

# CG920 Genomics

## Finishing Lesson 2

### Genes Identification

Jan Hejátko

**Functional Genomics and Proteomics of Plants,**  
Mendel Centre for Plant Genomics and Proteomics,  
Central European Institute of Technology (CEITEC), Masaryk University, Brno  
[hejatko@sci.muni.cz](mailto:hejatko@sci.muni.cz), [www.ceitec.muni.cz](http://www.ceitec.muni.cz)



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 Genes Identification**
  - Constructing gene-enriched libraries using methylation filtration technology
  - EST libraries
  - Forward and reverse genetics



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Forward Genetics

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

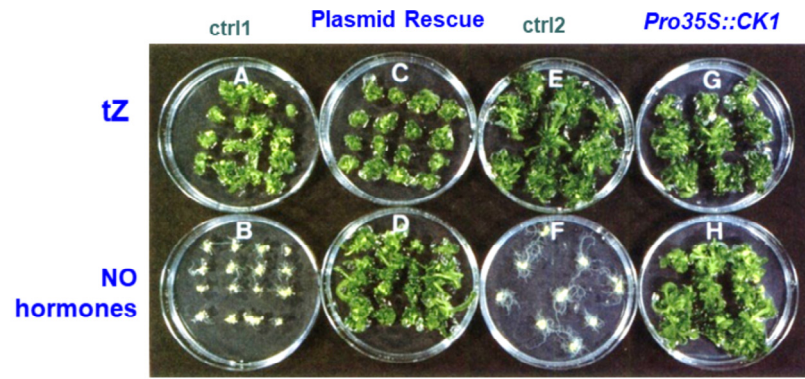


INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

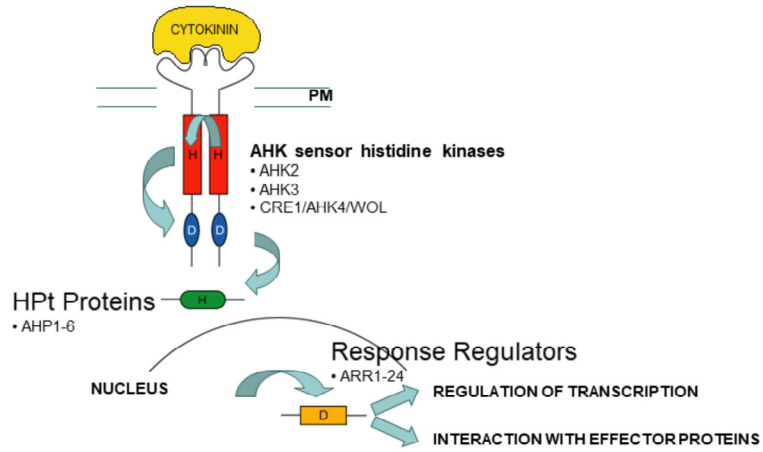
# Identification of *CKI1* via Activation Mutagenesis

- *CKI1* overexpression mimics cytokinin response



Kakimoto, *Science*, 1996

# Signal Transduction via MSP

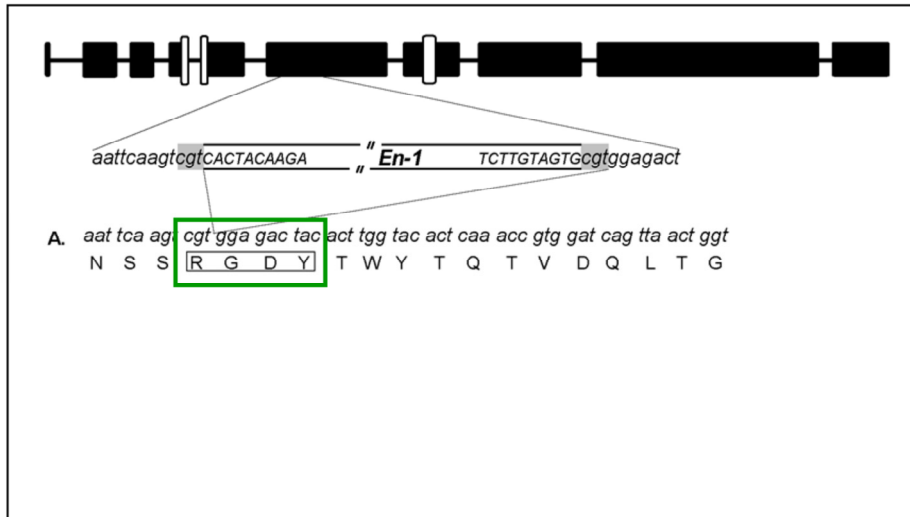




# Reverse Genetics

- 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



Hormonal regulations of plant development





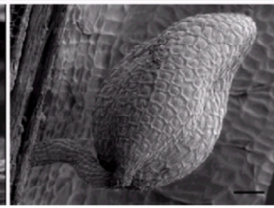
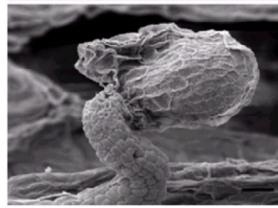
# CKI1 Regulates Female Gametophyte Development

- CKI1 is necessary for proper megagametogenesis in *Arabidopsis*

*CKI1/cki1-i*



*CKI1/CKI1*



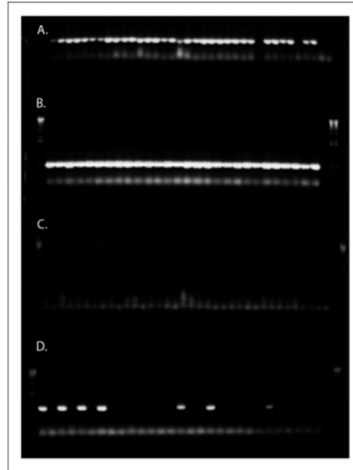
Hejätko et al., *Mol Genet Genomics* (2003)

Hormonal regulations of plant development



# 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*

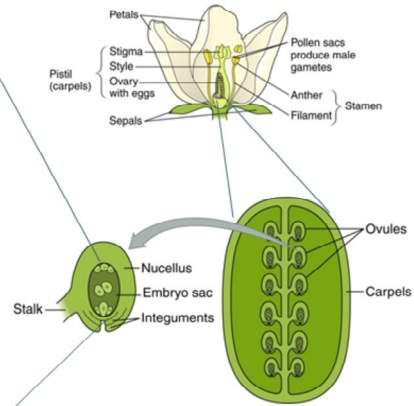
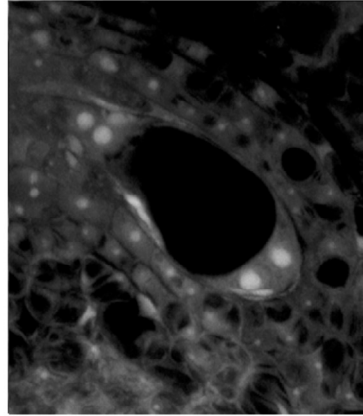


*cki1-i* specific primers

D. ♂ *CKI1/cki1-i* x ♀ wt

# CKI1 and Megagametogenesis

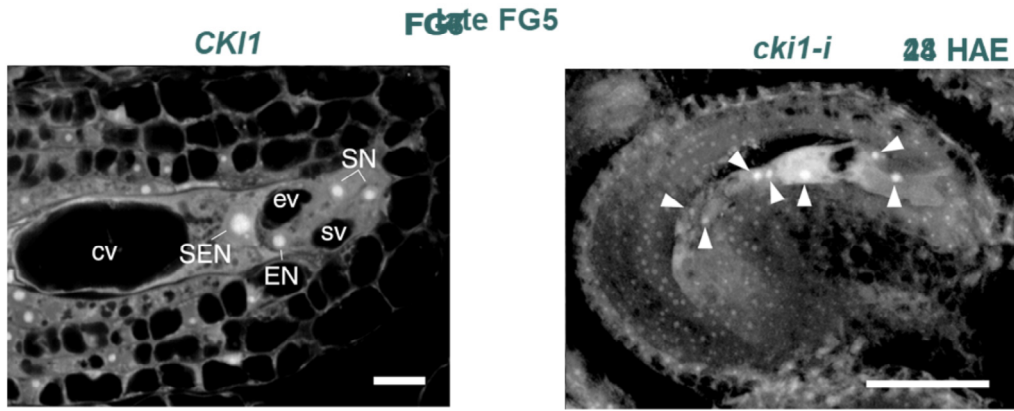
FG 4



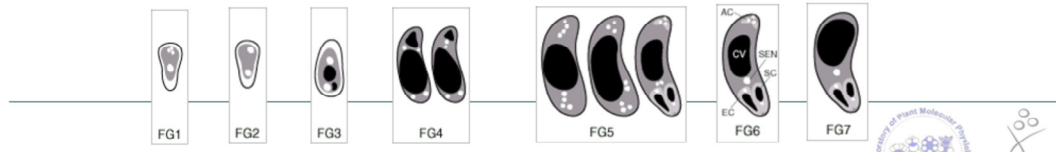
Hormonal regulation



# CKI1 and Megagametogenesis



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



Hormonal regulations of plant development

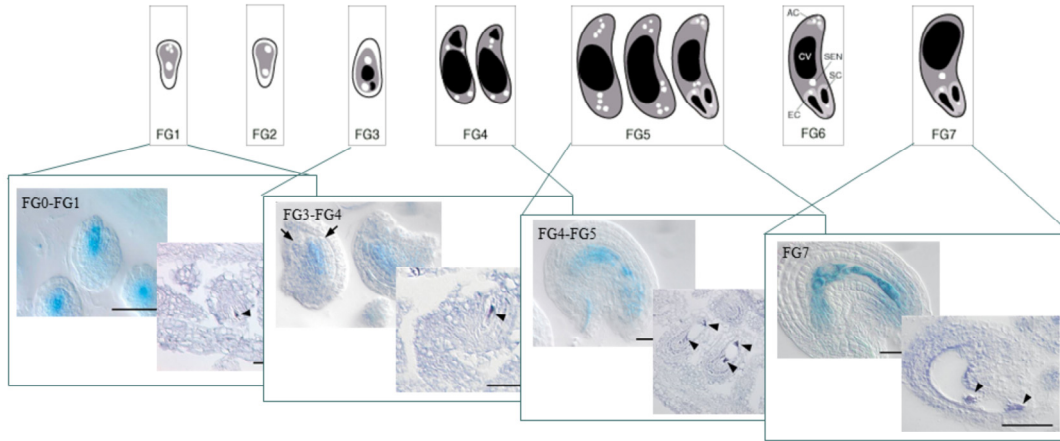




# Forward and Reverse Genetics

- 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



Hormonal regulations of plant development

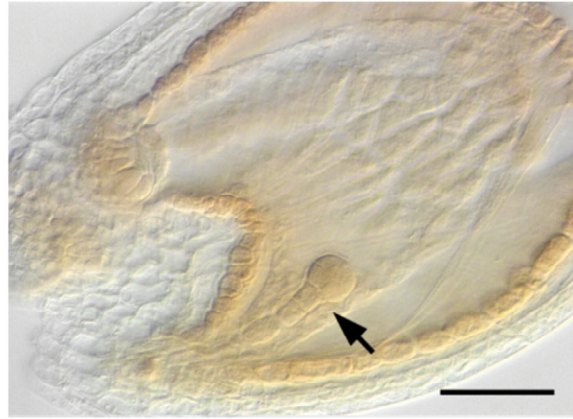


● ● ●

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

♀ wt x ♂ Pro*CKI1*:*GUS*

**22 HAP**  
(hours  
after  
pollination)



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

Hormonal regulations of plant development





**CG020 Genomics**  
**Bi7201 Genomics – a basic course**

**Lesson 3**  
**Reverse Genetics**

Jan Hejátko

**Functional Genomics and Proteomics of Plants,**  
Mendel Centre for Plant Genomics and Proteomics,  
Central European Institute of Technology (CEITEC), Masaryk University, Brno  
[hejatko@sci.muni.cz](mailto:hejatko@sci.muni.cz), [www.ceitec.muni.cz](http://www.ceitec.muni.cz)

---

Hormonal regulations of plant development





# Literature

- Literature sources for Chapter 03:

- **Bioinformatics and Functional Genomics**, 2009, Jonathan Pevsner, Wiley-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.



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

**„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



← T-DNA

„Reverse genetics“ approach

1. ISOLATION OF SEQUENCE-SPECIFIC MUTANT
2. IDENTIFICATION OF PHENOTYPE
3. PROOF OF CAUSAL RELATIONSHIP BETWEEN INSERTION AND PHENOTYPE



← (retro)transposons

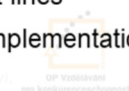


INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Outline

- Gene Silencing Using RNA Interference
  - Mechanism of RNA interference
- Genome Editing via CRISPR/Cas9



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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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)



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

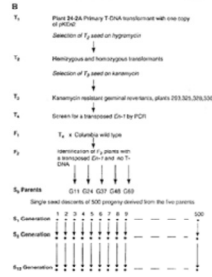
Tato prezentace je spolufinancována  
Evropským sociálním fondem  
a státním rozpočtem České republiky

# Libraries of Insertional Mutants (plants)

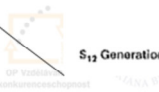
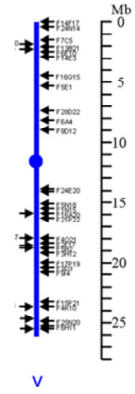
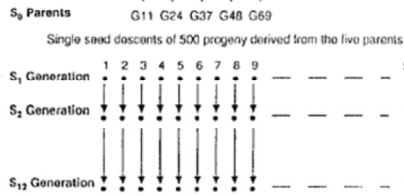
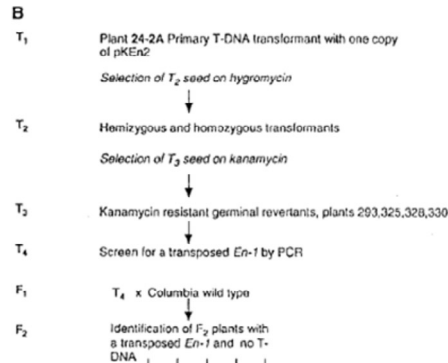


Preparation of transgenic plants

Creating the population of mutants

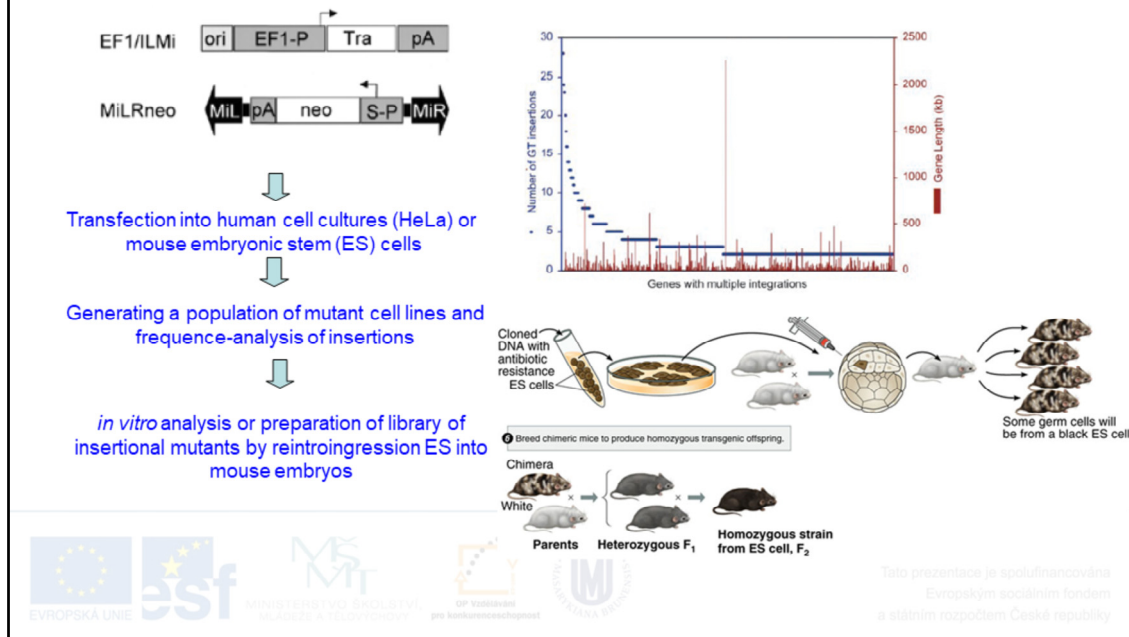


Searching for sequence-specific mutants by PCR



EVROPSKÁ UNIE  
je spolufinancována  
evropským sociálním fondem  
a státním rozpočtem České republiky

# Libraries of Insertional Mutants (animals)



Technologii inzerční mutagenese 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 nutno provést kotransfekci s tzv. helper plasmídem, 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  $\beta$ -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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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

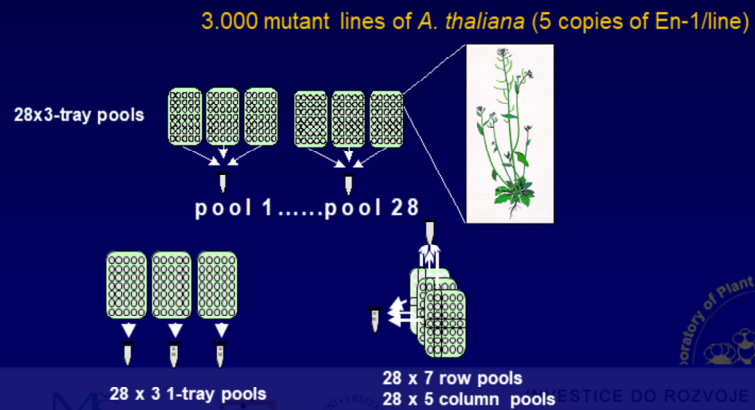


INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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)



ESTICE 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



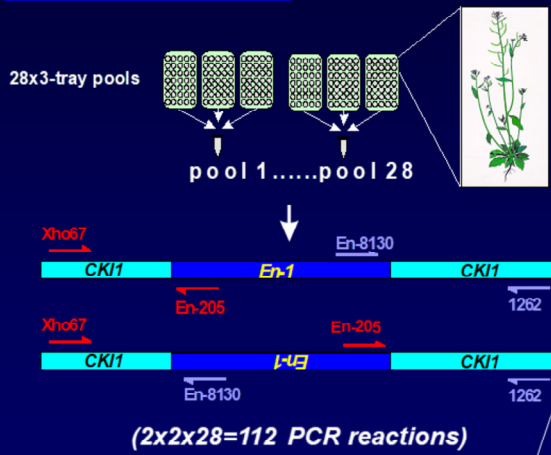
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

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
  - Identification of the positive line through identification of positive tray, row and column



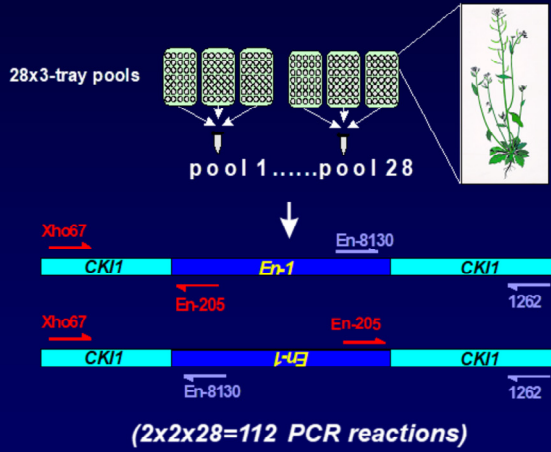
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

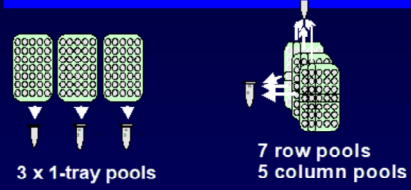
# Isolation of sequence-specific mutants

## 1. 3-tray pools screen

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



## 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

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
    - PCR-based three-dimensional screening
    - Hybridization with iPCR products on filters



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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



# 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>



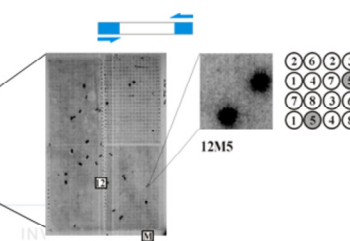
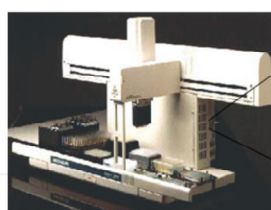
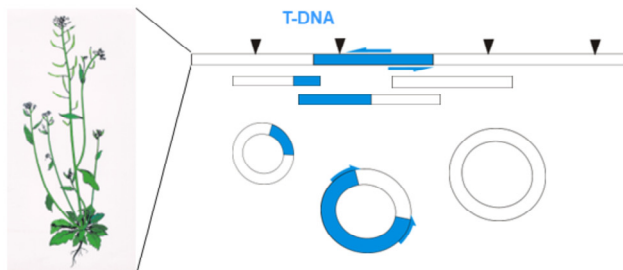
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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



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



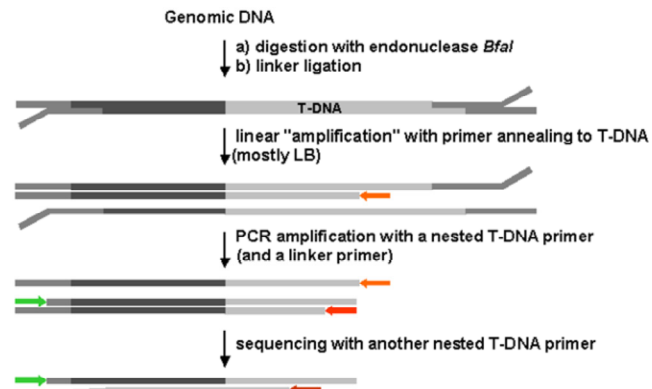
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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)

MINISTERSTVO ŠKOLSTVÍ, Mládeže a tělovýchovy

OP Vzdělávání pro konkurenceschopnost



EVROPSKÁ UNIE

EVROPSKÝ SOCIÁLNÍ FOND  
je spolufinancován  
evropským sociálním fondem  
a státním rozpočtem České republiky

# 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 attagagtttgattgaagtgtgttttatattgatagtgaggacctacttataaaaagc 1509
      |||
Sbjct: 459 attagagtttgattgaagocgcttttatattgatagtgaggacctacttataaaaagc 400

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

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

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

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

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

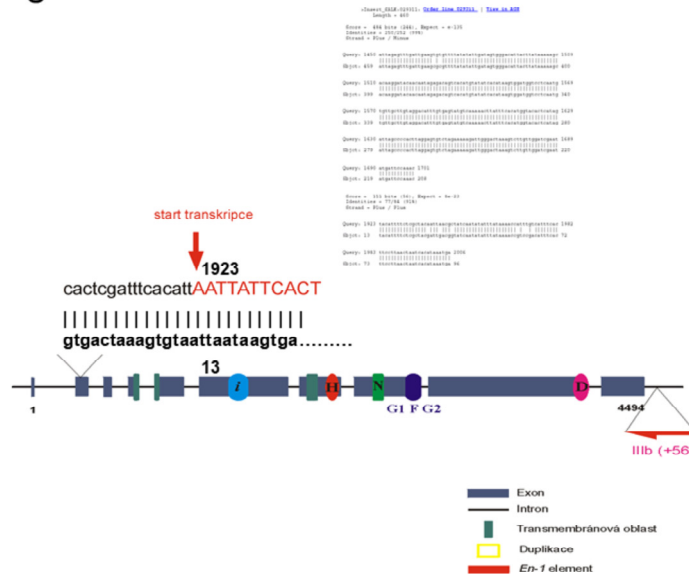
Query: 1923 tacattttctogtacaattaacggtatcnaatatttataaaaaccttggcatttccac 1982
      |||
Sbjct: 13 tacattttctogtacaattaacggtatcnaatatttataaaaaccttggcatttccac 72

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

EVROPSKÁ UNIE  **est** [EVROPSKÝ SOCIÁLNÍ FOND](#)  
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY  OP Vzdělávání pro konkurenceschopnost 

.NI  
šna  
Evropským sociálním fondem  
a státním rozpočtem České republiky

# Searching in electronic libraries of insertional mutants



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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



MINISTERSTVO ŠKOLSTVÍ,  
MLÁDEŽE A TĚLOVÝCHOVY



OP Vzdělávání  
pro konkurenceschopnost

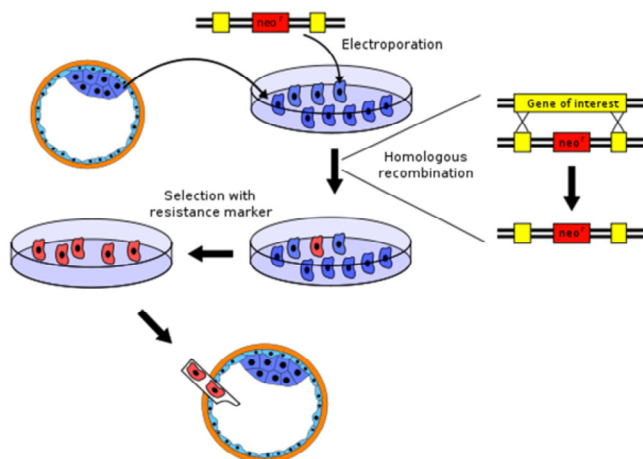


UNIVERZITA  
PAVLA DOBROŠE

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Knocking-Out the Gene



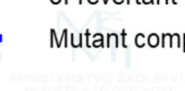
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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



# 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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

## 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)



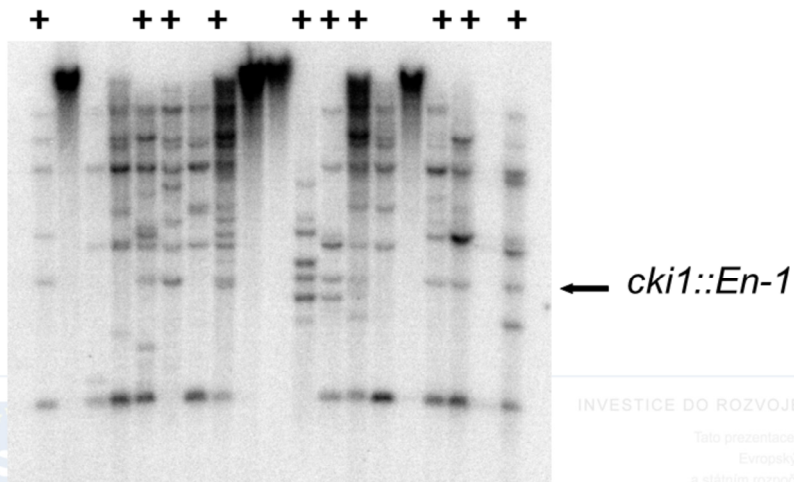
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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



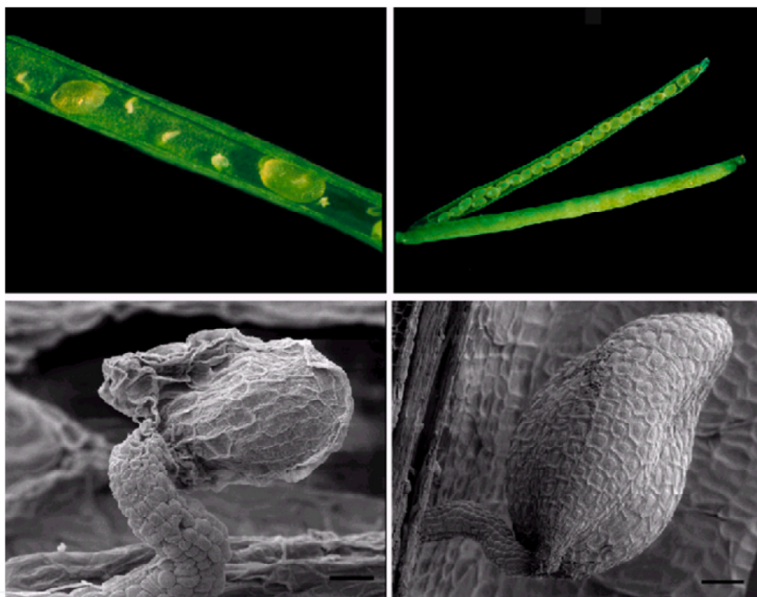
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

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

*cki1::En-1/CKI1*

*CKI1/CKI1*



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

## 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 acg 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 cgc gtg 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 .`



INVESTICE DO ROZVOJE VZDELÁVÁNÍ

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

# 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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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



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

`aattcaagtcgtCACTACAAGA "En-1" TCTTGTAGTcgtggagact`

**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 acg 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 cgc gtg 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 .`



INVESTICE DO ROZVOJE VZDELÁVÁNÍ

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

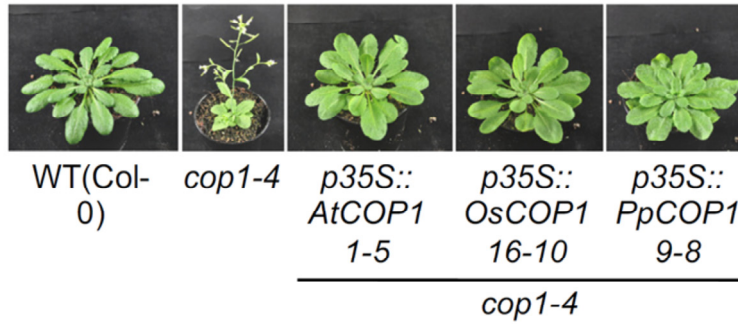
# Mutant Line Complementation



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Mutant Line Complementation



Ranjan et al., 2014



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Outline

- Gene Silencing Using RNA Interference
  - Mechanism of RNA interference



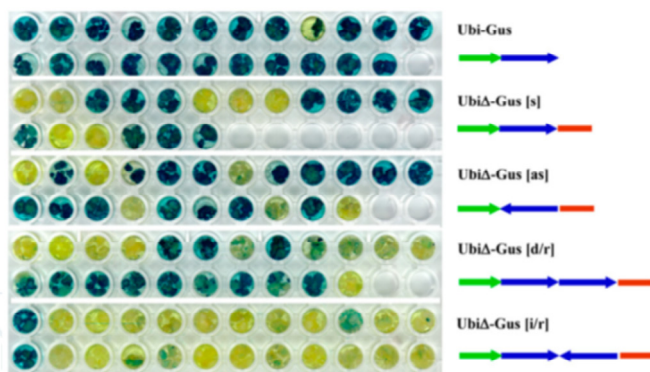
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# 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)

E VZDĚLÁVÁNÍ

je spolufinancována  
ym sociálním fondem  
a státním rozpočtem České republiky

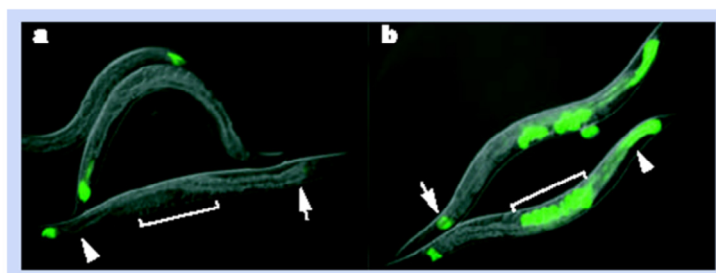
# RNA interference

- **Molecular basis of posttranscriptional gene silencing (PTGS)**

- dsRNA induction is dependent on its own genes – gene searching

*RNAi*

*rnai*



Mello and Conte, *Nature* (2004)



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# RNA interference

## ▪ Molecular basis of posttranscriptional gene silencing (PTGS)

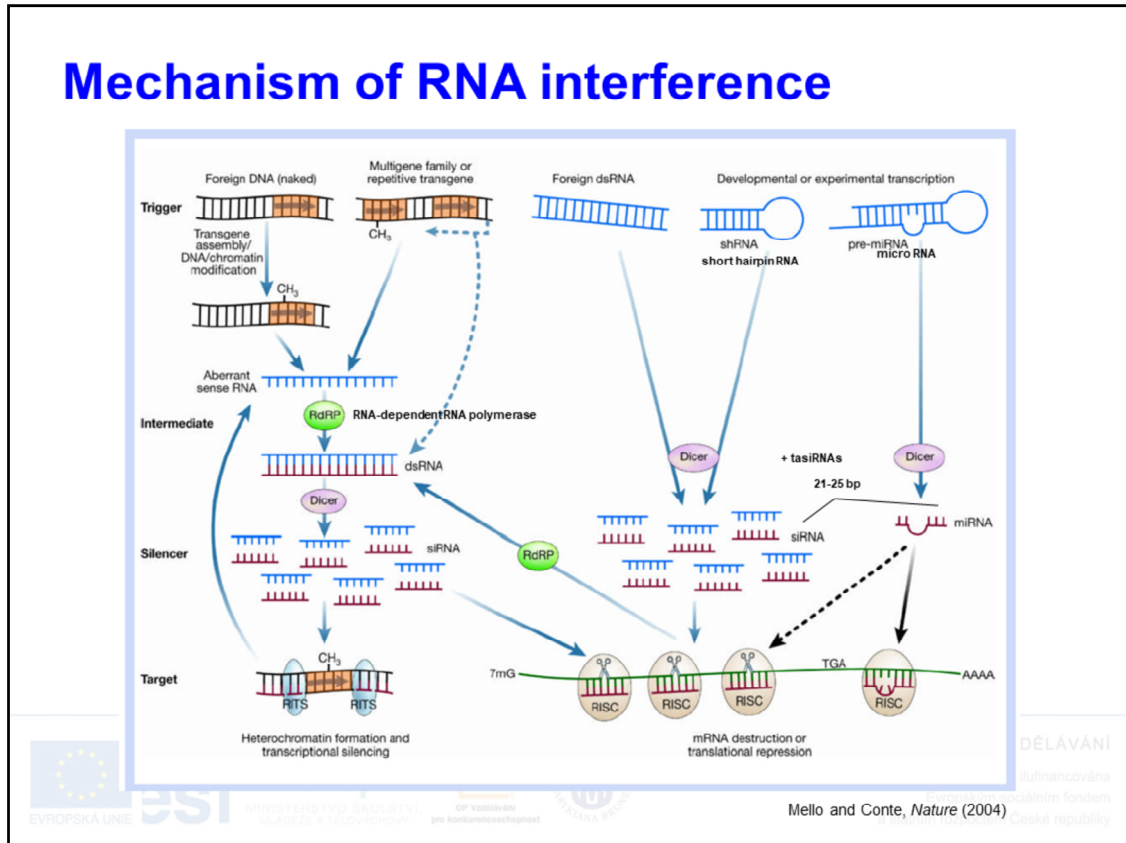
- RNAi found in *Coenorhabditis elegans* and in plants
- It is a **natural mechanism** of regulation of gene expression in all eukaryotes
- The principle is **creating dsRNA**, which can be triggered in several ways:
  - By presence of **foreign „aberrant“ DNA**
  - **Specific transgenes** containing **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 complex)
- **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)



DO ROZVOJE VZDĚLÁVÁNÍ

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

# Mechanism of RNA interference



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.

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.

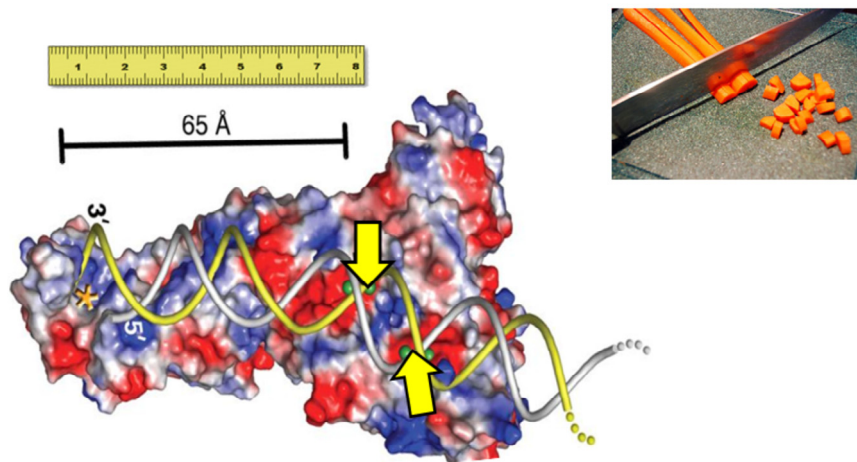
Developmental regulations via miRNAs are more often used in animals than in plants.

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).



## 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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

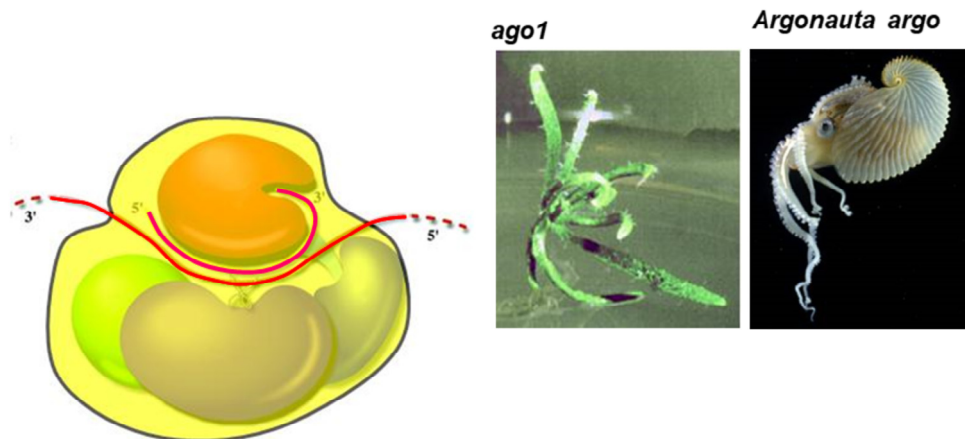
Tato prezentace je spolufinancována  
Evropským sociálním fondem  
a státním rozpočtem České republiky

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.

## Argonaute proteins



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.



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

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).

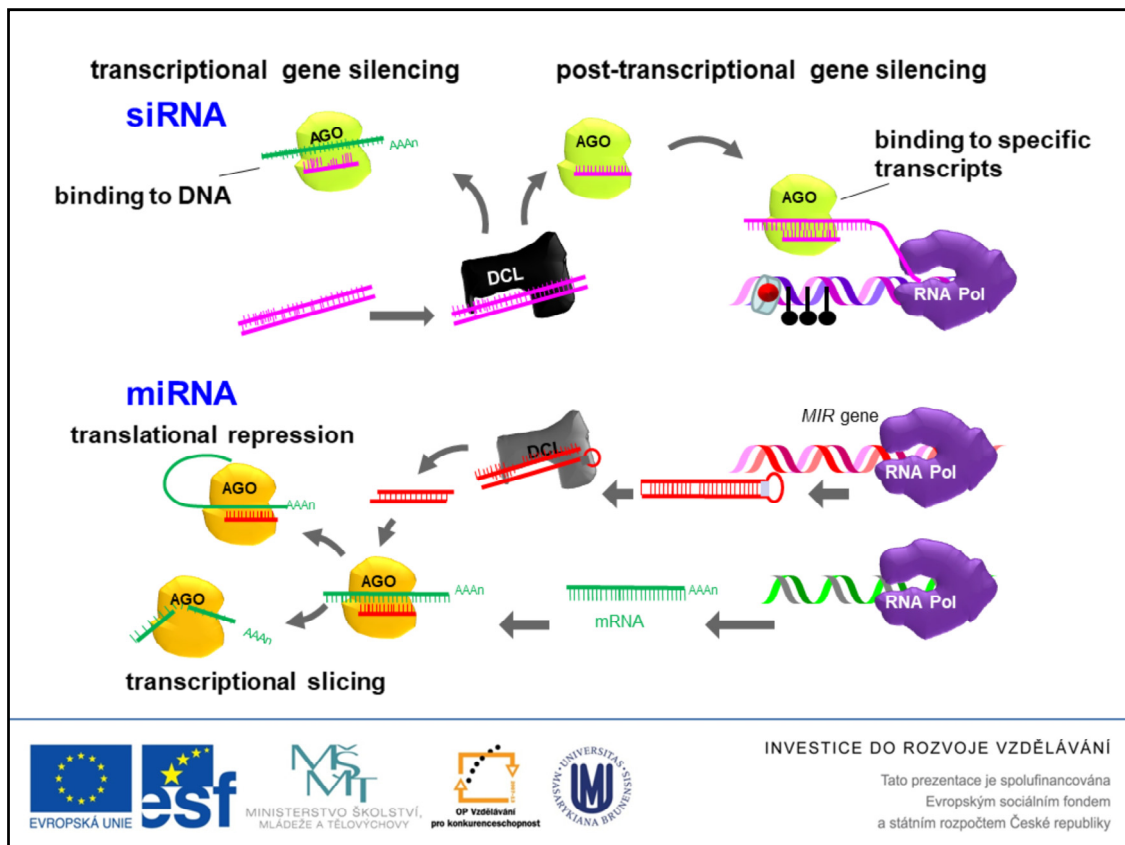
ARGONAUTE proteins were originally described as being important for plant development and for germline stem-cell division in *Drosophila melanogaster*.

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://youpreferanargonaute.com/2009/06/>



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



**Andrew Z. Fire**

USA

Stanford University  
School of Medicine  
Stanford, CA, USA

b. 1959



**Craig C. Mello**

USA

University of  
Massachusetts Medical  
School  
Worcester, MA, USA

b. 1960



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

In 2006, Andrew Z. Fire and Craig C. Mello were honored by the Nobel prize “for their discovery of RNA interference - gene silencing by double-stranded RNA”.

# Outline

- Gene Silencing Using RNA Interference
  - Mechanism of RNA interference
- Genome Editing via CRISPR/Cas9

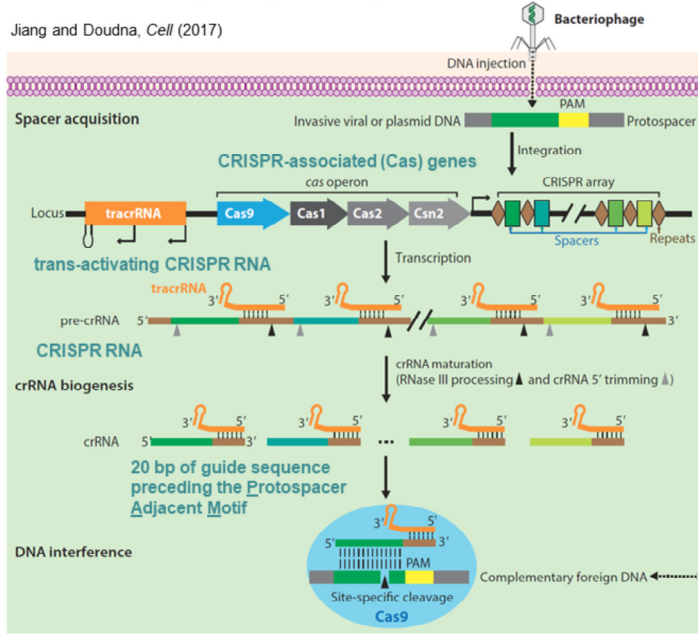


INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

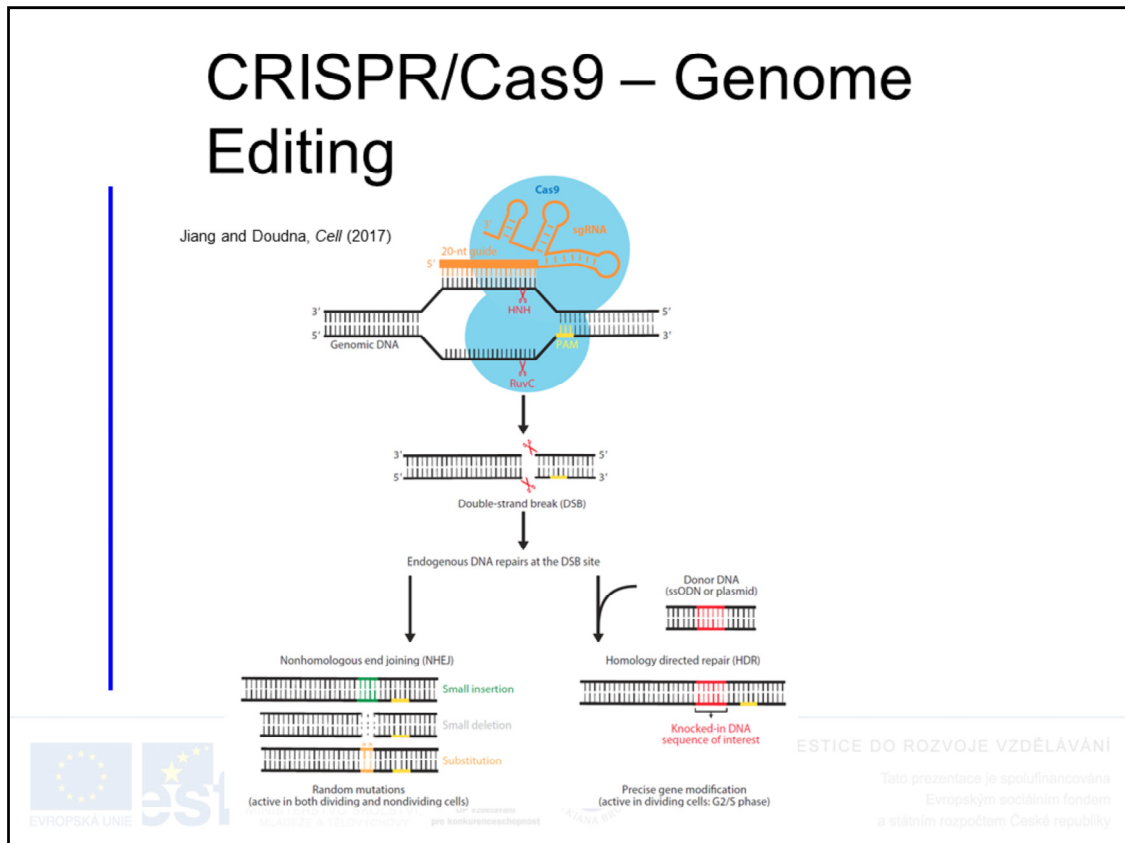
# CRISPR/Cas9 - Mechanism

- **Clustered Regularly Interspaced Short Palindromic Repeats**



CRISPR–Cas9-mediated DNA interference in bacterial adaptive immunity. A typical CRISPR locus in a type II CRISPR–Cas system comprises an array of repetitive sequences (repeats, brown diamonds) interspaced by short stretches of nonrepetitive sequences (spacers, colored boxes), as well as a set of CRISPR-associated (*cas*) genes (colored arrows). Preceding the *cas* operon is the trans-activating CRISPR RNA (*tracrRNA*) gene, which encodes a unique noncoding RNA with homology to the repeat sequences. Upon phage infection, a new spacer (dark green) derived from the invasive genetic elements is incorporated into the CRISPR array by the acquisition machinery (*Cas1*, *Cas2*, and *Csn2*). Once integrated, the new spacer is cotranscribed with all other spacers into a long precursor CRISPR RNA (pre-crRNA) containing repeats (brown lines) and spacers (dark green, blue, light green, and yellow lines). The *tracrRNA* is transcribed separately and then anneals to the pre-crRNA repeats for crRNA maturation by RNase III cleavage. Further trimming of the 5' end of the crRNA (gray arrowheads) by unknown nucleases reduces the length of the guide sequence to 20 nt. During interference, the mature crRNA–*tracrRNA* structure engages *Cas9* endonuclease and further directs it to cleave foreign DNA containing a 20-nt crRNA complementary sequence preceding the PAM sequence. Asterisks denote conserved, key residues for *Cas9*-mediated DNA cleavage activity. Abbreviations: Arg, arginine-rich bridge helix; crRNA, CRISPR RNA; CTD, C-terminal domain; nt, nucleotide; NUC, nuclease lobe; PAM, protospacer adjacent motif; REC, recognition lobe; *tracrRNA*, trans-activating CRISPR RNA.

# CRISPR/Cas9 – Genome Editing



The mechanism of CRISPR–Cas9–mediated genome engineering. The synthetic sgRNA or crRNA–tracrRNA structure directs a Cas9 endonuclease to almost arbitrary DNA sequence in the genome through a user-defined 20-nt guide RNA sequence and further guides Cas9 to introduce a double-strand break (DSB) in targeted genomic DNA. The DSB generated by two distinct Cas9 nuclease domains is repaired by host-mediated DNA repair mechanisms. In the absence of a repair template, the prevalent error-prone nonhomologous end joining (NHEJ) pathway is activated and causes random insertions and deletions (indels) or even substitutions at the DSB site, frequently resulting in the disruption of gene function. In the presence of a donor template containing a sequence of interest flanked by homology arms, the error-free homology directed repair (HDR) pathway can be initiated to create desired mutations through homologous recombination, which provides the basis for performing precise gene modification, such as gene knock-in, deletion, correction, or mutagenesis. CRISPR–Cas9 RNA-guided DNA targeting can be uncoupled from cleavage activity by mutating the catalytic residues in the HNH and RuvC nuclease domains, making it a versatile platform for many other applications beyond genome editing. Abbreviations: crRNA, CRISPR RNA; nt, nucleotide; PAM, protospacer adjacent motif; sgRNA, single-guide RNA; tracrRNA, *trans*-activating CRISPR RNA.

# CRISPR/Cas9 – Nobel Prize in 20..2x?



Francisco Mojica



Emmanuelle Charpentier



Jenifer Doudna



Martin Jinek

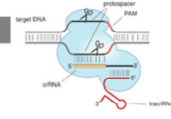
## RESEARCH ARTICLE

### A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity

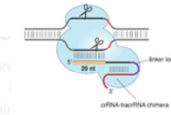
Martin Jinek,<sup>1,2\*</sup> Krzysztof Dyllinski,<sup>1,2\*</sup> Ines Fofana,<sup>2</sup> Michael Haer,<sup>2†</sup> Jennifer A. Doudna,<sup>1,2,3,4</sup> Emmanuelle Charpentier<sup>1,2</sup>

Jinek et al, *Science* (2012)

Cas9 programmed by crRNA:tracrRNA duplex



Cas9 programmed by single chimeric RNA

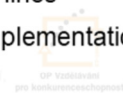
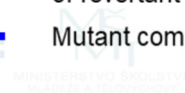


OP Vzdělávání pro konkurenceschopnost



# Summary

- **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



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Summary

- Gene Silencing Using RNA Interference
  - Mechanism of RNA interference
- Genome Editing via CRISPR/Cas9



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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

# Discussion



## INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

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