CG920 Genomics

Finishing Lesson 2

Genes Identification

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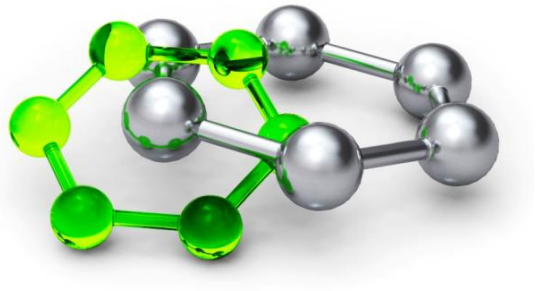


Outline

(finishing Lesson 02)

- Forward and Reverse Genetics Approaches
  - Differences between the approaches used for identification of genes and their function
- Identification of Genes *Ab Initio*
  - Structure of genes and searching for them
  - Genomic colinearity and genomic homology
- Experimental Genes Identification
  - Constructing gene-enriched libraries using methylation filtration technology
  - EST libraries
  - Forward and reverse genetics

Forward and Reverse Genetics

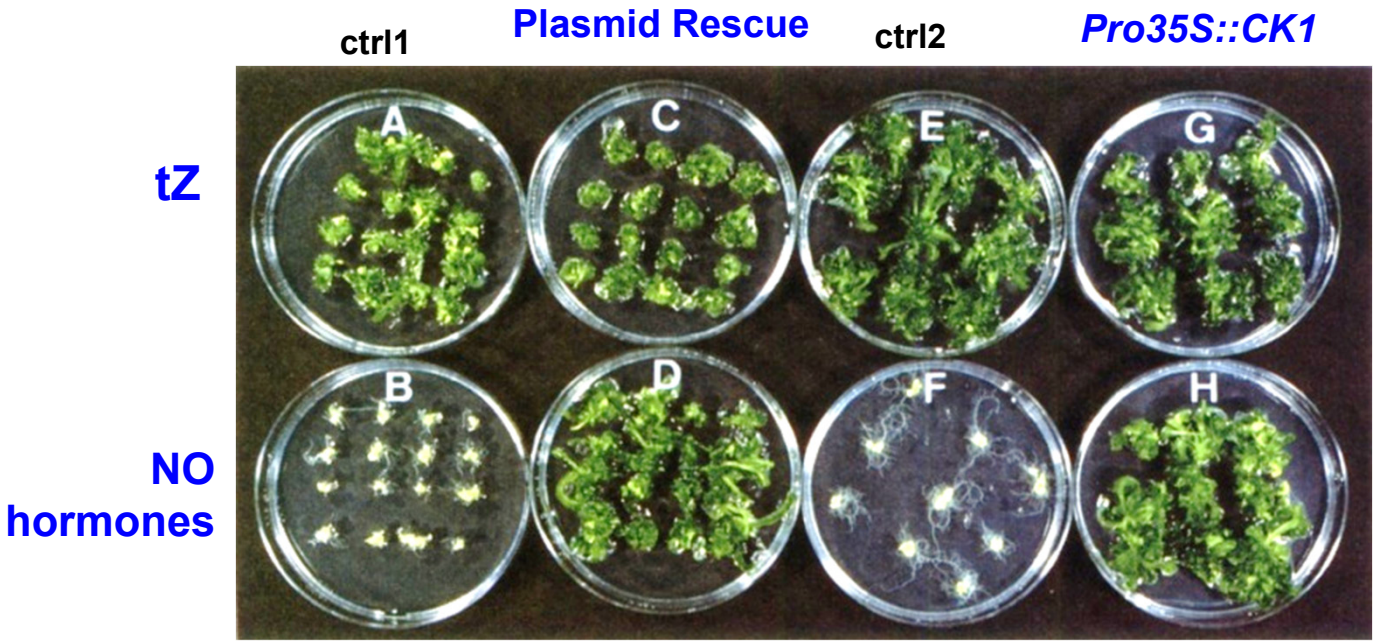
- Principles of experimental identification of genes using forward and revers genetics
  - Alteration of phenotype after mutagenesis
    - **Forward genetics**
  - Identification of sequence-specific mutant and analysis of its phenotype
    - **Reverse genetics**
  - Analysis of expression of a particular gene and its spatiotemporal specifity

Forward Genetics

- Principles of experimental identification of genes using forward and reverse genetics
  - Alteration of phenotype after mutagenesis
    - **Forward genetics**

Identification of *CKI1* via Activation Mutagenesis

- *CKI1* overexpression mimics cytokinin response



Kakimoto, *Science*, 1996

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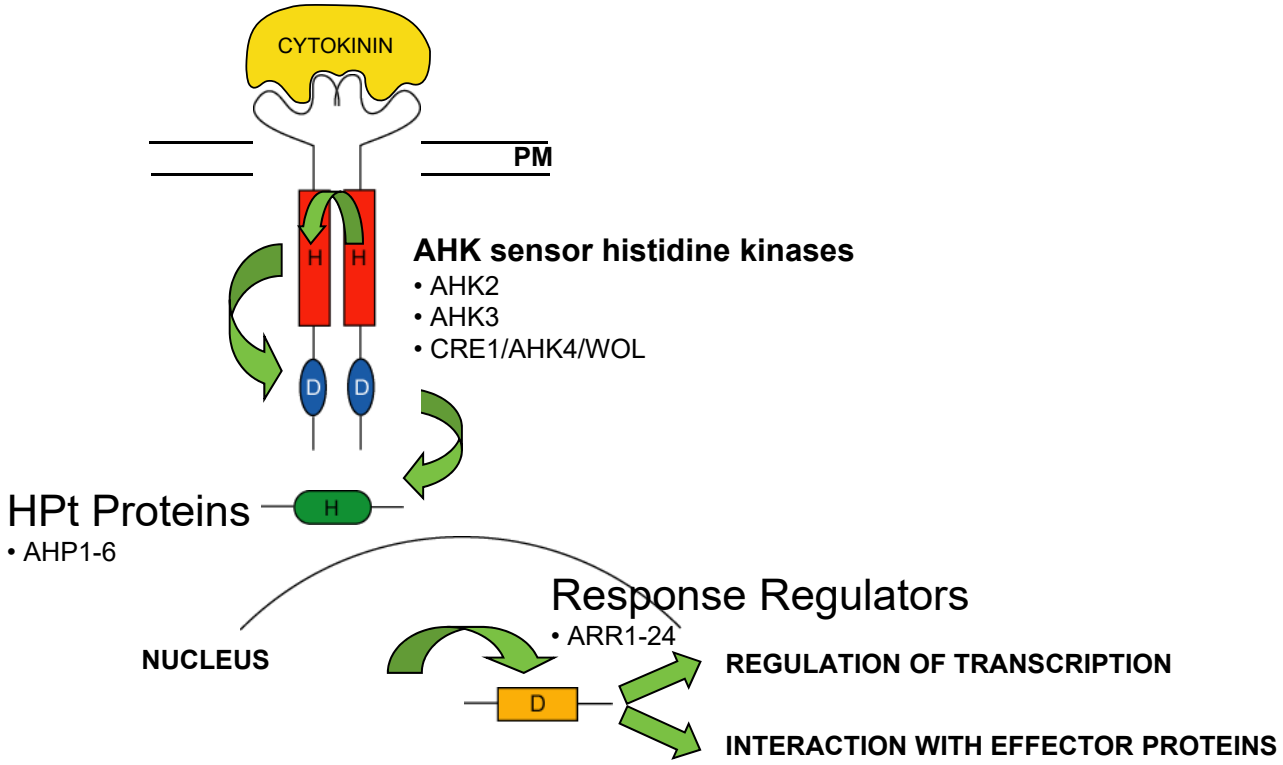
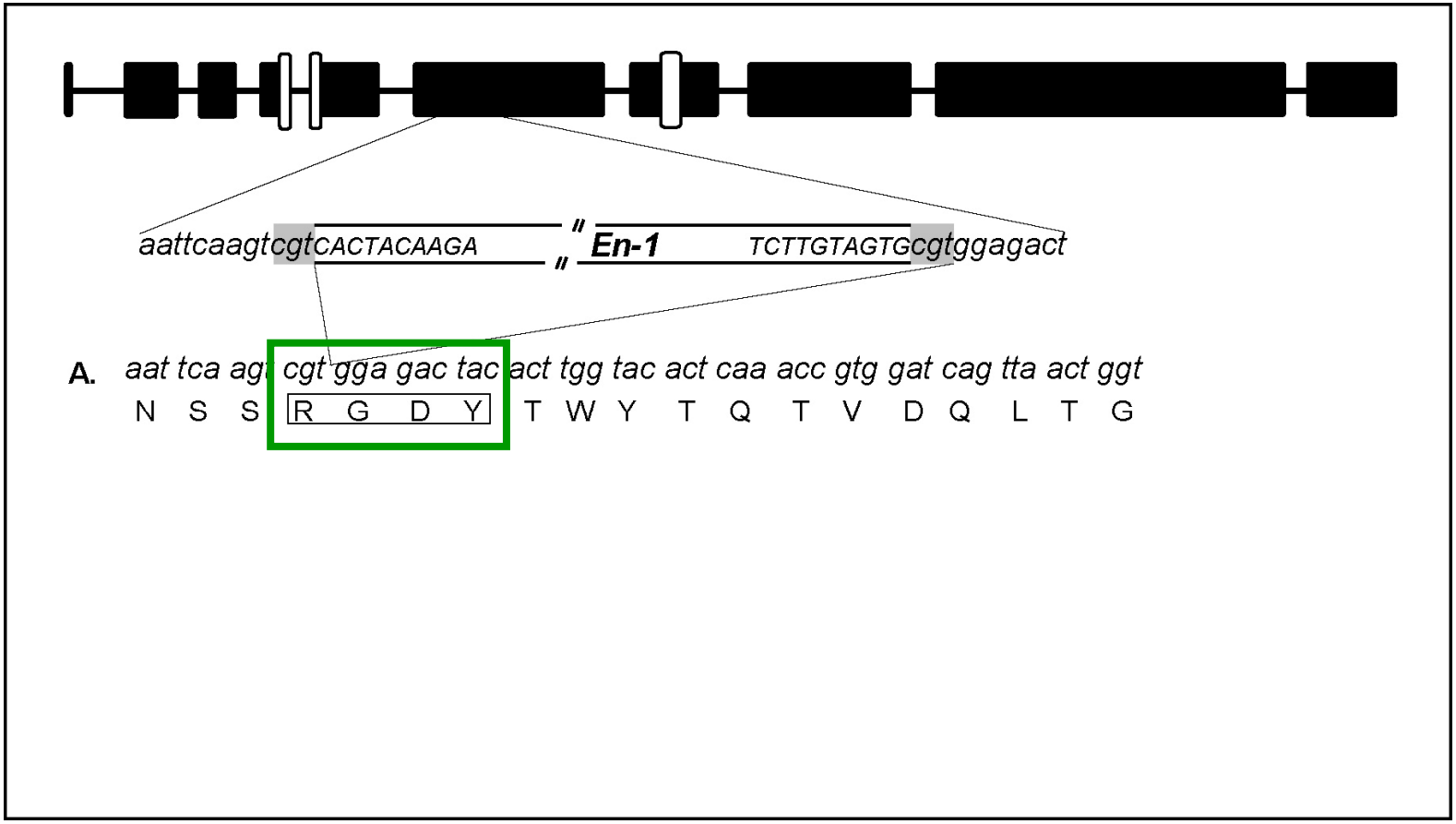
Signal Transduction via MSP



Reverse Genetics

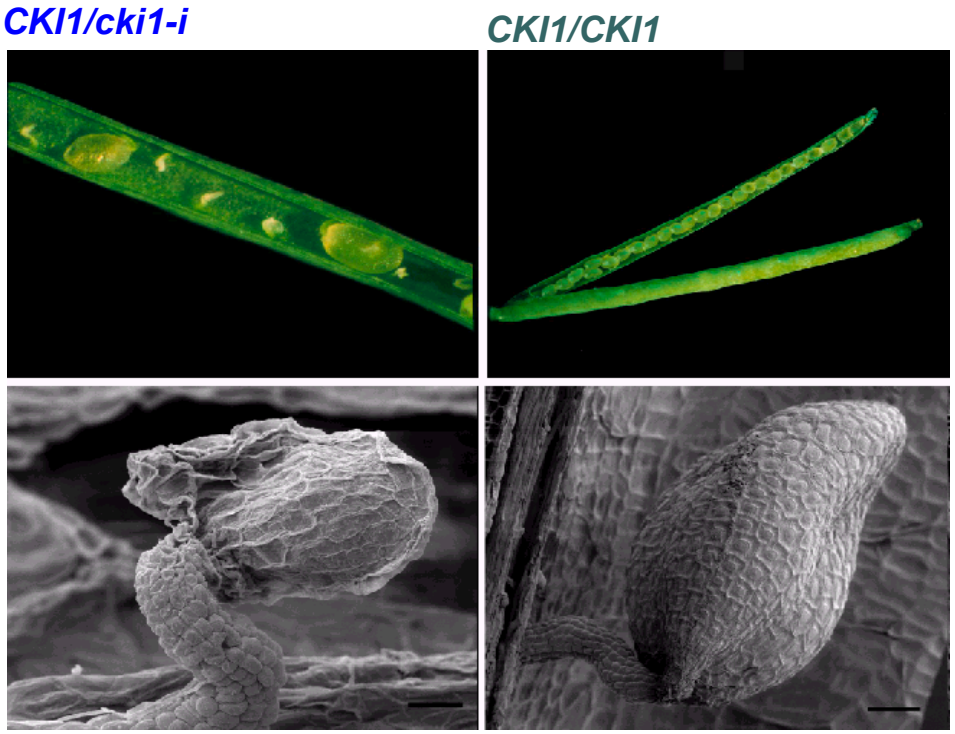
- Principles of experimental identification of genes using forward and revers genetics
  - Alteration of phenotype after mutagenesis
    - **Forward genetics**
  - Identification of insertional mutant and analysis of its phenotype
    - **Reverse genetics**

Identification of insertional *cki1* mutant allele



CKI1 and Megagametogenesis

- CKI1 is necessary for proper megagametogenesis in *Arabidopsis*



Hejátko et al., *Mol Genet Genomics* (2003)

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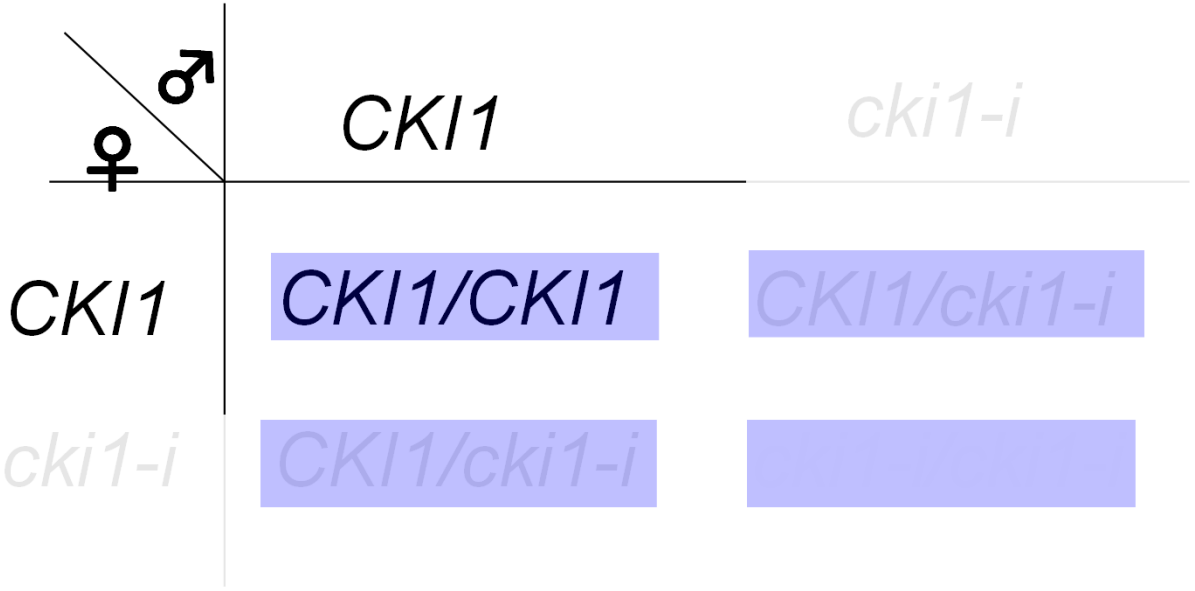
cki1-i reveals non-Mendelian inheritance

P *CKI1/cki1-i*

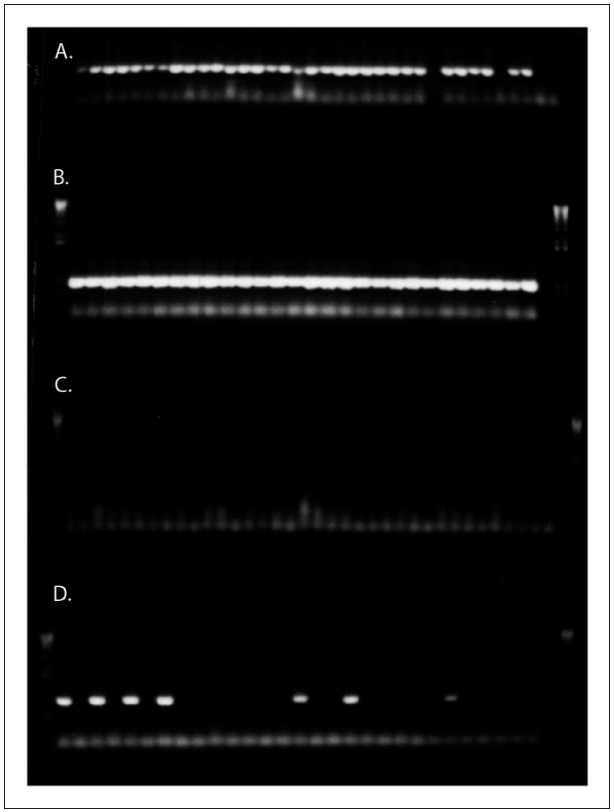
F1 Anticipated: **1** *CKI1* **: 2** *CKI1/cki1-i* **: 1** *cki1-i*

Observed: **1** *CKI1* : **1** *CKI1/cki1-i*



CKI1 and Megagametogenesis

- *cki1-i* is not transmitted through the female gametophyte



A. ♂ wt x ♀ *CKI1/cki1-i*

↕ ***CKI1* specific primers (PCR positive control)**

B. ♂ *CKI1/cki1-i* x ♀ wt

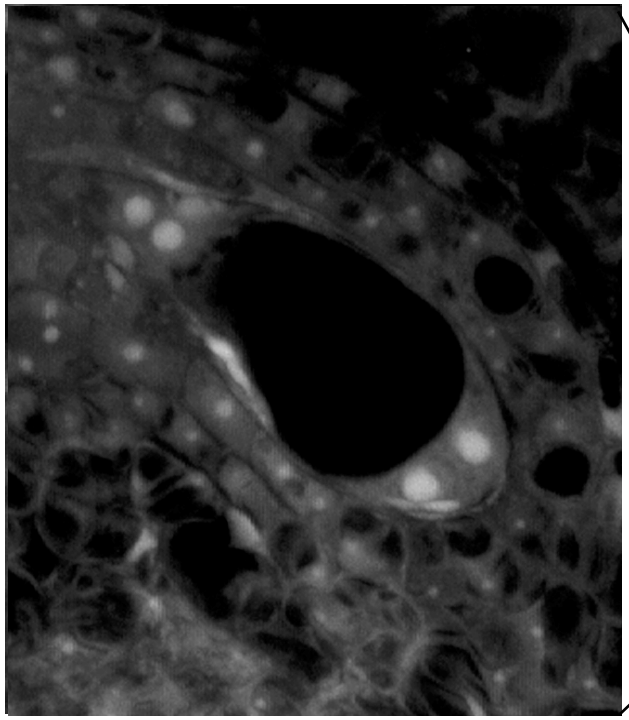
C. ♂ wt x ♀ *CKI1/cki1-i*

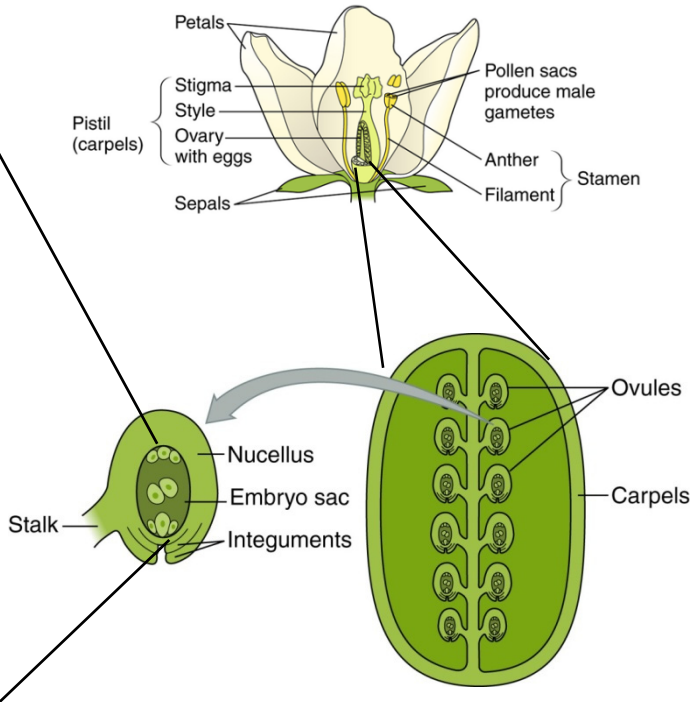
↕ ***cki1-i* specific primers**

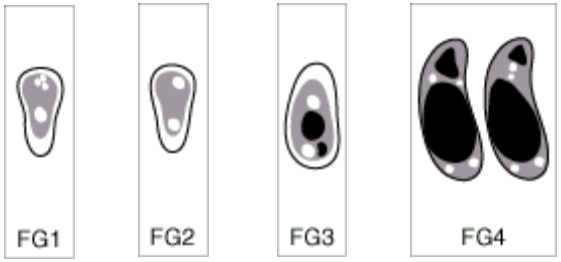
D. ♂ *CKI1/cki1-i* x ♀ wt

CKI1 and Megagametogenesis

FG 4







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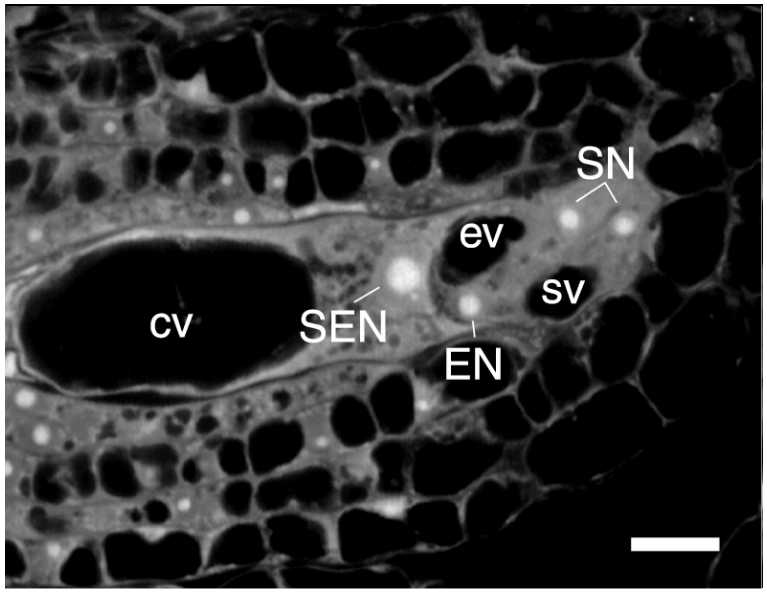
CKI1 and Megagametogenesis

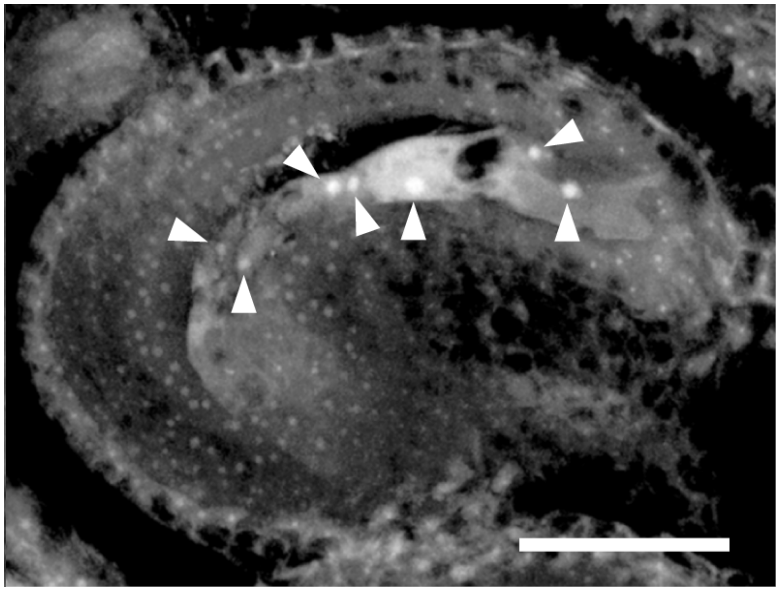
CKI1

late FG5

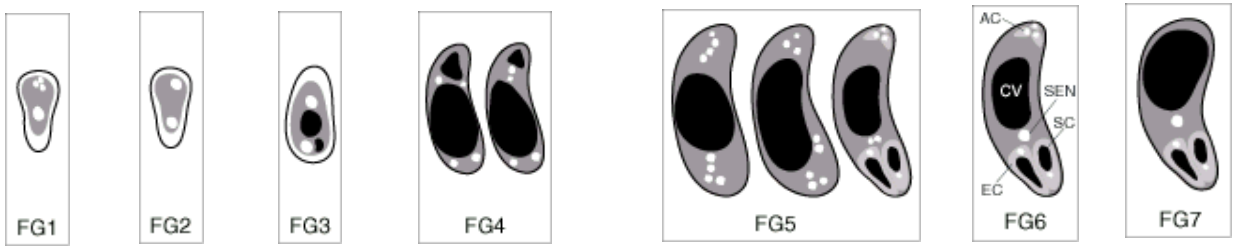
cki1-i

HAE





Hejátko et al., *Mol Genet Genomics* (2003)



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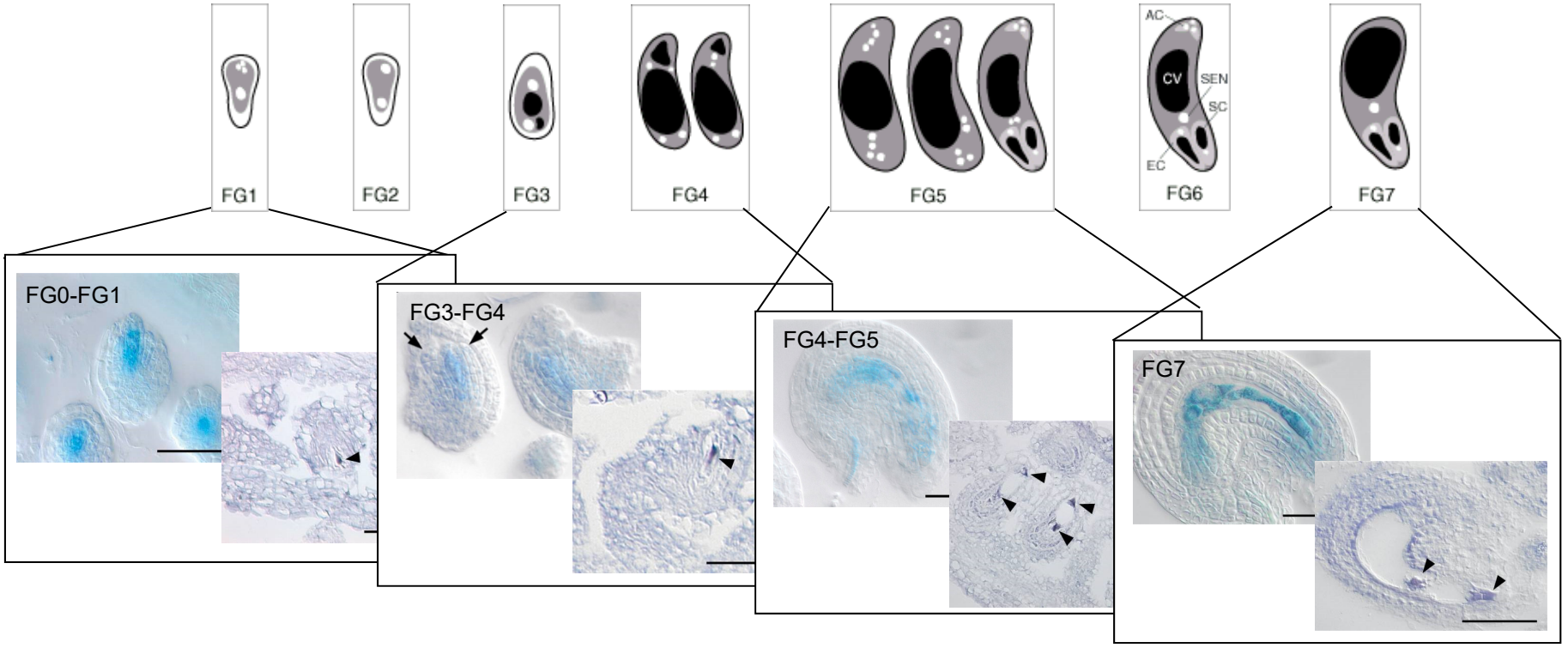
Forward and Reverse Genetics

- Principles of experimental identification of genes using forward and reverse genetics
  - Alteration of phenotype after mutagenesis
    - **Forward genetics**
  - Identification of insertional mutant and analysis of its phenotype
    - **Reverse genetics**
  - Analysis of expression of a particular gene and its spatiotemporal specifity

CKI1 is Expressed During Megagametogenesis



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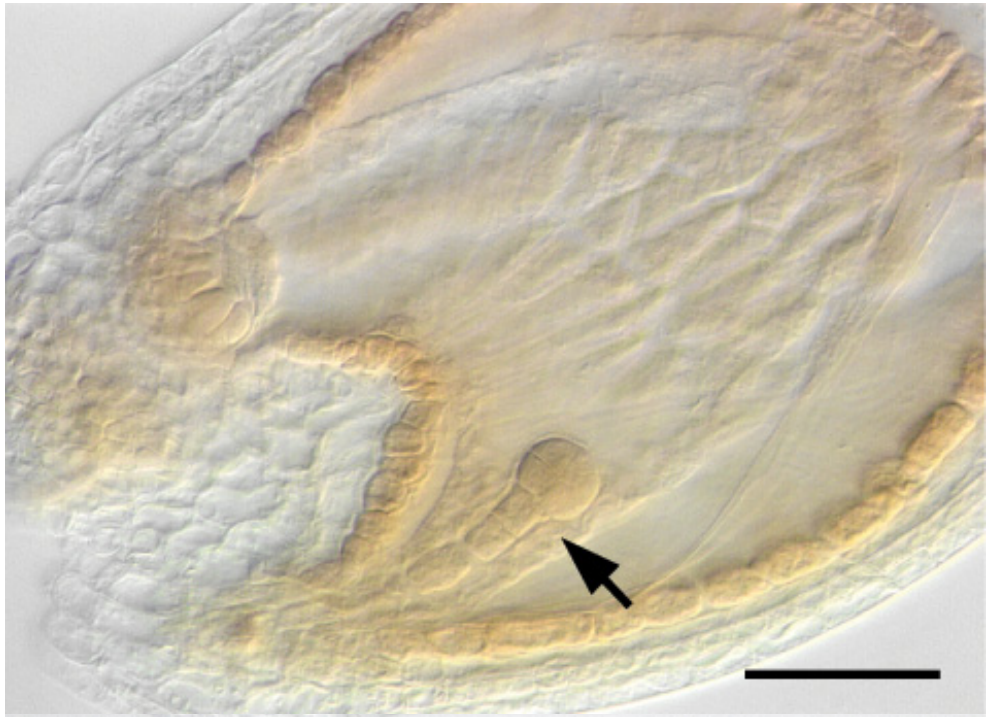
Paternal *CKI1* is Expressed in the *Arabidopsis* Sporophyte Early after Fertilization

♀ wt x ♂ Pro*CKI1:GUS*

72 HAP
(hours
after
pollination)



Hejátko et al., *Mol Genet Genomics* (2003)

CG920 Genomics

Lesson 3

Reverse Genetics

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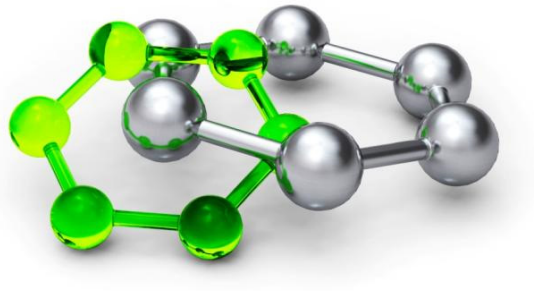


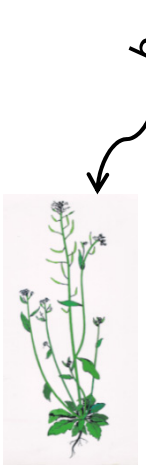
Literature

- Literature sources for Chapter 03:

  - **Bioinformatics and Functional Genomics**, 2009, Jonathan Pevsner, Willey-Blackwell, Hobocken, New Jersey
    http://www.bioinfbook.org/index.php
  - **Plant Functional Genomics**, ed. Erich Grotewold, 2003, Humana Press, Totowa, New Jersey
  - Mello, C.C. and Conte Jr., D. (2004) Revealing the world of RNA interference. *Nature*, **43**1, 338-342.
  - Klinakis et al.. (2000) Genome-wide insertional mutagenesis in human cells by the *Drosophila* mobile element *Minos*. *EMBO Rep*, **1**, 416.
  - Hansen et al.. (2003) A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome. *PNAS*, **100**, 9918.

„Classical“ genetics *versus* „reverse genetics“ approaches in functional genomics

RANDOM MUTAGENESIS



„Classical genetics“ approach

EMS

1. IDENTIFICATION OF PHENOTYPE

2. GENE MAPPING

3. GENE IDENTIFICATION
- position cloning

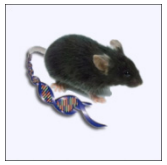
„Reverse genetics“ approach

T-DNA

1. ISOLATION OF SEQUENCE--SPECIFIC MUTANT

2. IDENTIFICATION OF PHENOTYPE

3. PROOF OF CAUSAL RELATIONSHIP BETWEEN INSERTION AND PHENOTYPE



(retro)transposons

Outline

- Methods for Identification of Sequence-Specific Mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
  - Searching for sequence-specific mutants in electronic databases
  - Knocking-out the gene using homologous recombinantion
- Analysis of Phenotype and Confirmation of Causality Between Phenotype and Insertional Mutation
  - Co-segregation analysis
  - Identification of independent insertional allele
  - Using unstable insertional mutagens and isolation of revertant lines
  - Mutant complementation by the transgene

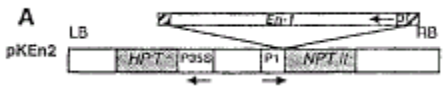
Outline

- Methods of identification of sequence-specific mutants
  - Preparation of mutants collection

Types of Insertional Mutagens

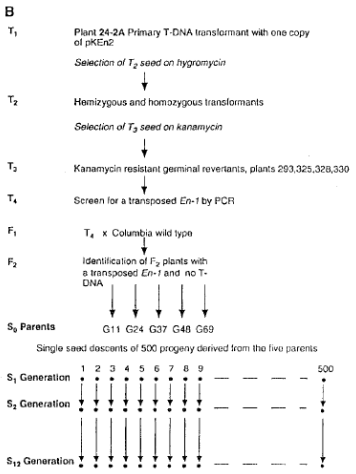
- Mobile elements
  - **Autonomous transposons (*En-1*)**
    - They contain a gene for transponase, enabling excision and reintegration into the genome
    - At both ends they contain short inverted repeat, which are recognized by transponase
- Stable elements
  - **Non-autonomous transposons (*dSpm*)**
    - mutant of En/Spm transposon, which has lost autonomy because of mutation in a gene for transponase
    - It can be activated by crossing with a line carrying the En/Spm transposon
  - **T-DNA**
    - completely stable, however, its insertion can lead to chromosome rearrangements (inversions, deletions, transpositions)

Libraries of Insertional Mutants (plants)

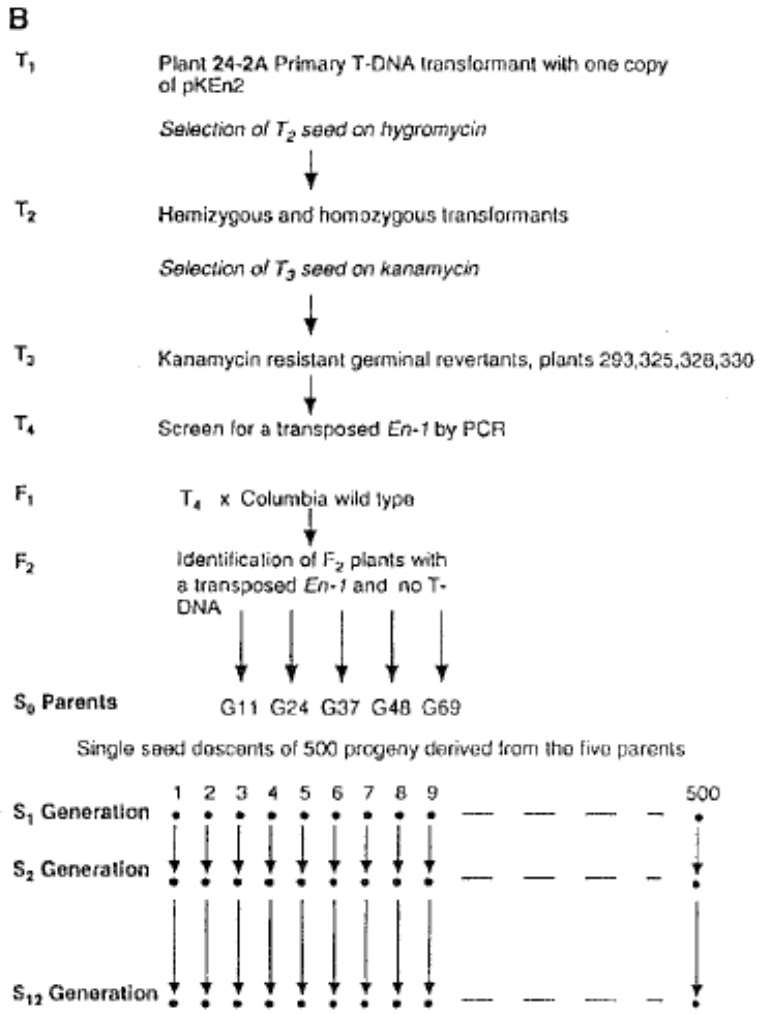


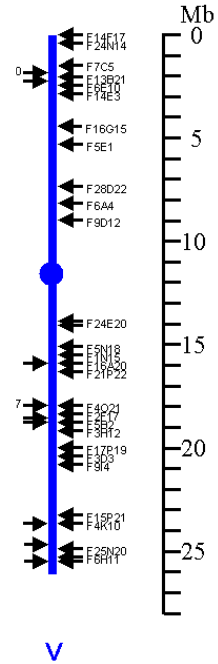
Preparation of transgenic plants

Creating the population of mutants

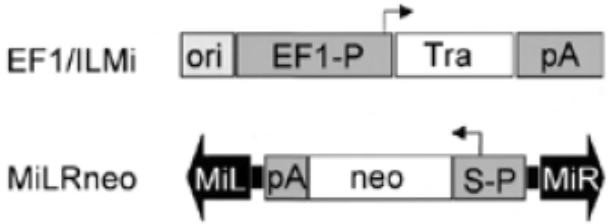


Searching for sequence-specific mutants by PCR





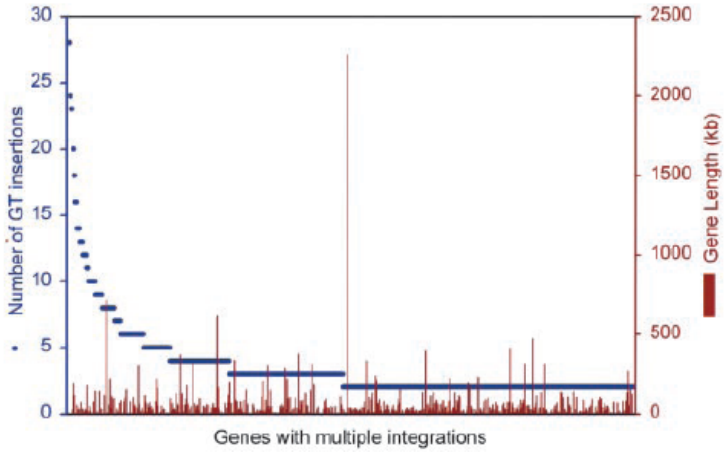
Libraries of Insertional Mutants (animals)

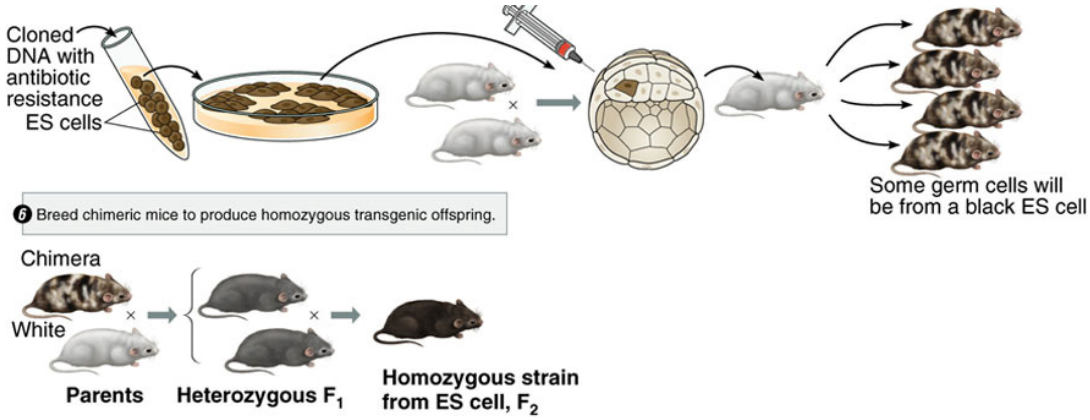


Transfection into human cell cultures (HeLa) or mouse embryonic stem (ES) cells

Generating a population of mutant cell lines and frequence-analysis of insertions

in vitro analysis or preparation of library of insertional mutants by reintroingression ES into mouse embryos





Outline

- Methods of identification of sequence-specific mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
    - PCR-based three-dimensional screening

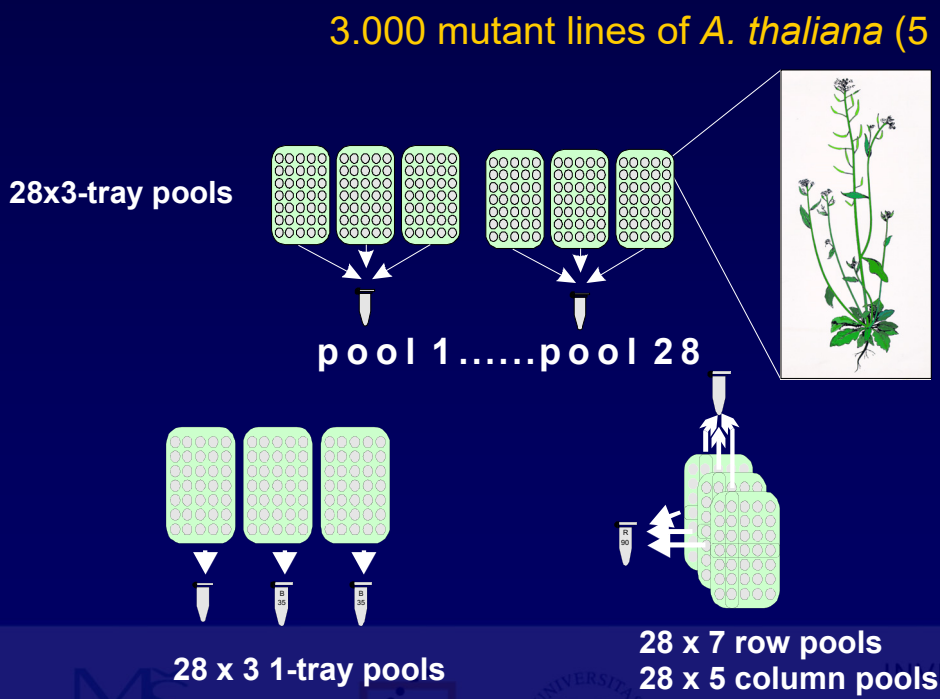
Isolation of sequence-specific mutants

1. Library of *En-1* insertional mutants

- autonomous En/Spm, without selection
- 3000 independent lines
- 5 copies per line on average
- PCR-based three-dimensional screening

Isolation of sequence-specific mutants

- PCR-based three-dimensional screening
  - Isolation of genomic DNA from the individual plants of mutant population and creating sets of DNA („triads“, rows and columns of triads and individual trays)



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ
Tato prezentace je spolufinancována Evropským sociálním fondem a státním rozpočtem České republiky

Isolation of sequence-specific mutants

- PCR-based three-dimensional screening
  - Isolation of genomic DNA from the individual plants of mutant population and creating sets of DNA („triads“, rows and columns of triads and individual trays)
  - Identification of positive „triad“ with PCR, blotting of PCR products and hybridization of the PCR products with gene-specific probe

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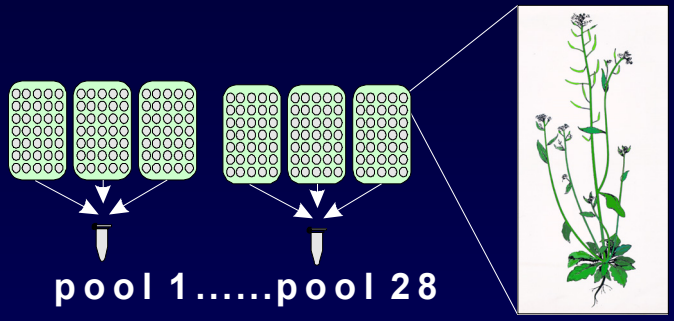
OP Vzdělávání pro konkurenceschopnost
UNIVERSITAS MASARYKIANA BRUNENSIS
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ
Tato prezentace je spolufinancována Evropským sociálním fondem a státním rozpočtem České republiky

Isolation of sequence-specific mutants

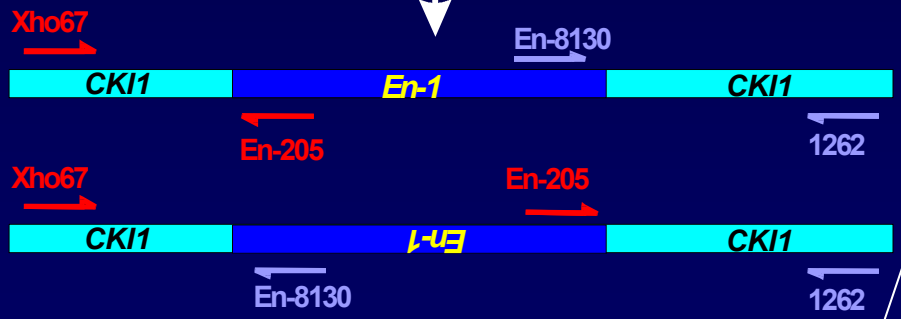
1. 3-tray pools screen

3.000 mutant lines of *A. thaliana* (5 copies of En-1/line)

28x3-tray pools

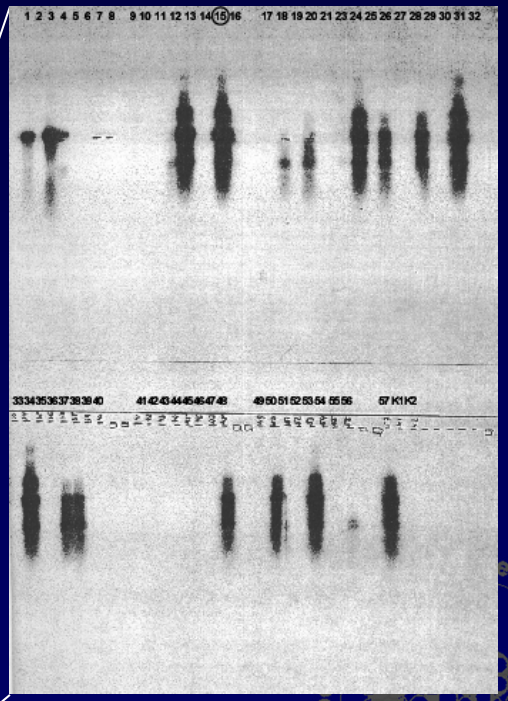


pool 1.....pool 28



(2x2x28=112 PCR reactions)

Identification of the PCR product by hybridization with a gene-specific probe



Isolation of sequence-specific mutants

- PCR-based three-dimensional screening
  - Isolation of genomic DNA from the individual plants of mutant population and creating sets of DNA („triads“, rows and columns of triads and individual trays)
  - Identification of positive „triad“ with PCR, blotting of PCR products and hybridization of the PCR products with gene-specific probe
  - Identification of the positive line through identification of positive tray, row and column

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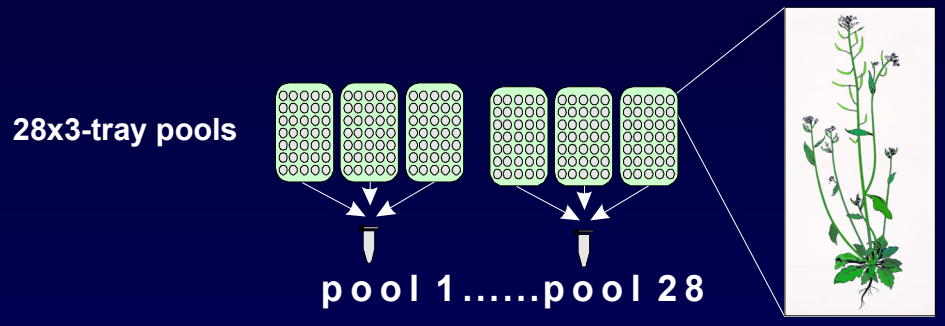
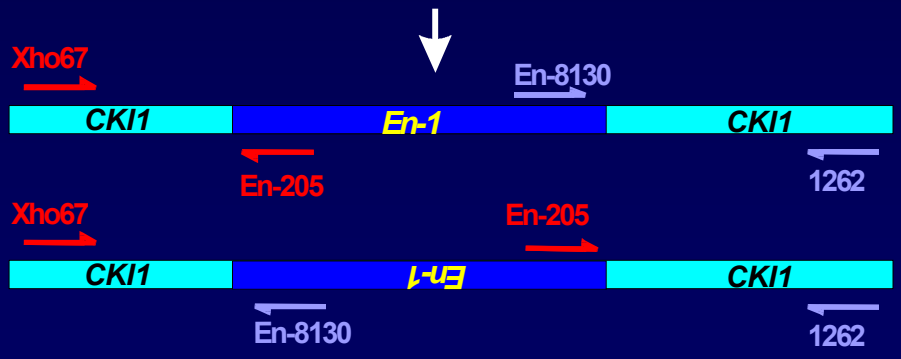
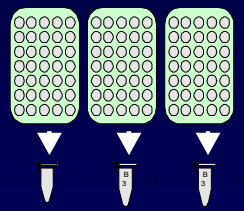


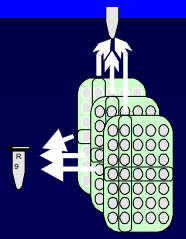


(2x2x28=112 PCR reactions)

2. Identification of line carrying the insertion



3 x 1-tray pools



7 row pools
5 column pools

(another 5+7+3=15 PCR reactions)

In total: 112+15=127 PCR reactions

Identification of the PCR product by hybridization with a gene-specific probe

Outline

- Methods of identification of sequence-specific mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
    - PCR-based three-dimensional screening
    - Hybridization with iPCR products on filters

Isolation of sequence-specific mutants

Insertion library of dSpm mutants

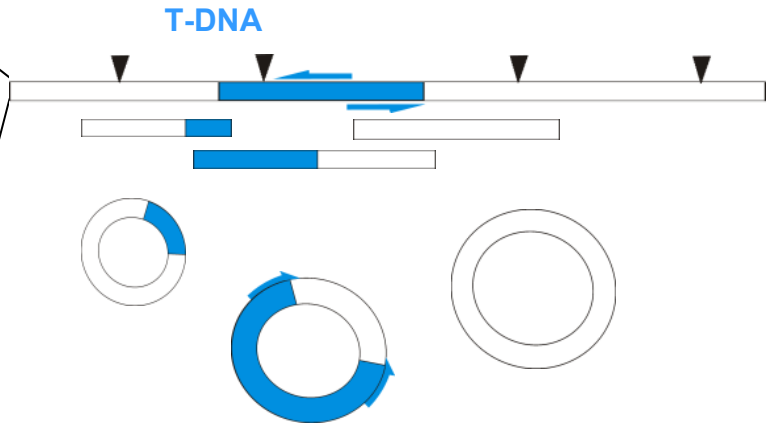
- The Sainsbury Laboratory (SLAT-lines), John Innes Centre, Norwich Research Park
- DNA and seeds in Nottingham Seed Stock Centre
- 48.000 lines
- 1.2 insertion per line on average
- non-autonomous transposon
- PCR searching or hybridization with iPCR filters
- SINS (sequenced insertion sites) database

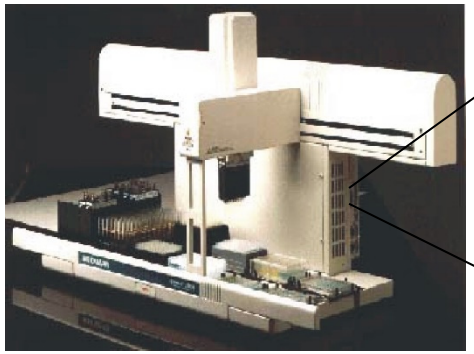
http://nasc.nott.ac.uk

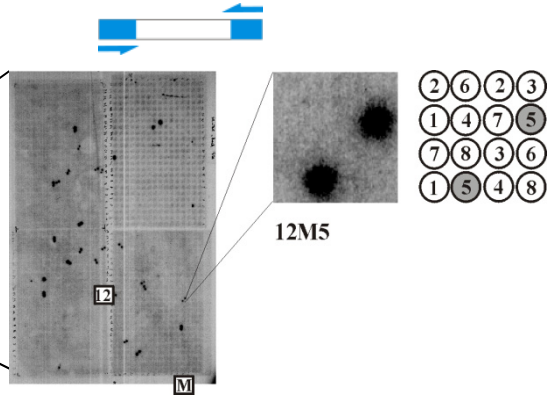
Isolation of sequence-specific mutants

- Hybridization with products of iPCR on filters

  - Isolation of genomic DNA from the individoul plants of mutant population
  - Restriction endonuclease cleavage
  - Ligation, formation of circular DNA
  - Inverse PCR (iPCR) using the T-DNA specific primers
  - Preparation of nylon filters with PCR products in the exact position using a robot
  - Hybridization with a gene-specific probe





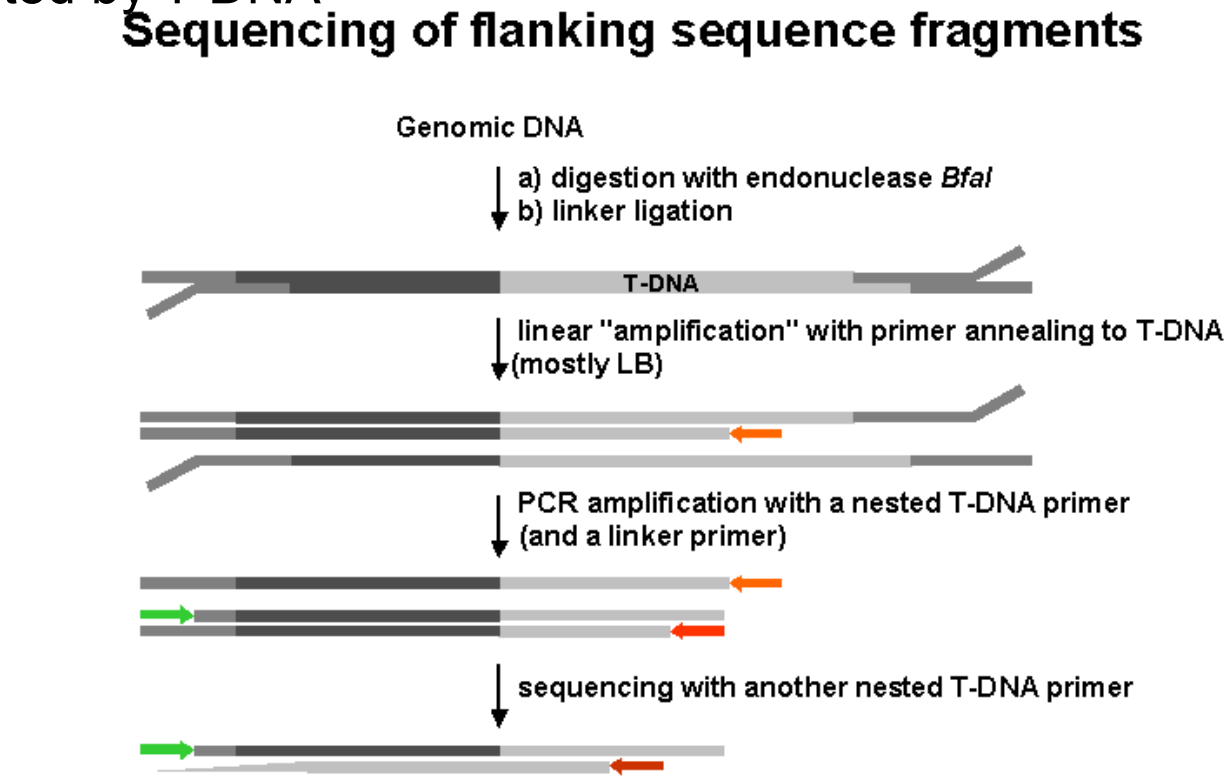


Outline

- Methods of identification of sequence-specific mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
  - Searching for sequence-specific mutants in electronic databases

Isolation of sequence-specific mutants

Preparation of librares from population of *A. thaliana* mutated by T-DNA



GABI-Kat (MPIZ, Köln)

Searching in electronic libraries of insertional mutants

>Insert_SALK:029311: Order line 029311 | View in AGR
Length = 460

Score = 484 bits (244), Expect = e-135
Identities = 250/252 (99%)
Strand = Plus / Minus

```
Query: 1450 attagagtttgattgaagtgtgttttatatattgatagtgggacattacttataaaaagc 1509
            |||||||||||||||| | ||||||||||||||||||||||||||||||||||||||||
Sbjct: 459  attagagtttgattgaagcgcgttttatatattgatagtgggacattacttataaaaagc 400


Query: 1510 acaaggatacaacaatagagacagtcacatgtatatcacataagtggatggtcctcaatg 1569
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 399  acaaggatacaacaatagagacagtcacatgtatatcacataagtggatggtcctcaatg 340


Query: 1570 tgttgcttgtaggacatttgtgagtatgtcaaaaacttatttcacatggtacactcatag 1629
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 339  tgttgcttgtaggacatttgtgagtatgtcaaaaacttatttcacatggtacactcatag 280


Query: 1630 attagccccacttaggagtgtctagaaaaagattgggactaaagtcttgttggatcgaat 1689
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 279  attagccccacttaggagtgtctagaaaaagattgggactaaagtcttgttggatcgaat 220


Query: 1690 atgattccaaac 1701
            ||||||||||||
Sbjct: 219  atgattccaaac 208
```

Score = 111 bits (56), Expect = 8e-23
Identities = 77/84 (91%)
Strand = Plus / Plus

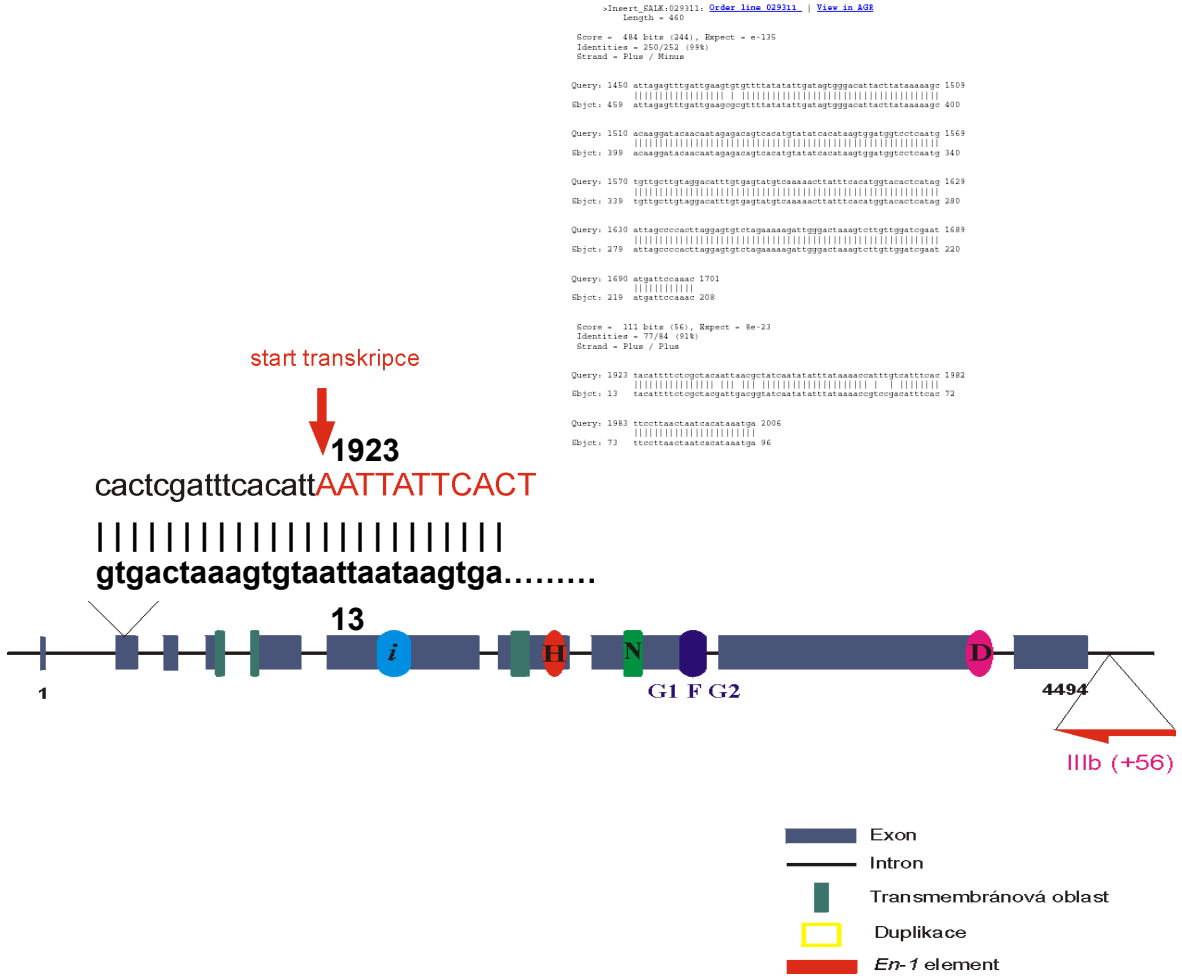
```
Query: 1923 tacattttctcgctacaattaacgctatcaatatatttataaaaccatttgtcatttcac 1982
            ||||||||||||||| ||| ||| |||||||||||||||||||| |   | ||||||||
Sbjct: 13   tacattttctcgctacgattgacggtatcaatatatttataaaaccgtccgacatttcac 72


Query: 1983 ttccttaactaatcacataaatga 2006
            |||||||||||||||||||||||
Sbjct: 73   ttccttaactaatcacataaatga 96
```

Sbjct: 292 ccagcttctagaagcttcttggtcaagtttccagtacgggacgatctcgagaatcaca 233

AGR insert page View detailed information on insert sequences in AGR

CEITEC

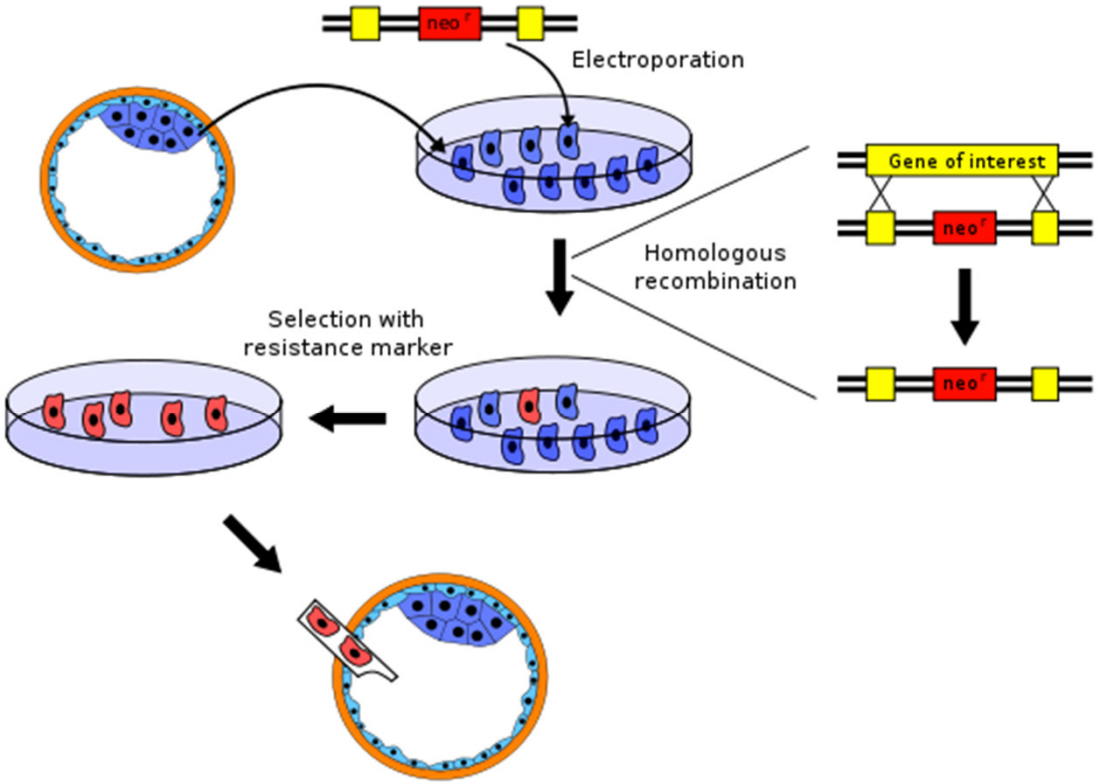
Searching in electronic libraries of insertional mutants



Outline

- Methods for Identification of Sequence-Specific Mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
  - Searching for sequence-specific mutants in electronic databases
  - Knocking-out the gene using homologous recombinantion

Knocking-Out the Gene



Outline

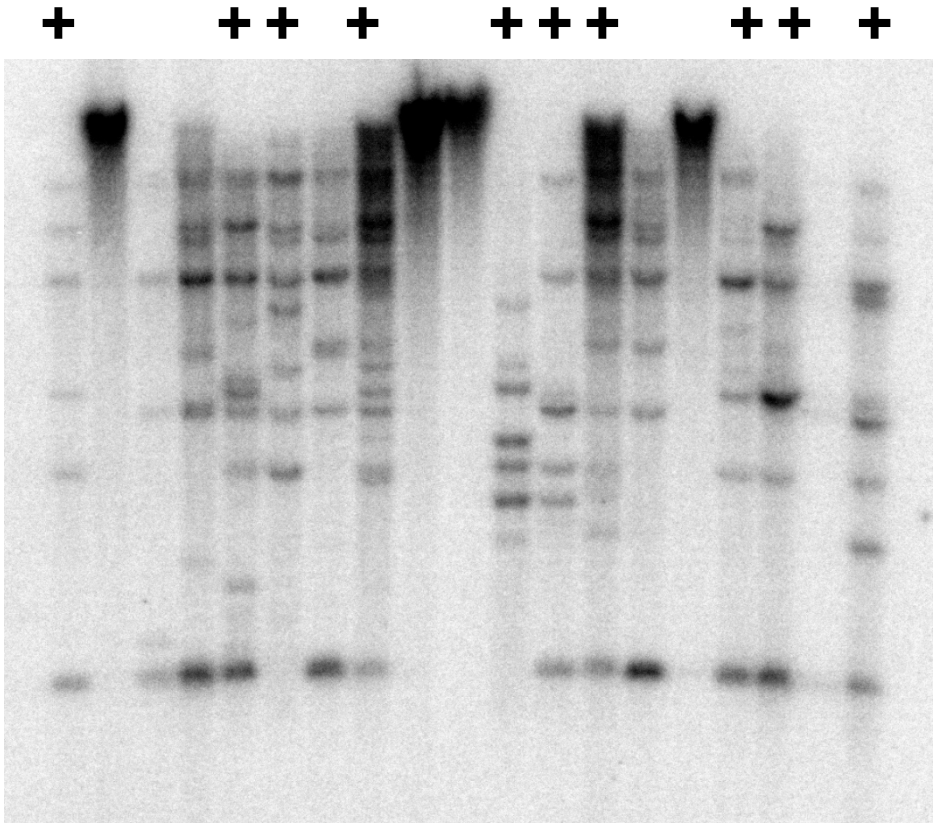
- Methods for Identification of Sequence-Specific Mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
  - Searching for sequence-specific mutants in electronic databases
  - Knocking-out the gene using homologous recombinantion
- Analysis of Phenotype and Confirmation of Causality Between Phenotype and Insertional Mutation
  - Co-segregation analysis
  - Identification of independent insertional allele
  - Using unstable insertional mutagens and isolation of revertant lines
  - Mutant complementation by the transgene

Why is it necessary to analyze the causality between the insertion and the observed phenotype?

- Presence of multiple insertions in one line
- Posibility of independent point mutation occurrence
- Insertions of T-DNA are often associated with chromosomal aberrations (duplications, inversions, deletions)

Causality between insertion and phenotype

- **Co-segregation analysis**
  - Co-segregation of specific fragment, e.g. after insertion of T-DNA (or exposure to EMS etc.) into the genome of the observed phenotype

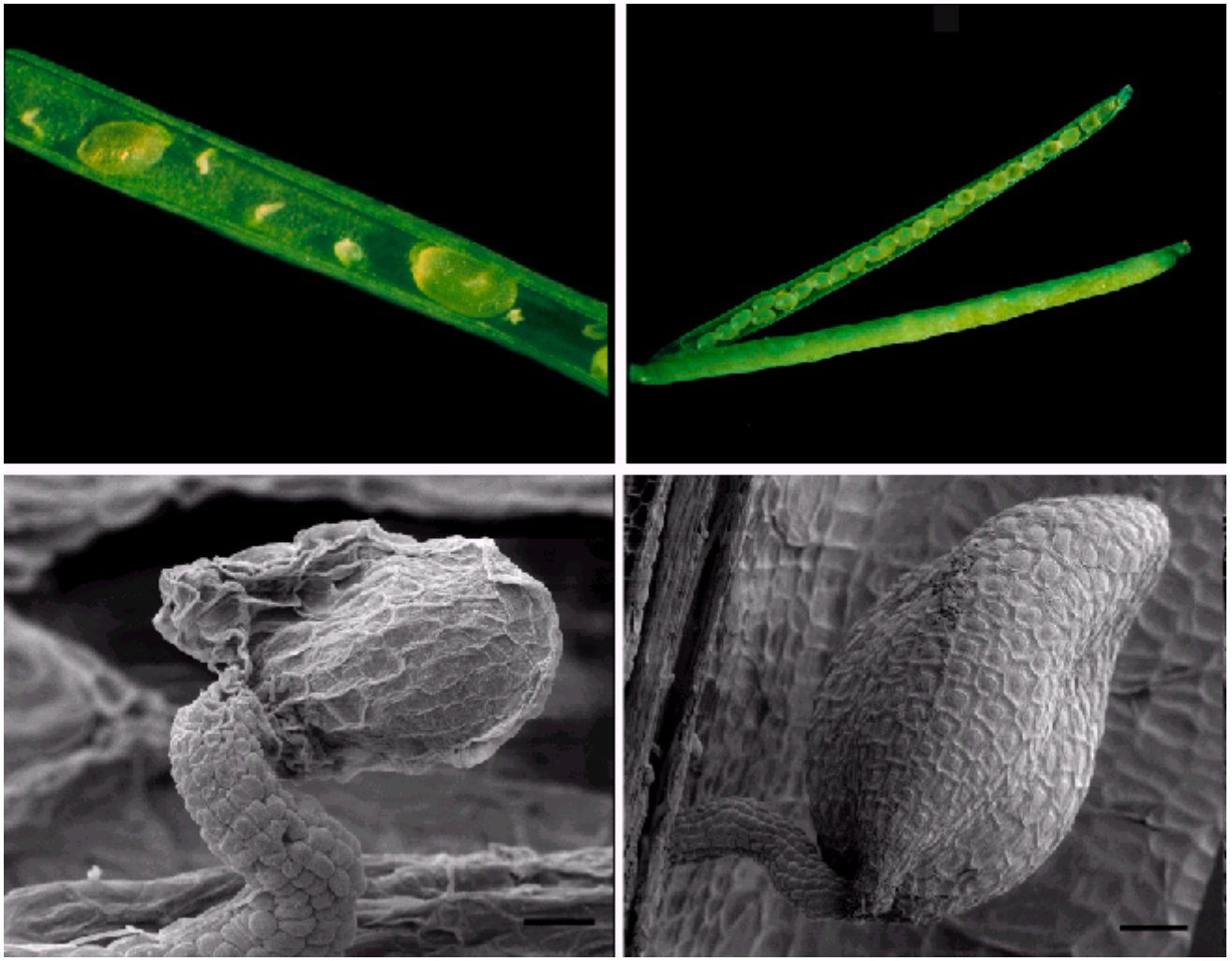


Use of autonomous transposons for the isolation of new stable mutations and of revertant lines

- Transposons are often characterized by excision and reinsertion into a nearby region – use for the isolation of new mutant alleles
- However, excision of transposons is not always entirely accurate – point mutations occurr – isolation of revertant lines with silent mutation, or even isolation of the stable mutants

cki1::En-1/CKI1 Phenotype

cki1::En-1/CKI1 *CKI1/CKI1*



Confirmation of phenotype *cki1::En-1/CKI1*

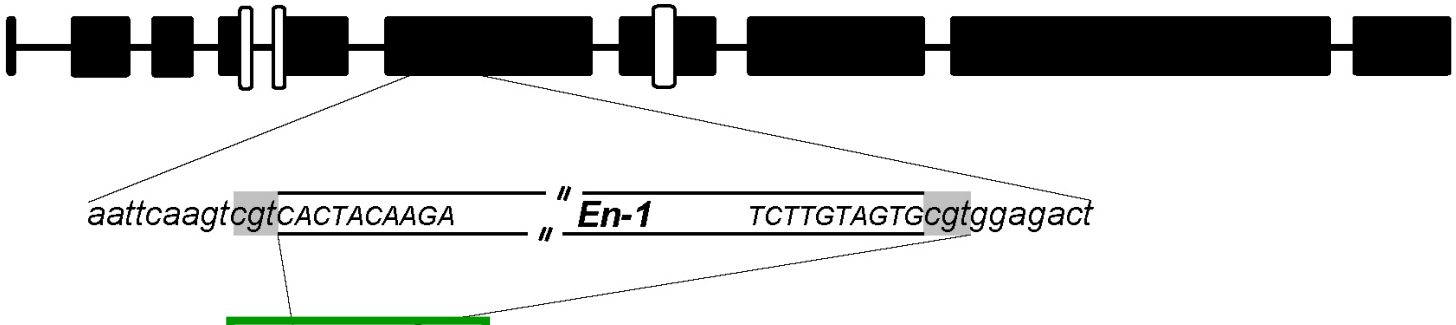
1. Isolation of revertant lines

- PCR-searching in 246 plants of segregating population
- from 90 *cki1::En-1* positive plants, 9 plants had both mutant and standard silicles

Offspring analysis

- confirmation of absention of insertion using PCR
- PCR amplification and cloning the part of the genomic DNA at the insertion site
- sequencing

Use of autonomous transposons for the isolation of new stable mutations and revertant lines



A. *aat tca agt cgt gga gac tac act tgg tac act caa acc gtg gat cag tta act ggt*
N S S R G D Y T W Y T Q T V D Q L T G

B. *aat tca agt **ggt acg** act tgg tac act caa acc gtg gat cag tta act ggt*
N S S G T T W Y T Q T V D Q L T G

C. *aat tca agt cgt **ac**g gag act aca ctt ggt aca ctc aaa ccg tgg atc agt taa*
N S S R T E T T L G T L K P W I S .

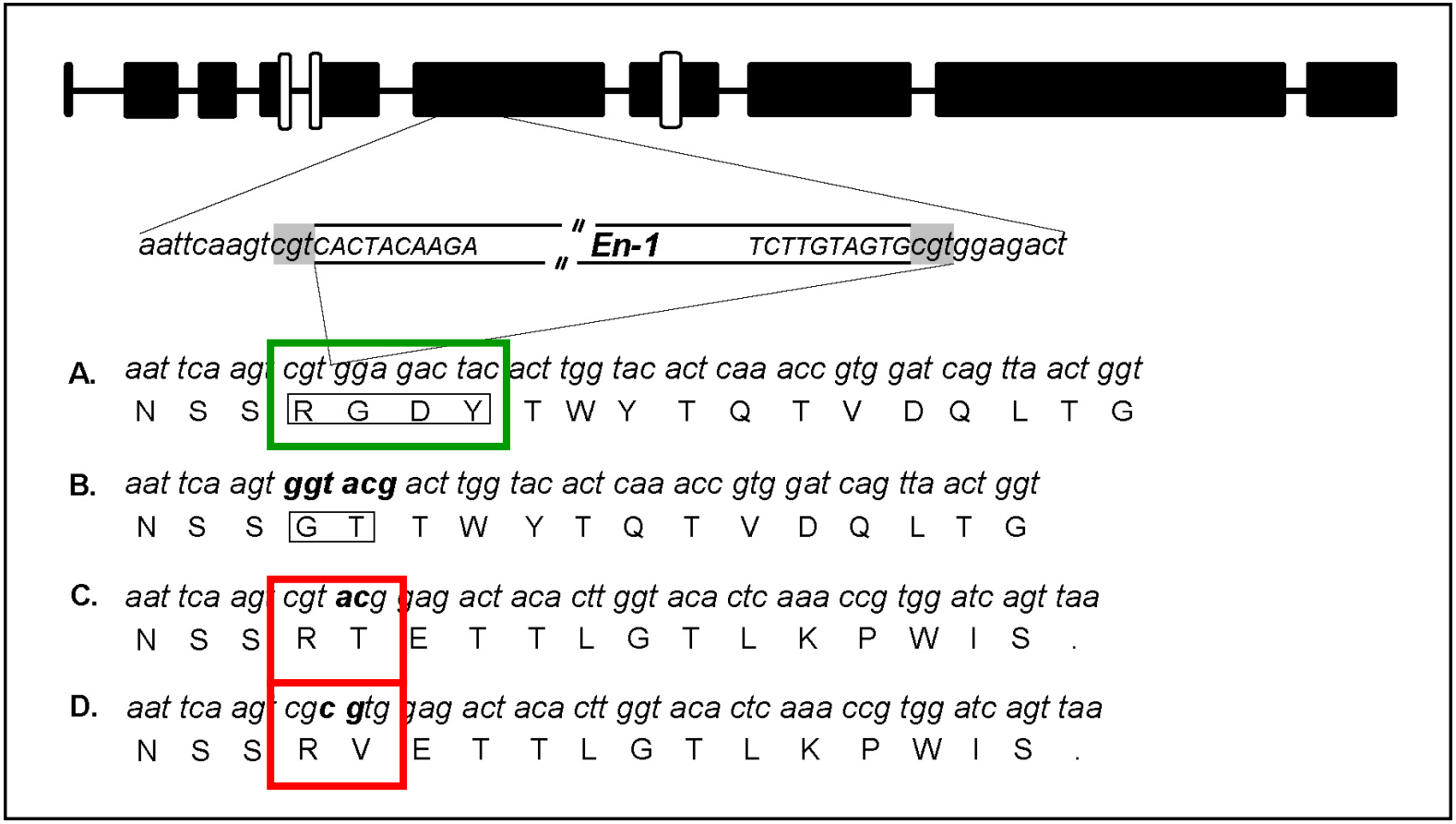
D. *aat tca agt cg**c** **g**tg gag act aca ctt ggt aca ctc aaa ccg tgg atc agt taa*
N S S R V E T T L G T L K P W I S .

Confirmation of phenotype *cki1::En-1/CKI1*

2. Isolation of a stable mutant line

- analysis of the phenotype of the segregating population (*CKI1/CKI1 CKI1/cki1::En-1)*
- PCR analysis of plants with the mutant phenotype – identification of plants without insertion
- PCR amplification and cloning the part of the genomic DNA at the insertion site
- sequencing

Use of autonomous transposons for the isolation of new stable mutations and revertant lines



Mutant Line Complementation

Mutant Line Complementation

WT(Col-0)	*cop1-4*	*p35S::AtCOP1 1-5*	*p35S::OsCOP1 16-10*	*p35S::PpCOP1 9-8*
		cop1-4		

Ranjan et al., 2014

Key Concepts

- How reverse genetics explores the gene and its role?
  - Targeted gene silencing
    - Searching in the insertion mutant libraries
    - Homologous recombination
  - Phenotype analysis
  - Confirmiong the causality between the observed phenotype and the insertion mutation
    - Co-segregation analysis
    - Identification of independent allele
    - Use of unstable insertion mutagenes and identification of revertant lines
    - Mutant line complementation by transgene

Discussion