CG920 Genomics

Lesson 4

Forward Genetics

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

Outline

- Forward vs. reverse genetics
- Use of libraries of insertional mutants in forward genetics
  - Searching in libraries of insertional mutants according to:
    - anatomically or morphologically detectable phenotype
    - metabolic profile
    - expression of genes of interest
  - Identification of the mutated locus
    - plasmid rescue
    - iPCR
- Use of libraries of point mutants in forward genetics
  - Positional cloning

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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

1. ISOLATION OF SEQUENCE--SPECIFIC MUTANT

2. IDENTIFICATION OF PHENOTYPE

3. PROOF OF CAUSAL RELATIONSHIP BETWEEN INSERTION AND PHENOTYPE

← (retro)transposons

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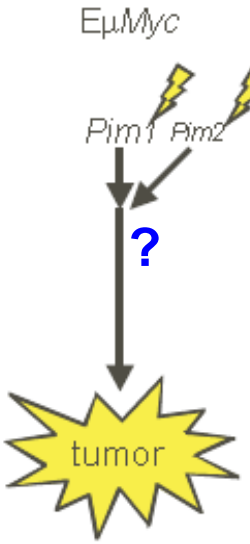
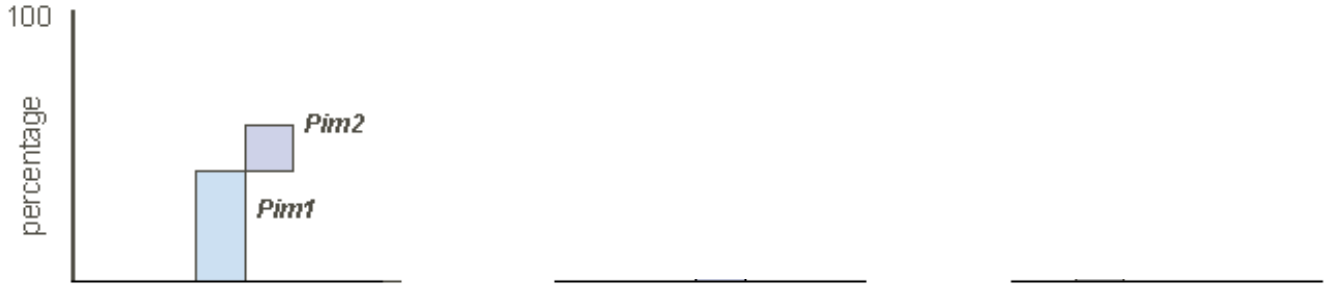
Insertional mutagenesis in forward genetics approaches

- Use of insertional mutagenesis for study of carcinogenesis
  - Infection of EμMyc mice by MoMuLV retrovirus leads to lymphomas formation, which arose due to activation of Pim kinases (40 % activation of Pim1, 15 % activation of Pim2), molecular targets of these kinases were unknown

a

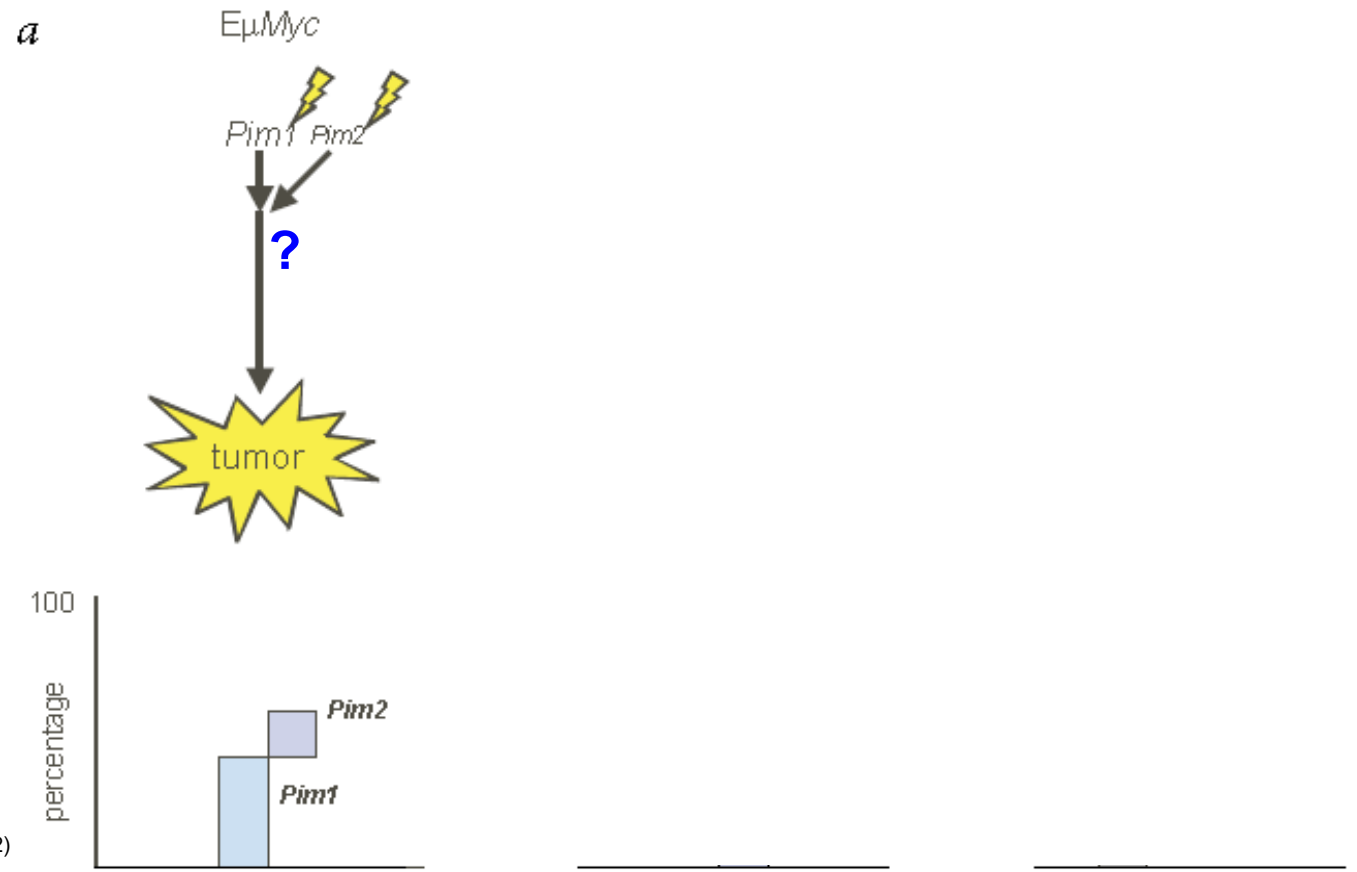




Mikkers et al., Nature Gen (2002)

Insertional mutagenesis in forward genetics approaches

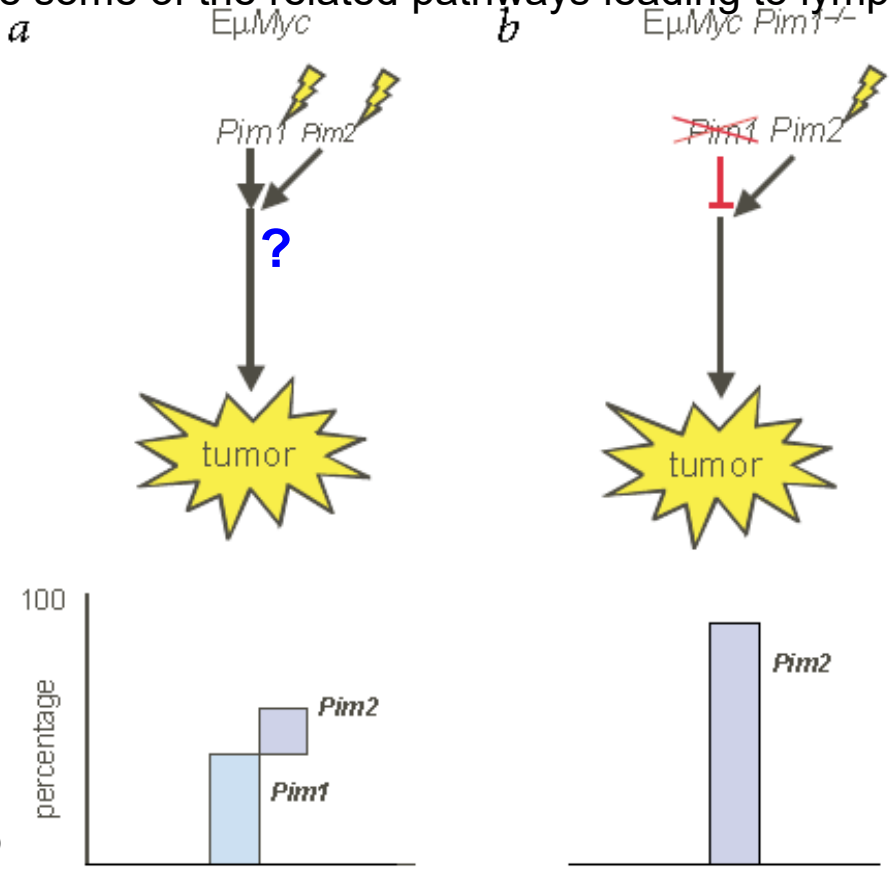
- Use of insertional mutagenesis for study of carcinogenesis
  - Infection of EμMyc *pim1* mutants by MoMuLV retrovirus leads to lymphomas formation, which in 90 % contain insertion nearby (activation) Pim2



Mikkers et al., Nature Gen (2002)

Insertional mutagenesis in forward genetics approaches

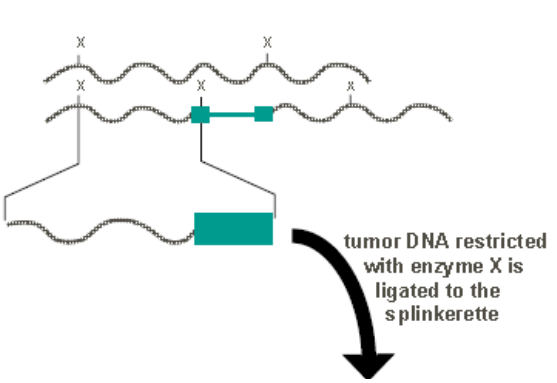
- Use of insertional mutagenesis for study of carcinogenesis
  - Infection of EμMyc double mutants *pim1, pim2* by MoMuLV retrovirus leads to lymphomas formation, which can be expected to activate either one of the signalling partner of Pim proteins (Y), one of the proteins of Pim signalling pathway (X) or to activate some of the related pathways leading to lymphomagenesis (Z).

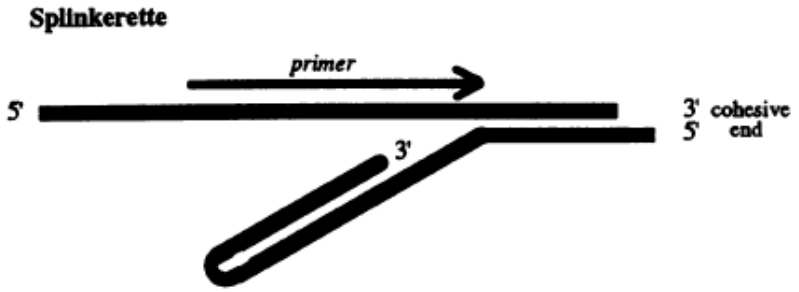


Mikkers et al., Nature Gen (2002)

Insertional mutagenesis in forward genetics approaches

- Isolation of genomic regions adjacent to the insertion site of the provirus
  - Cleavage of genomic DNA and ligation of special linkers, so-called *splincerettes* (increasing the specifity of amplification)



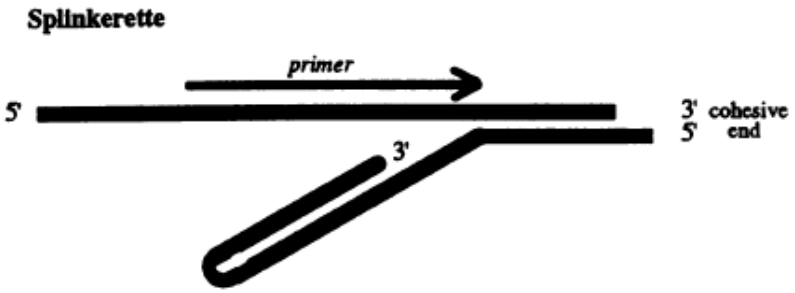


Devon et al., Nucl Acid Res (1994)

Mikkers et al., Nature Gen (2002)

Insertional mutagenesis in forward genetics approaches

- Isolation of genomic regions adjacent to the insertion site of the provirus
  - First amplification using specific primers



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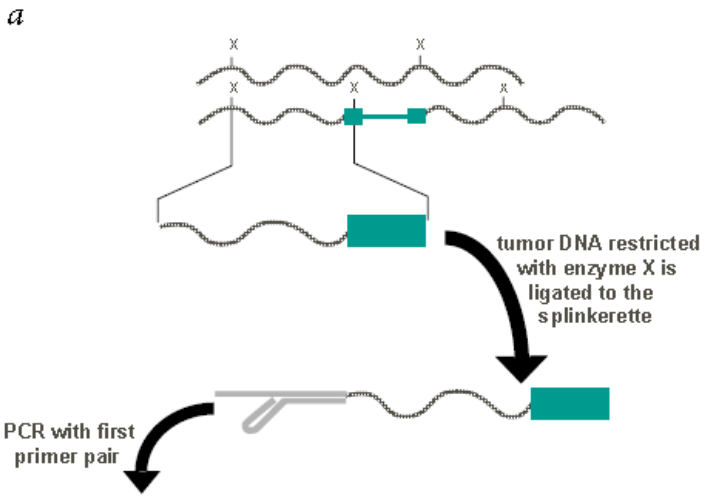
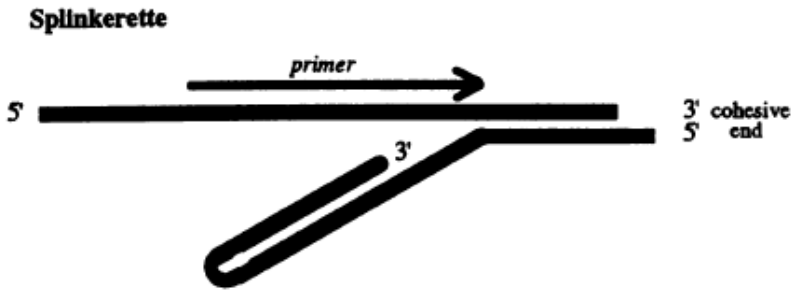


Mikkers et al., Nature Gen (2002)

Insertional mutagenesis in forward genetics approaches

- Isolation of genomic regions adjacent to the insertion site of the provirus
  - Second amplification using nested primers (increasing the specifity)



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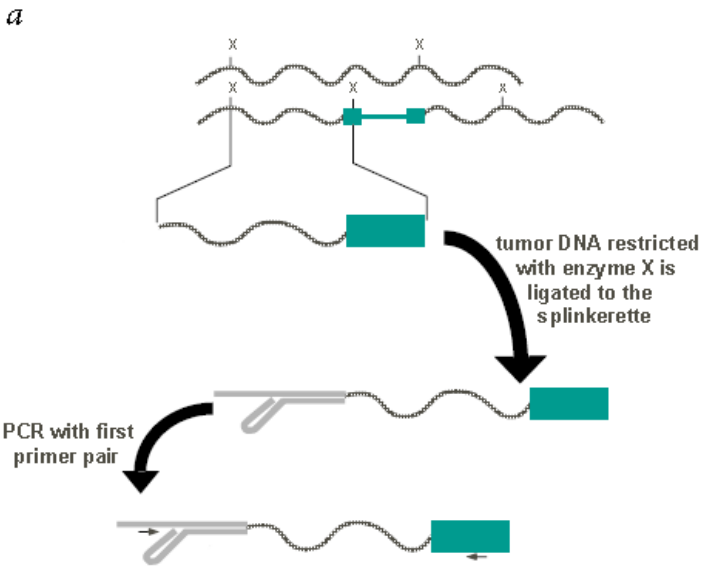
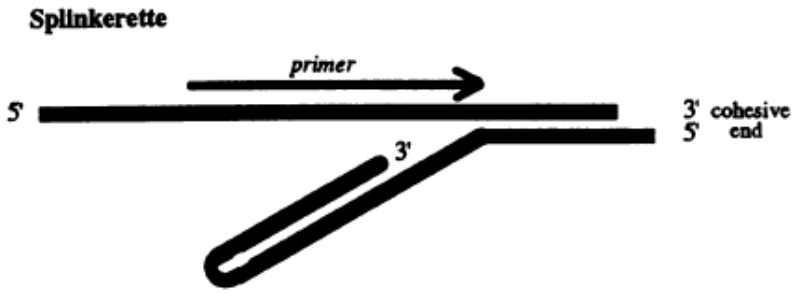


Mikkers et al., Nature Gen (2002)

Insertional mutagenesis in forward genetics approaches

- Isolation of genomic regions adjacent to the insertion site of the provirus
  - Sequencing and localization of regions adjacent to provirus by searching in annotated databases of mouse genome



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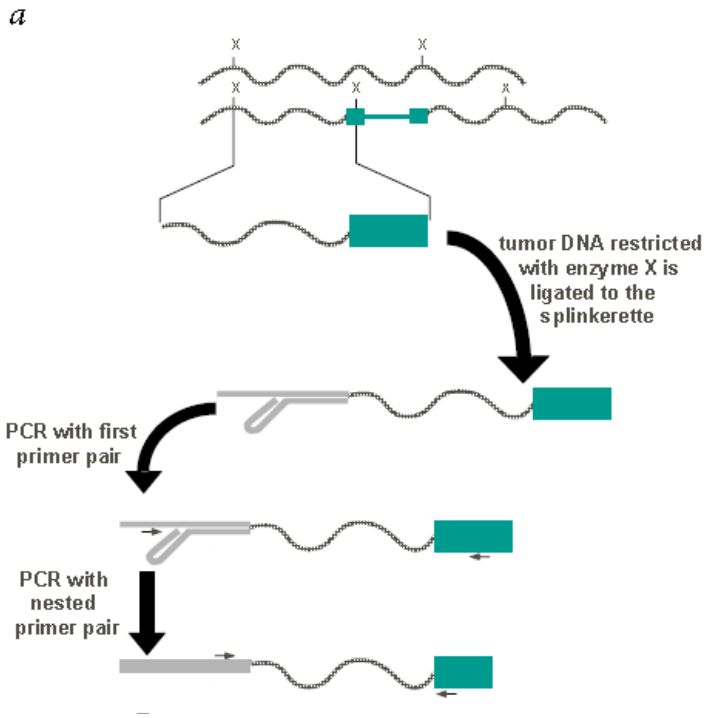

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In case of splincerette, the primer is of the same sequence as the top strand and therefore it is unable to act as a primer until the complement of this strand has been synthesized (from the insert-specific primer at the right-hand side).

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  - Searching in libraries of insertional mutants according to:
    - anatomically or morphologically detectable phenotype
    - metabolic profile

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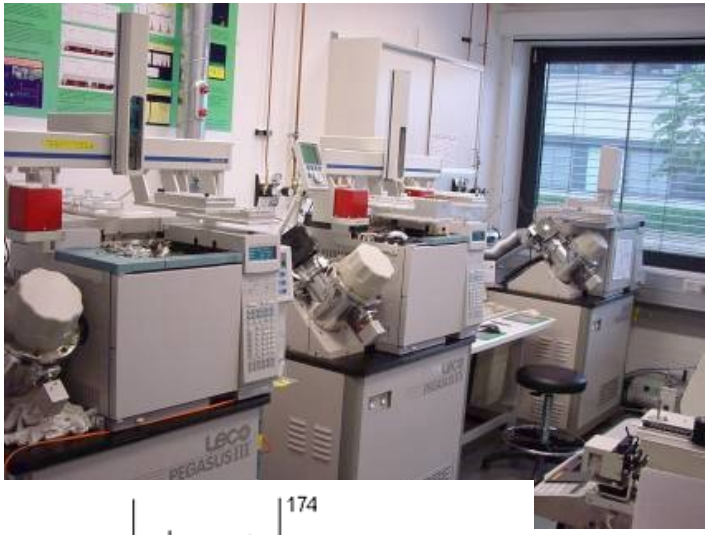
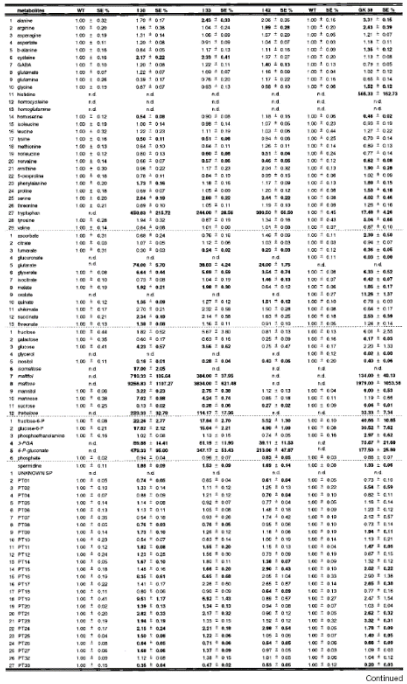
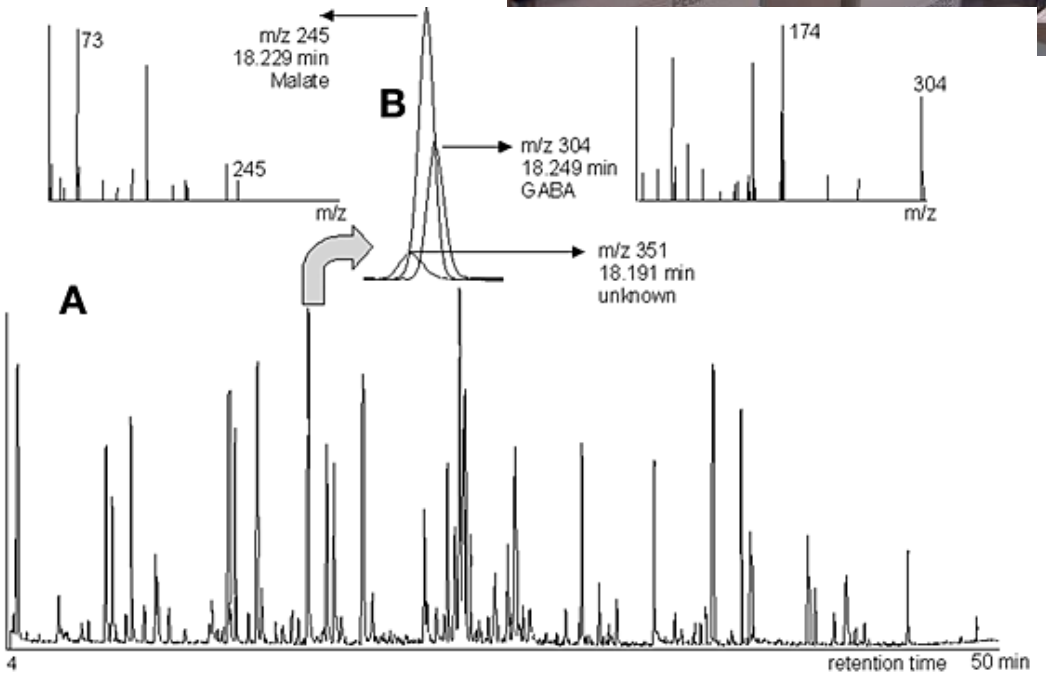
Metabolic profiling

- Metabolic profiling of plants
  - Mass and automated analysis of metabolites (up to 25.000) by GC-MS techniques in libraries of T-DNA mutants







Metabolic profiling

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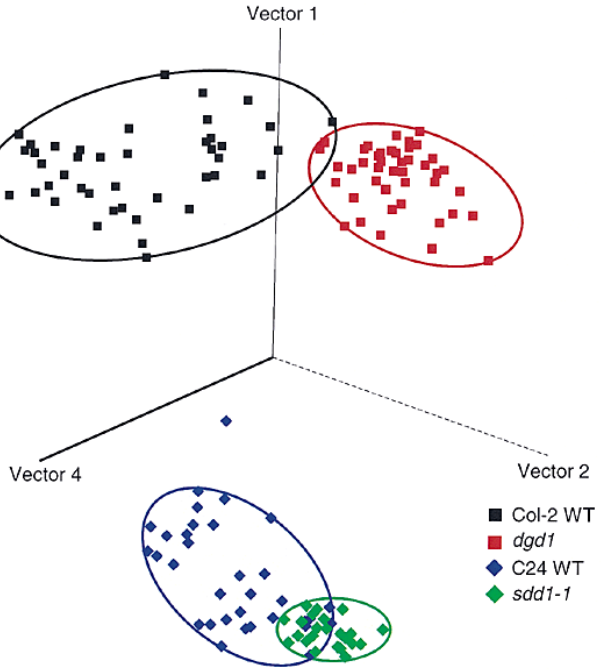
  - Identification of interesting (even comercially interesting) mutants

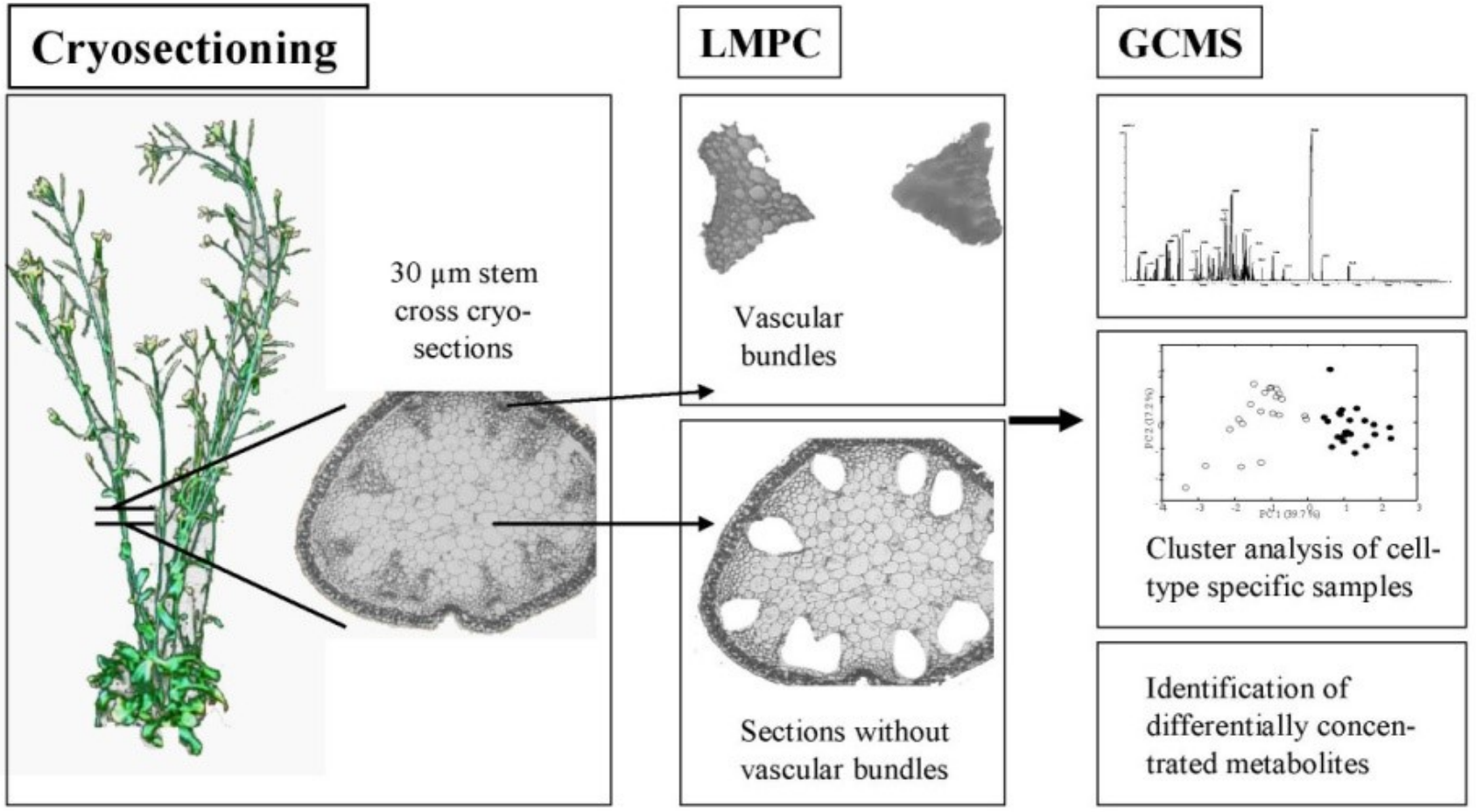


Metabolic profiling

- Metabolic profiling of plants
  - Mass and automated analysis of metabolites (up to 25.000) by GC-MS techniques in libraries of T-DNA mutants
  - Identification of interesting (even comercially interesting) mutants
  - Fast and easy isolation of genes through identification of sequences affected by T-DNA

Metabolic profiling

- Metabolic profiling of plants
  - Possibility to use special techniques, e.g. microdissection



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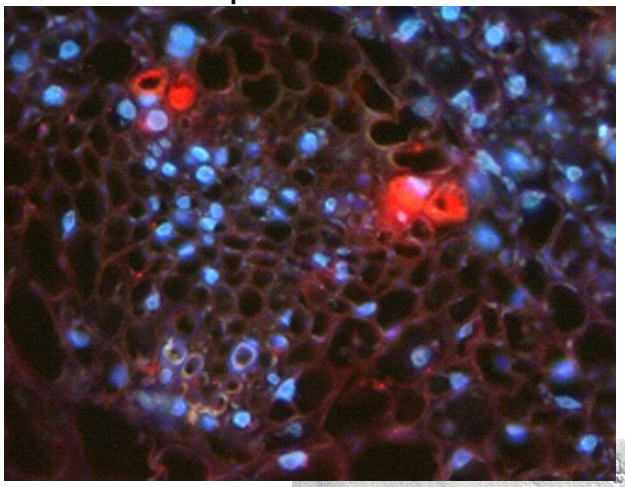
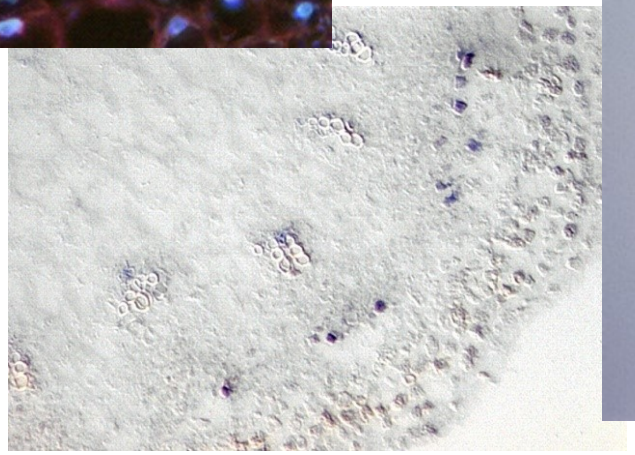
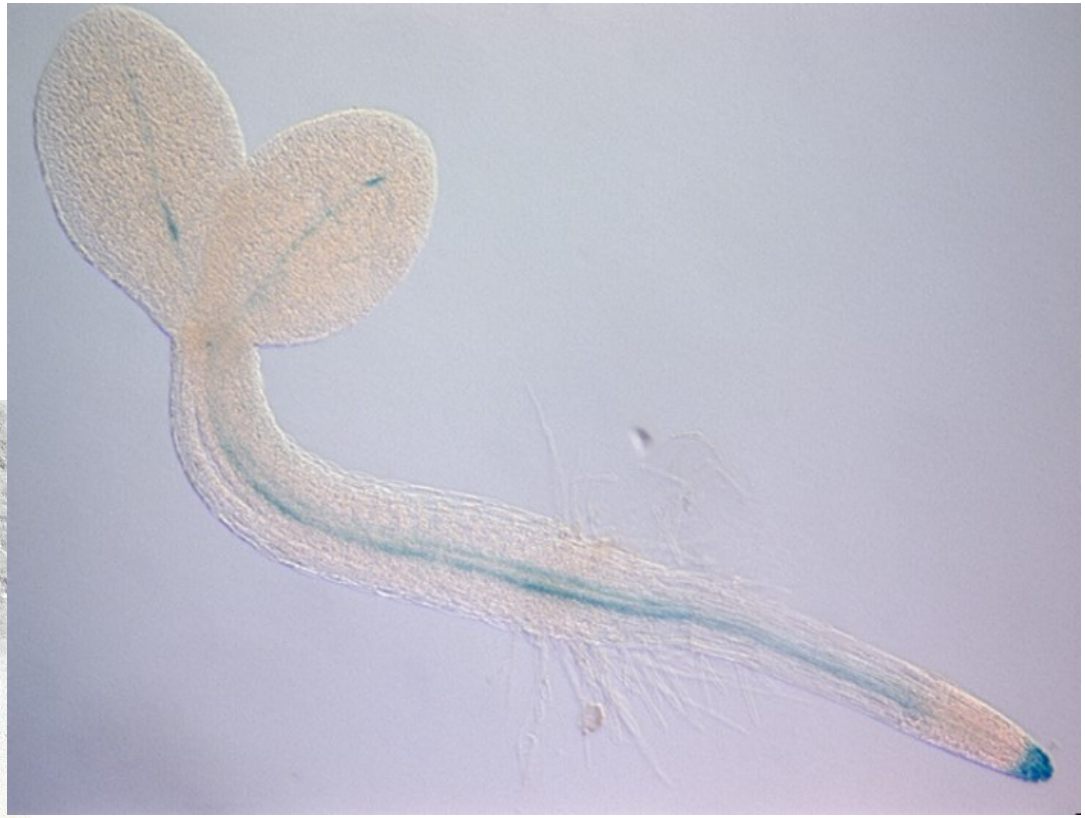
Expression profile

- Identification of mutants with a change in the expression profile
  - Analysis of expression profile (pattern) of the gene and identification of mutants with altered pattern of expression



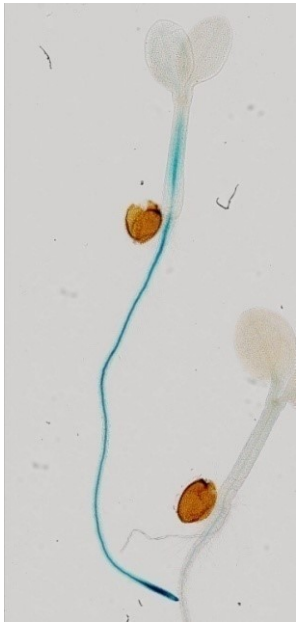


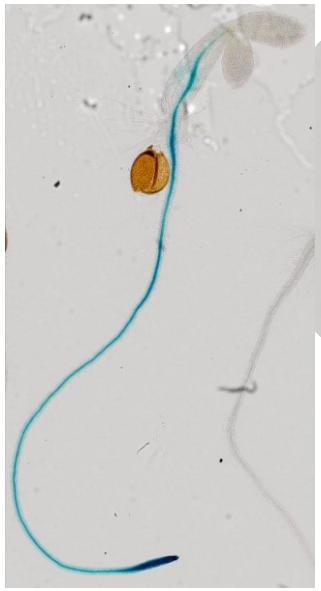


Expression profile

- Identification of mutants with a change in the expression profile
  - Analysis of expression profile (pattern) of the gene and identification of mutants with altered pattern of expression
  - Possibility of partial automation (virtual digital microscopy)

Expression profile





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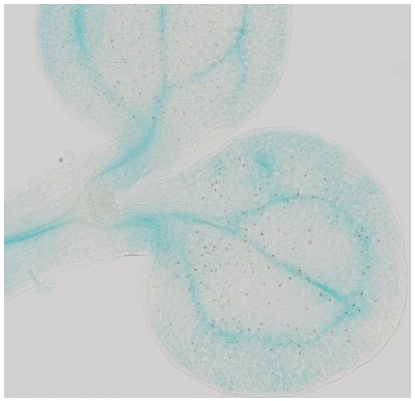

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Identification of mutated locus

- Identification of chromosomal rearrangements responsible for bushy phenotype of *Arabidopsis*
  - Description of phenotype

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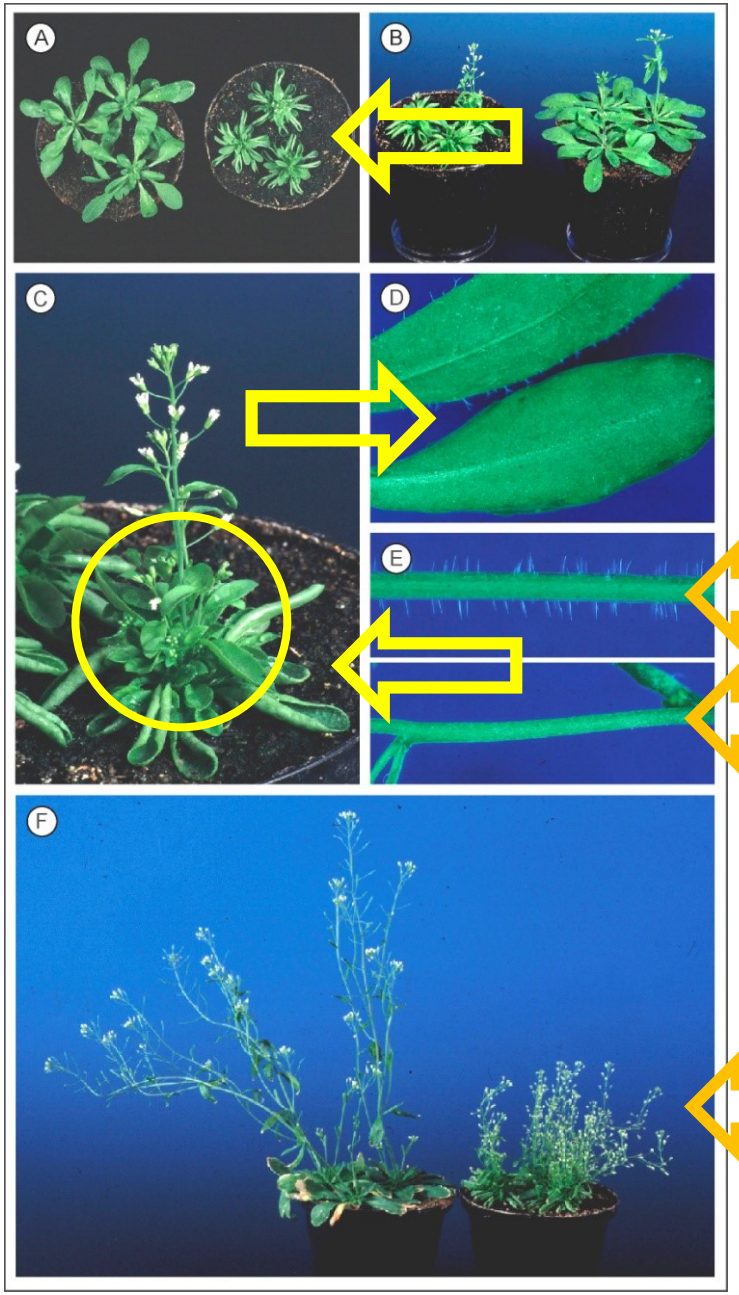
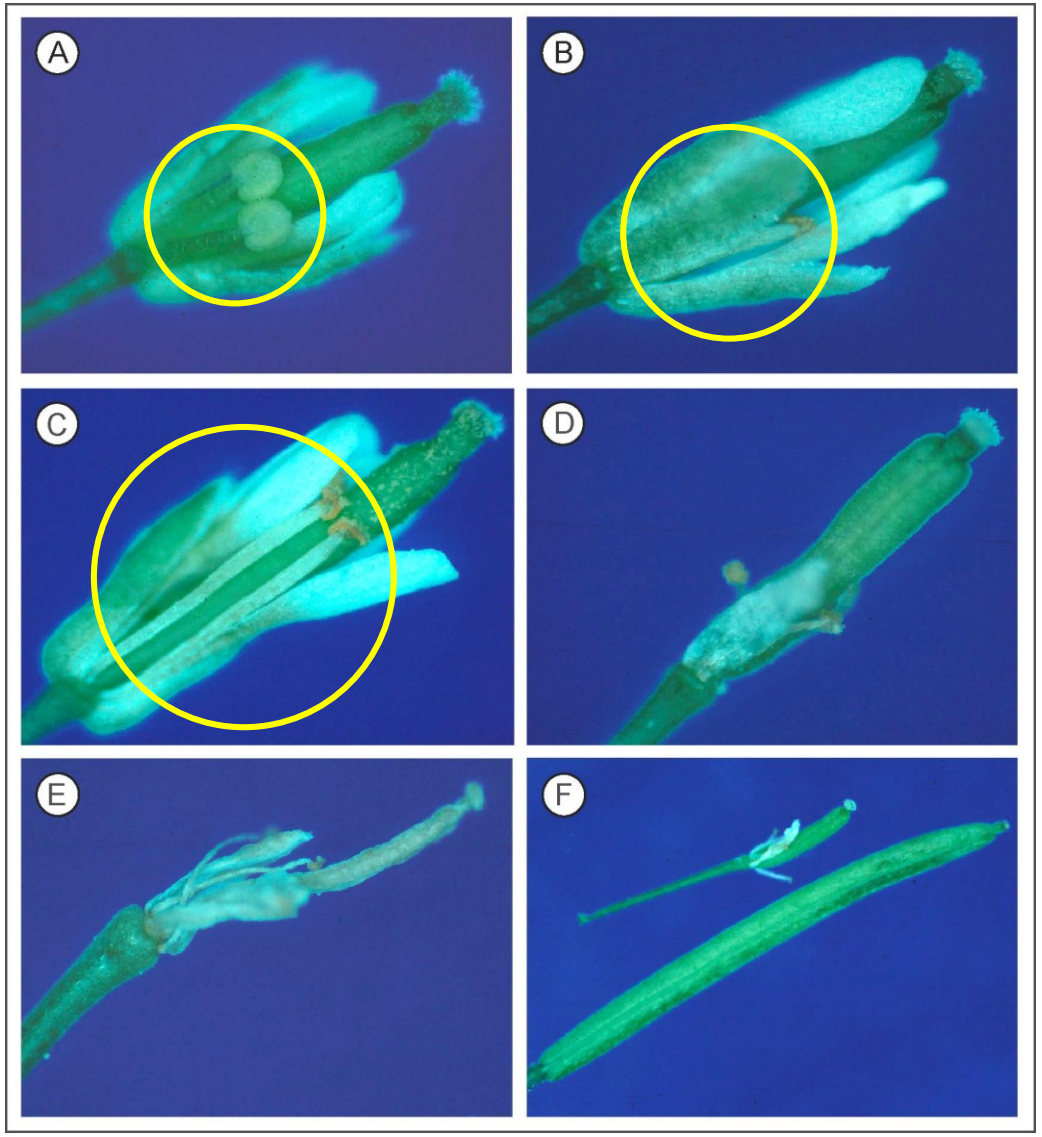
Identification of mutant



- Crinkled leaves
- Bushy phenotype (branching defective)
- No trichomes on leaves and stems
- Late senescence

Identification of mutant



- Male sterility, defects in stamen filament elongation (A,B)

  (compare with wild type C)

Identification of mutated locus

- Identification of chromosomal rearrangements responsible for bushy phenotype of *Arabidopsis*
  - Description of phenotype
  - Identification of T-DNA mutated region

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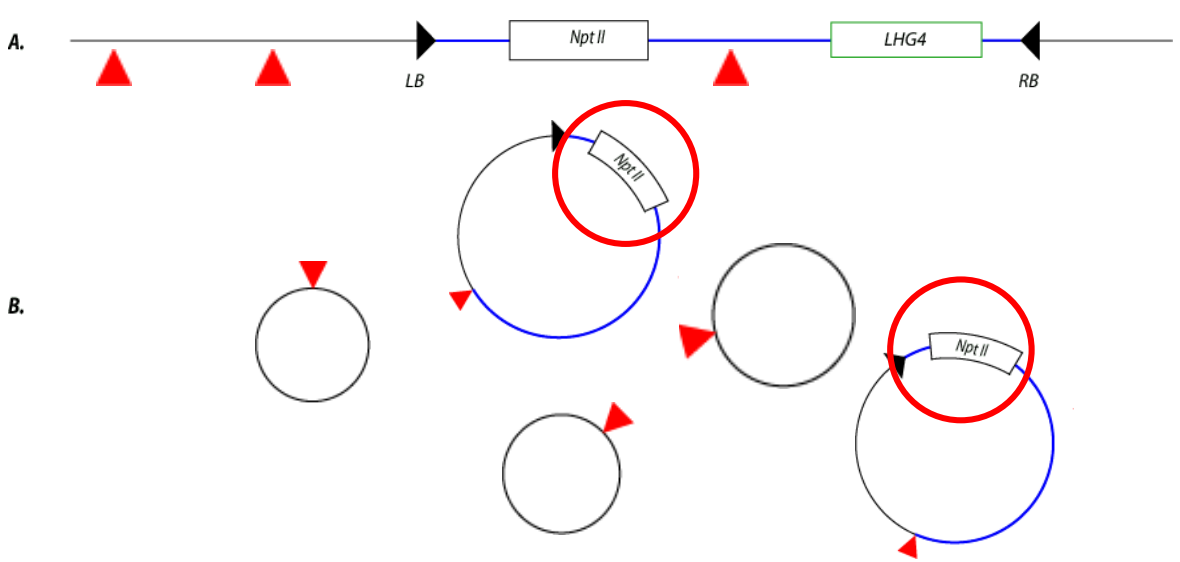
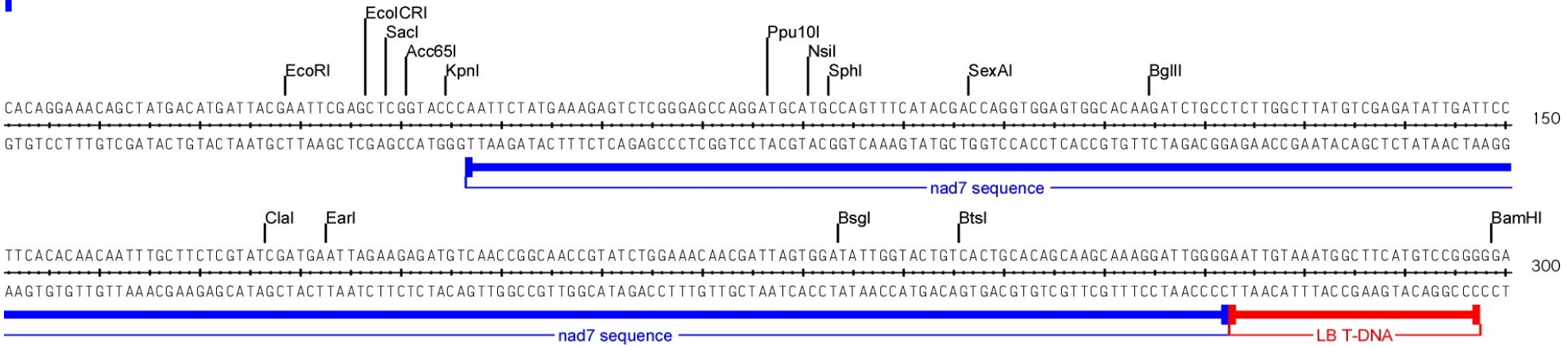
Identification of mutated locus

1. Identification of region of genomic DNA adjacent to the ***left border*** using ***plasmid rescue***

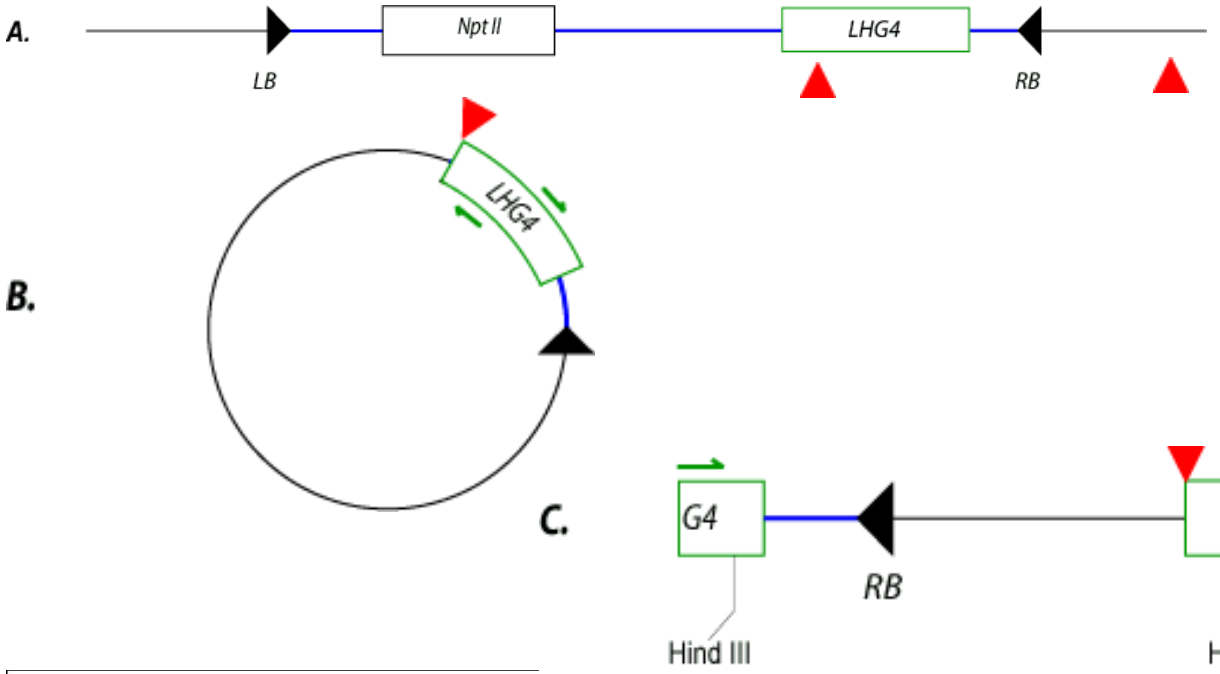


- Restriction digestion (*EcoRI*) of mutant genomic DNA
- Religation and transformation of *E. coli*
- Isolation of plasmid DNA from positively selected clones
- Identified sequence was identical to gene for *NAD7* coded by mtDNA

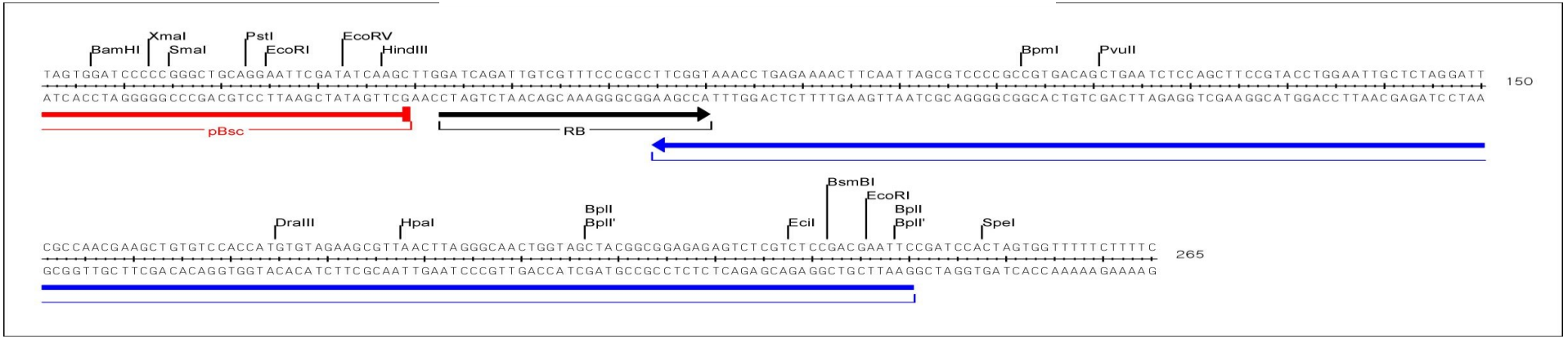


Identification of mutated locus

2. Identification of region of genomic DNA adjacent to the **right border** using **inversion PCR (iPCR)**



- Restriction digestion (*EcoRI)* of mutant genomic DNA
- Purification, religation and PCR using T-DNA specific primers
- Cloning and sequencing
- Identified sequence was not homologous to any sequences with known function



Identification of mutated locus

- Identification of chromosomal rearrangements responsible for bushy phenotype of *Arabidopsis*
  - Description of phenotype
  - Identification of T-DNA mutated region
  - Localization of T-DNA insertion site in *Arabidopsis* genome

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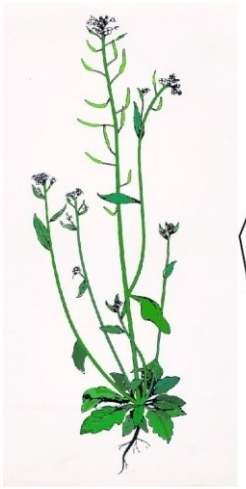
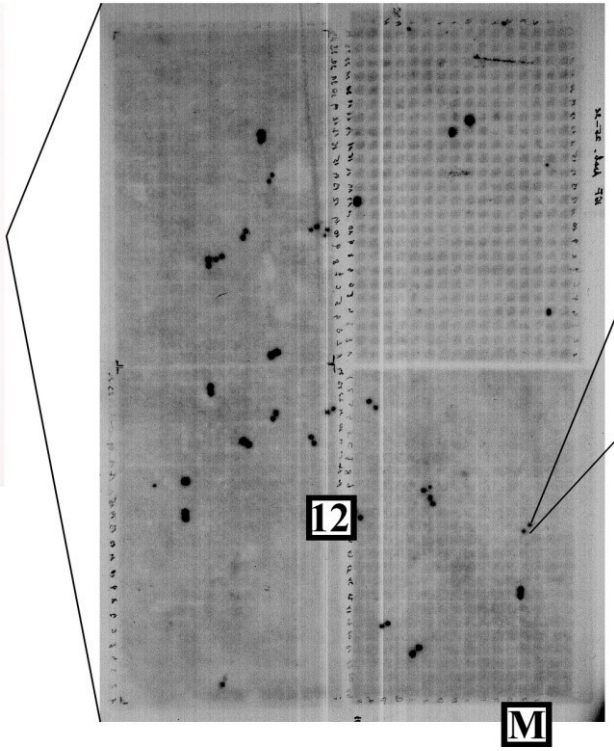
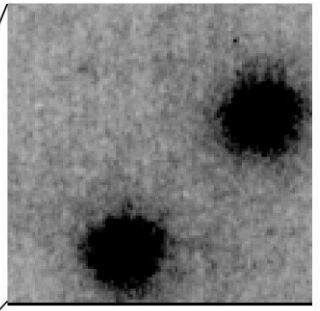
Searching in library IGF-BAC

- Genome library containing 10.752 clones with an average size of an insert of 100 kb
- Bacterial clones arranged in the microtiter plates
- Library loaded onto nylon filters for hybridization with the radiolabeled probe







F12M5

2	6	2	3
1	4	7	5
7	8	3	6
1	5	4	8

Mapping with IGF-BAC database

I. Sequences adjacent to the left border of T-DNA

- 28 positively hybridizing clones in total
- 19 of them located on chromosome 2
- 18 of them similar with mtDNA

II. Sequences adjacent to the right border of T-DNA

- 6 positively hybridizing clones in total
- all of them located on chromosome 2

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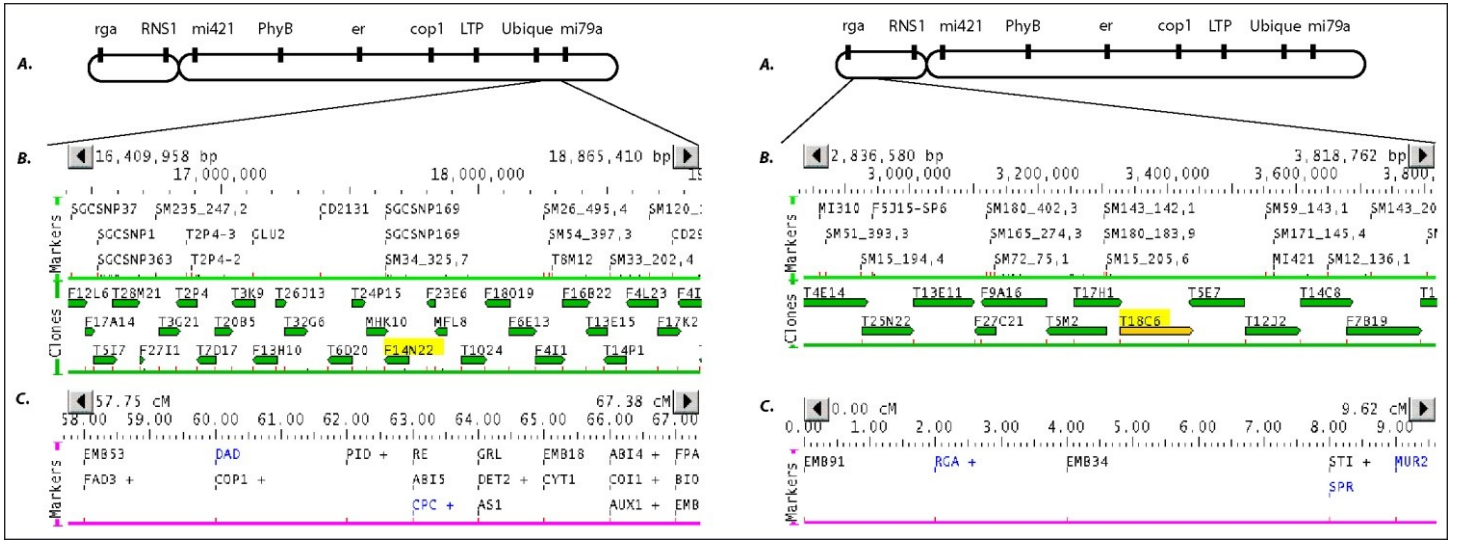
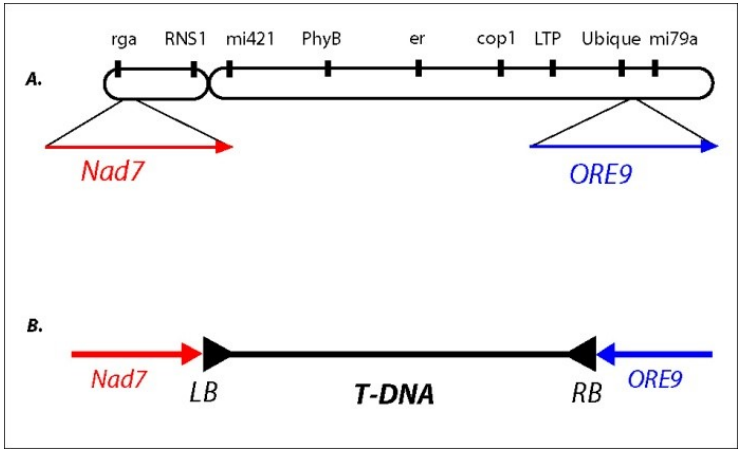
Localization of genomic T-DNA adjacent to both left and right T-DNA borders on chromosome 2

Sequences adjacent to ***right*** and ***left*** border of T-DNA



- There was probably an inversion of almost entire chromosome 2



Outline

- Forward vs. reverse genetics
- Use of libraries of insertional mutants in forward genetics
  - Searching in libraries of insertional mutants according to:
    - anatomically or morphologically detectable phenotype
    - metabolic profile
    - expression of genes of interest
  - Identification of the mutated locus
    - plasmid rescue
    - iPCR
- Use of libraries of point mutants in forward genetics
  - Positional cloning

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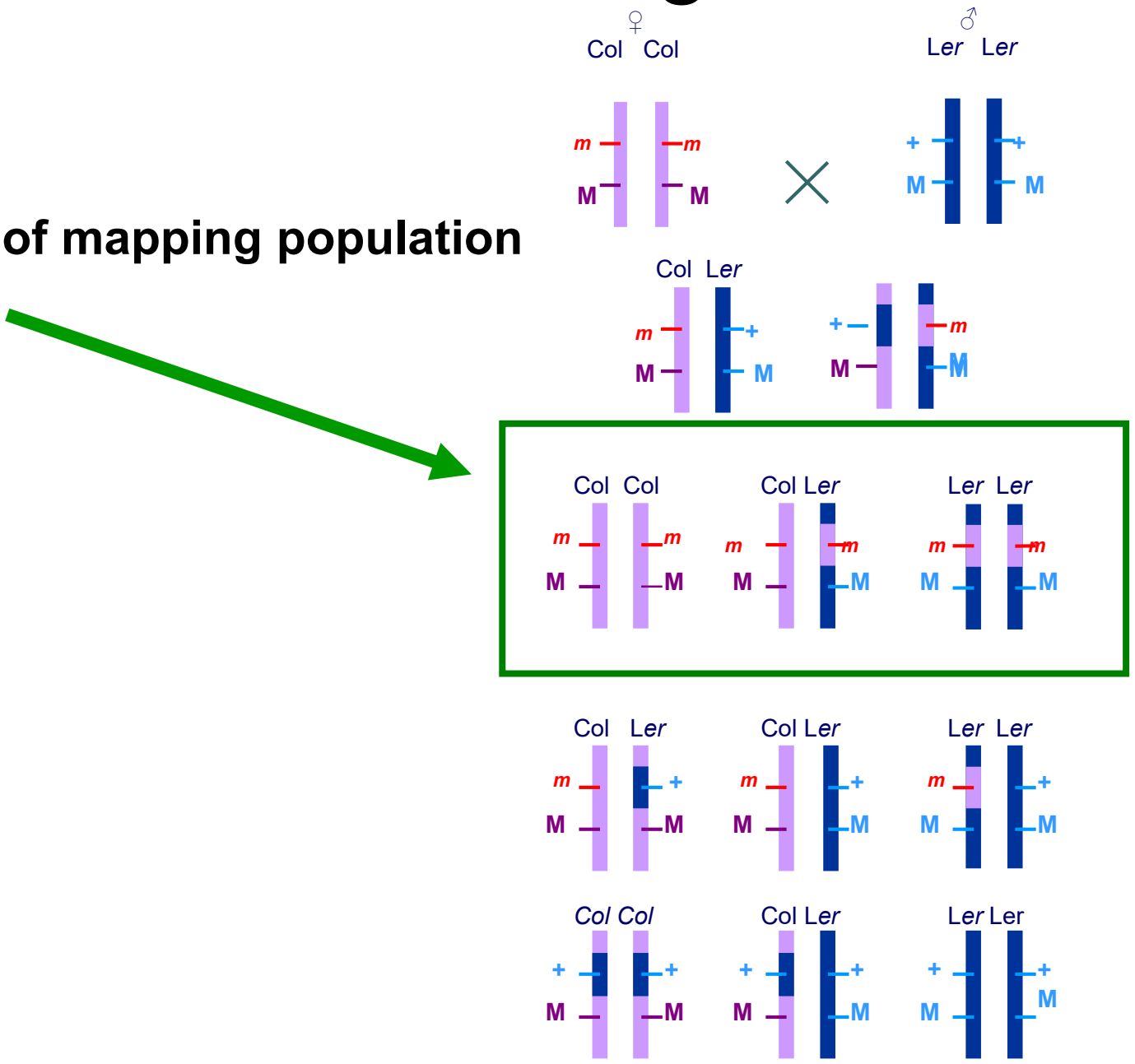
Identification of mutated locus

- Positional cloning
  - Principle: co-segregation analysis of segregating population (mostly of offspring of backcrosses) with molecular markers
    - **SSLP** (Simple Sequence Length Polymorphism)
      - Polymorphism of genome (PCR products) length, amplified using specific primers
    - **RFLP** (Restriction Fragment Length Polymorphism)
      - Detection by Southern blot (PCR after digestion of the genomic DNA and ligation of adapters)
    - **CAPS** (Cleaved Amplified Polymorphic Sequence)
      - Restriction fragment length polymorphism, genome segments amplified by PCR
    - **RAPD** (Randomly Amplified Polymorphic DNA)
      - Polymorphism of length of randomly amplified genome segments, using short 8-10bp primers

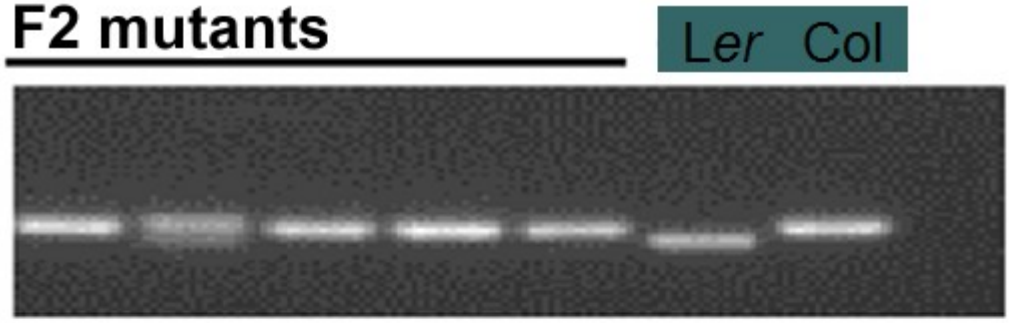
Positional cloning

Preparation of mapping population

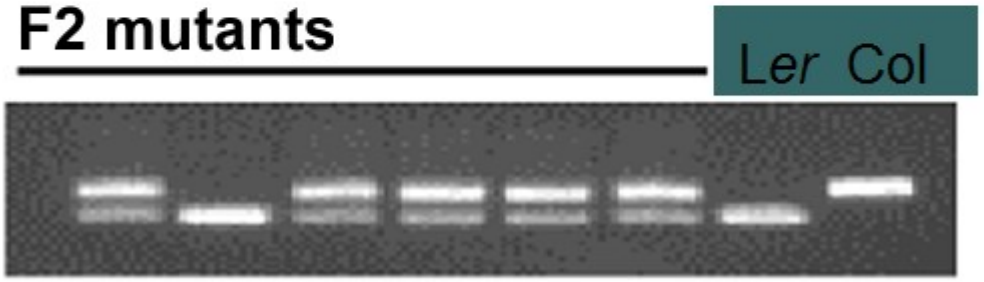


Recombinant analysis – determining the percentage of recombination between mutation and molecular marker

r [%] = number of chromosomes of *Col* / number of all the chromosomes × 100



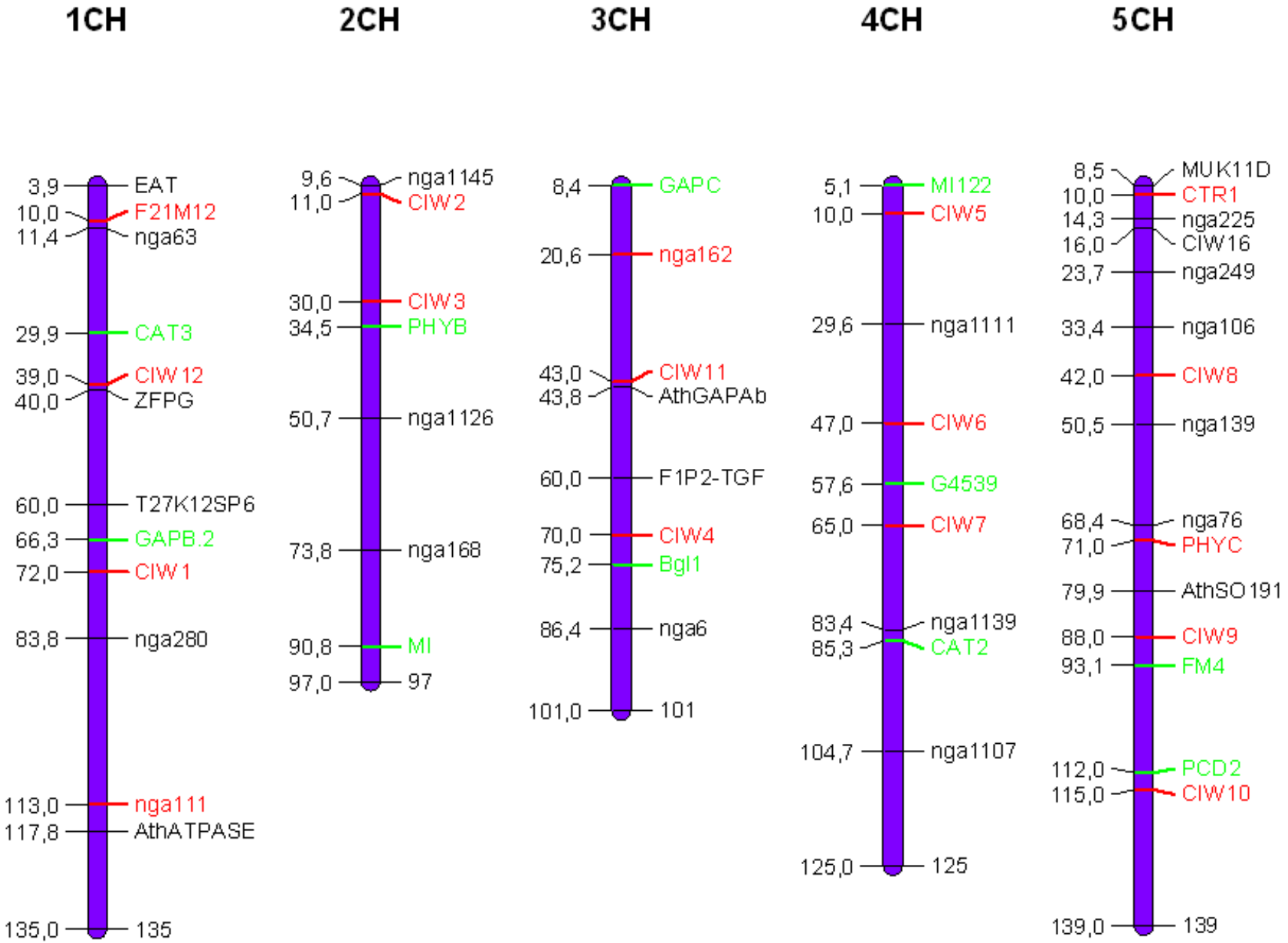
marker I – linked
5 mutants
1/10×100 = 10%



marker II - no linkage
6 mutants
7/12×100 = 58%

- Analysis of approximately 2000 mutant lines
- Determining the closest (still) segregating marker
- Identification of mutation by sequencing

Map of DNA molecular markers



Markers for fine mapping