

CG920 Genomics

Finishing Lesson 2

Genes Identification

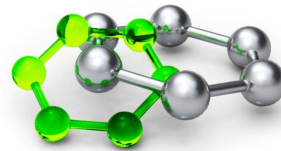
Jan Hejátko

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SCI

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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

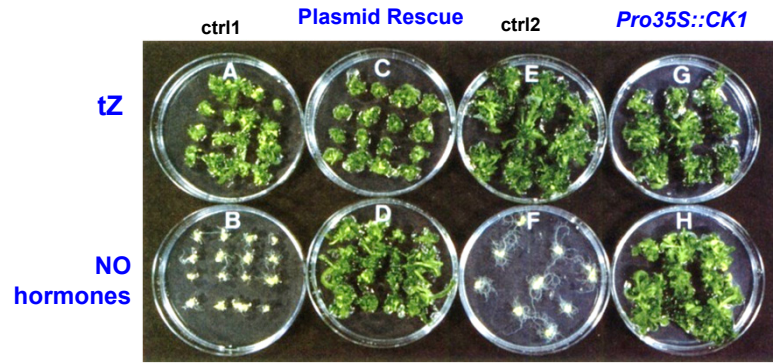
- Principles of experimental identification of genes using forward and reverse genetics
 - Alteration of phenotype after mutagenesis
 - **Forward genetics**
 - Identification of sequence-specific mutant and analysis of its phenotype
 - **Reverse genetics**
 - Analysis of expression of a particular gene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

Forward Genetics

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

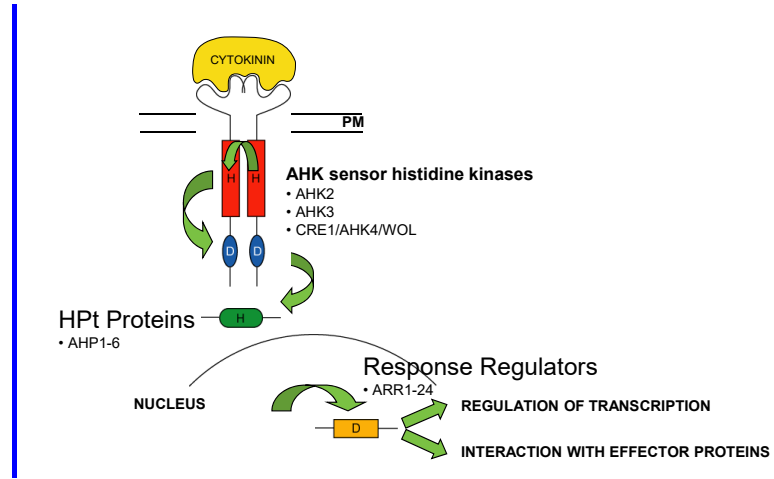
Identification of *CK1* via Activation Mutagenesis

- *CK1* overexpression mimics cytokinin response



Kakimoto, *Science*, 1996

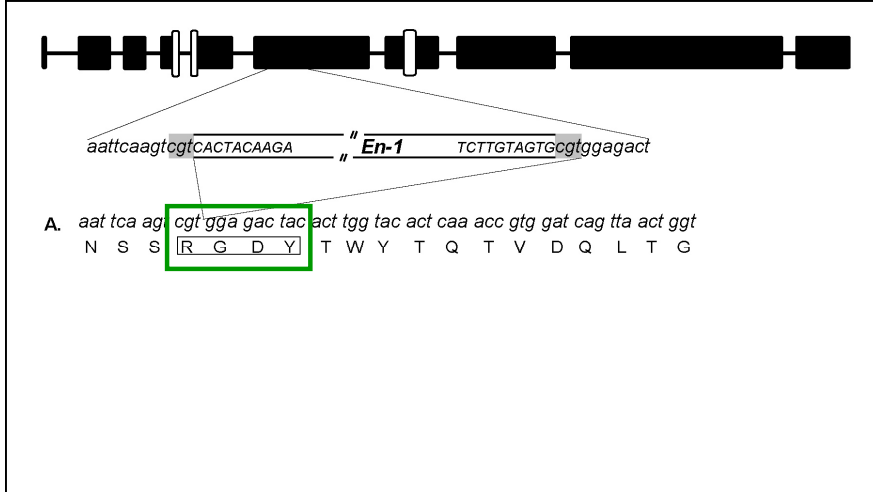
Signal Transduction via MSP



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**

Identification of insertional *cki1* mutant allele



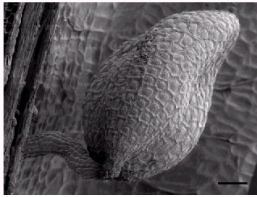
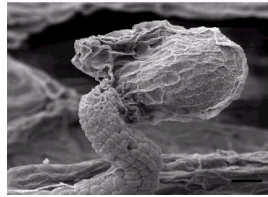
CKI1 and Megagametogenesis

- CKI1 is necessary for proper megagametogenesis in *Arabidopsis*

CKI1/cki1-i



CKI1/CKI1



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

cki1-i reveals non-Mendelian inheritance

P *CKI1/cki1-i*

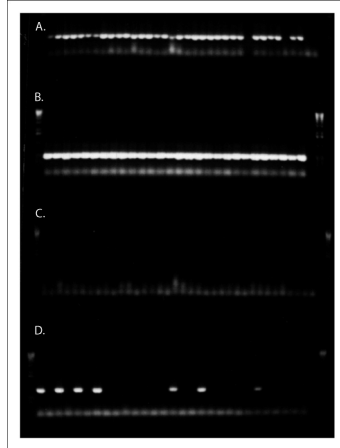
F1 Anticipated: 1 *CKI1* : 2 *CKI1/cki1-i* : 1 *cki1-i*

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

	♂	
♀	<i>CKI1</i>	<i>cki1-i</i>
<i>CKI1</i>	<i>CKI1/CKI1</i>	<i>CKI1/cki1-i</i>
<i>cki1-i</i>	<i>CKI1/cki1-i</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*

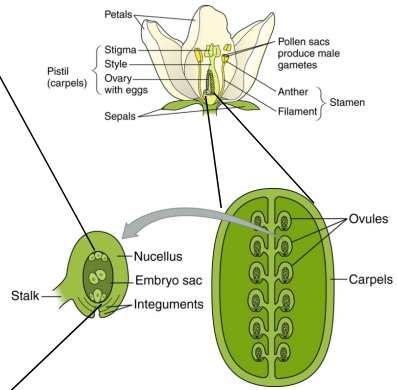
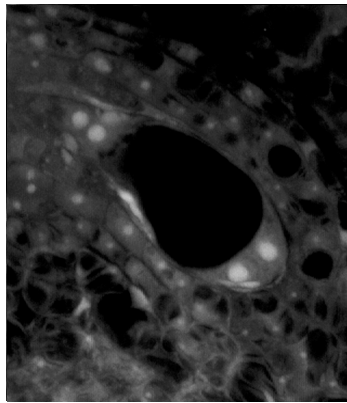


cki1-i specific primers

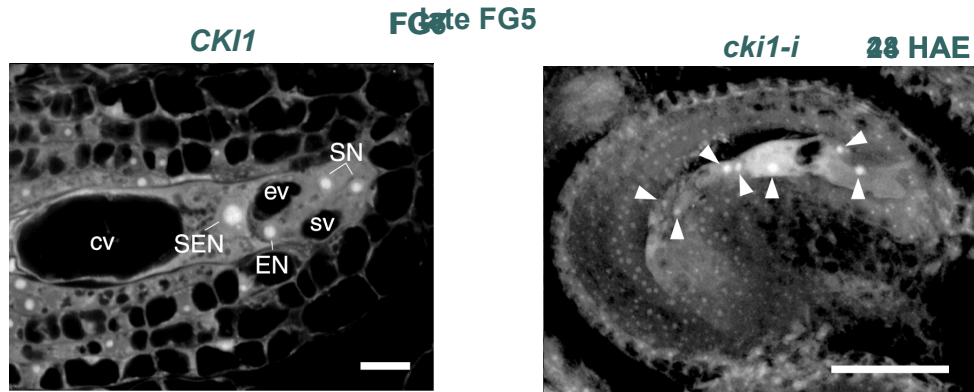
D. ♂ *CKI1/cki1-i* x ♀ wt

CKI1 and Megagametogenesis

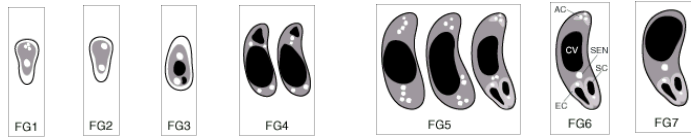
FG 4



CKI1 and Megagametogenesis



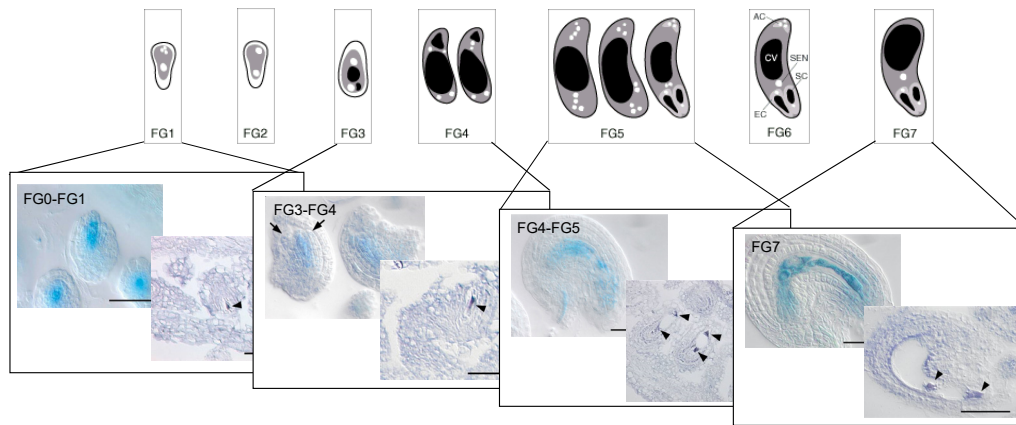
Hejätko et al., *Mol Genet Genomics* (2003)



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 specificity

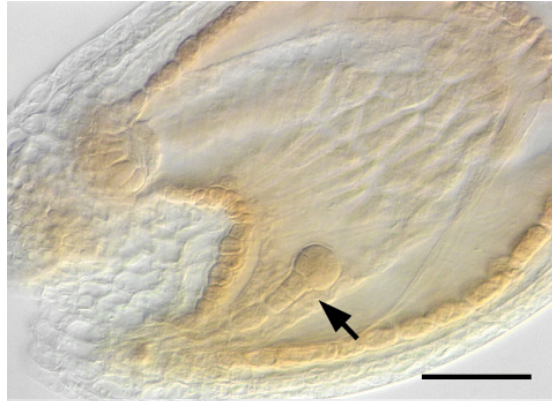
CKI1 is Expressed During Megagametogenesis



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

♀ wt x ♂ Pro*CKI1*:*GUS*

22 HAP
(hours
after
pollination)



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

CG920 Genomics

Lesson 3

Reverse Genetics

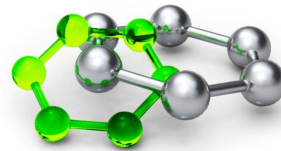
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Literature

- Literature sources for Chapter 03:
 - **Bioinformatics and Functional Genomics**, 2009, Jonathan Pevsner, Willey-Blackwell, Hoboken, 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*, **431**, 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 recombination
- **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

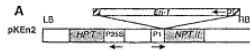
Outline

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

Types of Insertional Mutagens

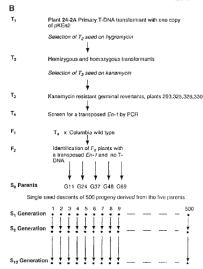
- Mobile elements
 - **Autonomous transposons (*En-1*)**
 - They contain a gene for transposase, enabling excision and reintegration into the genome
 - At both ends they contain short inverted repeat, which are recognized by transposase
- Stable elements
 - **Non-autonomous transposons (*dSpm*)**
 - mutant of *En/Spm* transposon, which has lost autonomy because of mutation in a gene for transposase
 - 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)

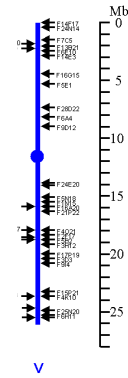
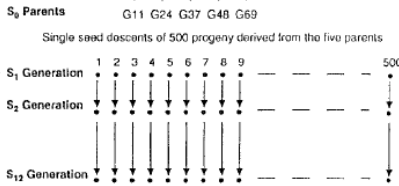
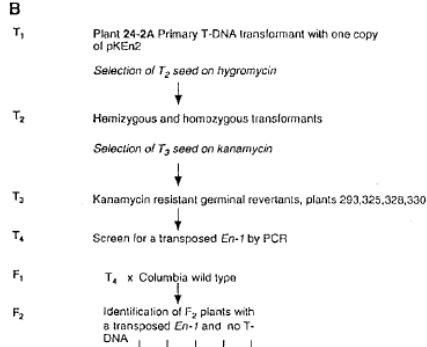


Preparation of transgenic plants

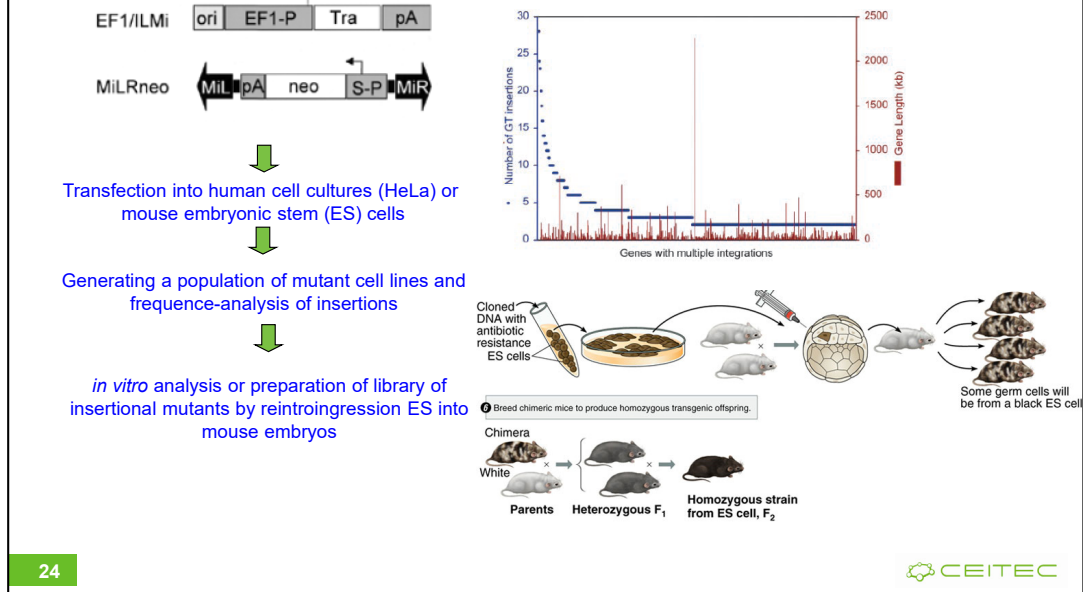
Creating the population of mutants



Searching for sequence-specific mutants by PCR



Libraries of Insertional Mutants (animals)



Technologii inzerční mutagenéze 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 nutné provést kotransfekci s tzv. helper plasmidem, kódujícím transponázu (neautonomní transpozon). Neo kóduje rezistenci k neomycinu, šipky ukazují směr transkripce řízený příslušnými 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 fúzi akceptorových míst sestřihu s ORF reportérového genu, např. lacZ-neo (bez AUG kodonu). Tento přístup umožňuje identifikovat inserce 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

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)
 - Identification of positive „triad“ with PCR, blotting of PCR products and hybridization of the PCR products with gene-specific probe



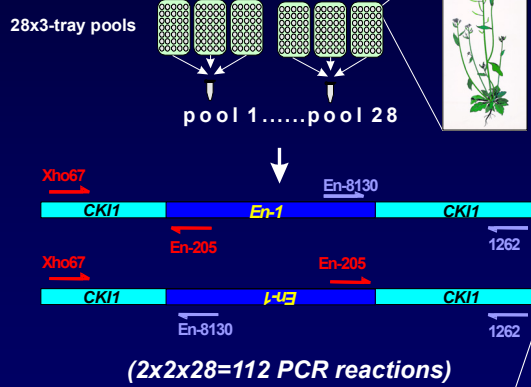
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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)



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

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 - 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



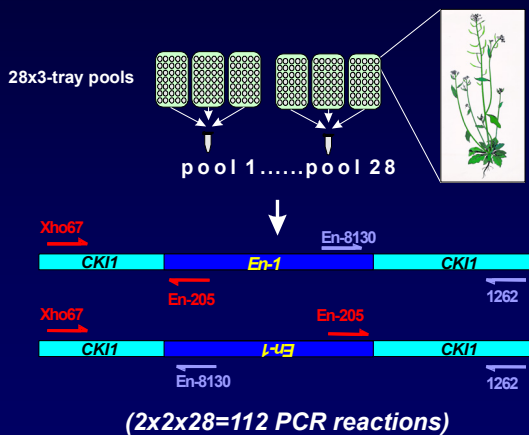
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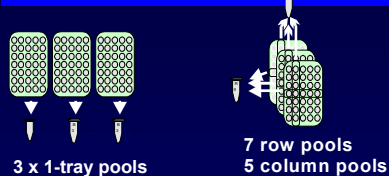
Isolation of sequence-specific mutants

1. 3-tray pools screen

3,000 mutant lines of *A. thaliana* (5 copies of En-1/line)



2. Identification of line carrying the insertion



(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

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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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

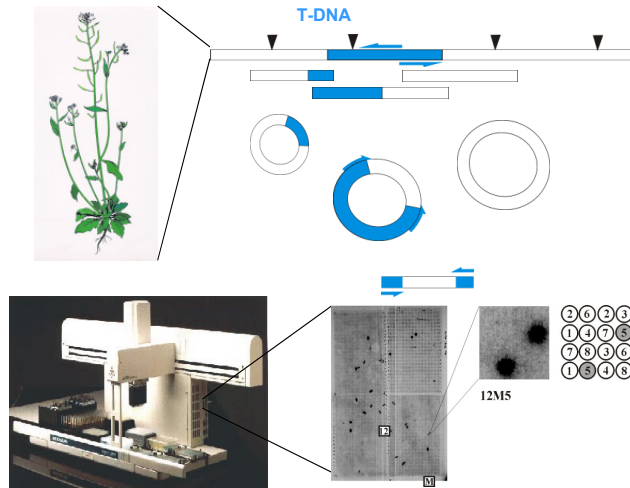
- The Sainsbury Laboratory (SLAT-lines),
John Innes Centre, Norwich Research Park
- DNA and seeds in Nottingham Seed Stock Centre
- 48.000 lines
- 1.2 insertion per line on average
- non-autonomous transposon
- PCR searching or hybridization with iPCR filters
- SINS (sequenced insertion sites) database

<http://nasc.nott.ac.uk>

Isolation of sequence-specific mutants

Hybridization with products of iPCR on filters

- Isolation of genomic DNA from the individual 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



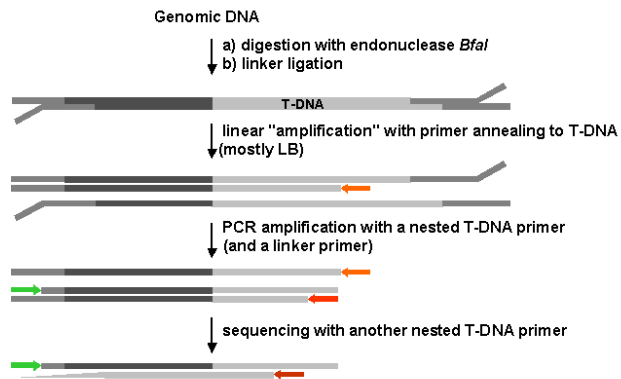
Outline

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 - Searching for sequence-specific mutants in electronic databases

Isolation of sequence-specific mutants

Preparation of libraries 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 AGE
Length = 460

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

Query: 1450 attagagtttgattgaagtgtgttttatattatattgatagtgggacattacttataaaaagc 1509
      |||
Sbjct: 459 attagagtttgattgaagcgttttatattatattgatagtgggacattacttataaaaagc 400

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

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

Query: 1630 attagcccaacttaggagtgctagaaaaagattgggactaaagtcttggatcgaaat 1689
      |||
Sbjct: 279 attagcccaacttaggagtgctagaaaaagattgggactaaagtcttggatcgaaat 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 tacattttctcgtacaaattaacgctatcaatatatttataaaacatttgcatttcaac 1982
      |||
Sbjct: 13 tacattttctcgtacgattgacggtatcaatatatttataaaacgctcgaatttcaac 72

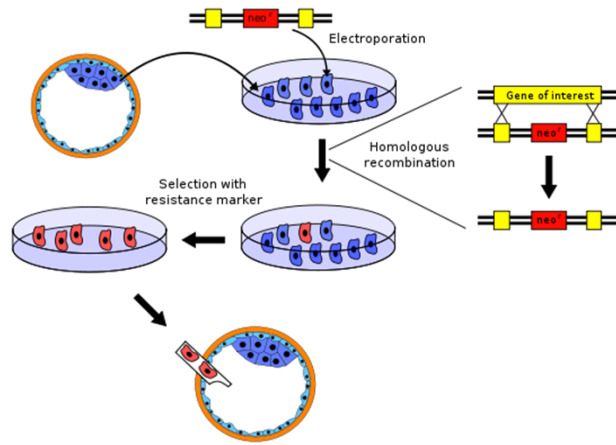
Query: 1983 ttccttaactaatcacataaatsga 2006
      |||
Sbjct: 73 ttccttaactaatcacataaatsga 96

Sbjct: 494 ccagctttagaagctcttggtaagtttcagtaacgggacagatctcagagatcaca 243
AMIK JENSEN ENRGE view detailed information on insert sequences in AMIK
```


Outline

- **Methods for Identification of Sequence-Specific Mutants**
 - Preparation of mutants collection
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 - **Knocking-out the gene using homologous recombination**

Knocking-Out the Gene



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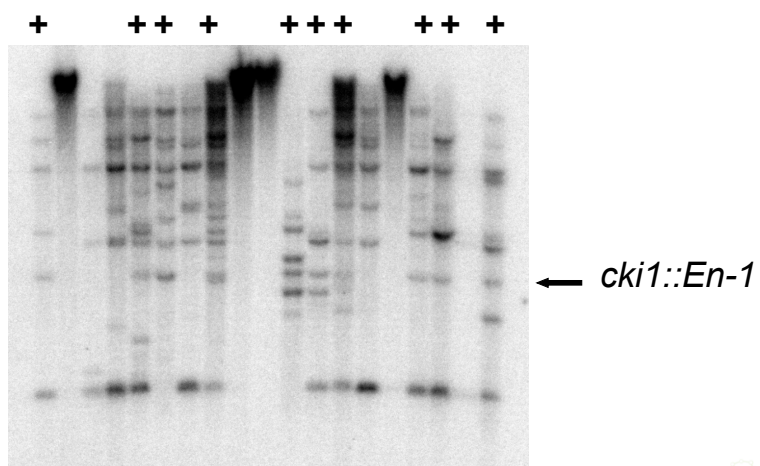
Why is it necessary to analyze the causality between the insertion and the observed phenotype?

- Presence of **multiple insertions** in one line
- Possibility 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

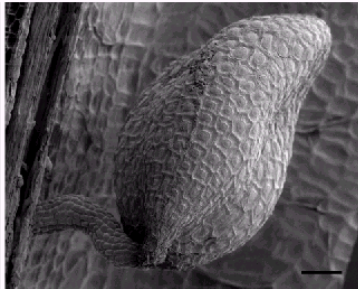
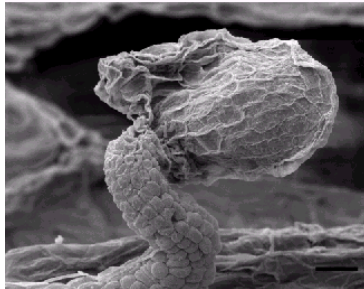
- Transposons are often characterized by excision and reinsertion into a nearby region – use for the isolation of new mutant alleles
- However, excision of transposons is not always entirely accurate – point mutations occur – isolation of revertant lines with silent mutation, or even isolation of the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

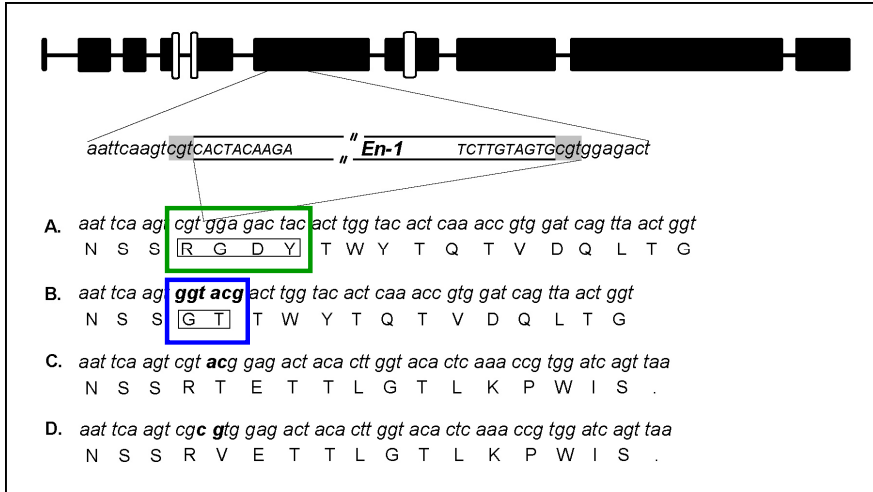
- PCR-searching in 246 plants of segregating population
- from 90 *cki1::En-1* positive plants, 9 plants had both mutant and standard silicles



Offspring analysis

- confirmation of absence 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

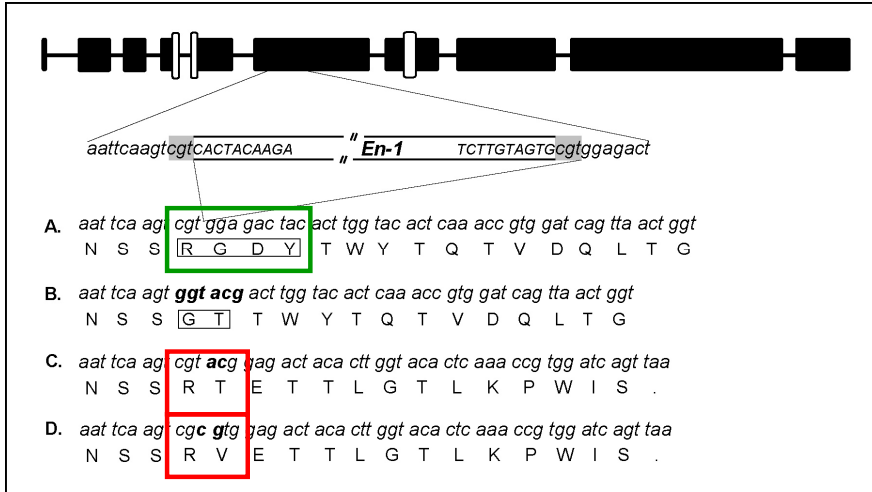


Confirmation of phenotype *cki1::En-1/CKI1*

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

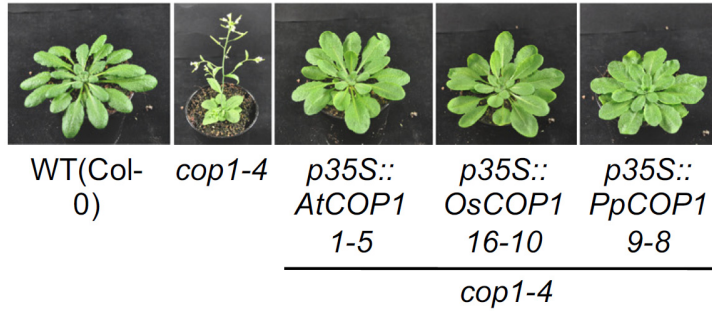
Use of **autonomous** transposons for the isolation of new stable mutations and revertant lines



Mutant Line Complementation



Mutant Line Complementation



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
 - Confirming 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