# How mutagenic is genetic modification of plants, using *Agrobacterium tumefaciens?*

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# Project for COGEM

COGEM: Commission on genetic modification

Advisory committee on approval of experiments and market introduction of GMOs in NL.

# Goals of experiments

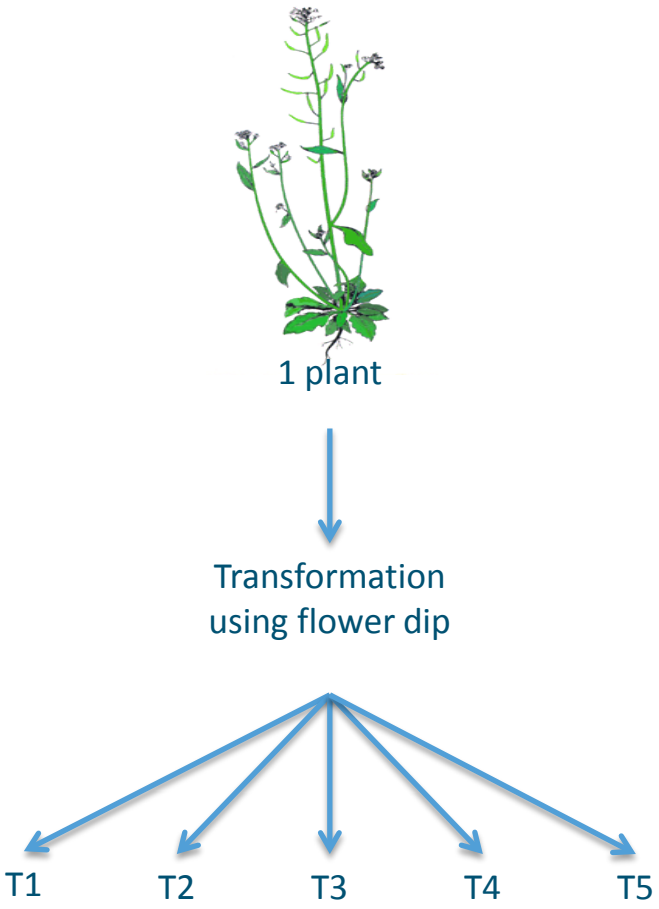
- Evaluate mutations due to
  - transformation;
  - tissue culture and regeneration (= somaclonal variation).
- Compare to the natural variation in cultivars and breeding material;

Experiments performed with:

- *Arabidopsis thaliana*
- Tomato

- *Arabidopsis* experiment
- ~~Tomato experiment~~

# 1. Experiment with *Arabidopsis thaliana*



- DNA of the 5 gm *A. thaliana* plants was isolated
- DNA sheared into short fragments ~ 500 bp
- Both ends of the fragments were sequenced, ~ 100 bp per end (HiSeq Illumina)
- Coverage > 25 X
- Sequence reads aligned to the reference genome of *A. thaliana* Colombia


| sample ID | Sequence reads | reads after trimming | Read Length | % mapped reads | Average Coverage |
|---|---|---|---|---|---|
| **At_T1** | 61683376 | 59480648 | 98.1 | 99.6% | 48.53 |
| **At_T2** | 33049036 | 32103568 | 98.42 | 99.5% | 26.25 |
| **At_T3** | 31968584 | 30902759 | 98.2 | 99.8% | 25.29 |
| **At_T4** | 56915326 | 54937185 | 98.1 | 99.5% | 44.77 |
| **At_T5** | 45006154 | 43359470 | 98.1 | 99.7% | 35.4 |

# Results

1. Genome wide mutation frequency
2. Positions of T-DNA inserts
3. Splinter
4. Deletions at insert sites
5. Structural variation

# Detection of mutations

## Filters:

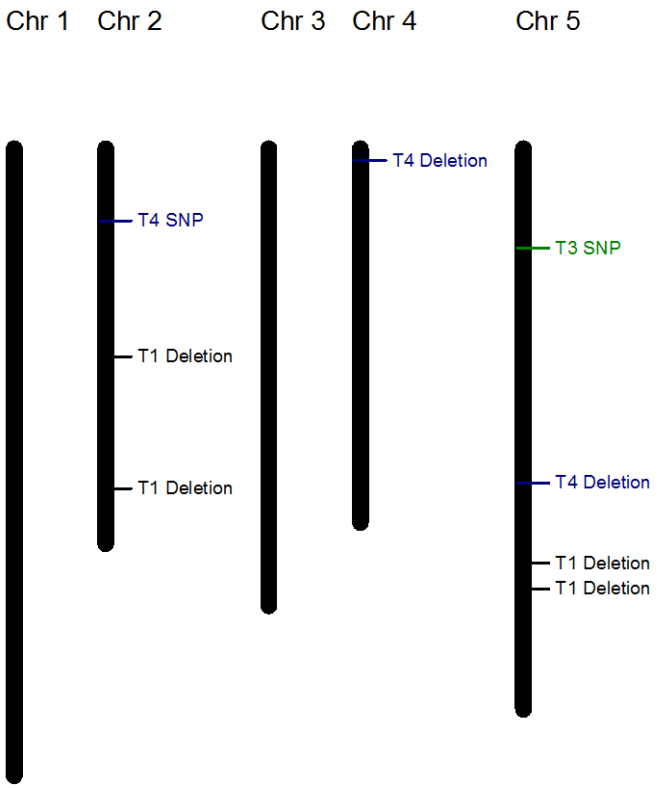
- Find SNVs (simple nucleotide variants) between transformant and reference genome
- Sufficient coverage of the locus (> 10 X), to exclude sequencing errors
- Ignore repetitive DNA
- Select heterozygous SNVs in transformants
- Select SNVs that are present in one transformant only
- For now, ignore the reads with T-DNA

## Check:

- Check each putative mutation visually to exclude false positives

# 1. Genome wide mutation frequency

- 8 mutations in 5 plants, when ignoring the T-DNA
- On the average 8/5 = 1.6 mutations per plant



# Natural mutation rate

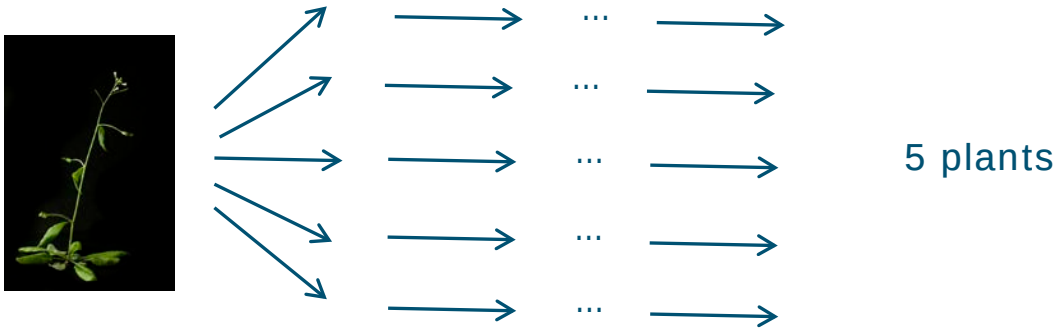
Ossowski et al. (2010) took one plant of *A. thaliana*.

They sowed 5 seeds from this plant.

Harvested seeds from each daughter plant, and sowed one seed per daughter plant: single-seed descendants

For 30 generations in a glasshouse.

Sequenced the 5 genomes.

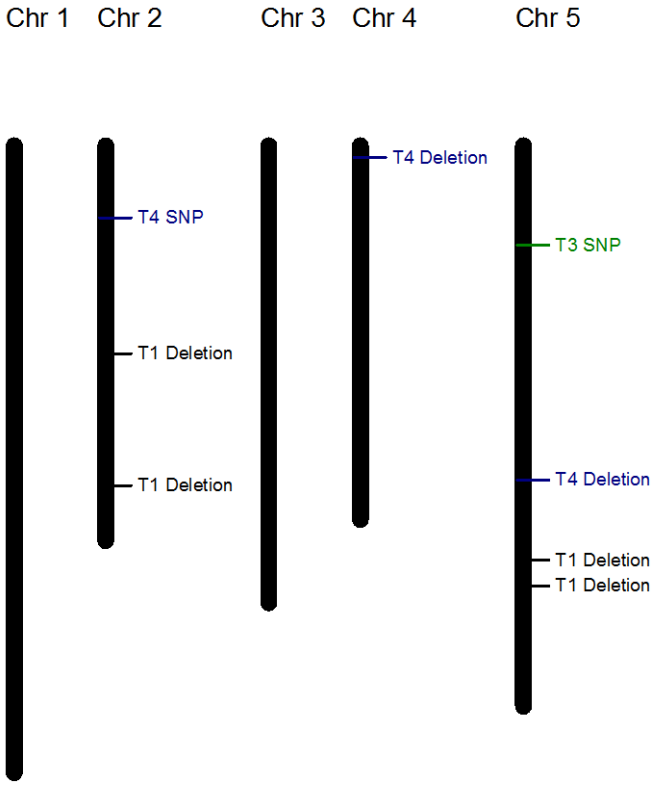


# Natural mutation rate (Ossowski et al. 2010)

- **2.3 spontaneous mutations per plant per generation**.
- 10 times more base substitutions (SNPs) compared to deletions
- Some large deletions (> 5000 bp)

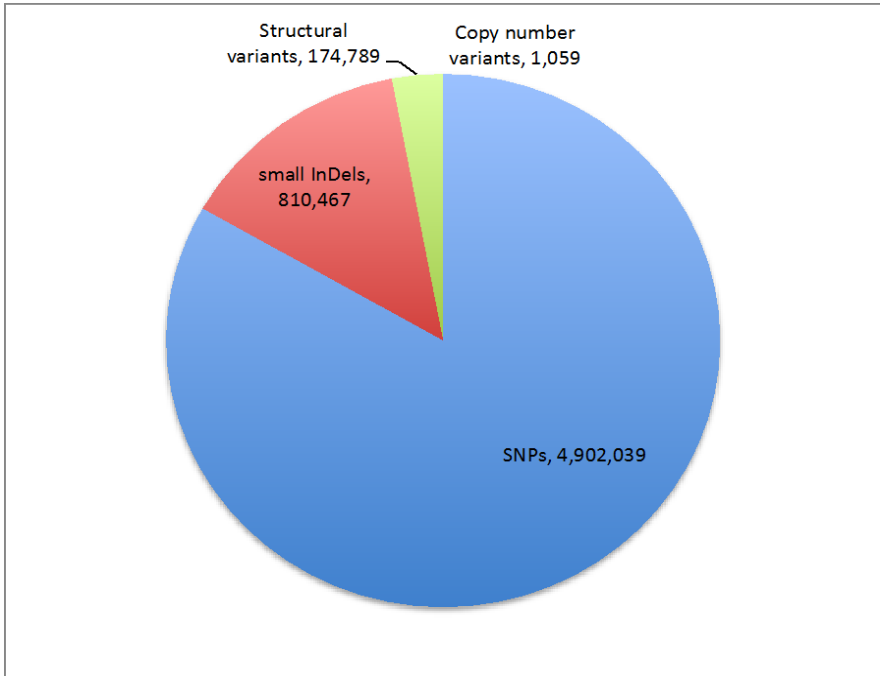
# Comparison mutation rate after trafo to natural mutation rate

- We found on the average 8/5 = 1.6 mutations per plant
  - Not significantly different from 2.3
  - More deletions?



# Baseline. Natural variation within the species

- Result of accumulated mutations during evolution of the species
- Cao et al. (2011) identified 5.9 million polymorphisms across 80 *A. thaliana* strains

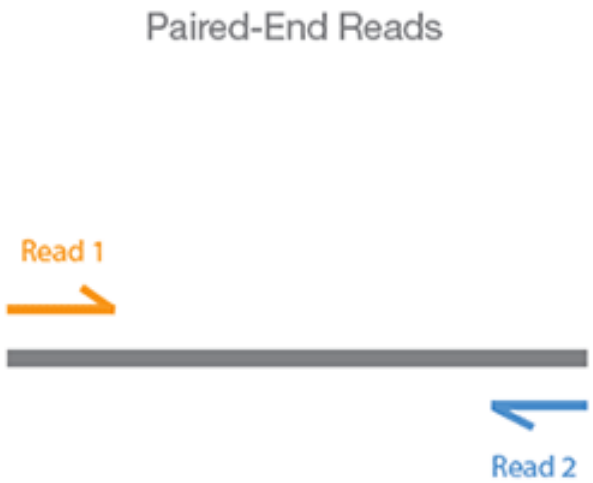



Conclusion:

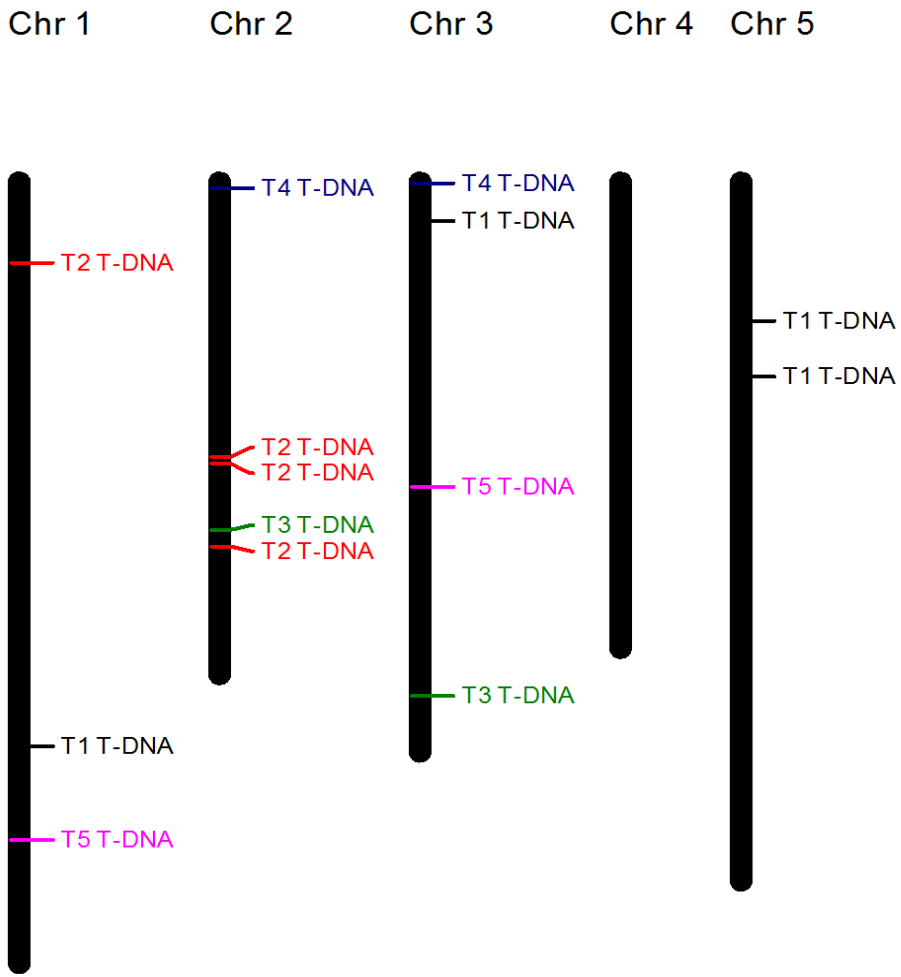
The number of mutations after trafo is very small compared to the existing natural variation (baseline), disregarding the T-DNA itself.

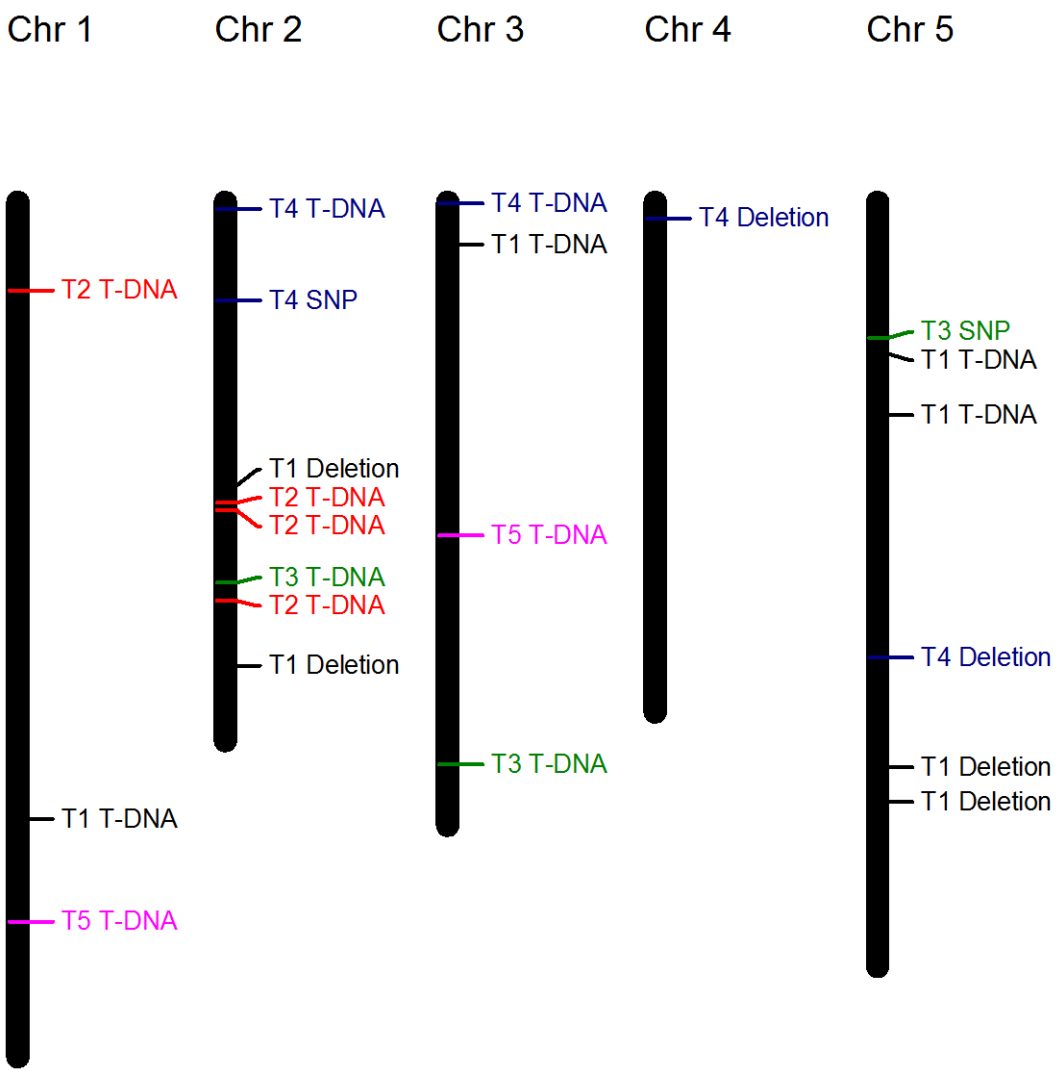
# 2. Positions of T-DNA inserts

- We aligned reads to reference genome of *A. thaliana* and to vector sequence of *A. tumefaciens* (incl. T-DNA)
- Searched for:
  - Reads that mapped to vector sequence (incl. T-DNA)
  - Broken pairs
  - Split reads




# Multiple inserts



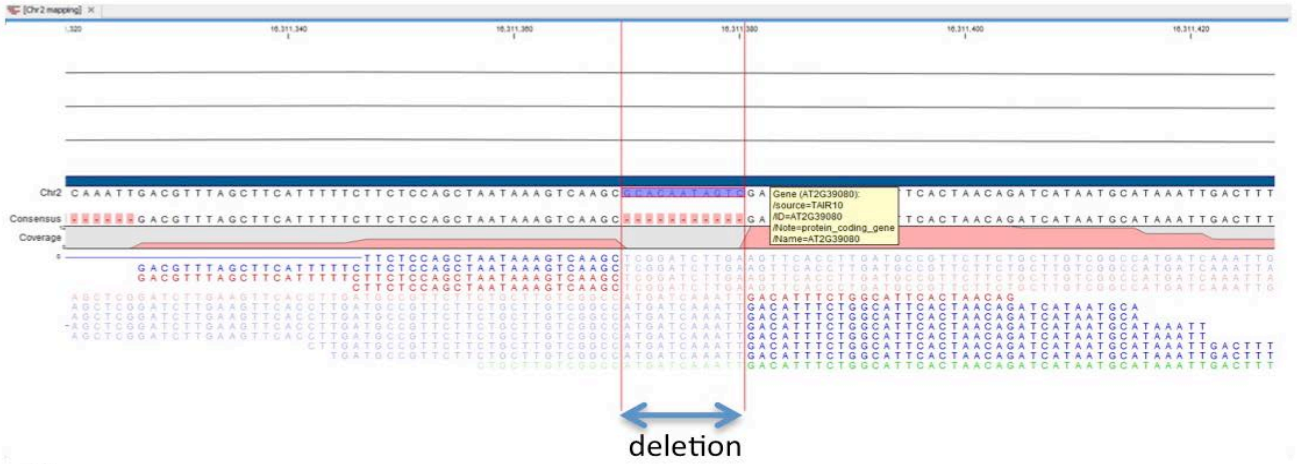

Chr 1
Chr 2
Chr 3
Chr 4
Chr 5
T2 T-DNA
T1 T-DNA
T5 T-DNA
T4 T-DNA
T4 SNP
T1 Deletion
T2 T-DNA
T2 T-DNA
T3 T-DNA
T2 T-DNA
T1 Deletion
T4 T-DNA
T1 T-DNA
T5 T-DNA
T3 T-DNA
T4 Deletion
T3 SNP
T1 T-DNA
T1 T-DNA
T4 Deletion
T1 Deletion
T1 Deletion

- No association between T-DNA insert sites and SNVs
  - Frequency
  - Position

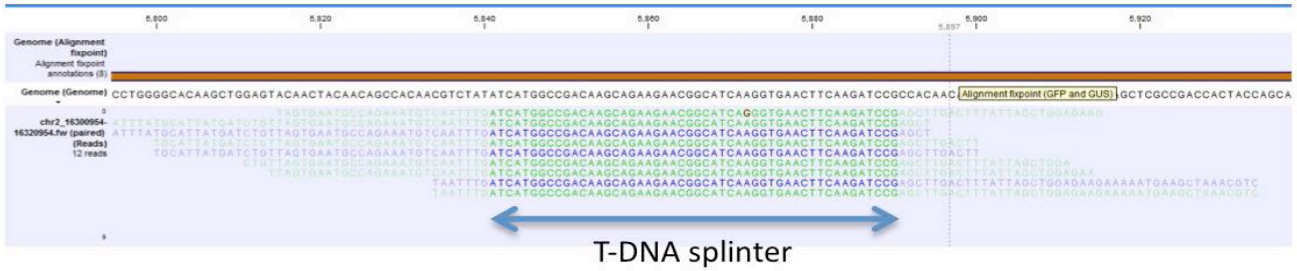
# 3. Splinter

- In one plant (T2) a T-DNA splinter of 50 bp
- A small fragment of *gfp* gene
- Remarkable: not near a border, but from the middle of the T-DNA
- Heterozygous
- Not in the other transformants

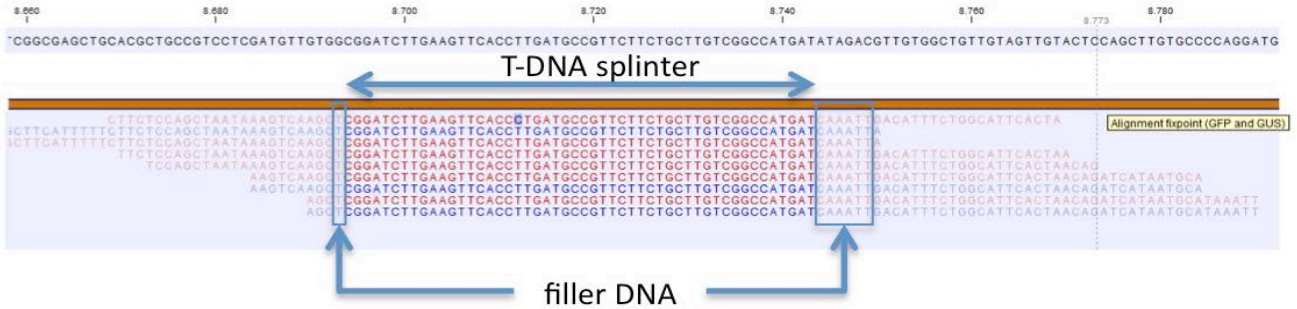
A.



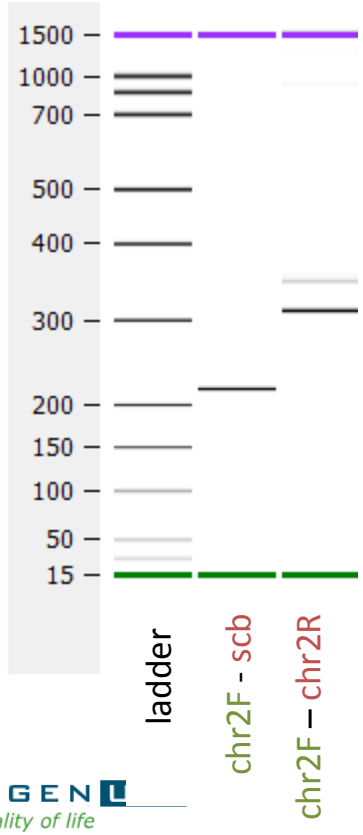

B.




C.



# PCR for confirmation of splinter


Bands were sequenced.
Confirmed the sequences of the bioinformatic analysis.

Agilent Bioanalyzer

# Splinter

- In an intron. Would be spliced out.
- Would probably be overlooked when using Southern blotting or genome walking kits
- NGS more sensitive than conventional methods

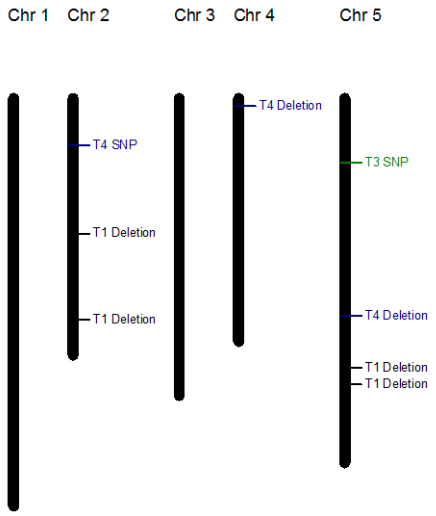
# Results

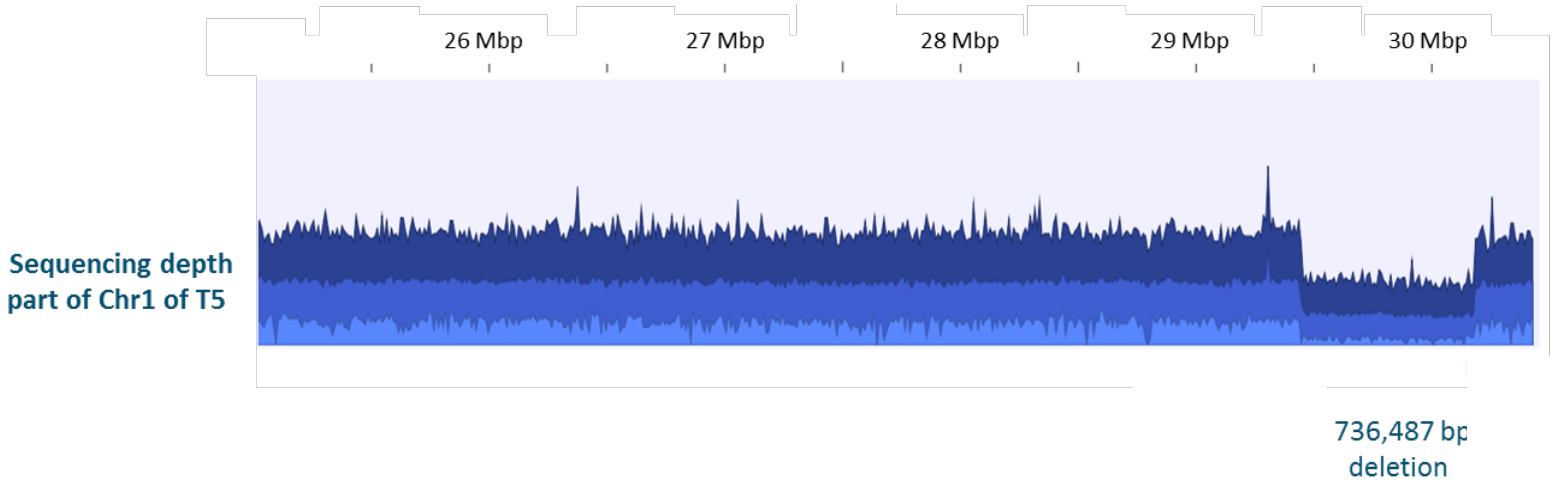
1. Genome wide mutation frequency
2. Positions of T-DNA inserts
3. Splinter
4. **Deletions at insert sites**
5. Structural variation

# 4. Deletions at insert site

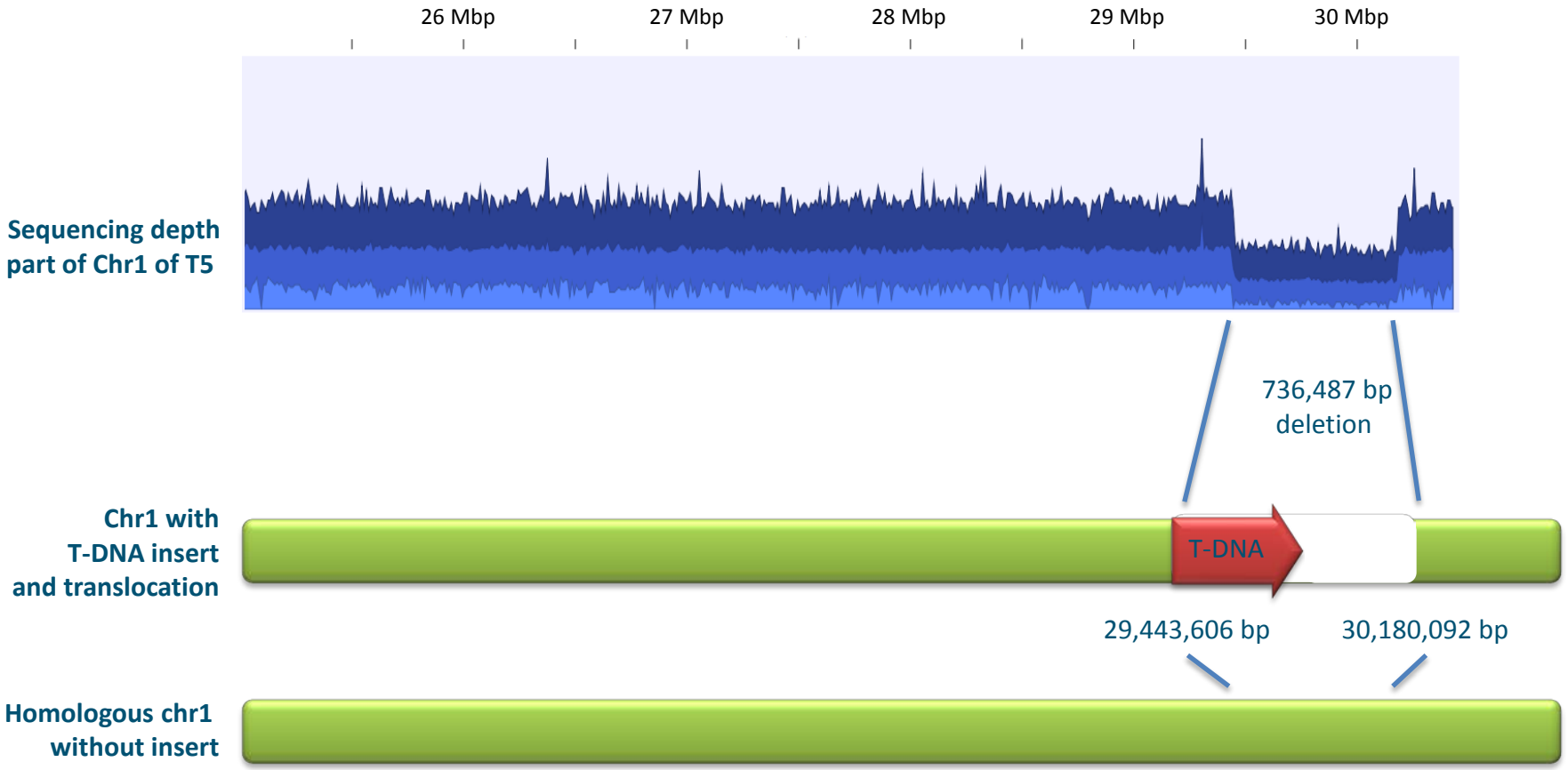
- 12 T-DNA insert sites detected
- In 8 of these sites, deletions were found in *A. thaliana*
- Usually small deletions (1 – 10 bp)
- One very large (>700 kb) deletion, flanking the T-DNA
- Conclusion: deletions in the genomic DNA in the T-DNA insert sites were common.
- These deletions were disregarded in

the slide on genome-wide mutations






- Deletion removed many genes
- Heterozygous
- Plant still looked normal



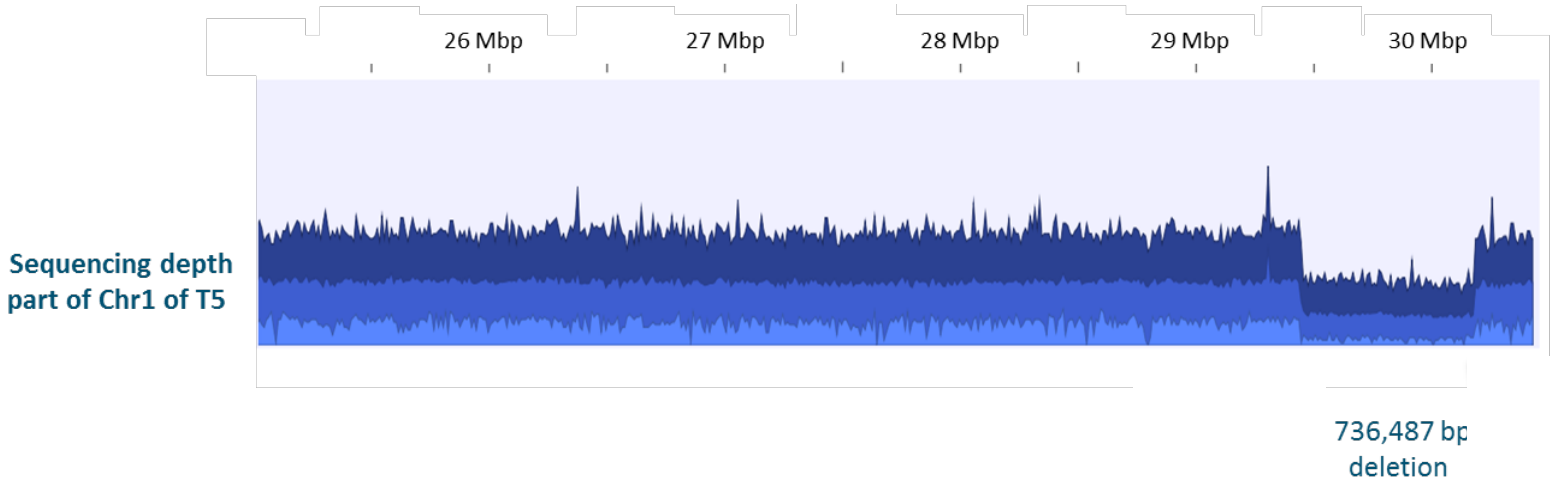

Although the **primary transformant** grew normally, the **next generation** that would contain the T-DNA homozygously, might not survive, due to the large deletion

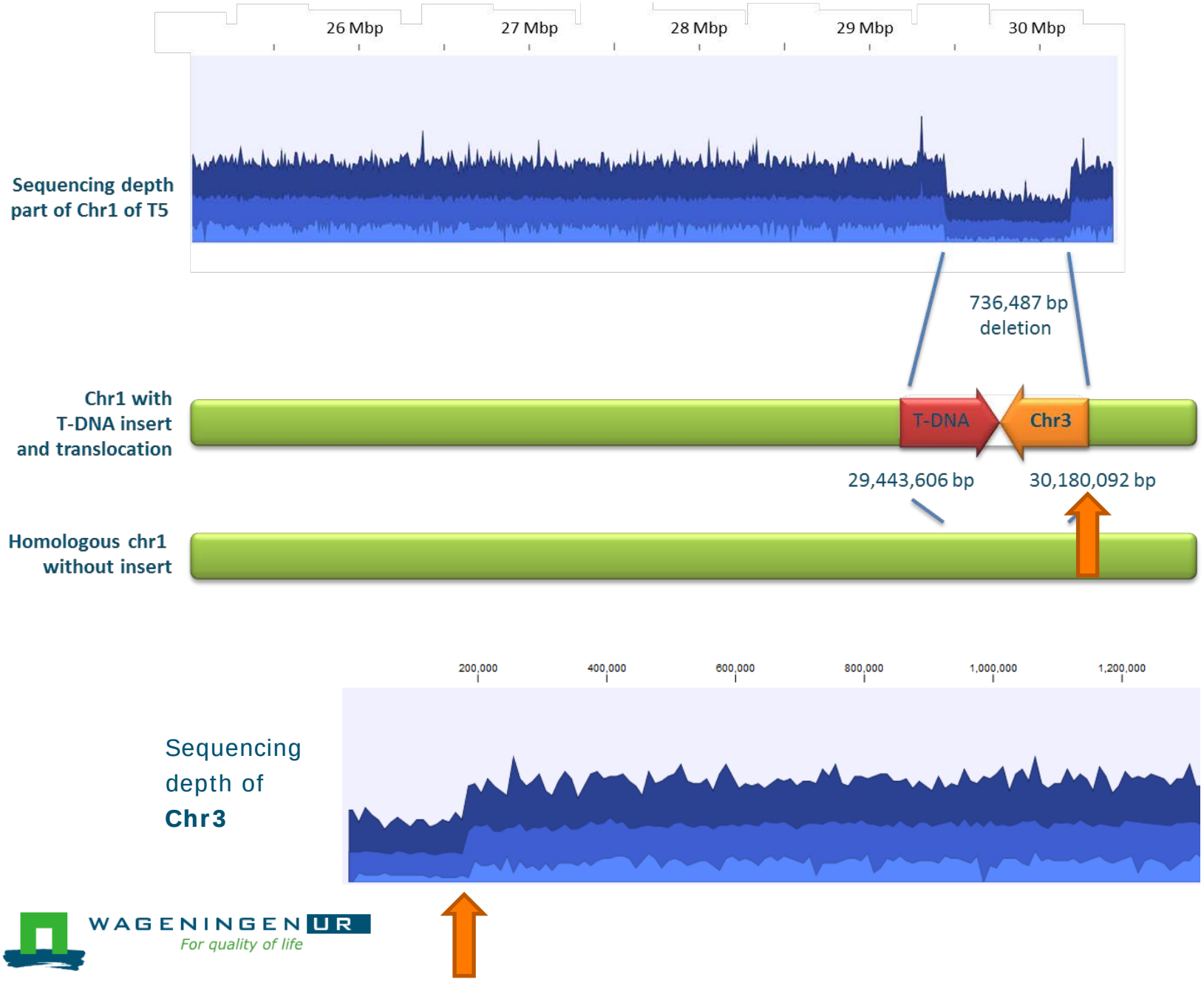
So, possibly loss of transgenic plants in second generation.

# 5. Structural variation

- In 4 out of 5 plants putative translocations detected:

Fragments from other chromosomes were flanking T-DNA inserts


26 Mbp
27 Mbp
28 Mbp
29 Mbp
30 Mbp
Sequencing depth
part of Chr1 of T5
736,487 bp
deletion

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# Structural variation (cont.)

- We found putative translocations, neighbouring T-DNA inserts, in 4 out of 5 transgenic plants
- Totally 12 T-DNA insert sites.

- Literature study: Similar rearrangements mentioned in transgenic *Arabiodopsis thaliana*, after floral dip.

- We re-sequenced 3 transgenic tomato plants. No indications for chromosomal rearrangements.
- Rearrangements increased when using floral dip?

# Conclusions from *Arabidopsis* experiment

- No increased frequency of small mutations, when disregarding T-DNA insert sites
- The transformation can induce deletions at the T-DNA insert sites
- Indications for translocations to insert sites after floral dip

# Co-workers

**Arabidopsis**

- Gerco Angenent
- Marian Bemer
- Jan Schaart

**Sequencing and bioinformatics**

- Bas te Lintel Hekkert
- Elio Schijlen
- Henri van de Geest
- Sofia Papadimitriou
- Gabino Sanchez Perez

**Discussion**

- René Smulders