Agriculture and Agri-Food Canada
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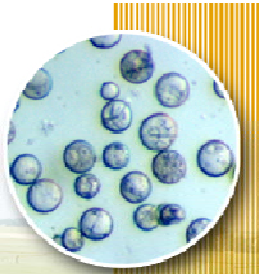

# Transfection of the Three Plant Organelles Using Cell Penetrating Peptides

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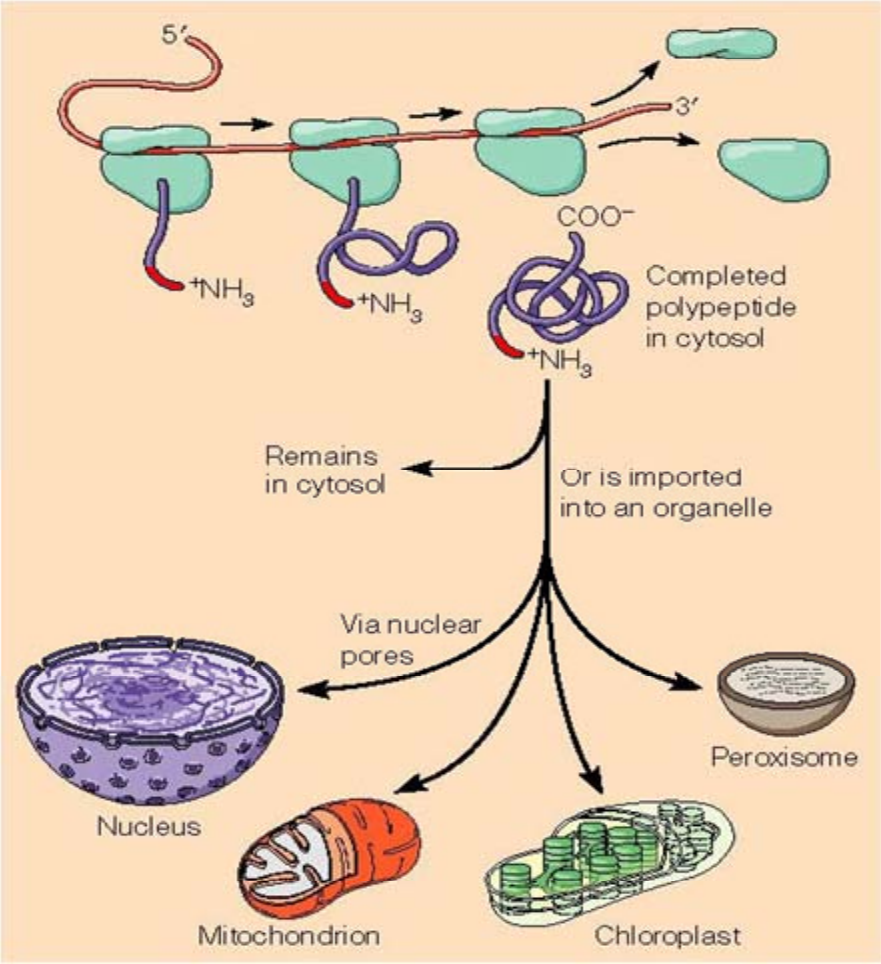
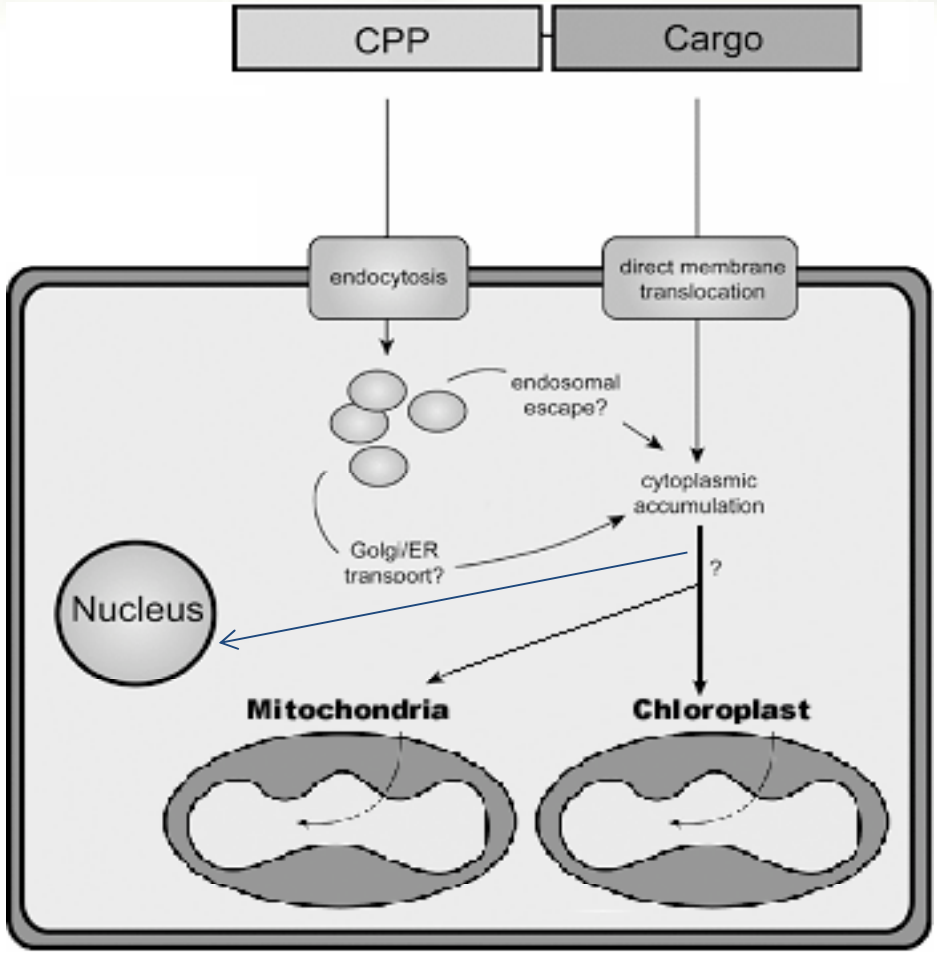
Canada

# What are Plant Organelle Targeting Cell Penetrating Peptides?




Protein Sorting Signals

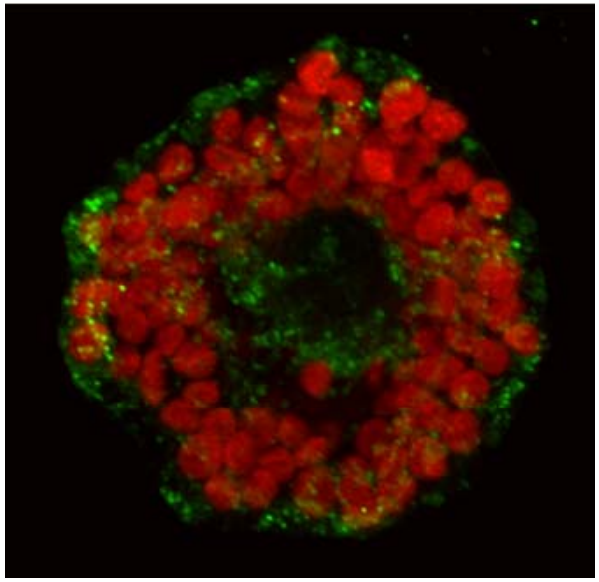



Cell Penetrating Peptides

# Mitochondrial targeting peptide

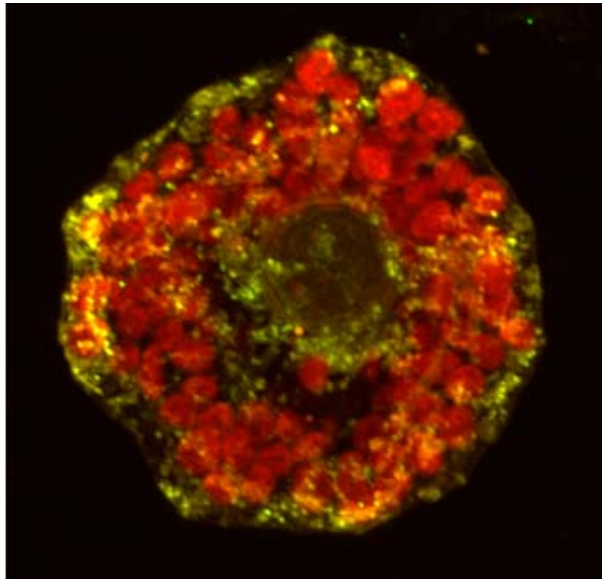
# Nuclear targeting CPP

**Peptide + Chloroplasts**

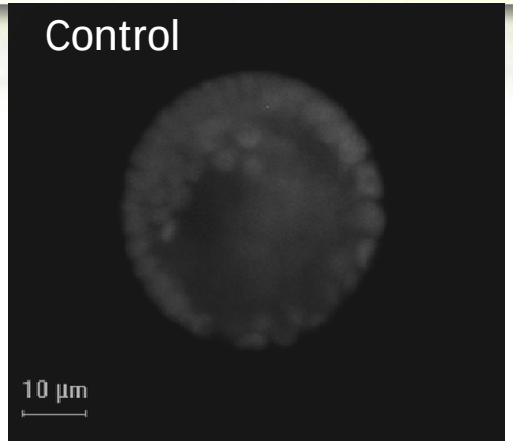


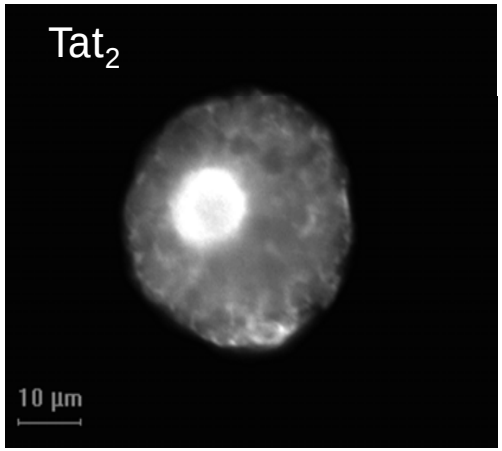
**Protoplast transfected with the green fluorescently labeled mTP peptide.**

**Peptide + Mitochondria + Chloroplasts**



**Protoplast transfected with the green fluorescently labeled mTP peptide co-localized with MitoTracker Orange.**



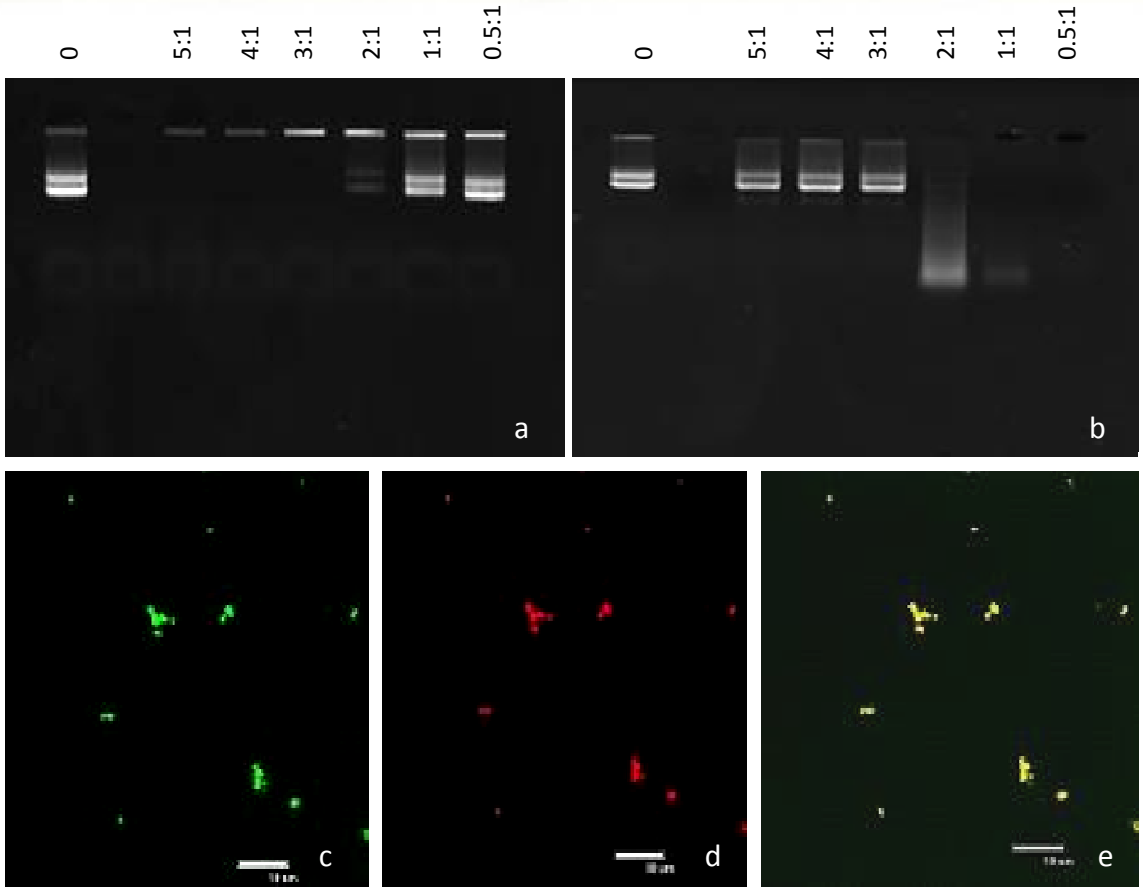




**Protoplast transfected with the fluorescently labeled nuclear CPP**

# CPPs complex and protect DNA

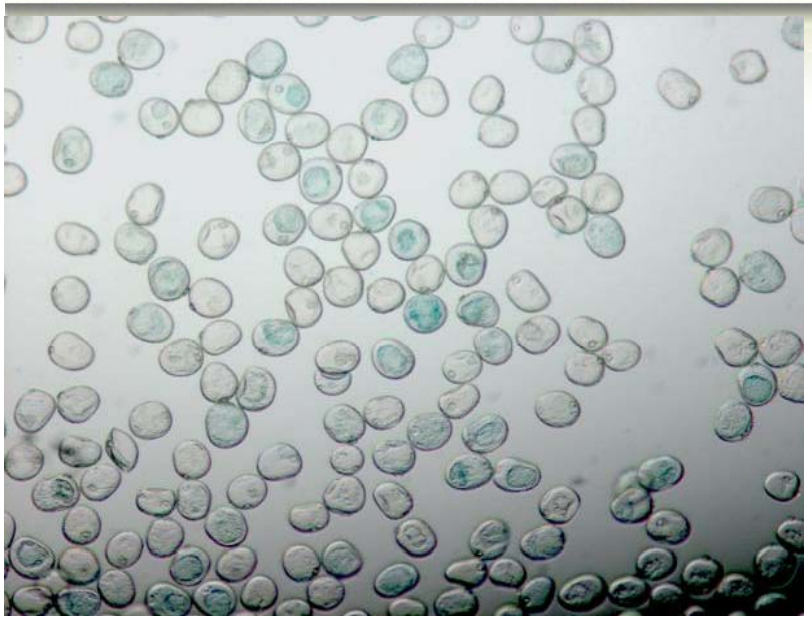
(A) Gel retardation assay
(B) DNaseI protection assay
(C) Confocal laser microscopy

Complexes of various sizes (in range 0.85–4 µm

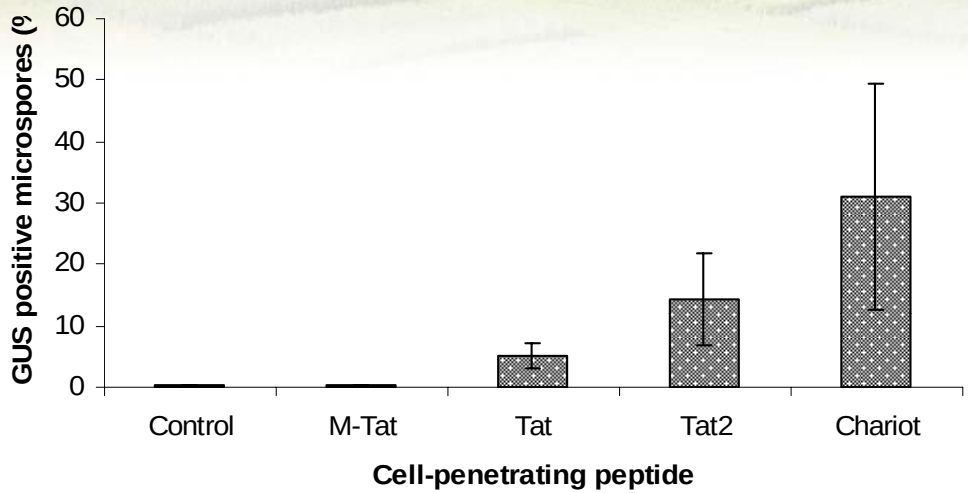


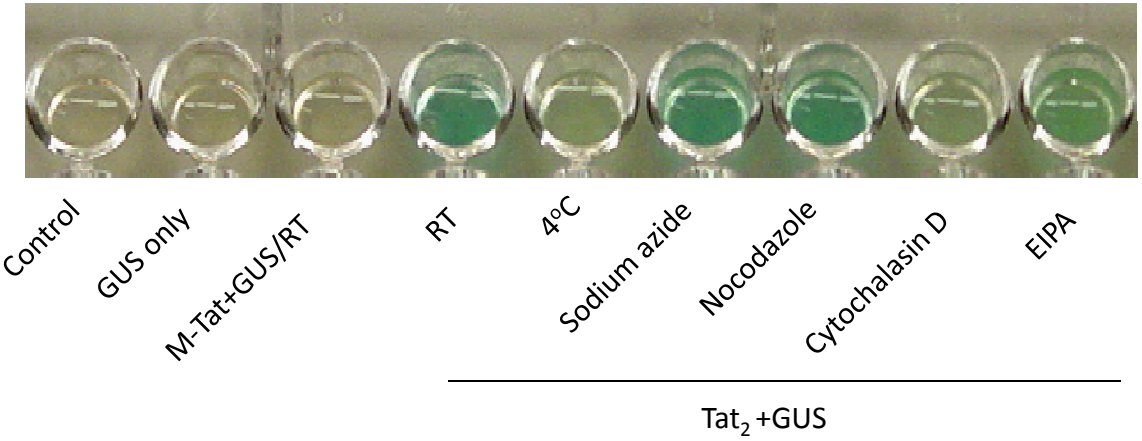

Tat2-fluorescein p*Act-1GUS*-rhodamine Merge

# CPP-Protein complex trafficking in microspore



GUS enzyme complexed with pep-1 (Chariot)

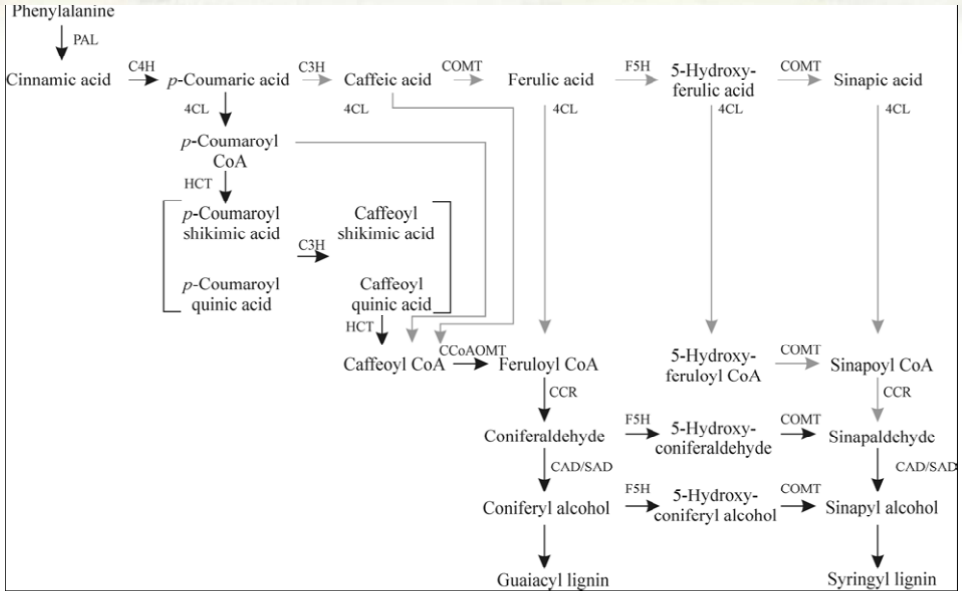


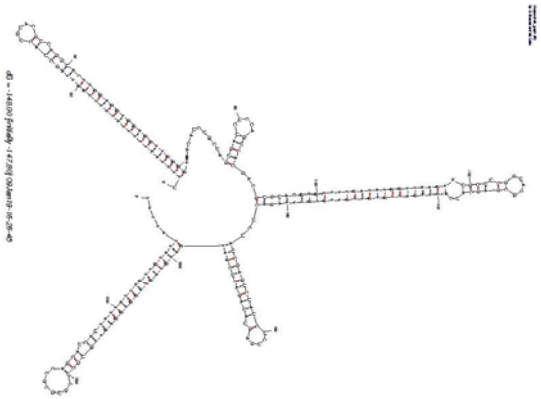




Active transport

# Nuclear delivery: Application to silencing triticale lignin pathway

- 4CL and CCoAOMT under constitutive promoter (Actin) and terminator (Nos)
- CAD and OMT
- Scrambled sequences
- Co-delivery double genetic constructs
- Linearized GUS construct to confirm the successful transfection
- dsDNA, Tat2, with or without RecA
- semi-automated production system (Rita Box) to produce candidate transgenic lines

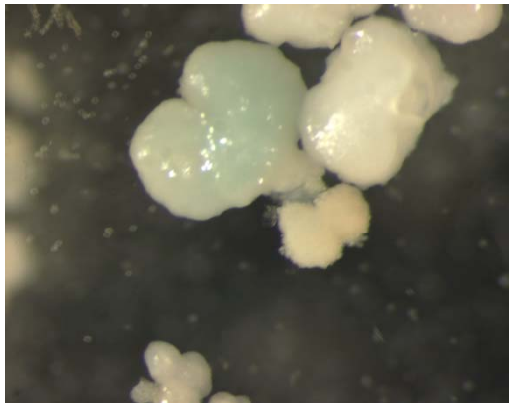


# Silencing triticale lignin pathway

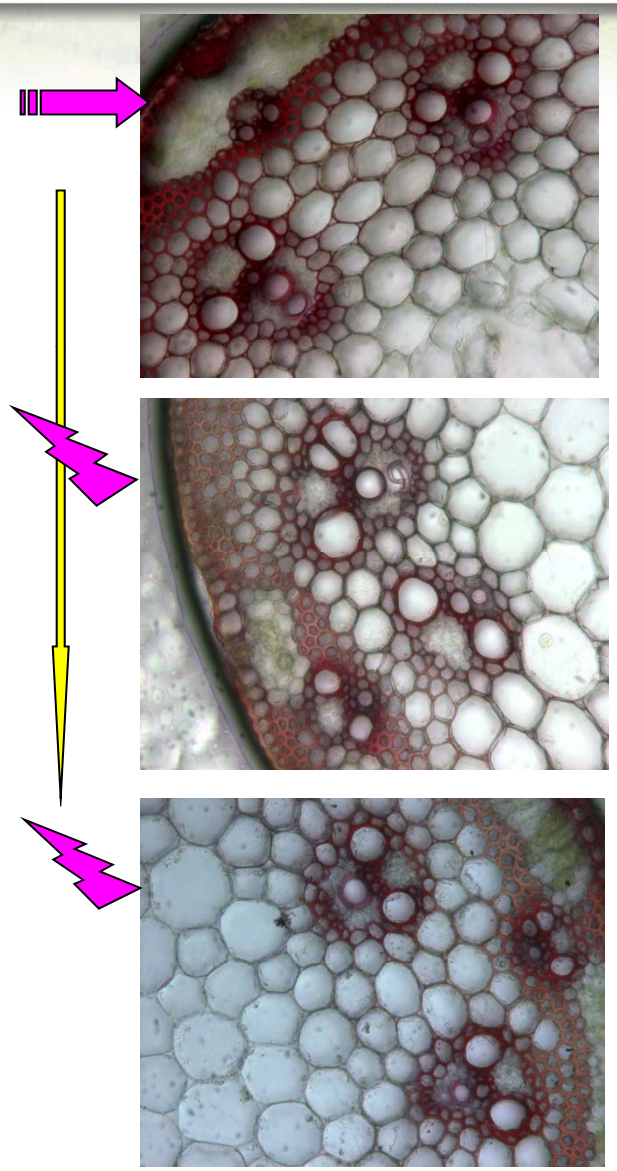
Results:

- 444 lines produced
- GUS histochemical assay of embryos confirmed the transfection is successful
- Rita Box for plant regeneration is time and labour saving and highly efficient
- The  stages qRT-PCR confirm silencing in 46 T0s (13.46%)
- RecA protein increased the transgene expression (close to 4-fold )
- The level of lignin reduction varied among the transformants to reach up to 50%
- Tissue sections prepared for imaging and quantification of lignin and cellulose.
- phenotypic difference was observed



# Silencing triticale 4cl with RNAi technology

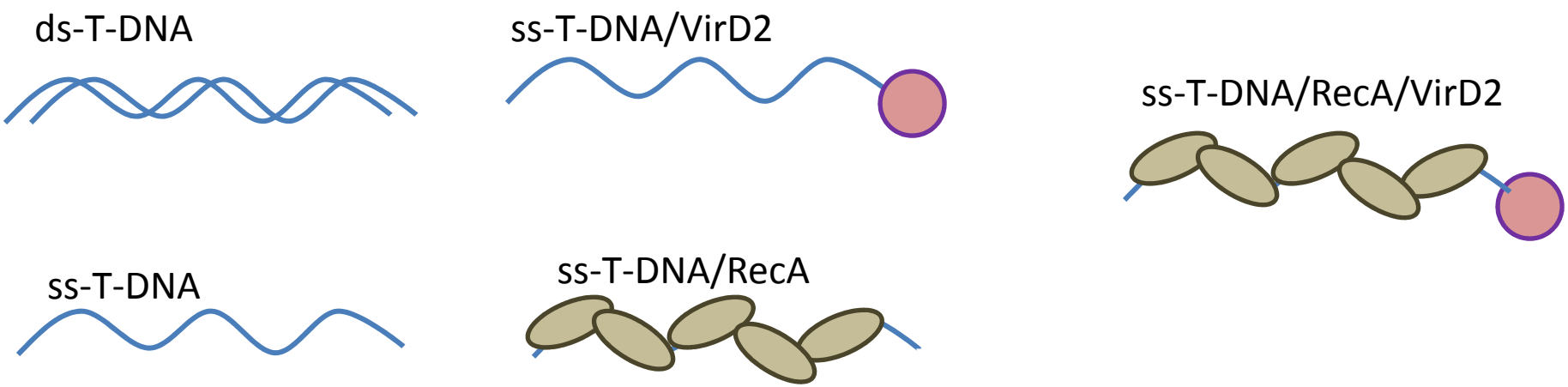
| Sample Name | T. Lignin | % of decreased lignin |
|---|---|---|
| Germplasm Test Samples | 15.79 | 0.00 |
| 4cl 052 | 15.67 | 0.76 |
| 4cl 053 | 15.47 | 2.03 |
| 4cl 129 | 14.73 | 6.71 |
| 4cl 101 | 14.65 | 7.22 |
| 4cl 142 | 14.59 | 7.60 |
| 4cl 151 | 14.23 | 9.88 |
| 4cl 079 | 13.8 | 12.60 |
| 4cl 070 | 13.51 | 14.44 |
| 4cl 054 | 12.65 | 19.89 |
| 4cl 133 | 12.55 | 20.52 |
| 4cl 099 | 12.4 | 21.47 |
| 4cl 074 | 12 | 24.00 |
| 4cl 065 | 11.99 | 24.07 |
| 4cl 125 | 11.45 | 27.49 |
| 4cl 114 | 11.39 | 27.87 |
| 4cl 094 | 11.16 | 29.32 |
| 4cl 096 | 10.56 | 33.12 |
| 4cl 081 | 10.22 | 35.28 |
| 4cl 089 | 9.71 | 38.51 |
| 4cl 095 | 9.39 | 40.53 |
| 4cl 100 | 9.38 | 40.60 |
| 4cl 119 | 8.8 | 44.27 |



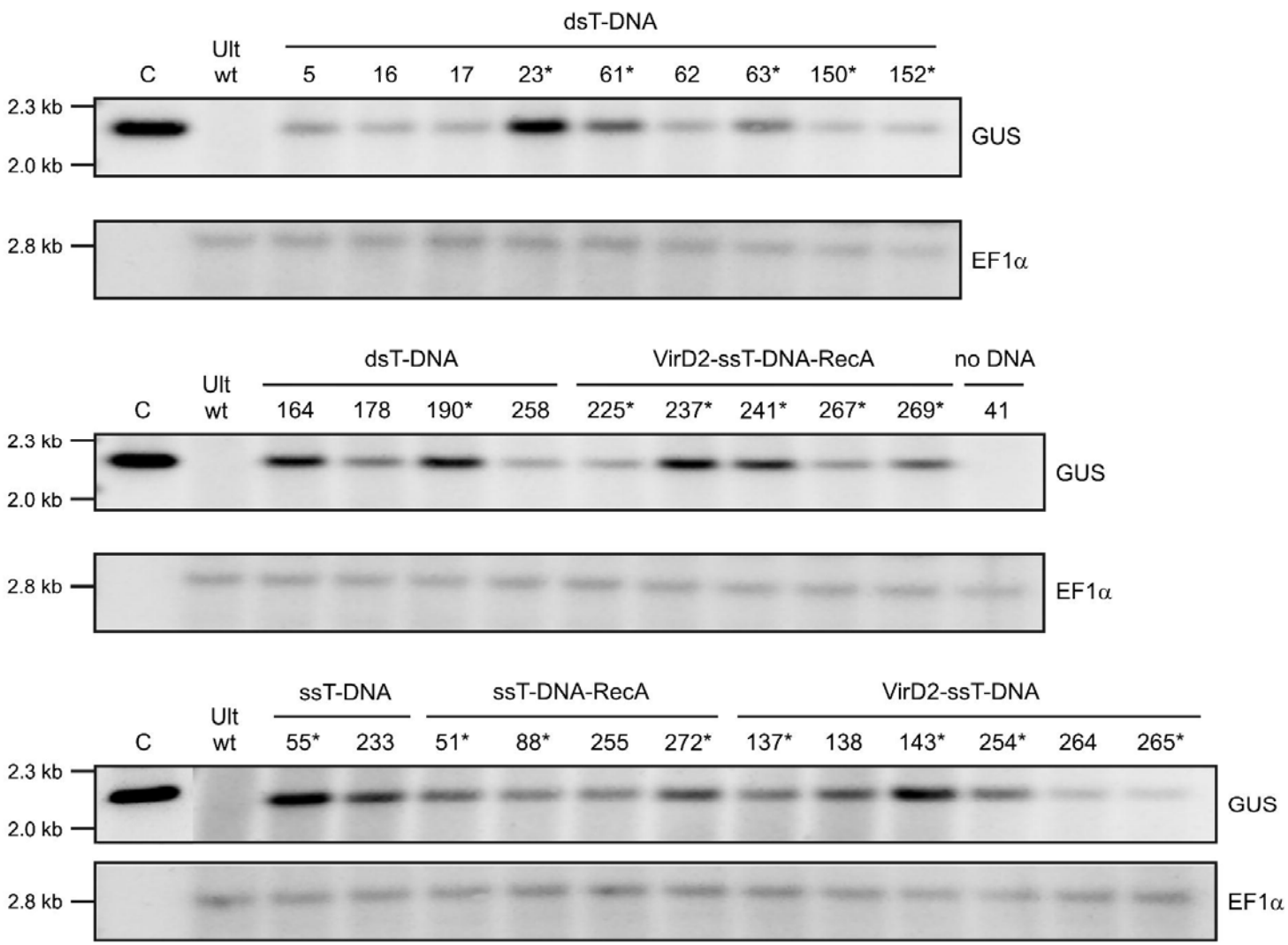
# Nuclear delivery: T-DNA delivery independent of *Agrobacterium*

We tested whether delivery of T-DNA protected with RecA protein and guided by VirD2 protein would result in better quality of transgene integrations.

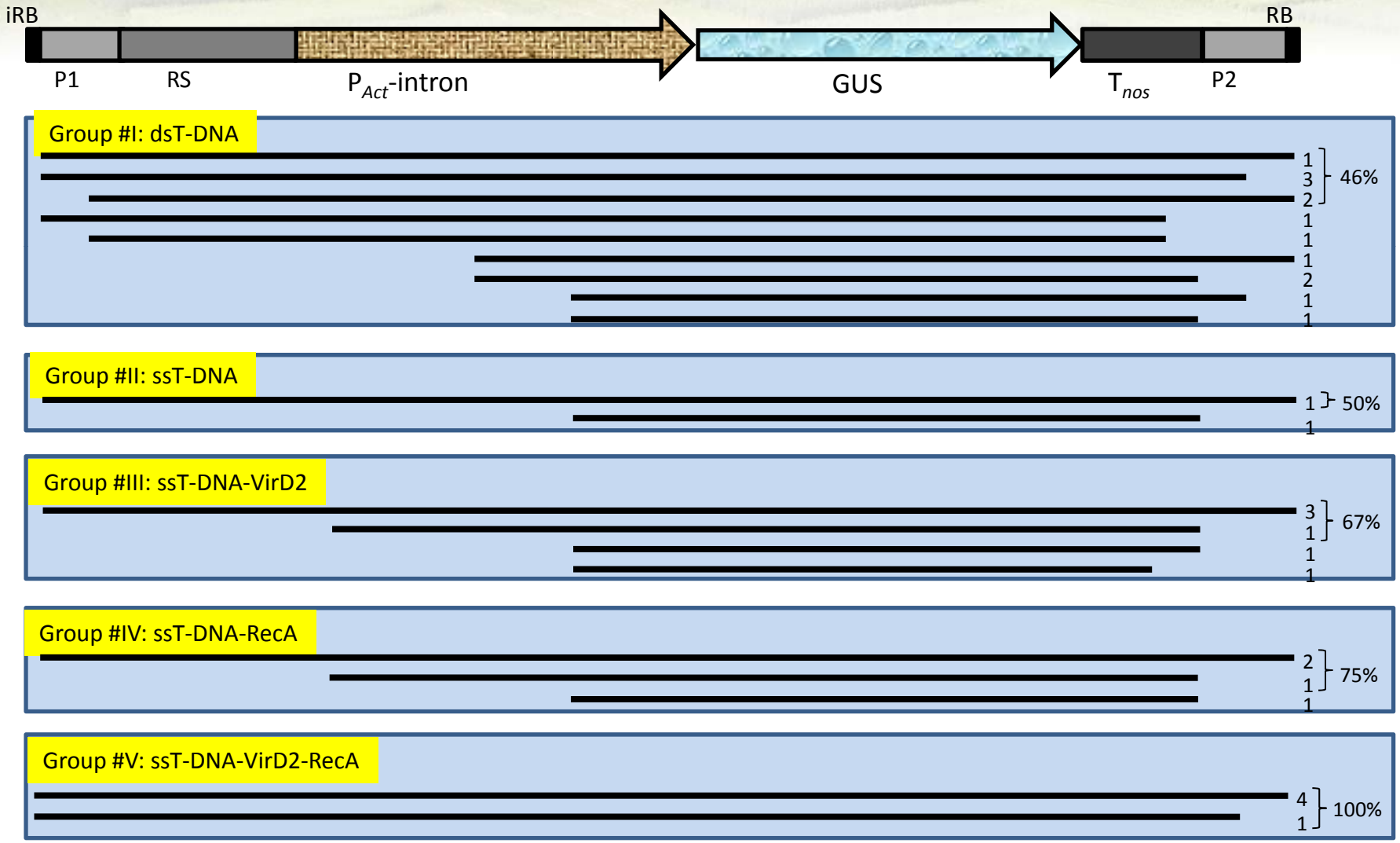
Five cargoes delivered by Tat2:



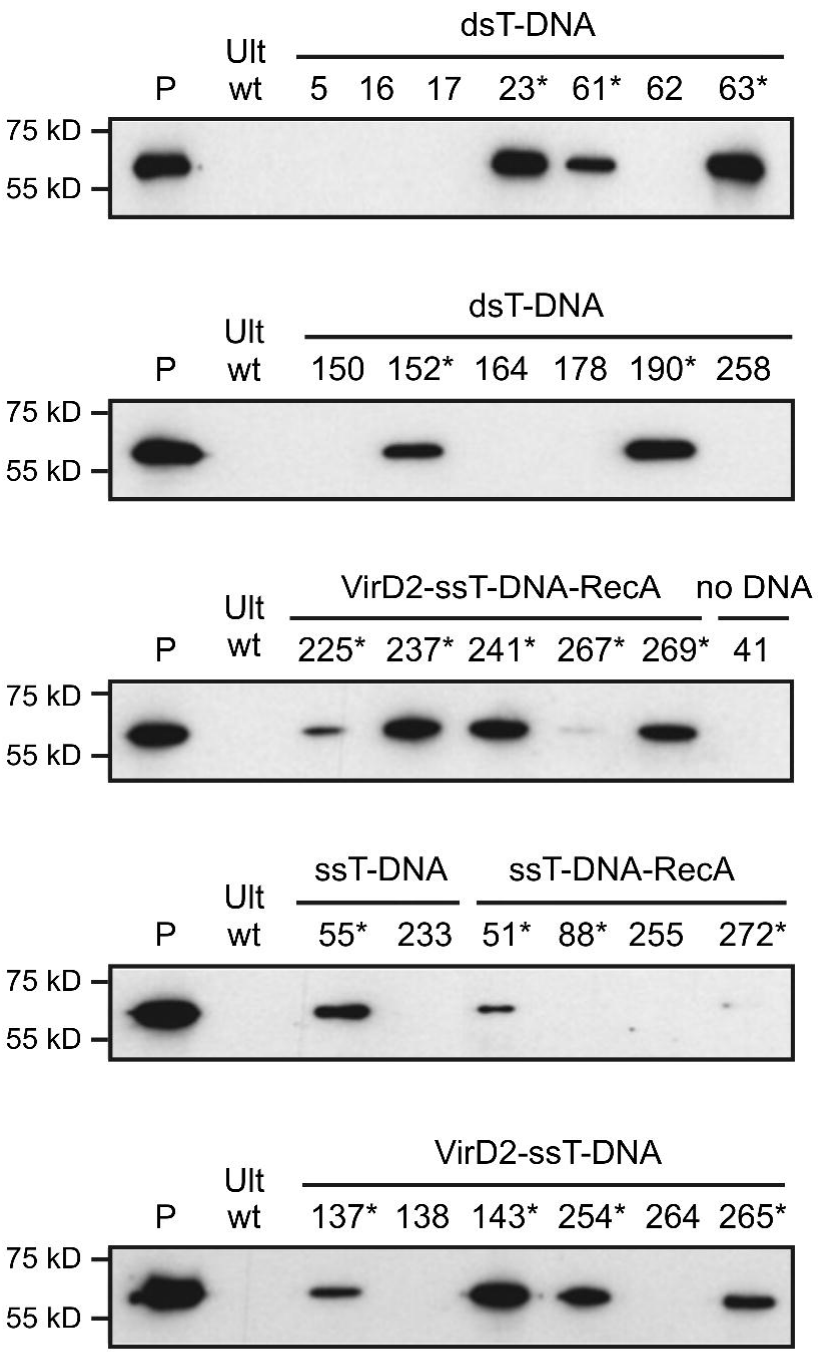
# Southern blot analysis of transgenic triticale



# Preserving the integrity of the transgene



# Western blot analysis on protein extract from GUS positive doubled haploids regenerated from microspores



# Microspore competency for nuclear homologous recombination

## HR related gene transcripts in microspores

| Sample | Cultivar | RAD 51A1 | RAD 51C | RAD 51D | Dmc1 | EF1a |
|---|---|---|---|---|---|---|
| Microspores | CDC Teal | 0.0 | 0.5 | 3.4 | 3.0 | 1000.0 |
| Microspores | Fielder | 0.1 | 0.5 | 3.2 | 2.6 | 1000.0 |
| Microspores | T036 | 0.0 | 1.1 | 3.9 | 2.0 | 1000.0 |
| Microspores | Ultima | 0.1 | 0.6 | 5.5 | 2.1 | 1000.0 |
| | | | | | | |
| Pre-treated microspores | CDC Teal | 0.1 | 0.7 | 5.2 | 13.6 | 1000.0 |
| Pre-treated microspores | T036 | 0.1 | 0.9 | 9.6 | 13.6 | 1000.0 |
| Pre-treated microspores | Ultima | 0.1 | 0.5 | 5.9 | 15.6 | 1000.0 |
| | | | | | | |
| Leaf | CDC Teal | 0.5 | 0.3 | 0.4 | 0.8 | 1000.0 |
| Leaf | Fielder | 0.0 | 0.4 | 0.2 | 0.8 | 1000.0 |
| Leaf | T036 | 3.4 | 2.6 | 0.1 | 0.9 | 1000.0 |
| Leaf | Ultima | 1.3 | 1.4 | 0.2 | 0.4 | 1000.0 |

# Site targeted point mutations – ALS locus

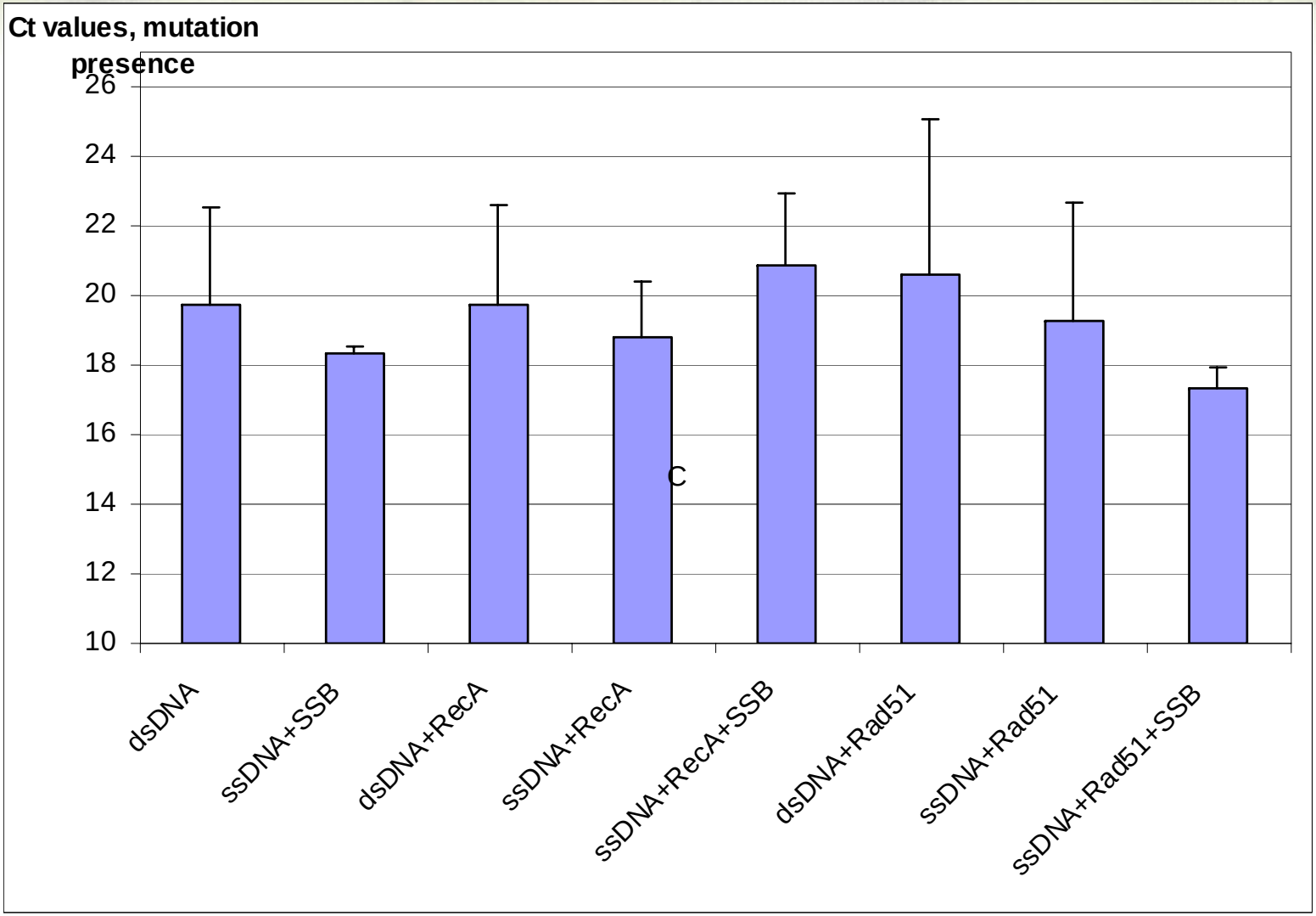
## ALS gene sequence target

```
CDC Teal sequence        GTTCGTGTGACGAAGAAGAGCGAAGTCACTGCAGCAATCAAGAAGATGCTTGA**GACCCCA** 420
Transfecting fragment    GTTCGAGTGACGAAGAAGAGCGAAGTCACTGCAGCAATCAAGAAGATGCTTGAGACCCCA 383
Sequenced contig         ----------------------------------------------------------------------------------------GACCCCA 7

CDC Teal sequence        **GGGCCATACTTG**TTGGATATCATCGTCCCGCATCAGGAGCACGTGCTGCCTATGATCCCA 480
Transfecting fragment    GGGCCATACTTGTTGGATATCATAGTCCCGCATCAGGAGCACGTGCTGCCTATGATCCCA 443
Sequencing contig        GGGCCATACTTGTTGGATATCATAGTCCCGCATCAGGAGCACGTGCTGCCTATGATCCCA  67

CDC Teal sequence        AGCGGTGGTGCTTTCAAGGACATGATCATGGAGG**GTGATGGCAGGACCTCGTAC** 534
Transfecting fragment    AACGGTGGTGCTTTTAAGGACATGATCATGGAGG------------------- 477
Sequencing contig        AACGGTGGTGCTTTTAAGGACATGATCATGGAGGGTGATGGCAGGACCTCGTAC 121
```

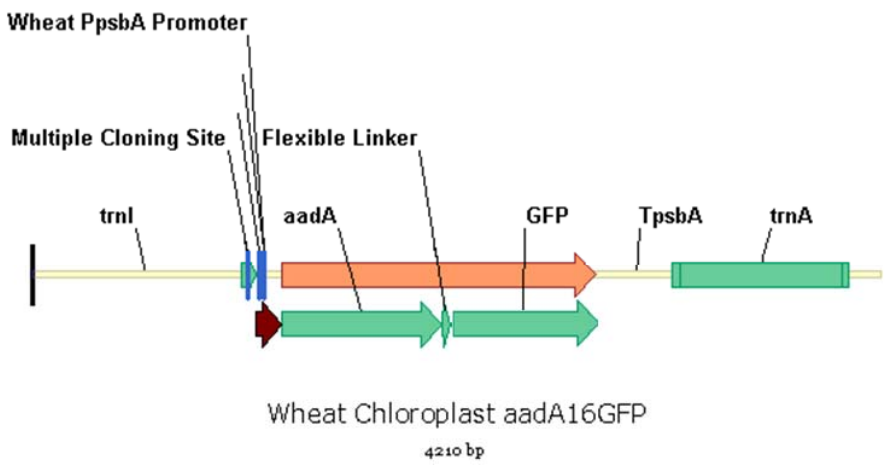
## Tracking product of homologous recombination

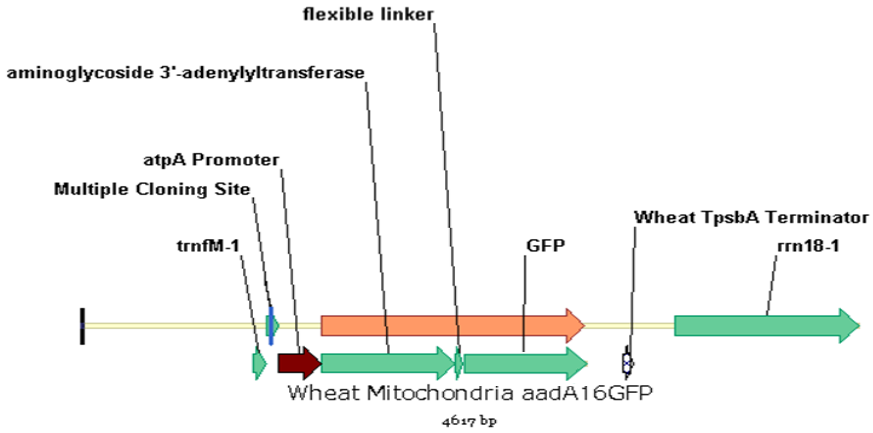
| Treatment | time after transfection | Material | extracted | Average Ct-values in RT with MGB probe |
|---|---|---|---|---|
| Control | 5 days | non-transfected microspores | DNA | Undet |
| Control | 5 days | non-transfected microspores | RNA | Undet |
| dsDNA+Tat2 | 5 days | living microspores | DNA | 21.42 |
| dsDNA+Tat2 | 5 days | living microspores | RNA | 29.26 |

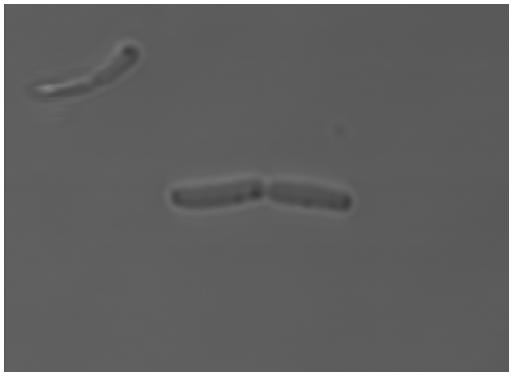
# Site targeted point mutations – ALS locus



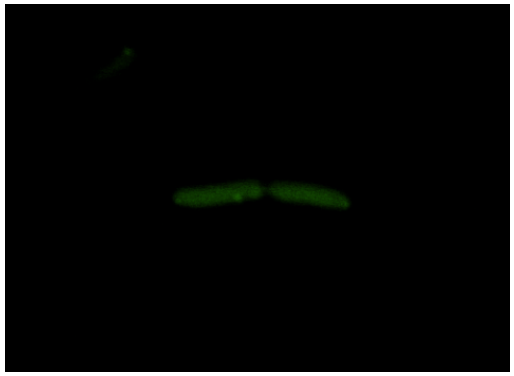
# Chloroplast and Mitochondrial DNA delivery



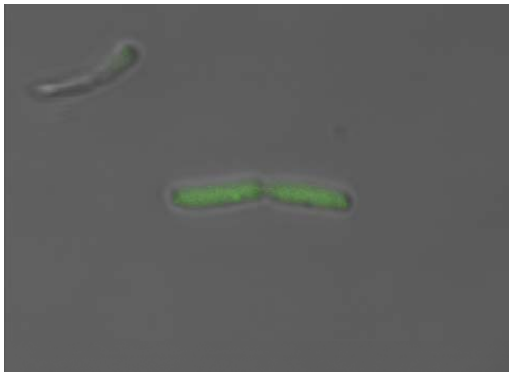



Rosetta confocal image DIC.



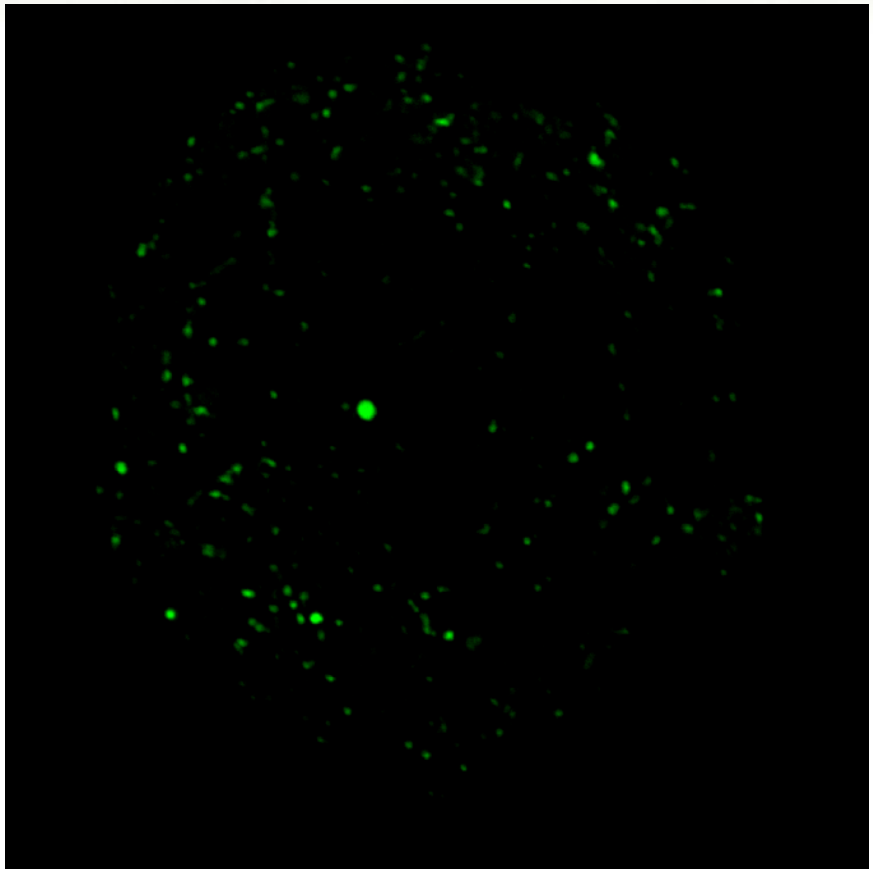
Rosetta confocal image GFP.



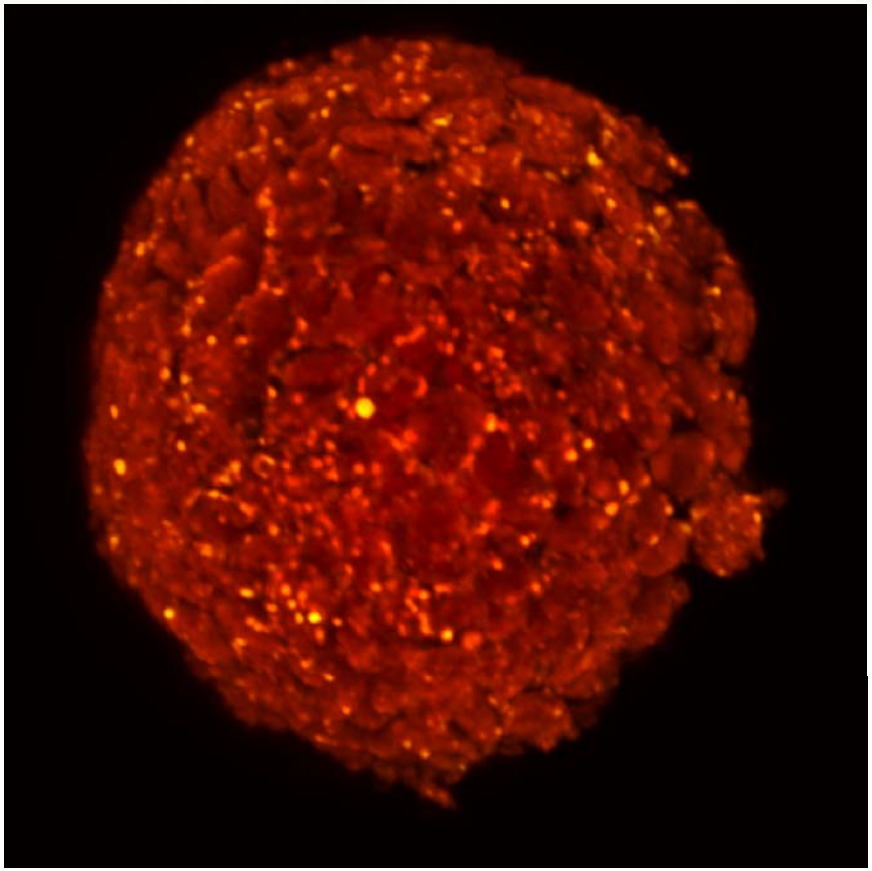
Rosetta confocal image DIC + GFP.

# Transfection of GFP reporter in mitochondria

**GFP expression**

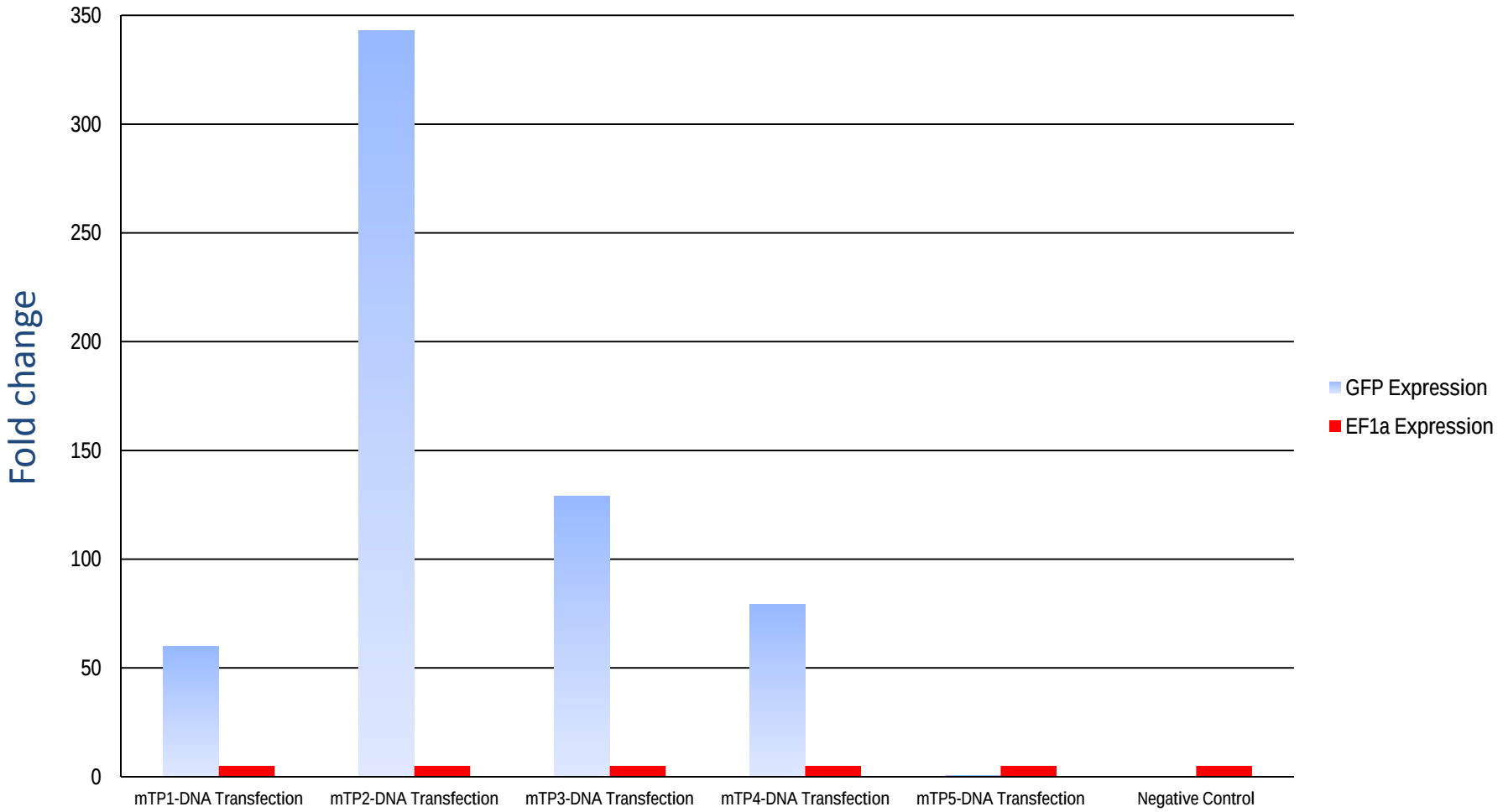


**Co-localization with mito-tracker orange**

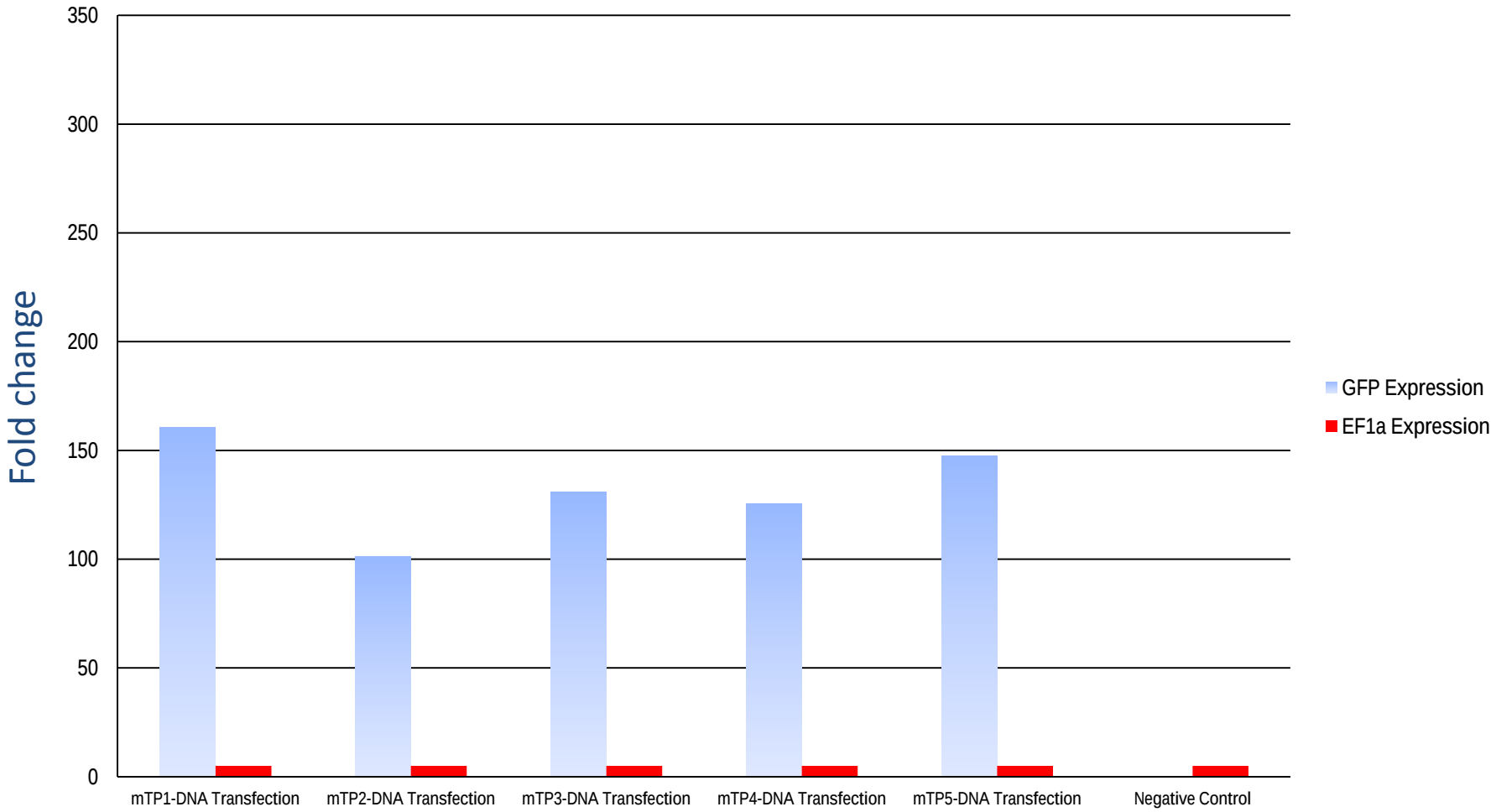


**Confocal image of a triticale/wheat protoplast transfected with WMaadA16GFPusing a mTP peptide.**

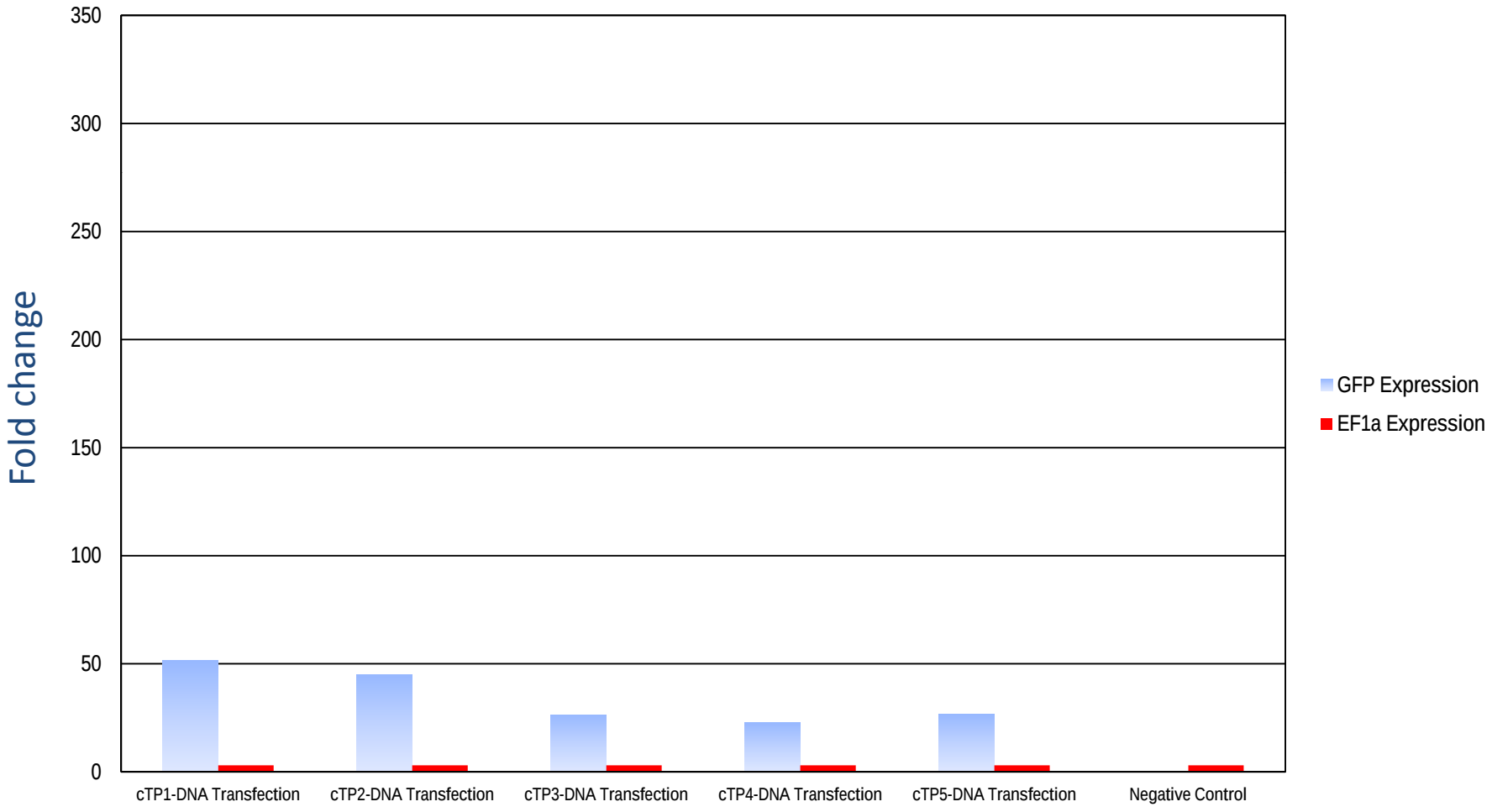
# GFP qRT-PCR Expression data from Mitochondrial Transfected Protoplasts



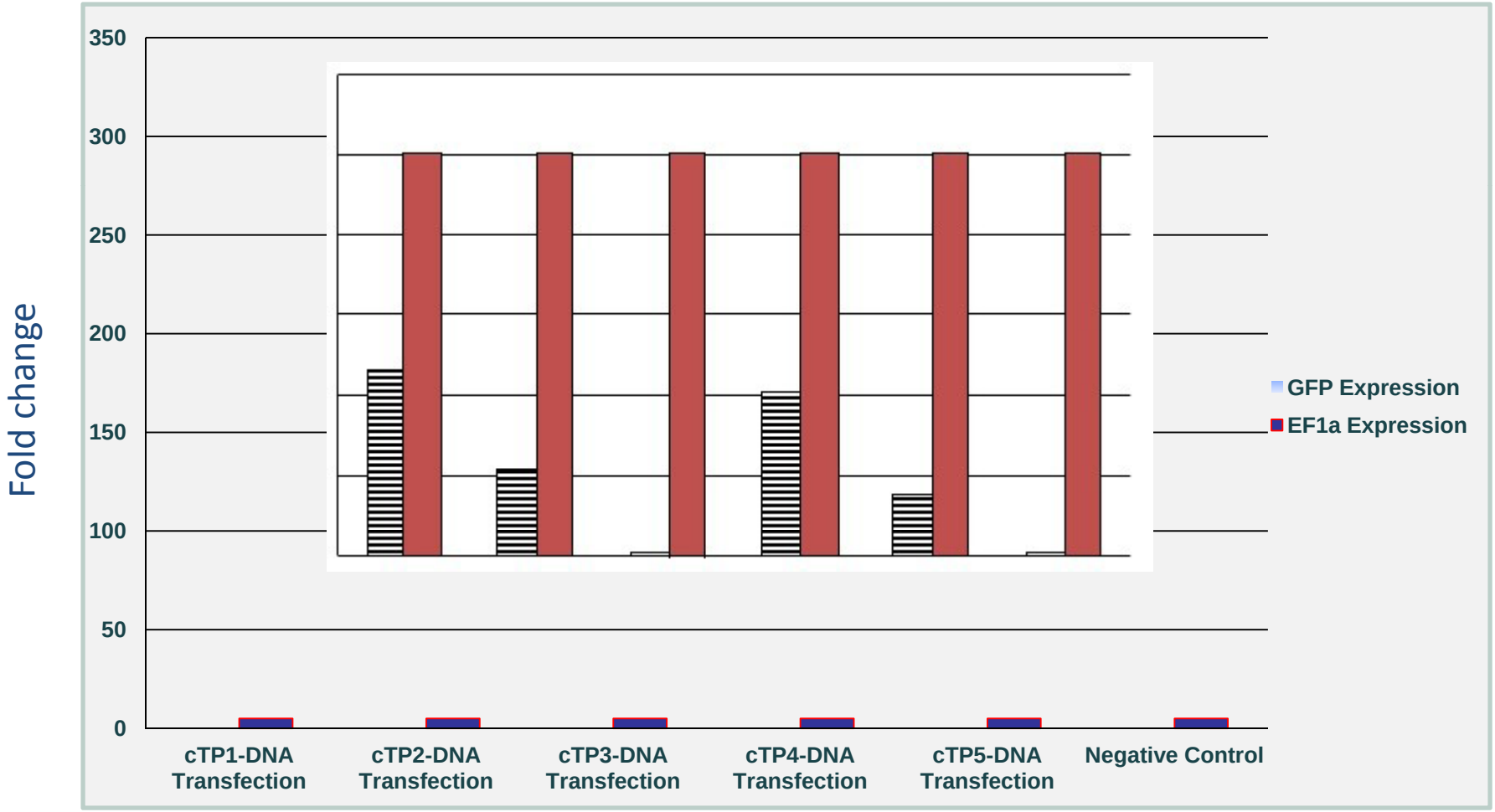
# GFP qRT-PCR Expression data from Mitochondrial Transfected Microspores



# GFP qRT-PCR Expression data from Chloroplast Transfected Protoplasts



# GFP qRT-PCR Expression data from Chloroplast Transfected Microspores



# Technology convergence for genome editing in microspores derived plants

- Haploidy (microspores)
- Nano-carriers for three plant organelles
- ssDNA-proteins cargoes
- DSB nucleases, e.g. Zinc Finger, meganuclease
- Genome editing

- Mitochondria and Chloroplast - CO2 and N2 fixation?

- These three CPP classes based transfection are being made available through a non-exclusive license agreement

# Acknowledgements


- Eric Amundsen
- Denise Nilsson
- Dr. Archana Chugh
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- Dr. Igor Kovalchuk
- Dr. Alicja Zimienovich

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