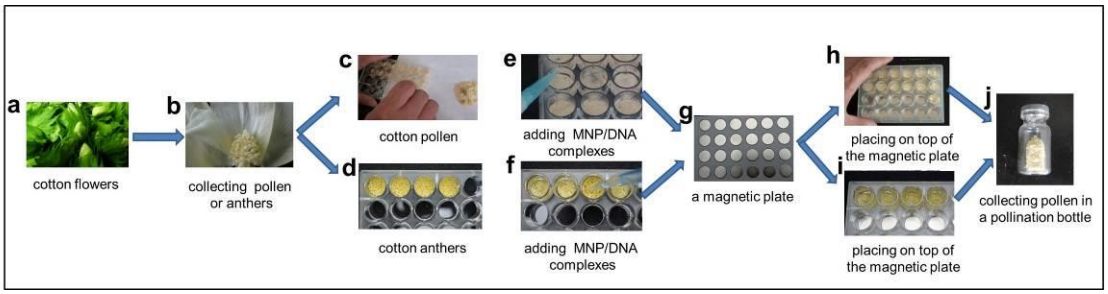
*Eco*RⅠrespectively, overnight, then was separated on a 0.8% agarose gel and transferred onto a Hybond-Nylon^{+} film (Amersham, USA). The PCR products were used as the template DNA for probes preparing. The DIG-labeled probe was prepared and hybridized according to the DIG High Prime DNA Labeling and Detection Starter Kit Ⅱ (Roche, USA) manufacturer's protocols.

ELISA analysis of BT protein. Cotton leaf samples (0.1g) were grinded and mixed with 500 μL of extraction buffer, and incubated for 10 minutes at 4℃. Then samples were centrifuged at 12000 rpm for 10 minutes at 4℃. 10 μL extracted solution was transferred into an EP tube and diluted 100 times by ddH_2O. 50 μL of each diluted sample were added into a 96 wells plate. The ELISA assay was performed using the QualiPlate Kit for Cry1Ab/Cry1Ac (EnviroLogix, USA, Catalog Number AP 003 CRBS).

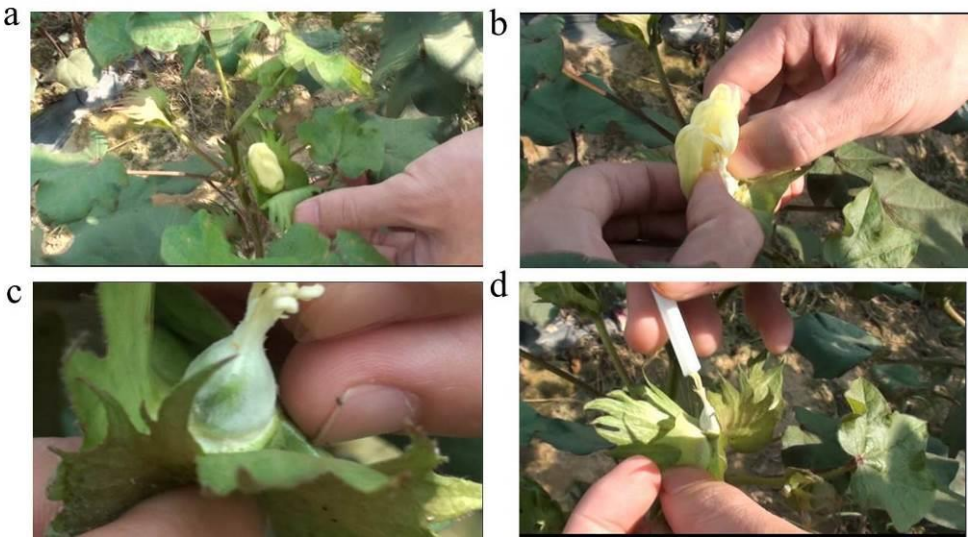
Supplementary Figures



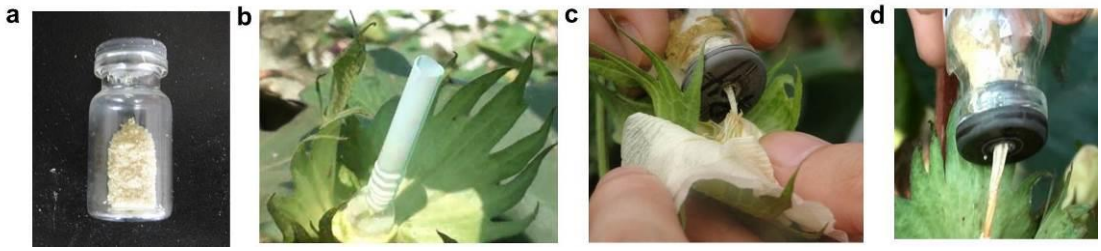
Supplementary Figure 1. SEM images of cotton pollen aperture.



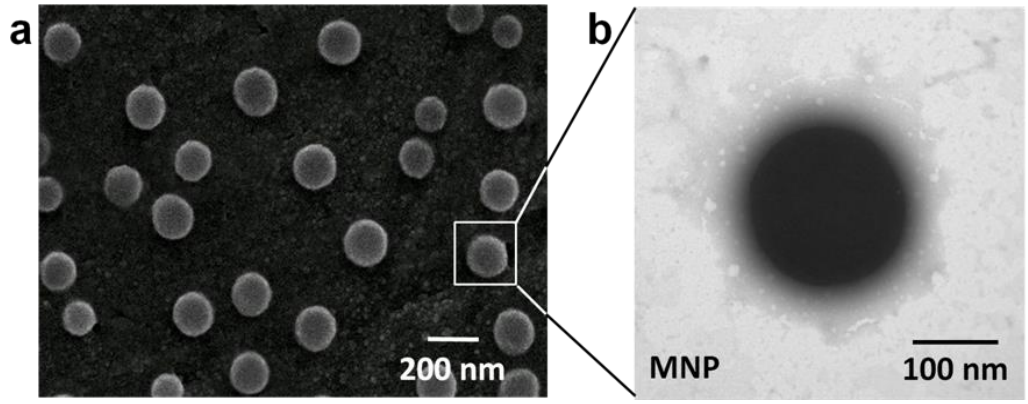
Supplementary Figure 2. Schematic illustration of pollen magnetofection experiment procedure. (a) Cotton flowers in 1 DBA; **(b)** Opening the flowers and collecting the pollen or anthers; **(c) (d)** Placing the pollen or anthers in a 24-well plate; **(e) (f)** Mixing with MNP/DNA complexes; **(g)** A magnetic plate; **(h) (i)** Placing the 24-well culture plate on top of the magnetic plate; **(j)** Collecting the magnetofected pollen in a pollination bottle.



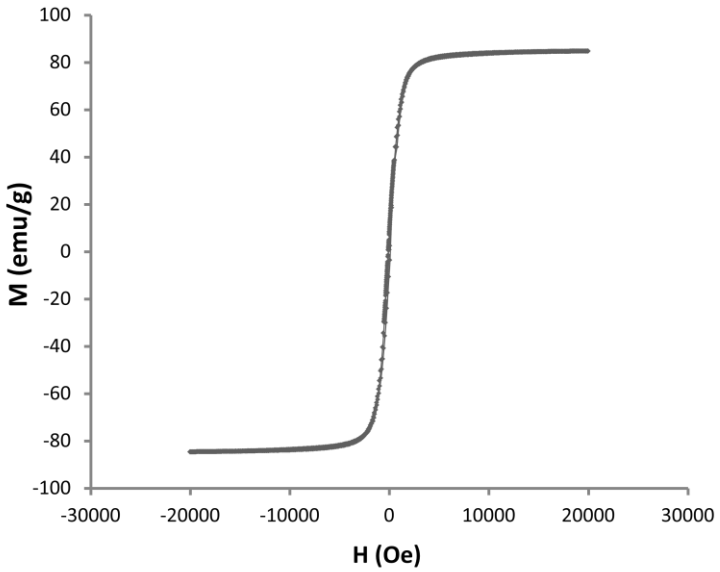
Supplementary Figure 3. The emasculation process of cotton flowers in 1 day before anthesis (DBA). (a) Selecting a cotton flower; **(b)** Opening the petals; **(c)** Removing the anther; **(d)** Casing protection of the stigma.



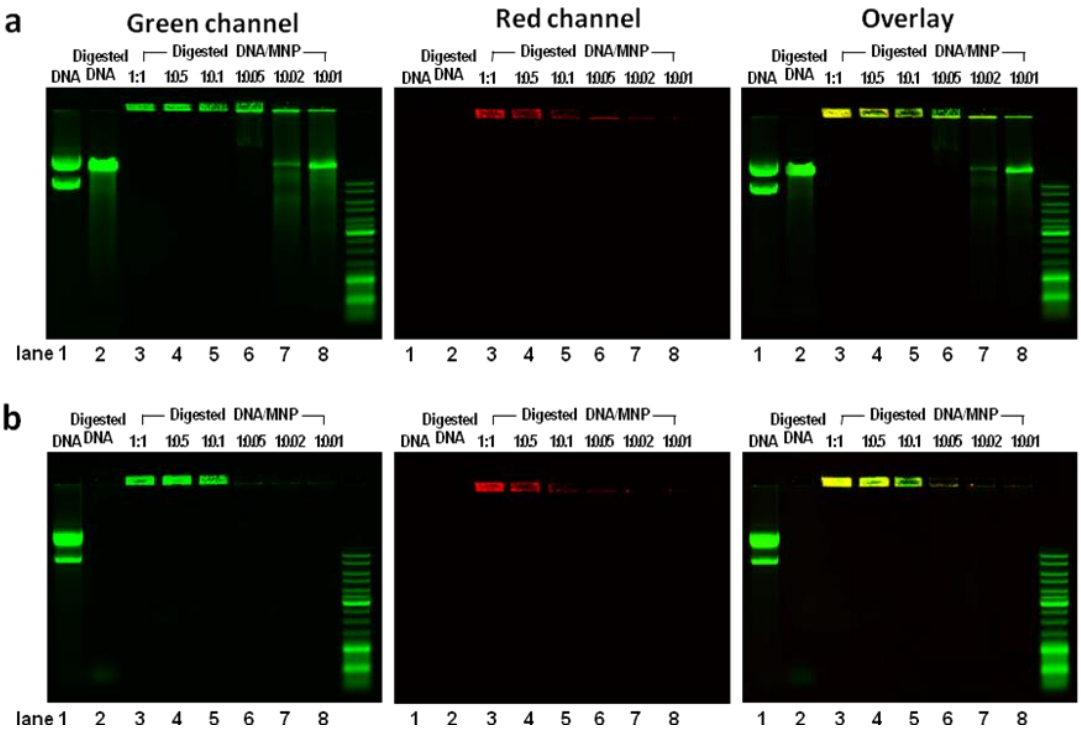
Supplementary Figure 4. Artificial pollination with magnetofected pollen. (a) Collecting the magnetofected pollen in a pollination bottle; **(b)** Taking out the casing pipe on the emasculated stigma; **(c) (d)** Artificial pollination on the stigmas with the pollination bottle.



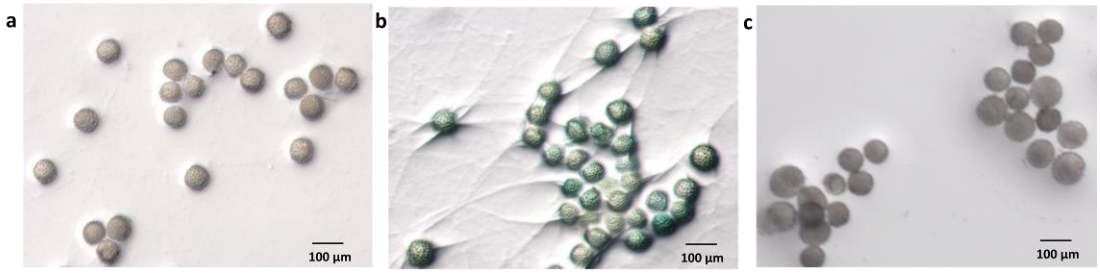
Supplementary Figure 5. SEM (a) and TEM (b) images of MNPs. The SEM image shows the spherical shape of the magnetic nanoparticles with diameter about 150 nm. Magnifying TEM image shows that MNPs are the core-shell structures of Fe_3O_4 cores coated with PEI shells.



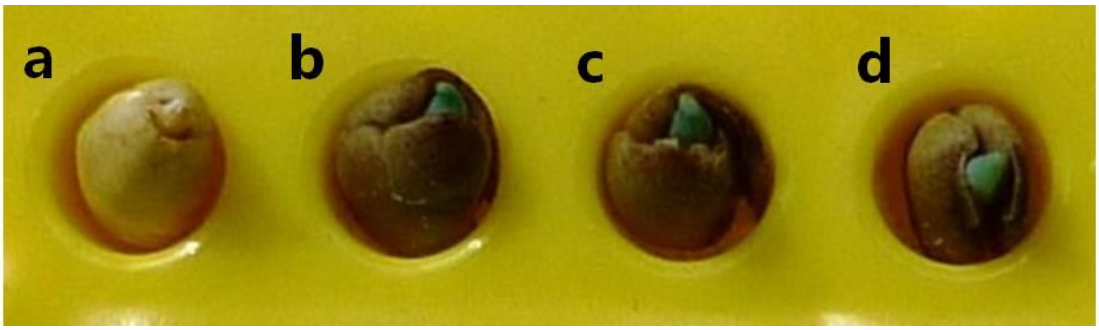
Supplementary Figure 6. Magnetization curve of MNPs. The magnetic properties of MNPs were measured by vibrating sample magnetometer (VSM). The magnetization curve showed a typical superparamagnetic behavior of MNPs.



Supplementary Figure 7. Agrose gel electrophoresis of MNP/DNA complexes. (a) Digested with *Hind* III. **(b)** Digested with *DNaseI*. Green channels were stimulated by 488 nm wavelength light; red channels were stimulated by 532 nm wavelength light. Lane 1: pure plasmid (control); lane 2: plasmid digested; lanes 3~8: MNP/DNA complexes digested at DNA/MNP mass ratio of 1:1, 1:0.5, 1: 0.1, 1: 0.05, 1: 0.02 and 1: 0.01. MNPs (red) can completely bind DNA (green) to form MNP/DNA complexes (yellow) and protect DNA against degradation in lanes 3~6. Excessive DNA is digested at the mass ratio more than 1:0.05.

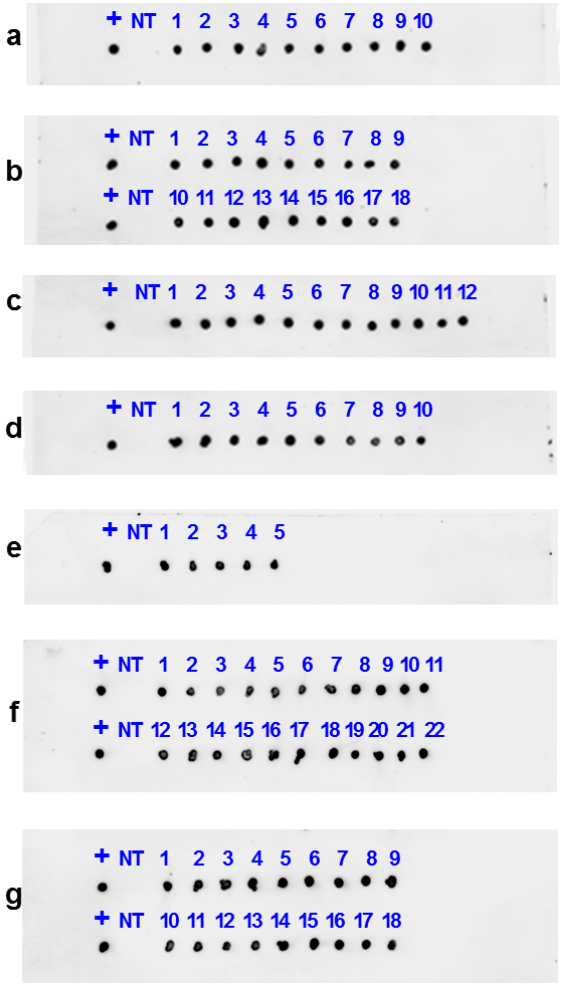


Supplementary Figure 8. The expression of exogenous *GUS* gene occurred in the magnetofected pollen. (a) non-treated control. (b) pollen magnetofected with MNPs. (c) pollen treated with magnetic particles (diameters 5-10 µm). If the GUS gene is successfully transformed and expressed, the GUS protein (β-Glucuronidase) is expressed in plant cells, which will be stained blue by X-gluc solution (5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronic acid). No GUS signal was examined in the control (a,c) while *GUS* gene strongly expressed in the magnetofected pollen (b). The GUS expression efficiency in cotton pollen was 38%.

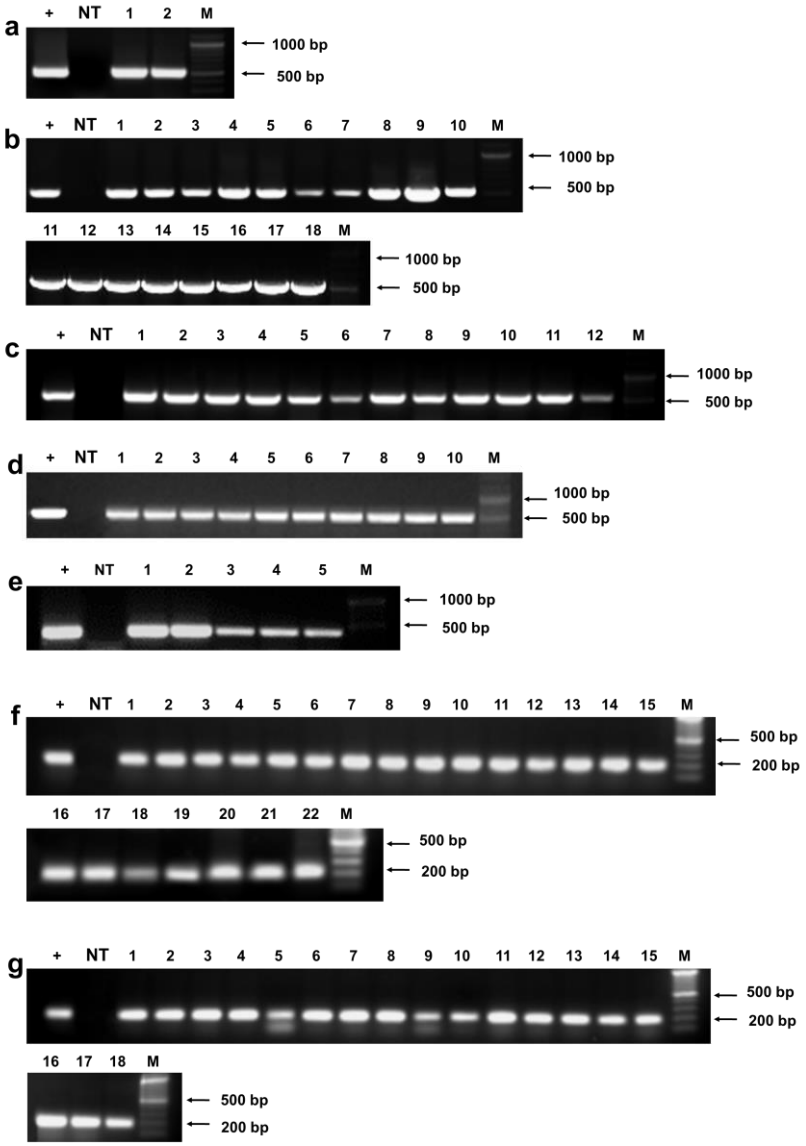


Supplementary Figure 9. The expression of exogenous *GUS* gene occurred in the transgenic seeds. No GUS signal was examined in the non-treated control (a) while *GUS* gene strongly expressed in the transgenic seeds (b, c, and d).

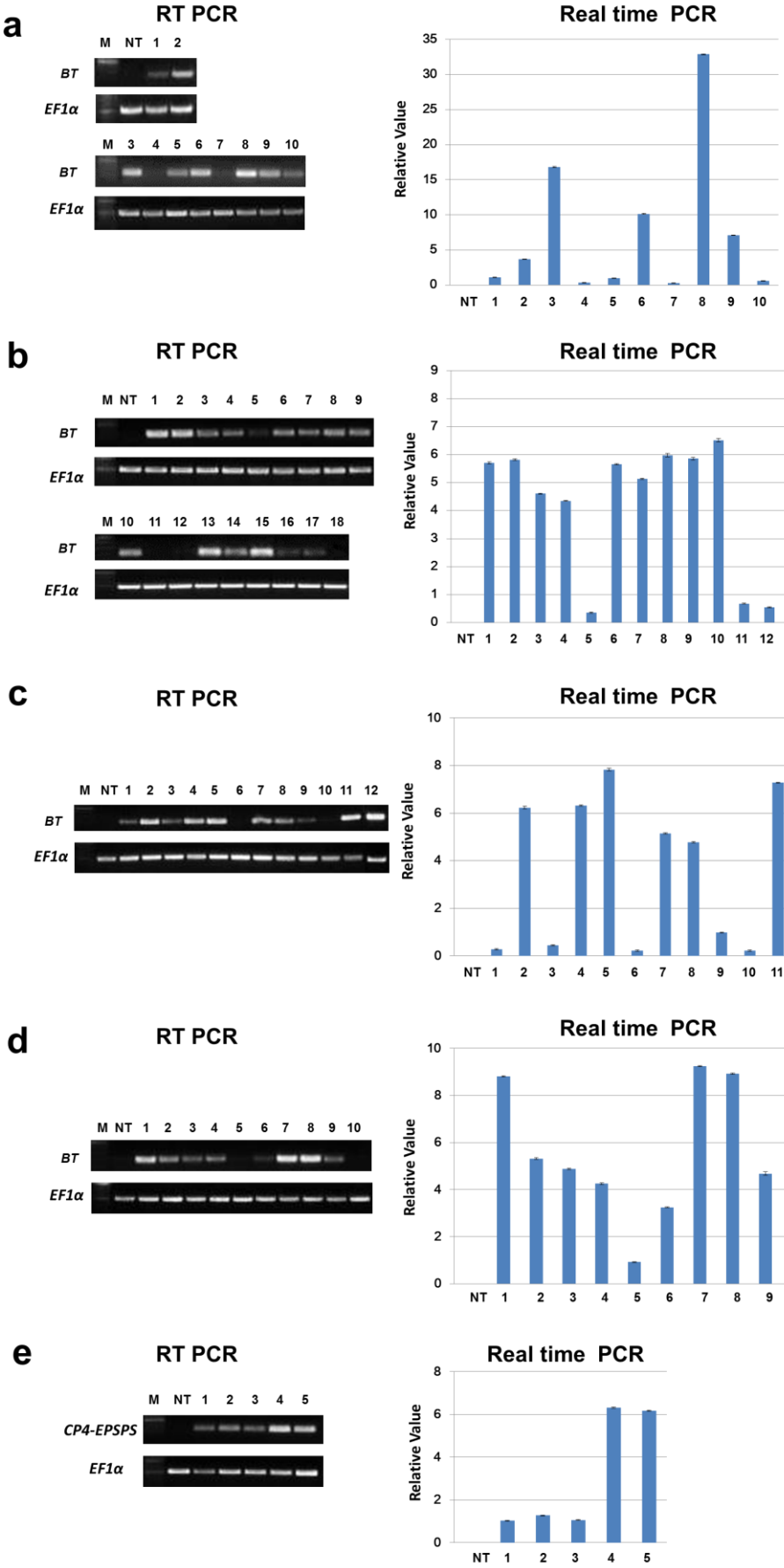
Supplementary Figure 10. Kanamycin screening for transgenic cotton. (a) The seedlings generated from transformed seeds were sprayed by 0.5% kanamycin at 2, 4 and 8 leaves stage to screen transgenic cotton. **(b)** The spots occurred on the non-transgenic leaves. **(c)** Four plants of transgenic cotton were finally obtained through three times screening of all T1 seedlings.

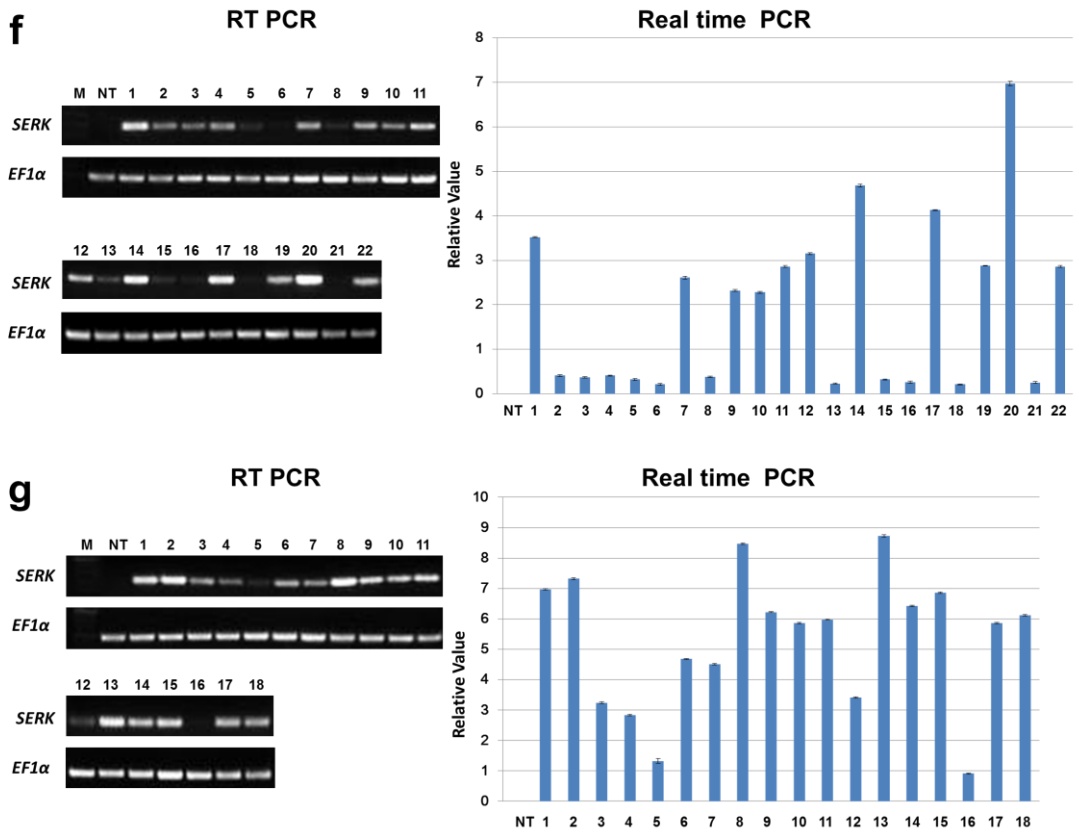


Supplementary Figure 11. Dot blot hybridization of transgenic cotton plants for seven exogenous genes. **(a)** Dots 1~2: transgenic plants of SU12 with pBI35SBTΔαCPTI; dots 8~10: transgenic plants of Y18 with pBI35SBTΔαCPTI. **(b)** Dots 1~9: transgenic plants of SU12 with pBI35SBTCPTI; dots 10~18: transgenic plants of Y18 with pBI35SBTCPTI. **(c)** Dots 1~12: transgenic plants of Y18 with pBI35SCTPBT. **(d)** Dots 1~10: transgenic plants of Y18 with pBI35SMTPBT. **(e)** Dots 1~5: transgenic plants of Y18 with CP4-EPSPS. **(f)** Dots 1~22: transgenic plants of SU12 with pBI35SGhSERK. **(g)** Dots 1~18: transgenic plants of SU12 with pBIArf1SERK. From (a) to (g), the symbol of "+" is used for plasmid DNA fragments (positive control), while "NT" for normal plant (negative control).

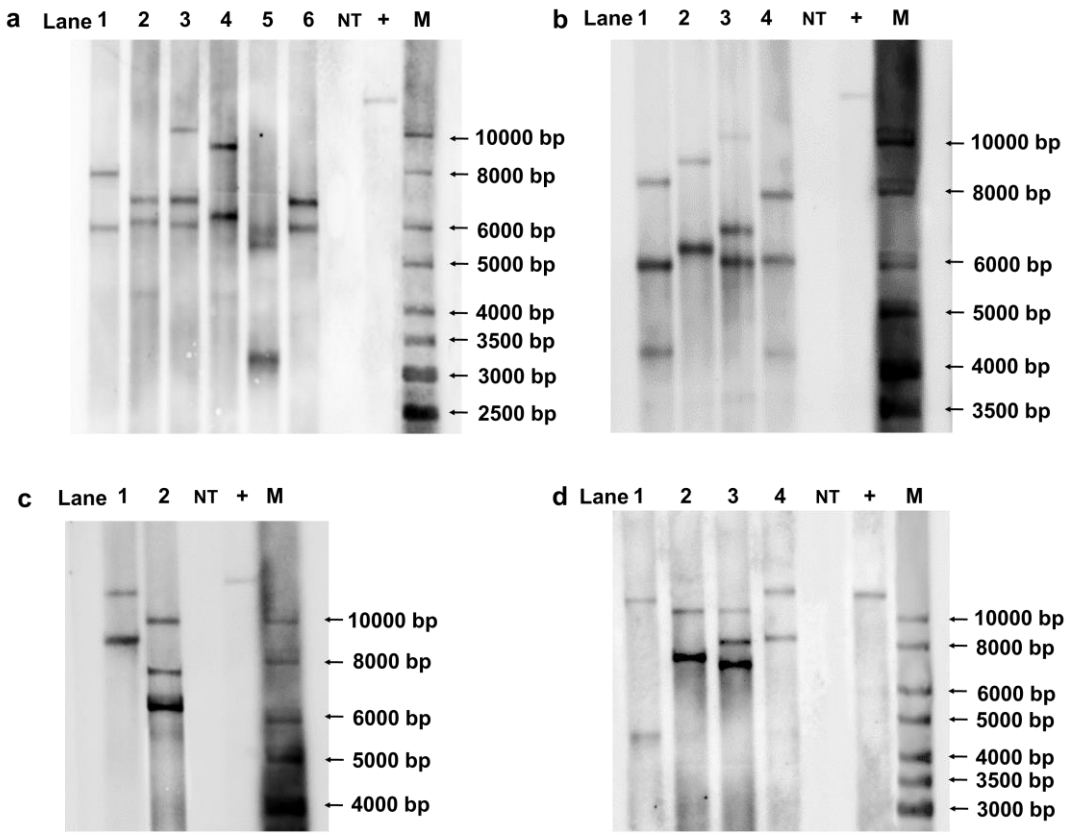


Supplementary Figure 12. PCR analyses of transgenic cotton plants for seven exogenous gene. (a) Transgenic plants with pBI35SBTΔαCPTI. lanes 1~2: transgenic plants of SU12. **(b) Transgenic plants with pBI35SBTCPTI.** lanes 1~9: transgenic plants of SU12; lanes 10~18: transgenic plants of Y18. **(c) Transgenic plants with pBI35SCTPBT**. lanes 1~12: transgenic plants of Y18. **(d) Transgenic plants with pBI35SMTPBT.** lanes 1~10: transgenic plants of Y18. **(e) Transgenic plants with CP4-EPSPS**. lanes 1~5: transgenic plants of Y18. **(f) Transgenic plants with pBI35SGhSERK**. lanes 1~22: transgenic plants of SU12. **(g) Transgenic plants with pBIArf1SERK**. lanes 1~18: transgenic plants of SU12. From (a) to (g), the symbol of "+" is used for plasmid DNA fragments (positive control), while "NT" for normal plant (negative control) and "M" for DNA ladder.

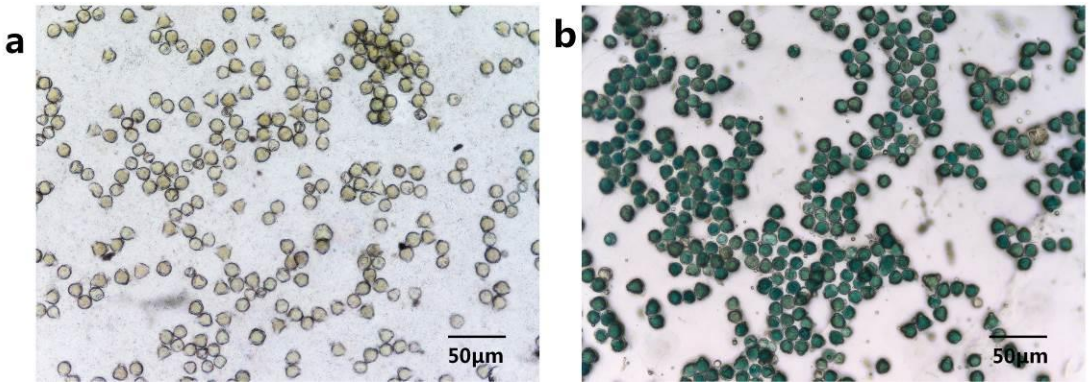

a
RT PCR
M NT 1 2
BT
EF1α
M 3 4 5 6 7 8 9 10
BT
EF1α
Real time PCR
Relative Value
0 5 10 15 20 25 30 35
NT 1 2 3 4 5 6 7 8 9 10
b
RT PCR
M NT 1 2 3 4 5 6 7 8 9
BT
EF1α
M 10 11 12 13 14 15 16 17 18
BT
EF1α
Real time PCR
Relative Value
0 1 2 3 4 5 6 7 8 9
NT 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
c
RT PCR
M NT 1 2 3 4 5 6 7 8 9 10 11 12
BT
EF1α
Real time PCR
Relative Value
0 2 4 6 8 10
NT 1 2 3 4 5 6 7 8 9 10 11 12
d
RT PCR
M NT 1 2 3 4 5 6 7 8 9 10
BT
EF1α
Real time PCR
Relative Value
0 2 4 6 8 10
NT 1 2 3 4 5 6 7 8 9 10
e
RT PCR
M NT 1 2 3 4 5
CP4-EPSPS
EF1α
Real time PCR
Relative Value
0 2 4 6 8
NT 1 2 3 4 5



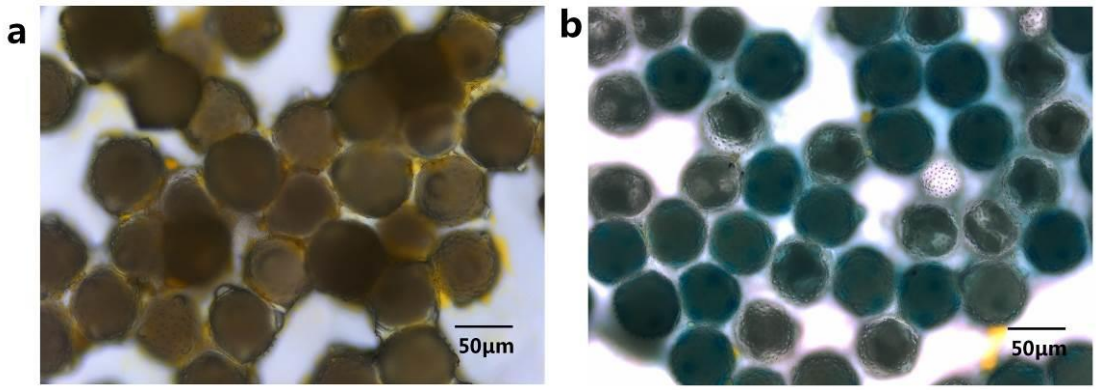
Supplementary Figure 13. RT PCR and real time PCR analyses of transgenic cotton plants for exogenous gene transcription. (a) Transgenic plants with pBI35SBTΔαCPTI. lanes 1~2: transgenic plants of SU12; lanes 8~10: transgenic plants of Y18. **(b) Transgenic plants with pBI35SBTCPTI**. lanes 1~9: transgenic plants of SU12; lanes 10~18: transgenic plants of Y18. **(c) Transgenic plants with pBI35SCTPBT**. lanes 1~12: transgenic plants of Y18. **(d) Transgenic plants with pBI35SMTPBT**. lanes 1~10: transgenic plants of Y18. **(e) Transgenic plants with linearized plasmid of CP4-EPSPS**. lanes 1~5: transgenic plants of Y18. **(f) Transgenic plants with pBIArf1SERK**. lanes 1~18: transgenic plants of SU12. **(g) Transgenic plants with pBI35SGhSERK**. lanes 1~22: transgenic plants of SU12. NT: non-transgenic plant (negative control); *EF1α* gene were used as the internal standard.



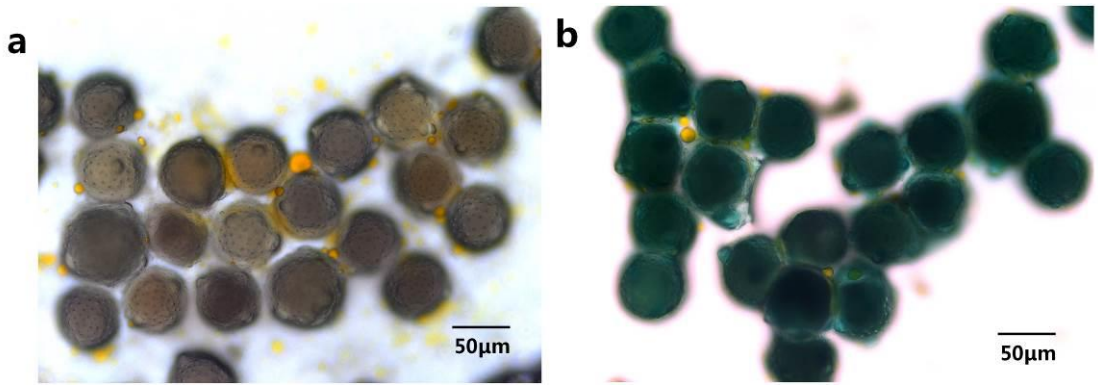
Supplementary Figure 14. Southern blot analyses of transgenic cotton plants for seven exogenous genes. **(a)** Lanes 1~2: transgenic plants of SU12 with pBI35SBTΔαCPTI; lanes 3~4: transgenic plants of SU12 with pBI35SBTCPTI; lanes 5~6: transgenic plants of Y18 with pBI35SBTCPTI. **(b)** Lanes 1~2: transgenic plants of Y18 with pBI35SCTPBT; lanes 3~4: transgenic plants of Y18 with pBI35SMTPBT. **(c)** Lanes 1~2: transgenic plants of Y18 with CP4-EPSPS linearized plasmid. **(d)** Lanes 1~2: transgenic plants of SU12 with pBI35SGhSERK; lanes 3~4: transgenic plants of SU12 with pBIArf1SERK. From (a) to (d), the symbol of "NT" is used for non-transgenic plant (negative control), while "+" for plasmid DNA fragments (positive control) and "M" for DNA ladder.



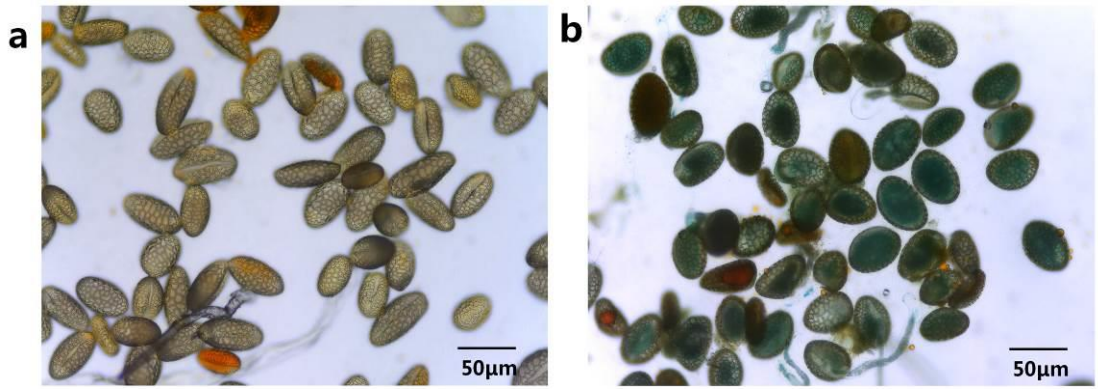
Supplementary Figure 15. The expression of exogenous *GUS* gene occurred in the magnetofected pollen of chili pepper. No GUS signal was examined in the non-treated control (a) while *GUS* gene strongly expressed in the magnetofected pollen (b). The GUS expression efficiency in pepper pollen was 66%.



Supplementary Figure 16. The expression of exogenous *GUS* gene occurred in the magnetofected pollen of pumpkin. No GUS signal was examined in the non-treated control (a) while *GUS* gene strongly expressed in the magnetofected pollen (b). The GUS expression efficiency in pumpkin pollen was 65%.

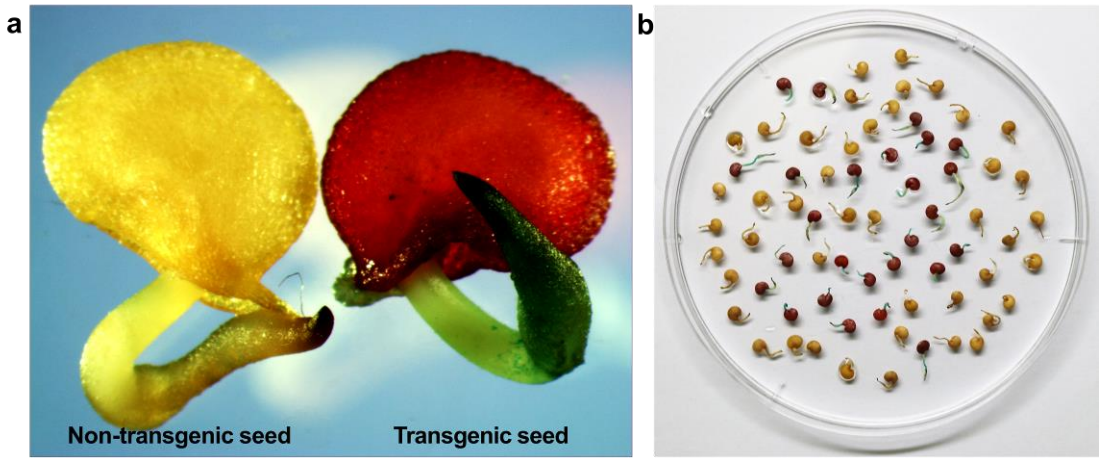


Supplementary Figure 17. The expression of exogenous *GUS* gene occurred in the magnetofected pollen of cocozelle. No GUS signal was examined in the non-treated control (a) while *GUS* gene strongly expressed in the magnetofected pollen (b). The GUS expression efficiency in pumpkin pollen was 53%.

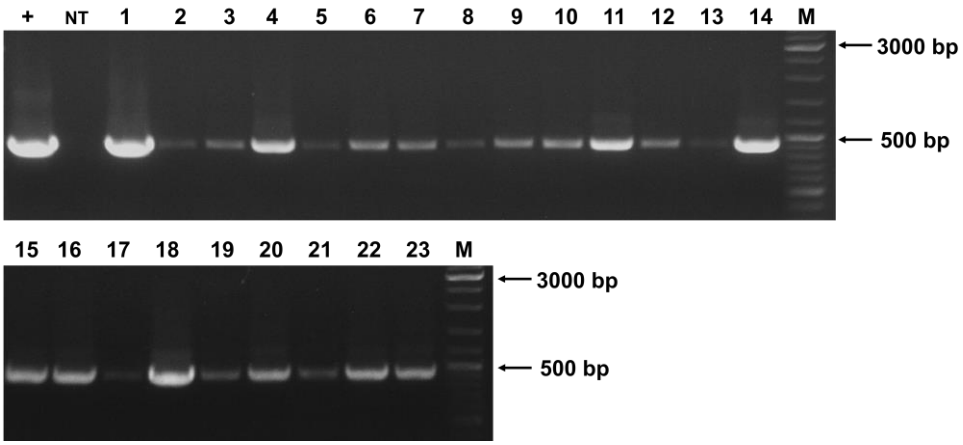


Supplementary Figure 18. The expression of exogenous *GUS* gene occurred in the magnetofected pollen of lily. No GUS signal was examined in the non-treated control (a) while *GUS* gene strongly expressed in the magnetofected pollen (b). The GUS expression efficiency in lily pollen was 90%.

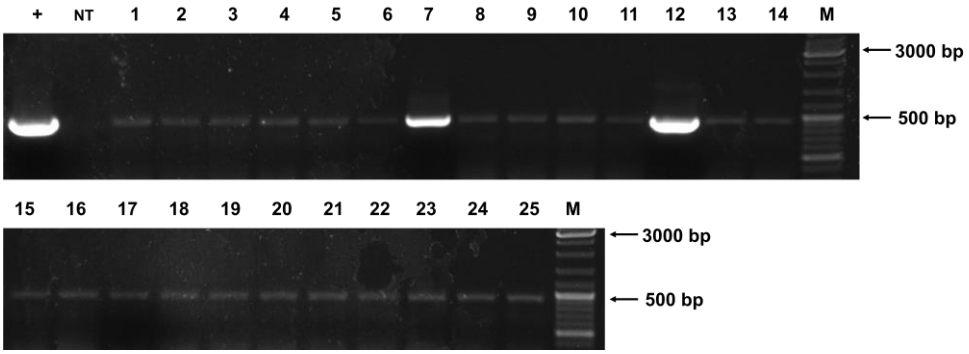
Supplementary Figure 19. The expression of exogenous *GUS* gene occurred in the T1 transgenic seeds of pumpkin. (a) A single non-transgenic seed and *GUS* transgenic seed. (b) All of the harvested T1 seeds of pumpkin. The blue seeds stained with X-gluc solutionindicated that exogenous *GUS* gene was successfully transformed in pumpkin.



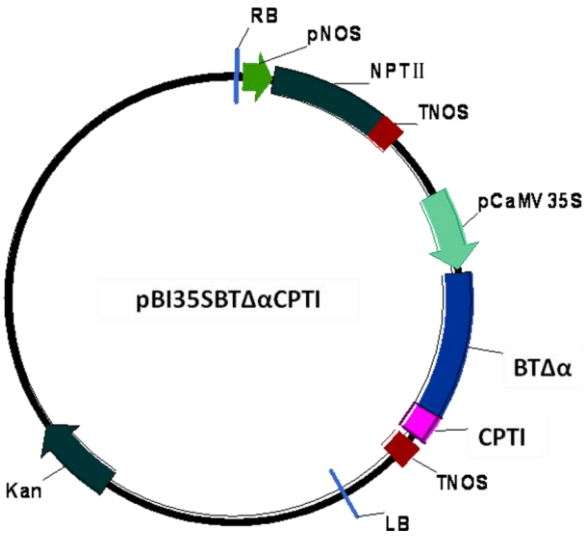
Supplementary Figure 20. The expression of exogenous *GUS* gene occurred in the T1 transgenic seeds of chili pepper. (a) A single non-transgenic seed and *GUS* transgenic seed. (b) All of the harvested T1 seeds of chili pepper. The blue seeds stained with X-gluc solution indicated that exogenous *GUS* gene was successfully transformed in pepper.



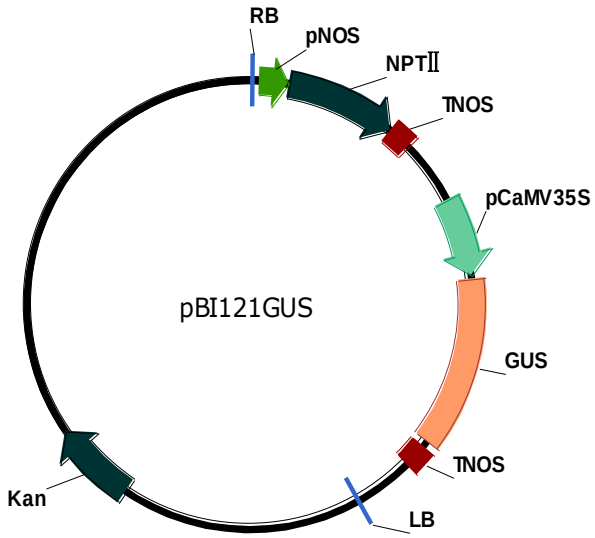
Supplementary Figure 21. PCR analyses of transgenic pumpkin plants for exogenous *GUS* gene. The symbol of "+" is used for plasmid DNA fragments (positive control), while "NT" for normal plant (negative control) and "M" for DNA ladder.



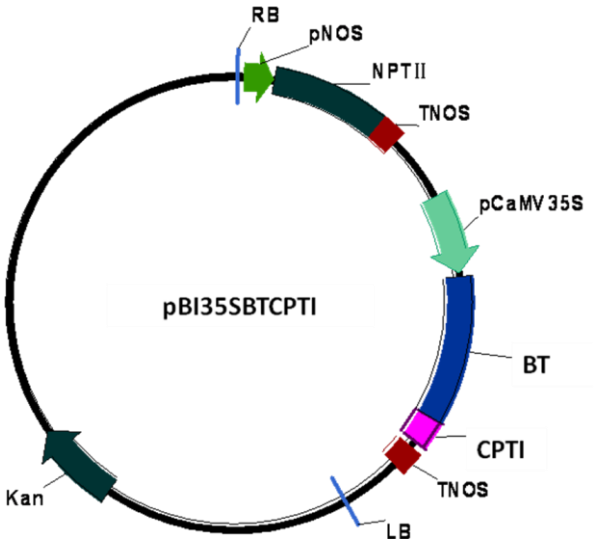
Supplementary Figure 22. PCR analyses of transgenic pepper plants for exogenous *GUS* gene. The symbol of "+" is used for plasmid DNA fragments (positive control), while "NT" for normal plant (negative control) and "M" for DNA ladder.



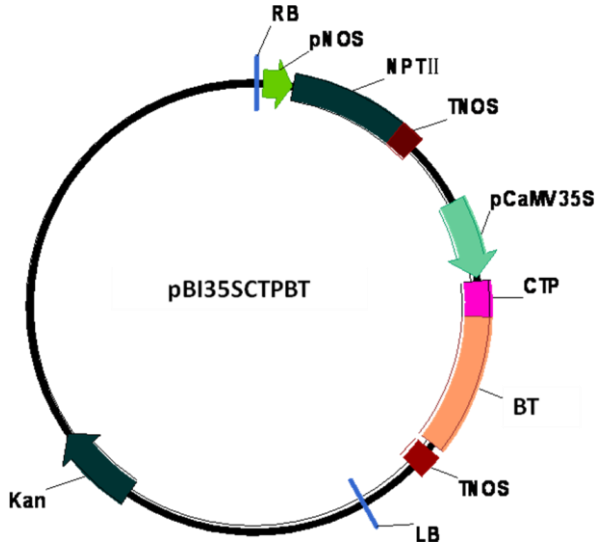
Supplementary Figure 23. Construction of plasmid pBI35SBTΔαCPTI



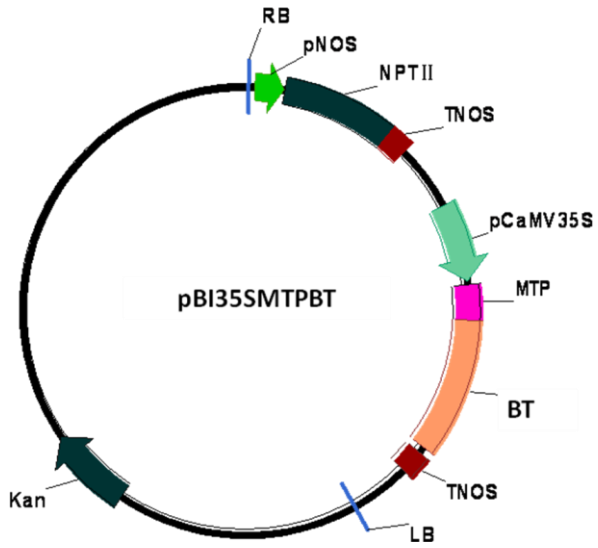
Supplementary Figure 24. Construction of plasmid pBI121GUS



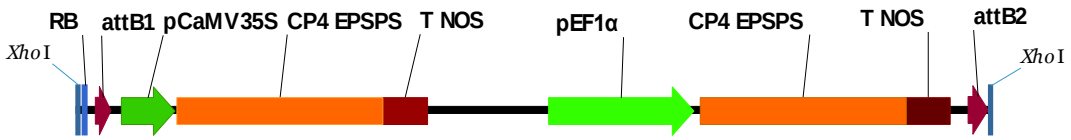
Supplementary Figure 25. Construction of plasmid pBI35SBTCPTI



Supplementary Figure 26. Construction of plasmid pBI35SCTPBT



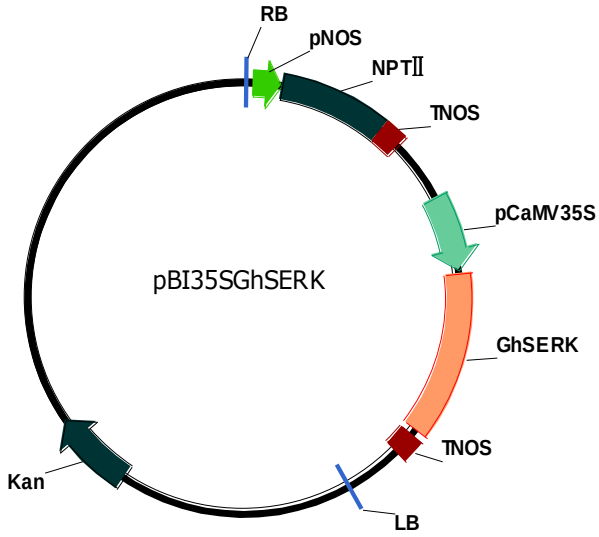
Supplementary Figure 27. Construction of plasmid pBI35SMTPBT



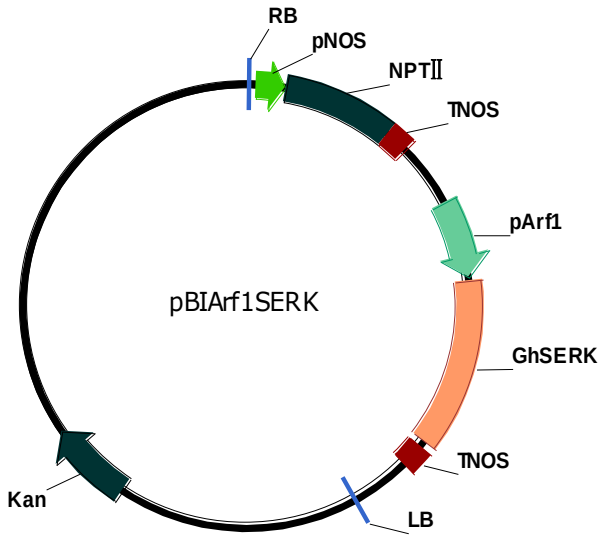
35s::CP4EPSPS::tNos-EF1α::CP4EPSPS::tNos

Supplementary Figure 28. Construction of linearized plasmid

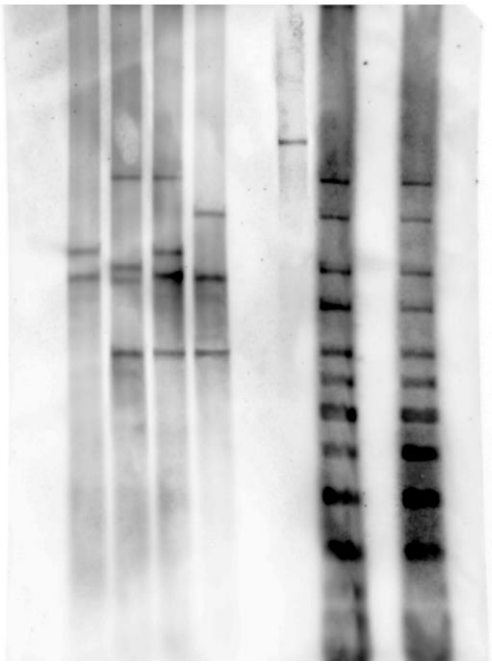
35S::CP4EPSPS::tNOS –EF1α::CP4EPSPS::tNOS



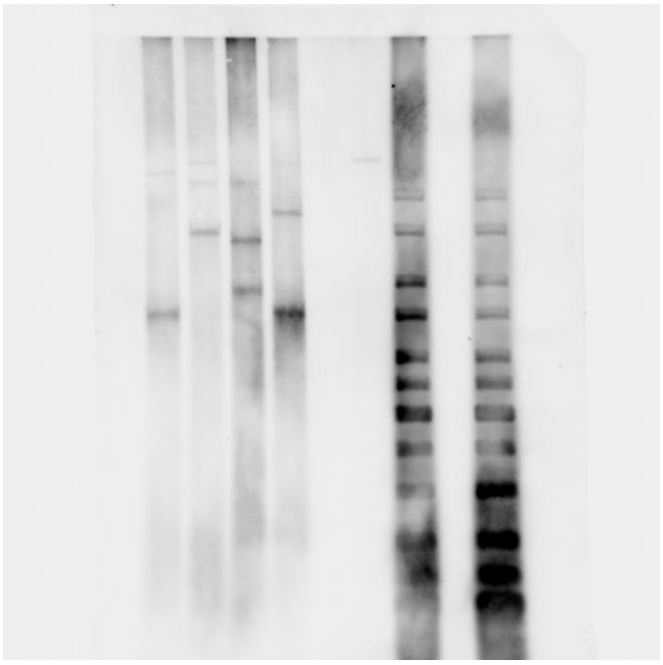
Supplementary Figure 29. Construction of plasmid pBI35SGhSERK



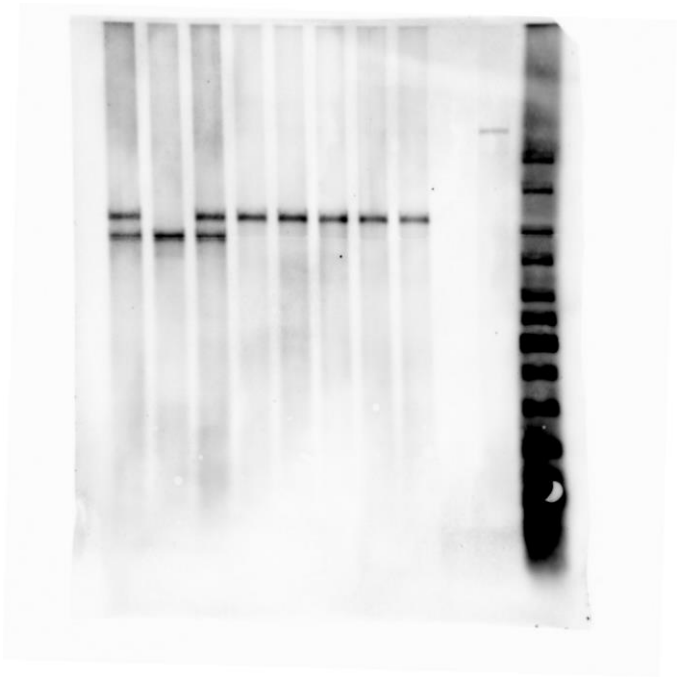
Supplementary Figure 30. Construction of plasmid pBIArf1SERK



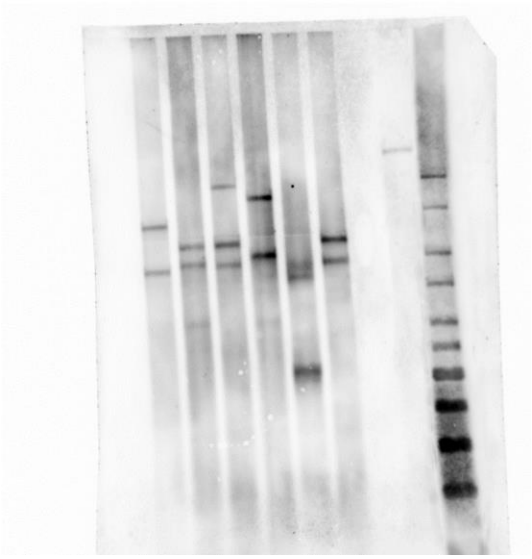
Supplementary Figure 31. The original and complete scans of southern blots in fig.4b.



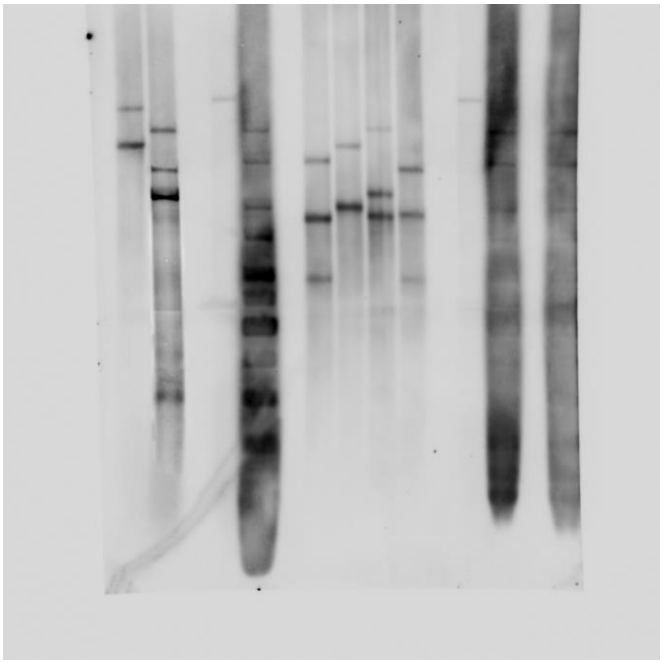
Supplementary Figure 32. The original and complete scans of southern blots in fig.4c.



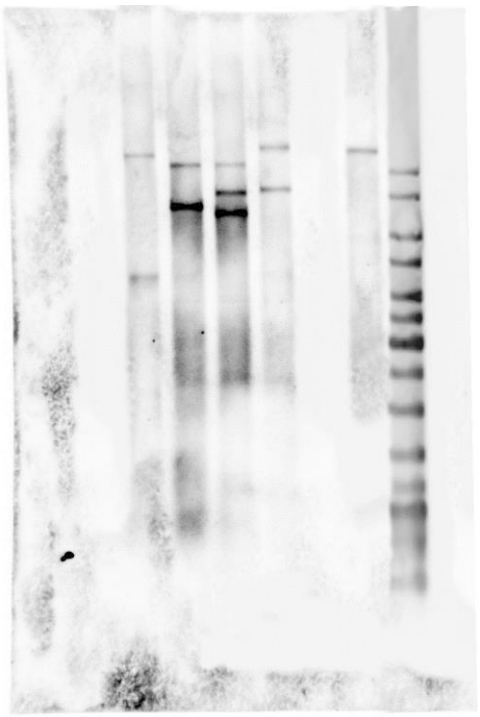
Supplementary Figure 33. The original and complete scans of southern blots in fig.5b.



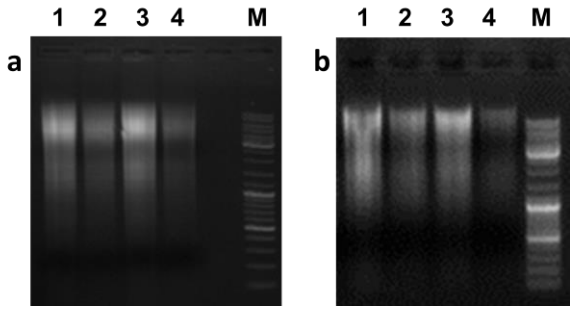
Supplementary Figure 34. The original and complete scans of southern blots in Supplementary Fig. 14a.



Supplementary Figure 35. The original and complete scans of southern blots in Supplementary Fig. 14b and c.



Supplementary Figure 36. The original and complete scans of southern blots in Supplementary Fig. 14d.



Supplementary Figure 37. Digested cotton genomic DNA on agarose gel. (a) genome digested with *Hind*Ⅲ; **(b)** genome digested with *Eco*RⅠ; lanes 1~4: cotton genome ; M: DNA ladder.

Statistical analyses

Experimental Data

In order to investigate the reproducibility of pollen magnetofection, we have repeated experiments of pollen magnetofection for 13 times during 2010-2012 with different DNA expression vectors and recipient materials. The original experimental data were shown in Supplementary Table 1.

Supplementary Table 1. Transformation efficiencies of different expression vectors with pollen magnetofection system

No.	DNA expression vectors	DNA size	DNA form	Year	Treatments	Recipient materials	Flowers	Seeds	Transgenic plants	Success rate[1]
1	35S::BTΔα CPTI::tNOS	16.8kb	circular	2010	CK1[2]	Y18	100	1380	0	0
				2010	CK2[3]	Y18	100	1332	0	0
				2010	magnetofection	Y18	100	1101	8	8%
2				2010	magnetofection	SU12	100	1109	2	2%
3				2011	CK1	Y18	100	1106	0	0
				2011	CK2	Y18	100	1223	0	0
				2011	magnetofection	Y18	100	1216	11	11%
4				2012	CK1	Y18	100	1300	0	0
				2012	CK2	Y18	100	1221	0	0
				2012	magnetofection	Y18	100	1208	10	10%
5	35S::BT · CPTI::tNOS	16.9kb	circular	2010	CK1	Y18	100	1413	0	0
				2010	CK2	Y18	100	1176	0	0
				2010	magnetofection	Y18	100	1020	9	9%
6				2010	magnetofection	SU12	100	1608	9	9%
7				2011	CK1	Y18	100	1311	0	0
				2011	CK2	Y18	100	1063	0	0
				2011	magnetofection	Y18	100	893	5	5%
8				2012	CK1	Y18	100	1208	0	0
				2012	CK2	Y18	100	1355	0	0
				2012	magnetofection	Y18	100	1305	11	11%
9	35S::CTPBT::tNOS	15.2kb	circular	2010	CK1	Y18	100	1302	0	0
					CK2	Y18	100	1140	0	0
					magnetofection	Y18	100	1200	12	12%
10	35S::MTPBT::tNOS	15.1kb	circular	2010	CK1	Y18	100	1315	0	0
					CK2	Y18	100	1220	0	0
					magnetofection	Y18	100	1244	10	10%

11	35S::CP4EPSPS::tNOS–EF1α::CP4EPSPS::tNOS	7.8kb	linear	2010	CK1	Y18	100	1120	0	0
					CK2	Y18	100	1035	0	0
					magnetofection	Y18	100	1028	5	5%
12	35S::GhSERK::tNOS	16.6kb	circular	2010	CK1	SU12	200	2650	0	0
					CK2	SU12	200	2235	0	0
					magnetofection	SU12	200	2380	22	11%
13	Arf1::GhSERK::tNOS	18.1kb	circular	2010	CK1	SU12	200	2414	0	0
					CK2	SU12	200	2220	0	0
					magnetofection	SU12	200	2120	18	9%

Notes:

[1] Success rate: the ratio of the number of transgenic plants to the number of pollinated flowers.

[2] CK1: Control treated with naked plasmids.

[3] CK2: Control treated with MNP/DNA complexes without a magnetic field.

Method of Statistical Analysis

We used a binomial regression analysis because the total number of pollinated flowers was fixed at either 100 or 200 in each experiment. The factors for this analysis are: 1) three types of treatment (control treated with naked plasmids, control treated with MNP/DNA complexes without a magnetic field, and pollen magnetofection); 2) seven DNA expression vectors; 3) three experiment years (2010, 2011, and 2012); 4) two types of recipient materials (Y18 and SU12). We use R^2 – a statistics software to build a binomial regression model on the counts of transgenic plants and test for the significance of the four factors with a significance level of 0.05.

Results of Statistical Analysis

The statistical analysis revealed the following results (Supplementary Table 2):

1. The effect of treatment types is significant with an extremely small p-value

(2e-5). Therefore pollen magnetofection treatment exhibits significant increase in transgenic success rates over the two control treatment types.

2. The effect of years is not significant with a p-value of 0.657.

3. The effect of recipient materials is not significant with a p-value of 0.238.

4. The effect of DNA expression vectors is significant with a p-value of 0.008. Using z-test for proportions, we further conduct pairwise comparisons of effect of the seven expression vectors on the transgenic success rates for the magnetofection treatment group. The linear plasmid has a significantly lower success rate than all other circular expression vectors, which are not different among themselves (See Supplementary Table 3).

Supplementary Table 2. Test of significance of main factors

Factor	Degree of Freedom	Deviance (Sum of Square)	AIC	Likelihood Ratio Test	P-Value
Treatment Type	2	277.4	348.7	269.8	2.00E-05
DNA Expression Vector	6	25	88.3	17.4	0.008
Year	2	8.4	79.7	0.8	0.657
Recipient Material	1	8.9	82.2	1.393	0.238

Supplementary Table 3. P-values of pairwise tests of significance of seven DNA expression vectors on success rates

	V1	V2	V3	V4	**V5**	V6	V7
V1	-	0.80	0.25	0.60	**0.02**	0.24	0.71
V2	0.80	-	0.37	0.78	**0.01**	0.40	0.96
V3	0.25	0.37	-	0.82	**0.00**	0.95	0.54
V4	0.60	0.78	0.82	-	**0.01**	0.95	0.94
V5	**0.02**	**0.01**	**0.00**	**0.01**	**-**	**0.00**	**0.01**
V6	0.24	0.40	0.95	0.95	**0.00**	-	0.62
V7	0.71	0.96	0.54	0.94	**0.01**	0.62	-

Notations:

V1=35S::BT.CPTI::tNOS,

V2=35S::BT.CPTI::tNOS,

V3=35S::CTPBT::tNOS,

V4=35S::MTPBT::tNOS,

V5=35S::CP4EPSPS::tNOSEF1::CP4EPSPS::tNOS,

V6=35S::GhSERK::tNOS,

V7=Arf1::GhSERK::tNOS.

We investigated the effects of magnetic exposure time and the concentrations of MNPs-DNA complexes on the success rate. The result was shown in Supplementary Table 4.

Supplementary Table 4. Effects of concentrations of MNP/DNA complexes and magnetic exposure time on success rates of pollen magnetofection

Concentration \ Exposure	0.25 h			0.5 h			1 h		
0.5 μg	Flowers	Transgenic plants	Success rate	Flowers	Transgenic plants	Success rate	Flowers	Transgenic plants	Success rate
	100	4	4%	100	9	9%	100	4	4%
1 μg	Flowers	Transgenic plants	Success rate	Flowers	Transgenic plants	Success rate	Flowers	Transgenic plants	Success rate
	100	5	5%	100	10	10%	100	4	4%
2 μg	Flowers	Transgenic plants	Success rate	Flowers	Transgenic plants	Success rate	Flowers	Transgenic plants	Success rate
	100	5	5%	100	7	7%	100	3	3%

We conducted a binomial regression analysis on the transgenic counts using two factors: 1) time of magnetic exposure (0.25, 0.5, and 1 hour); 2) concentration of MNPs (0.5, 1, and 2 μg). The test result was presented in Supplementary Table 5, from which we have following observations:

1. The effect of exposure time is significant with a p-value of 0.023. Further analysis shows that the exposure of half hour produces significantly high success rates than exposure of 15 minutes or one hour.

2. The effect of concentration is not significant with a p-value of 0.777.

Supplementary Table 5. Test of significance of the factors of time and concentration of exposure

Degree of Freedom	Deviance (Sum of Square)	AIC	Likelihood Ratio Test	P-Value
2	7.97	45.22	7.53	0.023
2	0.95	38.19	0.5	0.777

Comparisons of pollen magnetofection and other methods

While some traditional DNA transfection methods, including Agrobacterium incubation, biolistic bombardment, electroporation, and ultrasonication, have been reported in plant pollens, none of these conventional pollen-based methods have been widely adopted[3].

In particular for cotton, so far there was only one pollen-based successful case reported (using the biolistic bombardment)[4]; but the resultant transformation efficiency was extremely low, only at 0.075%.

Although Agrobacterium infection-tissue culture is a main transformation method, it requires regeneration[5]. Regeneration involves a complicated, long, and laborious process, taking about 1.5 years. Unfortunately due to the fact that most cotton varieties are difficult to regenerate, Agrobacterium infection rarely works in cotton. The only limited success cases started with and stemmed from G. hirsutum L. cv. Coker -- it took 3 or 4 years of backcrossing and generation to transfer the elite traits to other cotton varieties, severely limited the development of transgenic crops in general and cotton in particular.

We are also aware of the pollen tube pathway work; with this method DNA were microinjected into pollen tubes, which was neither an easy technique nor high efficient route. Other significant limitations of tube injection included the poor transformation efficiency, and more significantly, difficulty in reproducing results.

However, pollen magnetofection need to take extra two generations of self-pollination to obtain transgenic pure lines, due to more than one integration site in genome. Also circular DNA works better than linear DNA; this may be due to the fact that the linear plasmids were much less stable in the environment compared with the circular expression vectors, which leads to the lower transformation frequency.

In addition, the shape and size of pollen apertures also affected the success of pollen magnetofection. Most pollen grains possess apertures, they function as exits for the pollen tube in germination, as sites for the uptake of water, in the accommodation of volume changes and as sites for the transfer of recognition substances[4, 5]. There are two main shape types of apertures: pores and furrows. The size of pollen apertures varies with different pollen sizes. The pollen grains normally range from 10 μm to 300 μm[6]. However, inaperturate pollen, in which an obvious aperture is lacking, occurs in some groups, especially in early divergent angiosperms and monocots (present most in Zingiberales, Liliales, and some Asparagales)[7]. A few eudicots also include some taxa with inaperturate pollen (*Ranzania*，*Disciphania, Tiliacora*，*Souliea vaginata*，*Nepenthes*，*Atkinsonia*，*Balbisia, Wendtia*，*Baliospermum, Croton, Jatropha, Joannesia, Neoboutonia, Tritaxis*，*Anisadenia, Reinwardtia, Rafflesia, Rhizanthes, Sapria*，*Populus*，*Hydrostachys*，*Secamone*，*Asclepias, Ceropegia, Cynanchum, Matelea, Stapelia*，*Cephaelis, Hymenocoleus, Palicourea, some Psychotria, some Rudgea*，*Some Matthiola*)[8].

Furthermore, the transformation rate of pollen expression was higher than that of the transgenic events. The success rate of the transgenic events is was affected by multiple factors, including pollen viability (some pollen grains may have died before fertilization), pollination timing (affecting the pollen viability), pollination technique (determining the numbers of pollen adhering to the stigma), and environment conditions (affecting the seed setting rate), etc. Thus the success rate would be improved through optimizing experimental conditions and careful preparing.

We summarized the above discussions in Supplement Information Table 6.

Supplementary Table 6. Comparisons of pollen magnetofection and other methods

	Pollen magnetofection	Agrobacterium incubation with pollen	Agrobacterium infection-tissue culture	Pollen tube pathway	Biolistic bombardment with pollen	Electroporation with pollen	Ultrasonication with pollen
Success rate in Cotton transformation	2%-12%	-	15% *	15.3% and 1.9%**	0.075% ***	-	-
Require for regeneration from tissue culture	No	No	Yes	No	No	No	No
Genotype limitation	No	No	Start with and stemmed from only *G. hirsutum* L. cv. Coker	No	No	No	No
Process from transformation to obtaining transgenic germplasm	Less than 6 months	-	1.5 years	Less than 6 months	Less than 6 months	-	-
Easy to operate	Yes	Yes	Tissue culture, complicated, long, and laborious processes	Trained and skilled experimenters	Operate with gene gun	Yes	Yes
Require for equipment	No	No	No	No	Yes	Yes	No
Damage for pollen viability	Mild	Severe damage	-	-	Severe damage	Severe damage	Severe damage
Exogenous gene integration	Random	Random	Random	Random	Random	Random	Random
Money cost for each transgenic event	Less than US$ 7	-	about US$ 700	about US$ 170	about US$ 200	-	-

* Shuangxia J, et al. *Plant Cell Tissue Organ Cult.* 81, 229-237 (2005).

** Ali A, Bang S W, Chung S M, et al. *Plant Molecular Biology Reporter,* 33, 742-747 (2015).

*** Only one successful case was reported (Gounaris Y, et al. *J. Food Agric. Environ.* 3, 157-160 (2005)).

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