

Agrobacterium-mediated transformation of maize (*Zea mays*) with Cre-lox site specific recombination cassettes in BIBAC vectors

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Abstract The Cre/loxP site-specific recombination system has been applied in various plant species including maize (*Zea mays*) for marker gene removal, gene targeting, and functional genomics. A BIBAC vector system was adapted for maize transformation with a large fragment of genetic material including a herbicide resistance marker gene, a 30 kb yeast genomic fragment as a marker for fluorescence in situ hybridization (FISH), and a 35S-lox-cre recombination cassette. Seventy-five transgenic lines were generated from *Agrobacterium*-mediated transformation of a maize Hi II line with multiple B chromosomes. Eighty-four inserts have been localized among all 10 A chromosome pairs by FISH using the yeast DNA probe together with a karyotyping cocktail. No inserts were found on the B chromosomes; thus a bias against the B chromosomes by the *Agrobacterium*-mediated transformation was revealed. The expression of a cre gene was confirmed in 68 of the 75 transgenic lines by a reporter construct for

cre/lox mediated recombination. The placement of the cre/lox site-specific recombination system in many locations in the maize genome will be valuable materials for gene targeting and chromosome engineering.

Keywords *Agrobacterium*-mediated transformation · B chromosome · BIBAC vector · Cre/loxP · Maize

Introduction

Site-specific recombination systems have been widely used in plants. For example, marker genes can be efficiently removed by directly flanking the marker gene expression cassette with recombination sites, such as lox and FRT (Dale and Ow 1991; Lyznik et al. 1996) as one method among other (Komari et al. 1996) to make marker free transgenic crops. It has also been used for gene targeting to insert foreign genes precisely into a pre-existing site for more predictable expression of introduced genes than occurs with random integrations (Day et al. 2000). By placing lox sites into the genome, fragments of genomic DNA can be generated by the Cre recombinase in vitro for long range restriction mapping (Qin et al. 1995; Stuurman et al. 1996). Site-specific recombination based chromosome engineering with the Cre/lox, FLP/FRT and R/RS systems has generated a variety of chromosomal rearrangements, such as deletions, inversions, duplications and translocations (Kilby et al. 1993; Ow 1996; Sauer 1994).

Site-specific recombination systems can be integrated into the genome by genetic transformation. Both *Agrobacterium*- and biolistic-mediated transformations generate random integrations (Barakat et al. 2000). To make efficient placement of site specific recombination cassettes in the genome, both random transformation and the use of

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engineered transposons that carry the recombination system have been used (Osborne et al. 1995; Medberry et al. 1995; van Haaren and Ow 1993). In this work, we assembled a 35S-*lox-cre* cassette, a yeast genomic DNA fragment and a selectable marker gene in a binary bacterial artificial chromosome vector (BIBAC) (Hamilton 1997) to transform maize (*Zea mays*), and placed the *cre/lox* cassette in many locations in the maize genome.

The BIBAC system was designed by Hamilton and colleagues (Hamilton 1997; Hamilton et al. 1996) for the transformation of large T-DNA inserts into plant genomes. The BIBAC combines the T-DNA transfer function of the *Ti* plasmid with the capacity of maintaining large DNA fragments of the bacterial artificial chromosome (BAC). As with a BAC, it has a single copy origin of replication from the *E. coli* F plasmid for stable maintenance in *E. coli*. It also has a single copy origin of replication from the *Agrobacterium* Ri plasmid, and the T-DNA borders for T-DNA transformation. A similar system, transformation competent artificial chromosomes (TAC) with an *E. coli* bacteriophage P1 origin, has also been used in the transformation of large DNAs (Liu et al. 1999). Both BIBAC and TAC have been used for transformation of large DNAs to various plant species, such as tobacco (Hamilton 1997; Hamilton et al. 1996), Brassica (Cui et al. 2000), Arabidopsis (Liu et al. 1999), tomato (Frary and Hamilton 2001), rice (He et al. 2003, 2006; Zhou et al. 2005), and fungi (Takken et al. 2004). We demonstrate that the BIBAC system can be used to transform large DNA to maize using a modified transformation protocol. A 30 kb yeast genomic DNA was integrated together with the *cre/lox* system as a FISH tag to facilitate the localization of the transgenes and to determine useful transgene sites for chromosome engineering by the *Cre/lox* system.

Materials and methods

BIBAC construction

The plasmid pCH20 is the backbone of the BIBAC vector without a plant selectable maker (Hamilton 1997). The backbone has the minimal origin of replication region of the *E. coli* F plasmid and the minimal origin of the *Agrobacterium rhizogenes* Ri plasmid (Fig. 1a), and thus replicates as a single-copy plasmid in both bacteria. In order to build a monocot optimized BIBAC with an integrated *Cre-lox* system, the following steps were carried out. The pCH20 vector (Fig. 1a) was modified by deleting the *Bam* HI to *Swa* I fragment. The *Bam* HI site was reconstituted by adding a *Bam* HI linker at the *Swa* I site followed by *Bam* HI digestion and re-ligation. The resulting plasmid was named pJV06. A 3.4 kb *Hind* III/*Sal* I

fragment containing the 35S-*lox66-cre* gene expression cassette from pED97 (courtesy from Dr. D. Ow) was cloned into the *Bam* HI site by blunt end ligation to produce pJV08. A 400 bp *Arabidopsis* telomere sequence from pAtT4 (Richards and Ausubel 1988) and a 2.9 kb *ubiquitin* gene expression cassette from pAHC25 (Christensen et al. 1992) were assembled in pBluescript (Stratagene, La Jolla, California) and then cloned into the *Srf* I site of pJV08 by blunt end ligation to make pJV15 in which the telomere sequence was placed at the left border of the binary vector. The telomere sequence TTTAGGG was oriented towards the left border. Another 400 bp telomere sequence from pAtT4 was cloned into the *Pac* I site of pJV15 by blunt end ligation to generate a direct repeat of the 800 bp of telomere sequence at the LB to produce pJV20 (Fig. 1b). This region of pJV20 was sequenced to confirm the orientation of the telomere repeat. A 30 kb *Not* I fragment of yeast DNA from BIBAC1.Y30 (Hamilton 1997; Hamilton et al. 1996) was then cloned into the *Not* I site to produce pJV21 (Fig. 1b). The BIBAC pJV21 construct was transferred to *Agrobacterium* strain LBA4404 by electroporation of competent cells following the manufacturer's instructions (Gibco, Invitrogen Co., Carlsbad, California). Competent LBA4404 cells carrying pJV21 were prepared and electroporated with helper plasmid pCH32 (Hamilton 1997). The plasmid pCH32 carries additional copies of *A. tumefaciens* virulence genes, *virG* and *virE*, which increase the efficiency of transfer of large BIBAC T-DNAs (Hamilton et al. 1996). BIBAC plasmids were selected in *E. coli* by adding 40 mg/l kanamycin to the growth medium. In *Agrobacterium*, pCH32 was selected with 2 mg/l tetracycline and pJV21 was selected with 50 mg/l kanamycin.

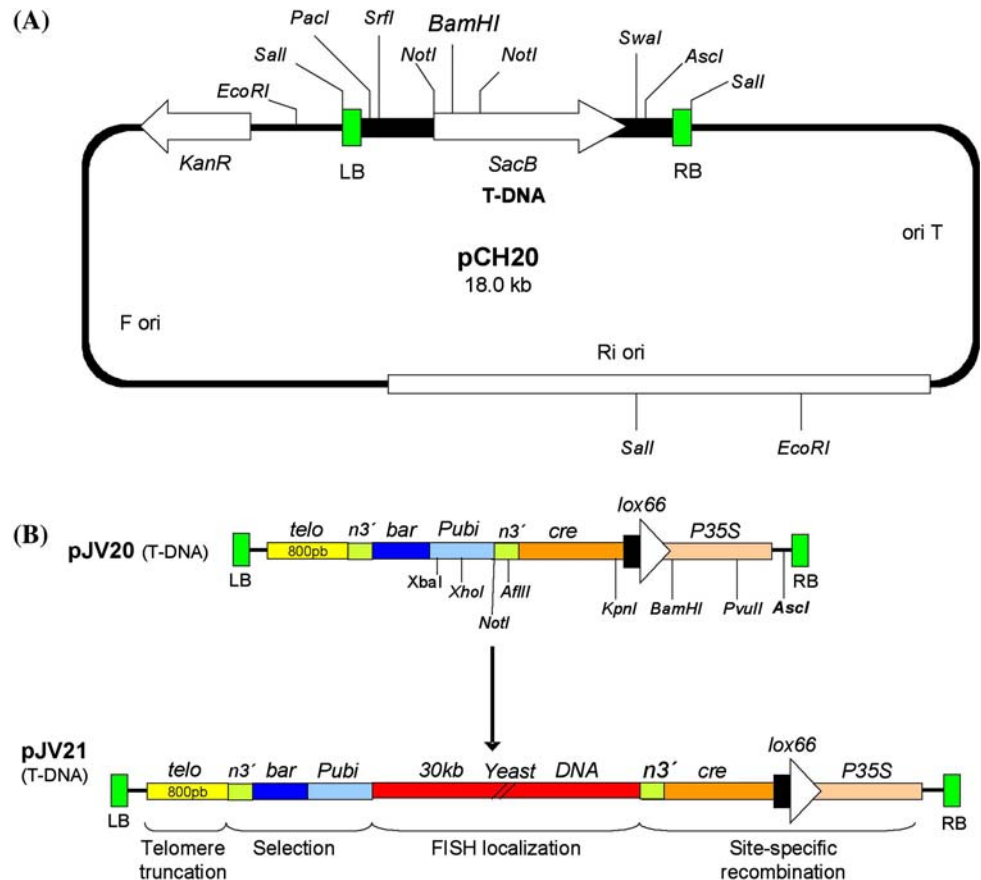
Introgression of B chromosomes to Hi II parents

B chromosomes were introgressed from the maize variety Black Mexican Sweet into the maize transformation line Hi II-A (Armstrong et al. 1991) and the maize inbred line B73 by repeated backcrossing and selection for B chromosomes. Hi II-A plants with a high number of B chromosomes were self pollinated or crossed as females by B73 plants carrying Bs to produce immature embryos for genetic transformation.

Gene transformation

Immature embryos, about 1.5 mm (1.0–1.8 mm) in size, were aseptically dissected from greenhouse-grown ears harvested 9–12 days after pollination. *Agrobacterium*-mediated genetic transformation was performed following

Fig. 1 Constructions and structure of pJV21. **(a)** The starting plasmid was pCH20. The left and right borders are shown in green to correspond to the modified constructs shown below. **(b)** The plasmid pJV20 was first constructed followed by the insertion of a 30 kb fragment of yeast DNA to produce pJV21. The four functional parts of JV21 are indicated by parentheses and labeled. LB, left border; RB, right border; *lox66* site; *cre*, *cre* recombinase gene; *bar*, bialaphos resistance gene; P35S, CaMV P35S promoter; *Pubi*, maize ubiquitin promoter; *n3'*, terminator sequence of the nopaline synthase gene; *telo*, 800 bp telomere repeats from Arabidopsis



the protocol of Zhao et al. (1999) with modifications (Vega et al. 2008). The key improvement was the use of low levels of N6 salt mixture in both inoculation and cocultivation media as described for maize transformation using standard binary vectors (Vega et al. 2008). Both 10 and 50% N6 salts were used for efficient transformation of maize with BIBAC pJV21. Embryos were subcultured on medium containing 1.5 mg/l bialaphos for herbicide selection of resistant transgenic calli.

Southern hybridization

Leaves from transgenic T0 plants were ground in liquid nitrogen and genomic DNA was isolated with the Plant DNAzol (Invitrogen) kit following the manufacturer's instructions. Southern hybridization was performed as described (Yu et al. 2006b). To assay the copy number of the left or right border sequences of pJV21 in the transgenic plants, 20 µg of genomic DNA was digested with *Afl* II or *Xba* I, respectively, separated on 0.8% agarose gel, transferred to a Qiabran nylon membrane (Qiagen), and then probed by ³²P-labeled *bar* gene or the *cre* gene sequences, respectively. To determine the terminal position of the J11-9a transgene, four restriction enzymes (*Xba* I,

Xho I, *Dra* I and *Hind* III) were used to digest the genomic DNA, and probed with a ³²P-labeled *bar* gene probe.

FISH

To identify the chromosome localization of the T-DNA insertions we performed FISH on root tip chromosomes of the transgenic lines with the 30 kb yeast DNA (Y30) as probe. DNA of BIBAC1.Y30 was isolated from an overnight bacterial culture using a Qiagen tip 500 plasmid maxi kit (Qiagen) and the protocol described by Kirschner and Stratakis (1999). Cy5-labeled Y30 probe was mixed with a modified cocktail of probes used previously to identify all maize chromosomes (Kato et al. 2004). The karyotyping cocktail used was: coumarin labeled 180 bp knob probe; fluorescein labeled CentC, NOR, 5S-rDNA and subtelomeric repeat (4-12-1) probes; and Texas red labeled Cent4 and AGT-microsatellite probes. The FISH procedure was essentially the method described by Kato et al. (2004, 2006). Four single channel (blue, green, red and infrared) images were captured in 8-bit depth black and white and were later superimposed in Photoshop software (Adobe Systems). The color assignments were blue for coumarin, green for fluorescein, red for Texas red, and white for Cy5.

Cre recombination assay

Cre activity in transformed plants was determined by bombarding T1 embryos with pHK52 (Srivastava et al. 1999), which exhibits β -glucuronidase (GUS) activity upon Cre-mediated inversion of the *gus* coding region. Immature embryos of the transformants crossed by a Hi II-A tester line were dissected aseptically, placed face down on an osmotic treatment media, and bombarded with pHK52 plasmid with a PDS 1000/He biolistic gun (BIO-RAD, Hercules, California) as described (Srivastava et al. 1999). Bombarded embryos were transferred to a callus induction medium and cultured at 28°C in the dark. The embryos were stained for GUS activity 48 h after bombardment as described (Jefferson et al. 1987).

Results

The rationale for the experiments described was to place site-specific recombination *lox* sites and a *cre* gene at multiple locations in the maize genome. The *Cre/loxP* system used was designed with two strategies to insure that future recombination events were not reversible (Albert et al. 1995). First, elimination of *cre* transcription by promoter displacement will occur following recombination. This is achieved by having the *lox* site located between the 35S promoter and the *cre* gene. Second, the asymmetric mutant *loxP* pair *lox66/lox71* was used. The mutant *lox66* cloned into pJV21 is capable of forward recombination with the mutant *lox71*, but once the double mutant is formed, the reverse reaction is not favored because of the low affinity for Cre recombinase (Albert et al. 1995). A 30 kb fragment of yeast DNA was included in pJV21 to localize the T-DNA insertion sites in the maize chromosomes using fluorescent in situ hybridization. Telomere sequences were included as a test for telomere truncation. The collection of transformants should be useful for targeted insertions to many locations in the maize genome as well as for potentially generating chromosomal rearrangements.

BIBAC construction

The monocot optimized BIBAC pJV21 was constructed as described in the material and methods (Fig. 1). In this construct, a 35S-*lox66-cre* cassette was cloned adjacent to the right border in order to place the *cre/lox* site-specific recombination system into the genome by gene transformation. To locate the transgenes, a 30 kb yeast DNA was cloned into the BIBAC vector as a cytological marker for FISH analysis. A *bar* (*bialaphos* resistance) gene under the control of the maize ubiquitin promoter (Christensen et al.

1992) was cloned into the construct as a selectable marker gene for transgenic plants, which confers resistance to the herbicide bialaphos. In addition, two direct repeats of the cloned *Arabidopsis* telomere sequence from pATt4 (Richards and Ausubel 1988) were placed at the left border in an attempt to truncate chromosomes based on telomere-mediated chromosomal truncation (Farr et al. 1991; Yu et al. 2006b).

Genetic transformation

BIBAC pJV21 and helper plasmid pCH32, carrying virulence genes (Hamilton 1997), were transferred to *Agrobacterium* strain LBA4404. This strain was used to transform immature embryos from Hi II-A and Hi II-A \times B73 crosses of plants with B chromosomes. A maize transformation protocol based on the use of low levels of N6 salt mixture in both inoculation and cocultivation media was followed (Vega et al. 2008). Both 10 and 50% N6 salts resulted in efficient transformation with BIBAC pJV21. Seventy-five transformed lines were recovered after selection of resistant calli on bialaphos containing media. Most of the transgenic lines were normal in morphology and fertility. No biological function of the 30 kb yeast DNA was recognized when integrated into the maize genome.

Characterization of transgenes

The transgenes were confirmed and characterized by Southern hybridization and FISH (Table 1). The transgene integrity was analyzed by Southern blot hybridizations at both the left and the right ends of the T-DNA region using probes of the *bar* gene and the *cre* gene, respectively. In the 75 lines that were analyzed, 52 transgenic lines were found to have the same copy number at both the left and the right border, which indicates that the transgenes in these cases are intact. Forty-six transgenic lines had single copy intact transgenes (Table 1). All the lines had at least one band on *bar* Southern hybridizations, as expected since the selection was performed with bialaphos. Only 8 of the 75 transgenic lines did not show bands in *cre* Southern hybridizations.

FISH on root tip chromosomes of the transgenic lines with the 30 kb yeast DNA (Y30) as a probe showed 63 transgenic lines with a single transgene, 9 lines with two transgenes, and 2 lines with three transgenes (Table 1). Only one line, J12-23, did not show FISH signals. Because this transgenic line was also negative for *cre* in Southern, the T-DNA inserted in this line is most probably not intact. Forty-five transgenic lines had the same transgene copy number by Southern and FISH characterization, and forty-one of them had single copy transgenes.

Table 1 Characterization of transgenic maize lines carrying pJV21 insertions

Transgenic line	No. B chr.	Southern		FISH Y30	Cre activity	Chromosome location
		<i>bar</i>	<i>cre</i>			
J10-1	4	1	1	1	+	1L middle
J10-2	5	1	1	1	+	1S
J10-3	1	1	1	1	+	1S distal
J10-4	3	1	1	1	+	1S
J10-5	3	1	2	1	+	5S distal
J10-6	4	1	0	1	+	6L middle
J10-7	2	1	0	1	–	10L distal
J11-1	4	1	1	1	+	7L distal
J11-2	0	1	1	2	+	11-2a, 2L distal 11-2b, 7L distal
J11-3	6	3	3	3	+	11-3a, 4S distal 11-3b, 5L middle 11-3c, 2 cent
J11-5	3	1	1	1	+	5L distal
J11-6	4	1	2	1	+	2S middle
J11-7	6	1	1	1	+	1L
J11-8	3	2	3	1	+	1S distal
J11-9	5	1	1	2	+	11-9a, 3L distal 11-9b, 5L distal
J11-10	8	1	0	1	–	10L distal
J11-11	3	2	1	1	+	7L middle
J11-12	1	1	1	1	+	1L distal
J11-13	2	1	1	1	+	7L distal
J11-14	4	1	1	1	+	1L proximal
J11-15	3	3	1	1	+	1S distal
J11-16	4	3	1	1	+	6L middle
J11-17	6	2	2	2	+	11-17a, 7S distal 11-17b, 7L proximal
J11-18	6	1	2	1	+	3S distal
J11-19	2	1	1	1	+	7L
J11-20	5	1	1	1	+	9S distal
J11-21	5	3	0	1	–	2 cent
J11-22	3	2	0	1	–	N.D.
J11-23	8	1	2	1	+	1L middle
J11-24	7	1	1	1	+	7L distal
J11-25	7	1	1	1	+	3L distal
J11-26	2	2	2	2	+	11-26a, 2L proximal 11-26b, 9L distal
J11-27	6	1	1	1	+	10L distal
J11-28	4	1	1	1	+	5L distal
J11-29	9	1	2	1	+	3S distal
J11-30	1	1	1	1	+	5L distal
J11-31	4	1	1	2	+	11-31a, 7L middle 11-31b, 10L distal
J11-32	2	1	1	1	+	4L distal
J12-1	6	1	1	2	+	12-1a, 2L middle 12-1b, 7S distal

Table 1 continued

Transgenic line	No. B chr.	Southern		FISH Y30	Cre activity	Chromosome location
		<i>bar</i>	<i>cre</i>			
J12-2	3	3	3	1	–	3S distal
J12-3	11	2	2	2	+	12-3a, 4L middle 12-3b, N.D.
J12-4	6	1	1	1	+	9L distal
J12-5	5	1	2	1	+	7L distal
J12-6	0	1	1	1	+	8L distal
J12-7	2	1	1	1	+	5L distal
J12-8	0	1	2	3	+	12-8a, 1L distal 12-8b, 3L distal 12-8c, 6L middle
J12-9	2	1	1	1	+	4S middle
J12-10	1	1	1	1	+	2S distal
J12-11	5	1	1	1	+	9L middle
J12-12	0	1	1	1	+	5L distal
J12-13	2	1	1	1	+	4S middle
J12-14	2	1	1	1	+	8L middle
J12-15	2	1	0	1	+	4L middle
J12-16	11	1	1	1	+	3L middle
J12-17	6	1	1	1	+	4L middle
J12-18	6	1	1	1	+	5L middle
J12-19	4	3	3	1	+	6L distal
J12-20	6	4	2	2	+	12-20a, 1L middle 12-20b, 10L distal
J12-21	0	2	1	1	+	2L middle
J12-22	3	2	1	1	+	3L middle
J12-23	1	1	0	0	–	N.D.
J12-24	7	1	1	1	+	8L middle
J12-25	4	1	2	1	+	2L distal
J12-26	5	1	1	1	+	10L distal
J12-27	9	1	1	1	+	1S middle
J12-28	3	1	1	1	+	N.D.
J12-29	2	1	1	1	+	3L middle
J12-30	5	1	0	1	+	1L distal
J12-31	4	1	1	1	+	6S middle
J12-32	4	1	1	1	+	3S distal
J12-33	2	1	1	1	+	3L middle
J12-34	3	1	1	1	+	2S distal
J12-35	4	1	1	1	–	8L proximal
J12-36	0	1	1	1	+	8L distal
J12-37	2	1	1	2	+	12-37a, 6L distal 12-37b, 1S distal

N.D., not determined

Position and distribution of transgenes in maize chromosomes

In order to profile the position and distribution of transgenes among maize chromosomes by FISH, the Y30

probe was mixed with a modified cocktail of probes used previously to identify all maize chromosomes (Kato et al. 2004, 2006). Transgenes were found on every chromosome of the karyotype (Table 1) and all chromosome arms were targeted except 8S and 10S. The transgene

locations within the chromosome were also determined (Table 1). From a total of 80 transgenes, 6 (7.50%) were located in the region proximal to the centromere, 27 (33.75%) were located near the middle of the chromosome arm, and 47 (58.75%) were located in the distal region near the telomere. The use of the karyotyping cocktail allowed us to visualize the main heterochromatic regions in the maize genome, and integration sites were found in telomeres, centromeres, heterochromatic knobs, NOR region and AGT microsatellite (Figs. 2, 3). However, no transgene was observed on B chromosomes although there were up to 11 B present (average 3.9) in the transformed plants (Table 1).

Terminal location of a transgene

One transgenic event J11-9a showed a smear pattern of Southern hybridization as compared to the others, which had discrete bands (Fig. 4). The smear pattern of hybridization is a hallmark of telomeres which result from natural telomere activity or newly seeded telomeres during telomere-mediated chromosomal truncation because the telomerase adds different numbers of the telomeric motif

(TTTAGGG) in different cells (Farr et al. 1991; Richards and Ausubel 1988; Yu et al. 2006b). To demonstrate the terminal nature of this transgene, multiple restriction enzymes that cut the distal region of the transgene just preceding the 800 bp telomere repeat were used to digest the J11-9a genomic DNA, and a Southern hybridization was performed using a ^{32}P -labeled *bar* gene probe as described (Yu et al. 2006b). The smear patterns of hybridization shift according to the distances between the three restriction enzyme cutting site (*Xba* I, *Xho* I and *Dra* I) and the telomeric end of the chromosome (Fig. 4a, b), as expected if the transgene seeded telomere formation. For example, the most proximal enzyme *Dra* I generated the largest sized smear pattern in the range of more than 6 kb, while the most distal one *Xba* I generated smear patterns in the range of 3.2–4.6 kb and the *Xho* I produced smears between 5 and 6 kb (Fig. 4b, lanes 5–7). In contrast, the cleavage at a more proximal *Hind* III site produced a discrete band (Fig. 4b, lane 8). A discrete band would be generated if there is a *Hind* III site distal to the *bar* gene probe. This *Hind* III site is potentially present at the natural telomere or was generated by a subtle rearrangement during transformation, because this site was not found in our sequence of the pJV21 construct. Alternatively, the larger

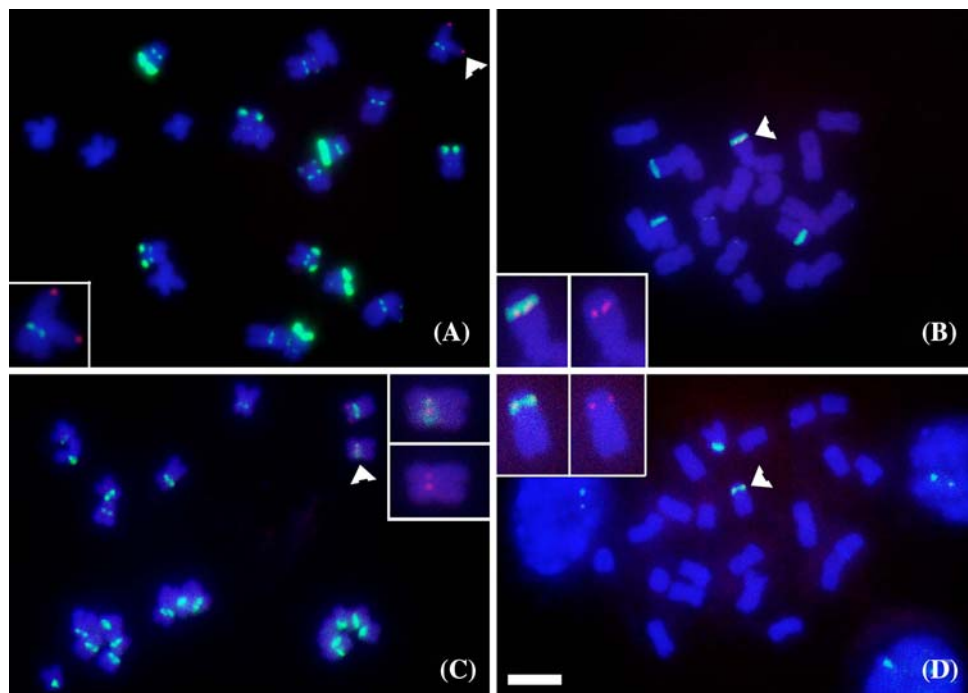
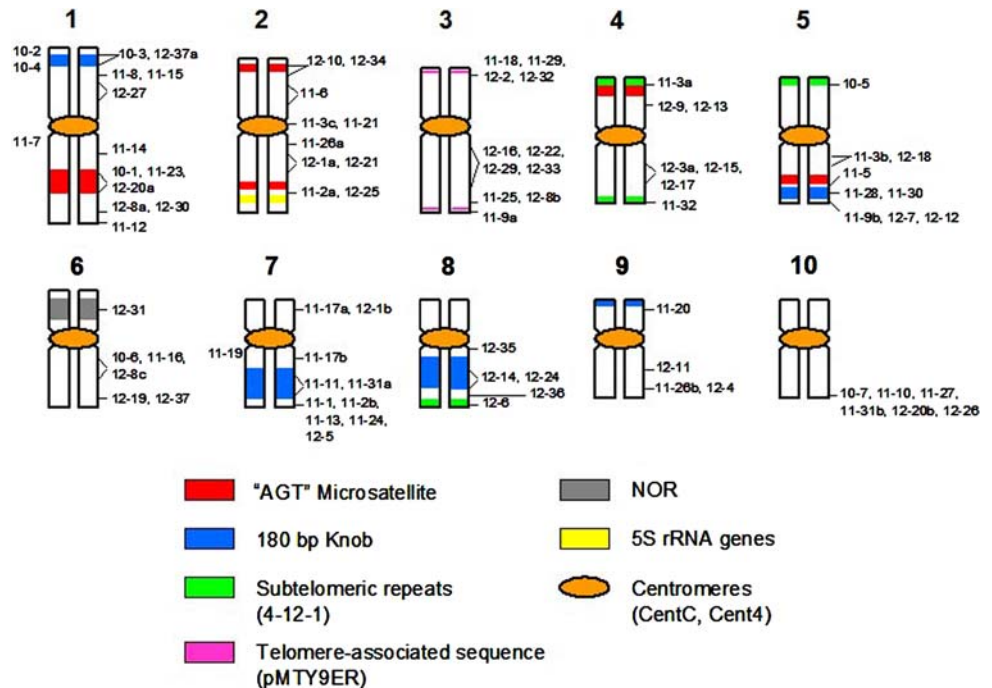


Fig. 2 FISH analysis of transformants of pJV21. (a) J11-9a with transgene (red) on two copies of chromosome 3. The inset shows a magnified chromosome 3 with the transgene localized at the end of 3L. Arrowheads denote the transgene positions. CentC and knob regions are labeled green. (b) J11-11 with a transgene (red) within the chromosome 7 knob (green) (arrowhead). The insets show the magnified chromosome with merged transgene and knob (left) and the

transgene only (right). (c) J11-3 with J11-3c transgene (red) in the centromere region (arrowhead) on chromosome 2. Centromeres were labeled with CRM probe (green). The insets show the magnified chromosome with merged transgene and CRM (top) and the transgene only (bottom). (d) J12-31 with transgene (red) in the NOR region (green) (arrow) on chromosome 6. The insets show merged transgene and NOR (left) and the transgene only (right). Scale bar = 10 μm

Fig. 3 Distribution of transgenes on the chromosomes of maize. Transgene localization was performed by FISH with the 30 kb yeast DNA probe mixed with the karyotyping cocktail. The components of the cocktail were color coded as shown



size of this fragment might obscure a heterogeneous nature. In addition, no abnormal growth or gametophyte sterility has been observed in the J11-9 plants, which indicated that there was no vital genetic material loss caused by this transgene. Taken together, the data indicate that this transgene is present at the very end of chromosome arm 3L with the telomere end located distally.

The end location of this transgene was also revealed by a cytological analysis. Chromosomes from J11-9a root tips were hybridized with a Texas Red—dUTP labeled 30 kb DNA probe mixed with a simplified karyotyping cocktail containing AlexaFluor488-dUTP labeled CentC and knob sequence (Yu et al. 2006a). The transgene was identified at the end of chromosome arm 3L (Fig. 5). Probing this material with the telomere-associated sequence pMTY9ER (Gardiner et al. 1996), which is prominent on 3L (Kato et al. 2004), reveals that this sequence is present in the transformant with the Y30 signal distal to it, indicating a very terminal location of the transgene (Fig. 5). The inclusion of telomere repeats in the BIBAC vector was intended to facilitate telomere truncation. Given the extremely terminal position of J11-9, it is not possible to establish whether truncation occurred or whether the transgene attached to the end of the chromosome and can be stable because of the presence of the telomere repeats.

Cre/lox site-specific recombination system

The transformation with the pJV21 BIBAC construct has placed 84 transgenes into the maize genome as shown by

FISH. These transgenes are mostly intact as indicated by Southern hybridizations. Eighty-seven transgenic sites were detected in the 75 transgenic lines by Southern hybridizations with a *cre* gene probe (Table 1). To demonstrate that these *cre* transgenes are expressed and functional, a reporter construct (pHK52) for *Cre/lox* site specific recombination (Srivastava et al. 1999) was biolistically delivered into the immature embryos of the progeny of the transgenic plants. The pHK52 construct contains an antisense oriented beta-glucuronidase (GUS) gene under the control of the maize ubiquitin promoter (Fig. 6a). The antisense GUS gene was flanked by inverted repeats of the *loxP* site. The antisense GUS gene can be reverted to the sense orientation and expressed in the presence of a Cre recombinase, which catalyzes the inversion of genetic elements flanked by inverted repeats of *lox* sites. A transient GUS assay was performed on bombarded embryos from all transgenic lines. We demonstrated that the *cre* gene was functional in 68 lines (Table 1). An example of embryos expressing GUS (blue color) following bombardment with the pHK52 construct is shown in Fig. 6c. The embryo population is segregating for the transgene in an outcross of J11-11 to the Hi II-A line. GUS expression was activated in 28 out of a total of 53 embryos, which indicated a near Mendelian 1:1 ratio. Interestingly, the transgene present in J11-11 is located within the knob region of chromosome 7 (Fig. 2b) and expresses a functional Cre. The Hi II-A tester line did not show any blue color embryos when bombarded with pHK52 (Fig. 6b) indicating the absence of Cre.

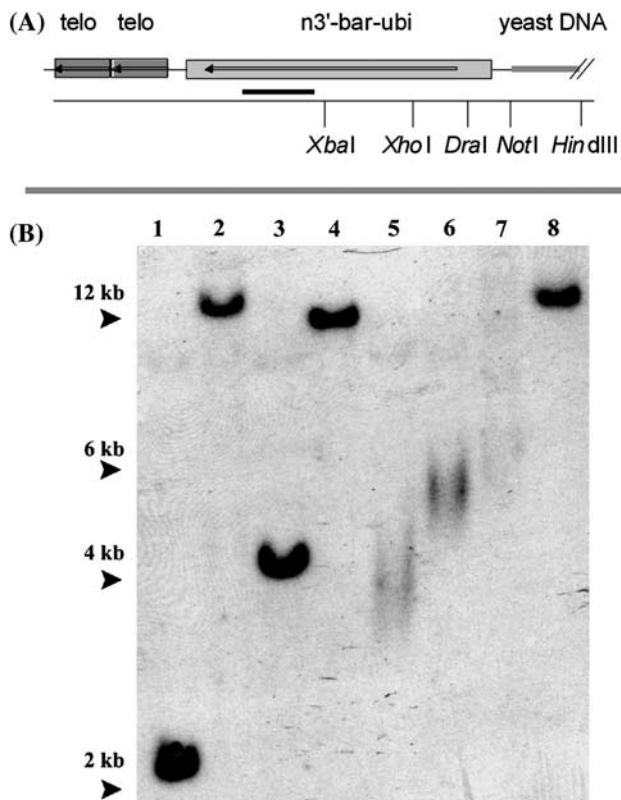


Fig. 4 Transgene J11-9a is present at a chromosomal terminal location. (a) Diagram of the left border region of pJV21. The 30 kb yeast DNA, *ubi-bar-n3'* selection gene expression cassette, and two copies of telomere repeats are labeled at the top. Restriction sites *Hind* III, *Not* I, *Dra* I, *Xho* I, and *Xba* I are labeled at the bottom in the order from the proximal to the distal region on the chromosome. *bar*, bialaphos resistance gene; *ubi*, maize ubiquitin promoter; *n3'*, terminator sequence of the nopaline synthase gene; *telo*, 400 bp telomere repeat from *Arabidopsis*. (b) Southern hybridization of genomic DNAs from transgenic plant lines digested with multiple enzymes. The molecular marker sizes are indicated by arrows at the left side. Lanes 1–4, J11-10; lanes 5–8, J11-9 genomic DNA digested with restriction enzymes in the order of *Xba* I, *Xho* I, *Dra* I and *Hind* III

Discussion

BIBAC and TAC are able to transfer up to 150 kb of genetic material to plants. An alternative method for high molecular weight DNA transformations is microprojectile bombardment. For example, the transformation of an 80 kb YAC into tomato and tobacco cells (Adam et al. 1997; Mullen et al. 1998; Van Eck et al. 1995) and the transformation of a 90 kb BAC containing 10 copies of the sorghum kafirin gene cluster into maize (Song et al. 2004) were reported. However, the direct transformation by bombardment was not able to transfer intact high molecular weight DNAs. For example, only one of 12 transgenic plants was demonstrated to have a continuously integrated transgene at the size of 45 kb when a 90 kb BAC was bombarded into the maize genome (Song et al. 2004).

Also, the bombardment method usually integrates multiple copies of transgenes which are prone to gene silencing. In contrast, we identified 52 of the 75 events that had intact integrations of the transgene as evidenced by intact left and right oriented sequences, and most of them had single copy transgenes. Cre gene expression was demonstrated in 68 of the 75 transgenic events. These transgenic events representing a high number of functional *Cre/lox* recombination units distributed across the maize genome are valuable for genetic and chromosome engineering. The localized transgenes can be used to induce chromosomal rearrangements, such as deletions, inversions and translocations. The functional *Cre/lox* system in these transgenic plants can also be used for gene targeting. In addition, the placement of the 30 kb yeast DNA in various locations of the maize genome provides valuable markers for genetic and cytogenetic mapping.

An interesting phenomenon observed from this work is that B chromosomes were not targeted by *Agrobacterium*-mediated transformation. Transgenes were found in every A chromosome and both euchromatic and heterochromatic regions were targeted. Although the maize B chromosome is mostly heterochromatic, regions of euchromatin are found in the proximal part and the long arm tip. In maize, each B chromosome accounts for about 4.5% of the genetic content of a diploid genome (Jones and Rees 1982), and the genetic content of B chromosomes in the transformation lines accounted for 17% based on an average of 3.9 B chromosomes per diploid genome. If the *Agrobacterium*-mediated T-DNA transfer was random, we would expect 17% of the 84 transgenes would be on B chromosomes. A potential explanation might come from the fact that T-DNA integration prefers genic regions (De Buck et al. 2004; Schneeberger et al. 2005). The maize B chromosome is basically inert, having no gene that will complement any known A chromosome mutation; thus, a low frequency of transformation on the B chromosomes might be predicted.

We also observed that transgenes can be expressed from different regions of the chromosome. For example, transgenes integrated in heterochromatic regions such as the centromere and the knob regions were expressed. The 30 kb yeast DNA provided a good cytological marker to identify transgenes and their positions. Two transgenes were located in the centromeres. Centromeres have long been considered to be gene poor, although sequence data have identified genes in the rice centromeres (Nagaki et al. 2004). Also, it has been proposed that functional centromeres are transcriptionally active (Yan et al. 2005, 2006), and the transcribed RNAs of both sense and antisense products from the centromeric repeat CentC and a retrotransposon CRM are involved in centromere kinetochore formation (Topp et al. 2004). The expression of transgenes from targeted maize centromeres provides additional evidence that genes can be

Fig. 5 Localization of transgene J11-9a. A line homozygous for the J11-9a transgene was probed with Texas red labeled yeast DNA (red) and fluorescein labeled telomere-associated sequence MTY9ER repeat and centromere satellite CentC (green). Chromosomes were counterstained with DAPI. At the right top are the merged images of the two chromosomes 3. At the middle right is the green channel only. The arrowheads denote the telomere-associated repeat signals. At the bottom right, the red channel illustrating the transgene site is shown

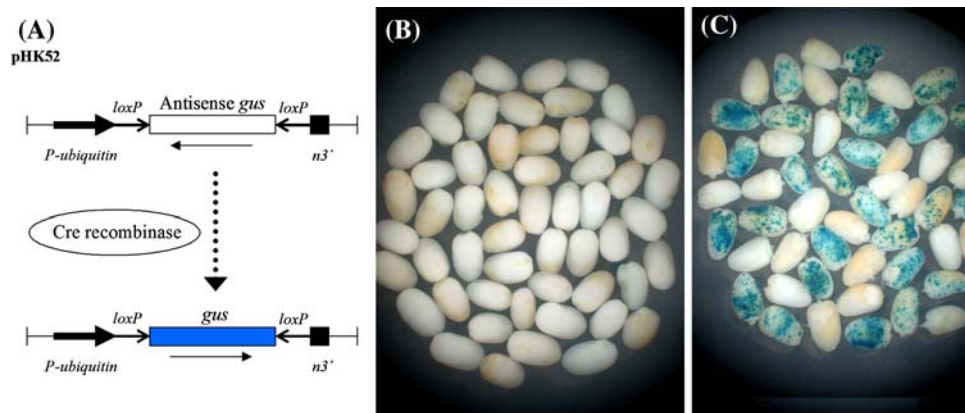
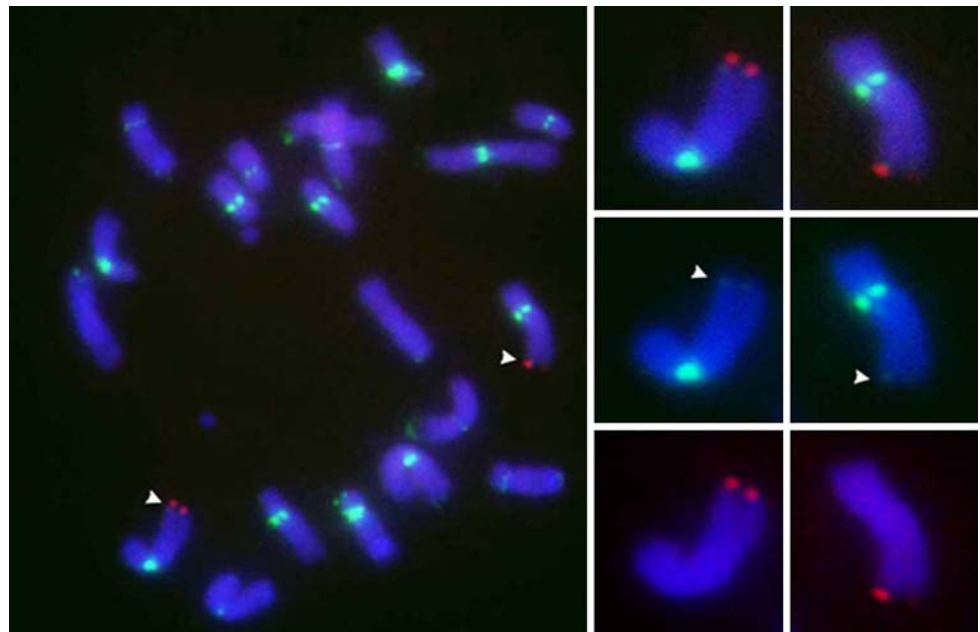


Fig. 6 Cre recombinase catalyzed recombination in transgenic maize embryos. **(a)** Diagram of Cre catalyzed recombination in maize immature embryos by a reporter construct pHK52 (Srivastava et al. 1999). An antisense GUS gene coding sequence flanked by inversely oriented *loxP* sequences was placed under the maize ubiquitin promoter. The action of Cre recombinase expressed from the transgenic plant upon this construct will flip the antisense GUS gene to the sense orientation. The expression can be monitored with a GUS stain on the pHK52 bombarded embryos (Jefferson et al. 1987). The

ubiquitin promoter, *loxP* sites, *gus*, antisense *gus*, Cre recombinase, and the *Agrobacterium* nopaline synthase terminator ($n3'$) are labeled. **(b)** Nontransgenic Hi II A embryos bombarded with pHK52 did not show Cre recombinase activity. **(c)** Example of embryos that inherited the *cre* gene (activating the GUS gene expression, blue color), and embryos that do not have the *cre* gene activity (no color) following bombardment of transformants with pHK52. The embryo population is segregating for the transformed construct in an outcross of the transgenic line J11-11 to the Hi II A tester line

active in centromeres. We also found eight transgenes that co-localized with the knob sequence, and four of them were apparently located within the large knobs from chromosomes 7 or 8. The heterochromatic knobs are variable among different maize inbred lines (Kato et al. 2004). For example, knobs are present on every chromosomes of the K10 line (Kato et al. 2004), but some lines have only few visible knobs (Adawy et al. 2004). It has been found that the heterochromatic knob is mainly composed of the 180 bp repeat (Peacock et al. 1981). Comparable to the heterochromatic knob, the B chromosome of maize is also mostly

heterochromatic and gene sparse, but apparently distinguishable by the transformation plasmid of *Agrobacterium*. One explanation for these observations could be that FISH on highly condensed chromosomes does not have the accuracy to place the transgenes within the knob sequence array and that they are in fact adjacent. Another explanation could be that the knob heterochromatic region is in fact transcribed and can be recognized by the *Agrobacterium*-mediated transformation.

In yeast, genes located near a telomere can be silenced by a telomere position effect, and this silencing can be

relieved depending on the distance between the gene and the telomere (Gottschling et al. 1990; Renauld et al. 1993). The telomere position effect is controlled by multiple genes such as SIR2, SIR3, SIR4, NAT1, ARD1 and HHF2 (Aparicio et al. 1991). However, in one report of a human fibroblast cell line, the expression of an introduced neomycin phosphotransferase gene was not affected by its distance from the telomere. No difference of expression level was found when the telomeres varied from 0.5 to 25 kb (Sprung et al. 1996). In this report, we identified a transgene event J11-9a, at the end of chromosome 3L. This transgene provided the ability to examine terminal position effects on gene expression. The expression of both the *bar* selection marker gene and the *cre* recombinase gene were not affected by its position. As was shown in Fig. 1, the *cre* and *bar* genes are separated by 30 kb of yeast DNA and are driven by two different promoters, but both of them are expressed. The absence of repression by the telomeres was also found in engineered telomeres, such as those created by telomere-mediated chromosomal truncations of both mammalian cells (Farr et al. 1991) and plants (Yu et al. 2006b).

The J11-9a chromosome has been demonstrated to undergo site-specific recombination with a minichromosome carrying a *lox71* site preceding a promoterless DsRed site (Yu et al. 2007). The recombination event activated the fluorescent protein gene and deactivated the *cre* expression by transferring the 35S promoter to DsRed via *lox* recombination. Thus, this construct could serve as a platform for targeting incoming DNA to a specific known location at the terminus of chromosome arm 3L.

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