Abschlussbericht

Zuwendungsempfänger: **Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA)**
Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit
Messeweg 11-12
38104 Braunschweig

Projektleiter: **Dr. Joachim Schiemann**

Förderkennzeichen: **0 312 627 E**

Vorhabenbezeichnung: **Verbundvorhaben: Gezielte Übertragung minimierter Transgensequenzen mit optimierter Funktion**
TP1: Methodenentwicklung zur Herstellung sicherer transgener Pflanzen der nächsten Generation
UP2: Markergen-Eliminierung mit dem Cre/lox-Rekombinationssystem durch transiente Expression des Rekombinasegens

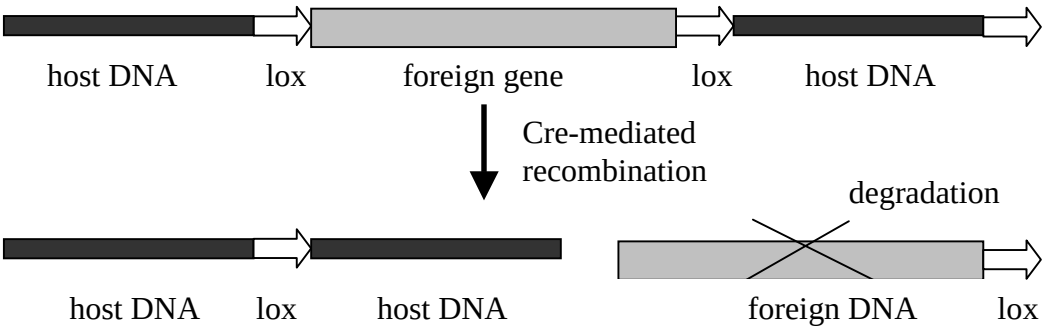
Laufzeit des Vorhabens: **01. April 2001 – 30. September 2004**

Content

1. Introduction and aim of the project

Selectable marker genes are required for creating transgenic plants, because only a small proportion of cells are transformed. These genes usually confer antibiotic or herbicide resistance and allow scientists to identify cells that have integrated DNA and regenerate plants from these cells. Once the transgenic plants have been generated, the selectable marker gene is not essential any more and can be eliminated from the plant genome. A number of methods for marker gene removal from transgenic plants have been reported in the literature including cotransformation of T-DNAs followed by segregation of the marker gene from the trait gene (McCormac et al., 2001), homologous recombination between direct repeats (Zubko et al., 2000) and site-specific recombination (Zhang et al., 2003). We applied in our project the Cre-*lox* site-specific recombination system, which is commonly employed for plant systems. This simple site-specific recombination system consists of two basic components: a recombination enzyme (Cre) and two small DNA recognition sites (*lox*) (Hoess et al., 1986). During recombination reaction, host DNA is cut at both *lox* sites by the Cre protein and then ligated back together. If two *lox* sites are in direct orientation, DNA between *lox* sites is excised and subsequently degraded (Fig. 1).

Figure 1. The Cre-*lox* site specific recombination system: the basic components and mode of action



Generally, an application of this system requires cross-breeding of two transgenic lines. One line carries the gene of interest flanked by *lox* sequences; the other one contains a *cre* transgene. Cre recombinase delivery can be also done transiently using the *A. tumefaciens* expression vector or chemically regulated promoters. A virus vector represents an alternative strategy for transient expression of *cre* recombinase. The plant virus vector is a full-length cDNA of virus. The virus vector performs all functions for virus replication, but it is modified to express a foreign gene during plant infection. At present, the most widely applied vectors for recombinant protein production are based on *Tobacco Mosaic Virus* (TMV) and *Potato*

Virus X (PVX). In our project we also used these viruses to develop Cre-virus vectors. These RNA viruses do not integrate into the plant genome, they express transiently their genes. The genomes of TMV and PVX can be easily manipulated with recombinant DNA techniques. The PVX expression vector can be useful for protein expression in potato plants and the TMV vector can infect about 200 plant species.

The second transient expression vector we used in this project is *A. tumefaciens* T-DNA. Most of the transferred T-DNA molecules are not integrated into the plant genome. They can persist in the nucleus for a period of time providing transient expression of genes from T-DNA.

The aim of our project was developing a new strategy to eliminate marker genes from transgenic plants by using transient expression of the *cre* recombinase. To fulfil this aim we had to solve several problems:

1. Designing *lox*-target plants;
2. Designing cre transient expression vectors;
3. Elimination of marker genes in lox-target plants;
4. Study of the next generation of marker-free plants;
5. Practical application of the PVX-Cre expression vector.

2. State of the art at the beginning of the project

2.1. Literature review

More than twenty years ago, the cre-*lox* system from bacteriophage P1 has been tested in eukaryotic organisms and shown to be functional in animal (Sauer and Henderson, 1988) and yeast (Sauer, 1987) cells. D. Ow in his pioneer work in 1990 demonstrated that *cre* recombinase expressed in tobacco protoplasts can catalyze deletion, inversion and integration events (Dale and Ow, 1990). The same group has also demonstrated that a marker gene flanked by two *lox* sites in direct orientation can be successfully removed from transgenic tobacco plants upon the *cre* recombinase expression (Dale and Ow, 1991). Different strategies have been designed to deliver Cre recombinase into plant cells. In one strategy, transgenic plants that carry a marker gene flanked by *lox* sites can be retransformed with a *cre* expression plasmid. The *cre* gene can then be segregated in a next generation (Dale and Ow, 1991; Russel et al., 1992). Alternatively, the *lox* plants can be crossed with plants that express Cre activity in which case marker excision occurs in the T_1 progeny, followed by loss of the cre gene by genetic segregation in T_2 generation (Bayley et al., 1992, Russel at al., 1992). Several difficulties appeared with these strategies. The applicability of these two strategies to vegetatively propagated species is somewhat limited. A second difficulty is that a chronic

expression of the *cre* recombinase gene can lead to some negative effects in animal and plant cells (Loonstra et al., 2001; Coppoolse et al., 2003). To overcome these problems, cre can be introduced transiently or by using autoexcision strategy. In autoexcision strategy the *cre* recombinase gene is placed under the control of a regulated transcription system. Zhang et al. (2003) combined the autoexcision strategy with a heat shock inducible promoter to eliminate marker genes from maize. Earlier, Zuo et al (2001) applied estradiol-regulated *cre* recombinase for gene excision from *Arabidopsis* plants. Several transient expression systems have been reported in the literature, including addition of purified cre recombinase to cultured cells (Will et al., 2002), cre translocation into plant cells using fusion between the recombinase and Vir proteins of *A. tumefaciens* (Vergunst et al., 2000), and infection of animal cells with Cre-virus vectors (Silver and Livingston, 2001). First animal-cre virus vectors were designed in the mid 1990s. To date, viral delivery of Cre recombinase provided a valuable method for investigation of virus genome replication (Thevenot et al., 2003), conditional gene expression (Ventura et al., 2004) and gene therapy (Goto et al. 2001) in mammalian cells.

For plant viruses, the construction of full-length cDNA clones of *Brome Mosaic Virus* (BTM), from which infectious transcripts could be obtained, was reported in 1984 (Ahlquist and Janda, 1984). Subsequently, full-length cDNA clones of many other RNA plant viruses have been produced. It opened the way to the development of several RNA viruses as vectors. The first plant-based expression vectors were simple „gene replacement“ vectors, in which a foreign gene of interest replaced the capsid protein gene of a virus (Takamatsu et al., 1987). These vectors could not move systemically in plants. Next generation of plant RNA viruses were constructed to express a foreign gene in addition to all required viral genes. Such vectors were able to move systemically with the foreign insert in host species. PVX and TMV-based vectors represent the second generation of plant viruses. A PVX-based vector was constructed in 1992 in the laboratory of D. Baulcombe (Chapman et al., 1992). A TMV-based vector was developed a bit earlier in 1991 in the laboratory of W.O. Dawson (Donson et al., 1991). PVX- and TMV-based vectors have become the most widely used vectors to produce recombinant proteins (Mor et al., 2003), therapeutics (Kumagai et al., 1993) and vaccines (McCormick et al., 2003) *in planta*. Nevertheless, the functional expression of the Cre recombinase by virus vectors has not yet been reported for plant systems.

2.2. Collaboration with other members of the cluster project

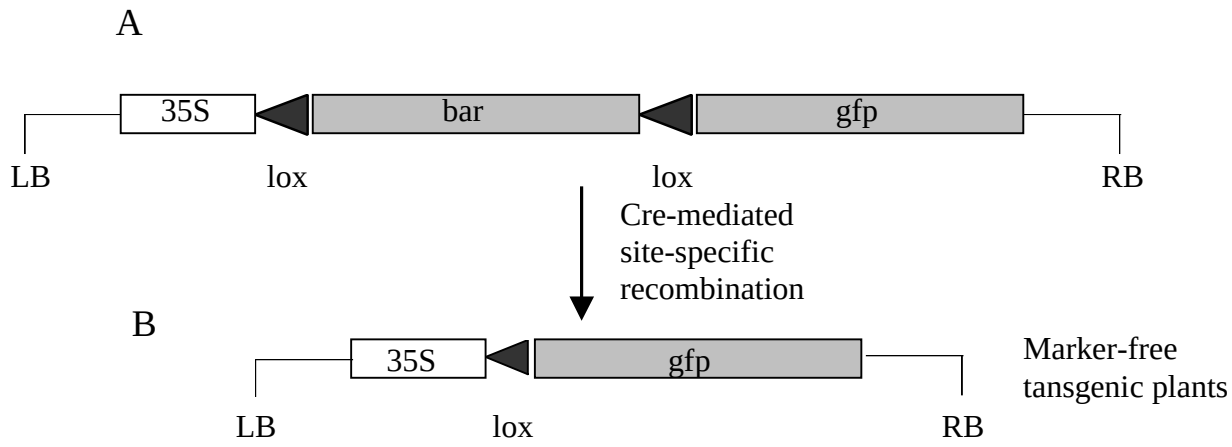
We worked in collaboration with Bioplant, Ebstorf to apply the PVX-Cre expression vector we developed for marker gene elimination from transgenic potato plants. The *gbssI* gene for potato transformation was received from Bioplant. Bioplant was also responsible for the biochemical analysis of amylose content in *gbssI* transgenic plants.

3. Scientific results

3.1 *Lox*-target transgenic *N. benthamiana* plants

Transgenic *N. benthamiana* plants were generated by *A. tumefaciens*-mediated transformation. These plants include a *lox* flanked marker gene (*bar*), which prevents the expression of *gfp* gene (Fig. 2). The introduction of *cre* recombinase by plant-cre virus or *A tumeaciens* vectors leads to the deletion of the marker gene (*bar)* and *gfp* fluorescence. 45 putative transformants were regenerated. Three independent lines (34, 44, 45) confirmed as transgenic by Southern analysis were used for marker gene elimination.

Figure 2. Schematic representation of the pLH-35S-*lox-bar-gfp* plasmid used for plant transformation before (A) and after (B) site-specific recombination

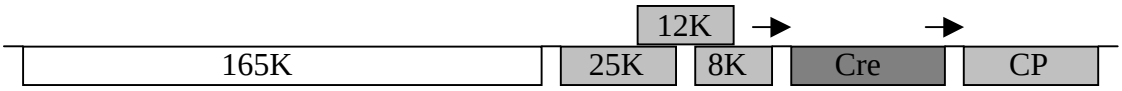


3.2 Cre expression vectors

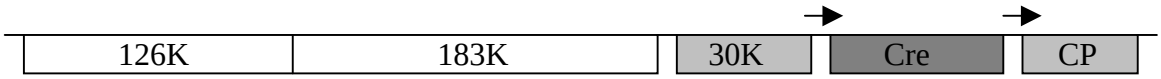
The second essential component of our strategy are the Cre-expression vectors. We designed three Cre-expression vectors: two virus-based vectors (PVX-Cre and TMV-Cre) and an *A. tumefaciens* expression vector. In PVX-Cre and TMV-Cre expression vectors the *cre* recombinase sequence was placed under the transcriptional control of the viral coat protein subgenomic promoter between movement and coat protein genes (Figure 3). *A. tumefaciens expression* vector includes *cre* and *gfp*, both genes are driven by the constitutive 35S promoter (Figure 3).

Figure 3. Cre-transient expression vectors

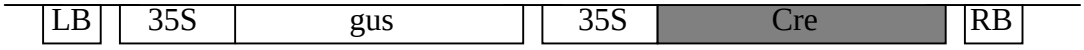
PVX-Cre



TMV-Cre



A. tumefaciens expression vector

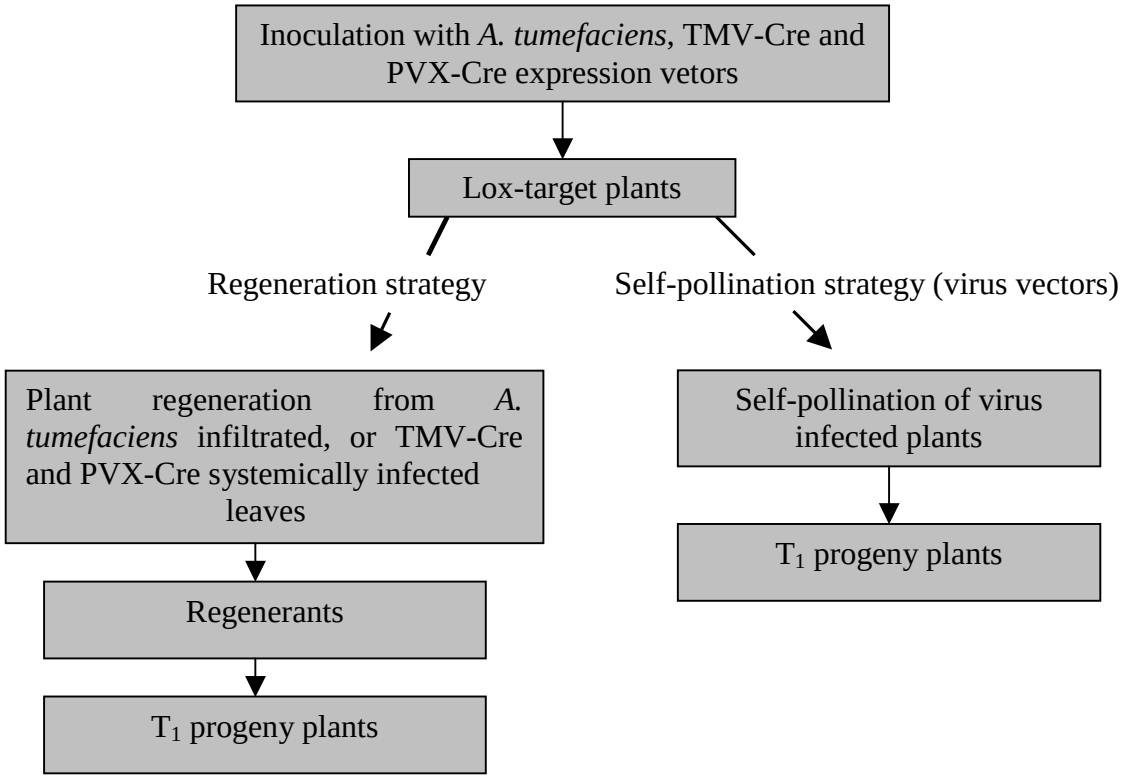


Open boxes indicate the following genes: 165K, 126K, 183K, RNA polymerase; CP, coat protein; Cre, *cre* gene; gus, *gus* gene; 35S, CaMV 35S promoter; arrows denote the subgenomic RNA coat protein promoter.

3.3 Elimination of marker gene in *lox*-target plants

Cre-transient expression vectors (TMV-Cre, PVX-Cre, *A. tumefaciens*) are not able to infect all cells and subsequently to excise the marker gene from the whole plant. Cre-mediated site-specific recombination events can be transferred from inoculated cells to the whole plant and then to the next generation via regeneration or self-pollination strategies (Figure 4). At the first step, *lox*-target plants were inoculated with PVX-Cre, TMV-Cre or *A. tumefaciens* expression vectors. In the regeneration strategy, PVX-Cre and TMV-Cre systemically infected or *A. tumefaciens* inoculated leaves were taken as explants for subsequent regeneration. The frequency of recombination was expressed as percentage of regenerated plants exhibiting marker gene excision. Marker-free regenerants were self pollinated and T_1 progeny was examined for the inheritance of the recombined state. In the second strategy, self progeny of TMV-Cre and PVX-Cre infected plants was obtained and examined for marker gene excision.

Figure 4. Two basic approaches to eliminate marker genes by transient expression of the *cre* recombinase



3.4 Efficiency of marker gene excision evaluated by using regeneration strategy

After inoculation of *lox*-target plants with cre-virus vectors, TMV-Cre and PVX-Cre systemically infected leaves were used for plant regeneration. The regeneration procedure was performed without selection pressure, but in the presence of 10 mg/l ribavirin to eliminate the virus from plant tissue. Applying ribavirin allowed us to eliminate PVX from 87% to 93% and TMV from 30% to 45% of regenerated plants, as was confirmed by Western blot analysis and RT-PCR (Table 1).

Table 1. Influence of ribavirin on the PVX and TMV infection in plants regenerated from TMV-Cre and PVX-Cre infected explants

Line	Expression vector	Regenerated plants			
		Tested	Infected	Uninfected	
				Number	%
34	PVX-Cre	15	1	14	93
44	PVX-Cre	38	3	35	92
45	PVX-Cre	15	2	13	87
34	TMV-Cre	27	19	8	30
44	TMV-Cre	33	18	15	45

The frequency of Cre-mediated marker gene excision was evaluated by PCR, using primers outside of the two *lox* sequences (Table 2). 48-61% of the regenerants for PVX-Cre expression vector and 47-57% regenerants for TMV-Cre expression vector did not contain *bar* gene in their genome. Total DNA of some *gfp* expressing plants were also investigated by Southern analysis, which confirmed precise site-specific excision of the selectable marker gene (*bar*). Each lox-target line (34, 44, 45) used in these experiments is an independent transformant in which the marker gene was integrated in different sites of the plant genome. Our results indicate that PVX-Cre and TMV-Cre mediated excision is independent of the genomic position of the *bar* gene.

Table 2. Efficiency of marker gene excision mediated by PVX-Cre and TMV-Cre virus vectors

Line	Expression vector	Regenerants			Recombination efficiency, %
		Tested	No excision	Excision	
34	PVX-Cre	44	23	21	48
44	PVX-Cre	52	9	43	82
45	PVX-Cre	49	19	30	61
34	TMV-Cre	115	61	54	47
44	TMV-Cre	104	45	59	57

Marker-free regenerants were allowed to self (T_1 generation) and progeny was examined by molecular and segregation analysis for the inheritance of the recombined loci. Seeds were germinated on medium with phosphinothricin. Analysis of 34-14, 44-16, 45-17 (PVX-Cre) and 34-111, 44-48 (TMV-Cre) lines identified that most of the investigated seedlings were *gfp* positive. No phosphinothricin-resistant seedlings for all examined lines were observed on the selective medium, confirming that excision of the *bar* sequence has been transmitted to the next generation for both Cre-virus vectors (Table 3). Molecular analysis of T_1 progeny plants (PCR and Southern) also proved the inheritance of the marker-free state.

Table 3. Segregation analysis of T_1 progeny derived from marker-free regenerants

Line	Expression vector	Examined plants			
		Total	Ph resistant	Ph sensitive	gfp expression
34-14	PVX-Cre	195	-	195	+
44-16	PVX-Cre	90	-	90	+
45-17	PVX-Cre	72	-	72	+
34-111	TMV-Cre	38	-	38	+
44-48	TMV-Cre	86	-	86	+

3.5 Efficiency of marker gene excision evaluated by using self-pollination strategy

If a virus can infect somatic cells from which generative cells can be developed later, self-pollination strategy may be useful to obtain marker-free transgenic plants directly from virus-infected plants. To test this opportunity, *lox*-target plants were inoculated with TMV-Cre and PVX-Cre virus vectors and self-pollinated. T_1 progeny seedlings were subjected to segregation analysis on the medium with phosphinothricin. After three weeks we counted resistant and sensitive seedlings and investigated GFP fluorescence of herbicide-sensitive plants (Table 4).

Table 4. Analysis of T_1 progeny derived from self-pollinated TMV-Cre and PVX-Cre infected plants

Line	Expression vector	Examined plants			
		Total	Pat resistant	Pat sensitive	Gfp expression
34-1	PVX-Cre	100	19	81	+
34-2	PVX-Cre	38	-	38	+
34-3	PVX-Cre	108	16	92	+
34-4	PVX-Cre	91	-	91	+
34-5	PVX-Cre	75	58	17	-
34-6	PVX-Cre	77	74	3	-
44-1	PVX-Cre	73	-	73	+
44-2	PVX-Cre	36	14	22	+
34-1	TMV-Cre	82	69	13	-
34-2	TMV-Cre	32	25	7	-
34-3	TMV-Cre	27	23	4	-
44-2	TMV-Cre	79	64	15	-
44-3	TMV-Cre	39	34	5	-
44-4	TMV-Cre	56	56	-	-

We tested the T_1 progeny of eight self-pollinated PVX-Cre infected plants. Segregation analysis and investigation of GFP activity in phosphinothricin sensitive plants showed that the progeny of six plants inherited the Cre-mediated recombination event. In contrast, for TMV-Cre expression vector, the ratio of herbicide resistant to herbicide sensitive seedlings was high and GFP activity was not observed in phosphinothricin sensitive plants of all investigated lines. These results demonstrate that the self-pollination strategy which is efficient for the PVX-Cre expression vector was not useful for the TMV-Cre expression vector.

3.6 *A. tumefaciens* expression vector

The second vector for transient expression of cre recombinase is based on *A. tumefaciens* T-DNA. We used the agroinfiltration technique to deliver Cre protein into *lox*-target plants. Recombinant *A. tumefaciens* harbouring pLH-*gus*-*cre* was infiltrated into plant leaves as a liquid culture. Agroinfiltration of *N. benthamiana* leaves was followed by plant regeneration on MS medium containing 500 mg/l ticarcillin to kill any remaining bacteria cells. PCR analyses of the regenerants showed that 34% of the plants were marker free (Table 5). To test whether Cre-mediated excision of the *bar* sequence was due to expression of *cre* from integrated pLH-*gus*-*cre* T-DNA or to transient expression, we applied DNA from *gfp* expressing plants to PCR analysis using primers specific to the internal sequence of the *cre* gene (Table 5). 14% of the regenerants were identified as having undergone site-specific *bar* gene excision via transient expression of the *cre* recombinase. Self progeny of a marker-free plant (44-3) inherited the recombined state, as was shown by PCR and Southern analysis. From 114 seeds tested on phosphinothricin containing medium in a seed germination assay none was resistant. Most of them (109 from 114) were *gfp* positive. Therefore, both phenotypic and molecular evidence indicate that the *A. tumefaciens* vector can be applied for marker gene elimination using the regeneration strategy.

Table 5. The frequency of site-specific excision event by using the *A. tumefaciens* expression vector

Line 44	No. regenerants				
	total	Bar positive	Bar negative		
			total	Cre positive	Cre negative
	73	48	25 (34%)	15 (20%)	10 (14%)

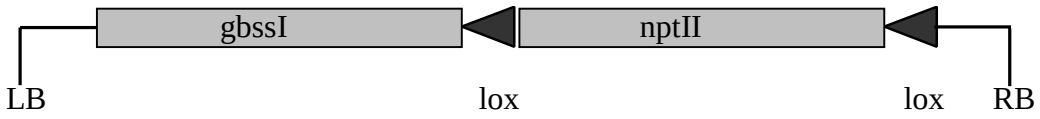
4. Expected utilization of the project results

4.1. Practical application of the PVX-Cre expression vector

The efficiency of marker gene excision from model *N. benthamiana* plants observed for the PVX-Cre expression vector strongly suggests a practical application of our strategy. The goal of these experiments which have been conducted in cooperation with Dr. Eckhard Tacke (BIOPLANT Biotechnologisches Forschungslabor GmbH, Ebstorf) is obtaining marker-free potato plants with reduced amylose content. Potato starch is composed of two polymers, amylose and amylopectin. The granule-bound starch synthesis (GbssI) takes part in the synthesis of amylose. Suppression of natural *gbssI* activity by transgenic *gbssI* gene can result

in a reduced content of amylose. Such amylopectin rich starch might be interesting for different practical purposes. Potato belongs to vegetatively propagated species and all experiments were performed according to the scheme for regeneration strategy (Fig. 4). As a first step to design amylopectin rich, marker-free potato plants, we constructed the pLH-*gbssI-lox-nptII-lox* vector (Fig. 5) and introduced it into potato plants.

Figure 5. pLH-*gbssI-lox-nptII-lox* construct



The pLH-*gbssI-lox-nptII-lox* vector for plant transformation includes the *nptII* marker gene surrounded by two *lox* sites in direct orientation and the *gbssI* gene which is placed under the control of *gbssI* promoter. After *A. tumefaciens* mediated transformation more than 100 transgenic potato lines were obtained, and tubers were tested for the synthesis of amylose. Three lines (25, 37, and 74) with decreased amylose content were selected for further experiments (Table 6).

Table 6. Amylose content in tubers of *gbssI* transgenic potato plants

Line	% Amylose
Tomensa, control	17,21
25	3,36
37	3,61
74	4,82

The functional expression of *cre* recombinase in PVX-Cre systemically infected potato leaves was shown by Western blot analysis. We received a large number of regenerants from PVX-Cre infected leaf explants of line 37. Molecular analysis of the regenerants is in progress.

4.2. Possible application of the Cre-transient expression vectors

We developed an alternative way to obtain marker free transgenic plants by using *A. tumefaciens* and plant-cre-virus (PVX-Cre and TMV-Cre) vectors. It was shown that this transient expression vectors can excise a marker gene from model *N. benthamiana* plants. Our strategy can be applied to vegetatively propagated genotypes that depend on organogenesis or somatic embryogenesis for regeneration, particularly potato and woody plants. PVX-Cre expression vector might be also useful to eliminate selectable markers from generatively

propagated species. Another practical application of plant-Cre-virus vectors can be a regulated expression of toxic products or viral proteins that normally are harmful for plant cells at high levels. Such proteins can be delivered into cells in a silent form and then expressed by infection with plant-Cre viruses.

4.3. Final conclusions

We developed a new approach to eliminate marker genes by transient expression of cre recombinase. This approach includes:

1. Efficient cre protein activity in transiently transformed plant cells. Cre delivery can be done by both plant-cre virus and *A. tumefaciens* expression vectors.
2. Transfer of the recombined state from plant cells to the whole plant. Our results demonstrate that two strategies are applicable: regeneration strategy and self-pollination strategy. Regeneration strategy can be employed for vegetatively propagated species. Excision of the selectable marker from generatively propagated genotypes can be performed by the PVX-Cre expression vector using the self-pollination strategy. Plant-cre virus vectors (PVX-Cre and TMV-Cre) were more efficient in comparison to the *A. tumefaciens* expression vector.
3. Transfer of the recombined state to the next generation. Self progeny of marker-free regenerants and self-pollinated PVX-Cre infected plants demonstrated marker-free genotype as was proved by segregation and molecular analysis.

We started to apply the PVX-Cre expression vector to potato. First experiments have shown promising results.

5. RD results of third parties

Until now, the new approach to eliminate marker genes by transient expression of cre recombinase developed in our laboratory was only published by us. Several laboratories asked for the published constructs to introduce the system into their further work.

6. Publications

Scientific results of the project have been published in the following articles:

1. Kopertekh, L., Jüttner, G. and Schiemann, J. 2004. Site-specific recombination induced in transgenic plants by PVX virus vector expressing bacteriophage P1 recombinase. Plant Science 166: 485-492.

2. Kopertekh, L., Jüttner, G. and Schiemann, J. 2004. PVX-Cre-mediated marker gene elimination from transgenic plants. Plant Mol. Biol. 55: 491-500.

3. Kopertekh, L. and Schiemann J. 2005. Agroinfiltration as a tool for transient expression of cre recombinase in vivo. Transgenic Research, in press.

7. Literature

1. Ahlquist, P. and Janda, M. 1984. cDNA cloning and *in vitro* transcription of the complete brome mosaic virus genome. Mol. and Cell Biol. 4: 2876-2882.

2. Bayley, C.C, Morgan, M., Dale, E.C. and Ow, D.W. 1992. Exchange of gene activity in transgenic plants catalyzed by the Cre-lox site-specific recombination system. Plant Mol Biol 18: 353-361.

3. Chapman, S., Kavanagh, T. and Baulcombe, D. 1992. Potato Virus X as a vector for gene expression in plants. Plant J 2 549-557.

4. Coppoolse, E.R., de Vroomen, M.J., Roelofs, D., Smit, J., van Gennip, F., Hersmus, B.J.M., Nijkamp, H. J. and van Haaren, M.J.J. 2003. Cre recombinase expression can result in phenotypic aberrations in plants. Plant. Mol. Biol. 51:263-279.

5. McCormac, A.C., Fowler, M.R., Chen, D.F, Elliot, M.C. 2001. Efficient co-transformation of *Nicotiana tabacum* by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. Trans Res 10:143-145.

6. McCormick, A.A., Reinl, S.J., Cameron, T.I., Vojdani, F., Fronefield, M., Levy, R. and Tuse, D. 2003. Individualised human scFv vaccines produced in plants: humoral anti-idiotype responses in vaccinated mice confirm relevance to the tumor Ig. J. Immunol. Methods. 278: 95-104.

7. Dale, E.C. and Ow, D.W. 1990. Intra and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. Gene 91:79-85.

8. Dale, E.C. and Ow, D.W. 1991. Gene transfer with subsequent removal of the selection gene from the host genome. Proc. Natl. Acad. Sci. USA 88:10558-10562.

9. Donson, J., Kearney, C.M., Hilf, M.E. and Dawson, W.O. 1991. Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. Proc. Natl. Acad. Sci. 88: 7204-7208.

10. Goto, H., Osaki, T., Kijima, T., Nishino, K., Kumagai, T., Funakoshi, T., Kimura, H., Takeda, Y., Yoneda, T., Tachibana, I. and Hayashi, S. 2001. Gene therapy utilising the Cre/lox system selectively suppresses tumour growth of disseminated carcinoembyonic antigen-producing cancer cells. Int J. Cancer. 94: 414-419.

11. Hoess, R.H., Wierzbicki, A. and Abremski, K. The role of the loxP spacer region in P1 site-specific recombination. 1986. Nucl. Acids Res. 14: 2287-2300.

12. Kumagai, M.H., Turpen, T.H. Weinzettl, H., Della-Cioppa, G., Turpen, A.M., Donson, J., Hilf, M.E., Grantham, G.L., Dawson, W.O., Chow, T.P. Piatak, M.J. and Grill, L.K .1993. Rapid, high-level expression of biologically active L-trichosantin in transfected plants by an RNA viral vector. Proc Natl Acad Sci USA 90:27-430.

13. Loonstra, A., Vooijs, M., Beverloo, H.B., Allak, B.A., van Drunen, E., Kanaar, R., Berns, A. and Jonkers, J. 2001. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc. Natl. Acad. Sci. USA 98:9209-9214.

14. Mor, T.S., Moon, Y.S., Palmer, K.E. and Mason, H.S. 2003. Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol. Bioeng. 81:430-437.

15. Russel, S.H, Hoopes, J.L. and Odell, J.T. 1992. Direct excision of a transgene from the plant genome. Mol Gen Genet 234: 49-59.

16. Sauer, B. 1987. Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. Mol Cell Biol. 7: 2087-2096.

17. Sauer, B. and Henderson, N. 1988. Site-specific recombnation in mammalian cells by the Cre recombinase of bacteriophage P1. Proc Natl Acad Sci USA 85:5166-5170.

18. Silver, D.P. and Livingston, D.M. 2001. Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol. Cell. 8:233-243.

19. Takamatsu, N., Ishikama, M., Meshi, T and Okada, Y. 1987. Expression of a bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. EMBO Journal 6: 307-311.

20. Tiscornia, G., Tergaonakar, V., Galimi, F. and Verma, M.I. 2004. Cre recombinase – inducible RNA interference mediated by lentiviral vectors. Proc Natl Acad Sci 101:7347-7351.

21. Thevenot E., Cote, F., Colin, P., He, Y., Leblois, H., Perricaudet, M., Mallet, J. and Vodjani, G. 2003. Targeting conditional gene modification into the serotonin neurons of the dorsal raphe nucleus by viral delivery of the Cre recombinase. Mol and Cell Neuroscience. 24: 139-147.

22. Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Parijs, L.V., Jaenisch, R. and Jacks, T. 2004. Cre-lox-regulated conditional RNA interference from transgenes. Proc. Natl. Acad. Sci. 101. 10380-10385.

23. Vergunst, A.C., Schrammeijer, B., den Dulk-Ras, A., de Vlaam, C.M.T., Regensburg-Tuink, T.J.G. and Hooykaas, P.J.J. 2000. Vir B/D4 – dependent protein translocation from *Agrobacterium* into plant cells. Science 290:979-982.

24. Will, E., Klump, H., Heffner, N., Schwieger, M. and Schiedlmeier, B. 2002. Unmodified Cre recombinase crosses the membrane. Nucl. Acids Res. 30:e 59.

25. Zhang, W., Subbarao, S., Addae, P, Shen, A., Armstrong, C., Peschke, V. and Gilbertson, L. 2003. Cre-lox-mediated marker gene excision in transgenic maize (Zea mays L.) plants. Theor Appl Genet 107: 1157-1168.

26. Zubko E., Scutt, C and Meyer P. 2000 Intrachromosomal recombination between *attp* regions as a tool to remove selectable marker genes from tobacco transgenes. Nat Biotechnol. 18: 422-445.

27. Zuo, J., Niu, Q-W., Moller, S.G. and Chua, N-H. 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. Nat. Biotechnol. 19:157-161.