CG920 Genomics

Finishing Lesson 2

Genes Identification

Jan Hejátko

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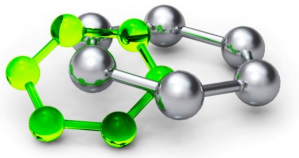
SCI



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

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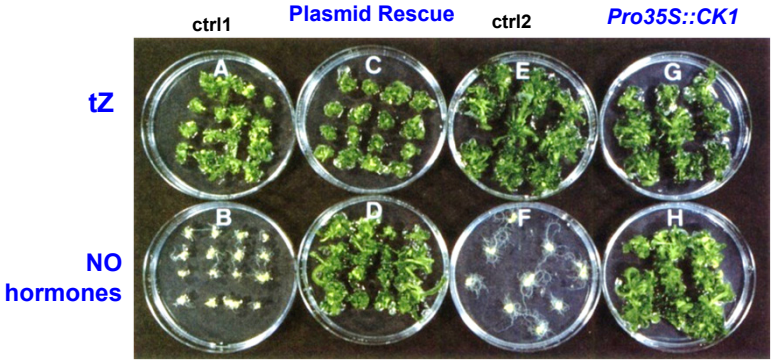
Forward Genetics

- 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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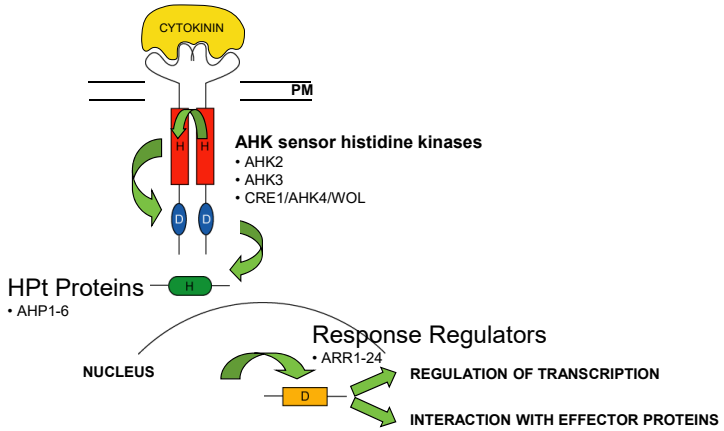
Signal Transduction via MSP



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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**

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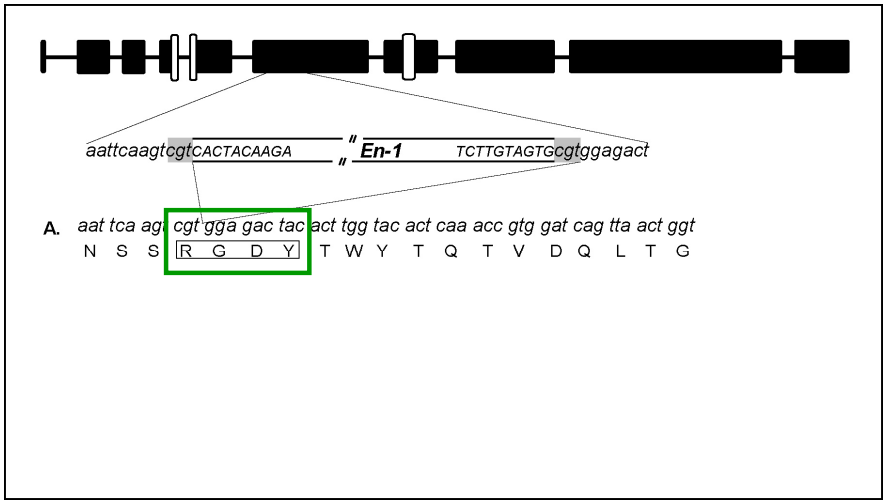
Identification of insertional *cki1* mutant allele



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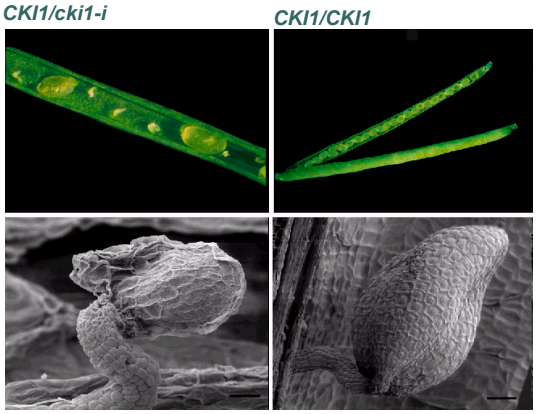
CKI1 Regulates Female Gametophyte Development

- CKI1 is necessary for proper megagametogenesis in *Arabidopsis*



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

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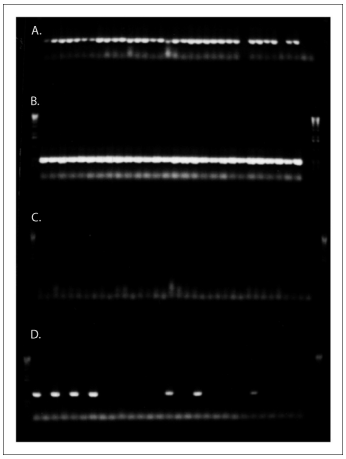
CKI1 and Megagametogenesis

- *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

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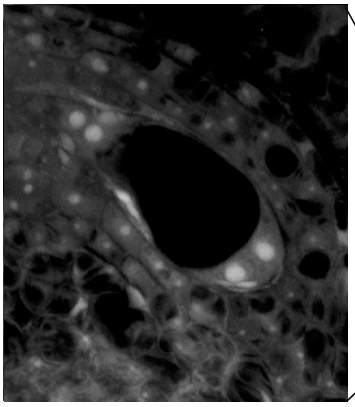
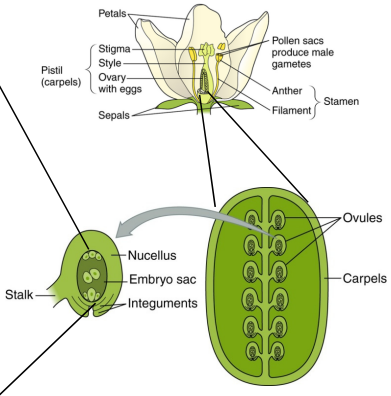
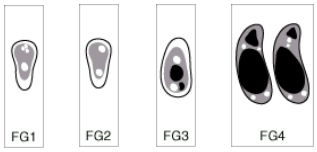
CKI1 and Megagametogenesis

FG







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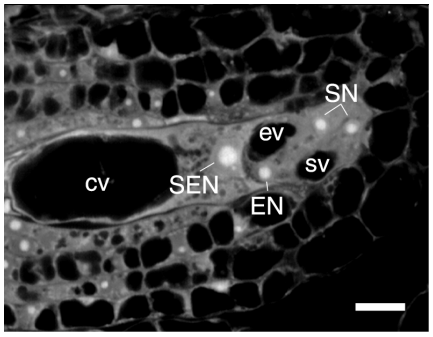
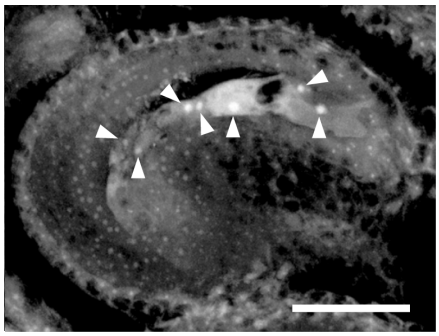
CKI1 and Megagametogenesis

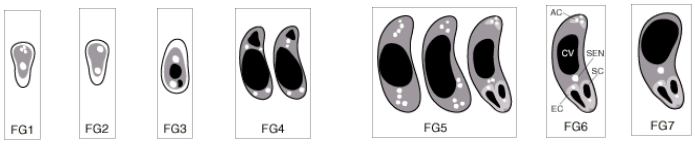
CKI1 FG6 late FG5



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

cki1-i 28 HAE





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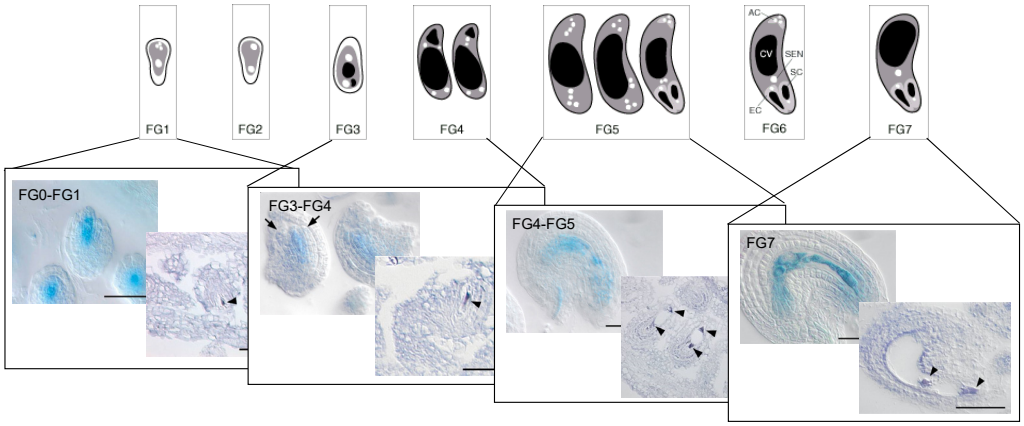
Forward and Reverse Genetics

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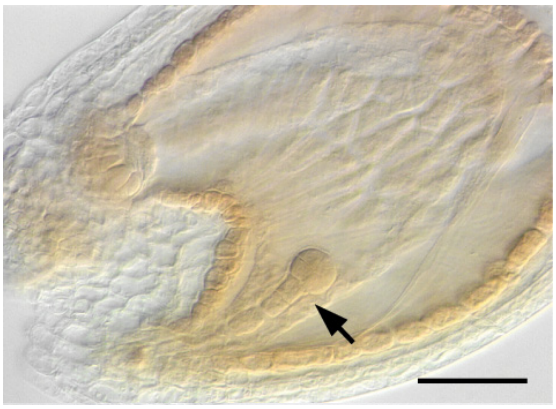
Paternal *CKI1* is Expressed in the *Arabidopsis* Sporophyte Early after Fertilization

♀ wt x ♂ Pro*CKI1:GUS*

~~12~~ ~~24~~ HAP
(hours
after
pollination)



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

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CG920 Genomics

Finishing Lesson 2

Genes Identification

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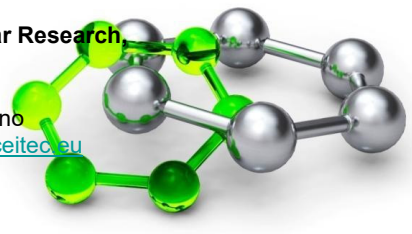

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2007–13
OP Výzkum a vývoj
pro inovace

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.

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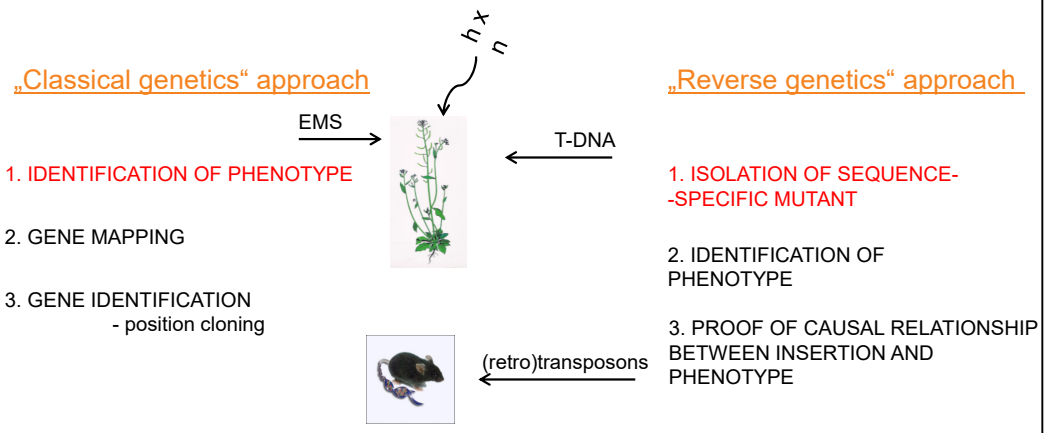
„Classical“ genetics *versus* „reverse genetics“ approaches in functional genomics

RANDOM MUTAGENESIS



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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

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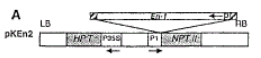
Types of Insertional Mutagens

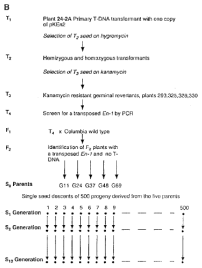
- Mobile elements
  - **Autonomous transposons (*En-1*)**
    - They contain a gene for transponase, enabling excision and reintegration into the genome
    - At both ends they contain short inverted repeat, which are recognized by transponase
- Stable elements
  - **Non-autonomous transposons (*dSpm*)**
    - 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)

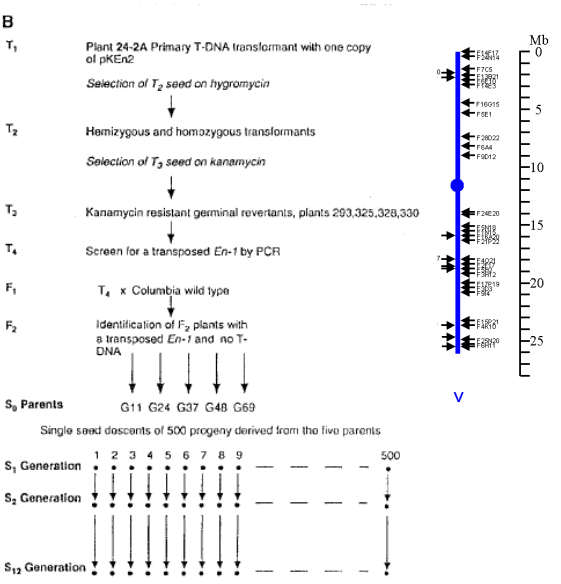


Preparation of transgenic plants

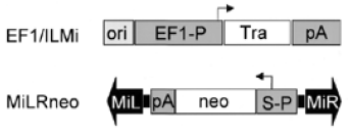
Creating the population of mutants



Searching for sequence-specific mutants by PCR



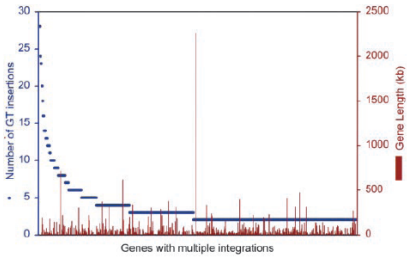
Libraries of Insertional Mutants (animals)

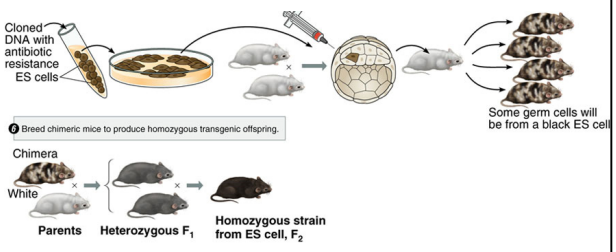


Transfection into human cell cultures (HeLa) or mouse embryonic stem (ES) cells

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





Technologii inzerční mutageneze lze využít i u živočichů. Zda se využívají např. transpozony odvozené z Drosophily (transpozon Minos, viz schéma vlevo nahoře (Klinakis et al., 2000). V tomto případě bylo nutne provést kotransfekci s tzv. helper plasmiem, kódujícím transponázu (neautonomní transpozon). Neo kóduje rezistenci k neomycinu, šipky ukazují směr transkripce řízený přislušnými promotory, pA je polyadenylační signál, ori je počátek replikace viru SV40, S-P je promotor téhož viru. Pro identifikaci inzercí „in frame“ se zasaženými geny lze využít transpozony, obsahující fůzi akceptorových míst sestřihu s ORF reportérového genu, např. lacZ-neo (bez AUG kodonu). Tento přístup umožňuje identifikovat inzerce do aktivních genů prostřednictvím selekce inzerčních mutantů na rezistenci k neomycinu, resp. vykazující β-galaktozidázovou aktivitu (Klinakis et al., 2000).

Outline

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

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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

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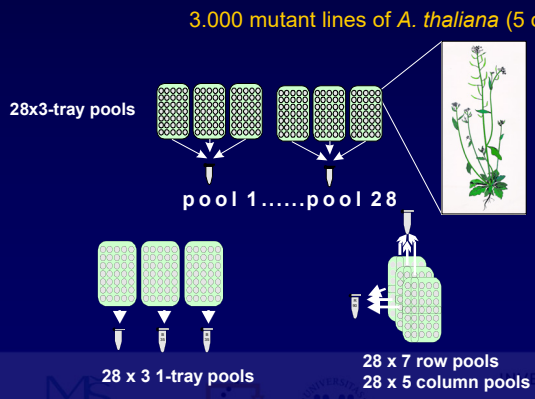
Isolation of sequence-specific mutants

- 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)



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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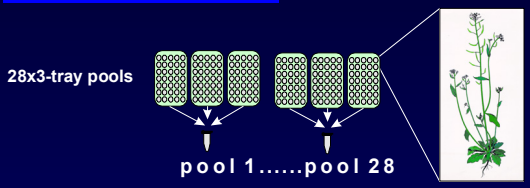
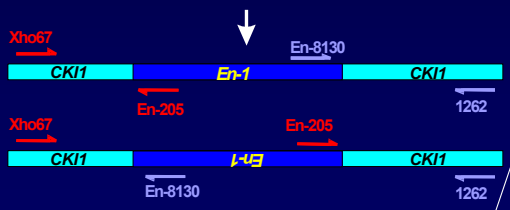
Tato prezentace je spolufinancována Evropským sociálním fondem a státním rozpočtem České republiky

Isolation of sequence-specific mutants

1. 3-tray pools screen

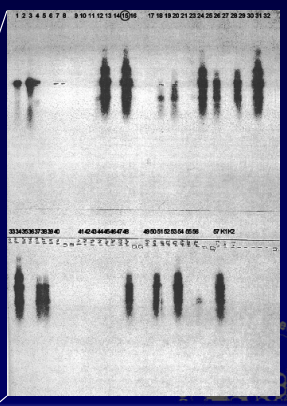
3.000 mutant lines of *A. thaliana* (5 copies of En-1/line)





(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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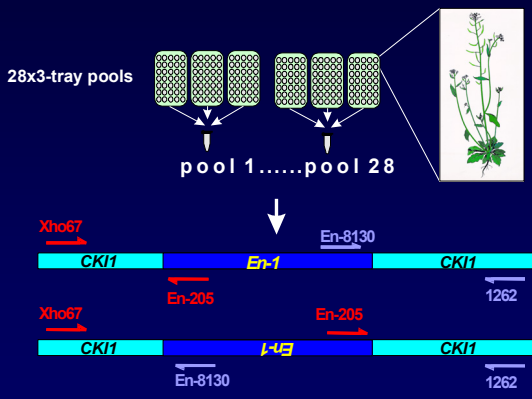
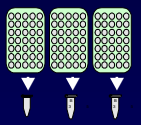
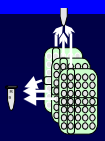


(2x2x28=112 PCR reactions)

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

2. Identification of line carrying the insertion





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

In total: 112+15=127 PCR reactions

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

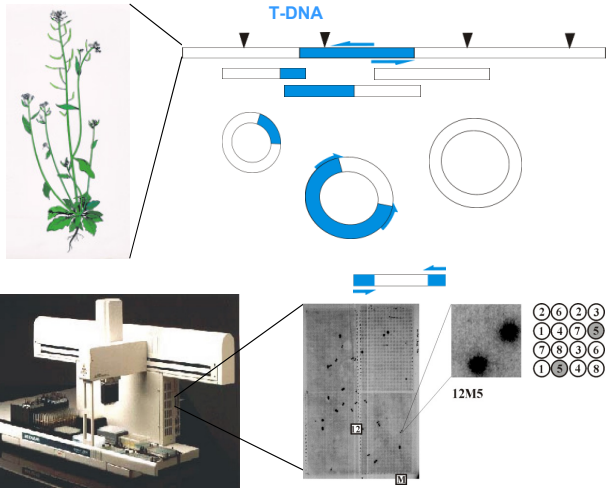
- The Sainsbury Laboratory (SLAT-lines), John Innes Centre, Norwich Research Park
- DNA and seeds in Nottingham Seed Stock Centre
- 48.000 lines
- 1.2 insertion per line on average
- non-autonomous transposon
- PCR searching or hybridization with iPCR filters
- SINS (sequenced insertion sites) database

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
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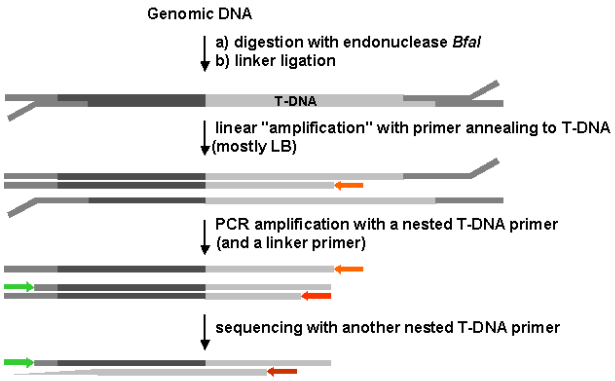
  - Searching for sequence-specific mutants in electronic databases

Isolation of sequence-specific mutants

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

Sequencing of flanking sequence fragments



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 ccagcttctagaagcttcttggtcaagtttccagtaccgggaccgatctcgagaatcaca 233

AGR insert page View detailed information on insert sequences in AGR

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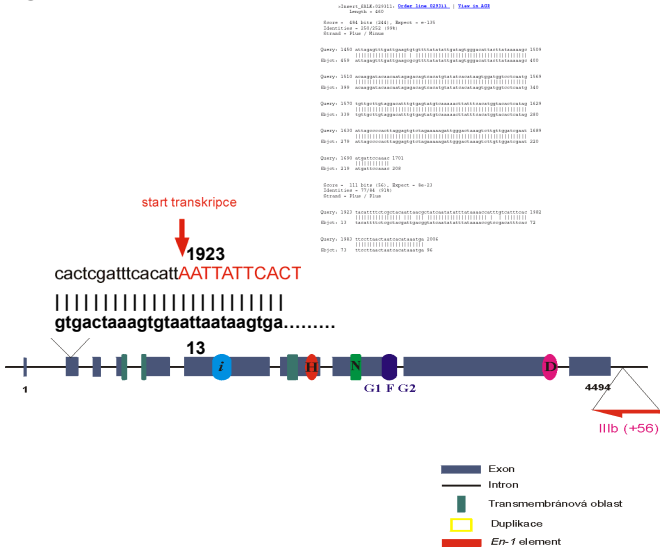
Searching in electronic libraries of insertional mutants



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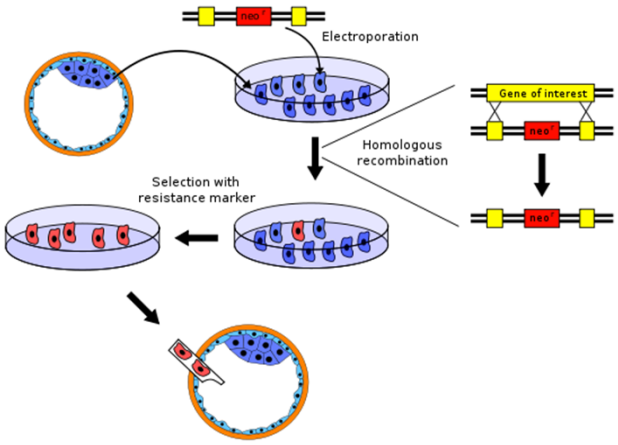
Outline

- 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



CEITEC

Outline

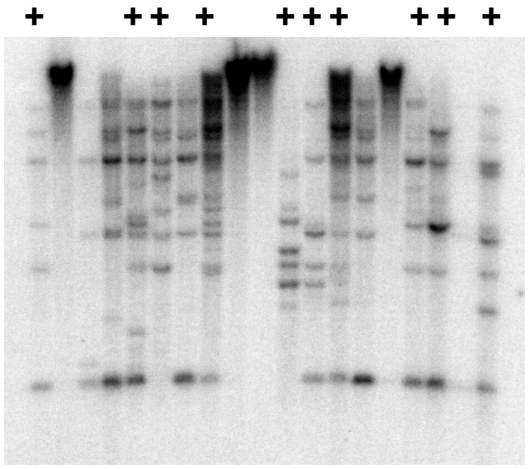
- Methods for Identification of Sequence-Specific Mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
  - Searching for sequence-specific mutants in electronic databases
  - Knocking-out the gene using homologous recombinantion
- Analysis of Phenotype and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

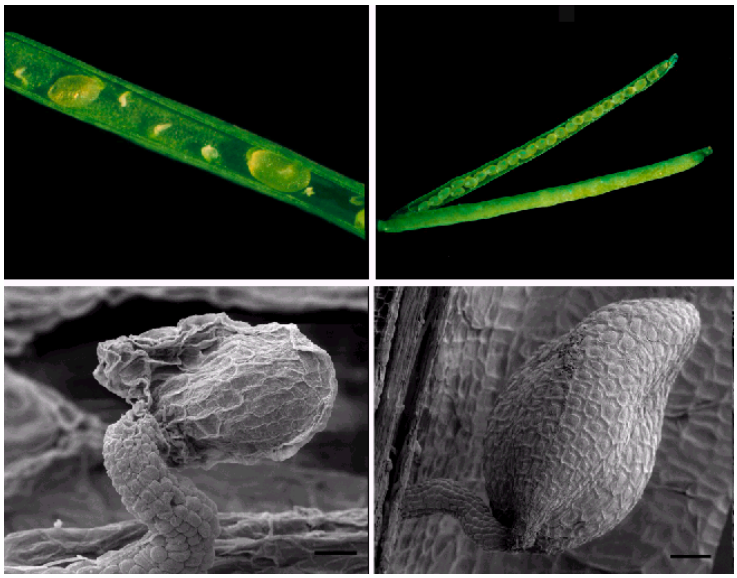


Use of autonomous transposons for the isolation of new stable mutations and of revertant lines

- 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

Phenotype of silicles *cki1::En-1/CKI1*

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

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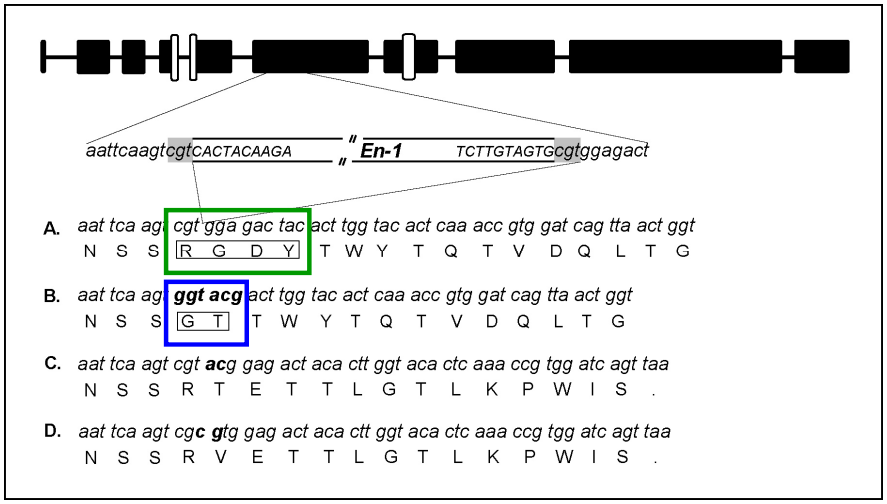
Use of autonomous transposons for the isolation of new stable mutations and revertant lines

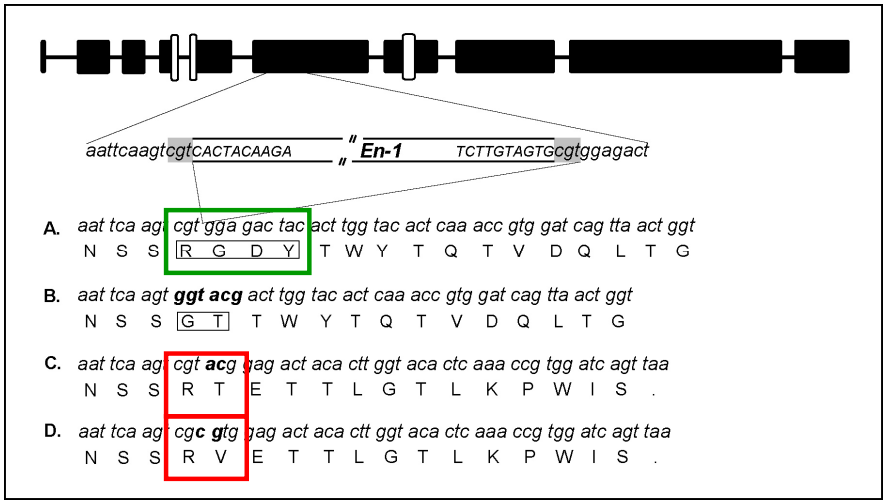


Confirmation of phenotype *cki1::En-1/CKI1*

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

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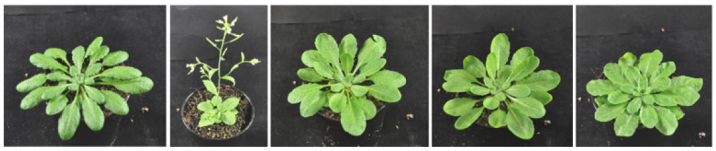
Mutant Line Complementation



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