Construction and behavior of engineered minichromosomes in maize

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Engineered minichromosomes were constructed in maize by modifying natural A and supernumerary B chromosomes. By using telomere-mediated chromosomal truncation, it was demonstrated that such an approach is feasible for the generation of minichromosomes of normal A chromosomes by selection of spontaneous polyploid events that compensate for the deficiencies produced. B chromosomes are readily fractionated by biolistic transformation of truncating plasmids. Foreign genes were faithfully expressed from integrations into normal B chromosomes and from truncated miniB chromosomes. Site-specific recombination between the terminal transgene on a miniA chromosome and a terminal site on a normal chromosome was demonstrated. It was also found that the miniA chromosome did not pair with its progenitor chromosomes during meiosis, indicating a useful property for such constructs. The miniB chromosomes are faithfully transmitted from one generation to the next but can be changed in dosage in the presence of normal B chromosomes. This approach for construction of engineered chromosomes can be easily extended to other plant species because it does not rely on cloned centromere sequences, which are species-specific. These platforms will provide avenues for studies on plant chromosome structure and function and for future developments in biotechnology and agriculture.

artificial chromosomes | FISH | genetic engineering | telomere truncation

Artificial chromosomes involving *de novo* centromere formation on an independently assembled unit and engineered minichromosomes produced by telomere truncation provide striking advantages over traditional methods of gene transformation in yeast and mammalian cells (1–5). The development of such chromosomes in plants would provide these advantages for many applications in basic studies, biotechnology, and agriculture. These chromosomes could be used as independent platforms for foreign gene expression without random integration into the normal chromosomes. Further additions of unlimited amounts of DNA could be added to these platforms in a sequential manner via different site-specific recombination cassettes. Genes introduced in this way would be present in a defined context and thus could be expressed at a more predictable level than through random integration (6). Hence, additional genes, multigene complexes, or even whole metabolic pathways could potentially be added to a genotype. Moreover, engineered or artificial chromosomes could be easily introduced or removed from a genotype by genetic crosses and would facilitate introgression of transgenes to different genetic backgrounds.

To extend engineered chromosome technology to plants, we developed a method of telomere-mediated chromosomal truncation in maize by *Agrobacterium*-mediated transformation of constructs with multiple copies of the telomere sequence (7). Here, we report the use of this technology to produce minichromosome platforms by truncating both normal A and supernumerary B maize chromosomes and at the same time introducing site-specific recombination cassettes for future manipulations. We describe the biological parameters involved with production of minichromosomes and the behavior of these small chromosomes.

Results

Minichromosomes from Truncated Maize A Chromosomes. Chromosomal truncation was conducted by *Agrobacterium*-mediated transformation by using two telomere-containing constructs, pWY76 and pWY86, that possess a bialophos herbicide resistance selectable marker (*bar*), Cre/lox or FLP/FRT site-specific recombination cassettes, and the telomere repeats (7). During the integration event, the telomere-containing transgene caps the broken chromosome and seeds the production of a telomere. FISH analysis of the primary transgenic plants (T_0) identified a minichromosome with a transgene signal at one end of the chromosome close to the centromere (Fig. 1). This minichromosome was derived from chromosome 7 by truncation of the long arm, as revealed by karyotyping probes. It was recovered in an otherwise tetraploid plant and was named R2 (Fig. 1*A*). The spontaneous production of a tetraploid likely allowed the selection of the minichromosome bearing a large chromosomal deletion and reveals the biological parameter in which minichromosomes can be recovered by telomere truncation. In fact, gametophyte abortion was not observed, and this minichromosome was recovered in the progeny after crossing the tetraploid by a diploid plant, which reduced the ploidy level in the progeny to triploid. The triploids that contained this minichromosome were again crossed by diploid plants of the transformation recipient inbred line to reduce the ploidy level to diploid. Five aneuploid plants that contained this minichromosome were rescued by embryo culture (8). These aneuploid plants contained one to four trisomes and were again crossed by normal diploid plants to produce diploid plants with the minichromosome (Fig. 1*B*).

When examined in this diploid background, R2 was never found to pair with chromosome 7 or any other chromosome during meiosis in >30 meiotic prophase cells examined (Fig. 1 *C–E*). The lack of pairing of R2 with its progenitor is probably because it is too small to synapse with the normal chromosome pair. This characteristic of small chromosomes has been reported previously (9). This fact illustrates that small chromosomes have minimal chance of recombination with the normal set and, thus, can be used as starting materials for plant engineered chromosomes. R2 is stable during both mitosis and meiosis, and homozygotes with a pair of R2 could be selected in the progeny of selfed plants (Fig. 1*F* and Table 1).

Minichromosomes from Maize B Chromosomes. Although we demonstrated the production of a minichromosome from an A chromosome, we were interested in B chromosome-based minichromosomes, because the B chromosome has many properties that make it preferable for engineered chromosomes. B chromosomes are

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Abbreviation: GUS, glucuronidase.

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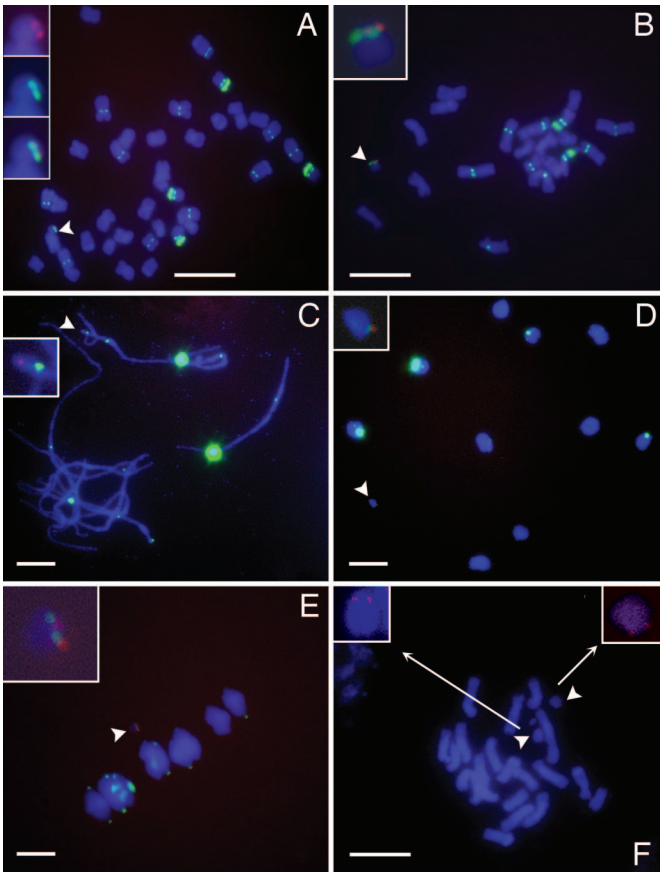

Fig. 1. Minichromosome R2 produced by telomere truncation of an A chromosome. (*A* and *B*) Minichromosome R2 in a tetraploid (*A*) and a diploid (*B*) plant. Centromere and NOR are labeled green; truncating transgene is labeled red. Arrowhead denotes R2. *Insets* in *A* shows transgene (*Top*), CentC (*Middle*), and the merged (*Bottom*) images of R2. *Inset* in *B* shows an enlarged merged image of R2. (*C–E*) R2 minichromosome in meiotic cells. CentC and knob are labeled green. Transgene is labeled red. R2 was not paired with other chromosomes at pachynema (*C*), diakinesis (*D*), and metaphase I (*E*). (*F*) Homozygote of R2. Enlarged images of R2 are shown in *Insets*. Transgenes are labeled red. Arrowheads denote chromosomes enlarged in *Insets*. (Scale bars, 10 μm.)

naturally occurring supernumerary chromosomes (10–12). The B chromosome is basically inert, and its presence at low number does not affect the phenotype of plants. In addition, recombination of B chromosomes with the A set has never been observed; thus B chromosome-based vectors will have minimal detrimental effects on the host genome.

Toward this end, a biolistic-mediated transformation was used for the genetic transformation of immature embryos with 0–12 (average 3.3) B chromosomes by using three telomere-containing plasmids, pWY76, pWY86 or pWY86-bar (a pWY86 derivative with a deletion of the selection *bar* gene expression cassette) plus a pAHC25 construct (13). The pAHC25 plasmid has a strong maize ubiquitin promoter driving the *bar* selection marker gene and also has a β-glucuronidase (GUS) gene expression cassette. This plasmid was cobombarded with the respective pWY76, pWY86, or pWY86-bar plasmids to recover truncations of the B chromosome in the event that the selection marker on the B might be routinely silenced: a circumstance that did not occur (see below). By screening 281 transgenic events regenerated from bialaphos-resistant calli [supporting information (SI) Table 2], we observed seven fragments from A chromosomes (SI Fig. 5*A*), 45 events with transgenes on 55 normal B or miniB chromosomes (Fig. 2) and seven A-B translocations (SI Fig. 5*B*). In addition, 10 truncated B chromosomes without transgene signals were identified (SI Fig. 5*C*). These latter events were likely produced when truncation occurred in such an orientation that the transgenes were retained on acentric fragments and lost during cell division. These miniB chromosomes were selected because other transgenes with the selection marker were present in the same event (SI Table 2). The higher frequency of truncation of B than A chromosomes is consistent with our previous conclusion that some A chromosomal truncations are selected against during the culturing and regeneration procedures of transformation (7).

All miniB chromosomes can transmit through meiosis. Their meiotic behavior is shown in Fig. 2. Interestingly, in contrast to normal chromosome pairs in which sister chromatids remain adhered at meiosis I, the small chromosomes exhibit sister-chromatid separation at this stage (Fig. 2*E*). Sister-chromatid separation at anaphase I of meiosis was also reported for a tiny A chromosome (9). However, this early separation did not adversely affect their transmission to the next generation (Fig. 2*F*).

Gene Expression from Normal B and MiniB Chromosomes. FISH screening of root tips of regenerated seedlings by using pWY96 (7) and pAHC25 probes separately revealed that both transgenes were present at the same locations in 84% of the transformants, as is often the case with cobombardment (14). There were 53 targeted B or miniB chromosomes that had both the pWY96 and pAHC25 transgenes (SI Table 2). The B chromosome derivatives can be distinguished by their shape and the presence of a B chromosome-specific repeat in and around the centromeric region (15). Thus, this circumstance resulted in the placement of a GUS reporter gene onto the B or truncated B minichromosomes.

The B chromosome of maize is basically inert, without any known active genes (10–12). However, it is not known whether the lack of gene activity on B chromosomes is caused by the absence of genes or by suppression of transcription because of its heterochromatic nature. The transformation of the B chromosome allowed us to determine whether there is suppression of

Table 1. Transmission of minichromosomes

Event	76-15a	86-4	86B23	86B155	R2
Chromosome size	1/2 B	1/5 B	1/16 B	3/4 B	NM
Transgene location	Distal	Distal	Distal	Distal	Distal
Cross	As male	As male	As male	Self	Self
Transmission (a)	14 of 36	3 of 25	7 of 29	9 of 18	15 of 46
Transmission (b)	30 of 36	6 of 25	8 of 29	16 of 18	16 of 46

Chromosome size was visually estimated by comparison of minichromosomes with normal B chromosomes in mitotic cells except for 86B23, whose size was estimated by comparison of pachytene chromosomes with the Image Gauge program (Fuji, Tokyo, Japan). NM, R2 size was not measured. The number of progeny that receive the minichromosome(s) is presented in row (a); the numbers of minichromosomes transmitted to each progeny is presented in row (b). Progeny can receive 2–4 minichromosomes from a male parent by nondisjunction during pollen mitoses in the presence of a normal B chromosome.

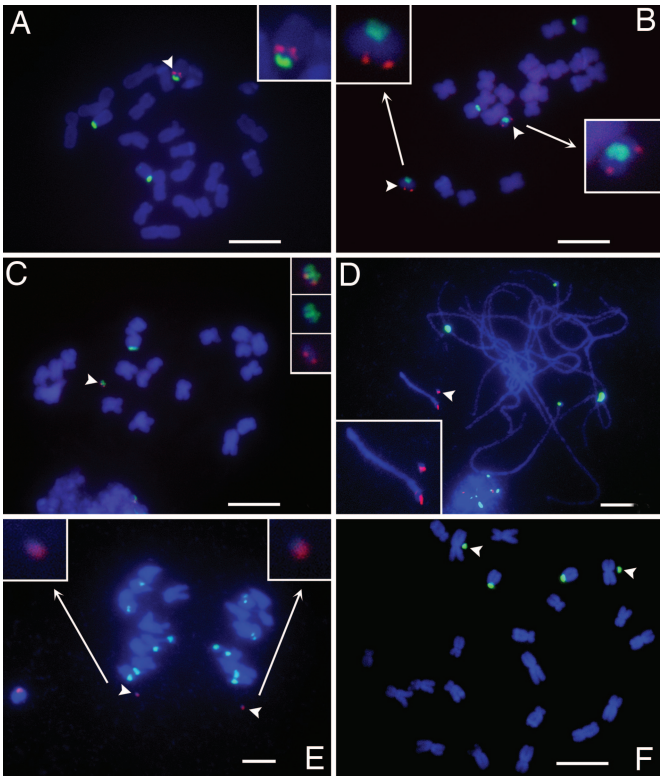

Fig. 2. Targeted normal B and miniB chromosomes. (*A–C*) Mitotic chromosomes of 86-14 (*A*), 86B93 (*B*), and 86B23 (*C*). Transgenes are labeled red, the B chromosome-specific repeat that identifies the centromeric region of B chromosome is labeled green, and chromosomes are stained blue with DAPI. Arrowheads denote intact or truncated B chromosomes with transgenes. *Inset* in *C* shows the merged (*Top*), B-repeat (*Middle*), and transgene (*Bottom*) images of 86B23. (*D* and *E*) Minichromosome 86B23 at pachynema (*D*) and anaphase I (*E*) of meiosis. B chromosome-specific repeat is labeled red and knobs are labeled green. Arrowheads denote minichromosomes. The sister chromatids of the minichromosome separate at anaphase I (*E*). (*F*) Progeny of the minichromosome 86B23. Two minichromosomes were transmitted to the F1 by the mechanism of nondisjunction at the second pollen mitosis when a male parent carrying the minichromosome and full-length B chromosomes was used in an outcross. B chromosome-specific repeat is labeled green. Arrowheads denote the minichromosomes. Enlarged images of minichromosomes (*A–E*) are shown in *Insets*. (Scale bars, 10 μm.)

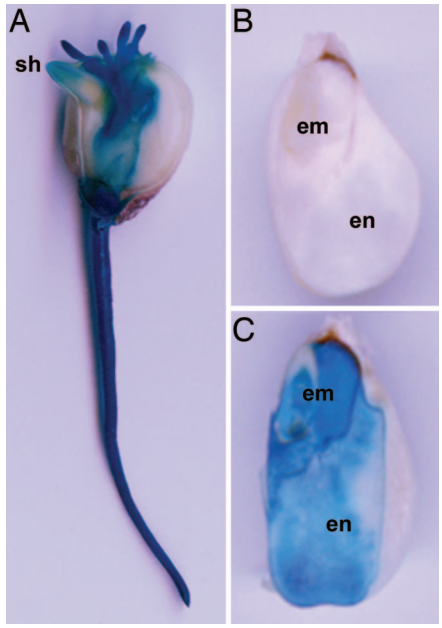

Fig. 3. GUS expression from minichromosome 86B23 with the pAHC25 transgene. sh, shoot; em, embryo; en, endosperm. (*A*) GUS expression in germinated seedling, root, and shoot (blue). (*B* and *C*) GUS expression in segregating mature kernels of a selfed plant with one copy of 86B23 minichromosome and one normal B chromosome. GUS expression is absent in kernel (*B*) but present in both embryo and endosperm of kernel (*C*). GUS expression was not found in the recipient Hill strain (not shown).

transgene expression. We recorded 17 transgenic events that had only transgenes on B chromosomes, which indicated that the *bar* selection marker gene was expressed from the B, although it is formally possible that fragments of transgenes on A chromosomes were present but were too small to be detected by the FISH method. However, the GUS gene expression cassette is ≈5 kb (13), well above our 3-kb FISH detection limit (16). We previously demonstrated the ability to detect routinely a *RescueMu* transgene (17) with a 3.0-kb pBluescript probe. The absence of FISH signal on chromosomes other than the B or miniB by using the pAHC25 probe makes the possibility highly unlikely that any other expressible GUS gene cassette exists in the genome. Also, a cosegregation analysis of transgenes and GUS expression on selected events is consistent with this conclusion (see below).

To test foreign gene expression from B or miniB chromosomes, GUS expression was confirmed in 9 of the 17 events by assaying the resistant calli (SI Table 2). The absence of GUS expression from the other 8 events can be attributed to either the silencing of the GUS gene or the absence of an intact GUS cassette because of rearrangement during the transformation process. GUS expression was also demonstrated in the segregating progeny of 7 events tested by assaying the primary roots (SI Table 3). For example, in the progeny of 76-15a, 76-15b, and 86-74, all individuals that had transgenes on the B or miniB chromosome (14, 11, and 3 individuals, respectively) expressed GUS, whereas those siblings without a modified B (22, 19, and 22 individuals, respectively) were GUS-negative. A χ^2 test rejected the null hypothesis of a random distribution of transgenes and GUS expression (d.f. = 3; $P < 0.001$). In the progeny of 86B23, 86B155, and 86-14, 92.0%, 75.0%, and 70.7% of individuals, respectively, that had transgenes on the B or miniB chromosomes expressed GUS. In these individuals, no other transgenes were detected in the genome. In the progeny of 76-10, only 2 of 12 individuals with the transgene showed GUS expression. This transgene might have undergone rearrangement or gene silencing, which occurs frequently in transformants recovered from biolistic-mediated gene transformation (14). In contrast, all individuals without a detectable transgene were negative for the GUS assay. These data demonstrate that transgenes inserted into the B chromosome can be capable of expression.

GUS expression was observed in all examined tissues of the plants such as leaves, roots, shoots, and mature kernel embryos and endosperm (Fig. 3). The finding that biologically functional GUS can be expressed from introduced genes on B and miniB chromosomes in both embryo and endosperm tissues has significant agricultural implications for engineered minichromosome platforms by demonstrating foreign gene expression in the kernel, the major harvested product.

Transmission of Minichromosomes. Minichromosomes produced by the truncation of both A and B chromosomes retained normal centromeres except in two cases, 86B136 (an A centromere was split, and two telocentric chromosomes were formed, SI Fig. 5*A*) and 86B23, in which the size of the B-specific repeat array in the centromeric region was reduced (Fig. 2 *C–F*). However, all

minichromosomes are transmissible (Table 1). The transmission rate of R2 is comparable with a previously reported trisomic chromosome (8). The meiotic transmission of B type minichromosomes varied from 12% (86-74) to 39% (76-15a) through the male parent, comparable with the transmission of miniB chromosomes generated by other means (15). Future developments might introduce pollen selection systems (18) to a minichromosome to increase the transmission frequency by allowing only grains with the minichromosome to develop. Such a configuration would produce a progeny with all individuals carrying the minichromosome.

B and miniB chromosomes allow dosage manipulations (19). As its accumulation mechanism, the maize B chromosome undergoes nondisjunction during pollen mitosis and preferential fertilization of the egg cell by the sperm with B chromosomes (10). This property requires the long arm of the chromosome to be present in the same cell as the centromere (10) and would be eliminated by truncation. Thus, truncated miniB chromosomes without the long arm distal region exhibit normal segregation, but the nondisjunction property can be restored by adding a full-length B chromosome to the genotype. Indeed, all B chromosome-derived minichromosomes were found to undergo nondisjunction in the presence of normal B chromosomes (Table 1). By nondisjunction, male parents can usually transmit two copies of a minichromosome to some of the offspring in the presence of normal B chromosomes. However, at a low frequency, the transmission of four copies of a minichromosome was also observed when nondisjunction occurred in both the first and second pollen mitoses as noted previously (20) (Fig. 2*F*, SI Fig. 5*D*, and Table 1). For example, by selfing a plant with one minichromosome and one normal B chromosome, five copies of a minichromosome can be obtained (one from the female parent, four from the male parent, SI Fig. 5*D*). The nondisjunction property of B type minichromosomes provides a mechanism to create a dosage series of the engineered construct for increased expression of the resident genes.

Recombination of Minichromosomes. To demonstrate the utility of using engineered minichromosome platforms, a Cre/*lox* site-specific recombination system on the R2 minichromosome was tested for its ability to undergo recombination. Unlike genetic recombination, which relies on chromosome pairing at meiosis, site-specific recombination can occur in somatic cells. Site-specific recombination systems have been demonstrated to be valuable tools for marker gene removal (21), gene targeting (22–24), and gene conversion (25). Such technologies could be applied to minichromosomes, for example, to add genes to the platform. A transgenic plant (J11-9) with a 35S-*lox66*-Cre expression transgenic cassette at the terminus of chromosome arm 3L (J.M.V., W.Y., F.H., and J.A.B, unpublished work) was crossed as a female by a plant carrying R2, which contains the promoterless *lox71*-DsRed gene (Fig. 4*A*). The J11-9 parent supplies the Cre recombinase and an alternative *lox* recombination site for introducing the 35S promoter to activate the DsRed gene. The recombination target sites, *lox66* and *lox71*, are mutated *lox* sequences that can recombine most favorably in the forward reaction (26). Successful exchange of the two transgenes will result in the transfer of the distal regions of the two transgenes, produce a red fluorescence protein by placing the promoterless DsRed gene under the control of the 35S promoter, and the addition of genetic material to the minichromosome from J11-9 (Fig. 4*A*). The Cre recombinase gene expression will also be inactivated by the recombination event. Such events were screened for by examining primary roots of germinated seedlings for red fluorescence and by using PCR amplification with primers flanking the predicted recombination region. A total of 10 plants exhibited red fluorescence from 120 total progeny examined from three crosses of the J11-9 stock by R2-containing plants (Fig. 4*B*).

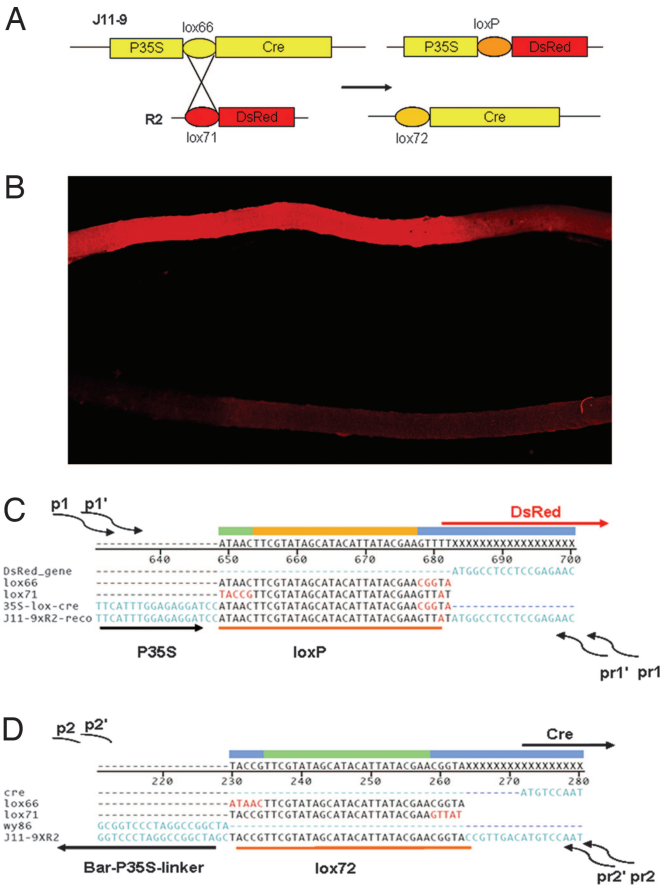

Fig. 4. Cre/lox-mediated site-specific recombination. (*A*) Diagram of recombination between the *lox71*-DsRed of the R2 minichromosome and a P35S-*lox66*-Cre transgene from J11–9. The CaMV 35S promoter (P35S), Cre gene, DsRed gene, and *lox* sites (*lox66*, *lox71*, *lox72*, *loxP*) are indicated. The recombination produces P35S-*loxP*-DsRed on the donor chromosome that activates the red fluorescence protein expression and *lox72*-Cre plus other sequences from the donor chromosome to the minichromosome. (*B*) Red fluorescence protein expression from the recombination of J11–9 and R2 in root tissue (upper) and the absence of red fluorescence protein in the R2 control (lower). (*C* and *D*) Sequence alignments of the two recombination products amplified by PCR with sequences of DsRed coding region, *lox66*, *lox71*, and P35S-*lox66*-Cre (*C*) and the sequences of Cre coding region, *lox66*, *lox71*, and pWY86 (*D*). p1, pr1, p2, and pr2 are outer primers for primary PCR. p1′, pr1′, p2′, and pr2′ are inner primers for nested PCR. The DsRed gene (red arrow), CaMV 35S promoter (P35S, black arrow), Cre gene (black arrow), the linker region between P35S-Bar gene (black arrow), and the *lox72* and *loxP*, which resulted from recombination (red lines), are labeled.

To confirm the site-specific recombination, genomic DNA was isolated from eight J11-9/R2 plants that expressed red fluorescence and was used as templates for PCR amplification across both of the predicted reciprocal recombination products. The amplified products were sequenced, and recombination at the *lox* site was confirmed in each case (Fig. 4*C*). The configurations of the sequenced regions matched the expected patterns. The recombination events were not inherited in the next generation resulting from self-pollination of the hybrid individuals but were recapitulated in 5 of 40 J11-9/R2 heterozygous individuals examined from among three F2 progenies as assayed by PCR. It was not possible to amplify recombinant fragments in 15 examined F_2 siblings that inherited only the J11-9 chromosome or from 17 examined siblings that inherited only the R2 minichromosome without J11-9. This observation and the variable pres-

ence of red fluorescence in J11-9/R2 hybrids indicate that these events are somatic and likely random, although regularly occurring in the J11-9/R2 combination. This result demonstrates that terminal *lox* sites are amenable for genetic manipulations through site-specific recombination systems. However, in this sample, either Cre recombinase expression was not sufficiently high or associations of the *lox* sites did not occur in the developmental lineages leading to the flowers, which resulted in no germinally transmitted recombinants. Nevertheless, these sites could be targeted by a variety of previously demonstrated methods (6, 21–25) for the recovery of germinal inserts for future genetic engineering using this technology.

Discussion

Engineered minichromosome platforms should have broad applications. For example, resistance genes could be stacked on the minichromosome to produce crops that are multiply resistant to viruses, insects, fungi, bacteria, and herbicides. Management of many resistance genes on different chromosomes by traditional gene transformation would be cumbersome. There are many other desirable genes that could also be placed on the minichromosomes, such as stress tolerance genes, antibodies, vaccines, and other pharmaceutical proteins of medicinal value (27). With the previous demonstration of transformation and site-specific integration of large inserts (23, 28, 29), it should be possible to target minichromosomes with a complex of foreign genes or even biochemical pathways to generate complicated products (30, 31) from the minichromosome platform.

The introduction of whole biochemical pathways into plants has the potential to confer new properties. Advantageous new properties would include those that would reduce, eliminate, or manipulate the use of chemical fertilizers and herbicides, provide insect or microbial resistance, allow adaptation to new environments, improve cultivation techniques, increase yield, or facilitate the development of biofuels (32). Engineered and artificial chromosome technology will also allow the use of plants as factories to generate multiple protein or metabolic products more inexpensively than by other methods.

Plant engineered chromosomes might also be used as a vector system for functional genomics. By transforming and recovering site-specific recombination sequences at a particular position on a normal chromosome and then recombining it with the vector, a segment of the chromosome could be translocated to the engineered chromosome, and its function could be assayed as are chromosomal translocations (10). Greater freedom for manipulation could occur in this system, because the recombination cassette could be easily recovered at many sites in the genome by either random transformation (33) or transposable elements that carry it (34).

The production of engineered minichromosomes by telomere truncation should be applicable to most plant species, given the widespread presence of the telomere structure (35, 36). This technology creates small chromosomes that can carry site-specific recombination or other sites (37) that will permit further additions to the chromosome. Different combinations of genes could be placed on a minichromosome either by cobombardment or subsequently by site-specific recombination, both of which have been demonstrated to occur in this study.

This procedure of engineered chromosome production does not rely on cloning centromere sequences from different plant species (38) and bypasses any complications of epigenetic components for centromere specification (39). Although the B chromosomes of various plant species (11) provide an attractive vehicle to produce engineered chromosomes, we present a proof of concept that minichromosomes can also be recovered from truncation events of A chromosomes. These chromosomes could be recovered in spontaneous polyploidy events as described above or by using polyploids as the starting material for transformation with the truncating transgenes. Alternatively, they could be derived by targeting trisomic or chromosome addition lines. Furthermore, truncation might also be applied to designer chromosomes introduced from other plant species (40). Once established as an extra chromosome in an otherwise diploid background, additional truncations should be recovered as efficiently as demonstrated above for the B chromosome, because there would be no selection against loss of portions of the extra chromosome. The ease of chromosomal truncation coupled with the simultaneous placement of other cassettes onto minichromosomes, including those demonstrated to accept incoming DNA, provides a foundation onto which future developments of plant genetic engineering can be built.

Materials and Methods

Plant Materials. HiII hybrid plants were generated by the cross of HiII parent A by parent B (41). HiII parent A line with B chromosomes was developed by recurrent back-cross of B chromosome containing plants to the HiII parent plants. HiII parent A line with B chromosomes was selfed to allow the accumulation of B chromosomes. Progeny with the multiple B chromosomes were self pollinated or crossed by HiII parent B to produce immature embryos for genetic transformation.

Plasmid Construction and Gene Transformation. Telomere truncation constructs, pWY76 and pWY86, were reported previously (7). Plasmid pWY86-bar was prepared by digesting pWY86 with PmlI/AvrII and self-ligation to delete the 35S-bar gene expression cassette. Plasmid pAHC25 (13) was provided by Z. Zhang from the Plant Transformation Core Facility at the University of Missouri.

Immature embryos between 1.2 and 2.0 mm were dissected aseptically in a flow hood. *Agrobacterium*-mediated transformation with pWY76 and pWY86 was performed as described (7). Biolistic-mediated gene transformation of maize immature embryos was conducted as described (42, 43).

FISH Analysis of Minichromosomes. T_0 transgenic plants were screened for minichromosomes by FISH (7). Probes of pWY96 (7) and pAHC25 (13) were labeled with Texas red-dCTP (PerkinElmer Life Sciences, Boston, MA) by nick translation (44) and hybridized to transgenes. B repeat sequence (45) was labeled with Alexa Fluor 488-dCTP (Invitrogen, Carlsbad, CA) and mixed with either pWY96 or pAHC25 transgene probes to screen transformed B chromosomes by biolistic-mediated transformation. CentC (46) and NOR (47) probes were labeled with Alexa Fluor 488-dCTP; CRM (48) was labeled with Cy5-dCTP (PerkinElmer). Minichromosomes were identified by their size as compared with normal A or B type chromosomes.

FISH of meiotic cells was performed as described (8, 49). B chromosome-specific repeat probe was labeled with Texas red-dCTP, and the knob sequence (50) was labeled green with Alexa Fluor 488-dCTP.

Embryo Rescue. The R2 minichromosome in triploids and aneuploids was rescued by embryo culture (8).

Histochemical GUS Assay. Histochemical assay of GUS gene expression was performed (51) by a GUS staining kit (Sigma, St. Louis, MO). Calli or cut roots 2–5 mm long were placed directly into a 50-μl GUS staining solution arrayed in a 96-well PCR plate. The plate was wrapped with Parafilm and incubated at 37°C for 1 h. For whole seedling staining, germinated seedlings were rinsed in tap water to remove soil and then submerged into GUS staining solution, degassed with a vacuum pump for 5 min, and incubated at 37°C for 1 h. For staining of mature kernels, dry seeds were cut in half with a razor blade and then submerged in

GUS staining solution and incubated at 37°C for 1 h. Images were scanned and processed in Photoshop.

Transmission Test of Minichromosomes. Plants with minichromosomes were selfed or cross-pollinated as a male to tester lines without the minichromosome. Kernels from each cross were germinated in moisturized Vermiculite (Therm-O-Rock, New Eagle, PA) for 2–3 days at 30°C. Root tip treatment and metaphase chromosome spreads were prepared as described (44). Progeny of minichromosome containing plants were screened either by chromosome counts (for R2) or by FISH with a B repeat probe (miniB chromosomes).

Recombination of Minichromosome with J11-9. Transgenic plants of J11-9 that had a P35S-lox66-Cre distal trangene on chromosome arm 3L were crossed by plants that contained minichromosome R2. To screen for recombination events, the following two methods were used. First, mature kernels were germinated in moisturized vermiculite (Therm-O-Rock) at 30°C for 2–3 days until the primary roots reached 2–3 cm. Roots were washed in tap water to remove vermiculite and then examined for red fluorescence by using a dissecting microscope with a Texas red filter. Secondly, the following pair of PCR primers were designed: 86F, 5′-TGCCCTTTGGTCTTCTGAGACTGT-3′, which is complementary to the transgene pWY86-Bar linker region, and Cre35Sr, 5′-GCCGCATAACCAGTGAAACAGCAT-3′, which is complementary to the Cre gene coding region. PCR screening with this pair of primers was performed with a Sigma Extract-N-Amp kit (Sigma, St. Louis, MO) as described in the manufacturer's instructions. PCR products were analyzed on a 1% agarose gel with ethidium bromide staining.

To sequence the regions of recombination, PCR products were amplified from leaf DNA. The primary and nested PCR primers for both the recombination products of P35S-loxP-DsRed and P35S (from a P35S-bar gene expression cassette, reverse oriented)-linker-lox72-Cre were designed as below for the amplification of the corresponding products.

Primers: p1: 5′-GCGGTACCACTGACGTAAGGGATGA-3′; pr1: 5′-GCCGCATAACCAGTGAAACAGCAT-3′; p1′: 5′-AAGGGATGACGCAGAATCCCACTA-3′; pr1′: 5′-GGTATGCTCAGAAAACGCCTGGCGA-3′; p2: 5′-GCAATGATGGCATTTGTAGGAGCC-3′; pr2: 5′-GGTCTAGACTACAGGAACAGGTGGTC-3′; p2′: 5′-AGATAGCTGGGCAATGGAATCCGA-3′; and pr2′: 5′-TCACCTTGTAGATGAAGCAGCCGT-3′

PCR was performed with a JumpStart REDTaq ReadyMix PCR mix (Sigma) according to the manufacturer's instruction. The first round of PCR cycling conditions were: 96°C for 30-sec denature, then 40 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.5 min, and then 72°C extension for 5 min. Nested PCR cycling conditions were: 96°C for 30-sec denature, then 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C or 30 sec, and then 72°C extension for 2 min. PCR products were sequenced at the DNA Core Facility of the University of Missouri. Sequences were aligned with a MegAlign program (DNASTAR, Madison, WI).

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