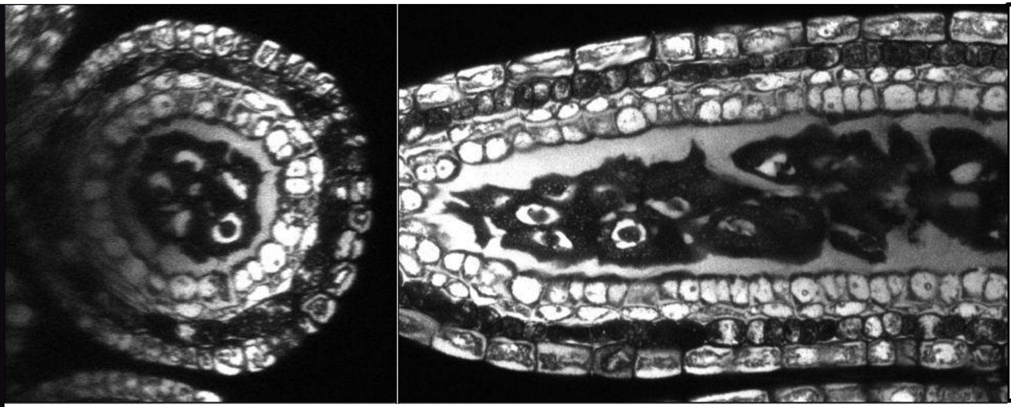
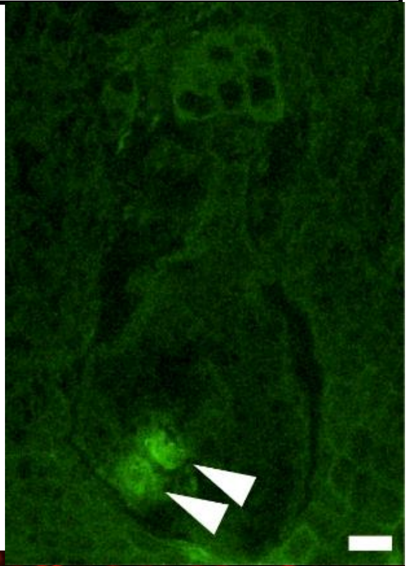


Reproductive Biology

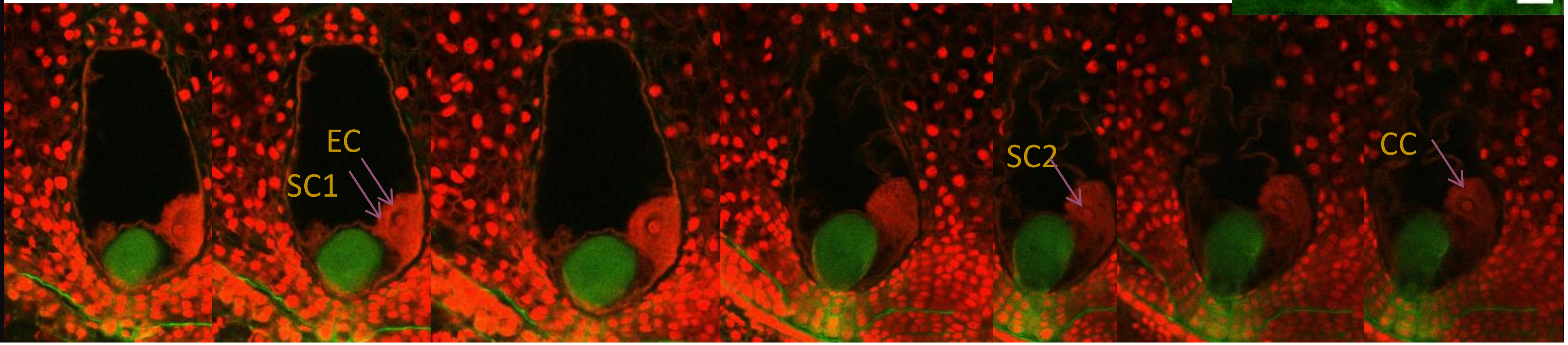


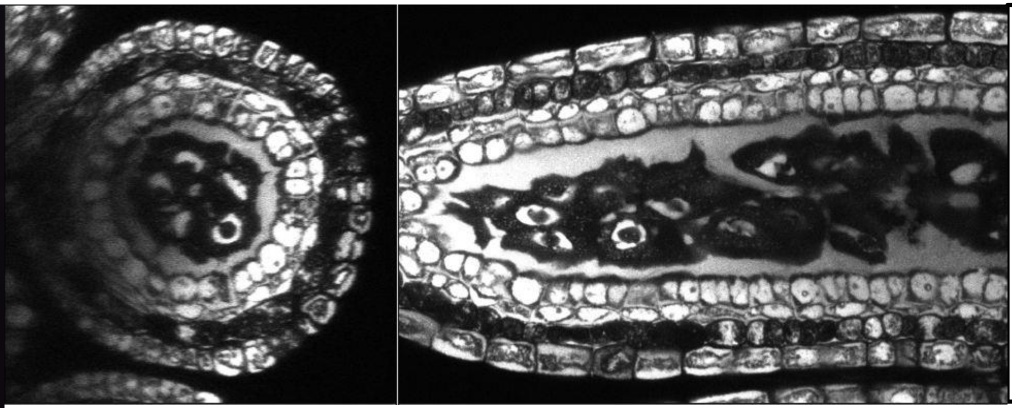
Fertile Ground for New Breeding Technologies

Tim Kelliher, Principal Scientist in Reproductive Biology

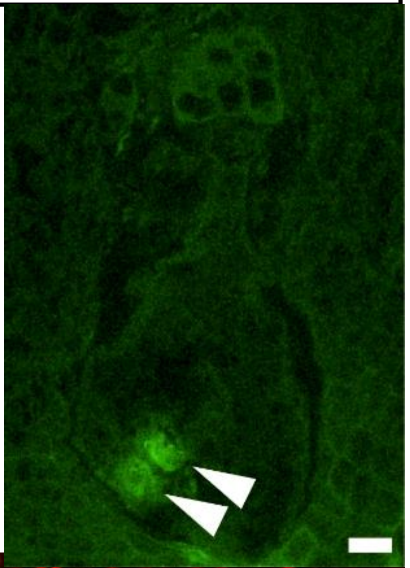
Seeds Research, Syngenta Crop Protection

February 9th, 2018



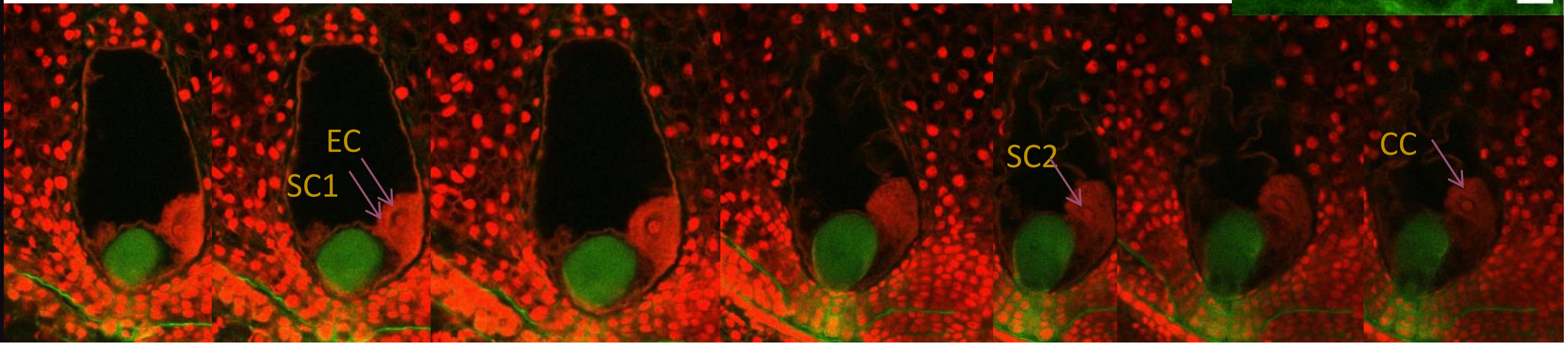


Reproductive Biology

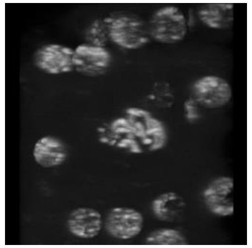


Improving the sex life of corn

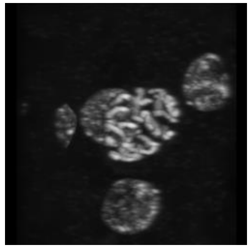
Tim Kelliher, Principal Scientist in Reproductive Biology
Seeds Research, Syngenta Crop Protection
February 9th, 2018



Haploids & doubled haploids

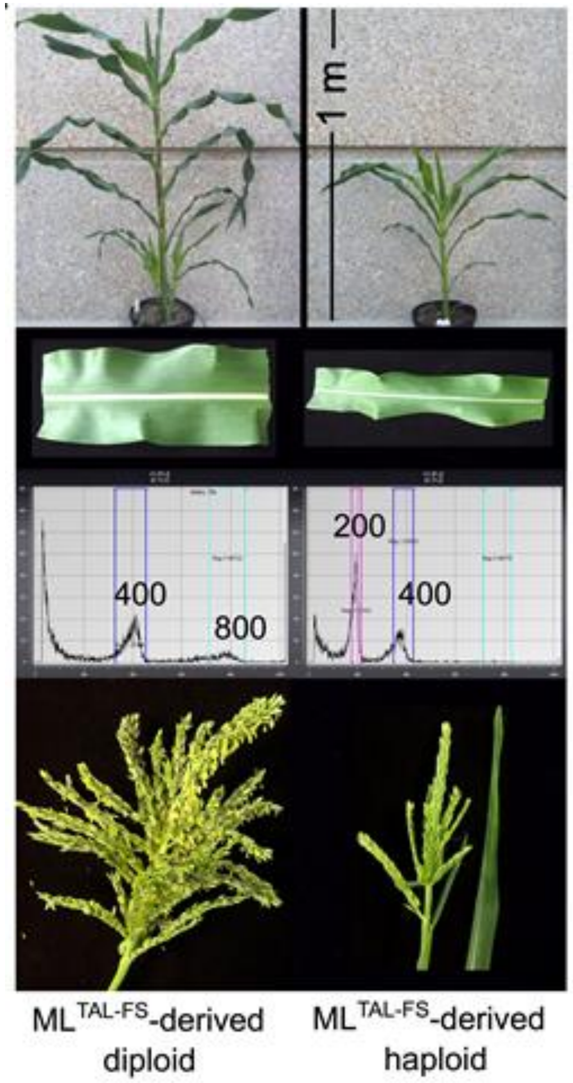


Haploid
10C



Diploid
20C

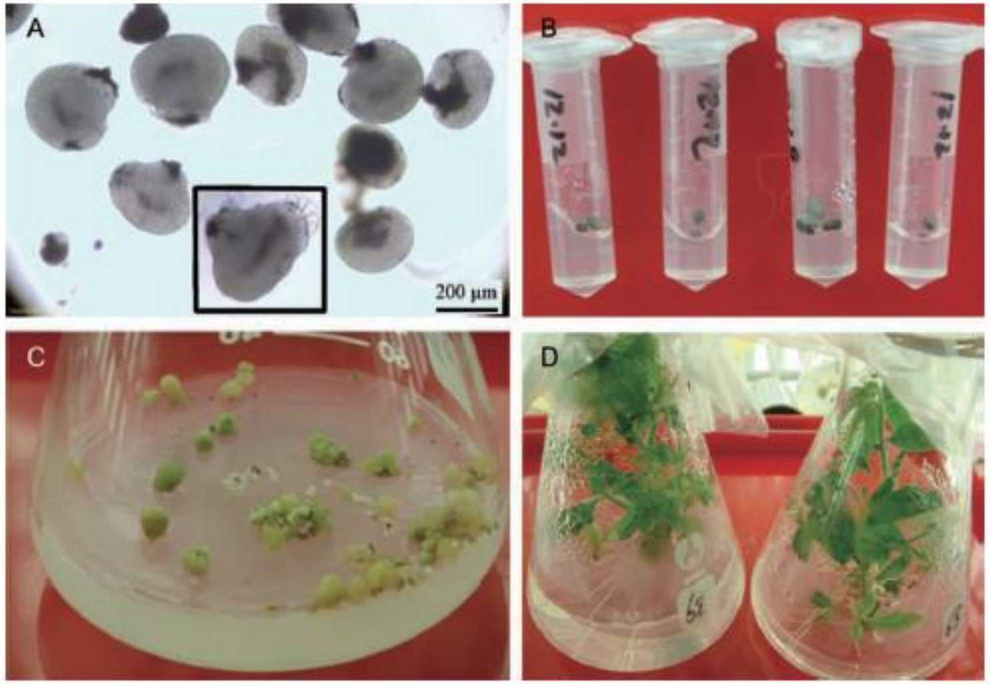
Haploid individuals have the gametic chromosome number (n) in their somatic cells



3D Images by Judith Sheldon, Syngenta Microscopy Core

Haploid Induction Vary in Different in Different Crops

- Brassicas; many other species: Anther, microspore, or



Fertilized (3n)
endosperm

Ovule culture, Zhao, J. Int Ag, 2014

Piosik, L. *et al*, Euphytica 2015

Kasha, K.J. & Kao, K.N. High frequency haploid production in barley (*Hordeum vulgare L*.). ***Nature*** 225, 874-876 (1970)

Burke L. G., et al., Maternal haploids of *Nicotiana tabacum L*. from seed ***Science*** 206, 585 (1979)

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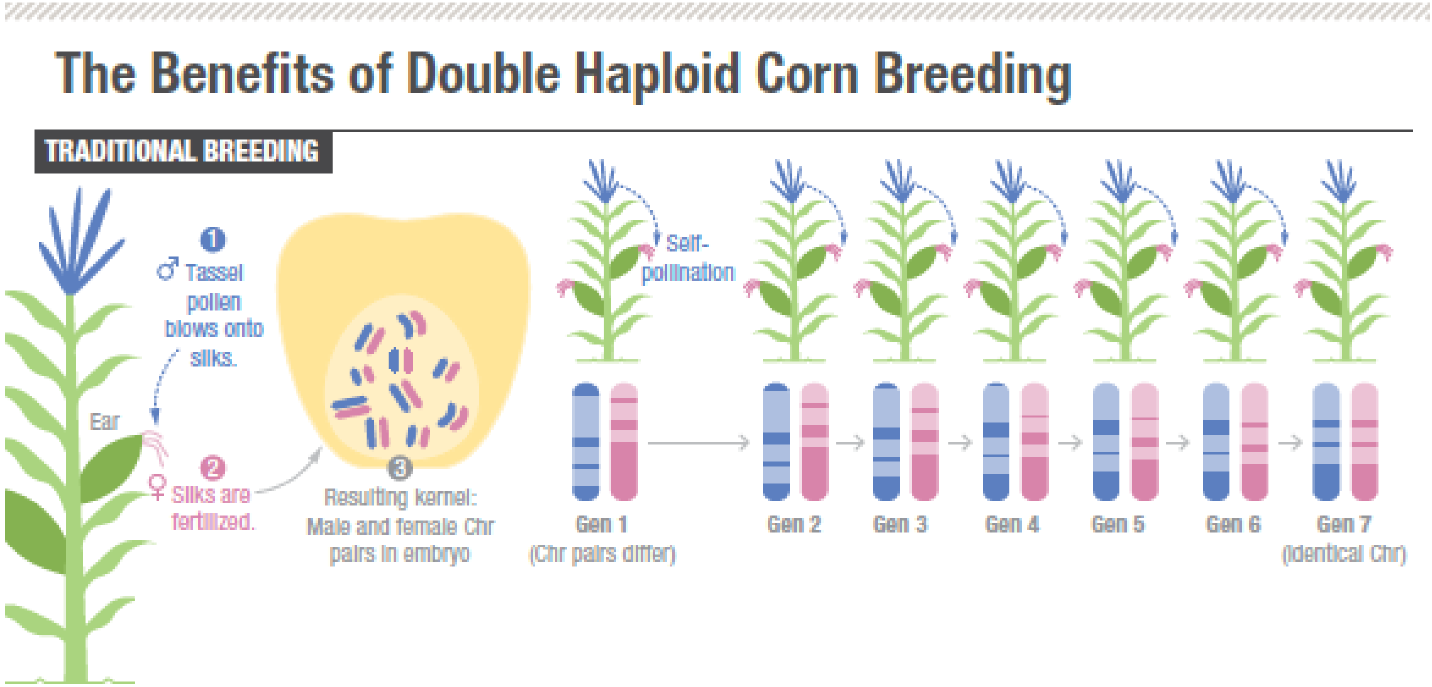
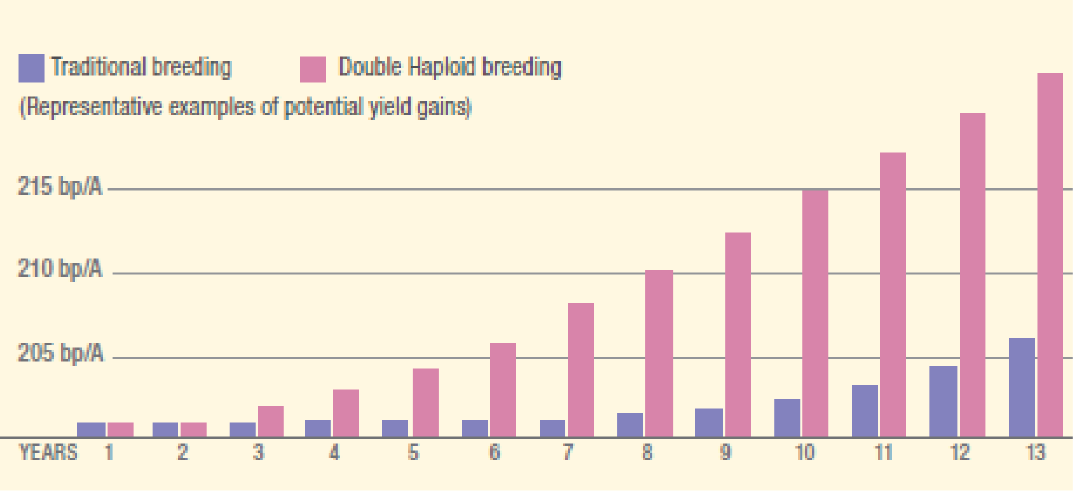
- DH = Rapid production of inbred lines
  - Inbreds help us understand the connection between genes and traits
  - They are also needed to produce hybrids





1950s: Discovery of the first haploid inducing line (maize)

Vol. XCIII, No. 873 The American naturalist November-December, 1959

LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

A LINE OF MAIZE WITH HIGH HAPLOID FREQUENCY

Chase (1949) has reported considerable variation in monoploid frequencies among different lines of maize, dependent upon both the maternal and paternal parents. The highest frequency found (0.688 per cent haploids from a particular single-cross hybrid crossed by a particular inbred pollen parent) is well above the average frequency of 0.111 per cent for all crosses used in the study. It is generally accepted that a haploid frequency of 0.1 per cent is usual.

A genetic inbred with an unusually high frequency of haploids has been found. Data accumulated over several years on self-pollinated progenies of an inbred designated as "stock 6" show 343 haploids in 10,616 observed plants, giving an over-all frequency of 3.23 per cent. Relatively few of these haploids have been verified by chromosome counts on root tips, as they are field-grown plants; however, the uniformity of stock 6 and the striking features of its haploids permit clear classification within the line with-

Advances in plant breeding

- Conventional Breeding
  - Introduce genes from the same or related species (wide cross introgression)
  - Induce variation (chemical mutagenesis, mutant screening)

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RWK was obtained from Univ. Hohenheim; mapping initiated

Satya Chintamanani

Brent Delzer

Initial Mapping

NP2391 X RWK	NP2460 X RWK
↓	↓
F1	F1
↓ X RWK	↓ X RWK
BC1	BC1
↓⊗	↓⊗
BC1F2	BC1F2
↓⊗	↓⊗
BC1F3	BC1F3
↓⊗	↓⊗
BC1F4 x 2 Testers	BC1F4 x 2 Testers

Mapped the QTL to Bin1.04

QTL = Quantitative Trait Locus

Qhir1 → 66% of variation

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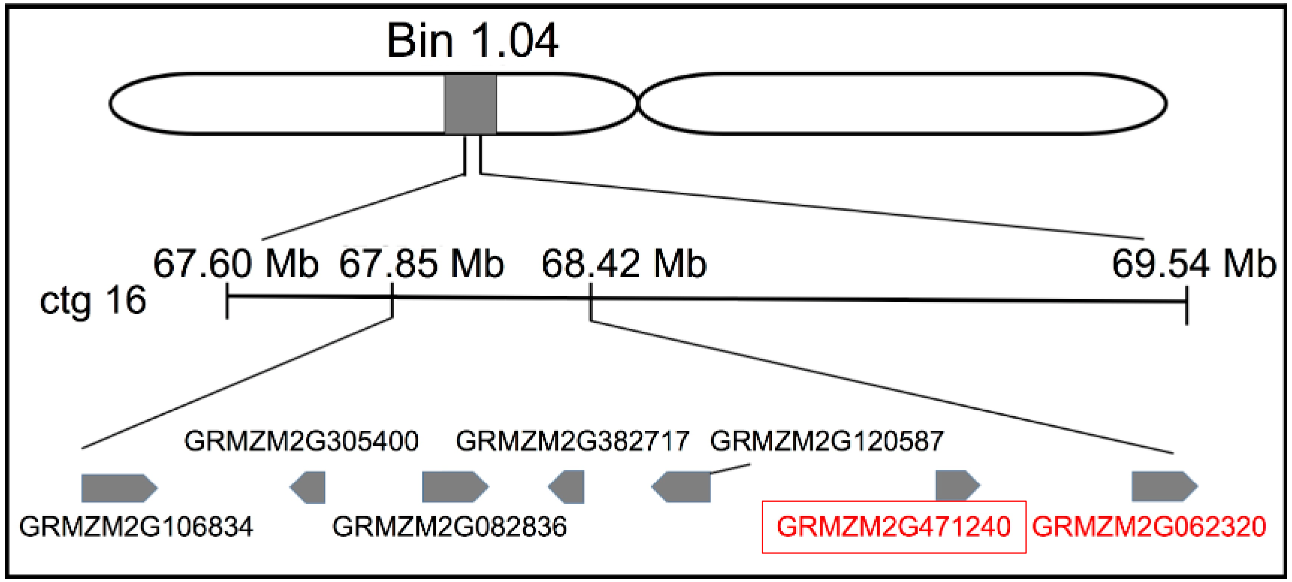
2012: Seven genes in QTL sequenced in inducer (RWK, Stock6) and non-inducer lines (RWK-NIL, B73)



Research Triangle Park

- Mike Nuccio
- Bob Dietrich

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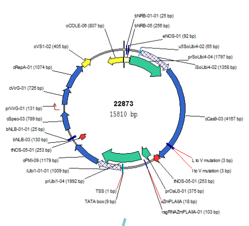
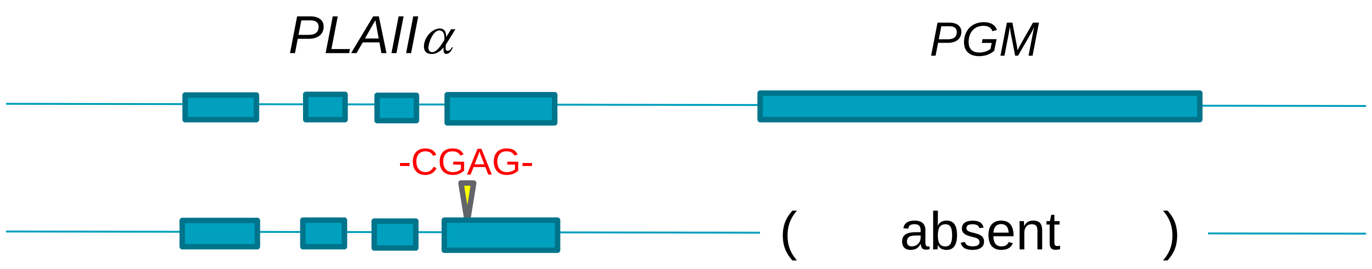
2013-2014: Complementation test, transgenic experiment

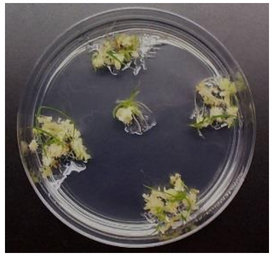


B73 

RWK 



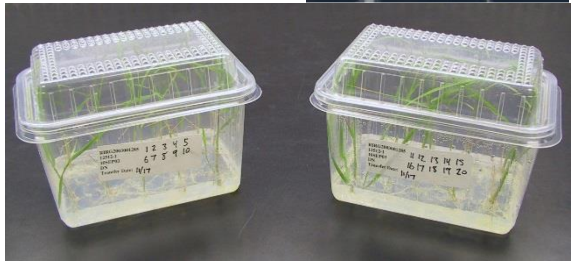
Pollen source	# ears	seed set	embryos	haploids	HIR
Control inducer	4	133/ear	255	26	**10.20%**
Control Inducer + *WT-PLA2* / *WT-PLA2*	16	357/ear	3047	7	**0.23%**



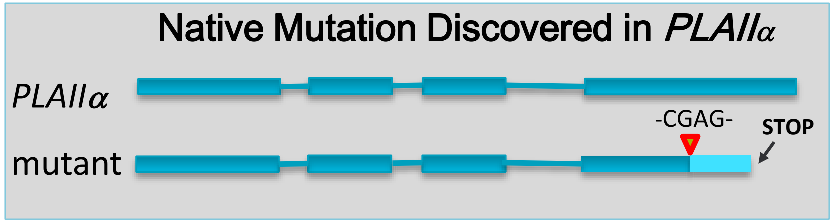
PLAIIα

Michael Nuccio
Dakota Starr
Anna Prairie

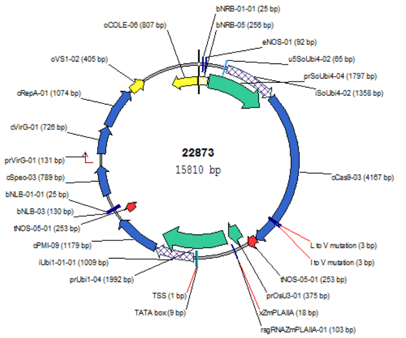
Lee Richbourg
Heng Zhong
David Bradley

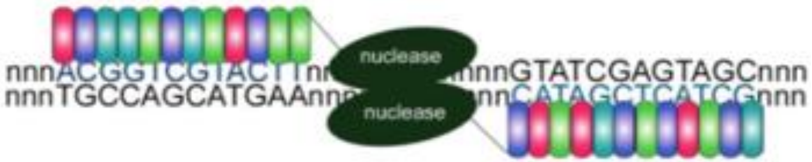


2015: TALEN editing provides the final proof



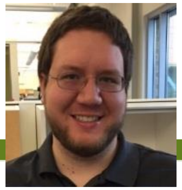
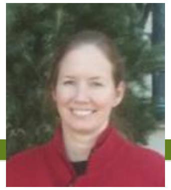
TALEN construct targeting native 4bp insertion site, induced several mutations





Pollen Source	Mutations	Ears	% Abort	Embryos	Putative Haploids	Confirmed Haploids	Induction Rate
AX	None	8	0.7%	1432	1	1	< 0.1%
Control inducer	4bp insertion	4	47.6%	531	56	54	10.2%
TAL-3954	-13bp; -28bp	4	44.10%	579	37	35	6.04%
TAL-3924	-8bp; -5bp	2	50.40%	128	18	16	12.50%
TAL-3932	-13bp	2	43.90%	169	18	15	8.88%
TAL-3317	-13bp	2	37.10%	343	19	19	5.54%
TAL-3303	-13bp	1	34.60%	176	7	7	3.98%
TAL-4108	-11bp; -5bp	4	40.10%	379	28	26	6.86%

Dakota Starr
Jamie McCuiston
Zhongying Chen
Shujie Dong





Tara Liebler
Sam Nalapalli
Siva Elumalai
Wenling Wang

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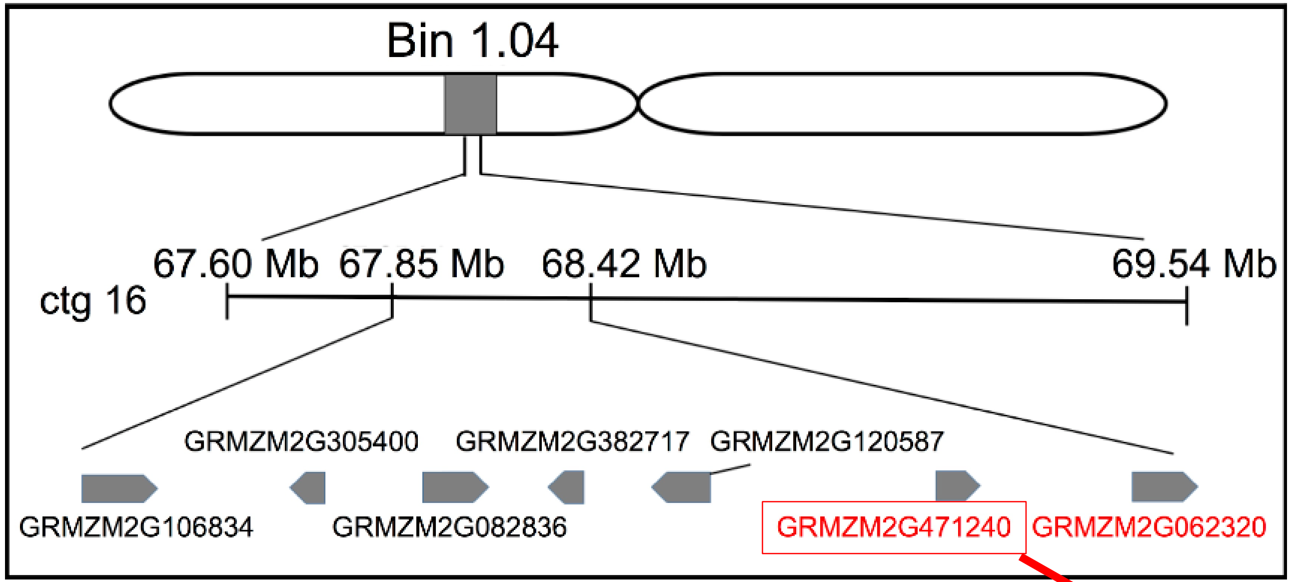
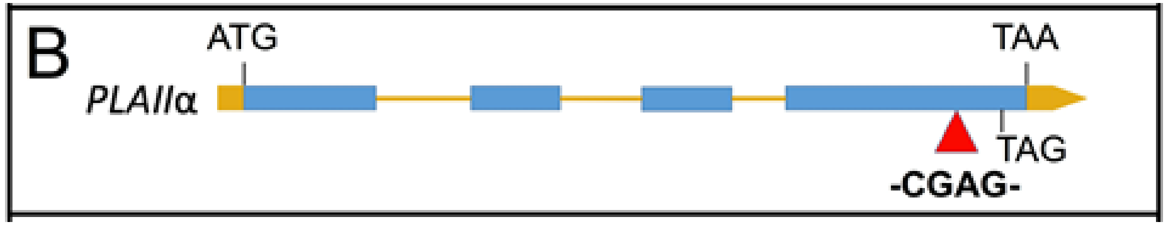


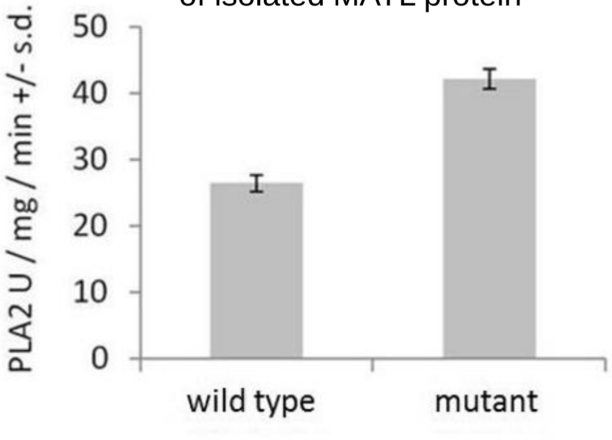


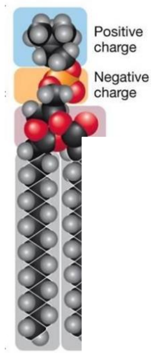
PLAIIα renamed *MATRILINEAL*

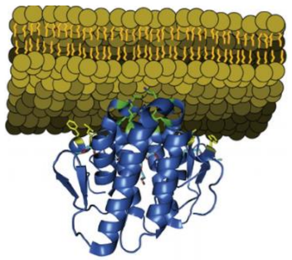
How does MATRILINEAL induce haploids?

Phospholipases cleave fatty acid chains off of membrane phospholipids

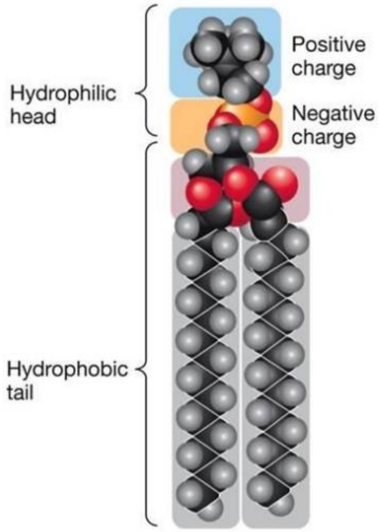
In vitro phospholipid cleavage assay of isolated MATL protein







MATL



Lyso-phospholipid
→ signaling, membrane remodeling

Free fatty acid → prostaglandins (animals)
oxylipins (plants)

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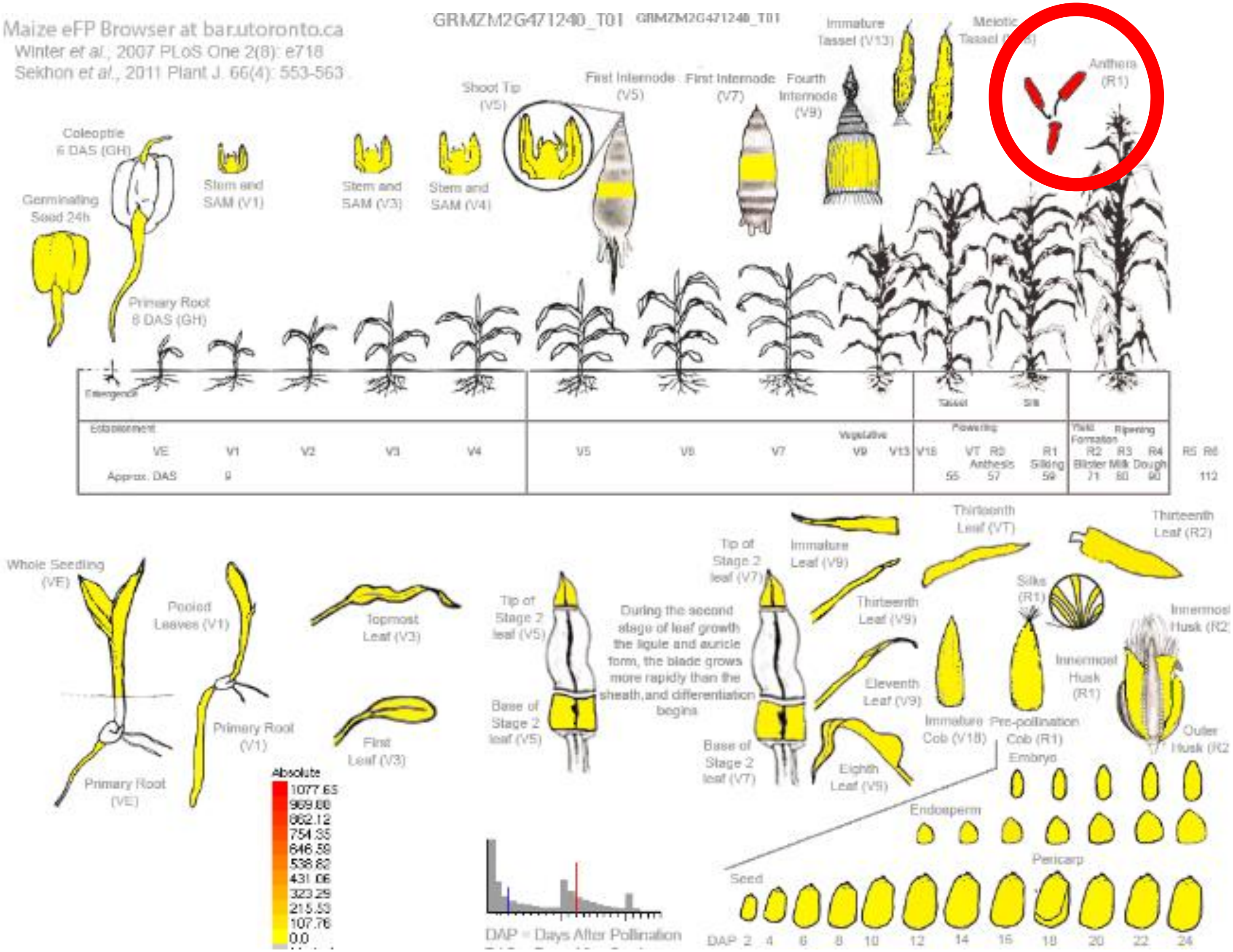
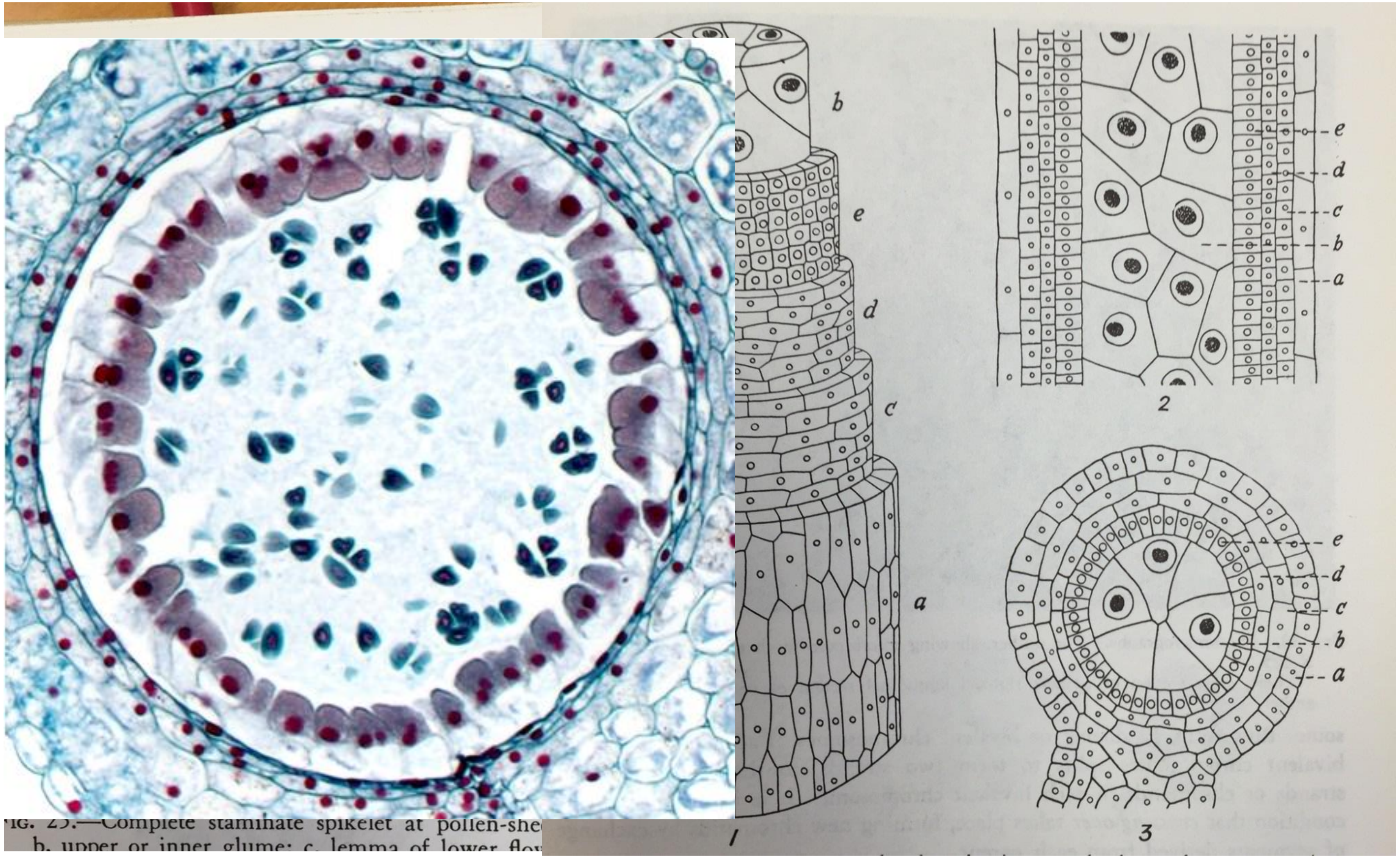
How does MATRILINEAL induce haploids?

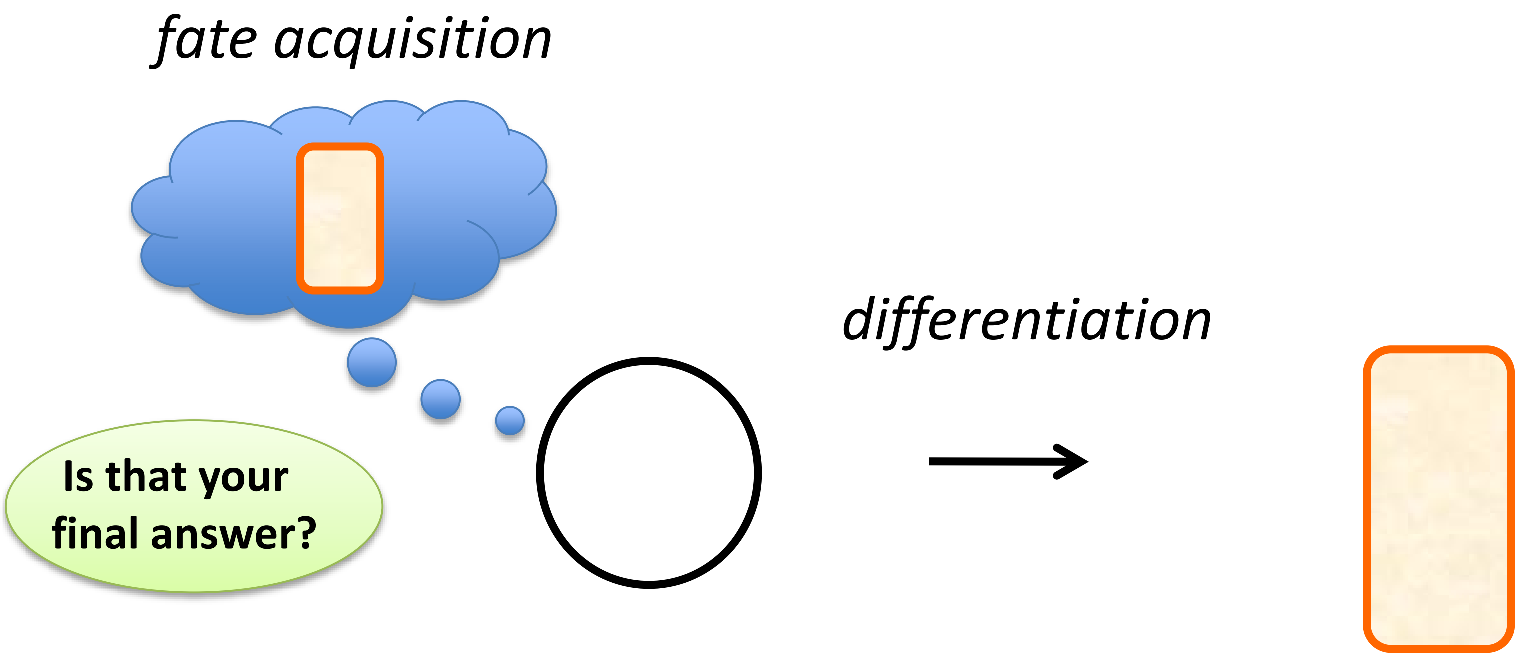
Expression pattern of *Matrilineal* RNA – anthers only!

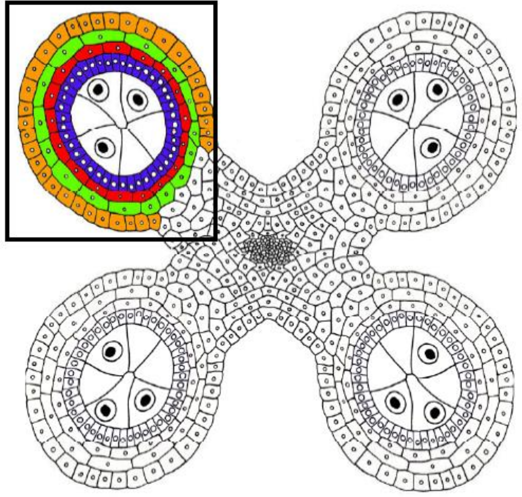


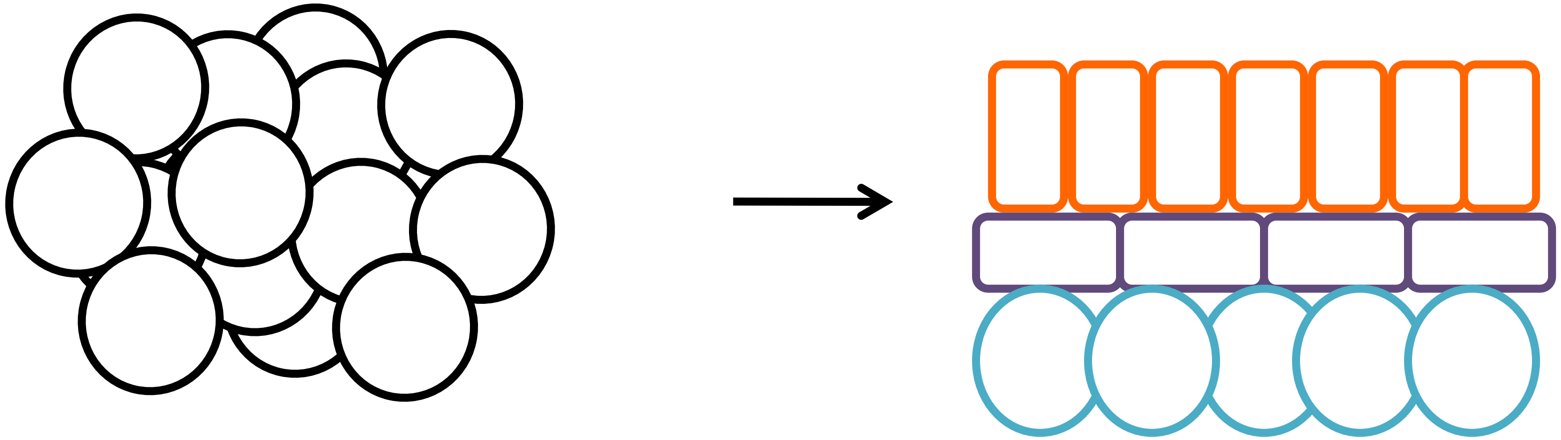
Formation of the male gametophyte (pollen)



Theodore Kiesselbach, 1949   The structure and reproduction of corn
University of Nebraska, Agricultural Experimental Station

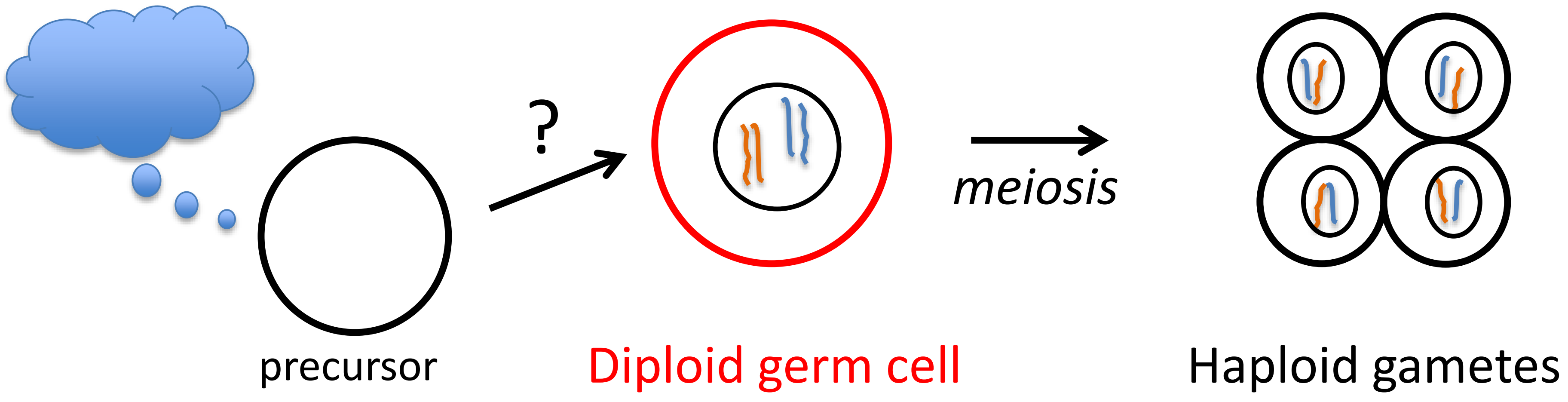






Cell walls → Fixed positions → get to know your neighbors!

tissue composition and arrangement



Germ cells: the ultimate totipotent cell

All others (somatic): “dead end” lineages

Germ cell specification in animals and plants

early sequestration, quiescence

late specification

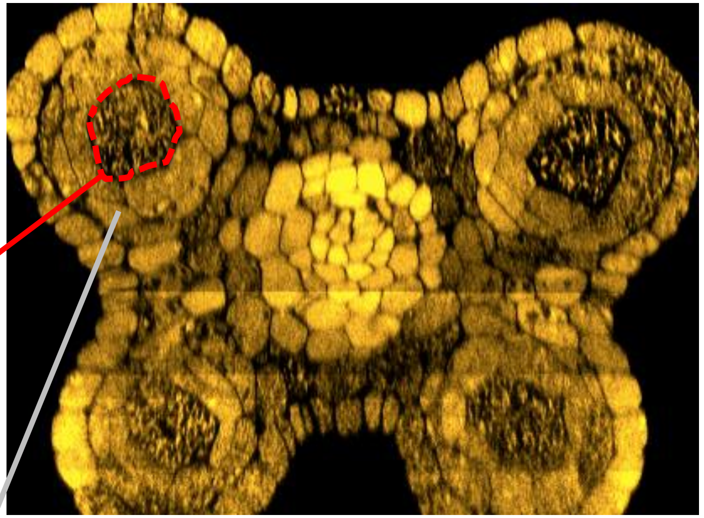
need for protection from DNA damage

induction by somatic cells

continuous production

When and where do germ cells arise?

anther

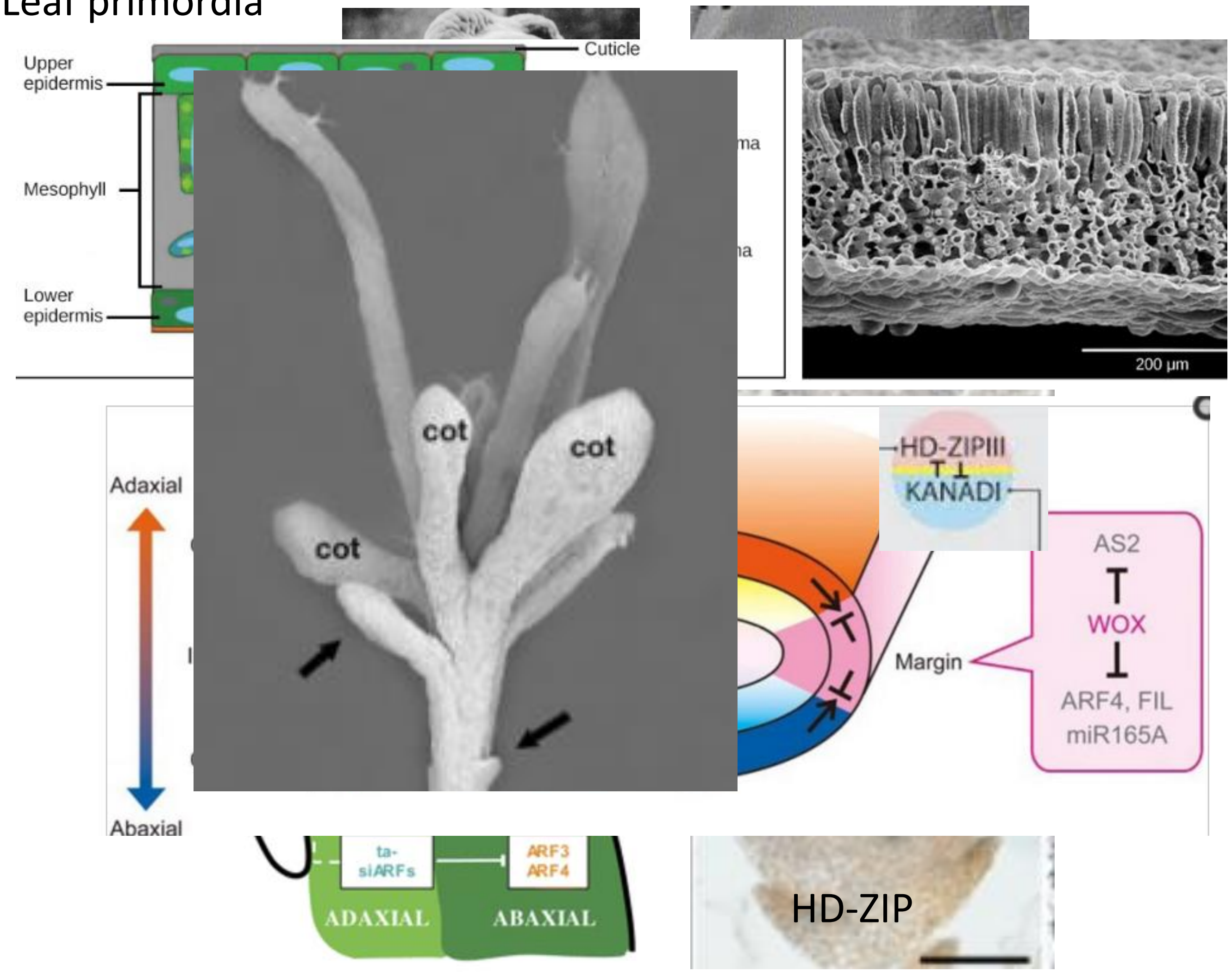


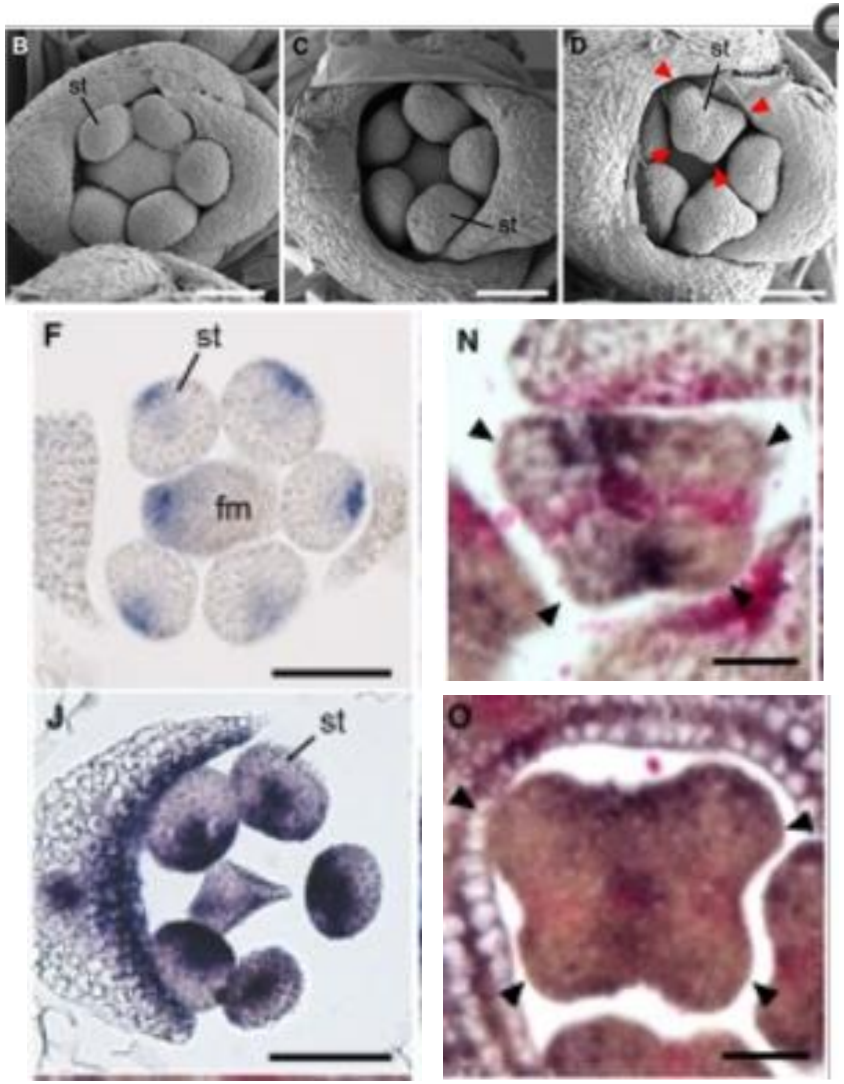
germ cells

somatic “nurse” cells

Leaves vs. Anthers

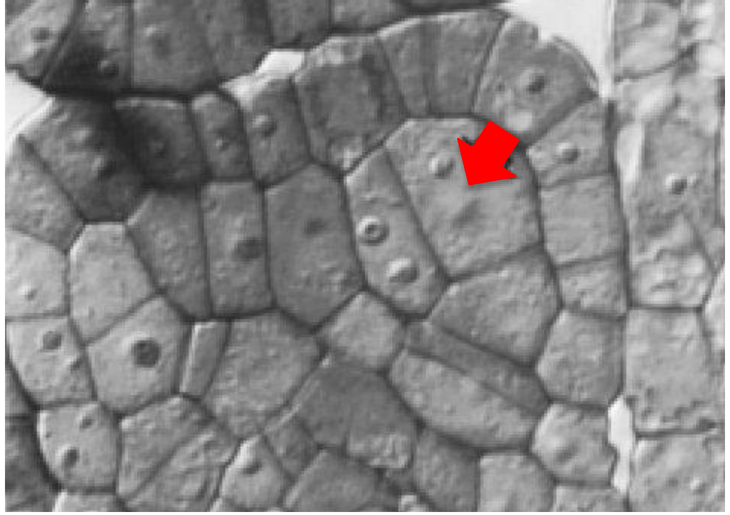
Shoot apical meristem
Leaf primordia





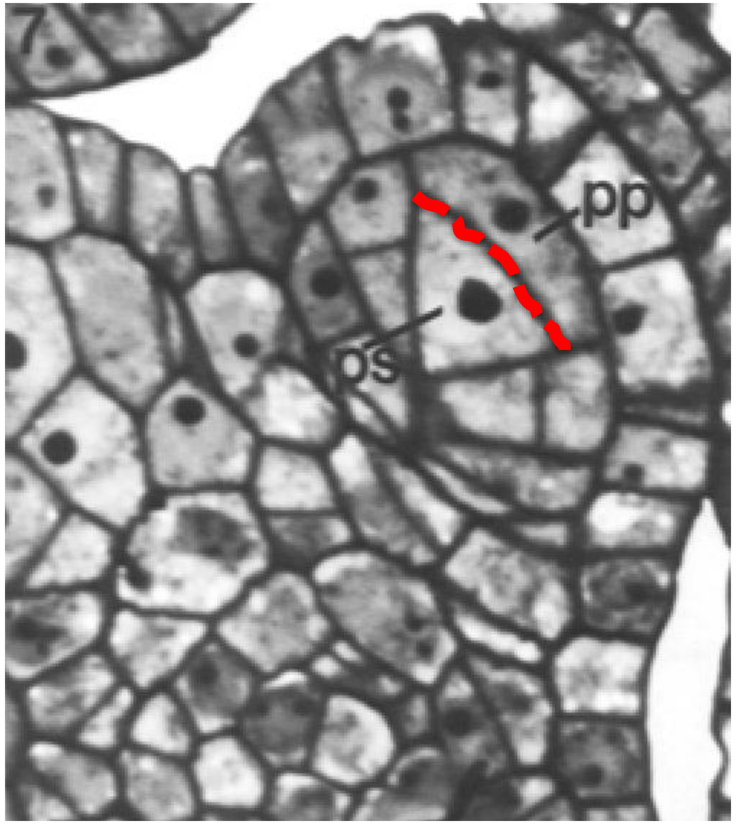
TEXTBOOK VIEW: lineage defines germinal fate

asymmetric cell division (ACD) is the mechanism

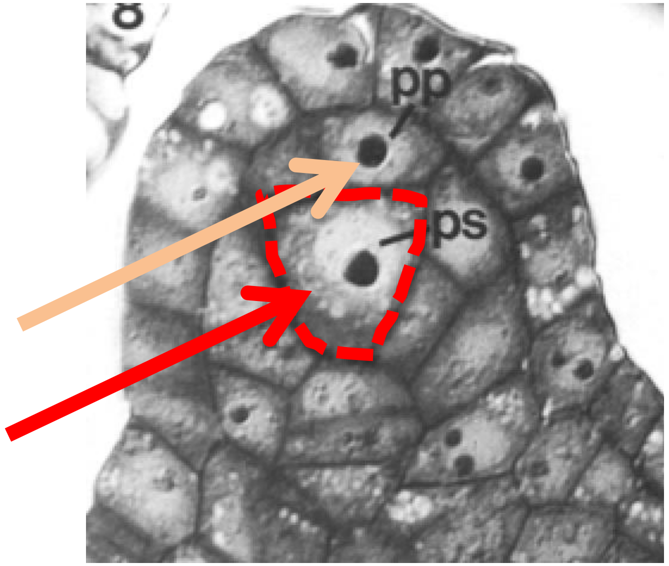


Raghavan, V., 1988. *Am J. Bot.* 75(2) (rice)

hypodermal cell



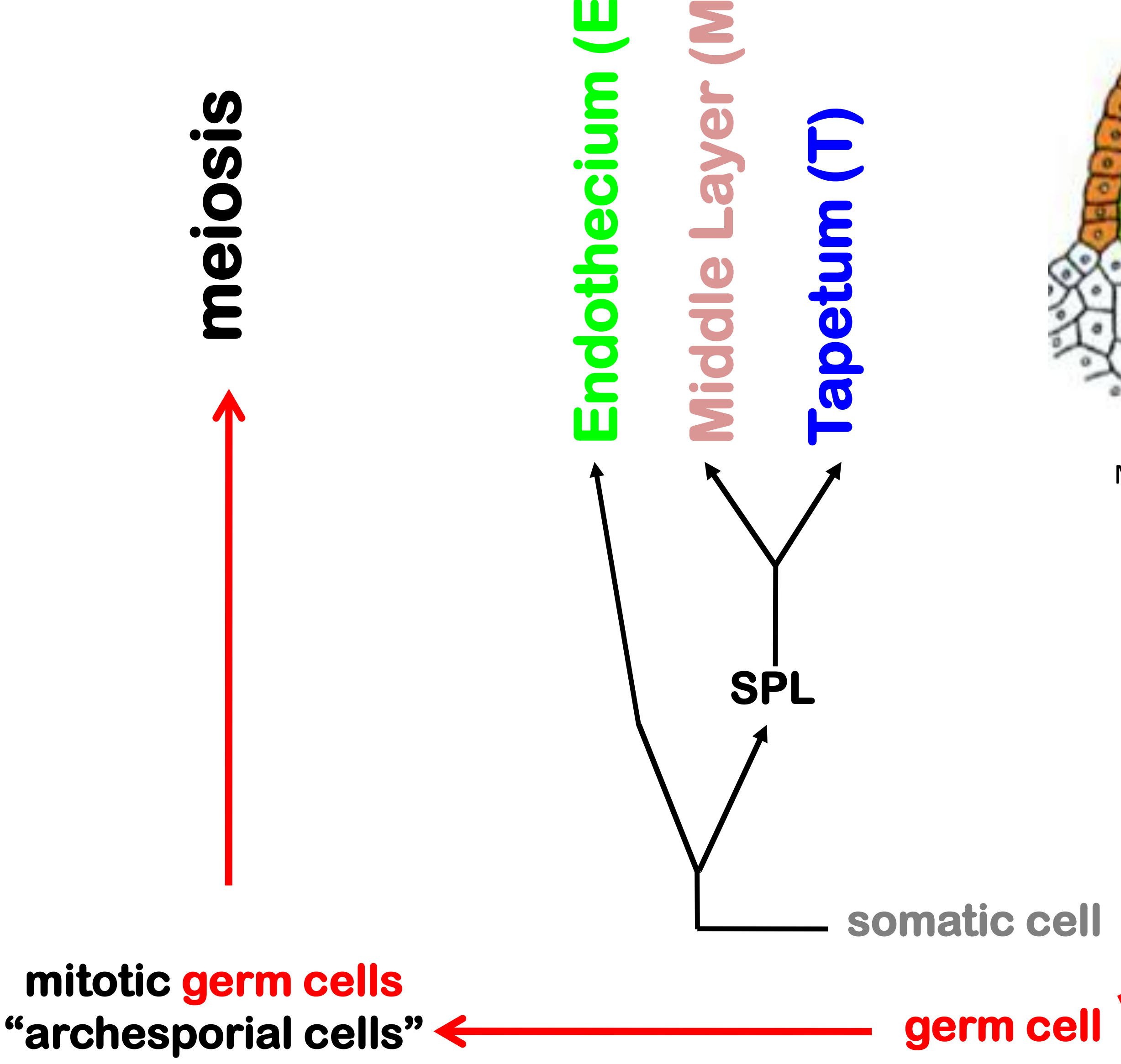
undergoes ACD

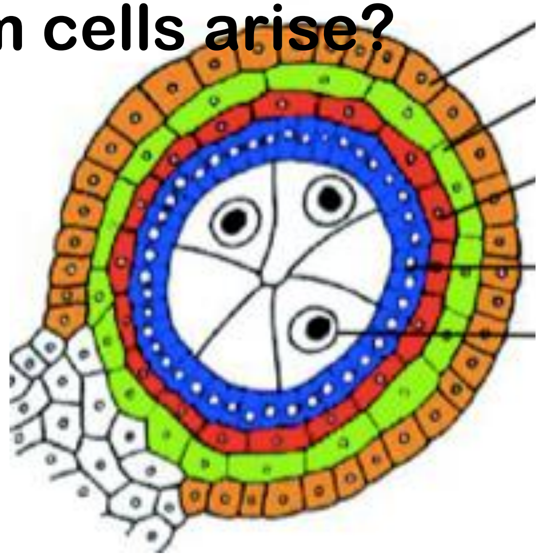


somatic cell

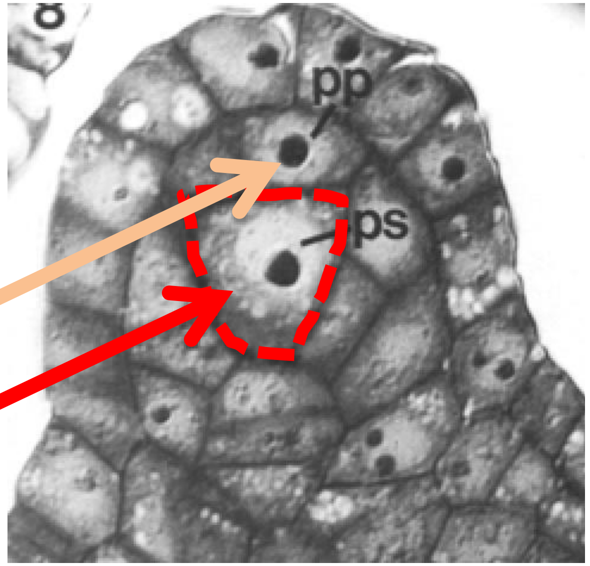
germ cell

When and where do germ cells arise?



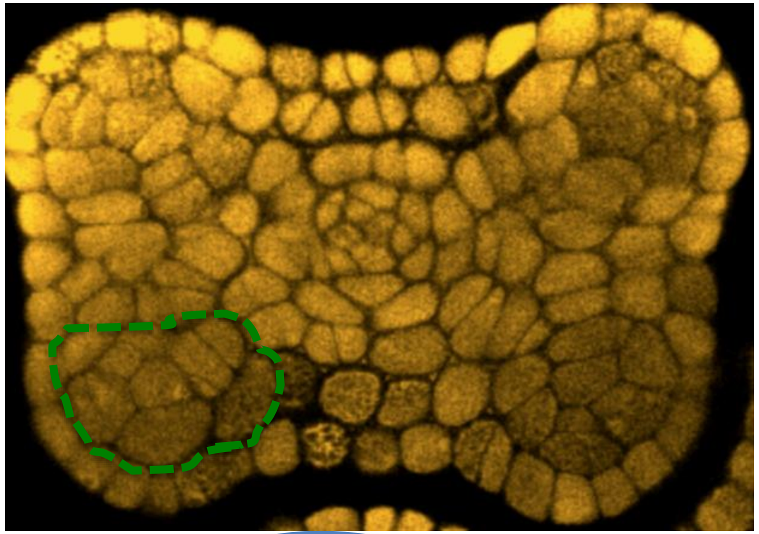


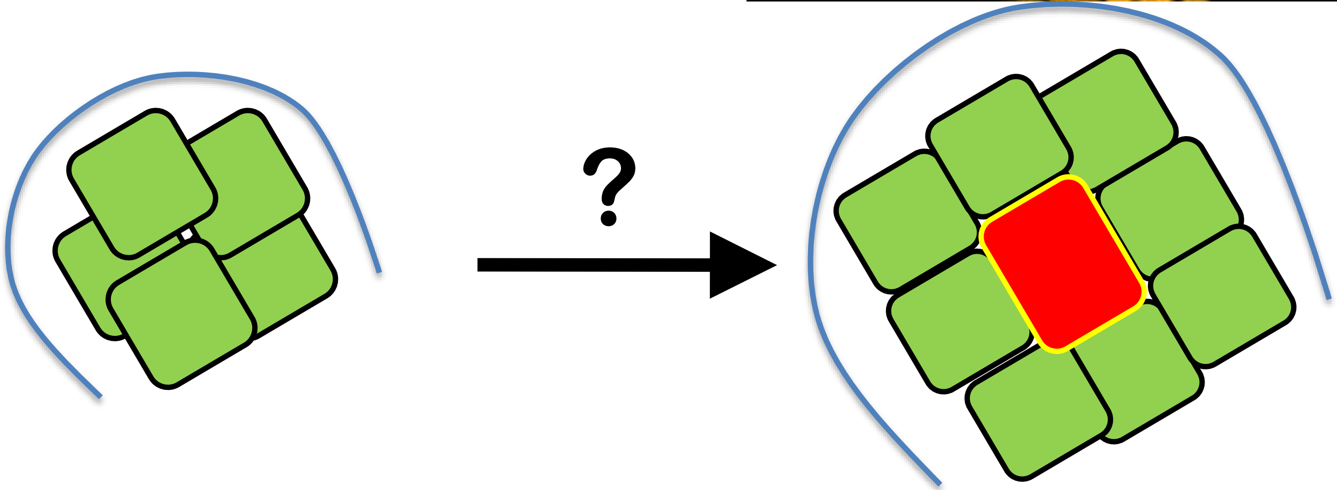
Ma, *et al.*, 2008. *Genome Biology*



When and where do germ cells arise?

~ 30-40 morphologically equivalent L2-d cells per lobe

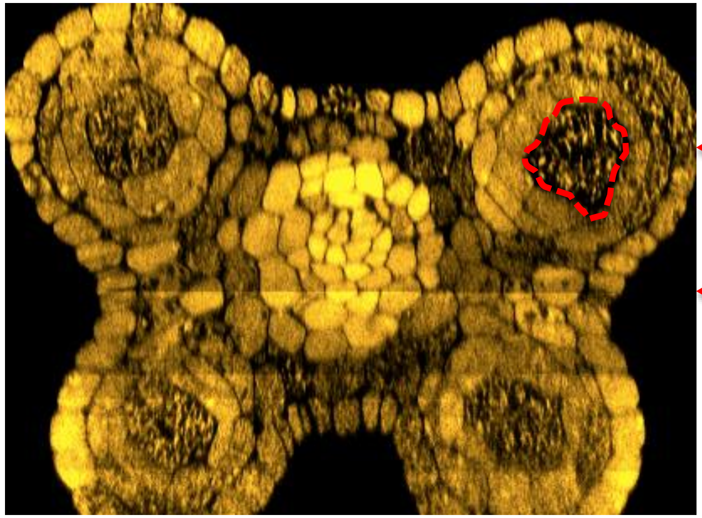


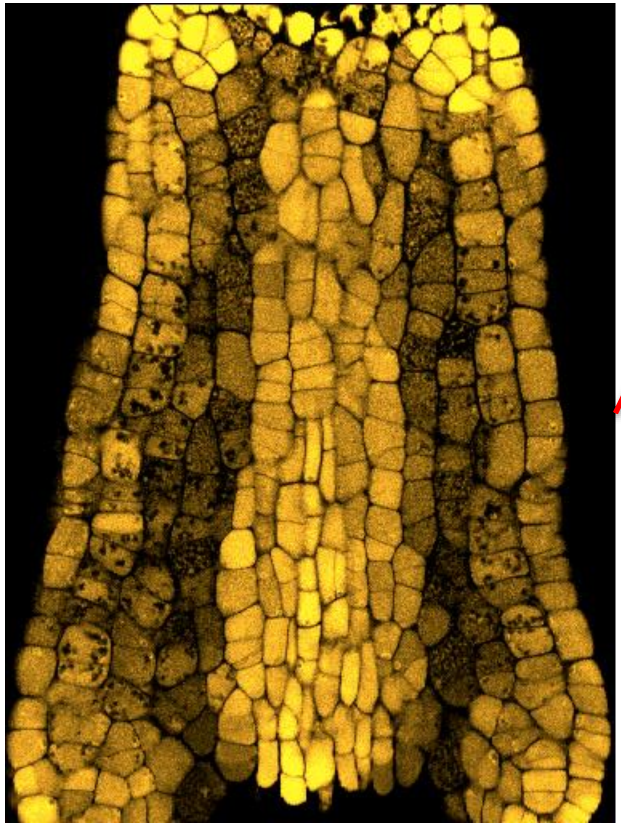


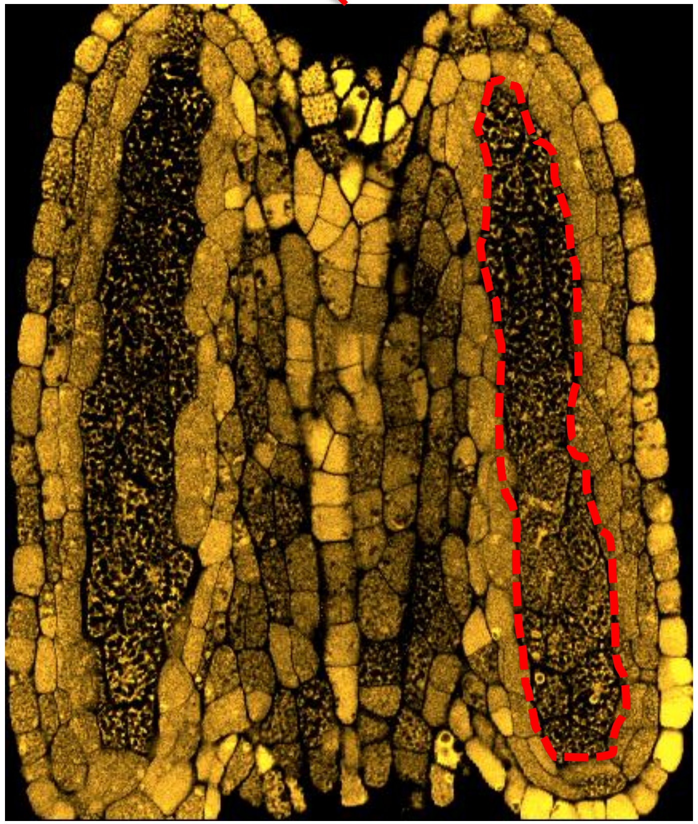
L2-d cells

AR (germ line)
+ L2-d cells (somatic niche)

NEW APPROACH: confocal microscopy





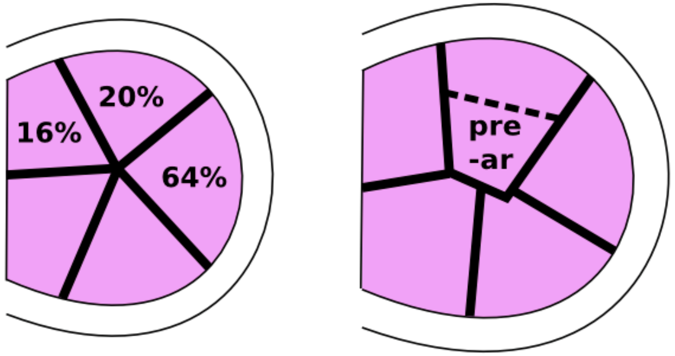


Characteristics of germ cells

Darker stain
Rounded shape
Central position

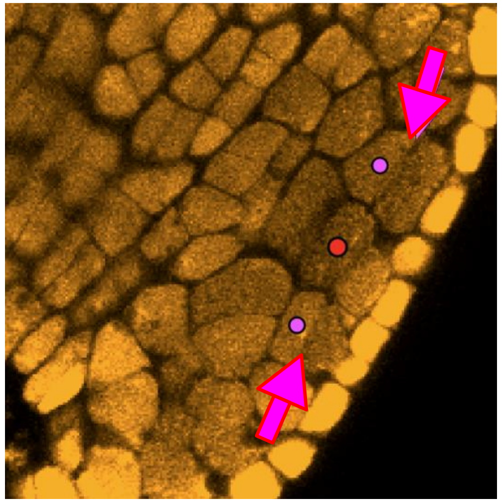
Kelliher & Walbot, 2011 *Dev. Biol.*

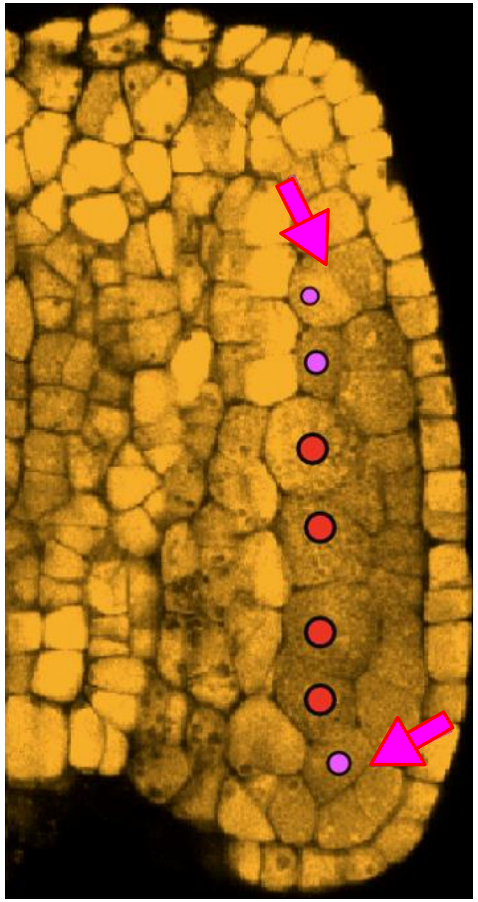
When and where do germ cells arise?

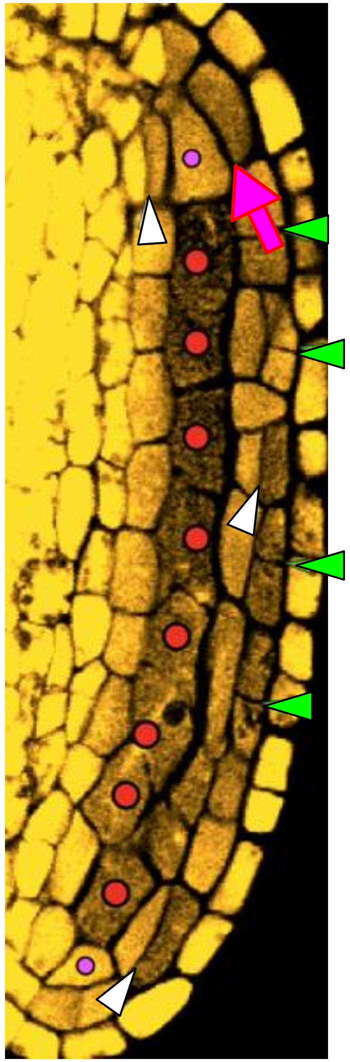


- multiple progenitors
- center more advanced
  than base and tip

presumptive germ cell
germ cell (AR cell)





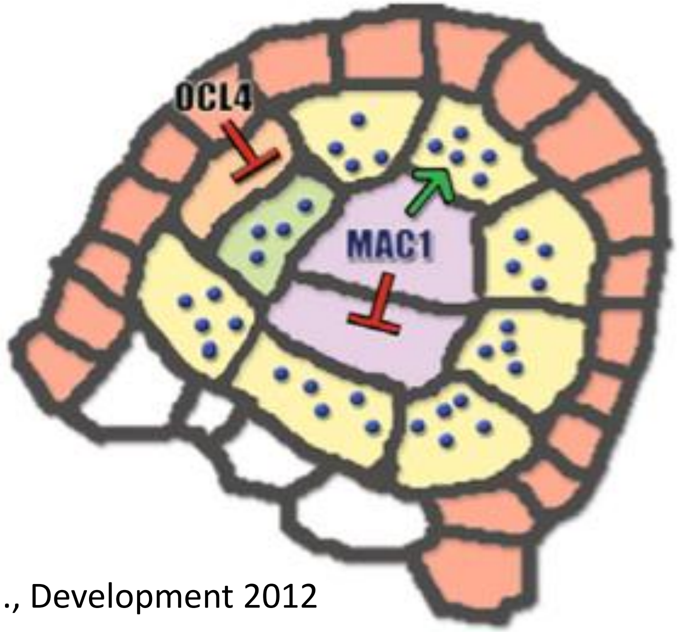


Hypoxia as
Positional cue

Science, 2012

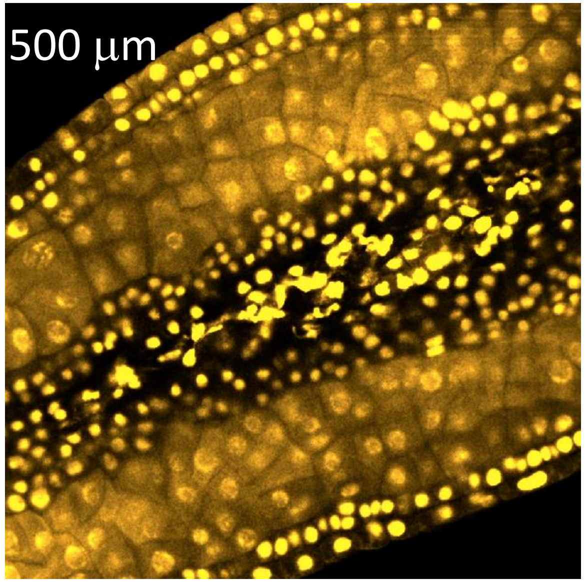
AR express MAC1, a secreted ligand

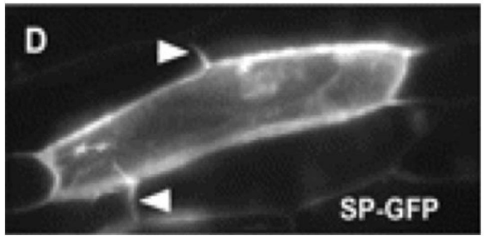
fertile



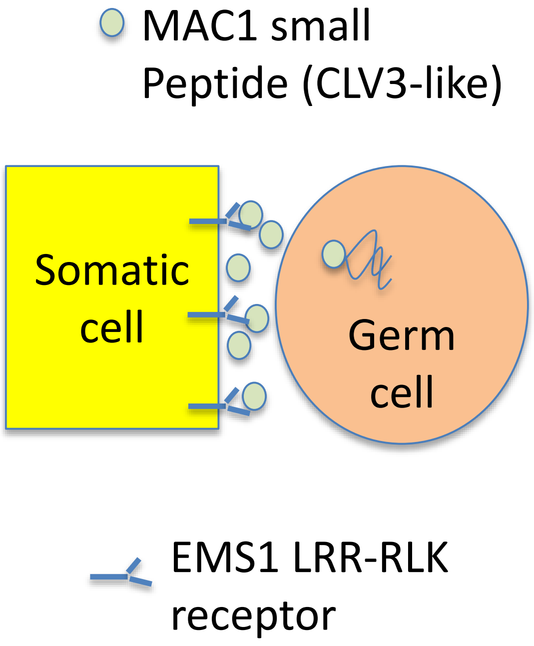
Wang, et al., Development 2012

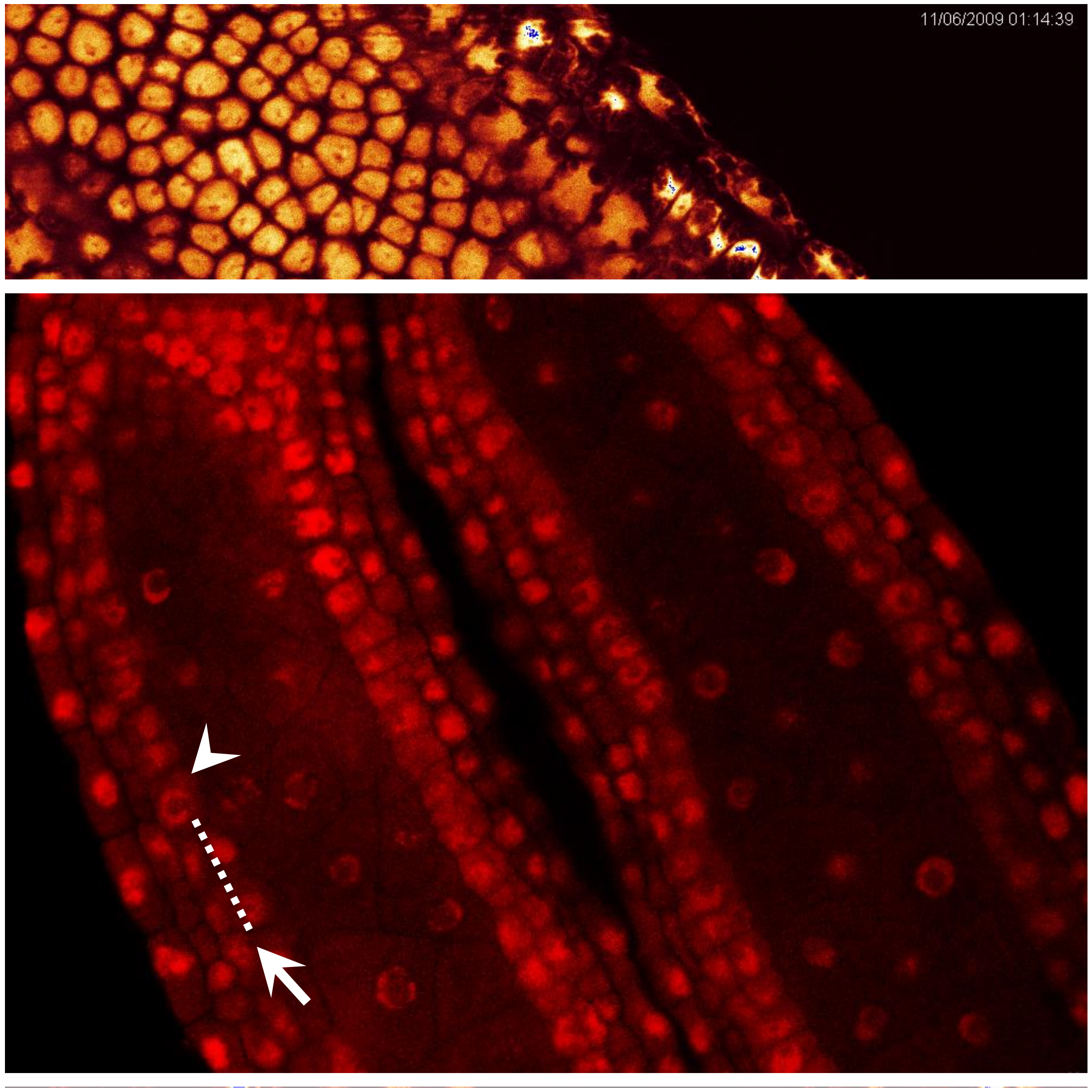
mac1





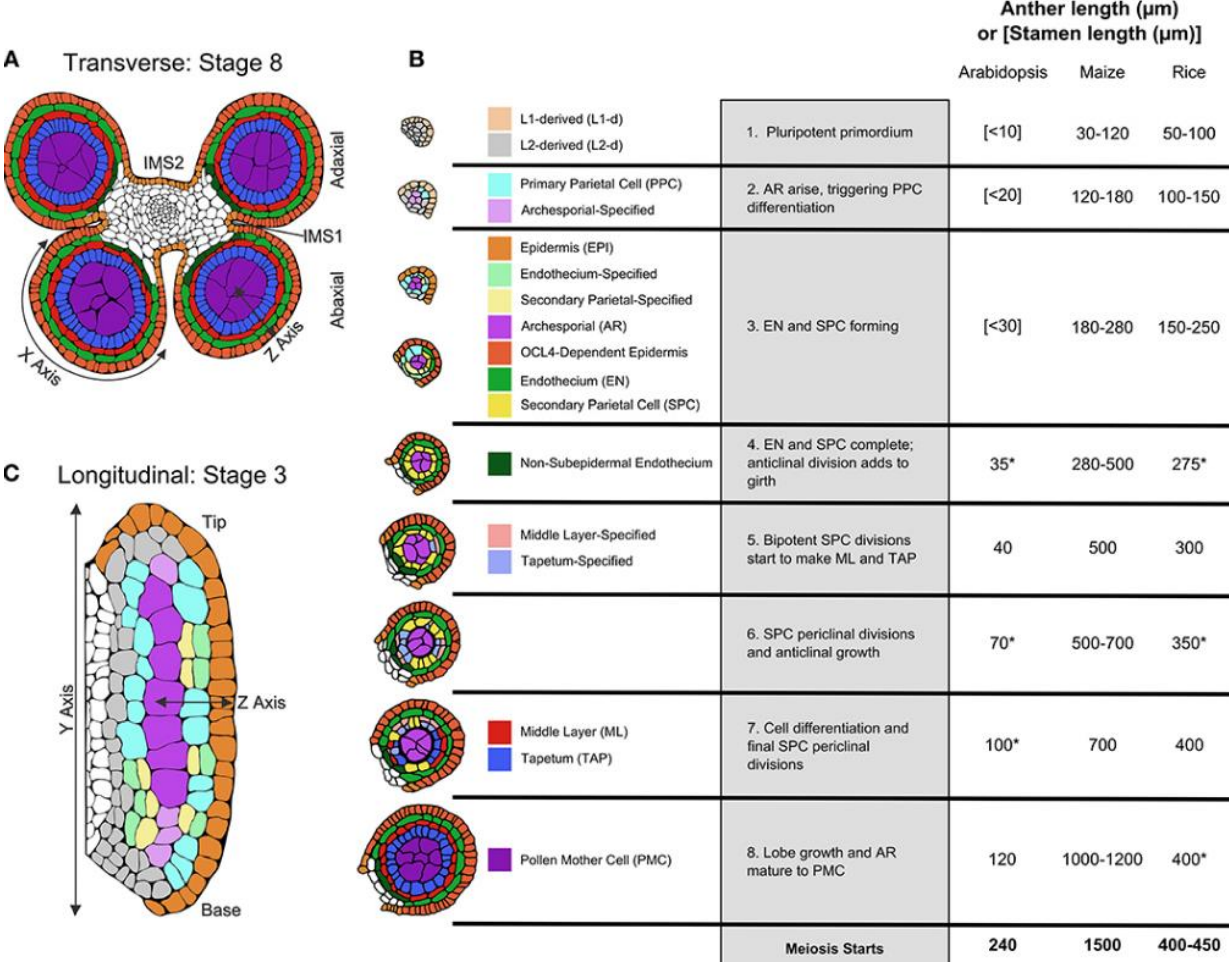
Excess germ cells
Faulty somatic development





Receptor mutant has never been found → gene editing!

Kelliher & Walbot, 2011 *Dev Biol*



Cell types	Stage	Arabidopsis	Maize	Rice
		Anther length (μm) or [Stamen length (μm)]		
L1-derived (L1-d); L2-derived (L2-d)	1. Pluripotent primordium	[<10]	30-120	50-100
Primary Parietal Cell (PPC); Archesporial-Specified	2. AR arise, triggering PPC differentiation	[<20]	120-180	100-150
Epidermis (EPI); Endothecium-Specified; Secondary Parietal-Specified; Archesporial (AR); OCL4-Dependent Epidermis; Endothecium (EN); Secondary Parietal Cell (SPC)	3. EN and SPC forming	[<30]	180-280	150-250
Non-Subepidermal Endothecium	4. EN and SPC complete; anticlinal division adds to girth	35*	280-500	275*
Middle Layer-Specified; Tapetum-Specified	5. Bipotent SPC divisions start to make ML and TAP	40	500	300
	6. SPC periclinal divisions and anticlinal growth	70*	500-700	350*
Middle Layer (ML); Tapetum (TAP)	7. Cell differentiation and final SPC periclinal divisions	100*	700	400
Pollen Mother Cell (PMC)	8. Lobe growth and AR mature to PMC	120	1000-1200	400*
	Meiosis Starts	**240**	**1500**	**400-450**

Formation of the male gametophyte (pollen)

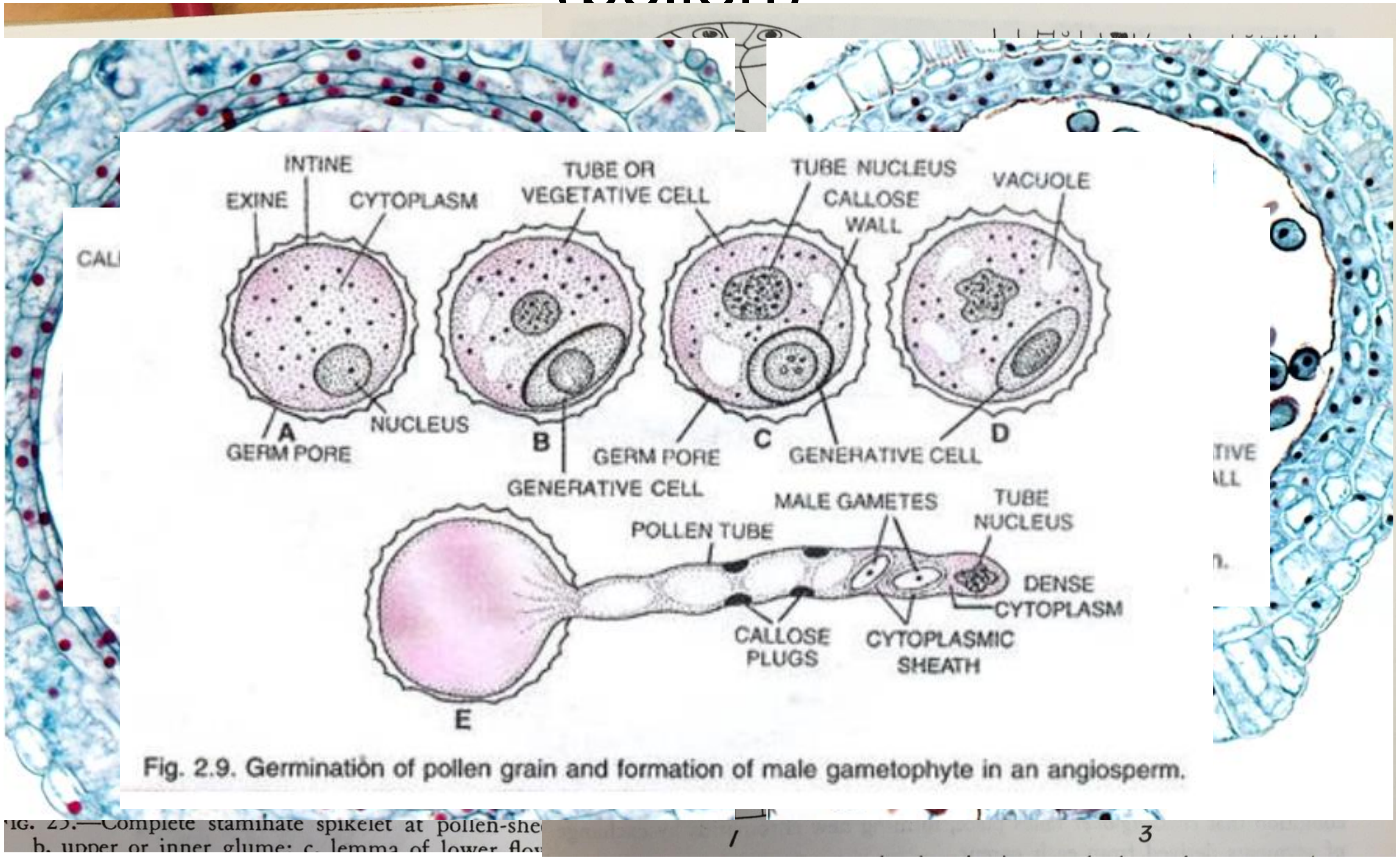


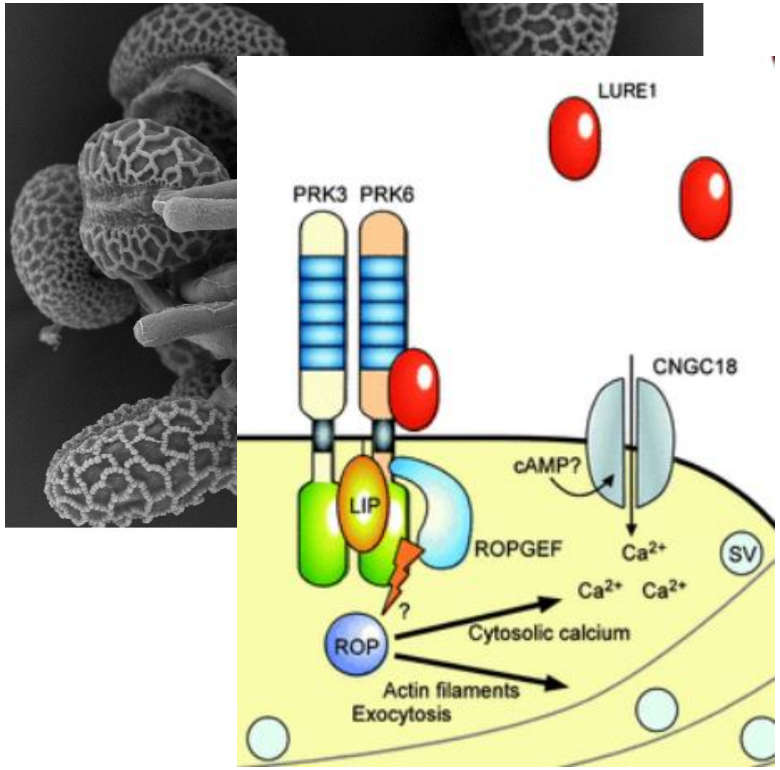
Fig. 2.9. Germination of pollen grain and formation of male gametophyte in an angiosperm.

Theodore Kiesselbach, 1949 The structure and reproduction of corn

Formation of the female gametophyte (embryo sac)

Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells.

Tetsuya Higashiyama,
Nagoya Univ.



PRK3/6 are the pollen tube tip-localized receptors of the LUREs

[In vitro pollination, Torenia]

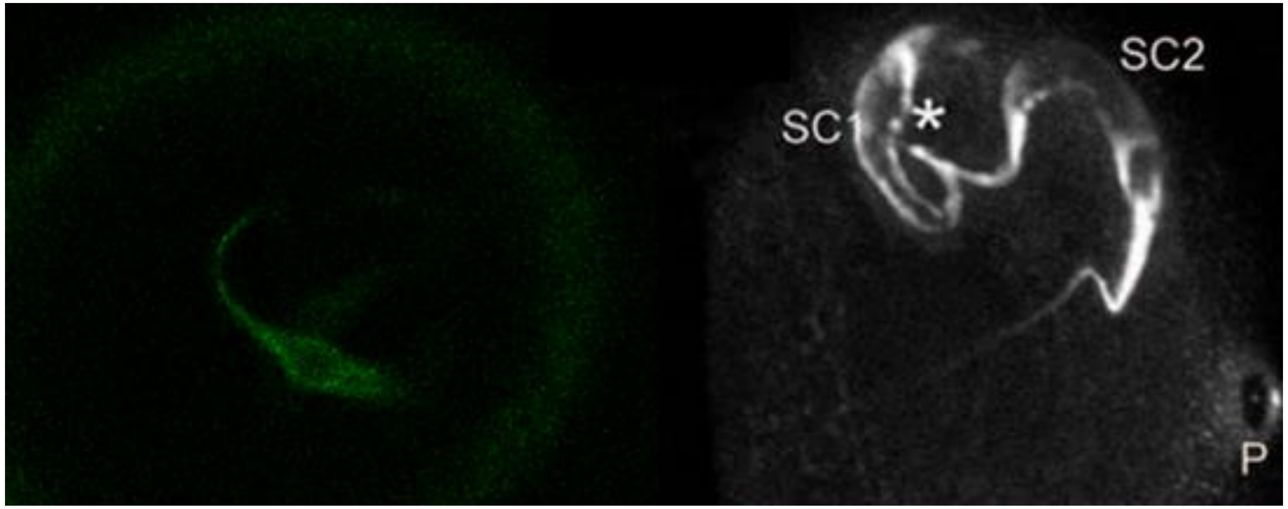
[Torenia - demonstrating guidance cues]

[Guidance cues alternative link (youtube)]

LUREs delivered by pipette

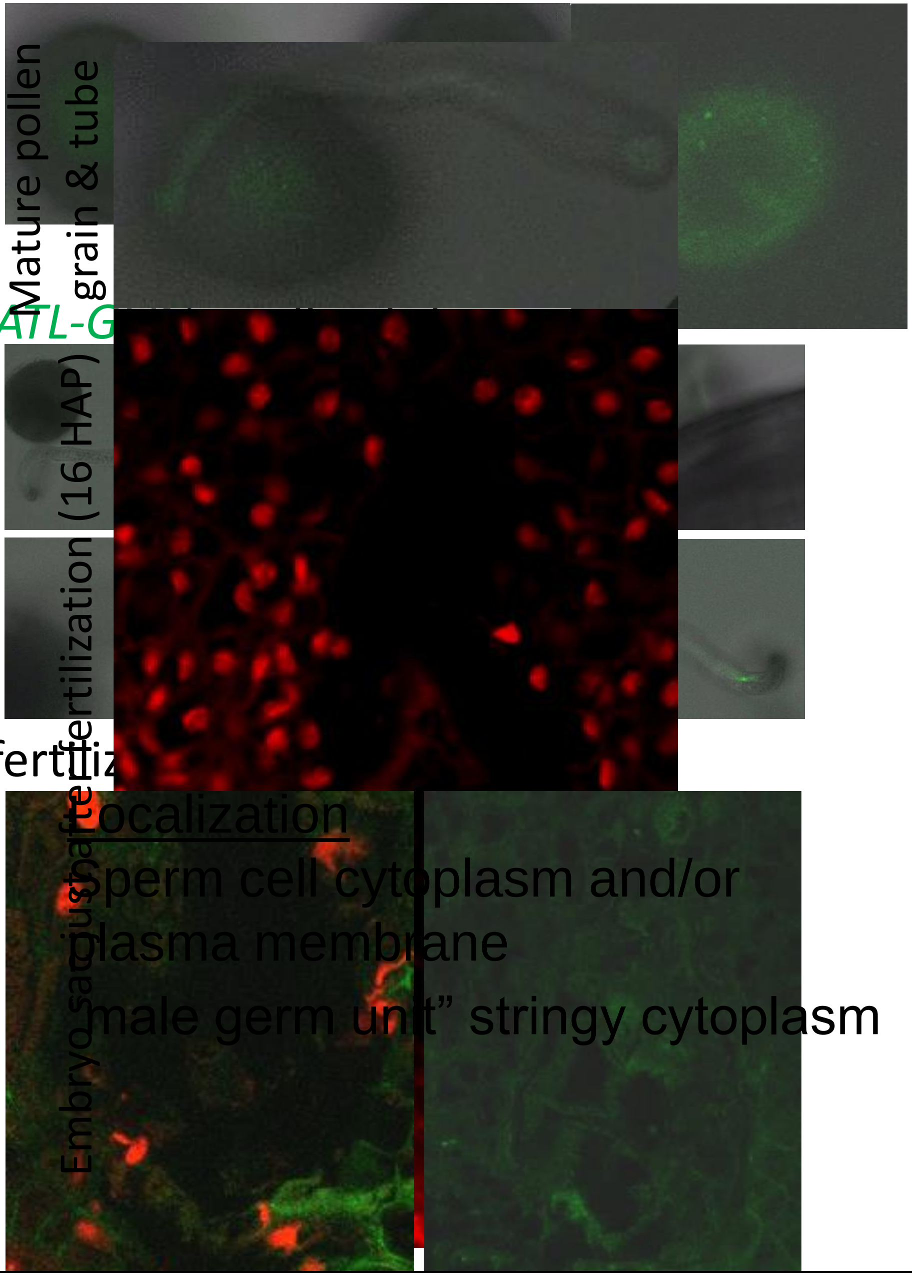
nature07882-s2 (Converted).mov

MATL protein localizes to sperm cell membranes in pollen



matl-GFP: *no signal*

MATL-GFP in sperm cells (SCs)



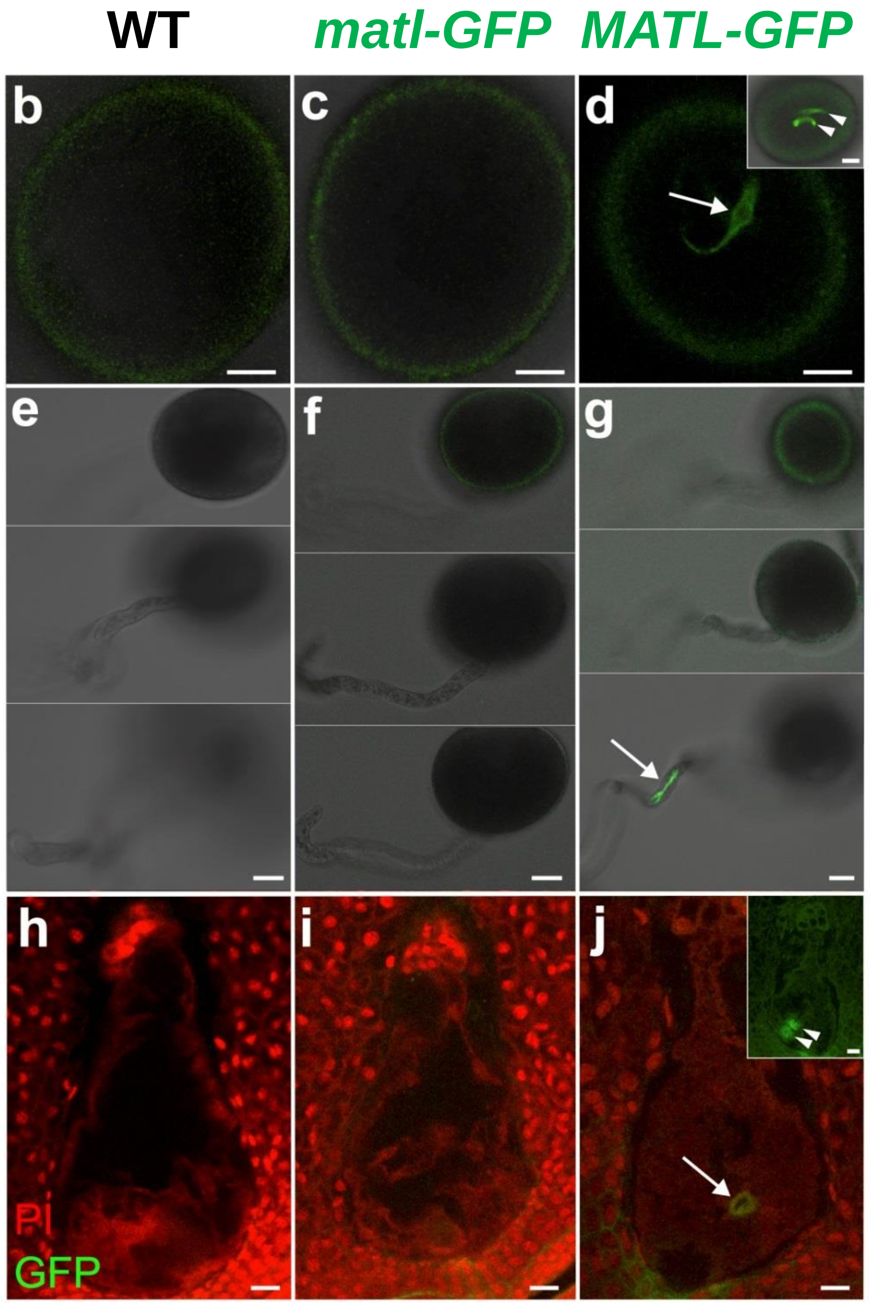
MATL-G

ES fertiliz

Localization

sperm cell cytoplasm and/or plasma membrane

"male germ unit" stringy cytoplasm



…signal missing shortly after syngamy; not found in any maternal or zygote cell types

Mechanism --

Single nucleus sequencing reveals spermatid chromosome fragmentation as a possible cause of maize haploid induction

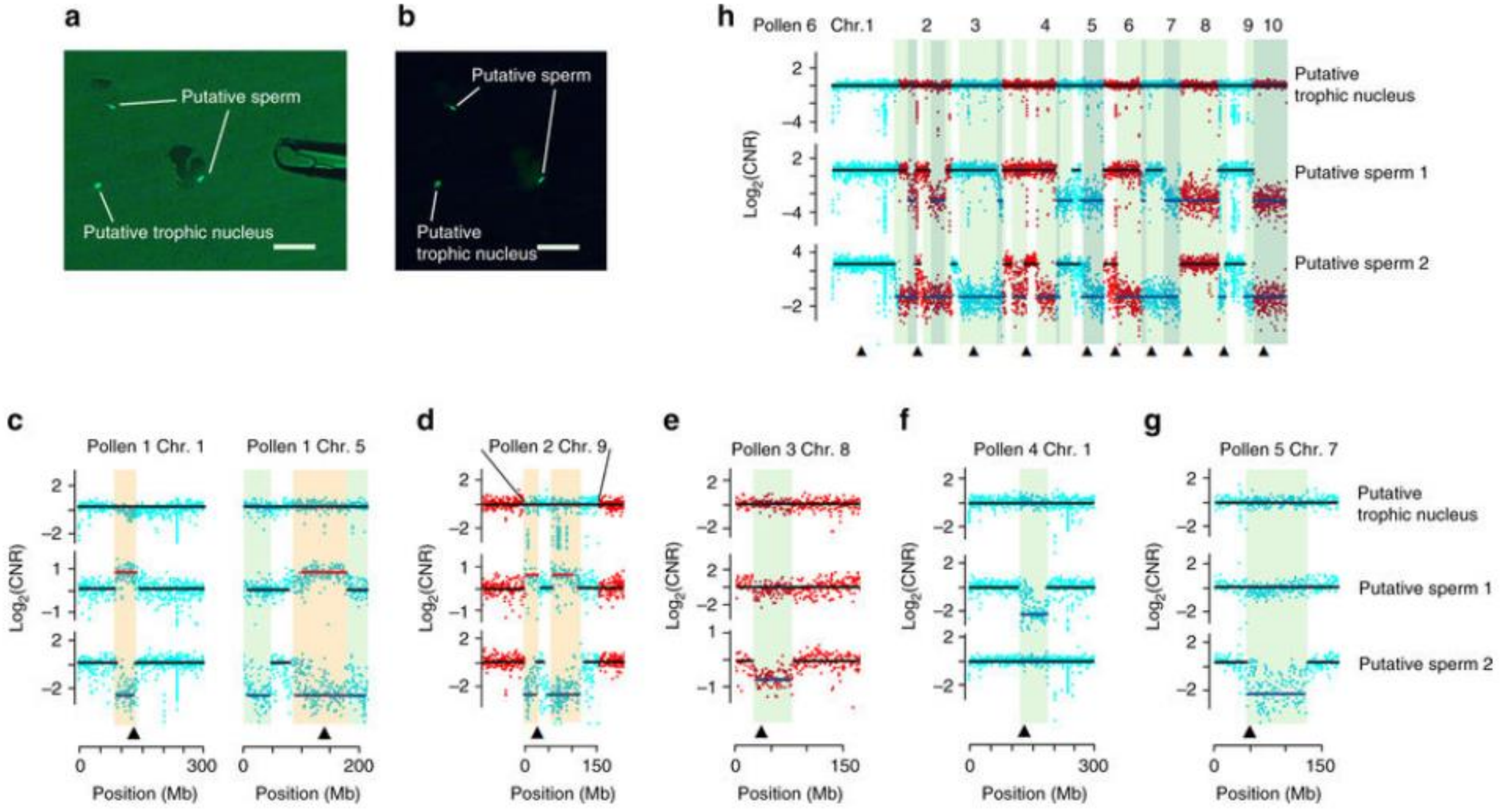
Xiang Li, Dexuan Meng, Shaojiang Chen, Haishan Luo, Qinghua Zhang, Weiwei Jin & Jianbing Yan

Nature Communications **8**, Article number: 991 (2017)
doi:10.1038/s41467-017-00969-8

Received: 04 January 2017
Accepted: 09 August 2017
Published online: 23 October 2017

Different sperm cells have different pieces of chromosomes missing → a sign of chromosome fragmentation

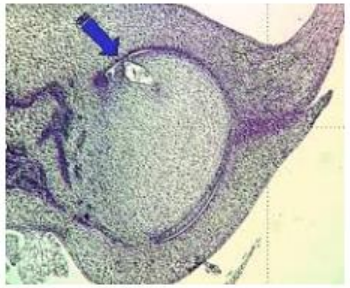
Fragmentation only in sperm cells!



Not in vegetative nucleus… suggests pollen carrying defective sperm can ride out development → fertilization may be defective or not… if not, sometimes male chromosomes will be lost in embryo afterwards

Conclusions: DNA fragmentation is progressive during late pollen development

Stage of pollen development	Frequency of aneuploidy
• Tetrad stage (uninucleate microspore):	Few
• Mature pollen:	~15% of sperm cells

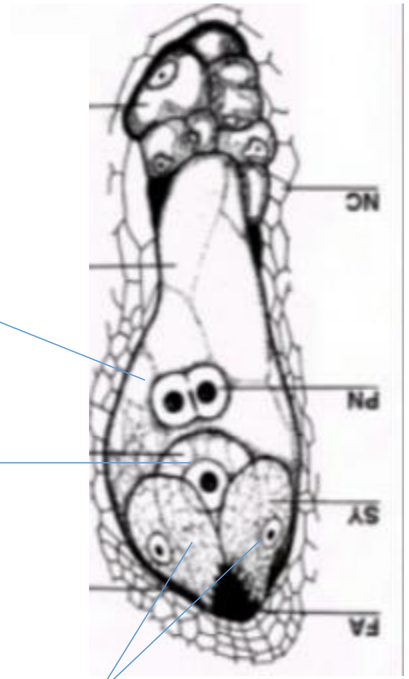


Hetero-fertilization (HF)

- Fertilization of egg and central cell by two different pollen grains
- Occurs very rarely in nature, but when it happens it indicates a fertilization defect

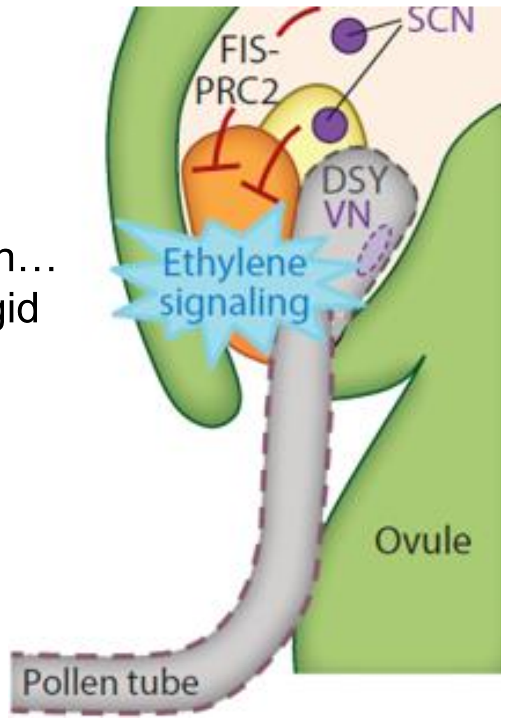
Central Cell

Egg Cell

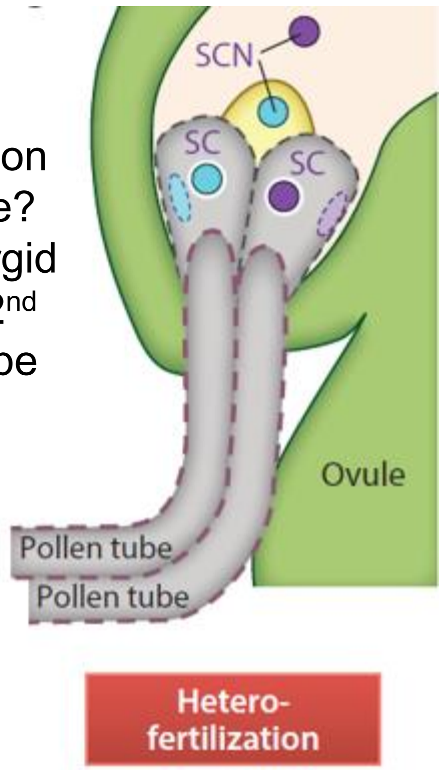


Synergids

Normal fertilization… 2nd synergid is blocked



Fertilization challenge? 2nd synergid attracts 2nd pollen tube



Hetero-fertilization rates are 4X higher in crosses with haploid inducer

- 9 & 11 am
- Both at 10
- 11 & 9 am

- 9 & 11 am
- Both at 10
- 11 & 9 am

- 9 & 11 am
- Both at 10
- 11 & 9 am

Progeny rates

~ 99 % same

~ 1 % different

74)

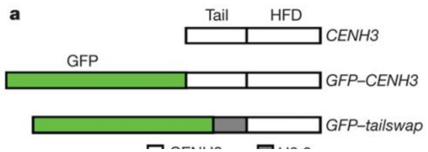
Confirmed in recent publication: Liu, C., *Euphytica* **213**, 174 (2017)

Experiments to prove post-fertilization genome elimination

- **Hyp #2: Chromosome elimination**: Male DNA is lost post-fertilization
  - Prediction: Male DNA is present in early embryo, then lost
  - Well documented in wheat x pearl millet and other wide crosses
  - Supported by evidence in literature
    - Aneuploid plants and chimeras seen after haploid induction crosses
    - Need precise tracking of male DNA markers in early embryos during HI
    - We have new data that supports this hypothesis also

Major crops that are lacking doubled haploids

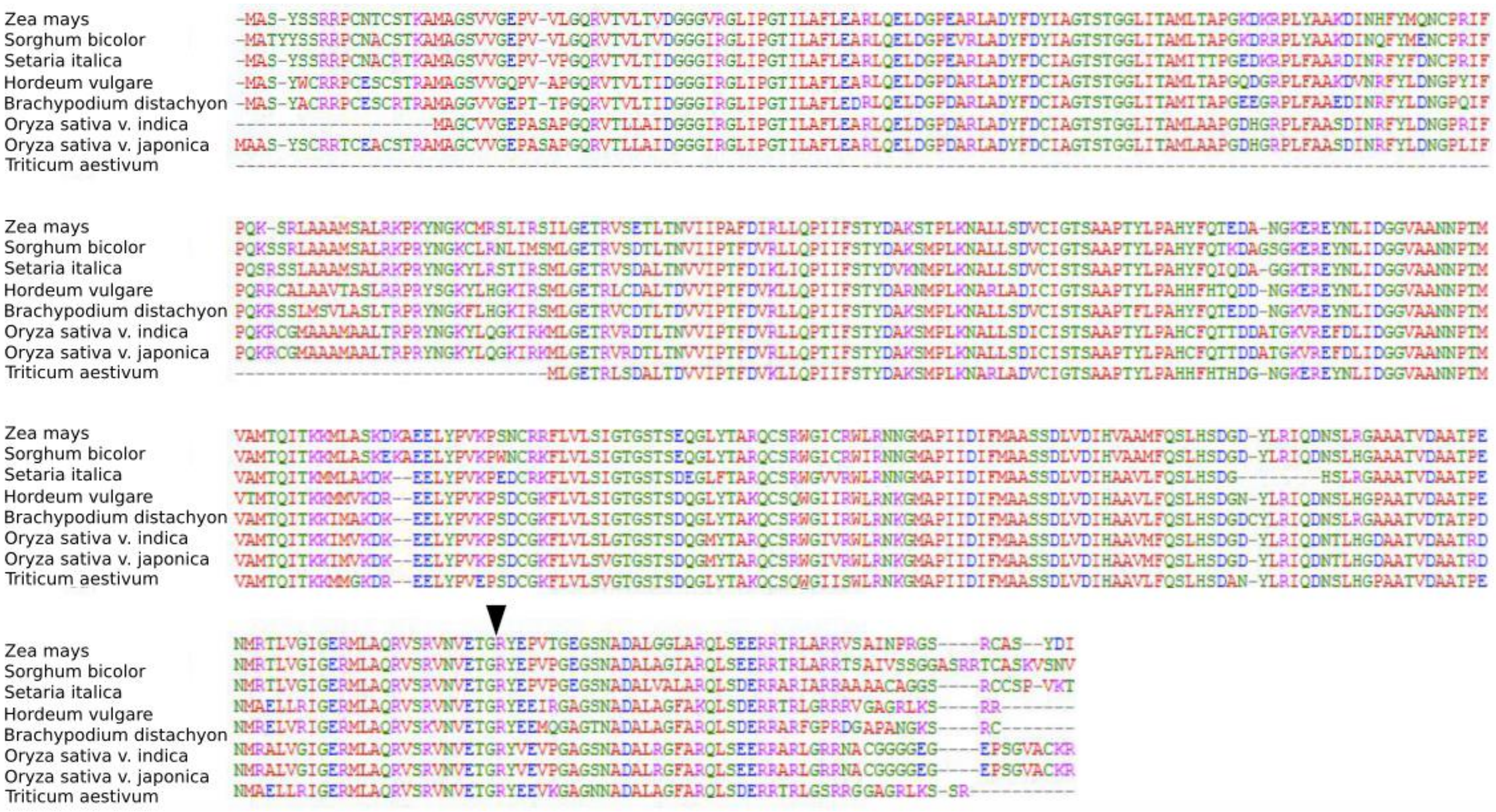
Arabidopsis (CENH3-tailswap) leads to haploids



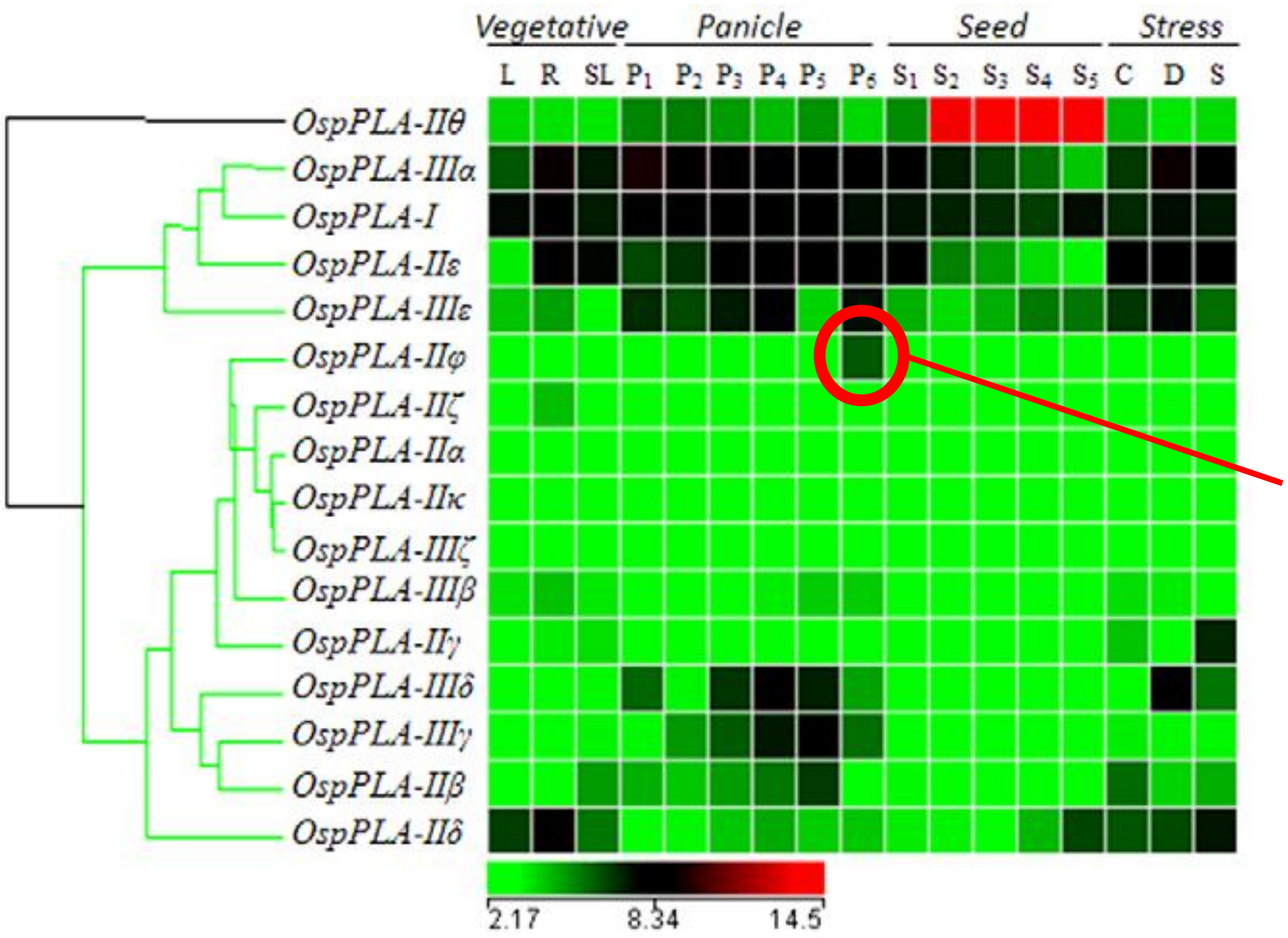
- CENH3 binds centromeric DNA; helps chromosomes segregate in division
- Aberrant alleles of CENH3 produce 3-30% haploid seed (genome elimination)

Haploid plants produced by centromere-mediated genome elimination (*Nature*, 2010)

Bringing Haploid Induction to new crops



OsPLAψ is the closest homolog in rice



Os03g27610

Closest homolog to *MATL*

Also pollen-specific

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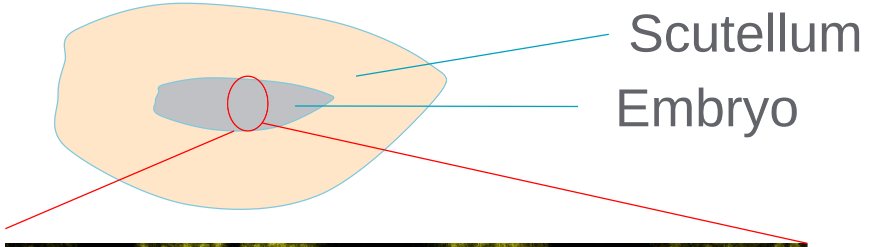
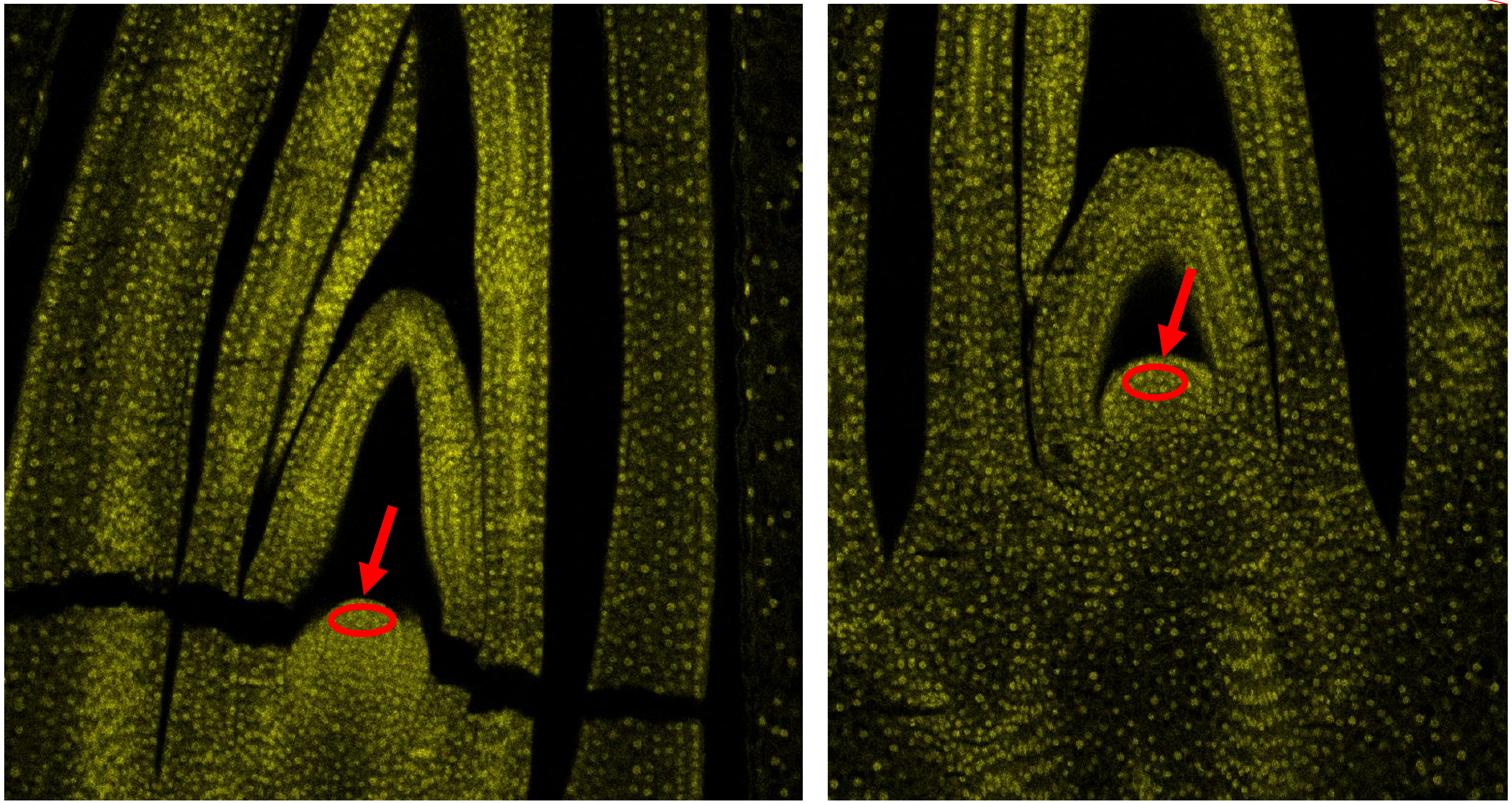
Synchronize the 4-8 meritem cells that → give rise to pollen

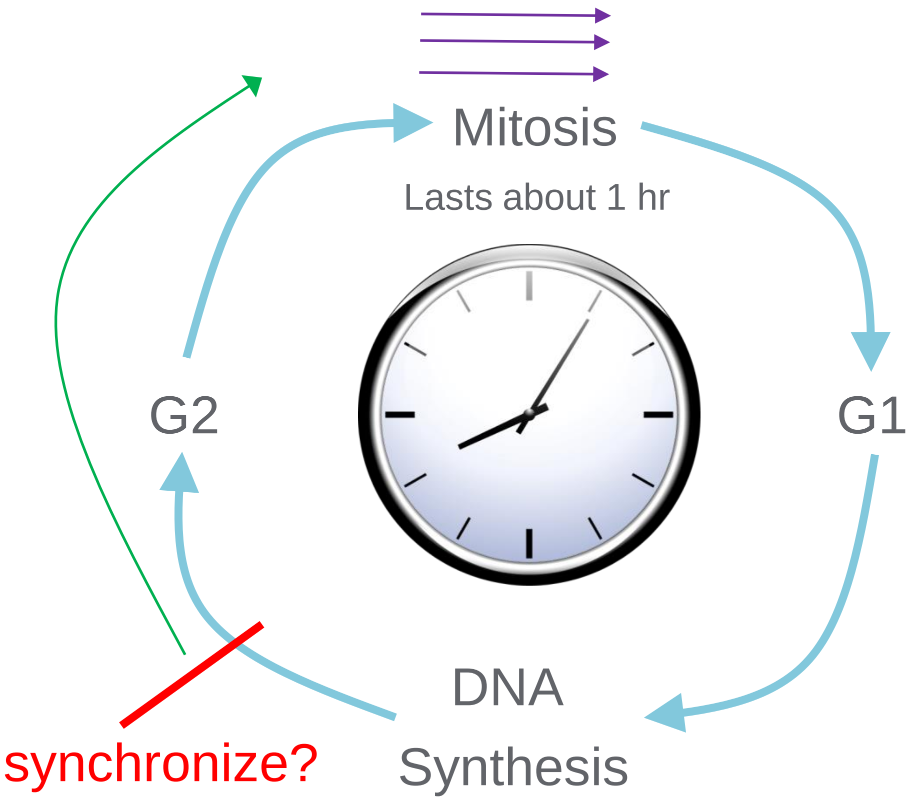


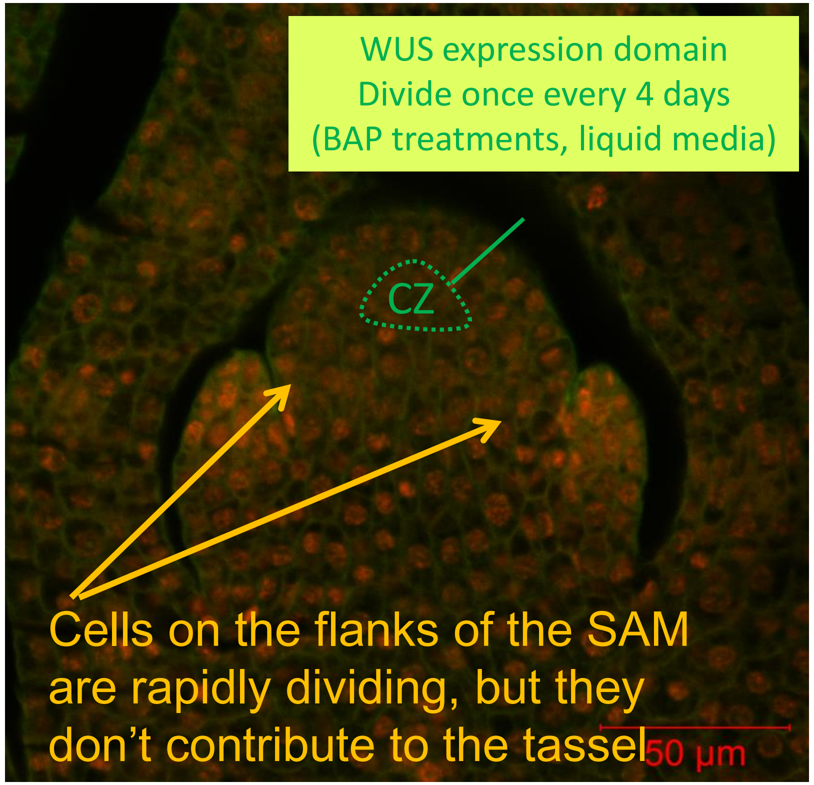


Find treatments to boost / synchronize cell division rate of SAM CZ cells

DISSECT → COLOR SORT → SYNCHRONIZE → RECOVER → COLCHICINE

24 hr | 24-72 hr | 24 hr





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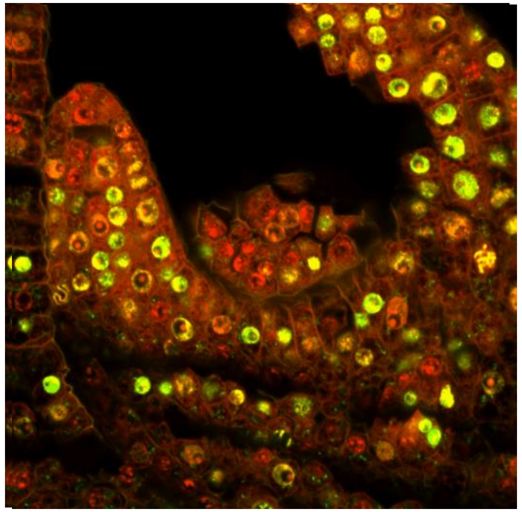
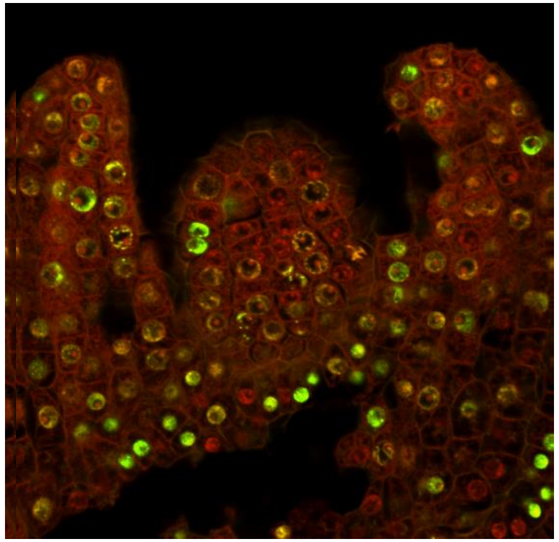
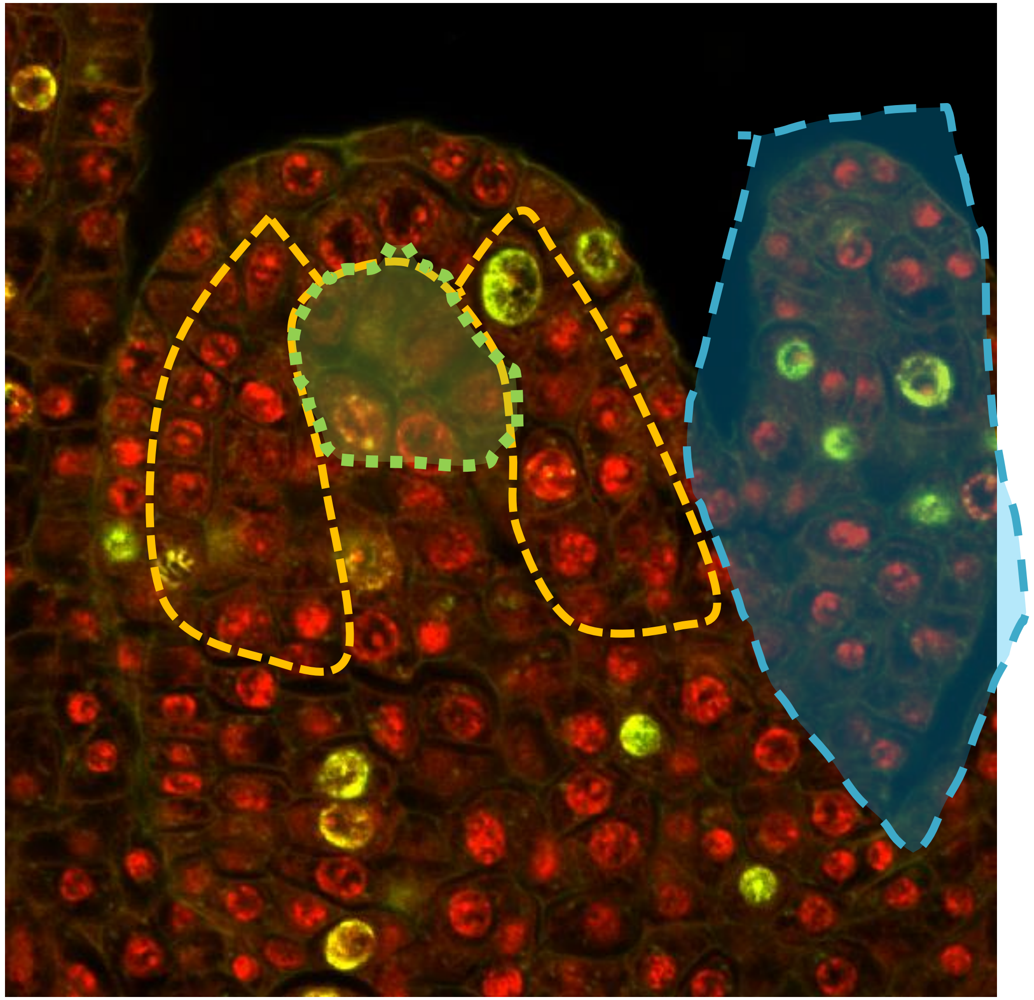
EdU: Incorporated into dividing cells during DNA synthesis







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- Performance Traits: Ian Jepson, Dave Skibbe, Erik Dunder, Paul Bullock
- Chris Leming, Stacy Miles

Research Triangle Park site founded in 1984 by Dr. Mary-Dell Chilton – Queen of Agrobacterium

- Led research study in '70s and '80s that resulted in the first biotech plants
- With Dr. Chilton's technical leadership, Syngenta was the first company to commercialize a biotech trait in corn
- Dr. Chilton named 2013 World Food Prize laureate. In 2015, inducted to the National Inventors Hall of Fame, USDA Hall of Heroes and National Academy of Inventors

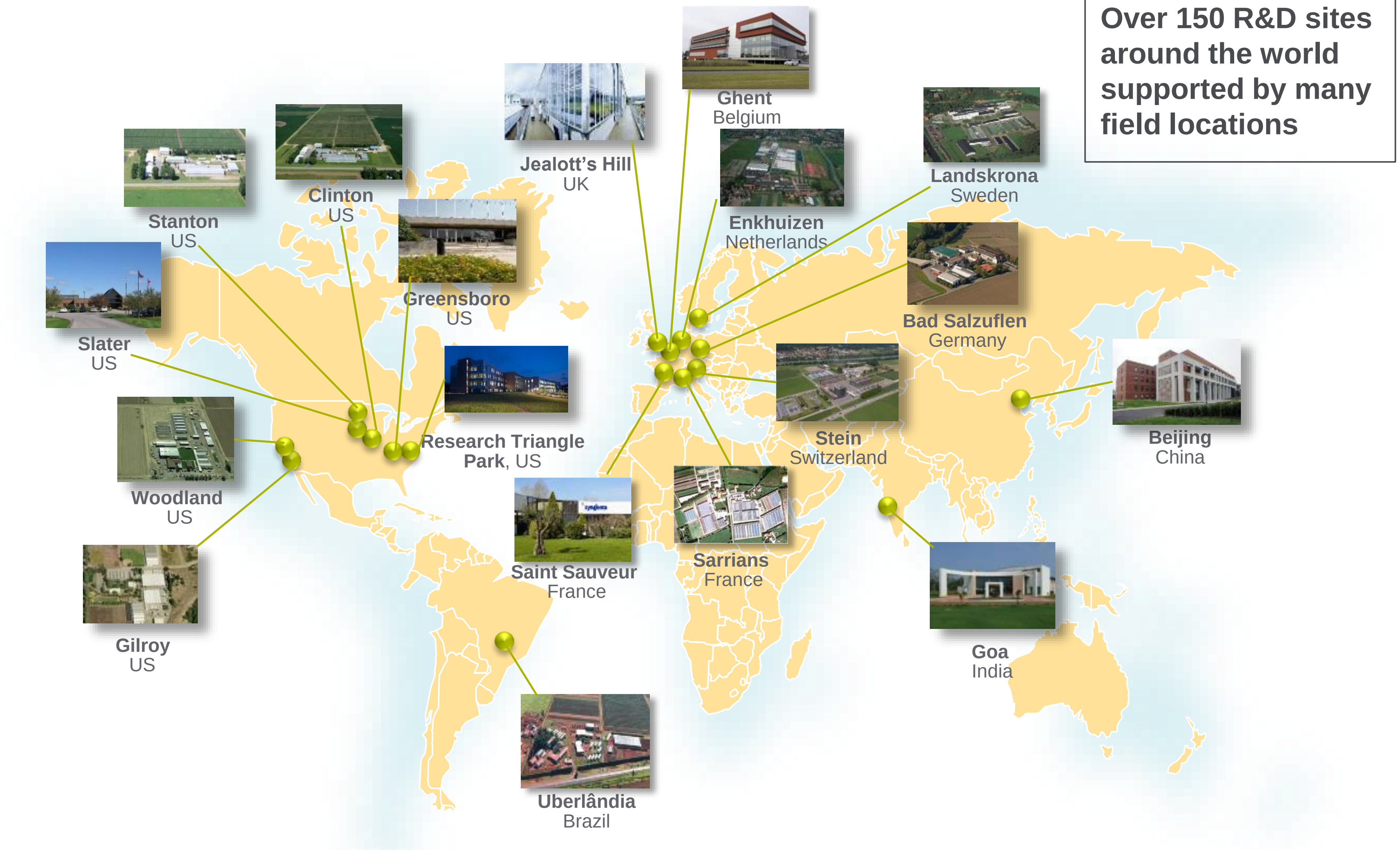
* Clive James, 2012

Classification: PUBLIC

Key R&D centers across the world

Unrivalled global breadth

Over 150 R&D sites around the world supported by many field locations



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The Syngenta RTP Innovation Center

- Phase I: Construction of the Advanced Crop Lab started Q4/2011
  - Construction completed Q1/2013
  - Investment of $72 million

- Phase II: Construction of the RTP Innovation Center started Q4/2013
  - Construction completed Q2/2016
  - Investment of $94 million

The Syngenta RTP Innovation Center: lab and office wings

- Lab space (136,000 sq. ft. total)
  - 100,000 sq. ft. of labs
  - 36,000 sq. ft. of offices and collaboration spaces
  - Flexible, fit-for-purpose, open laboratory environments
  - State-of-the-art fermentation suite

- Office space
  - 64,000 sq. ft.
  - Multi-workplace office environments to enable interaction between disciplines

- Total Innovation Center
  - 200,000 sq. ft.

- Houses approximately 475 employees.
  375 Syngenta employees
  100 resident contractors.

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The Syngenta RTP Innovation Center: Advanced Crop Lab

- 340,000 sq. ft. of State-of-the-art facilities

  The Advanced Crop Lab (140,000 sq. ft.)
  - Under Glass: 40,000 sq. ft.
  - Headhouse + Chambers: 35,000 sq. ft.
  - Support Areas: 65,000 sq. ft.

- **Greenhouses**
  - Positioned to receive optimal sunlight
  - Made of low carbon glass with antireflective coating and prismatic haze patterns.
  - Independently controlled room temperature and humidity
  - Control of day light exposure and gases such as carbon dioxide

- **Growth Chambers**
  - Designed specifically for Syngenta using technology developed by the International Space Program to study the impact of different atmospheres on crop performance.
  - Ability to precisely control all environmental variables (light, temperature, moisture, etc.)
  - Ability to precisely measure plant responses

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