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Comparison of different methods for the establishment of RNA silencing in plants

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Introduction

- **Application of RNAi for crop improvement requires profound knowledge of the target genes and their function**

- **Transient silencing systems (e. g. VIGS or agroinfiltraion) have proved to be simple, rapid and versatile tools for gene function studies**

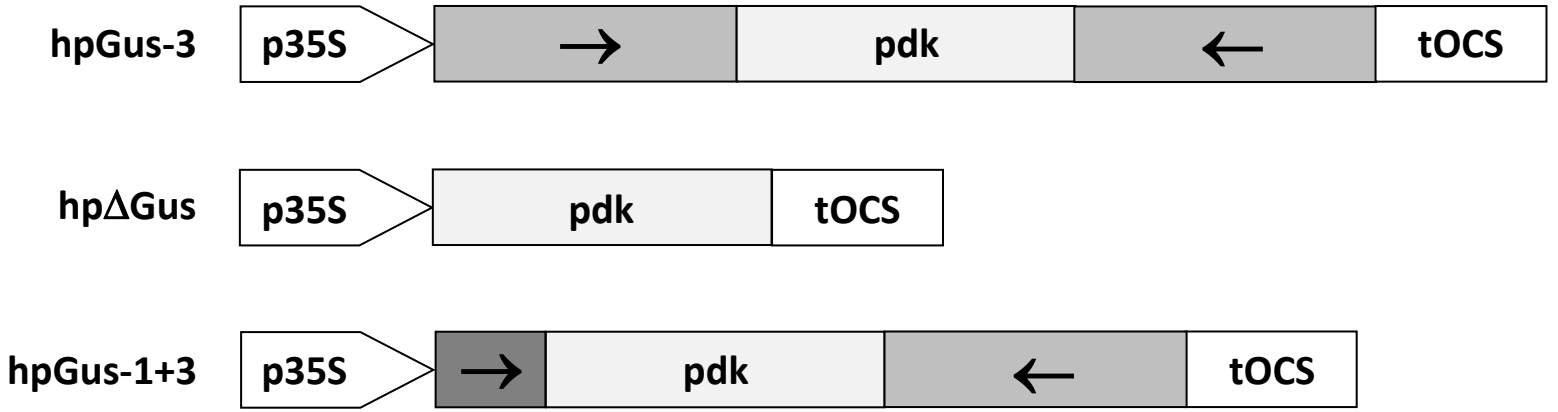
Are there differences in the applicability of stable and transient silencing systems for gene silencing studies?

→ Comparison of different silencing systems expressing a silencing-inducing hairpin sequence

Constructs

(a) Target gene: β-glucuronidase (*gus*) gene

(b) Test constructs:

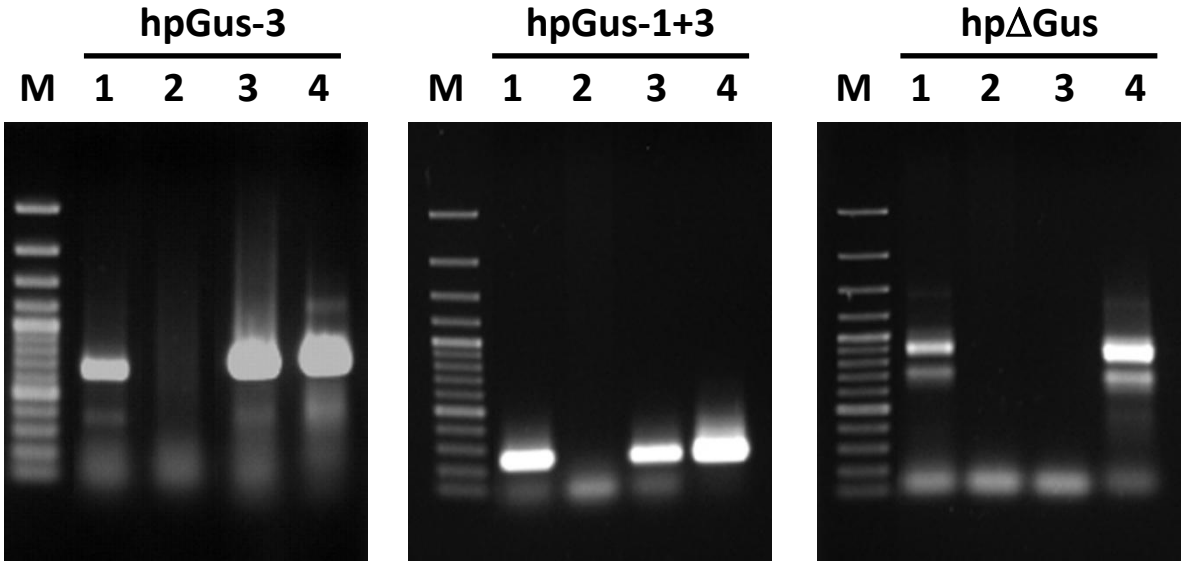


Expression cassettes were integrated in binary vectors and transferred into *A. tumefaciens*

Functional testing: Test construct expression

Expression of mRNAs from test constructs

- **Agroinfiltration of *Nicotiana tabacum* (N.t.) leaves with hairpin constructs**
- **Sampling 5 days after infiltration**
- **RT-PCR analysis with construct specific primers**



M: 100 bp DNA Ladder plus

1: cDNA from agroinfiltrated N.t.

2: cDNA from non-infiltrated N.t.

3: cDNA from *gus-int* transgenic N.t.

4: Construct specific plasmid DNA

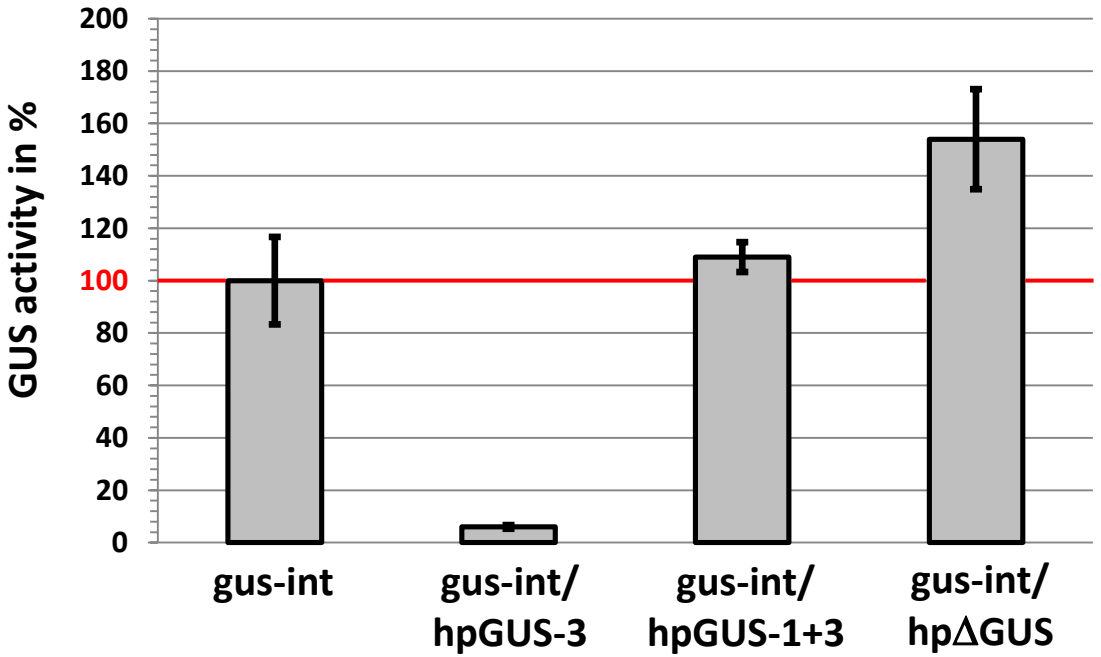
Functional testing: Silencing capacity

Silencing capacity of RNAs from test constructs

- **Simultaneous expression of test constructs and *gus-int* gene after co-agroinfiltration of *Nicotiana tabacum* plants**
- **For reference values: Expression of *gus-int* gene without test construct**
- **Infiltration of 10 plants for each test construct**
- **Sampling 5 days after infiltration**
- **Quantification of the GUS enzyme activity**

RNA silencing: Comparison of different methods

Functional testing: Silencing capacity



- **Functional hairpin construct:** **GUS activity significantly reduced** → **silencing**

- **Non-functional constructs:** **GUS activity similar or increased** → **no silencing**

Testing of different expression systems

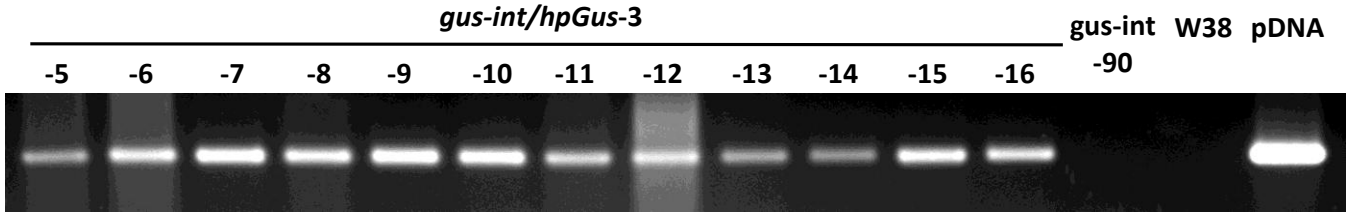
1. **Stable expression of GUS-3 hairpin in *gus-int* transgenic plants (*Agrobacterium*-mediated transformation)**

2. **Transient expression of GUS-3 hairpin in *gus-int* transgenic plants**

RNA silencing: Comparison of different methods

Stable system

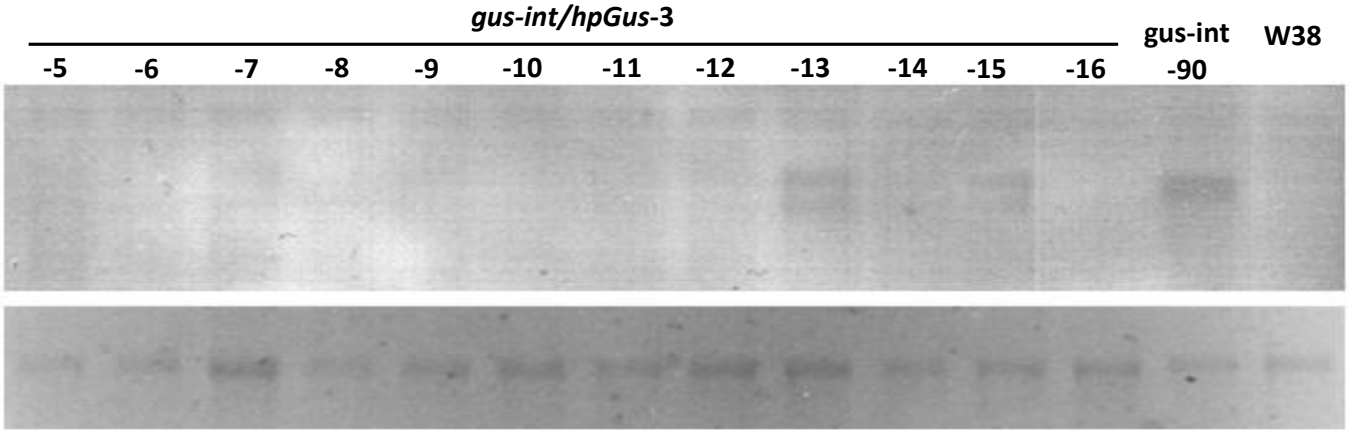
RT-PCR analysis of *hpGUS-3* RNA



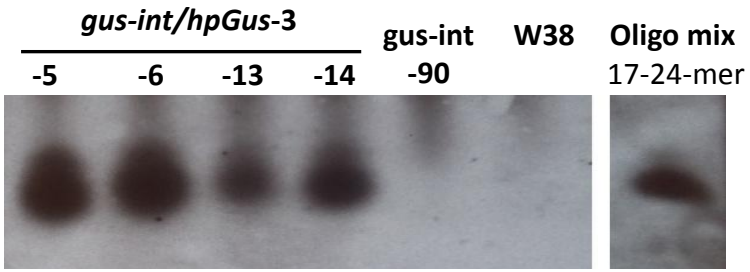
Analysis of GUS activity (nmol NP/mg protein/min)

gus-int/hpGus-3												gus-int -90	W38
-5	-6	-7	-8	-9	-10	-11	-12	-13	-14	-15	-16		
0.21	0.20	0.16	0.07	6.40	0.49	0.31	0.44	8.70	1.31	5.58	0.07	35.5	0.02

Northern analysis of *gus-int* mRNA



Northern analysis of *gus int* specific siRNA



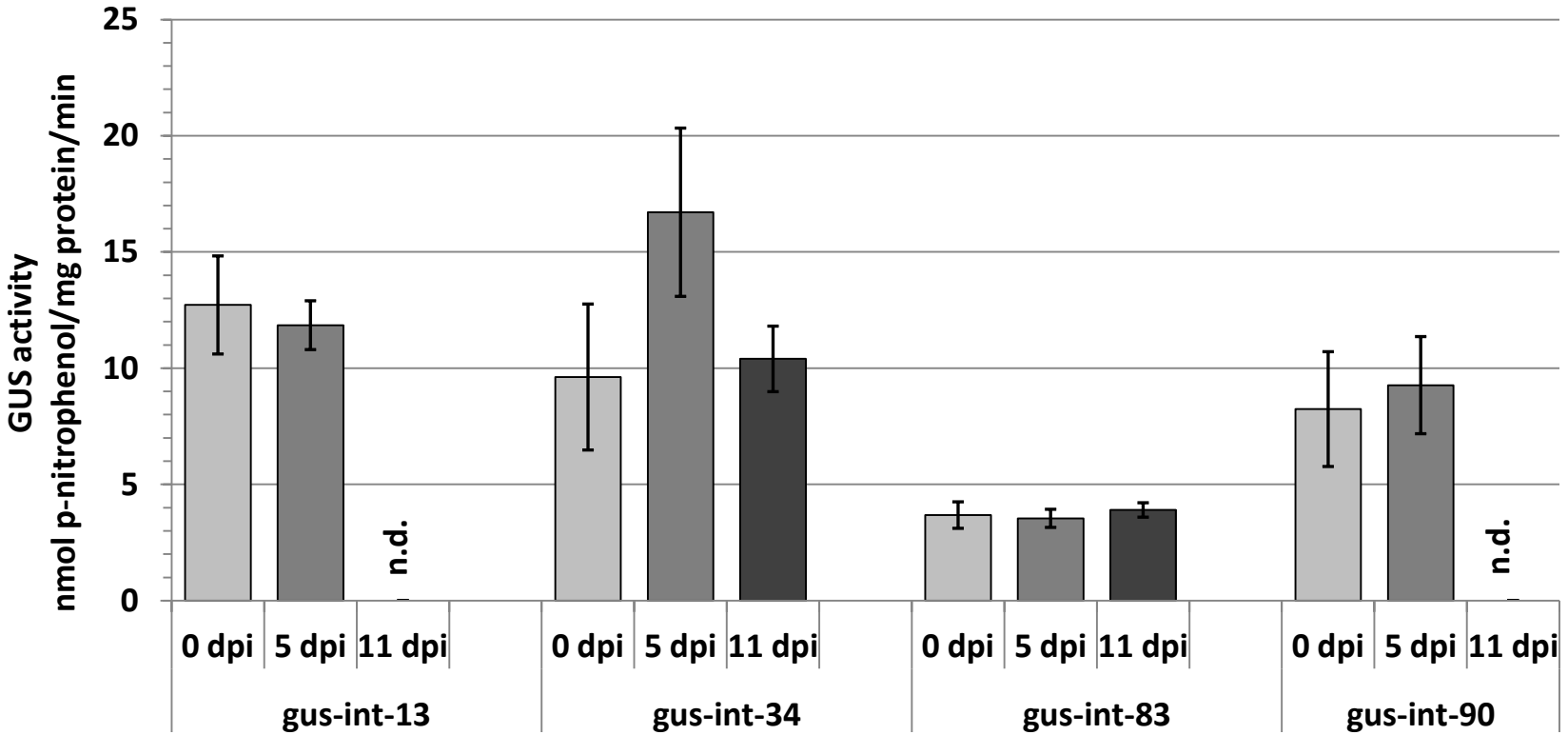
Transient system

Transient expression of hpGUS-3 in *gus-int* transgenic plants

- **4 independent *gus-int* transgenic *Nicotiana tabacum* lines**
- **Agroinfiltration of 6 plants per line with hpGUS-3**
- **Sampling before infiltration, 5 and 11 days after infiltration**
- **Quantification of the GUS enzyme activity**

RNA silencing: Comparison of different methods

Transient system

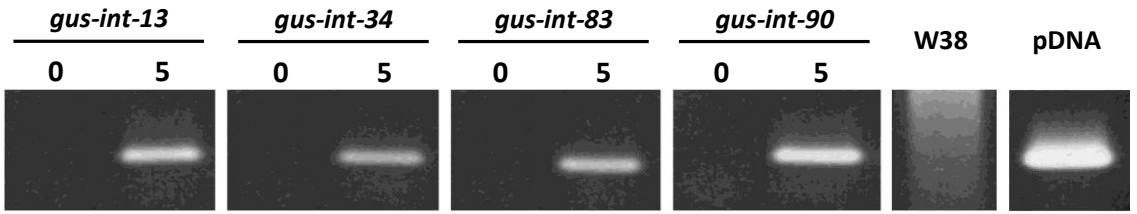


- **GUS activity similar or increased → no silencing**

Transient system

Detailed analysis of hpGUS-3 agroinfiltrated *gus-int* transgenic plants

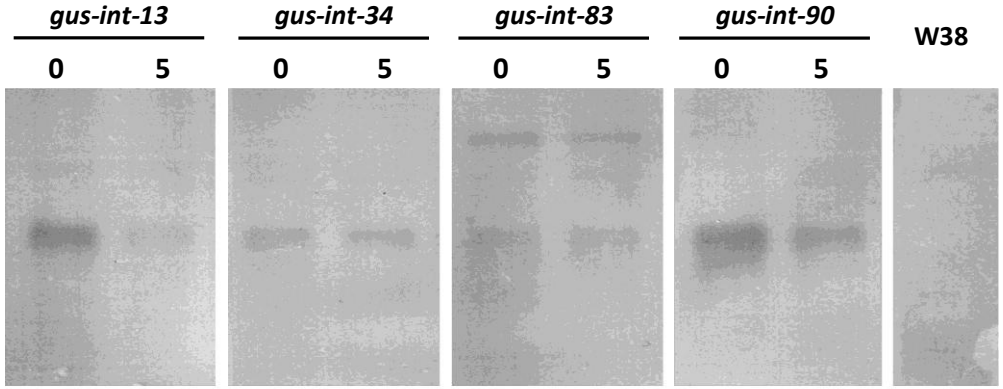
RT-PCR analysis of *hpGUS-3* RNA (Primers: gus2/ocs3)



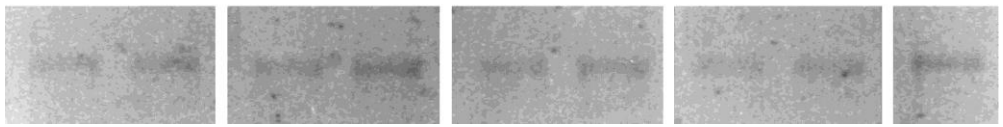
Analysis of GUS activity

gus-int-13		gus-int-34		gus-int-83		gus-int-90		W38
0	5	0	5	0	5	0	5	
22.20	26.04	10.51	30.00	12.56	10.64	29.52	29.56	-0.28

Northern analysis of *gus-int* mRNA



rRNA



Relevance of the chronological order of hairpin and target gene expression

Way of expression		Chronology of expression gus-int : hpGUS-3	Silencing effect
gus-int	**hpGUS-3**		
stable	stable	**simultaneous**	**+**
stable	transient	gus-int **prior to** hpGUS-3	**(-)**
transient	stable	gus-int **subsequent to** hpGUS-3	**+**
transient	transient	**simultaneous**	**+**
		gus-int **prior to** hpGUS-3	**-**
		gus-int **subsequent to** hpGUS-3	**+**

Interpretation of results

Transiently expressed hpGUS-3 was not able to trigger silencing of a pre-existing *gus* expression

- **high stability of the GUS protein**

  → silencing effect is masked on the protein level and may only be apparent on mRNA level

  however: reduced level of *gus-int* specific mRNA only in 2 out 4 agroinfiltrated *gus-int* lines

- **duration of hairpin expression is limited**

  → abundant levels of hairpin RNA only expected within a short period of time

  → sufficient to shut off incipient *gus* expression

  but not enough to trigger silencing of a pre-existing *gus* expression

Summary

There are differences in the applicability of different gene silencing methods

- **Stable silencing systems are laborious but result in reliable silencing of a target gene**

- **Transient silencing systems are very fast and easy to handle but their application may be limited**

  → temporary expression of the silencing inducer may not be sufficient for silencing induction

Specific properties of the applied method as well as individual characteristics of the target gene and its product have to be considered