

Elimination of Marker Genes and Targeted Integration of Transgenes via the *FLP/FRT*-Recombination System



Matthias Fladung, Tobias Schenk,
Horst Lörz, Dirk Becker

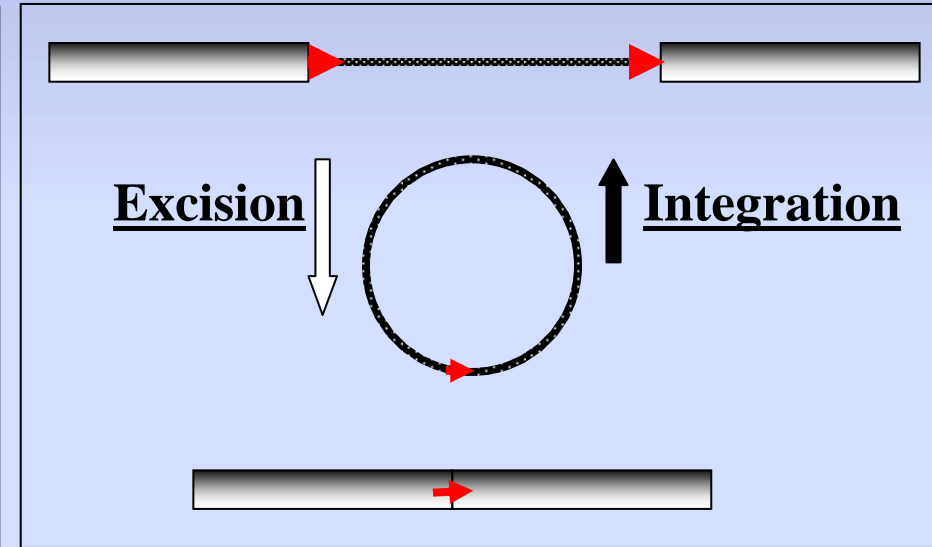
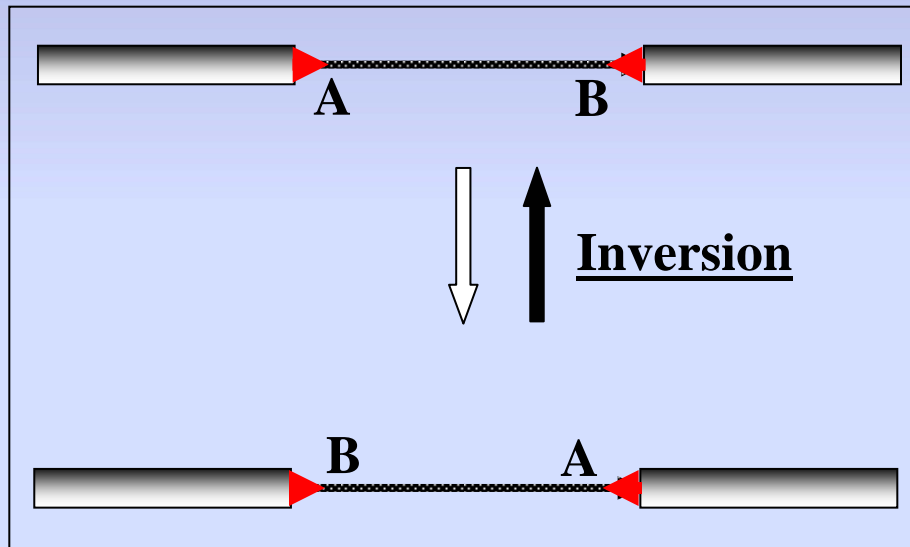
vTI, Institute for Forest Genetics, Grosshansdorf, Germany
University of Hamburg, Biozentrum Klein Flottbek, EBBT, Germany

FLP/FRT is a site-specific recombination system

- = non-homologous recombination
- Sequence-specific
- Needs only short DNA sequences as recognition sites
- Two fundamental reactions
 - Inversion of DNA-fragment
 - Excision/integration of DNA-fragment
- Examples:
 - *FLP/FRT* from yeast
 - *Cre/lox* from bacteriophage P1

Schematic overview

Site-specific recombination



- Inverted recognition sites (◄◄) : A-B fragment between these two sites is turned around
- Direct recognition sites (►►) : Excision of the DNA-fragment. The reverse reaction induces integration

Examples of successful site-specific recombination in plants

- *Cre/Lox*-System (from Phage P1)
successful in tobacco, tomato, *Arabidopsis* etc.
- *FLP/FRT* System (from yeast)
successful in tobacco, *Arabidopsis*, maize, barley etc.
- It works also in trees:
 - Ebinuma et al. (2001) (*Cre/lox*)
 - Fladung et al. (2005) (*Cre/lox*; *FLP/FRT*)
 - Schenk, Becker, Fladung (unpublished) (*FLP/FRT*)

Examples of successful site-specific recombination in plants

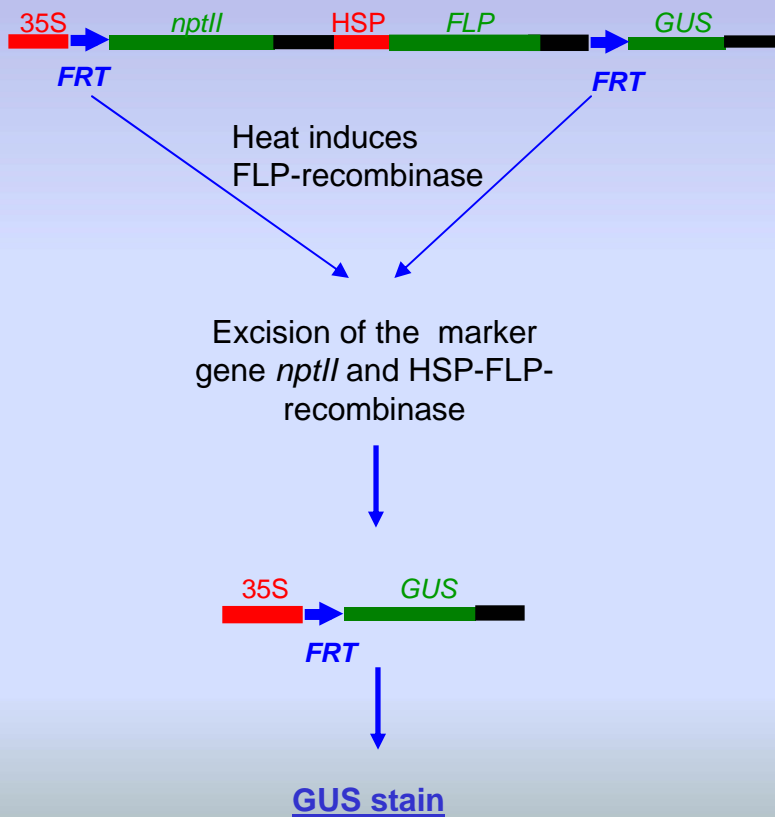
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Excision and integration in *Populus*

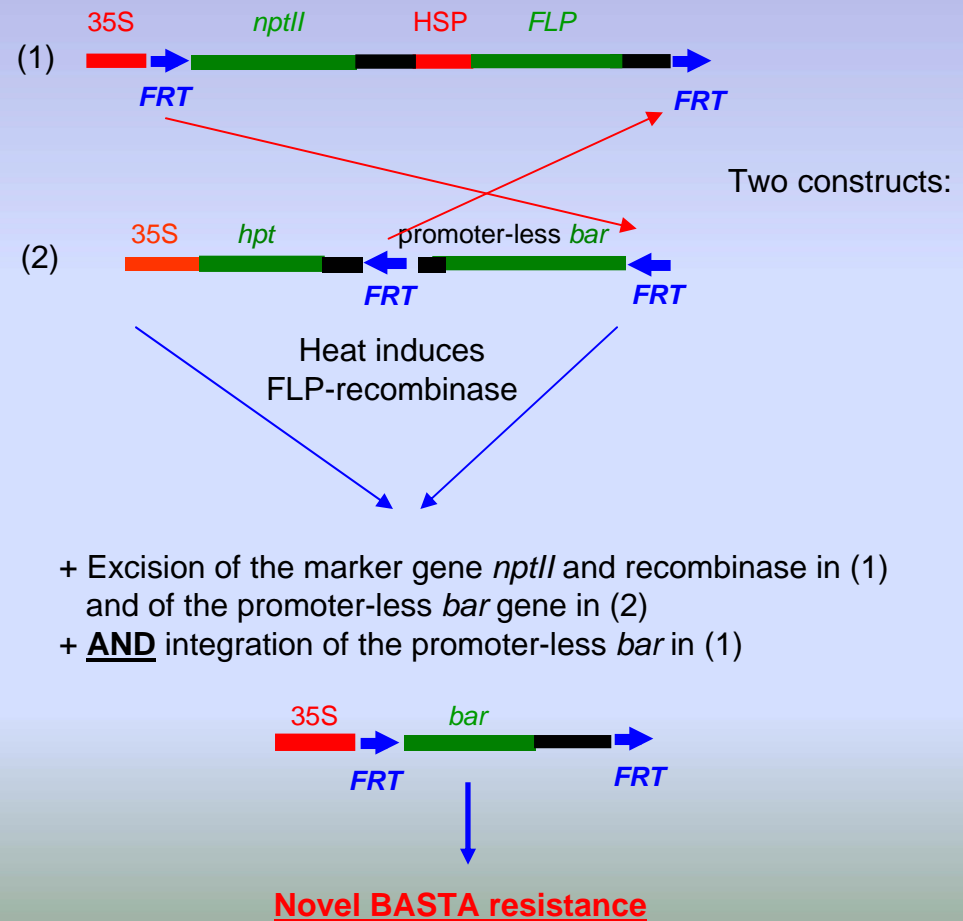
- Using the *FLP/FRT* system from yeast
- Heat-shock-inducible expression of the *FLP* recombinase
- Excision:
 - One construct was used
- Integration
 - Two constructs were used

Excision and **integration** in *Populus*

Excision



Integration



Excision in *Populus*

Why excision of DNA in *Populus*

- Elimination of antibiotic and herbicide genes
(selection marker) in transgenics
(selection marker are needed only for selection during
transformation process)
- ➔ Increase of public acceptance for GM trees
- Repetitive transformations are feasible using the
same marker gene

Excision in *Populus*

Excision



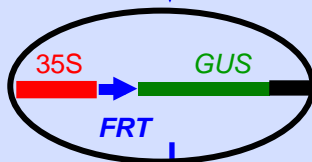
Excision in *Populus*

Excision



Heat induces
FLP-recombinase

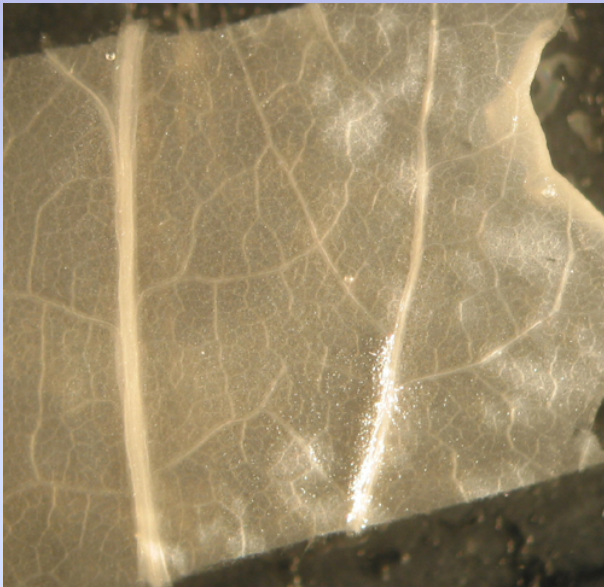
Excision of the marker
gene *nptII* and HSP-FLP-
recombinase



GUS stain

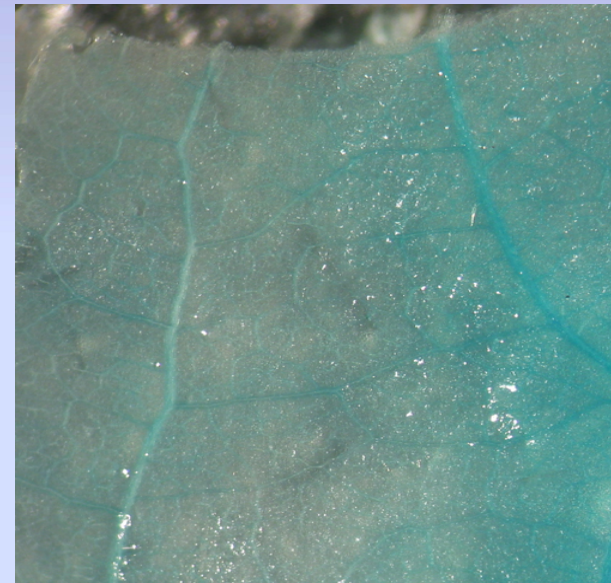
Excision in *Populus*

Without heat shock and in controls



No active GUS gene

Following heat shock



Excision: induction of the GUS gene

- Excision of DNA fragment is also confirmed by PCR and Southern-Blot experiments

Integration in *Populus*

Integration

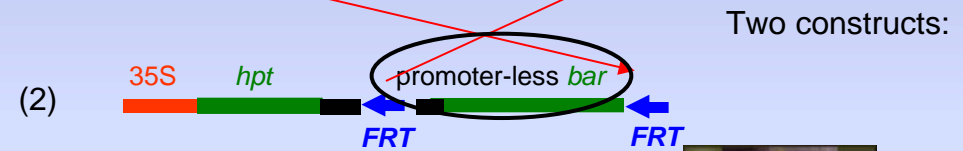
- Targeted integration of genes following transformation process
 - Transfer of a recognition target into the genome
 - Identification of “safe heavens” in the genome (no pleiotropic effects, stable transgene expression)
 - Excision of the recognition target and transfer of the desired gene to these “safe heavens”

- Stable transgene expression is important in particular for trees (long generation cycles (10 to 100 years) including long vegetative phases)

Integration in *Populus*



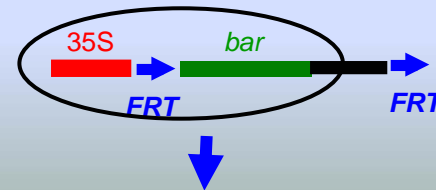
Integration



Heat induces FLP-recombinase

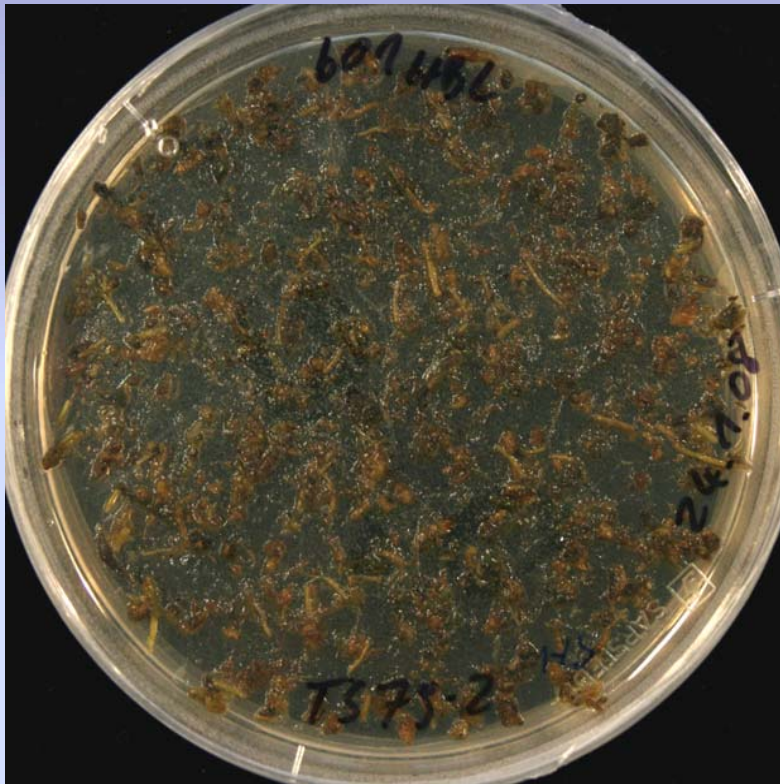


- + Excision of the marker gene *nptII* and recombination in (1) and of the promoter-less *bar* gene in (2)
- + **AND** integration of the promoter-less *bar* in (1) behind the 35S

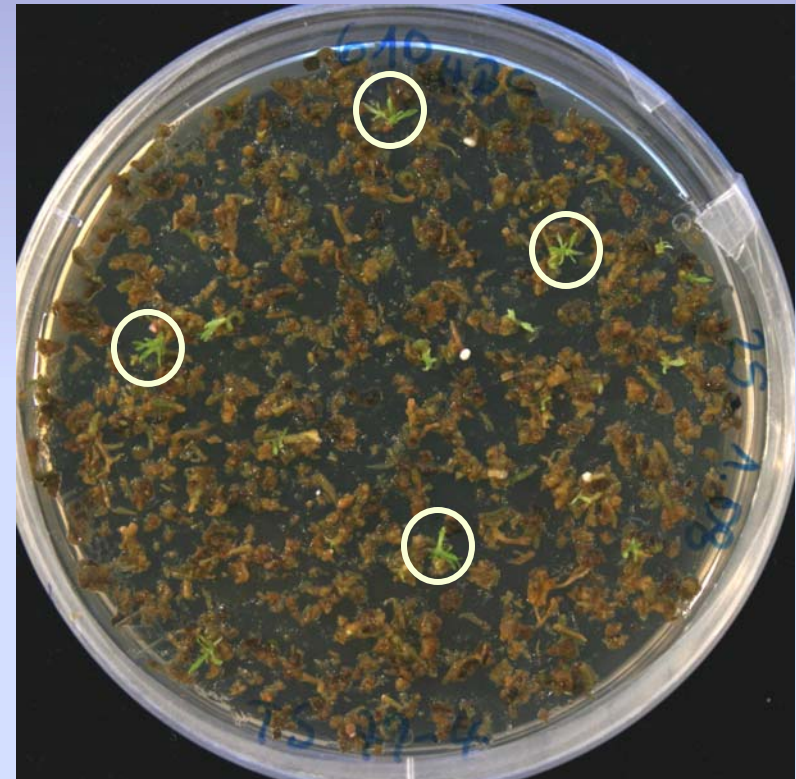


Novel BASTA resistance

Selection on BASTA containing media



No integration: cells are BASTA-sensitive and die on BASTA-containing media



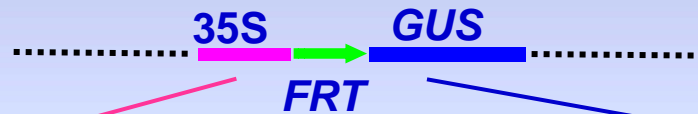
Integration: few cell lines are BASTA-resistant and survive on BASTA-containing media

Gene targeting of Bar gene

| Double-transgenic line | Number of small explants | Number of regenerated calli (%) | 35S-bar PCR positive from xy tested calli (%) | Gesamt % |
|------------------------|--------------------------|---------------------------------|---|----------|
| TS 71-1 | 42.088 | 45 (0,1%) | 2/3 (66%) | 0.1% |
| TS 71-3 | 51.392 | 244 (0,5%) | 10/21 (47%) | 0.2% |
| TS 79-1 | 39.663 | 2946 (7,4%) | 86/100 (86%) | 6.4% |
| TS 79-2 | 25.939 | 0 (0%) | // | 0% |
| TS 79-4 | 23.814 | 485 (2,0%) | 70/100 (70%) | 1.4% |
| TS 79-5 | 31.058 | 372 (1,2%) | 46/85 (54%) | 0.7% |
| TS 85-1 | 39.003 | 412 (1,1%) | 4/20 (20%) | 0.2% |
| TS 85-2 | 16.422 | 190 (1,2%) | 2/15 (13%) | 0.2% |

Molecular proofs of gene targeting

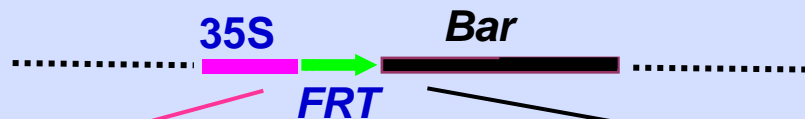
- PCR and Southern blot analyses reveal presence of 35S-bar fragment
- Sequencing: Original sequence:



CATTCATTTGGAGAGGACGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCgttaccggccaatatatcctg

Sequence after targeting:

Schenk, Becker, Fladung (unpublished)



CATTCATTTGGAGAGGACGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCATGGGCCCCAGAACGACGCC

Conclusions

- Site-specific recombination systems are working in trees
- Excision leads to elimination of selection marker genes (antibiotic and herbicide genes) in transgenics
- Following identification of “safe heavens” in the genome (no pleiotropic effects, stable transgene expression) a targeted transfer of a gene-of-interest to these “safe heavens” is feasible
- Transfer of different “test-genes” to the same position in the genome allows reproducible gene expression analyses

Many thanks to

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attention !**