CG920 Genomics

Finishing Lesson 2

Genes Identification

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INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

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 identification of genes
  - Constructing gene-enriched libraries using methylation filtration technology
  - EST libraries
  - Forward and reverse genetics

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Forward and 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**
    - Analysis of expression of a particular gene and its spatiotemporal specifity

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Forward and reverse genetics – summary

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

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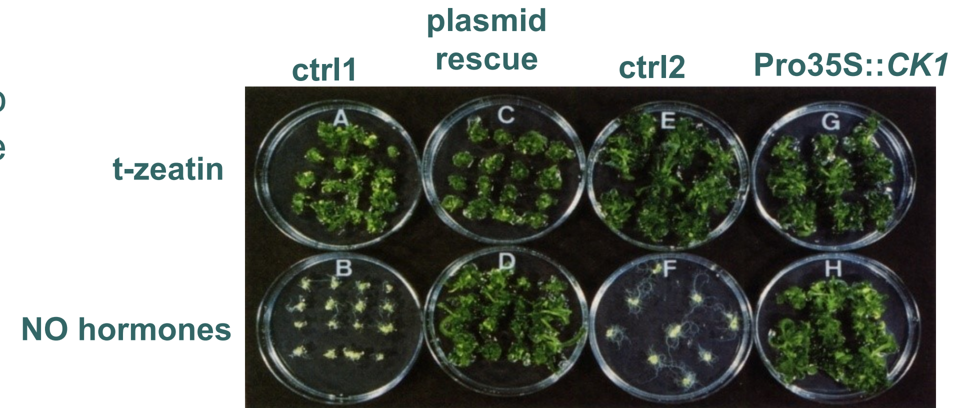
Cloning of CKI1

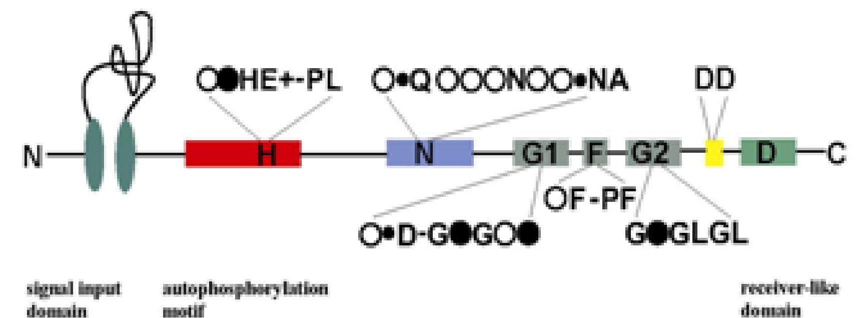
- CKI1 was identified via activation mutagenesis in *Arabidopsis*

  - Overexpresion of *CKI1* leads to CK-like response in the hypocotyl explants



Kakimoto, *Science* (1996)

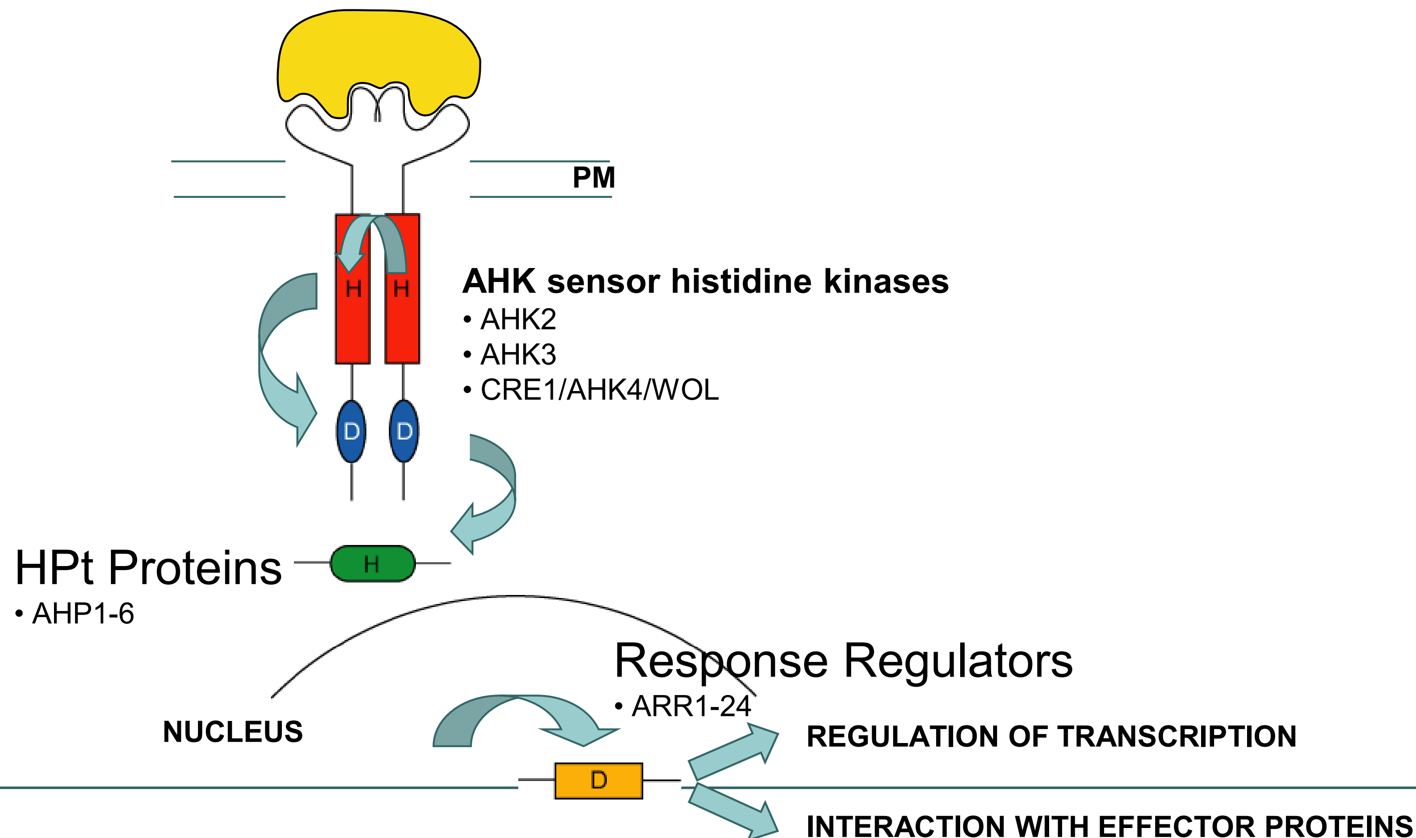
  - *CKI1* encodes a protein with similarity to bacterial histidine klinases



○ *non-polar amino acids*
● *polar amino acids (a.a.)*
\+ *basic a.a.*
\- *acidic or amidic a.a.*
• *positions with less than 50% conservation*

Signal Transduction via MSP

Recent Model of the CK Signaling via Multistep Phosphorelay (MSP) Pathway



Forward and reverse genetics

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  - Identification of insertional mutant and analysis of its phenotype
    - **Reverse genetics**

Identification of insertional *cki1* mutant allele

*aattcaagtcgt*CACTACAAGA // **En-1** // TCTTGTAGTG*cgtggagact*

A. *aat tca agt cgt gga gac tac act tgg tac act caa acc gtg gat cag tta act ggt*

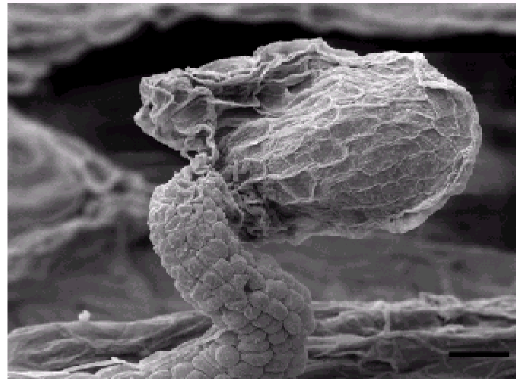
N S S R G D Y T W Y T Q T V D Q L T G

CKI1 Regulates Female Gametophyte Development

- CKI1 is necessary for proper megagametogenesis in *Arabidopsis*

CKI1/cki1-i

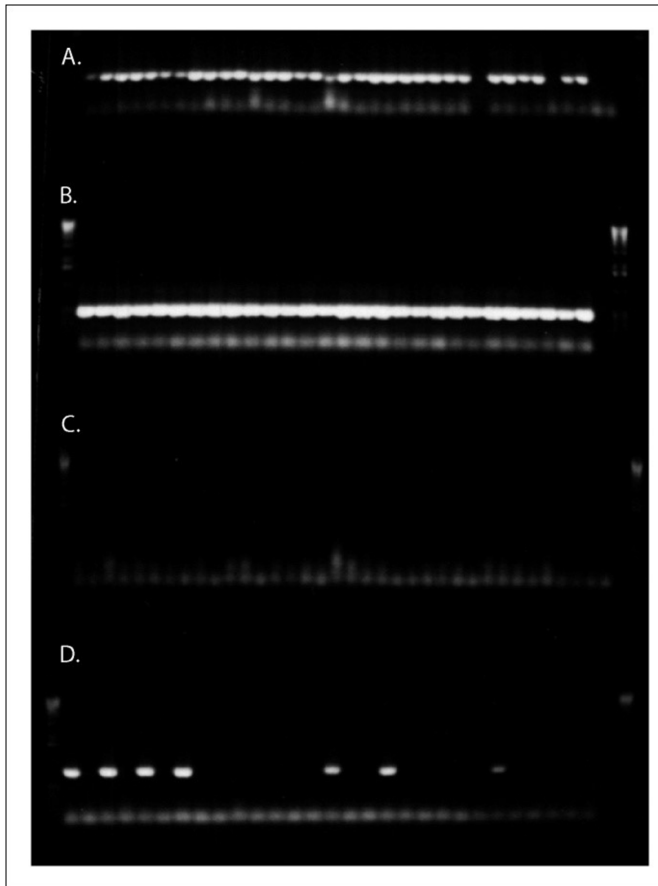
CKI1/CKI1



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

CKI1 and megagametogenesis

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



A. ♂ wt x ♀ *CKI1/cki1-i*

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

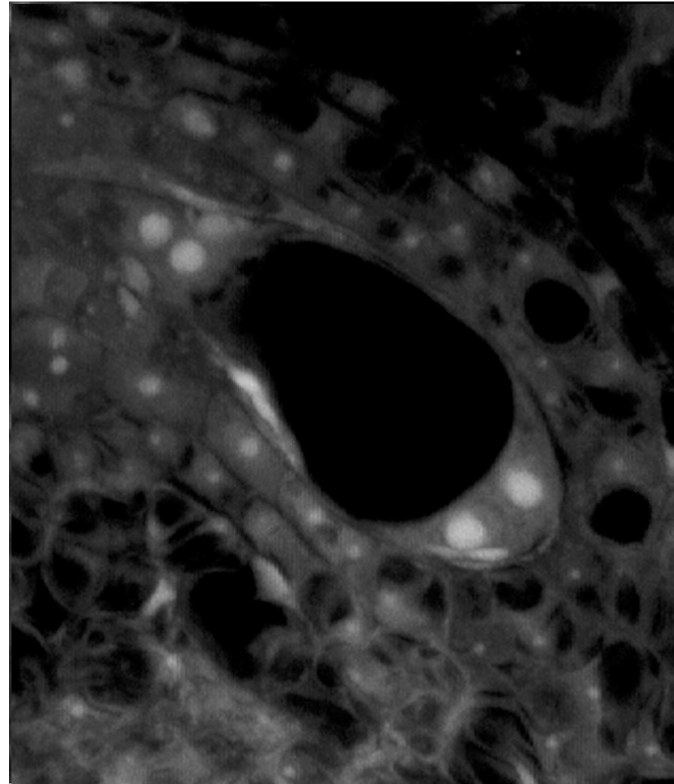
B. ♂ *CKI1/cki1-i* x ♀ wt

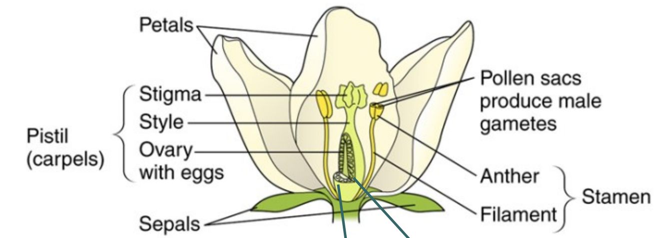
C. ♂ wt x ♀ *CKI1/cki1-i*

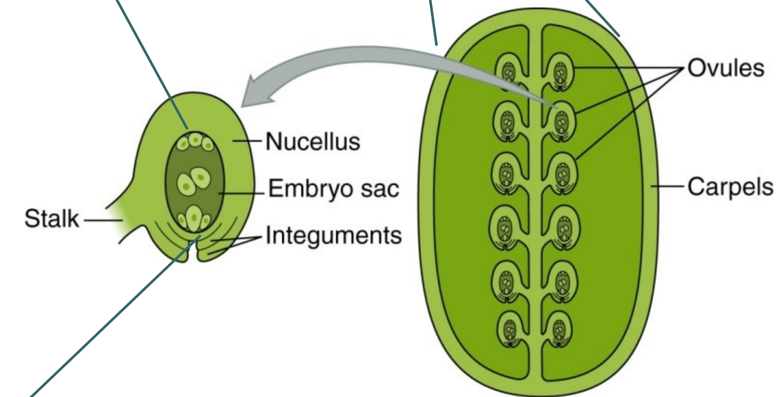
***cki1-i* specific primers**

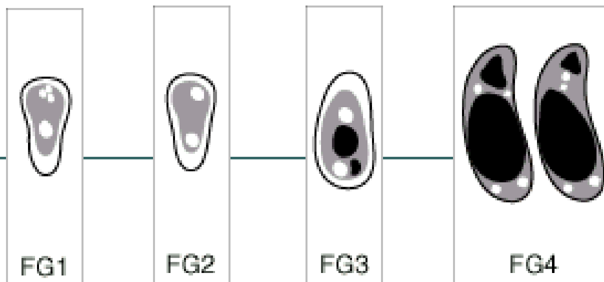
D. ♂ *CKI1/cki1-i* x ♀ wt

CKI1 and megagametogenesis





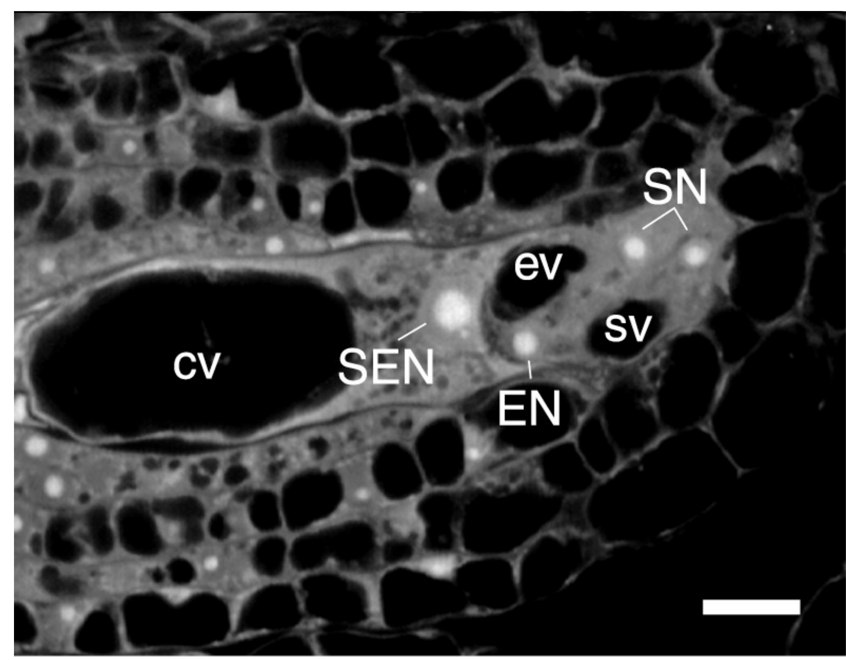




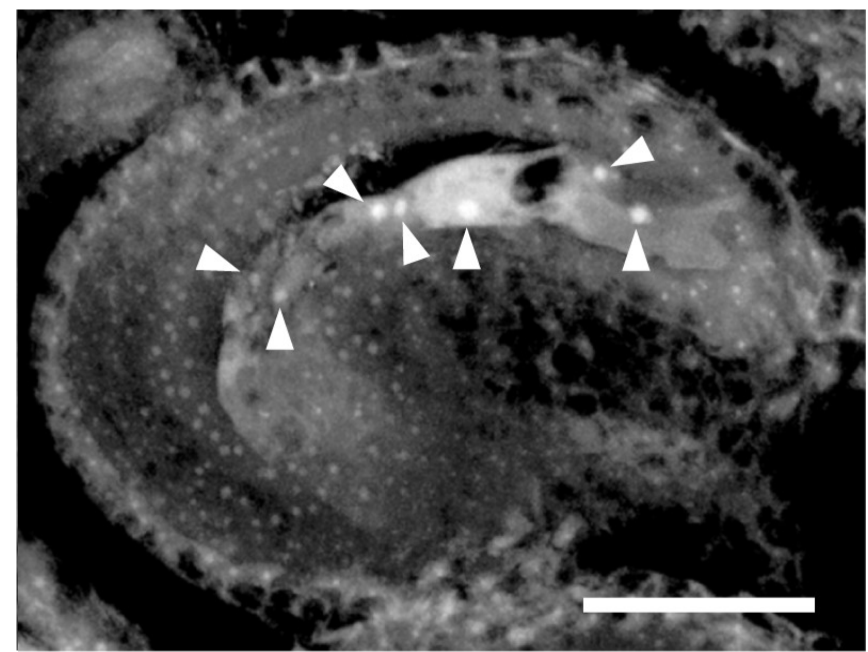
CKI1 and megagametogenesis

FG6 late FG5

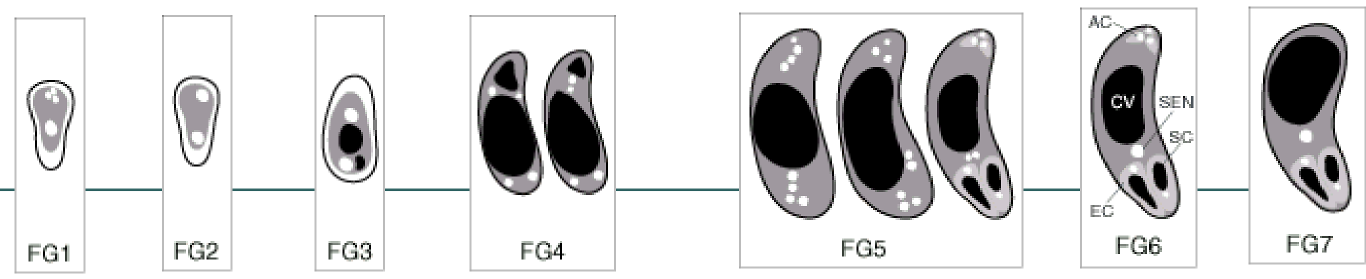
CKI1



cki1-i 24 48 HAE



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



Forward and reverse genetics

- Principles of experimental identification of genes using forward and revers genetics
  - Alteration of phenotype after mutagenesis
    - **Forward genetics**
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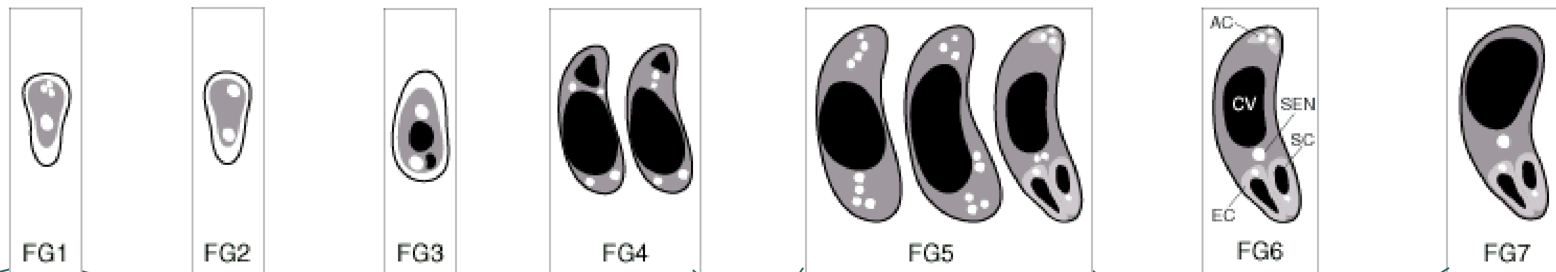
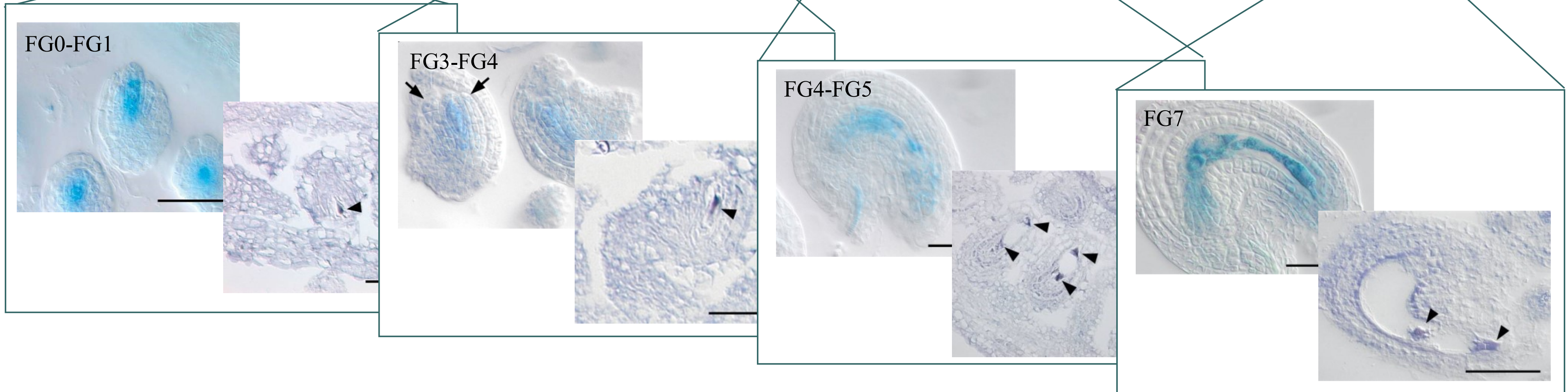
CKI1 is Expressed During Megagametogenesis

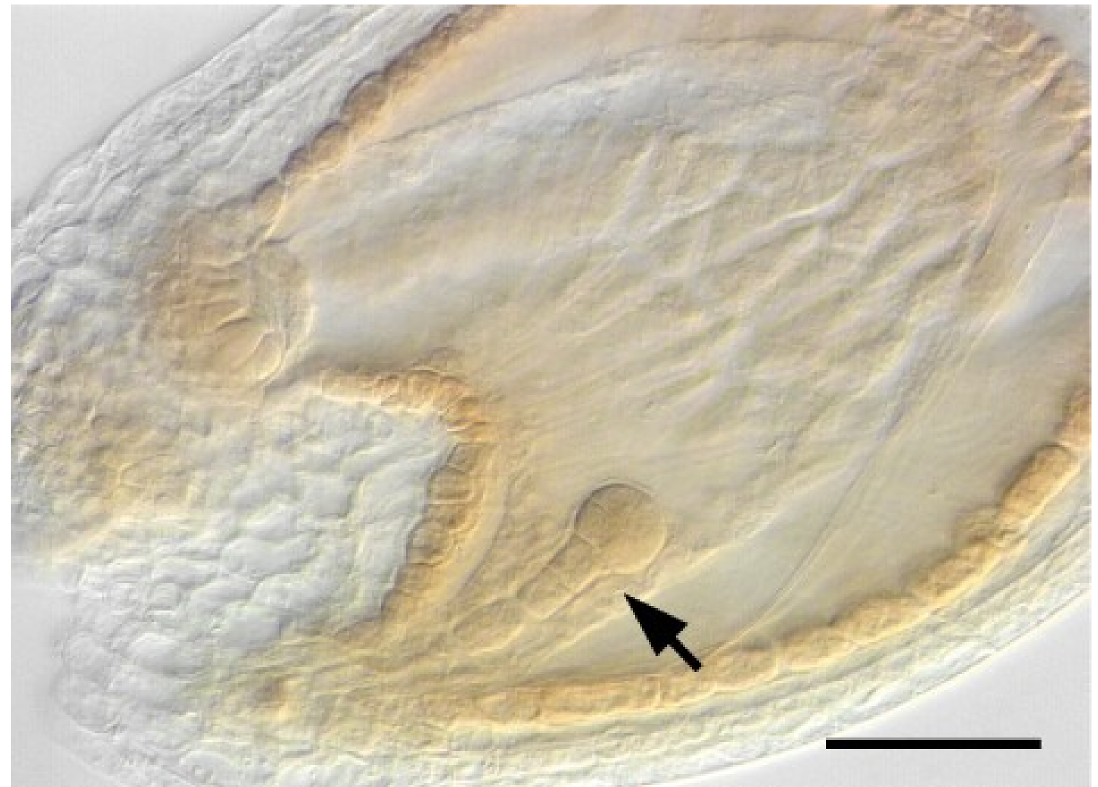




Paternal *CKI1* is Expressed in the *Arabidopsis* Sporophyte Early after Fertilization

♀ wt x ♂ Pro*CKI1:GUS*

72 HAP
(hours after pollination)



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

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.

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

RANDOM MUTAGENESIS

h x n

„Classical genetics" approach

EMS →

1. IDENTIFICATION OF PHENOTYPE

2. GENE MAPPING

3. GENE IDENTIFICATION
   - position cloning

„Reverse genetics" approach

← T-DNA

← (retro)transposons

1. ISOLATION OF SEQUENCE-
-SPECIFIC MUTANT

2. IDENTIFICATION OF PHENOTYPE

3. PROOF OF CAUSAL RELATIONSHIP BETWEEN INSERTION AND PHENOTYPE

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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
- 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
- Gene silencing using RNA interference
  - Mechanism of RNA interference

Outline

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

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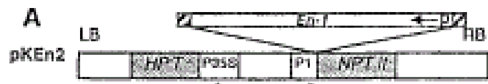
Types of insertional mutagens

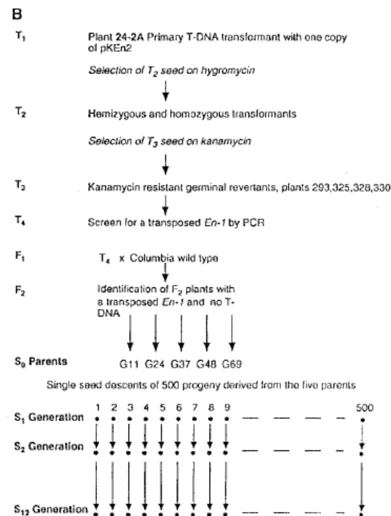
- Mobile elements
  - **Autonomous transposons (*En-1*)**
    - They contain a gene for transponase, enabling excision and reintegration into the genome
    - At both ends they contain short inverted repeat, which are recognized by transponase
- Stable elements
  - **Non-autonomous transposons (*dSpm*)**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

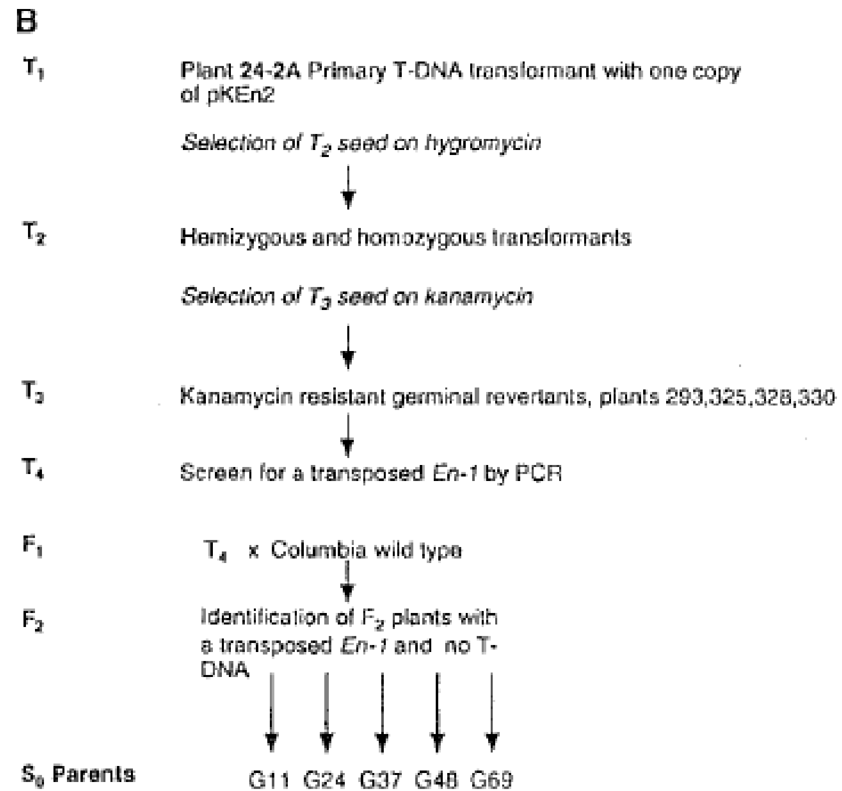


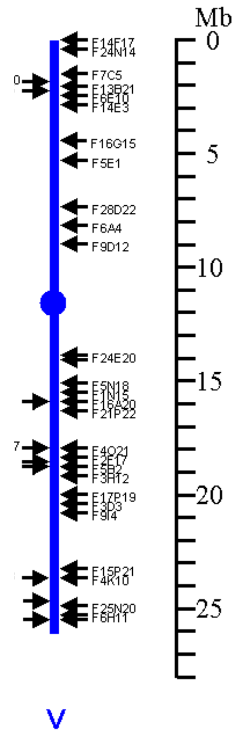
Preparation of transgenic plants

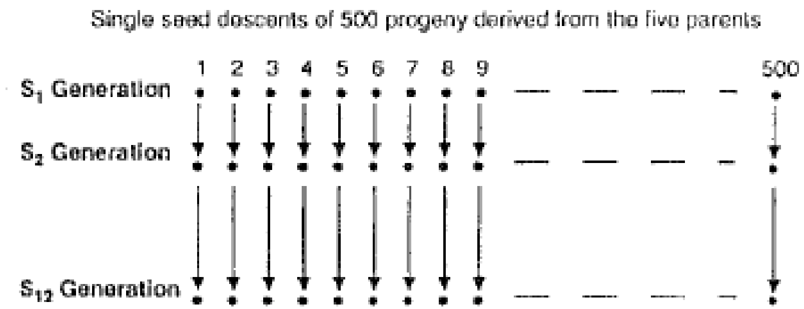
Creating the population of mutants



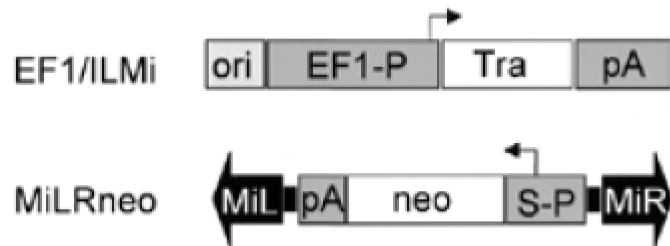
Searching for sequence-specific mutants by PCR







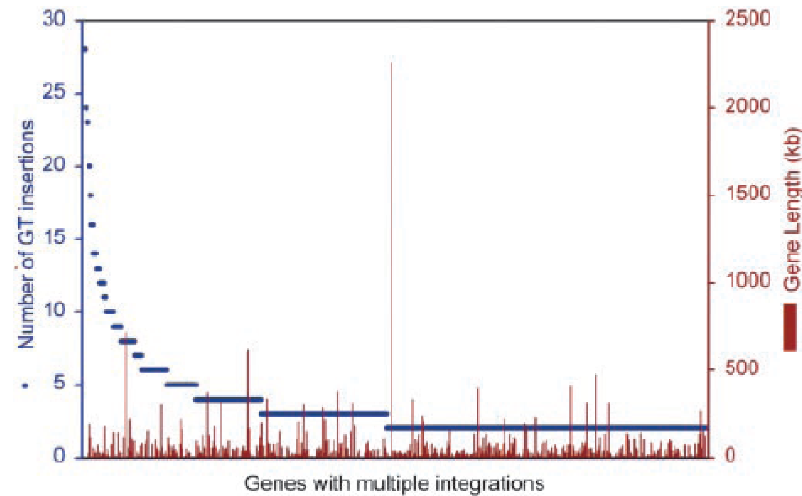
Libraries of insertional mutants (animals)

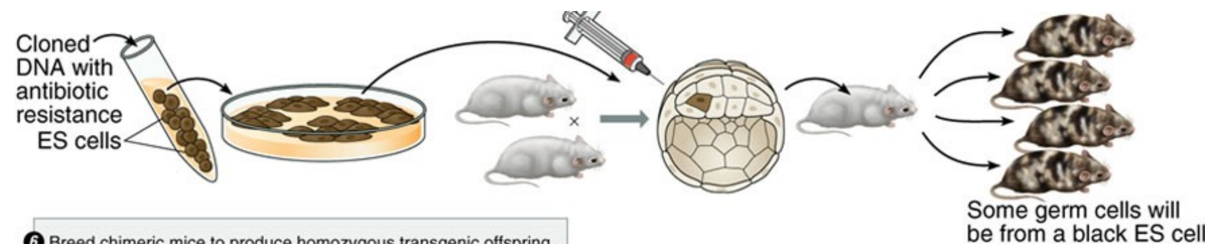


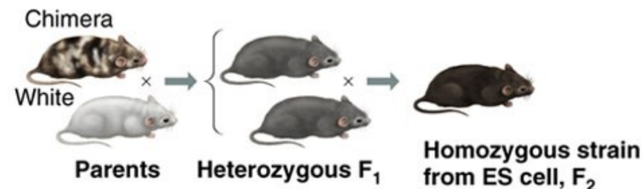
Transfection into human cell cultures (HeLa) or mouse embryonic stem (ES) cells

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







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

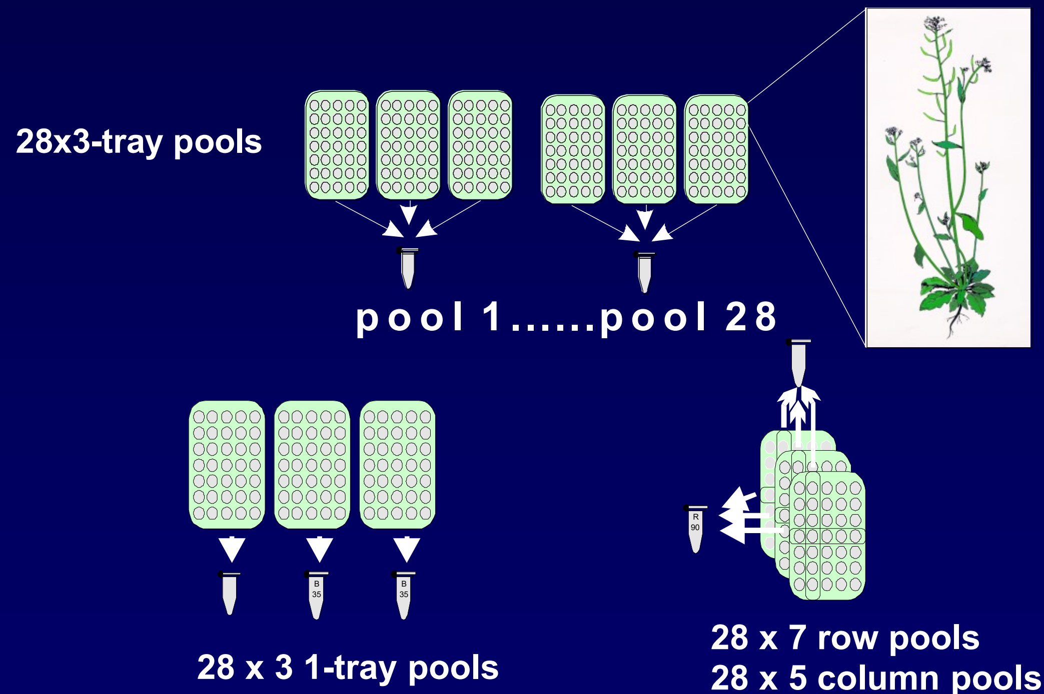
1. Library of *En-1* insertional mutants

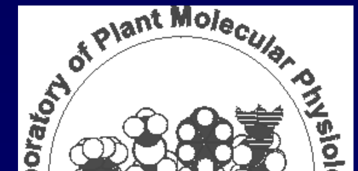
- autonomous En/Spm, without selection
- 3000 independent lines
- 5 copies per line on average
- PCR-based three-dimensional screening

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)

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





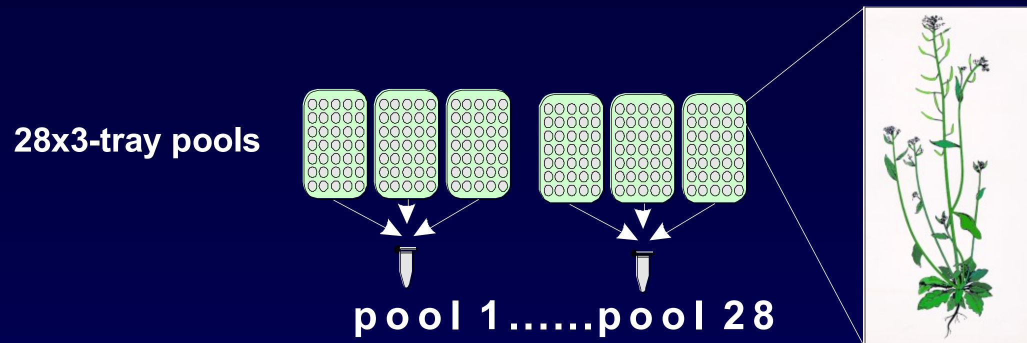
Isolation of sequence-specific mutants

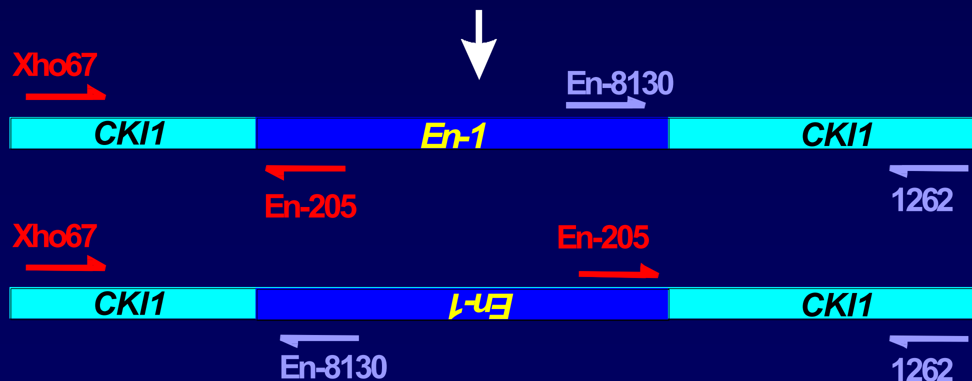
- PCR-based three-dimensional screening
  - Isolation of genomic DNA from the individual plants of mutant population and creating sets of DNA („triads“, rows and columns of triads and individual trays)
  - Identification of positive „triad“ with PCR, blotting of PCR products and hybridization of the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

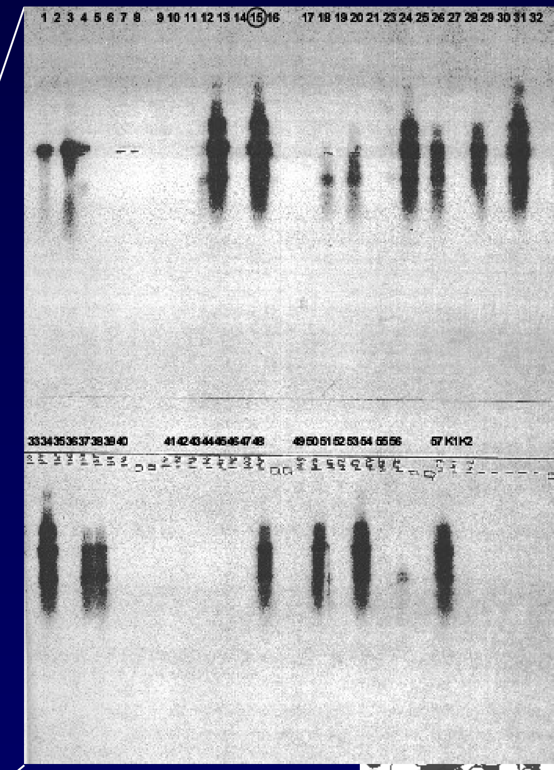
Isolation of sequence-specific mutants

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

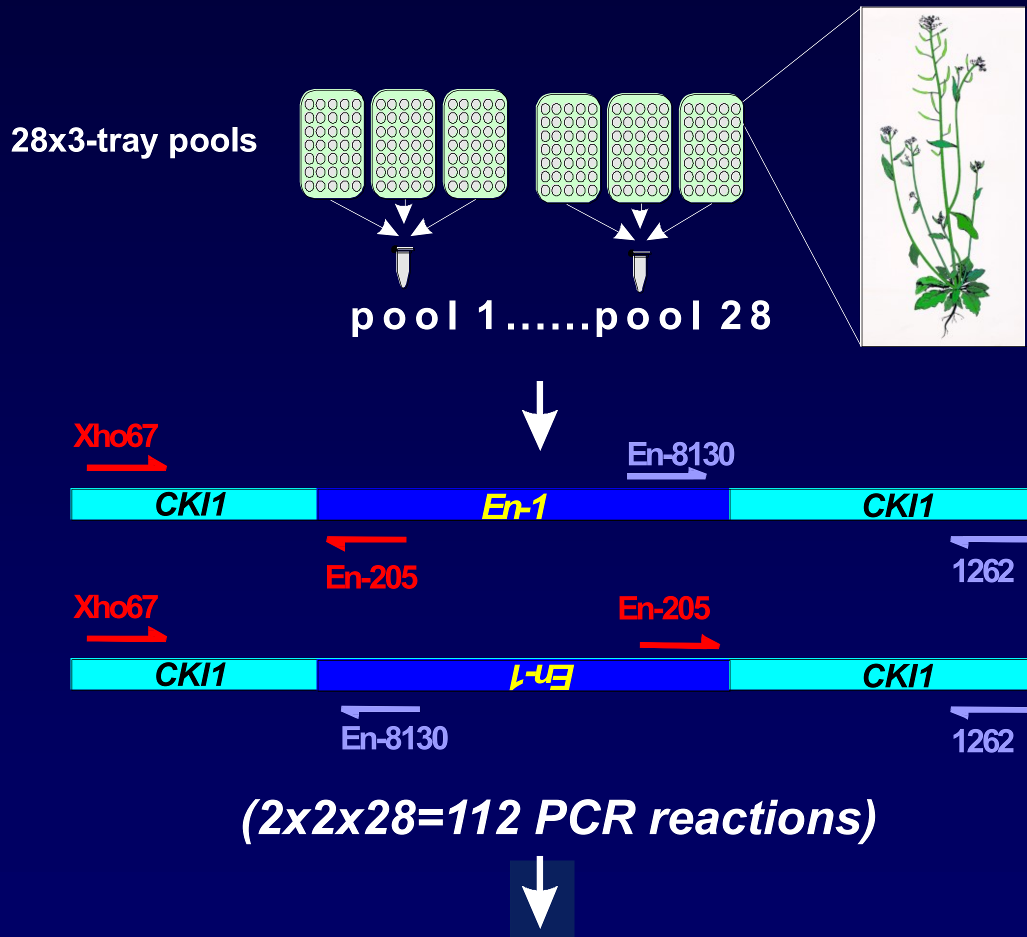
Isolation of sequence-specific mutants

- 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

Isolation of sequence-specific mutants

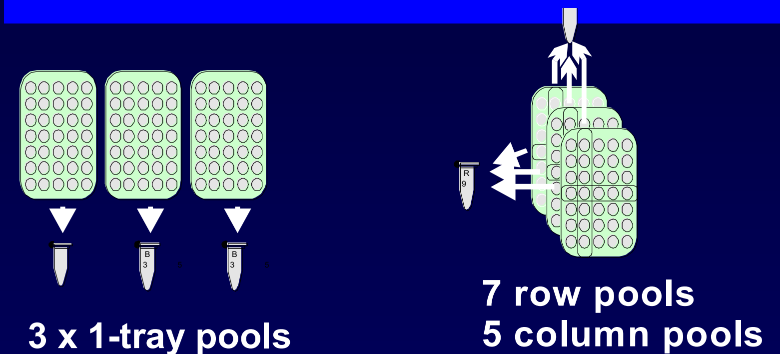
1. 3-tray pools screen

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



(2x2x28=112 PCR reactions)

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

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

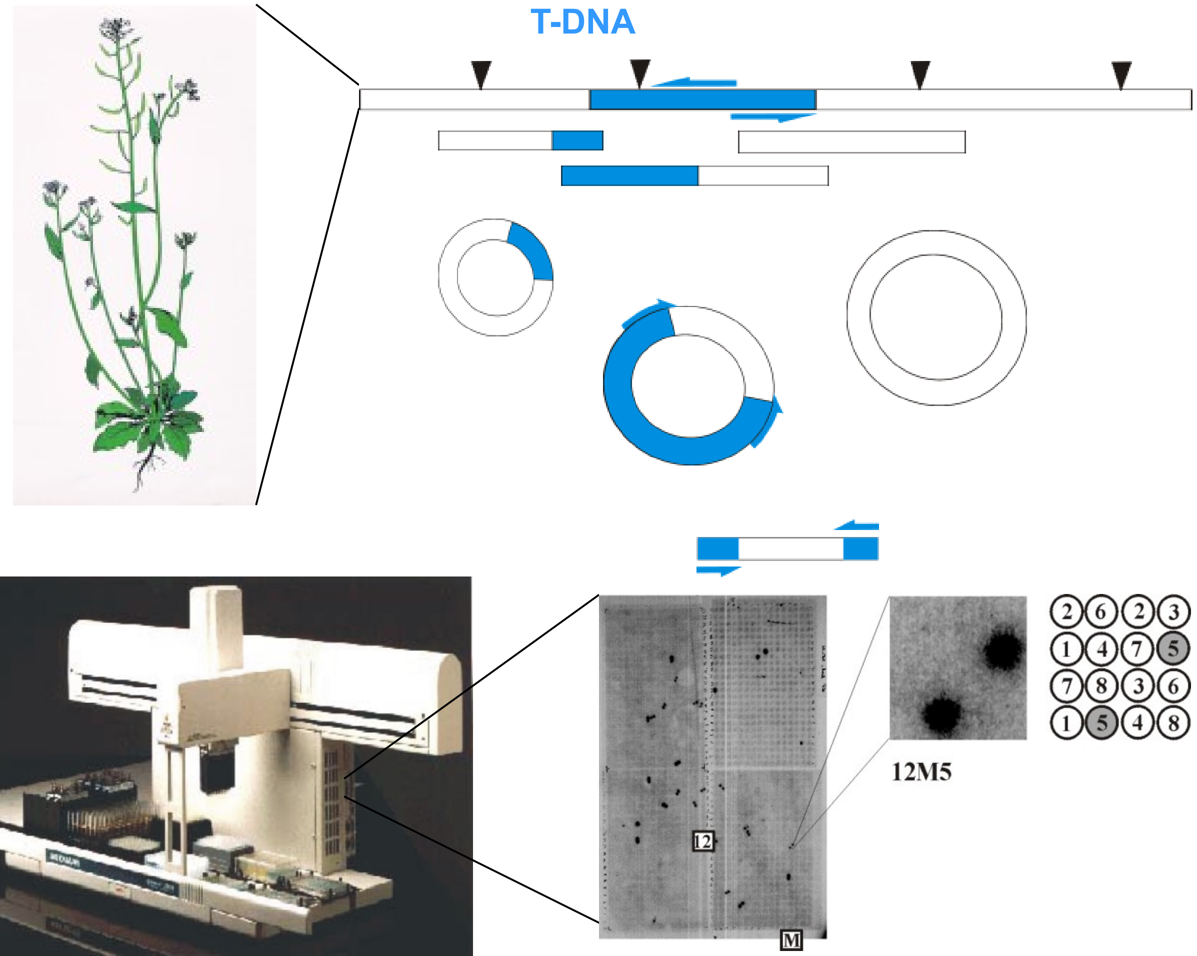
- The Sainsbury Laboratory (SLAT-lines),
  John Innes Centre, Norwich Research Park
- DNA and seeds in Nottingham Seed Stock Centre
- 48.000 lines
- 1.2 insertion per line on average
- non-autonomous transposon
- PCR searching or hybridization with iPCR filters
- SINS (sequenced insertion sites) database

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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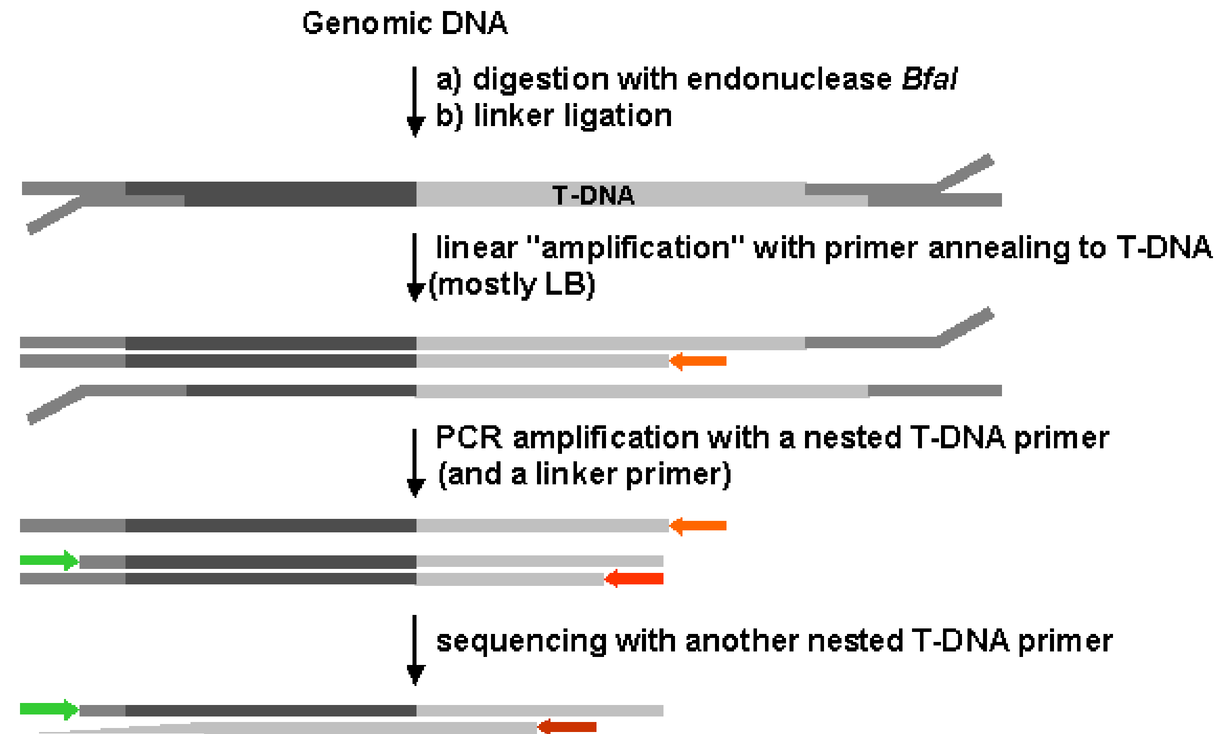
  - Searching for sequence-specific mutants in electronic databases

Isolation of sequence-specific mutants

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



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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MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
OP Vzdělávání pro konkurenceschopnost
SARYKIANA BRUNEN
NÍ
ána
Evropským sociálním fondem
a státním rozpočtem České republiky

Searching in electronic libraries of insertional mutants

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>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

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            |||||||||||||||||| | |||||||||||||||||||||||||||||||||||||||
Sbjct: 459  attagagtttgattgaagcgcgttttatatattgatagtgggacattacttataaaaagc 400

Query: 1510 acaaggatacaacaatagagacagtcacatgtatatcacataagtggatggtcctcaatg 1569
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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
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 Strand = Plus / Plus

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            ||||||||||||||| ||| ||| ||||||||||||||||||||| |  | ||||||||
Sbjct: 13   tacatttctcgctacgattgacggtatcaatatatttataaaaccgtccgacatttcac 72

Query: 1983 ttccttaactaatcacataaatga 2006
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Sbjct: 73   ttccttaactaatcacataaatga 96
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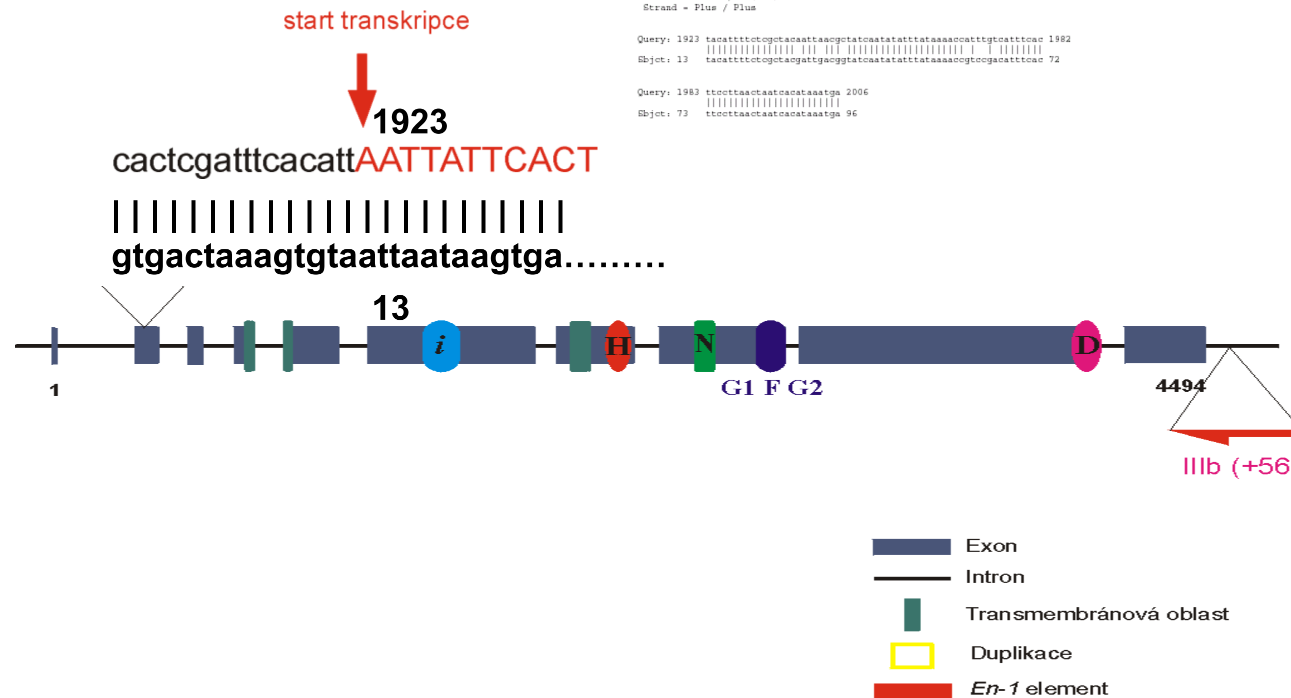

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INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

Outline

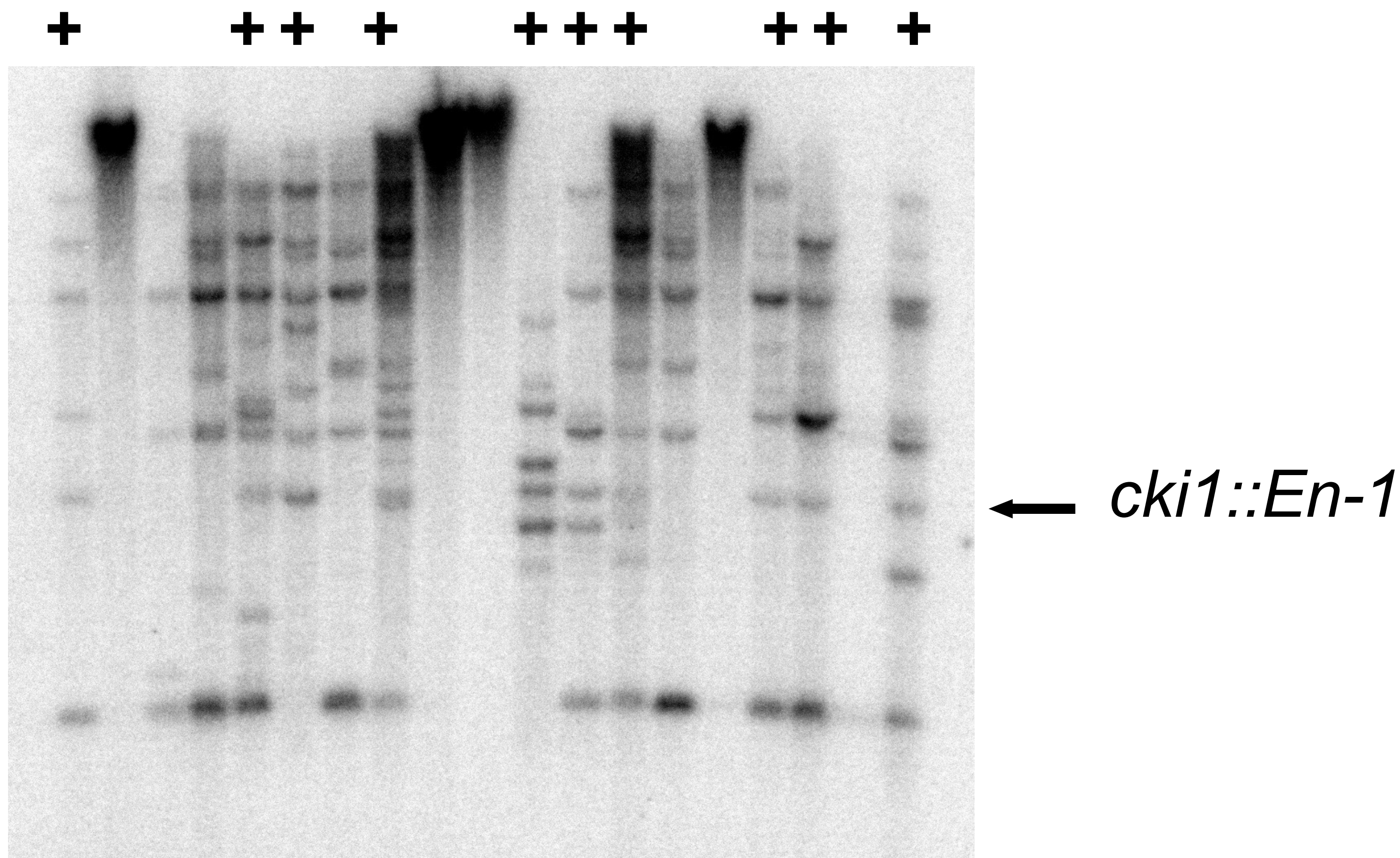
- Methods of identification of sequence-specific mutants
  - Preparation of mutants collection
  - Searching for sequence-specific mutants using PCR
  - Searching for sequence-specific mutants in electronic databases
- Analysis of phenotype and confirmation of causality between phenotype and insertional mutation
  - Co-segregation analysis
  - Identification of independent insertional allele
  - Using unstable insertional mutagens and isolation of revertant lines

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

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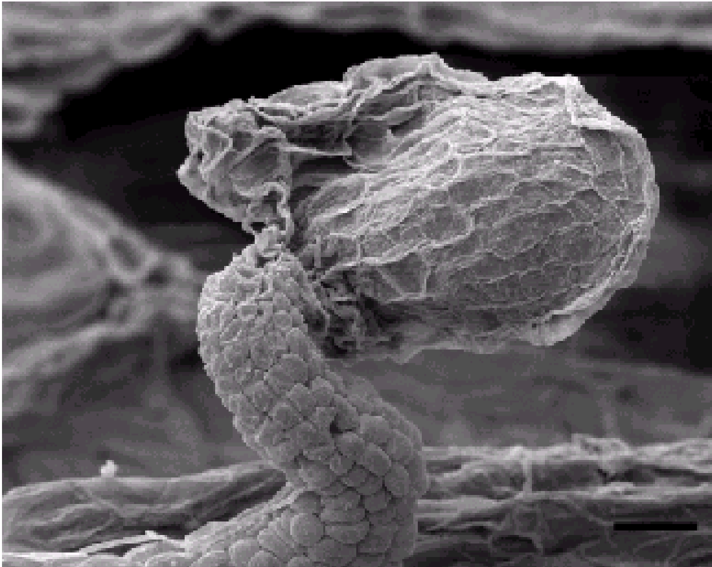
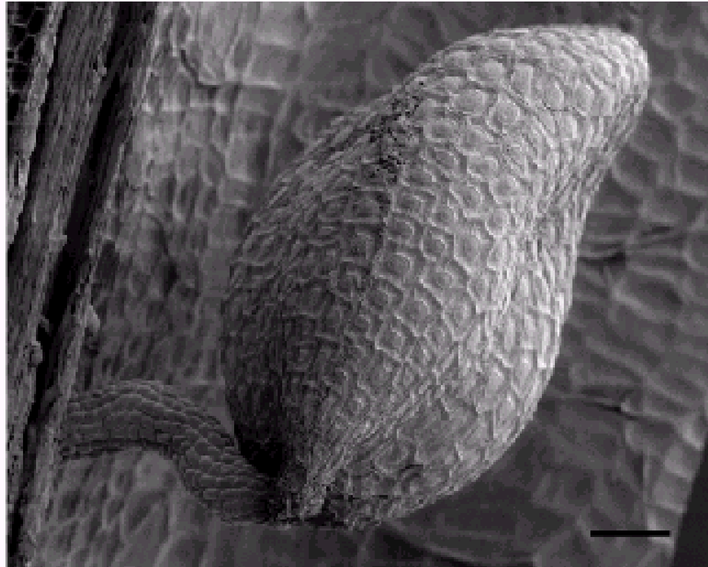
Phenotype of silicles *cki1::En-1/CKI1*

cki1::En-1/CKI1

CKI1/CKI1

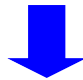




Confirmation of phenotype *cki1::En-1/CKI1*

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

aattcaagtcgtCACTACAAGA // ***En-1*** *TCTTGTAGTGcgtggagact*

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

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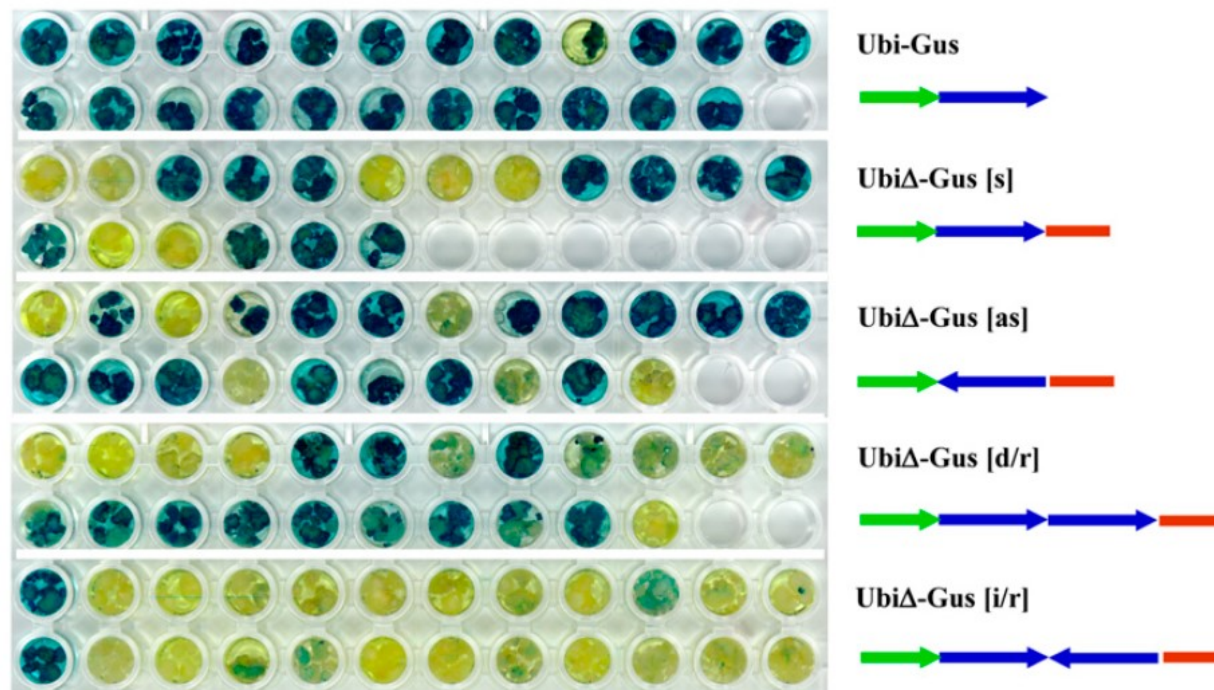
- Gene silencing using RNA interference
  - Mechanism of RNA interference

RNA interference

- **Molecular basis of posttranscriptional gene silencing (PTGS)**
  - RNAi found in plants and in *Coenorhabditis elegans*
    - Silencing was induced by both sense and antisense RNA (probably contamination by both during *in vitro* transcription)
    - dsRNA induced silencing about 10-100 times more effectively

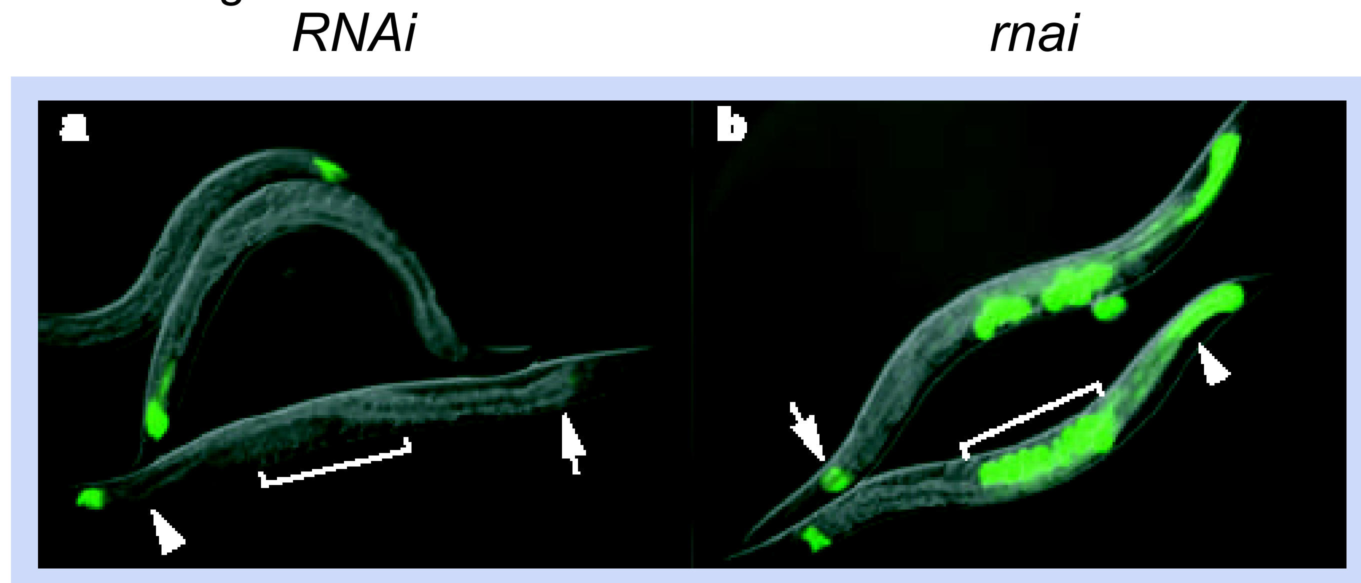


Waterhaus et al., *PNAS* (1998)

RNA interference

- **Molecular basis of posttranscriptional gene silencing (PTGS)**
  - dsRNA induction is dependent on its own genes – gene searching

RNAi *rnai*

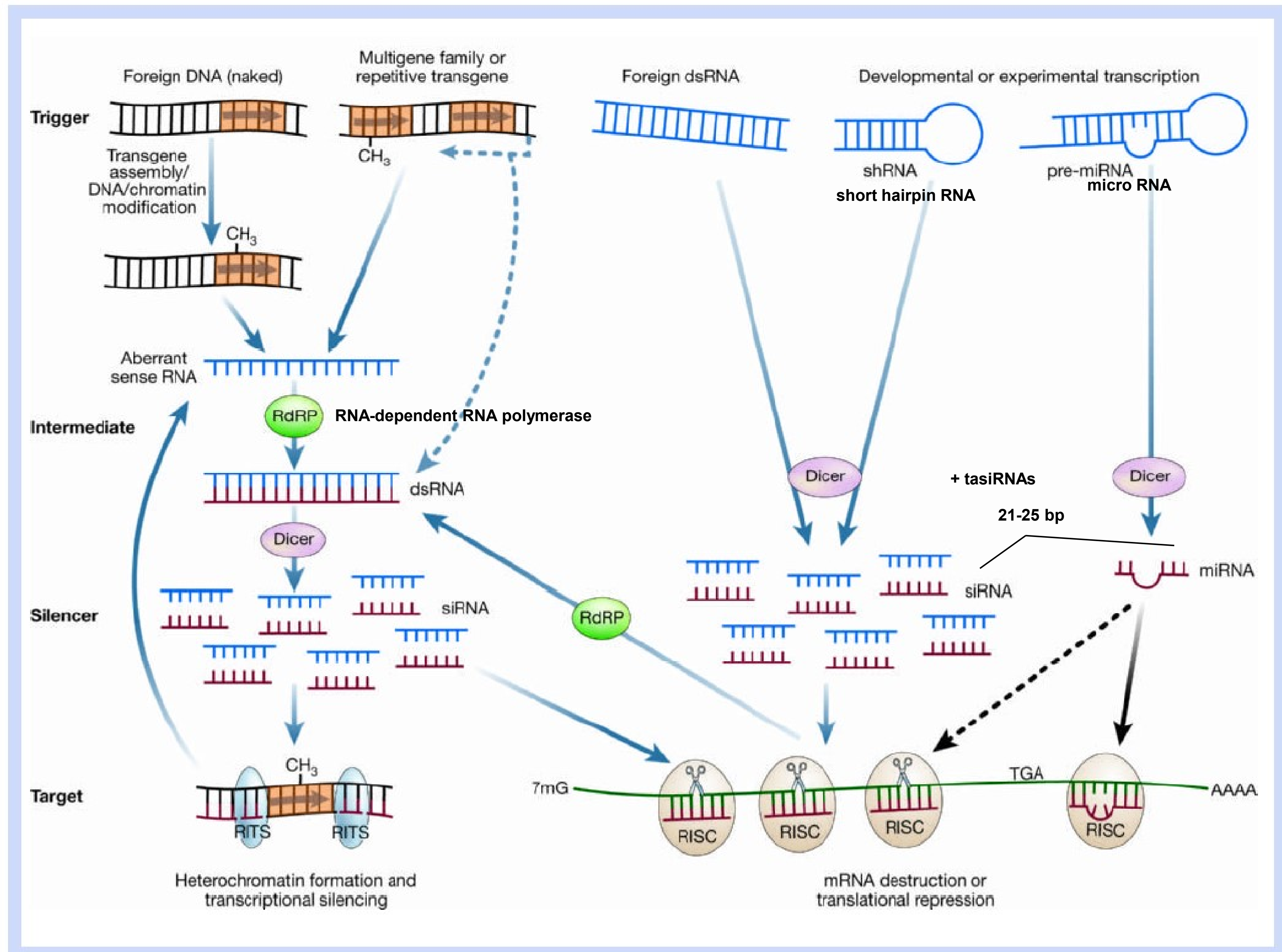


Mello and Conte, *Nature* (2004)

RNA interference

- **Molecular basis of posttranscriptional gene silencing (PTGS)**
  - RNAi found in *Coenorhabditis elegans* and in plants
  - It is a **natural mechanism** of regulation of gene expression in all eukaryotes
  - The principle is **creating dsRNA**, which can be triggered in several ways:
    - By presence of **foreign „aberrant“ DNA**
    - **Specific transgenes** containing **inverted repeats** of the cDNA parts
    - Transcription of own genes for **shRNA** (short hairpin RNA) or **miRNA** (micro RNA, endogenous hairpin RNA)
  - dsRNA is processed by enzyme complex (DICER), which leads to the formation of **siRNA** (short interference RNA), which is then bound to enzyme complex **RITS** (RNA-induced transcriptional silencing complex) or **RISC** (RNA-induced silencing komplex)
  - **RISC** mediates either **degradation of mRNA** (in case of full similarity of siRNA and the target mRNA) or leads only to **termination of translation** (in case of incomplete homology, e.g. as in the case of miRNA)
  - **RITS** mediates **reorganization of genomic DNA** (heterochromatin formation and inhibition of transcription)

Mechanism of RNA interference



Mello and Conte, *Nature* (2004)

It has been found that dsRNA might be either an intermediate or a trigger in PTGS.

In the first case, dsRNA is formed by the action of RNA-dependent RNA polymerases (RdRPs), which use specific transcripts as a template. It is still not clear, how these transcripts are recognized, but it might be e.g. abundant RNA that is a result of viral amplification or transcription of foreign DNA.

It is not clear, how the foreign DNA might be recognized, possibly, lack of bound proteins on the foreign "naked" DNA and its subsequent "signature" (e.g. by specific methylation pattern) during packing of the foreign DNA into the chromatin structure might be involved.

The highly abundant transcripts might be recruited to the RdRPs by the defects in the RNA processing, e.g. lack of polyadenylation.

In the case when dsRNA is a direct trigger, there are two major RNA molecules involved in the process: Short interference RNA (siRNA) and micro RNA (miRNA), both encoded by the endogenous DNA.

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These two functionally similar molecules differ in their origin:

siRNAs are dominantly product of the cleavage of the long dsRNA that are produced by the action of cellular or viral RdRPs. However, there are also endogenous genes, e.g. short hairpin RNAs (shRNAs) allowing production of the siRNA (see the figure).

miRNAs are involved in the developmental-specific regulations and are product of transcription of endogenous genes encoding for small dsRNAs with specific structure (see the figure).

In addition to siRNAs, there are trans-acting siRNAs (tasiRNAs) that are a special class of siRNAs that appear to function in development (much like miRNAs) but have a unique mode of origin involving components of both miRNA and siRNA pathways.

Developmental regulations via miRNAs are more often used in animals then in plants.

The dsRNAs of all origins and pre miRNAs are cleaved by DICER or DICER-like (DCL) enzyme complexes with RNAse activity, leading to production of siRNAs and miRNA, respectively.

These small RNAs are of 21-24 bp long and bind either to RNA-induced transcriptional silencing complex (RITS) or RNA-induced silencing complex (RISC).

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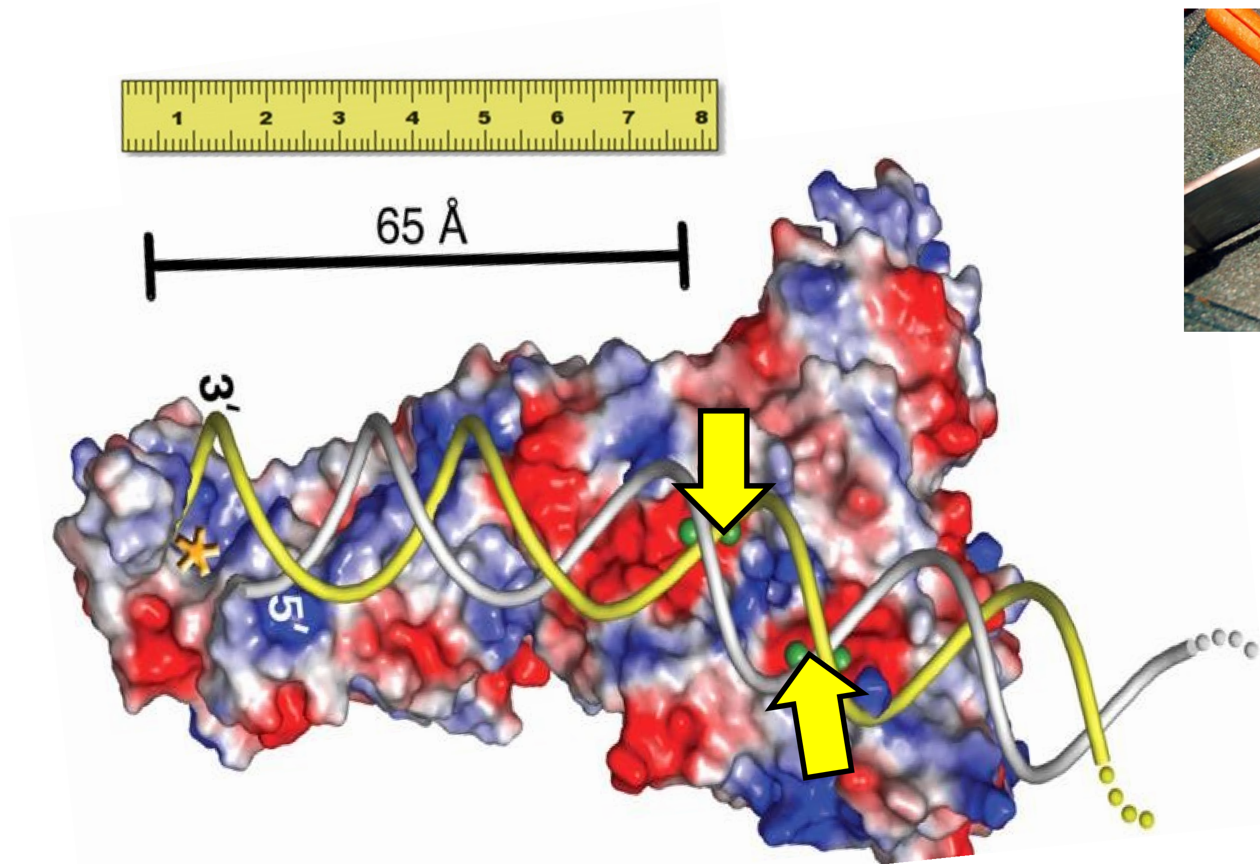
Dicer and Dicer-like proteins



From MacRae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.., Adams, P.D., and Doudna, J.A. (2006) Structural basis for double-stranded RNA processing by Dicer. Science 311: 195 -198. Reprinted with permission from AAAS. Photo credit: Heidi

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In siRNA and miRNA biogenesis, DICER or DICER-like (DCL) proteins cleave long dsRNA or foldback (hairpin) RNA into ~ 21 – 25 nt fragments.

Dicer's structure allows it to measure the RNA it is cleaving. Like a cook who "dices" a carrot, DICER chops RNA into uniformly-sized pieces.

Note the two strands of the RNA molecule. The cleavage sites are indicated by yellow arrows.

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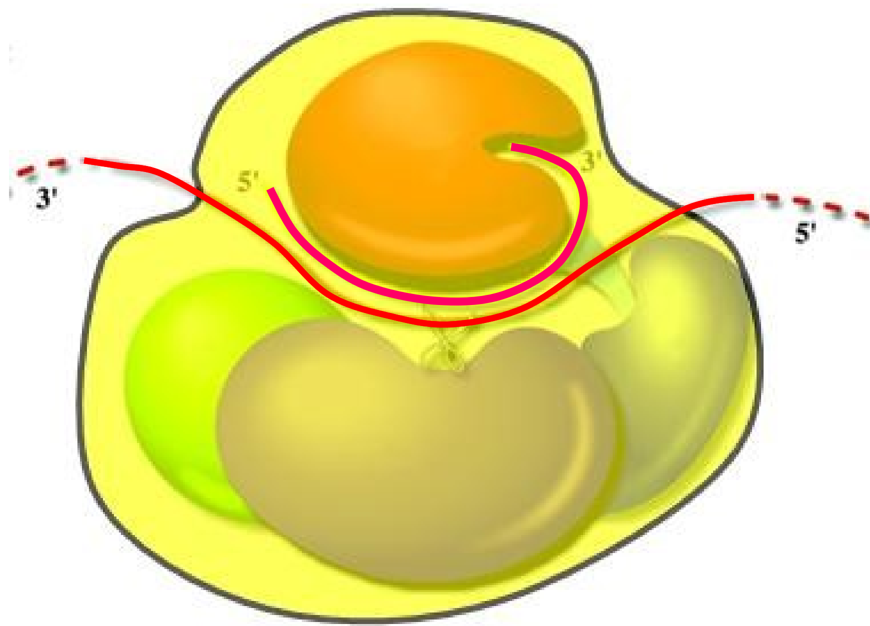
Argonaute proteins



ago1

Argonauta argo

Reprinted by permission from Macmillan Publishers Ltd: EMBO J. Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998) *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. EMBO J. 17: 170–180. Copyright 1998; Reprinted from Song, J.-J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. Science 305: 1434 – 1437. with permission of AAAS.

ARGONAUTE proteins bind small RNAs and their targets and it is an important part of both RITS and RISC complexes.

ARGONAUTE proteins are named after the *argonaute1* mutant of *Arabidopsis*; *ago1* has thin radial leaves and was named for the octopus *Argonauta* which it resembles (see the figure).

ARGONAUTE proteins were originally described as being important for plant development and for germline stem-cell division in *Drosophila melanogaster.*

ARGONAUTE proteins are classified into three paralogous groups: Argonaute-like proteins, which are similar to *Arabidopsis thaliana AGO1;* Piwi-like proteins, which are closely related to *D. melanogaster PIWI* (P-element induced wimpy testis); and the recently identified *Caenorhabditis elegans*-specific group 3 Argonautes.

Members of a new family of proteins that are involved in RNA silencing mediated by Argonaute-like and Piwi-like proteins are present in bacteria, archaea and eukaryotes, which implies that both groups of proteins have an ancient origin.

The number of Argonaute genes that are present in different species varies. There are 8 Argonaute genes in humans (4 Argonaute-like and 4 Piwi-like), 5 in the *D. melanogaster genome (2 Argonaute-like and* 3 Piwi-like), 10 Argonaute-like in *A. thaliana, only* 1 Argonaute-like in *Schizosaccharomyces pombe and at* least 26 Argonaute genes in *C. elegans (5 Argonaute-like,* 3 Piwi-like and 18 group 3 Argonautes).

http://youdpreferanargonaute.com/2009/06/

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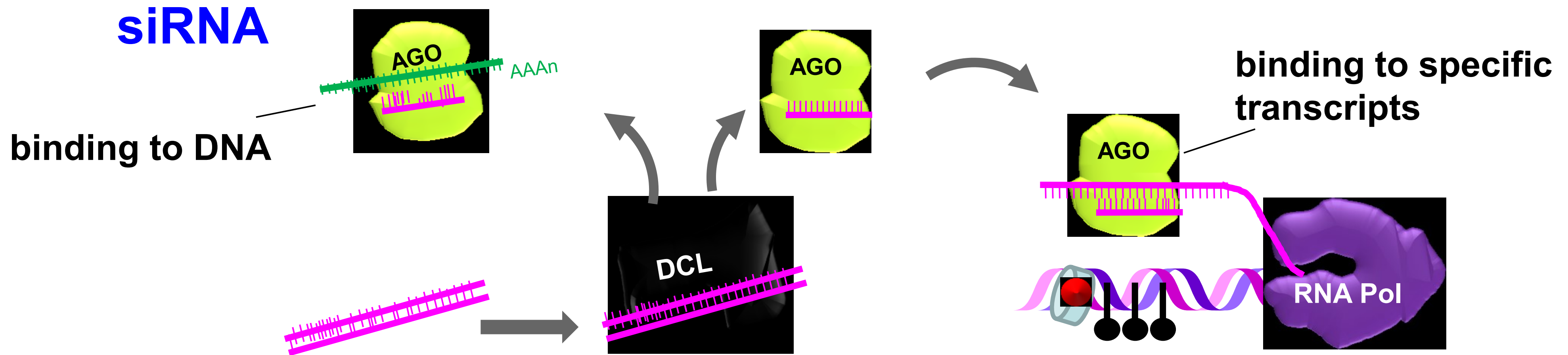
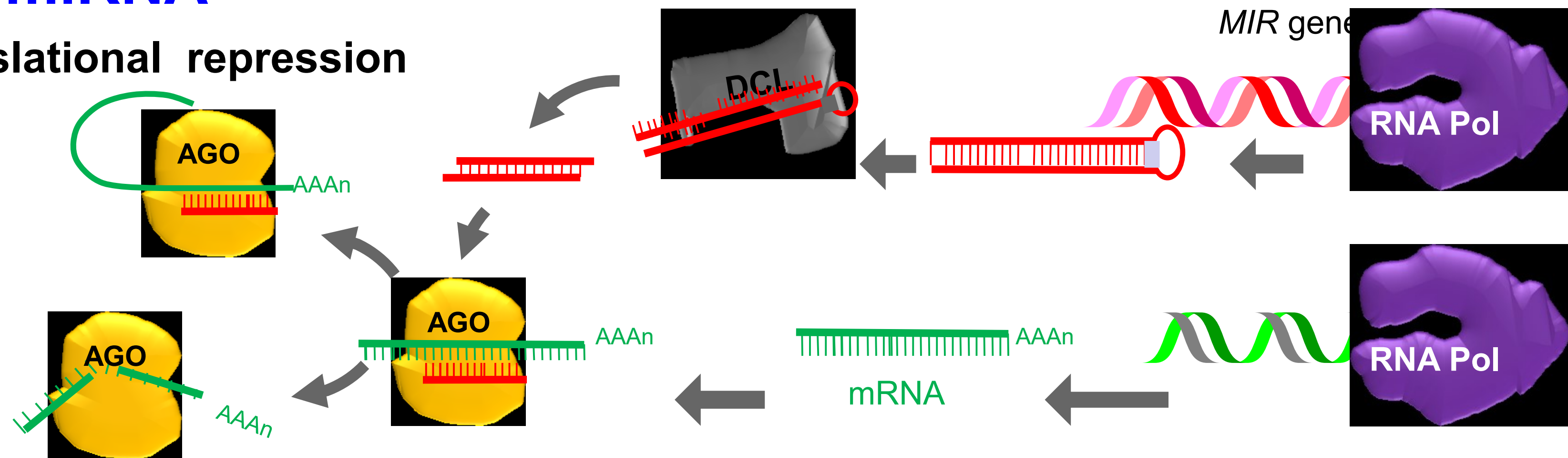
transcriptional gene silencing **post-transcriptional gene silencing**



miRNA

translational repression



transcriptional slicing

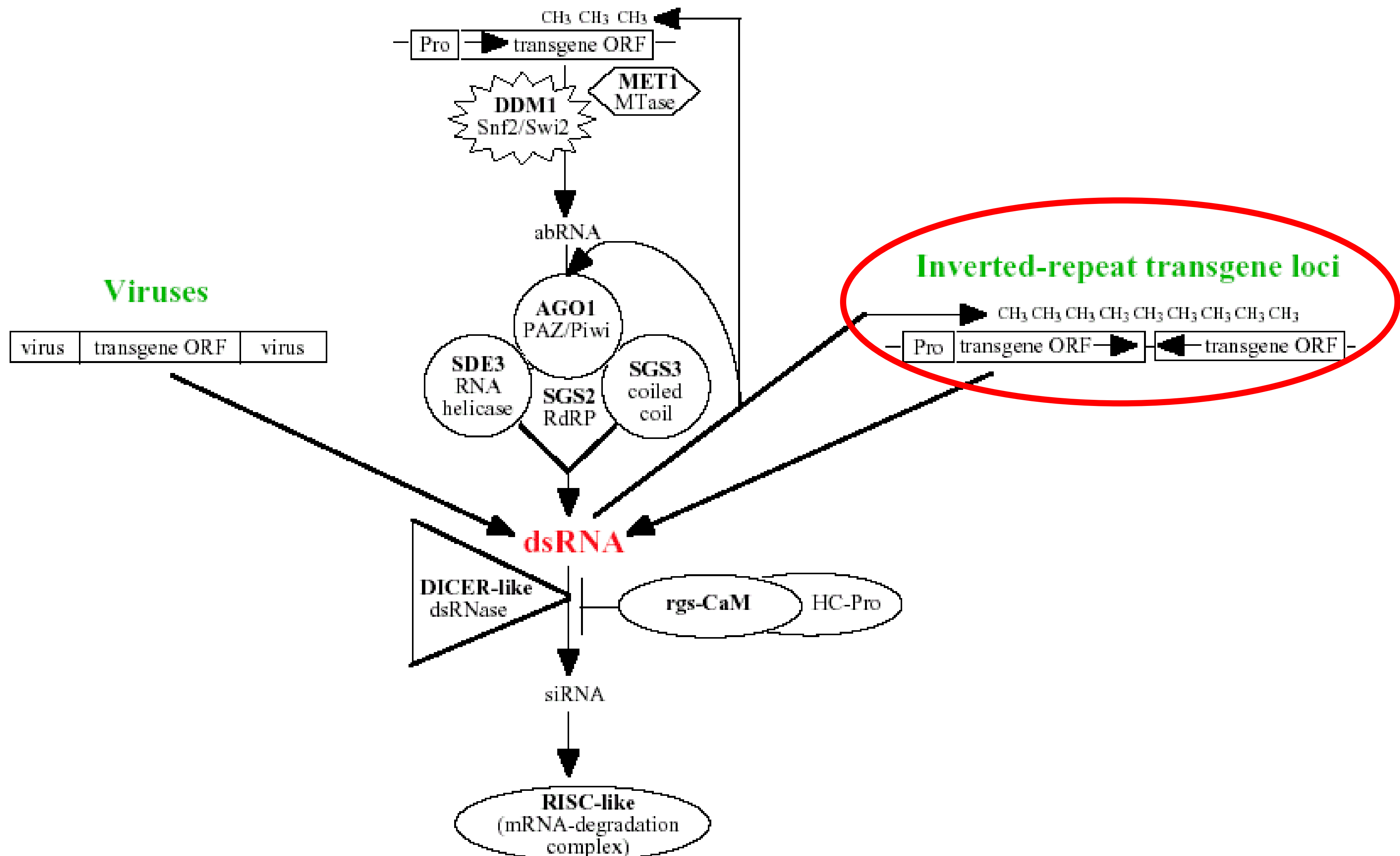
MicroRNAs are encoded by MIR genes, fold into hairpin structures that are recognized and cleaved by DCL (Dicer-like) proteins.
In summary, **siRNAs**-mediates silencing via post-transcriptional and transcriptional gene silencing, while **miRNAs** -mediate slicing of mRNA and translational repression.

The Nobel Prize in Physiology or Medicine 2006

“for their discovery of RNA interference - gene silencing by double-stranded RNA“

Andrew Z. Fire	**Craig C. Mello**
USA	USA
Stanford University School of Medicine Stanford, CA, USA	University of Massachusetts Medical School Worcester, MA, USA
b. 1959	b. 1960

Mechanism of posttranscriptional gene silencing by RNA interference (iRNA)



RNAi approach using regulated expression system