Theor Appl Genet (2005) 111: 1608–1616
DOI 10.1007/s00122-005-0092-2

ORIGINAL PAPER

S. Kerbach · H. Lörz · D. Becker

Site-specific recombination in *Zea mays*

Received: 28 February 2005 / Accepted: 22 August 2005 / Published online: 1 October 2005

Abstract The elimination of marker genes after selection is recommended for the commercial use of genetically modified plants. We compared the applicability of the two site-specific recombination systems Cre/lox and Flp/FRT for marker gene elimination in maize plants. The selection marker gene *pat* surrounded by two identically directed lox or FRT sites was introduced into maize. Sexual crossing with plants harboring the corresponding constitutively expressed recombinase led to the precise and complete excision of the lox-flanked marker gene in the F_1 progeny, whereas Flp-mediated recombination of FRT sequences occurred rarely. Further examination of site-specific integration was done by biolistic bombardment of immature embryos harboring only one lox site with a lox.*uid*A sequence with results indicating directed integration.

Introduction

Genetic engineering of plants offers the possibility of precise modification of the genome for application in agriculture or research. Nevertheless, some problems accompany the advantages of genetic engineering. Nearly all transformation approaches work with selectable markers, such as antibiotic-resistance genes. These sequences are not needed further for the function of the target transgene. Removal of the selection marker allows its reuse for the stacking of genes. The most frequently used marker genes confer resistance against antibiotics, which are widely spread in microorganisms, so it is reasonable to eliminate these sequences to respond to public concern.

Besides the excision of unwanted sequences the second challenge in plant transformation is the random integration of the introduced transgene by various transformation approaches, which can lead to unpredictable position effects (Meyer 2000). For reliable expression of the transgene, directed integration is recommended. The use of site-specific recombination systems can respond to these difficulties.

Different methods are available for the removal of marker genes, such as cotransformation, transposon- or recombinase-mediated marker elimination. With cotransformation, unlinked integration of marker and target genes into the genome of various plant species results in the successful elimination of marker genes by segregation (Ebinuma et al. 2001). The separation of transgene and selection marker gene was also achieved by transposition. The transposase catalyzes the transposition of a marker gene, flanked by recognition sites and leads to marker-free plants after segregation (Goldsbrough et al. 1993). Comparatively, site-specific recombination systems provide a more efficient and precise tool for marker removal. Recombination between two homologous recognition sites flanking the marker gene results in the elimination of the marker sequence (Ow 2002).

A number of very basic site-specific recombination systems are known from phages, prokaryotes and eukaryotes, such as Cre/lox from the bacteriophage P1 (Sternberg and Hamilton 1981), Flp/FRT from the 2 μ plasmid of *Saccharomyces cerevisiae* (Broach et al. 1982) or R/RS from *Zygosaccharomyces rouxii* (Araki et al. 1985). Common in these systems are the recombinases Cre, Flp and R, which all belong to the integrase type, mediating recombination between two homologous recognition sites (lox, FRT, RS). The recognition site is palindromic, except for the inner asymmetric spacer sequence. This spacer determines the direction of the recognition site and therefore the type of reaction. Opposite-directed recognition sites lead to the inversion

Communicated by P. Langridge

S. Kerbach · H. Lörz · D. Becker (✉)
Biocenter Klein Flottbek, Section Developmental Biology and Biotechnology, University of Hamburg,
Ohnhorststraße 18, 22609 Hamburg, Germany
E-mail: becker@botanik.uni-hamburg.de
Tel.: +49-40-42816284
Fax: +49-40-42816229

of the flanked DNA, whereas recombination between sites directed in the same orientation results in the excision of intervening DNA within one molecule or in the integration reaction between two molecules. The recombination systems described above were successfully adapted to various plant species such as tobacco, *Arabidopsis thaliana*, rice, wheat and mediate site-specific excision of DNA (Odell and Russell 1994, Ow 2002, Lyznik et al. 2003).

Besides the precise excision of marker genes, site-specific recombination systems offer the possibility of directed integration of foreign DNA. Hence, the random integration achieved by plant transformation approaches available so far can be minimized.

Although approaches for direct transgene integration by homologous recombination have been conducted, the frequency of integration in higher plants is too low for a valuable application, as shown in *Lotus japonicus* (Thykjaer et al. 1997) or in tobacco (Kovalchuk et al. 2003). Only in the moss *Physcomitrella patens* was a high rate of integration event found (Schaefer and Zryd 1997).

Site-specific integration of the transgene into a previously determined locus in the genome mediated by recombination systems can supply a promising means for the prevention of position effects. Until now, site-specific gene integration in plants has been reported by PEG-mediated protoplast transformation of tobacco (Dale and Ow 1990; Albert et al. 1995), *Agrobacterium*-mediated transformation of *A. thaliana* (Vergunst and Hooykaas 1998; Vergunst et al. 1998, 2000) and transformation of rice by biolistic bombardment (Srivastava et al. 2004).

For the application of site-specific recombination in *Zea mays* we enable excision events by crossing maize plants harboring the selection marker gene *pat*, which is flanked by two recognition sequences lox or FRT directed in the same orientation, with plants expressing the respective recombinase Cre or Flp. To our knowledge, this is the first report of transgenic maize plants expressing the Flp recombinase.

F_1 progeny which were freed of the marker gene *pat* should contain only one recognition site and the recombinase gene. Hence, we promoted the integration of a *uid*A gene associated with one recognition site into the genomic site. First evidence confirmed the site-specific integration in transient expression assays.

Methods

Transformation vectors

For the stable transformation of maize two constructs for each system were used which encode for the recombinases Cre and Flp and contain the recognition sites lox and FRT, respectively. The cloning of the transformation vectors was carried out as described by Sambrook et al. (1989).

The plasmid pUbi::*cre* carries the Ubiquitin1 promoter from maize, the *cre* coding sequence from the bacteriophage P1 and the nos-terminator from *Agrobacterium tumefaciens*. A *Hin*dIII/ *Pst*I Ubiquitin1 promoter fragment from the plasmid pUbi.cas (D. Becker, University of Hamburg) was integrated into the plasmid pMM23 (Dale and Ow 1990).

The transformation vector pAct1::*flp* encloses the *flp* coding sequence under the control of the constitutively expressing Actin1-F promoter from rice and the nos-terminator. Therefore a 1.2 kbp fragment containing the complete *flp* coding sequence was amplified out of commercially obtainable baking yeast with the primers Flp1 (5′-atagctggatccagatcggcgctaagcatg-3′) and Flp2 (5′-aggtacaggatccgaacggcatagtgcgtgttta-3′) derived from the sequence of the 2 µ plasmid of *S. cerevisiae* (NC 001398). This PCR product was cloned into the vector pZeroBlunt® TOPO® (Invitrogen, USA). Following this, the *Pst*I/ *Kpn*I-flp fragment was introduced into the vector pAct1.cas (McElroy et al. 1990) carrying the Actin1-F promoter and the nos-terminator.

The *cre* gene was cotransferred with the selection marker gene *pat* from the construct p35SAcS under the control of the CaMV 35S promoter and terminator (Eckes et al. 1989) into maize. For the construction of lox- and FRT-containing vectors two 34 bp lox or FRT sites were placed around the *pat* coding sequence and nos-terminator by PCR with a Ubiqutin1 promoter upstream (pUbi.2lox.*pat*, pUbi.2FRT.*pat*). By a standard PCR approach using the primers P1 (5′-tccggtccatggttagggccc-3′) and lox2rv (5′- cccggggatccataacttcgtataatgtatgctatacgaa gttattctagagtcgacctgcagaagtaacaccaaac-3′), which contains the wild-type lox sequence, a lox fragment with the restriction sites for *Nco*I and *Bam*HI was generated. The *Nco*I/*Bam*HI-lox fragment was introduced between the Ubiquitin1 promoter and the nos-terminator into the vector pUbi.cas, derived from pUC18. The resultant vector pUbi.lox.nos was used for the integration of the second lox site downstream of the nos-terminator, which was created by a similar PCR approach with the primers P3 (5′-ggtaccgagctcgaatttccccgatcgttcaaa catttggc-3′) and lox4rv (5′- tagtacgaattcataacttcgtataatgtatgctatacgaagtta tccgatctagtaacatagatgacaccgcgc-3′). The resulting *Sst*I/ *Eco*RI-lox fragment was inserted into the former vector pUbi.lox.nos generating the vector pUbi.2lox.nos with two lox sites. Finally, a blunt end *pat* fragment *Sal*I, cleaved from p35SAcS and filled in, was integrated into the *Sma*I restriction site downstream from the Ubiquitin1 promoter and 5′ lox site, resulting in pUbi.2lox.*pat*.

The production of the transformation vector pUbi.2FRT.*pat* was carried out in a similar way, using the primer FRT2rv (5′- cccggggatccgaagttcctatactttctagagaa taggaacttctctagagtcgacctgcagaagtaacac caaac-3′) instead of lox2rv for introducing the first recognition site and the primer FRT4rv (5′- tagtacgaattcgaagttcctatactttctagag aataggaacttcccgatctagtaacatagatgacaccgcgc-3′) instead of lox4rv for introducing the second recognition site. Compared to the wild-type FRT sequence with three Flp

binding sites, the 34 bp FRT sequence contained only two Flp binding sites.

To test the site-specific integration, we generated a promoterless *uid*A construct. For this purpose a *Bam*HI fragment, carrying the *uid*A coding sequence, was inserted into the vector pUbi.lox.nos containing one lox site under the control of the Ubiquitin1 promoter and the nos-terminator described above. After restriction with *Pst*I the Ubiquitin1 promoter was removed. Religation completed construction of the promoterless *uid*A vector, plox.*uid*A.

Plant transformation

The stable transformation of maize was carried out by biolistic bombardment of immature zygotic embryos (A188 × H99) as described by Brettschneider et al. (1997), with the following modifications. The explants were bombarded 7 days after isolation with a gas pressure of 1,350 psi using the Bio-Rad PDS 1000-He particle delivery system.

Molecular analyses

Genomic DNA was isolated from 200 mg frozen leaf material by a slightly modified protocol of Pallotta et al. (2000). For Southern blot analyses, 15–25 µg genomic DNA was cleaved with restriction enzymes in a total volume of 50 µl, separated on a 0.8% agarose gel, blotted on a nylon membrane (Amersham Biosciences Europe, Germany) and hybridized with ^{32}P-labeled DNA probes using the HexaLabel™ DNA Labeling Kit (Fermentas, Germany).

Total RNA for northern blot analyses was isolated from 200 mg frozen leaf material with peqGOLD TriFast™ using manufacturer's instructions (peqLab, Germany). On a denaturing MOPS-formaldehyde gel, 15 µg total RNA was separated (Sambrook et al. 1989) and blotted on a positively charged nylon membrane (Amersham Biosciences Europe) and hybridization carried out according to Church and Gilbert (1984) with ^{32}P-labeled DNA probes.

Total protein was extracted from 300 mg frozen leaf material with 300 µl buffer (125 mM Tris, pH 6.8; 10% SDS; 25% glycerol; 12.5% β-mercaptoethanol). The samples were boiled for 5 min. After centrifugation 25 µl of the supernatant was directly used for 12% polyacrylamide gel electrophoresis as described by Laemmli (1970). After semidry electroblotting (Bio-Rad, Germany) the proteins were transferred to a Hybond-ECl membrane (Amersham Biosciences Europe). Cre bands were visualized by a 1:1,000 diluted polyclonal rabbit antibody against Cre (Novagen, USA). Detection of the Cre antibody was carried out using a 1:1,000 diluted monoclonal antirabbit horseradish peroxidase antibody (Sigma, USA) and the SuperSignal®-System (Pierce, USA) as described in the manufacturer's protocol.

PCR reactions were performed according to the manufacturer's instruction (Fermentas, Germany). F_1 progeny were analyzed by a PCR approach using the primers Ubi-for (5′-atgccagcctgttaaacgcc-3′) and 18rev-75 (5′-cttccggctcgtatgttgtgtgg-3‘), which bind upstream and downstream of the recombination spots, respectively. After purification of PCR fragments from the agarose gel with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences Europe) the DNA was sequenced.

Transient expression tests

For transient expression studies zygotic maize embryos were isolated 17 days after fertilization. The explants were incubated on osmotic N6 medium (D'Halluin et al. 1992) and bombarded 1 day after isolation differing from the protocol for stable transformation with 83 µg gold particles and 167 ng DNA per shot.

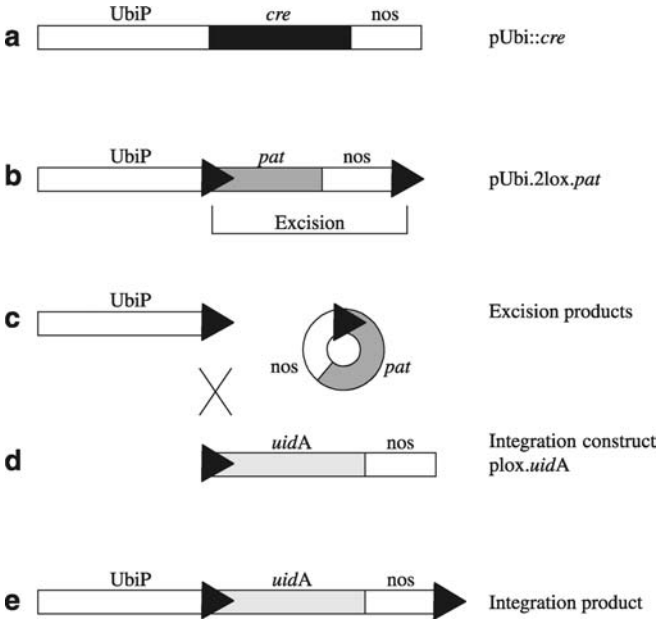

Fig. 1 Molecular strategy for site-specific Cre/lox recombination. **a** pUbi::*cre*, which is transferred into maize, provides the constitutively expressed recombinase under the control of the Ubiquitin1 promoter and the nos-terminator. **b** The transformation vector pUbi.2lox.*pat* presents the substrate for Cre-directed recombination. Two lox sites depicted as *triangles* orientated in the same direction surround the selection marker *pat* and the nos-terminator under the control of the Ubiquitin1 promoter. **c** The resulting excision products are the genomic Ubiquitin1 promoter with a lox site downstream and a free circular lox-*pat*-nos fragment. **d** The construct plox.*uid*A is used for site-directed integration. **e** Successful site-specific recombination between the genomic lox site downstream of the promoter and the lox site of the integration construct plox.*uid*A leads to the expression of the reporter gene *uid*A. *UbiP* Ubiquitin1 promoter from maize, *pat* phosphinothricin-*N*-acetyl transferase gene from *Streptomyces viridochromogenes*, *nos* nopaline synthetase terminator from *Agrobacterium tumefaciens*, *uid*A β-glucuronidase gene from *Escherichia coli*. *Filled triangles* represent lox sites

To prove the activity of β-glucuronidase histochemical staining was carried out as described by Jefferson et al. (1987) using the following modifications. Scutellar tissue was incubated 3 days after bombardment for 15 h at 37°C in X-Gluc staining solution [1.5 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuron acid; Duchefa, Netherlands); 100 mM NaH_2PO_4, pH 7.0; 10 mM EDTA, pH 7.0; 0.5% Trition X-100; 100 µg chloramphenicol/ml]

Results

Experimental strategy

In this study the two site-specific recombination systems Cre/lox and Flp/FRT were compared in terms of their applications in genetic engineering of maize plants. The Cre/lox recombination system is shown in Fig. 1 as an example for the general strategy of site-specific recombination.

Three constructs were designed for stable transformation of maize and transient expression tests using the Cre/lox system. Two constructs were introduced independently into maize within stable transformation experiments: pUbi::*cre*, containing the constitutively expressed *cre* gene under the control of the Ubiquitin1 promoter and the nos-terminator, and pUbi.2lox.*pat*, containing the *pat* gene and nos-terminator surrounded by two lox sites downstream from the Ubiquitin1 promoter. After sexual hybridization the Cre recombinase catalyzes the site-specific recombination between the two homologous lox sites, leading to the excision of the flanked DNA sequence. A lox site remains in the maize genome.

In order to investigate site-directed integration a third construct for transient expression analyses was performed. plox.*uid*A contains a promoterless *uid*A coding sequence downstream of one single lox site. By biolistic bombardment of embryos harboring a single genomic lox site and *cre* recombinase gene we expected a site-specific recombination between the genomic lox site downstream of the promoter and the plasmid-located lox site. Site-specific recombination places the *uid*A coding sequence adjacent to the Ubiquitin1 promoter. The integration of the promoterless *uid*A coding sequence downstream of the Ubiquitin1 promoter enables the expression of the *uid*A gene, whose activity is detectable by histochemical staining.

The components of the Flp/FRT system, *flp* recombinase gene (pAct1::*flp*) and the FRT-flanked *pat* sequence (pUbi.2FRT.*pat*), were transformed independently in maize.

Development of parental plants

Maize embryos (A188 × H99) were stably transformed either with the *pat* gene cassette flanked by lox or FRT sites (pUbi.2lox.*pat*, pUbi.2FRT.*pat*) or with the plasmids pUbi::*cre* or pAct::*flp* by particle bombardment of scutellar tissue. Since the maize lines containing the recognition sites already express the *pat* gene and the recombinase lines, formerly cotransformed with the *pat* gene, selection of all transgenic plants occurred via application of the phosphinothricin-containing herbicide Basta™.

With a transformation efficiency of 2.2–3.2% we obtained 19 independent maize lines harboring the full integrated lox-containing selection marker gene *pat*, 10 lines with the complete Ubi.2FRT.*pat* sequence as well as 12 lines with a complete integration of the *cre* recombinase gene and for the first time we report 5 maize lines with the complete *flp* recombinase gene. The presence of the complete transgenes in the primary transformants was confirmed by genomic DNA gel blot analyses. Transcription and translation of the *cre* and *flp* recombinase genes were shown by northern and western blot (only Cre) analyses. Furthermore the stable inheritance of the transgenes in the T_1 progeny was confirmed by segregation and Southern blot analyses.

Crossing of recombinase lines and recognition sites harboring maize

The obtained T_0 plants, either expressing the recombinase genes or harboring recognition sites flanked marker genes, were crossed to enable site-specific recombination. To optimize the identification of recombined DNA in the progeny the plants used were chosen according to a low copy number of integrated transgenes and a simple restriction pattern. In total, we generated 12 F_1 progeny plants with *cre* and lox sequences and for the first time report 7 F_1 plants harboring *flp* and FRT sequences. Table 1 summarizes the successful hybridization of Cre- and lox- as well as Flp- and FRT-lines. The presence of both components of the site-specific recombination system in one plant enables

Table 1 Successful hybridization of recombinase (Cre, C; Flp, F) and recognition sites (lox, FRT) harboring plants

	Crossing notation	Maternal parent	Paternal parent	Number of hybridized plants
Cre/lox hybridization	11	C 9-16	lox 9-3A	11
	12	lox 9-3A	C 9-17	1
Flp/FRT hybridization	A	F 6-9	FRT 30-5	1
	F	F 1-5A	FRT 30-5	2
	G	FRT 30-5	F 1-5A	4

the site-specific excision of the lox or FRT-flanked marker gene.

Gene excision in the F_1 progeny

To obtain molecular evidence of recombinase-mediated excision events the F_1 progeny was investigated by Southern blot analyses. The detection of the transgenes of the Cre/lox system was conveniently carried out using the same Ubiquitin1 promoter-directed DNA probe in Southern blot analyses, since both components were expressed under the control of the Ubiquitin1 promoter. Site-specific recombination led to the excision of the lox-flanked *pat* marker gene and therefore to a shortening of the introduced transgene cassette by the length of the eliminated DNA sequence. This length reduction was detectable by comparison of non-recombined parental plant DNA with DNA of the F_1 progeny, in which site-specific recombination occurred. By treatment of the plant DNA with endonucleases cutting out the complete 2.45 kbp lox transgene cassette Ubi.2lox.*pat*, followed by electrophoresis, blotting and Southern hybridization, the complete excision of the marker gene was confirmed (Fig. 2). In all progeny plants with the *cre* recombinase gene and lox sites together, the shortening of the former introduced lox cassette was observed. Altogether 12 F_1 progeny plants originating from two independent crosses (Table 1) presented a shorter 1.5 kbp Ubiquitin1 promoter fragment instead of the 2.45 kbp Ubi.2lox.*pat* cassette. No re-integration was detected in the tested plants.

To verify site-specific excision events the region of the recombination spots was amplified by PCR. The PCR products of recombined progeny were 1,050 bp in size, stretching from 943 bp upstream of the lox site to 75 bp downstream of the 34 bp lox site, whereas non-recombined DNA was 1.9 kbp in size (Fig. 3). Sequencing the PCR products from the recombined progeny plants revealed an exact excision of the lox-flanked sequence and the identical, predicted junction compared with the non-recombined parental lines.

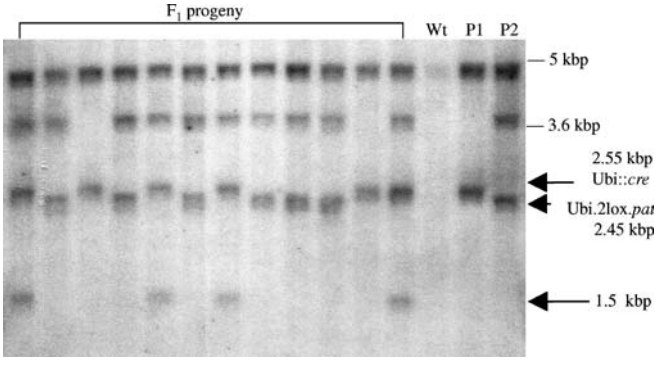

Fig. 2 Southern blot analysis of the F_1 progeny (C 9-16 × lox 9-3A). *Hin*dIII/*Eco*RI cleaved genomic DNA of 25 µg from 12 individual F_1 plants were separated on an agarose gel, blotted and hybridized with a ^{32}P-labeled Ubiquitin1 promoter DNA probe. *Wt* wild type, *P1* maternal *cre*-parent, *P2* paternal Ubi.2lox.*pat* parent

Flp-mediated recombination events between FRT sites which surround the selection marker gene *pat* have not been detected by Southern blot analyses to date. The first successful excision event has been found in one F_1 plant by PCR. The sequence of the PCR product obtained corresponded to that expected by the action of Flp (Fig. 3).

Marker excision is stable and inheritable

In order to prove that marker excision is inheritable, we propagated five F_1 plants from two independent crosses showing successful site-specific recombination by self-pollination. Thirty F_2 progeny plants per F_1 parent were grown and examined by phosphinothricin selection and Southern blot analyses. In all progeny plants the same restriction pattern as the parental F_1 plants showing a recombined excised lox transgene was detected or the pattern of the origin starter lines harboring only *cre* or lox transgenes. Hence, the state of completely excised marker sequence was stably inherited in all examined lines.

Site-specific integration

After site-specific recombination, the 12 F_1 plants contained the *cre* transgene and one single lox site downstream of the Ubiquitin1 promoter, remaining from the excision of the lox-flanked sequence. In order to carry out site-directed integration tests all available immature embryos derived from the self-pollination of five recombined F_1 plants with *cre* and lox sequences were cultivated on media and bombarded with gold particles coated with the promoterless *uid*A construct plox.*uid*A. Site-specific integration places the *uid*A coding sequence adjacent to the Ubiquitin1 promoter and facilitates the expression of the *uid*A gene. The activity of the resulting *β*-glucuronidase is then visualized by histochemical staining.

The comparison of embryos derived from Cre/lox-F_1 plants with parental type embryos should clarify if site-specific integration occurred more often than random integration of the promoterless *uid*A gene downstream of any endogenous promoter. As shown in Table 2, some progeny embryos showed a significantly higher number of signals than the corresponding negative control embryos. In one case the average signal number was 0.75 whereas the parental type embryos showed only 0.02–0.03 signals per embryo. Nevertheless, other progeny embryos revealed the same level of *β*-glucuronidase activity comparable with the activity of the parental type embryos. These results indicate the successful site-specific integration events in maize.

Discussion

Directed excision of DNA sequences by various site-specific recombination systems such as Cre/lox or Flp/

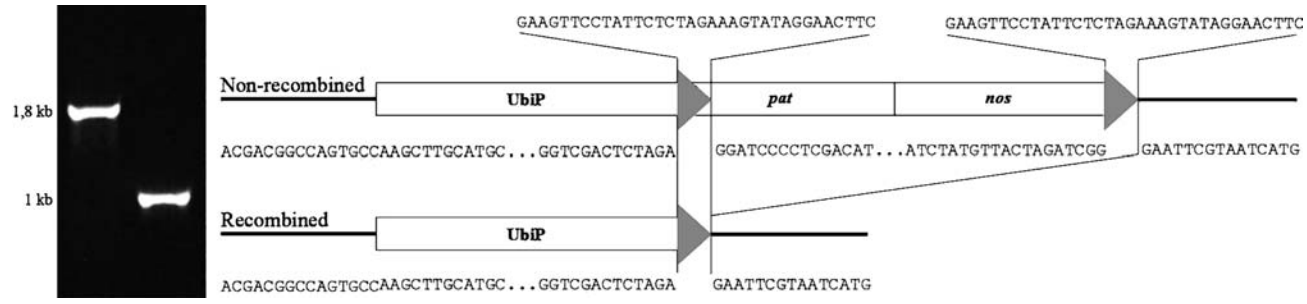

Fig. 3 PCR analysis and sequencing results of DNA sequences of recombined and non-recombined Flp/FRT progeny. *Grey arrows* represent FRT sites

Table 2 β-Glucuronidase activity in bombarded embryos derived from self-pollinated Cre/lox-F_1 plants (C 9-16 × lox 9-3A) for site-directed integration in comparison to the bombardment of parental type embryos served as negative controls

Origin of bombarded progeny embryos	Total number of bombarded embryos	Total number of signals for β-glucuronidase activity	Number of signals per embryo
No. 22	43	1	0.02
No. 23	45	0	0
No. 27	33	6	0.18
No. 28	68	19	0.30
No. 34	52	39	0.75
lox parent (male)	67	2	0.03
Cre parent (female)	42	1	0.02

FRT has been demonstrated in different plant species including tobacco (Odell et al. 1990; Russell et al. 1992; Lloyd and Davis 1994; Gidoni et al. 2001), *A. thaliana* (Sonti et al. 1995; Luo et al. 2000), wheat (Srivastava et al. 1999), rice (Hoa et al. 2002) and maize (Zhang et al. 2003). Whereas the Flp recombinase was successfully expressed for instance in *Nicotiana tabacum* (Kilby et al. 1995) its expression in monocotyledonous plant species has been reported only for *Oryza sativa* (Radhakrishnan and Srivastava 2005). Here we describe the comparative application of the site-specific recombination systems Cre/lox and Flp/FRT for specific elimination of selection markers in maize plants. The use of these site-specific recombination systems requires the presence and activity of a recombinase and the corresponding recognition sites at the same place and time. We achieved this by crossing maize lines carrying either the recombinase gene or the selection marker gene *pat* surrounded by identically orientated recognition sites and generated F_1 plants harboring Cre and lox as well as Flp and FRT sequences. This is the first report of transgenic maize plants containing Flp recombinase and FRT sites.

The chosen strategy of crossing maize lines carrying either the Cre recombinase or a lox construct results in a complete and precise excision of the lox-flanked DNA. All F_1 plants harboring cre and lox sequences showed by Southern and PCR analyses had successful site-specific excision events. Likewise, a recombination efficiency of Cre/lox recombination of 95% (Russell et al. 1992) and up to 100% (Odell et al. 1990) has been observed in tobacco. Flp-mediated recombination showed excision of the FRT-flanked sequence in only one maize plant, but was as precise as the Cre/lox recombination. To our knowledge, this is the first maize plant with a stable site-specific recombined sequence.

Besides sexual crossing retransformation is another possibility of combining the recombinase and the recognition sites in one plant (Odell et al. 1990; Russell et al. 1992; Gleave et al. 1999). The complete excision indicates that the site-specific recombination takes place early in the germ cells, as described by Russell et al. (1992) and Bayley et al. (1992). Somatically occurring recombination events display a mosaic pattern, which has not been observed here since neither non-recombined DNA with *cre* nor lox sequences were detected in the progeny plants. The early recombination events and therefore complete excision of the lox-flanked sequences is one special advantage of the crossing strategy used. Different results have been shown by Mlynarova and Nap (2003), who reported a low recombination frequency, if the *cre* recombinase is constitutively expressed. On the other hand, these authors detected a high-frequency, precise site-specific excision, if the *cre* recombinase was transiently expressed. There are numerous options to limit the presence of the recombinase. Various groups have enabled the excision of the recombinase gene by its own directed recombination via an autoexcision approach as for example in maize (Zhang et al. 2003), tobacco (Mlynarova and Nap 2003) or *A. thaliana* (Albert et al. 1995; Vergunst et al. 1998, 2000). A stable integrated *cre* gene could be activated in various plant species by inducible systems, such as induction by heat shock (Hoff et al. 2001; Zhang et al. 2003) or by estrogen (Zuo et al. 2001).

As mentioned earlier the recombinase catalyzes the excision as well as the integration reactions. The wild-type lox sites used in this study favor the excision reac-

tion. However, the free circular excision product with one single lox site could possibly re-integrate into the single genomic lox site. But no F_1 plants were observed containing both recombined as well as non-recombined DNA in the experiments reported here.

Contrary to our expectations PCR analysis revealed Flp/FRT recombination events only in one of the seven progeny plants, although effective site-specific recombination between the used wild-type minimal FRT sequences has already been shown in various plants or plant cells (Kilby et al. 1995; Lloyd and Davis 1994). The lower recombination ability of the Flp/FRT system in maize lines used here could originate from a lower affinity of the Flp recombinase to the FRT sites, compared with the Cre/lox system. Ringrose et al. (1998) presented gel mobility shift assays which identify a much higher affinity of the Cre recombinase for its target lox than the Flp recombinase to FRT. In contrast numerous authors observed a high frequency of Flp-mediated site-specific recombination in tobacco (Lloyd and Davis 1994; Kilby et al. 1995), *A. thaliana* (Kilby et al. 1995) and maize cells (Lyznik et al. 1996). Furthermore, both recombinases differ in their temperature optimum. Flp recombinase, having a temperature optimum of 23–30°C, showed a more thermolabile manner than the Cre recombinase in in vitro assays (Buchholz et al. 1996). Little activity was detectable above 39°C whereas the Cre recombinase was active at temperatures up to 46°C. The crossing of recombinase maize lines and plants harboring the selection marker flanked by recognition sites was performed in mid-summer of 2003. Although the tested plants were grown in a greenhouse under standard conditions, high temperature and intensive sun light in the summer of 2003 might explain the much higher enzyme activity of the Cre recombinase and the instability of the Flp recombinase.

Other approaches for the elimination of unwanted marker sequences such as transposition or cotransformation do not provide the outstanding advantages shown for the Cre/lox recombination. Whereas transposition often leads to changes in the DNA sequence (Scholz et al. 2001), site-specific recombination by the Cre/lox system provides exact excision. By choosing transgenic plants with one single integration one can assure the complete elimination of the undesired marker gene. The cotransformation approach implies the rarely occurring unlinked integration of marker and target genes. In comparison to the particle bombardment, *Agrobacterium*-mediated transformation offers the possibility for unlinked transgene integration by the use of different *Agrobacterium* strains, separate Ti-plasmid or T-DNAs (De Neve et al. 1997; Daley et al. 1998; Komari et al. 1996) followed by the separation of marker and target genes by segregation in the next generation. Thus, site-specific recombination systems enable a higher rated excision of unwanted sequences.

Besides the precise excision, site-specific recombination systems offer the possibility of directed integration of transgenes at characterized loci and therefore a more predictable expression level for advancing the genetic engineering of monocotyledonous crop plants. Such directed transgene integration is reported only in a few plant species, such as *A. thaliana* (Vergunst et al. 1998) and also rice (Srivastava et al. 2004), where the authors bombarded transgenic rice calli containing lox sites with an integration construct and were able to generate integrant plants. In this study we investigated a similar strategy for directed transgene integration in maize plants. Embryos harboring the *cre* gene and a single lox site in the genome were transiently transformed with the integration construct plox.*uid*A by microprojectile bombardment. The results of these experiments indicate a successful site-specific integration of the lox construct, since the site-specific integration rate was significantly higher than the random integration of the promoterless lox.*uid*A construct adjacent to an endogenous promoter used as a negative control. The putatively directed integration could be the first step for specific and targeted transgene integration.

Future work will investigate the site-specific integration of selection marker genes into a genomic recognition site followed by the in vitro regeneration of integrant plants. Further, it could be necessary to stabilize the integration state since both recombination reactions, excision as well as integration, occur at the same time and sites. To stabilize the integration state Albert et al. (1995) analyzed the recombination frequency between differently mutated lox sites and identified the sequences lox76/75, which favor the integration reaction and were used by Srivastava et al. (2004) for stabilizing the integration state in rice plants.

Through a combination of different recombination systems, such as Flp/FRT or R/RS as suggested by Srivastava and Ow (2004), and the appropriate assembly of the recognition sites, including the orientation, it is possible to combine excision and integration, i.e. excision of unwanted selection marker sequences and integration of the target gene.

As shown in this comparative study, the site-specific recombination system Cre/lox is well suited for high-frequency marker elimination in maize. Considerable advantages, such as high accuracy and efficiency, the need of only very short recognition sites, the complete and early recombination reaction, make the Cre/lox system a useful tool in the genetic engineering of crop plants.

Acknowledgements The Cre recombinase vector was kindly provided by Dr. Schiemann, Federal Biological Research Centre for Agriculture and Forestry, Institute for Plant Virology, Microbiology and Biosafety, Braunschweig, Germany.

References

Albert H, Dale EC, Lee E, Ow DW (1995) Site-specific integration of DNA into wild-type and mutant *lox* sites placed in the plant genome. Plant J 7:649–459

Araki H, Jearnpiparkul A, Tatsumi H, Sakurai T, Ushio K, Muta T, Oshima Y (1985) Molecular and functional organization of yeast plasmid pSR1. J Mol Biol 182:191–203

Bayley CC, Morgan M, Dale EC, Ow DW (1992) Exchange of gene activity in transgenic plants catalyzed by the Cre-lox site-specific recombination system. Plant Mol Biol 18:353–361

Brettschneider R, Becker D, Lörz H (1997) Efficient transformation of scutellar tissue of immature maize embryos. Theor Appl Genet 94:737–748

Broach JR, Guarascio VR, Jayaram M (1982) Recombination within the yeast plasmid 2 mu circle is site-specific. Cell 29:227–234

Buchholz F, Ringrose L, Angrand PO, Rossi F, Stewart AF (1996) Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. Nucleic Acids Res 24:4256–4262

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Dale EC, Ow DW (1990) Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. Gene 91:19–85

Daley M, Knauf VC, Summerfelt KR, Turner JC (1998) Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Rep Publ 17:489–496

De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J 11:15–29

D'Halluin K, Bonne E, Bossut M, De Beuckeleer M, Leemans J (1992) Transgenic maize plants by tissue electroporation. Plant Cell 4:1495–1505

Ebinuma H, Sugita K, Matsanuga E, Endo S, Yamada K, Komamine A (2001) Systems for the removal of a selection marker and their combination with a positive marker. Plant Cell Rep 20:383–392

Eckes P, Vijtewaal B, Donn G (1989) Synthetic gene confers resistance to the broad spectrum herbicide L-phosphinothricin in plants. J Cell Biochem Suppl 13D:334

Gidoni D, Bar M, Gilboa N (2001) FLP/FRT-mediated restoration of normal phenotypes and clonal sectors formation in *rol*C transgenic tobacco. Transgenic Res 10:317–328

Gleave AP, Mitra DS, Mudge SR, Morris BA (1999) Selectable marker-free transgenic plants without sexual crossing: transient expression of *cre* recombinase and use of a conditional lethal dominant gene. Plant Mol Biol 40:223–235

Goldsbrough AP, Lastrella CN, Yoder JI (1993) Transposition mediated re-positioning and subsequent elimination of marker genes from transgenic tomato. Bio/Technology 11:1286–1292

Hoa TT, Bong BB, Huq E, Hodges TK (2002) Cre/lox site-specific recombination controls the excision of a transgene from the rice genome. Theor Appl Genet 104:518–525

Hoff T, Schnorr KM, Mundy J (2001) A recombinase-mediated transcriptional induction system in transgenic plants. Plant Mol Biol 45:41–49

Jefferson RA, Kavanagh TA, Bevan MW (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Kilby NJ, Davies GJ, Snaith MR, Murray JAH (1995) FLP recombinase in transgenic plants: constitutive activity in stably transformed tobacco and generation of marked cell clones in *Arabidopsis*. Plant J 8:637–652

Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174

Kovalchuk I, Kovalchuk O, Kalck V, Boyko V, Filkowski J, Heinlein M, Hohn B (2003) Pathogen-induced systemic plant signal triggers DNA rearrangements. Nature 423:760–762

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685

Lloyd AM, Davis RW (1994) Functional expression of the yeast FLP/FRT site specific recombination system in *Nicotiana tabacum*. Mol Gen Genet 242:653–657

Luo H, Lyznik LA, Gidoni D, Hodges TK (2000) FLP-mediated recombination for use in hybrid plant production. Plant J 23:423–430

Lyznik LA, Rao KV, Hodges TK (1996) FLP-mediated recombination of *FRT* sites in the maize genome. Nucleic Acids Res 24:3784–3789

Lyznik LA, Gordon-Kamm WJ, Tao Y (2003) Site-specific recombination for genetic engineering in plants. Plant Cell Rep 21:925–932

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2:163–171

Meyer P (2000) Transcriptional transgene silencing and chromatin components. Plant Mol Biol 43:221–234

Mlynarova L, Nap JP (2003) A self-excising Cre recombinase allows efficient recombination of multiple ectopic heterospecific lox sites in transgenic tobacco. Transgenic Res 12:45–57

Odell JT, Russell SH (1994) Use of site-specific recombination systems in plants. In: Paszkowski J (ed) Homologous recombination and gene silencing in plants. Kluwer, Dordrecht, pp 219–265

Odell JT, Caimi P, Sauer B, Russell SH (1990) Site-directed recombination in the genome of transgenic tobacco. Mol Gen Genet 223:369–378

Ow DW (2002) Rekombinase-directed plant transformation for the post-genomic era. Plant Mol Biol 48: 183–200

Pallotta MA, Graham RD, Langridge P, Sparrow DHB, Barker SJ (2000) RFLP mapping of manganese efficiency in barley. Theor Appl Genet 101:1100–1108

Radhakrishnan P, Srivastava V (2005) Utility of the FLP–FRT recombination system for genetic manipulation of rice. Plant Cell Rep 23:721–726

Ringrose L Lounnas V, Ehrlich L, Buchholz F, Wade R, Stewart AF (1998) Comparative kinetic analysis of FLP and cre recombinases: mathematical models for DNA binding and recombination. J Mol Biol 27:363–384

Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234:49–59

Sambrook F, Fritsch EF, Maniatis F (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Schaefer DG, Zryd JP (1997) Efficient gene targeting in the moss *Physcomitrella patens*. Plant J 11:1195–1206

Scholz S, Lörz H, Lütticke S (2001) Transposition of the maize transposable element *Ac* in barley (*Hordeum vulgare* L.). Mol Gen Genet 264:653–661

Sonti RV, Tissier AF, Wong D, Viret JF, Signer ER (1995) Activity of the yeast FLP recombinase in *Arabidopsis*. Plant Mol Biol 28:1127–1132

Srivastava V, Ow DW (2004) Marker-free site-specific gene integration in plants. Trends Biotechnol 22:627–629

Srivastava V, Anderson OD, Ow DW (1999) Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc Natl Acad Sci USA 96:11117–11121

Srivastava V, Ariza-Nieto M, Wilson AJ (2004) Cre-mediated site-specific gene integration for consistent transgene expression in rice. Plant Biotechnol J 2:169–179

Sternberg N, Hamilton D (1981) Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. J Mol Biol 150: 467–486

Thykjaer T, Finnemann J, Schauser L, Christensen L, Poulsen C, Stougaard J (1997) Gene targeting approaches using positive–negative selection and large flanking regions. Plant Mol Biol 35:523–530

Vergunst AC, Hooykaas PJ (1998) Cre/lox-mediated site-specific integration of *Agrobacterium* T-DNA in *Arabidopsis thaliana* by transient expression of *cre*. Plant Mol Biol 38:393–406

Vergunst AC, Jansen LE, Hooykaas PJ (1998) Site-specific integration of Agrobacterium T-DNA in *Arabidopsis thaliana* mediated by Cre recombinase. Nucleic Acids Res 26:2729–2734

Vergunst AC, Jansen LE, Fransz PF, De Jong JH, Hooykaas PJ (2000) Cre/lox-mediated recombination in *Arabidopsis*: evidence for transmission of a translocation and a deletion event. Chromosoma 109:287–297

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

Zuo J, Niu QW, Moller SG, Chua NH (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. Nat Biotechnol 19:157–161