# Development of genotype independent cotton transformation protocol

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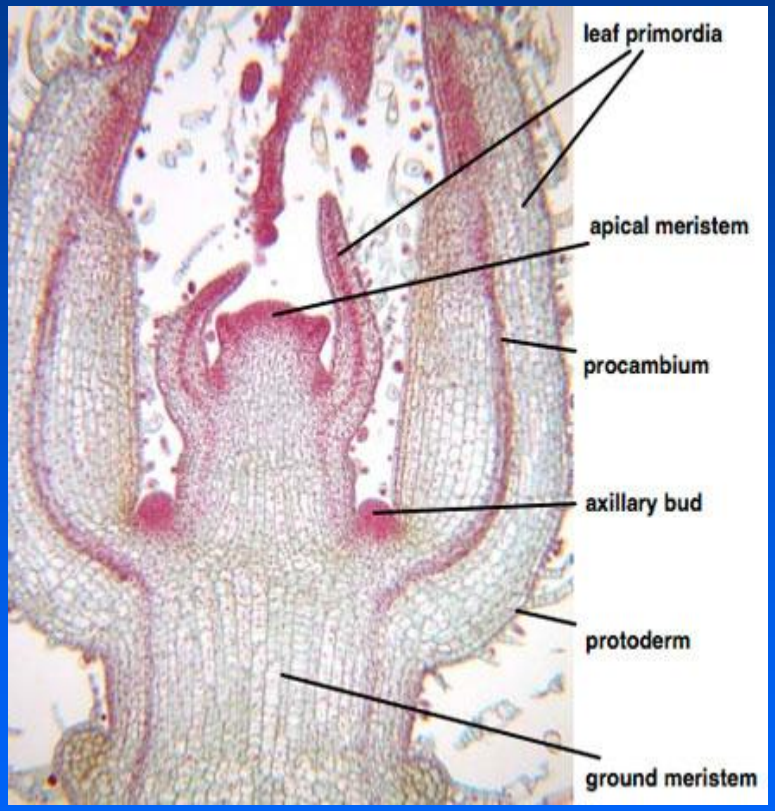
# What are our goals?

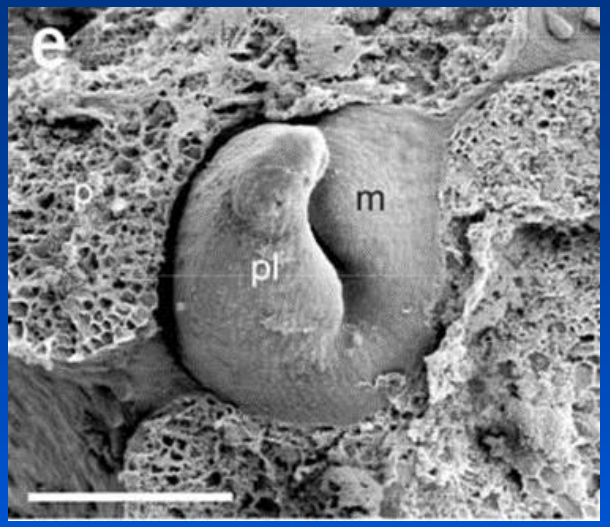
- Robust and reproducible procedures
- Practical and adaptable protocols
- Acceptable transformation efficiency
- Protocols available in public domain

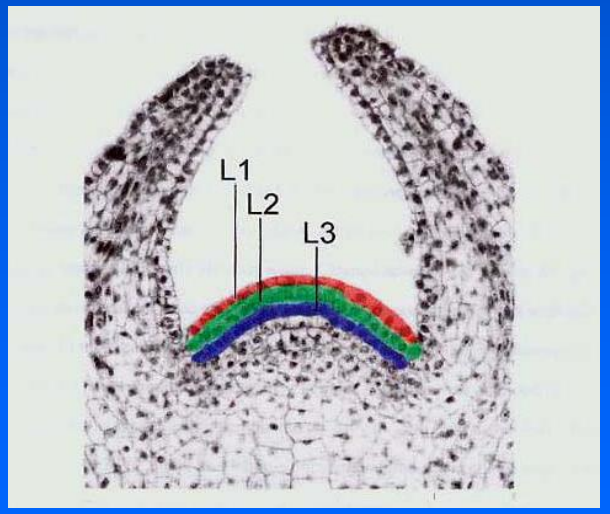
# Common strategies for genotype-independent transformation of cotton:

- *Agrobacterium*-mediated transformation targeting meristems in shoot or embryo apex
- *In Planta* pollen-mediated transformation
- *In Planta* pollen-tube pathway transformation

# *Agrobacterium*-mediated transformation of meristems in shoot or embryo apex




L1 gives rise to the epidermis, L2 gives rise to ground tissue, L3 forms the body of new tissues, including vasculature and germline tissue.

Adaptation of cotton shoot apex culture to Agrobacterium-mediated transformation Gould JH and Magallanes-Cedeno M Plant Mol. Biol. Rep. (1998), 16, pp 1-10

Transformation of a Texas cotton cultivar by using Agrobacterium and the shoot apex Zapata C, Park SH, El-Zik KM, Smith RH, Theor Appl Genet, 1999, 98: pp 252-256

The current status of gene transformation in cotton. Sakhanokho HF, Chee PW *SAAS Bull*. of *Biochem*. and *Biotechnol (2002), 15,* pp *34-46*

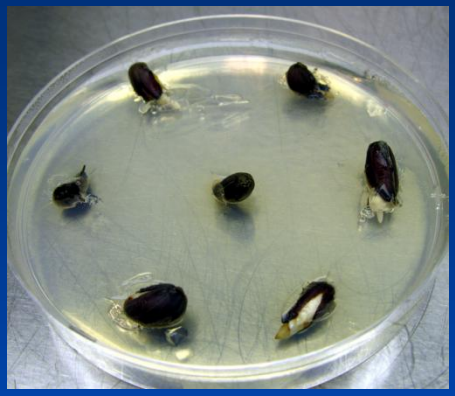
Germ line genetic transformation in cotton (*Gossypium hirsutum* L.) by selection of transgenic meristematic cells with a herbicide molecule Aragao F, Vianna G, Carvalheira S, Rech E Plant Science (2005), 168, pp 1227–1233

Tissue culture-independent *In Planta* transformation strategy: an *Agrobacterium tumefaciens*-mediated gene transfer method to overcome recalcitrance in cotton (*Gossypium hirsutum* L.) Keshamma E, Rohini S, Rao KS, Madhusudhan B, Kumar MU The Journal of Cotton Science (2008), 12, pp 264-272

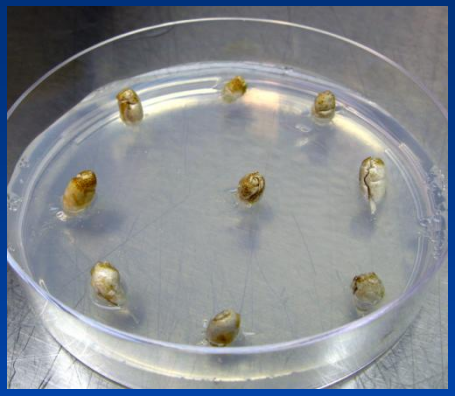
Multiple shoot regeneration of cotton (*Gossypium hirsutum* L.) via shoot apex culture system Bazargani MM, Tabatabaei B, Omidi M African Journal of Biotechnology (2011) Vol. 10(11), pp. 2005-2011

*Agrobacterium*-mediated transformation of cotton shoot apex with *SNC1* gene and resistance to cotton fusarium wilt in $T_1$ generation Lei J, Wang D, Shao L, Wei X, Huang L Cotton Genomics and Genetics (2012), Vol.3, No.1, pp 1-7

Transgenic Indian cotton (*Gossypium hirsutum*) harboring rice chitinase gene (Chi II) confers resistance to two fungal pathogens Ganesan M, Bhanumathi P, Ganesh Kumari K, Lakshmi Prabha A, Pill-Soon Song, Jayabalan N American Journal of Biochemistry and Biotechnology (2009), 5 (2), pp 63-74 ???

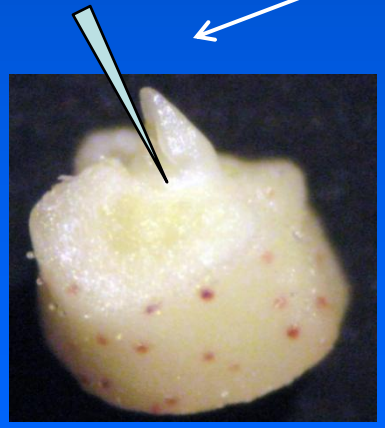


Seed sterilization



Seed germination for 48 h

5-10 days old seedlings



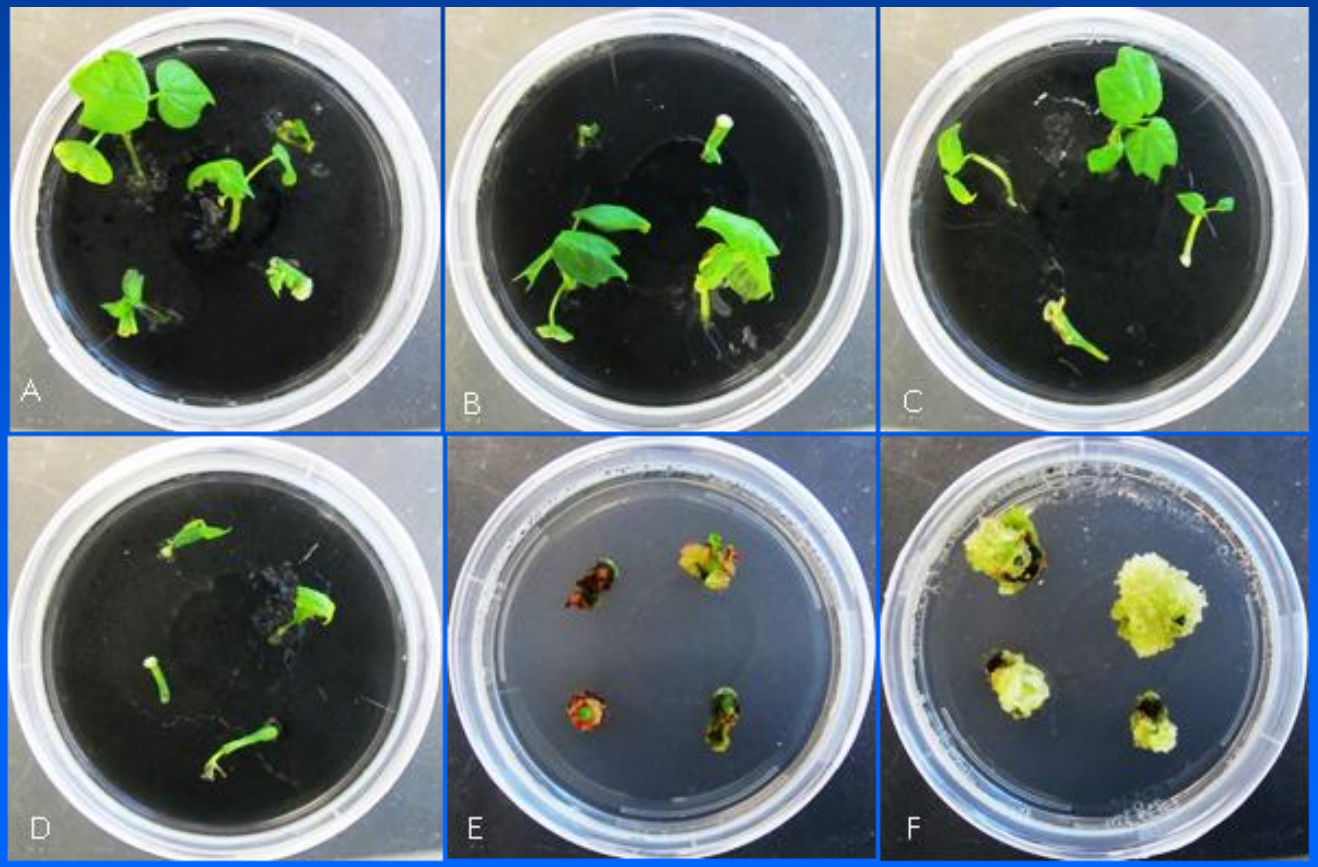
Needle wounding of apical meristem

Meristem wounding with sand and vortexing

Cut shoot apex

Split shoot apex

High-frequency regeneration via multiple shoot induction of an elite recalcitrant cotton (*Gossypium hirsutum* L. cv Narashima) by using embryo apex Pathi KM and Tuteja N Plant Signaling & Behavior (2013), 8:1, e22763, pp 94-99

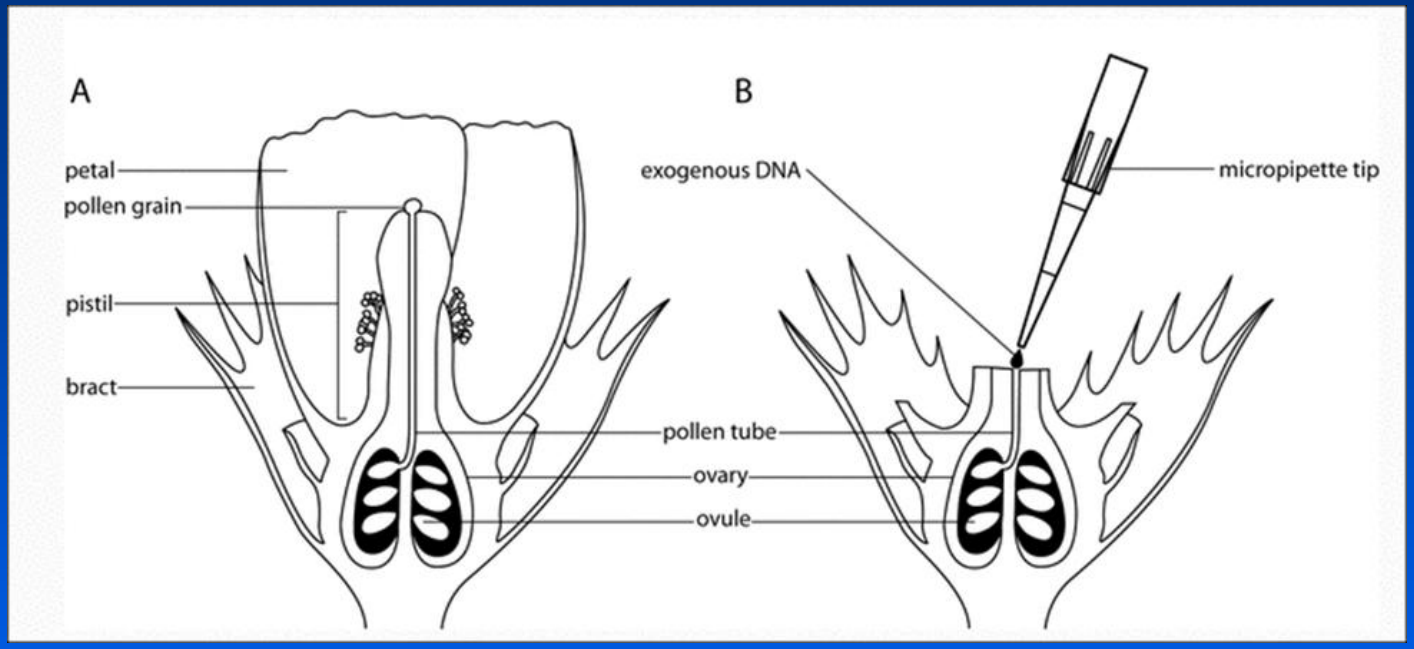


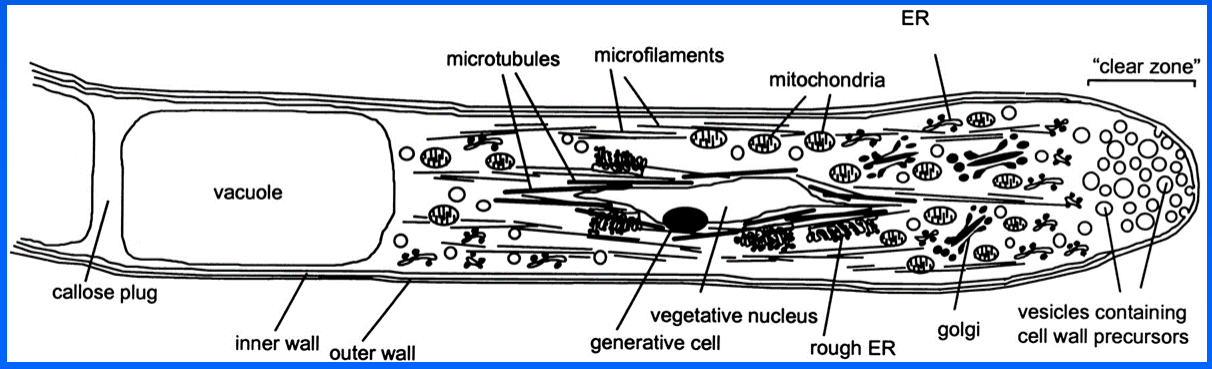
# *In Planta* pollen-mediated transformation

The preliminary study on transformation of cotton pollen using *Agrobacterium* - mediated vacuum infiltration Zhang Y, Huang L, Zhou X, Wang D Cotton Science (2008), 20(5), pp 354-358


Pollen-mediated genetic transformation of cotton with the *Arabidopsis thaliana hmgr* cDNA using the particle gun Gounaris Y, Galanopoulou S, Galanopoulos N, Ladopoulos A, Michailidis Z, Theophilous Z Food, Agriculture and Environment (2005), Vol. 3, Issue 2, pp 157-160 1.6%

# *In Planta* pollen-tube pathway transformation



Evaluation of Cotton Transformation Via Pollen-Tube Pathway Kohel RJ and Yu J In Vitro Cell Dev Biol (2001) 42, p.498–501

Direct transformation of higher plants through pollen tube pathway Peffley E, Allen R, Song P, Shang X Patented in 2003, Texas Tech University

Application of a transformation method via the pollen-tube pathway in agriculture molecular breeding Song X, Gu Y, Qin G Life Science Journal (2007), 4(1), pp 77-79

Establishment of transgenic cotton lines with high efficiency via pollen-tube pathway Zhang H, Zhao F, Zhao Y, Guo C, Li C, Xiao K Front. Agric. China (2009), 3(4), p. 359–365

Cotton transformation via pollen tube pathway Wang M, Zhang B, Wang Q Methods Mol Biol. (2013), 958, p,71-77

Development of transgenic CryIA(c) + GNA cotton plants via pollen tube pathway method confers resistance to Helicoverpa armigera and Aphis gossypii Glover Liu Z, Zhu Z, Zhang T Methods Mol Biol. (2013), 958, p.199-210

Pistil drip following pollination: a simple in planta Agrobacterium-mediated transformation in cotton Chen TianZi, Wu ShenJie, Zhao Jun, Guo WangZhen, Zhang TianZhen Biotechnol Lett (2010) 32:547–555

Protocol outline:

Pistil drip 10-12 hours after pollination resulted in 0.07–0.17% of Basta-resistant plants/viable seeds generated.

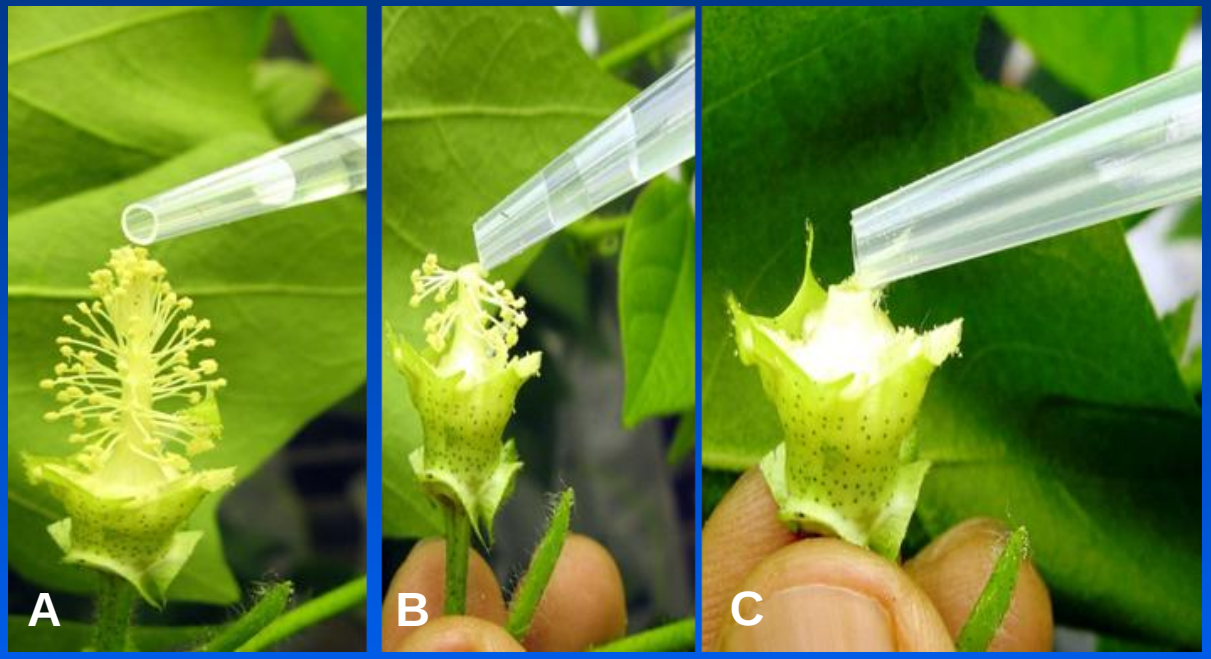
Stigma excision prior to pistil drip 10-12 hours after pollination provided transformation efficiency of 0.46–0.93%.

Stigma excision prior to pistil drip 24-26 hours after pollination resulted in only 0.04-0.06% of transformation efficiency.

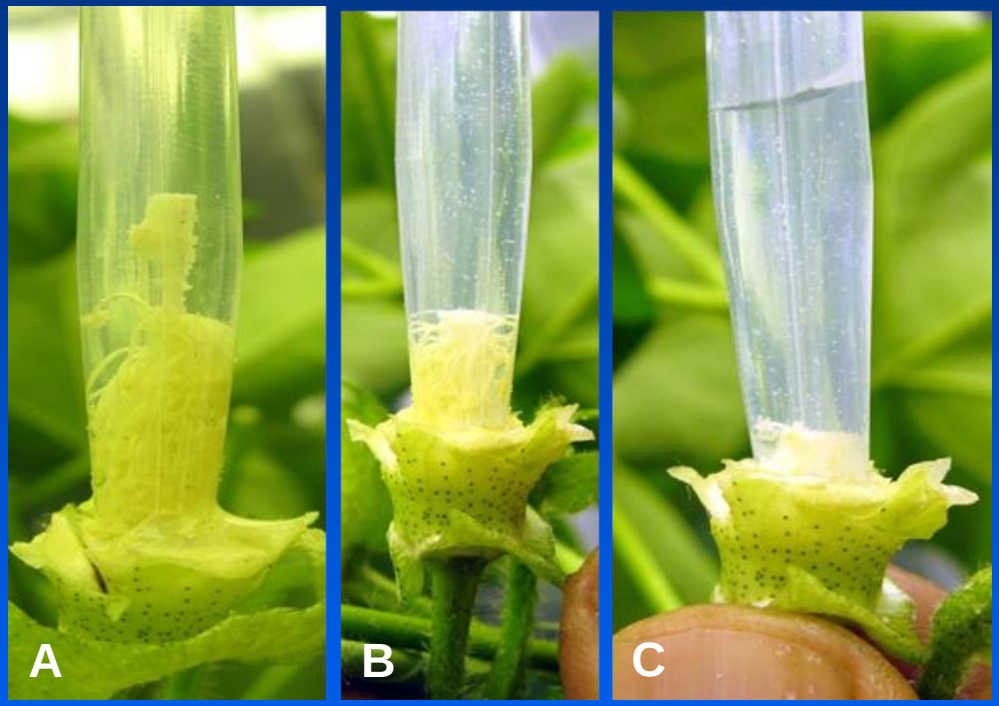
# *Agrobacterium-mediated* floral transformation

**A**, flower 6-8 h after hand pollination; **B**, flower after 22-24 h after hand pollination; **C**, flower with cut off sepals and petals before *Agrobacterium* treatments.

Agrobacterium injected into the style of flower

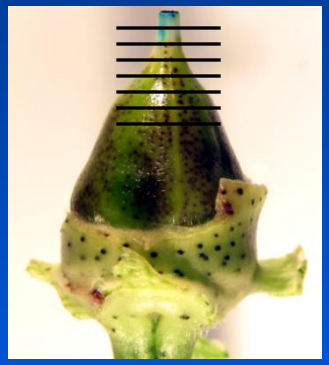



**A**, on the top of style (stigma was removed); **B**, on the half cut style (stigma with upper style was removed); **C**, on the basal part of style (entire style was removed).

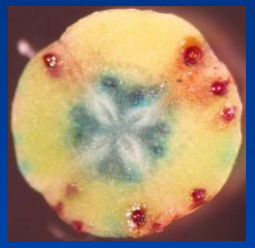


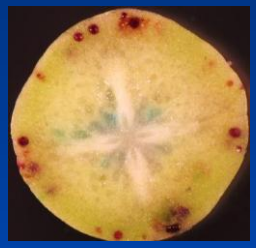

**A**, on the top of style (stigma was removed); **B**, on the half cut style (stigma with upper style was removed); **C**, on the basal part of style or tip of the boll (entire style was removed).

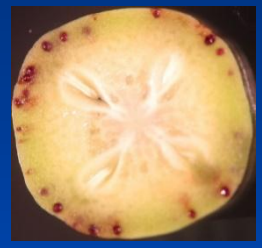
# Toluidine staining of cotton flowers



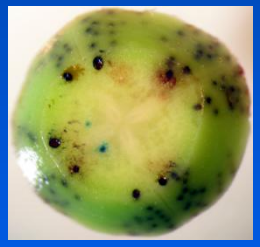
Toluidine staining and slicing



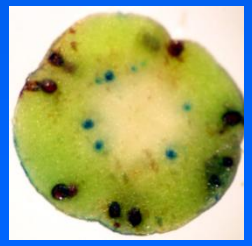


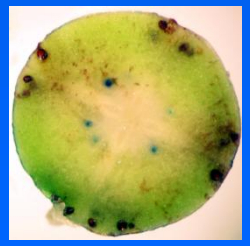


Flowers stained 3-4 hours after pollination



Flowers stained 7-8 hours after pollination

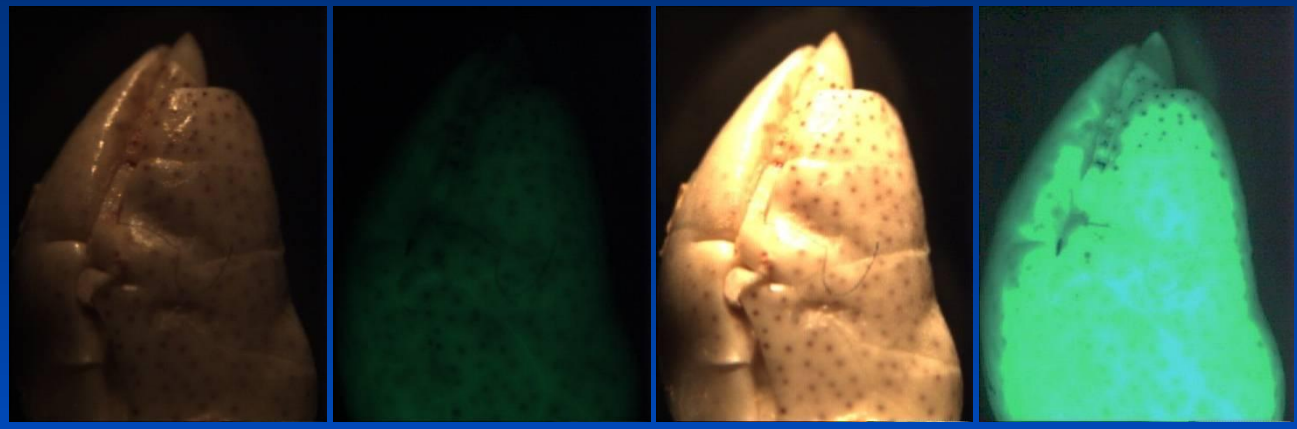




Flowers stained 23-24 hours after pollination

| | Agro applied 2-4 h after pollination on uncut stigma | Agro applied 24-26 h after pollination on cut style | Agro applied 10-12 h after pollination on uncut stigma | Agro applied 10-12 h after pollination stigma excised |
|---|---|---|---|---|
| No. of flowers treated | 664 | 785 | 605 | 263 |
| Bolls harvested | 55 | 645 | 339 | 54 |
| Viable seeds | 786 | 16136 | 8880 | 1218 |
| Transformed seeds | 0 | 2 | 4 | 7 |
| Boll setting rate | 8.28% | 82.17% | 23.33% | 21.34% |
| Transformation efficiency | 0% | 0.01% | 0.05% | 0.57% |

# Effect of $GA_3$ on boll and seed development

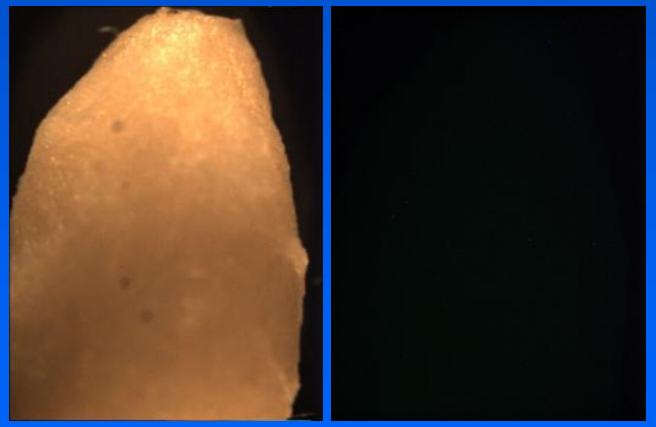



Bright 1x 0.320 sec  Blue 1x 0.320 sec  Bright 1x 1.0 sec  Blue 1x 1.0 sec

**T2 mature seeds transformed with TN211 construct**

Bright 1x 0.98 sec  Blue 1x 0.98 sec

**20-22 days old seeds collected from the flowers treated with *Agrobacterium* carrying TN211 construct**



Bright 1x 2.0 sec  Blue 1x 2.0 sec

**20-22 days old seeds collected from untransformed Coker 312 flowers**

# Summary of floral transformations experiments

| Hours after pollination | Number of flowers treated | Number of bolls analyzed | Results |
|---|---|---|---|
| 8 hours | 162 | 101 | No GFP expression |
| 24 hours | 111 | 95 | No GFP expression |

# Acknowledgement

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