ESTABLISHING A FOUNDATION FOR LARGE DNA TRANSFER TO ARTIFICIAL MINICHROMOSOMES AND B INSERT PLATFORMS IN MAIZE

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This is for you, Drew

I miss you

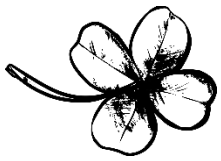

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

CHAPTER 5:

CHAPTER 6:

APPENDIX 1:

APPENDIX 2:

APPENDIX 3:

APPENDIX 4:

APPENDIX 5:

LIST OF TABLES

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

CHAPTER 5:

APPENDIX 3:

APPENDIX 5:

Abstract

In plants, conventional genetic engineering methods limit the number of available traits that could potentially improve the quality of agriculture. *Agrobacterium*-mediated transformation and biolistic bombardment are tools used in transferring genes into plant cells, both of which result in random integrations into host genomes. The absence of targeting machinery, together with low DNA carrying capacity on most plasmid vectors, limit researchers to a few genes in a single modification experiment, a process that takes ~1 year in most plant species. While stacking traits from independent genetic modifications allow for an increase in the number of transgenes in a single plant, recovery of all genes in subsequent generations becomes increasingly difficult due to independent segregation in meiosis. Alternatively, the use of binary bacterial artificial chromosomes (BiBACs), large insert cloning vectors, can maintain and transfer up to 300 kps, but are also subject to random integrations. Therefore, establishment of a BiBAC targeting system would be advantageous for researchers focusing on creating plant lines that contain several genes that work together to express complex traits, such as disease resistance clusters or whole biosynthetic pathways. Additionally, BiBAC targeting to a location outside the native chromosomal sets, such as an artificial minichromosome or B chromosome platform, would enable researchers to stack traits without disrupting endogenous sequences.

A necessary component in creating a BiBAC targeting system is establishing the gene targeting framework, which has been accomplished using site-specific recombinase technology. Recombinases are enzymes that have previously been demonstrated to efficiently activate strand switching reactions between two DNA recognition sites in a number of different plant species, including soybean, rice, and tobacco. Depending on recognition site location and orientation, reactions result in the integration, excision, or inversion of specific sequences. In maize, we have established and demonstrated functionality of five different recombinases (Cre, R, Flp, phiC31 Integrase, and phiC31 Excisionase), that function as tools for genome editing (Chapter 2). By engineering recombinase recognition sites into BiBAC transfer DNA, researchers could direct precise modifications of transgenic lines through breeding or bombardment. Additionally, if recombinase sites are oriented in a strategic manner, cyclical stacking of multiple BiBAC inserts into a single locus could be possible.

In this work, we have created two artificial chromosome platforms located outside the native chromosomal sets maize, an engineered minichromosome and B chromosome insert (Chapter 3). In parallel, we created a plant line that contains a stable insertion of a ~30 kb BiBAC T-DNA molecule (Chapter 4). Using the site-specific recombinase genome editing toolkit established in Chapter 2, we explore the possibility of inducing sequential circularization and targeting of BiBAC inserts to predetermined locations through breeding or bombardment strategies (Chapter 5). Additionally, we describe and establish the framework for

an alternating BiBAC targeting system that could be used to sequentially stack large DNA inserts on artificial minichromosomes or B chromosome inserts in the future (Chapter 4).

Chapter 1: Minichromosomes: Vectors for Crop Improvement

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Summary

Minichromosome technology has the potential to offer a number of possibilities for expanding current biofortification strategies. While conventional genome manipulations rely on random integration of one or a few genes, engineered minichromosomes would enable researchers to concatenate several gene aggregates into a single independent chromosome. These engineered minichromosomes can be rapidly transferred as a unit to other lines through the utilization of doubled haploid breeding. If used in conjunction with other biofortification methods, it may be possible to significantly increase the nutritional value of crops.

Introduction

While efforts to reduce global hunger have been successful, one in nine humans still suffer from malnourishment (FAO; IFAD; WFP, 2014). Such a statistic is not exclusively dependent on plant nutritional content, but arises from compounded factors in food security, with developing countries being greatly impacted (FAO; IFAD; WFP, 2014). Many of these developing regions depend on few staple crops for full nutrient intake, which often lack important dietary components, such as essential amino acids, carbohydrates, and minerals (Hirschi, 2009). With limited options and access to nutritional supplements, many individuals in these countries suffer from micronutrient malnutrition (FAO; IFAD; WFP, 2014). Utilization of biofortification strategies may not be the “solve all” answer to global hunger problems. However, these techniques not only offer an opportunity to significantly reduce malnourishment in developing regions, but improve human health worldwide.

Biofortification aims to improve or supplement crop nutritional content through fertilizer application, conventional breeding, and/or genetic engineering (Hirschi, 2009). The technique to be used would depend on the specific micronutrient being manipulated; however, complementary utilization of strategies could enhance nutritional output (Bruulsema et al., 2012). Inexpensive and simple fertilization strategies are not effective in most scenarios because of several disadvantages including differential effects on crop variants, inability to target edible plant components, and the fact that this approach only modulates

the minerals and not the genotype (Zhu et al., 2007). Conventional breeding is an alternative technique that utilizes favorable characteristics that exist in natural variants and introduces them into commercial lines. The conventional breeding strategy, however, is time consuming and limited by genetic resources that are available (Hirschi, 2009; Zhu et al., 2007). Alternatively, genetic engineering/biotechnology, is a powerful tool that can be used to directly manipulate the genetic code of specific crop variants to alter metabolic processes or increase mineral uptake (Zhu et al., 2007).

In 1983, researchers demonstrated that an isolated gene fragment could be transformed into a plant species Bevan et al., 1983; Herrera-Estrella et al., 1983; Fraley et al., 1983; Murai et al., 1983). Since then, biotechnology has been making significant contributions to several fields of scientific study, enabling timely alterations to genetic codes without lengthy introgression processes. Additionally, genetic engineering expands the possibilities that are available with conventional breeding by not relying on natural variations, and allowing increased control over gene expression with diverse promoters that are publically available. Because gene delivery is accomplished through either *Agrobacterium* mediated transformation or particle bombardment, transgene integration is random, which creates a number of limitations with this technology. Notable limitations include, but are not limited to: (1) disruption of endogenous gene function; (2) affected expression from regulatory elements; (3) difficulty of

separation from closely associated genes; and (4) inefficient recovery of multiple transgenic events in each successive generation (Yu et al., 2007).

With many metabolic processes and nutrient accumulation mechanisms requiring multiple gene products, current genetic engineering methods are not an efficient strategy to accomplish the goals of nutrient accumulation in plants. Minichromosome technology, if applied to this problem, offers a unique solution to these limitations through the creation of an autonomous element that works as a platform for transgene stacking and can be transferred efficiently to subsequent generations (Gaeta et al., 2012). Coupled with doubled haploid breeding, transfer of these engineered chromosomes into other varieties could be expedited, allowing rapid analysis of gene aggregates in several crop lines. Here, the basics of minichromosome technology are discussed in the hope of illustrating the potential utility in a number of such applications.

Discussion

Structure Overview:

In order for minichromosomes to function properly, they must contain the necessary components required to be successfully propagated during cell division. All eukaryotic chromosomes must contain a centromere for kinetochore formation, origins of replication to maintain proper chromosome numbers during cell division, and telomeres to protect the chromosome ends from degradation. Interestingly, in plants, the telomere is the only component that can be

synthesized, as the centromere is epigenetic in nature, and the origin of replication has not been elucidated. As a result, minichromosomes must be created by utilizing endogenous centromeres and telomeres in a process known as the top–down method.

Centromeres:

The centromere is required in order for chromosomes to segregate properly during cell division. The region is responsible for recruitment of kinetochore proteins, and the ultimate attachment of the chromosome to microtubules for proper movement through the cell cycle. While some organisms only require a short sequence to form a centromere, those of plants are much more complex. Plants have regional centromeres, which are composed of satellite repeats, and can vary widely in number between species and even chromosomes (Kanizay et al., 2009; Burrack et al., 2012; Kato et al., 2004). CENH3, the histone H3 variant of plants typical of active centromeres, associates with this repeat region (Zhong et al., 2002); however, studies have shown that the entire region may not interact (Zhong et al., 2002). Many plant centromeres are additionally interspersed with retrotransposon sequences, in maize known as CRM elements (Wolfgruber et al., 2009). CRM elements and satellite repeats both interact with CENH3 and are found throughout the centromere region (Zhong et al., 2002; Wolfgruber et al., 2009; Jin et al., 2004). Not all plant

centromeres rely on such repeat regions; wheat, for example, does not possess any tandem repeats within its centromere (Liu et al., 2008).

Despite most centromeres within a genome containing similar sequences, multiple studies have found that new centromeres can form in regions that are unique in sequence structure (Nasuda et al., 2005; Gong et al., 2009; Fu et al., 2013). Additionally, active centromeres that are formed over repeat regions can be inactivated, as was shown by the recovery of structurally dicentric B chromosomes in maize (Han et al., 2009). The realization that both centromere inactivation and de novo formation occur regularly illustrates an epigenetic component to centromere specification rather than a determination by the DNA sequence (Han et al., 2009; Birchler et al., 2011; Henikoff et al., 2010). As a result, simply including centromeric repeats on a potential minichromosome construct is unlikely to induce kinetochore formation when introduced into a cell.

Origins of Replication:

The timely duplication of the genome is an important step in the cell reproduction process. DNA synthesis is strictly regulated by a variety of mechanisms that determine the time and location of replication fork assembly (Leonard et al., 2013). The number of replication origins in a genome is mostly dependent on chromosome size (Leonard et al., 2013). In most prokaryotes, there exists only one origin of replication on the circular chromosome. Replication origins have been identified in many prokaryotes and there are even tools for

predicting their locations. Eukaryotes, generally, have many origins of replication that assemble at different times, which allows for the replication of the large linear eukaryotic chromosomes.

S. cerevisiae is the only eukaryote where the replication origin has been identified. Neither a specific location nor consensus sequence has been found for origins of replication in any other yeast strains, nor in higher eukaryotes (Leonard et al., 2013). In metazoans there are many origins of replication, which are not all active in every cell (Leonard et al., 2013). With regard to minichromosomes, this situation eliminates any concern for specifically identifying replication origin constitution.

Telomeres:

The telomere of the chromosome is a sequence repeat, and accompanying protein complex, that protects the end from damage or chromosome end-to-end fusions. While the majority of the telomere is a heterochromatic region, the extreme chromosome terminus is protected by a single strand overhang that forms a protective loop, known as a G loop (Makarov et al., 2001). The sequence that confers telomere function is highly conserved, with most plants having a repeat sequence of TTTAGGG that can be extended with the enzyme telomerase (Fajkus et al., 1998). The telomerase is required for telomere extension as DNA polymerase can only extend after a primer template, leading to a loss of sequence at the end of the chromosome during replication.

This restriction in synthesis leads to a shorter telomere sequence with each chromosomal replication event. While telomerase has the ability to extend shortened telomeres, telomerase expression varies widely between tissue types, showing little activity in mature tissues (Fitzgerald et al., 1996; Kilian et al., 1998).

For the purposes of minichromosome creation, synthetically produced telomere sequences are introduced with the desired transgene. In most instances, the introduced telomere will be shorter than the endogenous telomere that it replaces. In order to be functional, these repeats must be extended and the end modified to include a G loop. While the exact process that occurs when a synthetic telomere is introduced is unknown, there are studies that suggest that telomere length is monitored, and can be extended in some organisms when the repeat number is too low (Hemann et al., 2001). Additionally, studies of Arabidopsis telomeres show that the repeat number can vary between individual chromosomes, and different ecotypes suggesting that the amount of telomere is being actively regulated (Shakirov et al., 2004). As a result, despite minichromosomes not containing the required amount of telomere upon introduction, the plant contains the machinery required to extend the copy number to the essential number.

Utilization of B Chromosomes:

B chromosomes are supernumerary chromosomes found in many species of plants. B chromosomes are dispensable. They contain no genes essential to

the survival of a plant (Jones et al., 2003), and show little to no effect on fitness except at high copy number (Randolph, 1941; Staub, 1987). B chromosomes are maintained in populations by a drive mechanism. This process consists of nondisjunction of the B chromosome. This results in more B chromosomes in the progeny than in the parents. In maize (Zea mays) nondisjunction happens at the second pollen mitosis, which makes the two maize sperm. A plant with one B chromosome produces pollen with sperm containing two B chromosomes or containing none. The sperm with the B chromosomes then preferentially fertilizes the egg cell rather than the polar nuclei. (Jones et al., 2003). In rye (*Secale cereale*) nondisjunction occurs at the first pollen mitosis and the two B chromosomes are directed to the generative nucleus resulting in both sperm carrying two B chromosomes (Jones et al., 2003). This directed nondisjunction also occurs during the formation of the egg. In maize, two regions are necessary for the nondisjunction of the B centromere. One is located in the proximal euchromatic region, while the other is at the distal end of the long arm (Roman, 1947). Both act in trans. In rye, only a region on the distal end of the long arm is necessary for nondisjunction.

B chromosomes are useful in the creation of an engineered minichromosome because telomere mediated truncation involves the removal of some or most of at least one chromosome arm. If an A chromosome is truncated, this will generally lead to a detrimental monosomic condition, though rare events of minichromosomes with an A chromosome centromere and otherwise normal

complement of chromosomes have been reported (Gaeta et al., 2013). Truncating a B chromosome has no detrimental effect on the phenotype of a plant and therefore does not lower the rate of recovery of truncation events.

B chromosomes do not pair or recombine with A chromosomes (Jones et al., 2003). This could allow multiple transgenes to be kept together as a unit instead of requiring intensive breeding strategies to stack multiple transgenes in a single plant. Because B chromosomes do not contain any vital genes, there is no chance of linkage of a transgene to an unfavorable allele or knockout of an important gene.

The accumulation mechanism of the B chromosome can be used to increase the copy number of minichromosomes and increase their dosage (Masonbrink et al., 2012). Because the truncation of a B chromosome usually removes the distal end of the long arm, it is necessary to have an unreduced B in the background, which can act in trans to restore the nondisjunction property.

Alternative Methods for Engineering Minichromosomes:

B chromosomes are not found in some agriculturally important crops. In order to create minichromosomes in these crops an A chromosome must be truncated to provide an active centromere. In a euploid individual the truncation of an A chromosome would be detrimental. For that reason telotrisomic lines are a good candidate for telomere truncation. A telotrisomic individual has a normal complement of chromosomes plus a chromosome consisting of one chromosome

arm. Telotrisomics can be found by screening the progeny of a triploid plant for trisomic individuals. From trisomics, centromere misdivision events, or splitting, can produce telotrisomics. A complete set of telotrisomics was developed for rice (Cheng et al., 2001).

Aneuploid plants are less vigorous than euploid; generally the less of the genome for which a plant is aneuploid, the less negative the phenotype. Optimally, a minichromosomes should contain as little of the A genome as possible. Telotrisomics are good candidates for creating minichromosomes because they require only one chromosome arm truncation to remove much of the genic region from the A chromosome for which they are trisomic and relieve the aneuploid phenotype.

The effects of aneuploidy can also be lessened in a higher ploidy background. In maize, a truncated A chromosome was rescued in a spontaneous tetraploidy event (Yu et al., 2007). Tetraploid plants have also been used for A chromosome truncation in Arabidopsis thaliana (Teo et al., 2011) and barley (*Hordeum vulgare*) (Kapusi et al., 2012). The truncated chromosome can then be transferred to a diploid background by successive crosses of the tetrapliod by a diploid to produce a triploid and again by a diploid to recover the truncated minichromosome as an extra entity in a diploid background (Yu et al., 2007).

Stability in Plants:

Minichromosomes derived from the B chromosome of maize are heritable, but transmitted at varying frequencies. Many minichromosomes fail to pair by metaphase 1 of meiosis. Other irregularities such as early sister chromatid separation and lagging chromosomes during division have also been observed. These meiotic abnormalities appear to be correlated with minichromosome size (Han et al., 2007). Nondisjunction can also occur in somatic tissues, especially at high minichromosome copy number. When mitotic metaphase cells were examined in maize root meristems, B chromosome and minichromosome copy number varied between cells when high numbers were present (Masonbrink et al., 2012).

Stable transmission of minichromosomes can be achieved in multiple ways. Firstly, larger B minichromosomes tend to pair better and behave more normally in meiosis than smaller B-derived minichromosomes. A minichromosome derived from a truncation of only the distal tip of a B chromosome will behave like an A chromosome if there are two copies to allow pairing and there are no normal B chromosomes present (Han et al., 2007). To improve transmission of smaller, less stable minichromosomes or minichromosomes in a species without B chromosomes, a gametophytic selection mechanism has been proposed. Cytoplasmic male sterile (cms) plants have a mitochondrial mutation which causes pollen abortion. A nuclear restorer of fertility (Rf) gene prevents pollen abortion in cms plants (Chase and Gabay-

Laughnan, 2004). Inserting Rf onto a minichromosome would allow any pollen grains with a minichromosome to develop, while any pollen grains without a minichromosome would abort (Birchler et al., 2010).

Transgene Expression:

Random integration of genetic fragments can lead to varying levels of expression, due to possible silencing events that occur at different loci throughout the host genome. This phenomenon, coupled with repressed transcription of repeated or homologous sequences, is an obstacle in the field of genetic engineering (Ye and Signer, 1996; Matzke and Matzke, 1998). Artificial minichromosome technology offers an autonomous platform for sequential transgene integration events at a specific locus that has been demonstrated to successfully express genes at detectable levels. However, utilization of repeated genetic elements needs to be avoided when designing minichromosomes due to possible homologous gene silencing events (Matzke and Matzke, 1998). While still in preliminary stages of development, the use of synthetic promoters will be a valuable tool in avoiding sequence repeats (Liu et al., 2013). As both artificial minichromosomes and synthetic promoter technologies progress in parallel, complementary use of both systems will work synergistically to expand limits of genetic engineering and avoid possible silencing events that occur from sequence homology.

Creating Large Minichromosomes with BiBACs:

Genetic engineering has, so far, focused mostly on introducing single or a few genes into crops. While these crops have been successful, more complex traits such as improved nutrition will rely on stacking multiple genes (Halpin, 2005). Thus far, introduction of multiple genes has relied on complex breeding to combine separately introduced genes, or co-introducing multiple genes during one transformation. As a result, the ability to introduce multiple traits on one T-DNA is preferred.

Vectors capable of carrying large (>300 kb) DNA fragments, known as bacterial artificial chromosome (BAC) vectors, were first created for use in E. coli (Shizuya et al., 1992). Shortly after, the vectors were modified to have the ability to be maintained in *Agrobacterium* tumefaciens as transformation vectors (Hamilton et al., 1996). The result, known as binary bacterial artificial chromosome (BIBAC) vectors, have been shown to transfer large intact DNA fragments in a variety of different plant species (Hamilton et al., 1996; Vega et al., 2008; Hamilton et al., 1999). By including telomere sequences near the right border of a BIBAC vector, it should be possible to create minichromosomes while introducing large DNA fragments. These minichromosomes would allow multiple traits to be introduced at a single locus, and would only require a single selection for the entire transgene array.

In Vivo Modification of Minichromosomes:

As previously stated, traditional genome manipulation tools, such as *Agrobacterium* mediated transformation and biolistic bombardment, impose a number of notable limitations. Introducing multiple traits into a single background will require several rounds of transformation, which can be labor intensive and time consuming. Through the utilization of genome targeting mechanisms, minichromosome technology offers a novel solution to these constraints. Precise targeting is derived from a number of site-specific recombination systems that add, remove, or modify specific genetic elements, allowing the assembly of large gene aggregates in a regulated manner.

A diverse number of site-specific recombination systems are publically available for research purposes (Grindley et al., 2006; Wang et al., 2011). While unique in sequence recognition, all systems follow the same basic steps: (1) Expressed recombinase enzymes bind to respective sites; (2) Bound enzymes form a synaptic complex, with sites assembled in a parallel orientation; (3) Recombinases catalyze the crossover and fusion of genetic material between recognized cassettes; (4) Disassembly of the synaptic complex [53]. The outcome of the recombination reaction is dependent on location and orientation of the recognition sites. Integration, inversion, and excision of specific genetic elements are all possible, so careful planning of experimental design needs to be carried out to ensure anticipated outcomes. It should be noted that all enzymes

fall under a particular sub-category of recombinases that reflect the nature of the system (Wang et al., 2011). These categories differ in a number of characteristics, including size and directionality (Wang et al., 2011). Such a collection of recombinases can be exploited for the purpose of large-scale genetic manipulations, specifically the creation of artificial minichromosomes.

Regulation of recombinase expression is of most importance in assembling gene aggregates on minichromosomes. Several strategies to control the timing and duration of recombinase activity have been demonstrated (Gidoni et al., 2008). With sexually propagating plants, backgrounds that are actively expressing a recombinase enzyme can be bred with lines containing a minichromosome that is to be modified. The recombinase gene could simply be crossed out in the next generation, leaving a background that only contains a modified minichromosome. Organisms that reproduce vegetatively, or require lengthy germination cycles, could favorably use tactics that transiently express the recombinase enzyme. An example of one of these methods is the auto-excision strategy. Under the control of an inducible promoter, recombinases can be activated by a number of factors, including heat-shock, chemical, or developmental cues (Gidoni et al., 2008). If flanked by respective recombination sites, induction of an inducible promoter will lead to expression of the recombinase, followed by its removal via intra-molecular recombination (Wang et al., 2011; Gidoni et al., 2008).

Taken together, minichromosome construction will require strategic utilization of several site-specific recombination systems. Each respective recognition site is assembled in a specific orientation to allow integration of gene fragments and removal of selectable markers in a successive manner (Ow, 2004). The proposed transgene stacking strategy exploits recombinase directionality, which enables recombination site and selectable marker recycling with each round of integration (Ow, 2004). Before this process can be initiated, however, the minichromosome generated must contain a specific sequence that is to be acted upon by a well-characterized recombination system for the purpose of integration. A background that contains a minichromosome, and actively expresses a recombinase, will be introduced to a gene fragment that contains gene(s) of interest, recombination sites, and a selectable marker. The expressed recombinase will form a synaptic complex between its respective recognition sites on the minichromosome and the gene fragment, initiating strand exchange and integration. Transgene orientation upon insertion is predictable and unidirectional, due to the nature of the specific recombination system used. The selectable marker, now bordered by recombination cassettes, is used to identify a successful integration event. Depending on the method of reproduction, a different site-specific recombination system, for the purpose of excision, will be activated through genetic crosses or transient expression. This recombinase will identify the flanking recognition sequences and remove the selectable marker,

restoring a single recombination site to be used in the next round of modifications.

Transfer to Minichromosomes with Haploid Induction:

It may be possible to transfer minichromosomes to multiple lines rapidly through haploid induction. If a haploid inducing line containing a minichromosome is used to produce haploid embryos it should be possible to recover haploids with one or more minichromosomes as follows. In maize, high haploid induction rate lines have been derived from a line called Stock 6 (Coe, 1959), and its derivatives. When used as a pollen donor Stock 6 derived haploid inducer lines produce maternal haploids at a high rate. Many groups have noted the transfer of markers from the inducer line to the maternal haploids (Zhang et al., 2008; Zhao et al., 2013), and the transfer of complete B chromosomes has also been reported (Zhao et al., 2013). Minichromosomes introduced into the inducer line should be able to be transferred in an analogous fashion.

Once a haploid plant with a minichromosome has been obtained, its ploidy can be doubled by colchicine or high-pressure nitrous oxide gas. These treatments produce diploid sectors in the plant which are fertile and the plant can be self-pollinated. This results in a doubled haploid line, which is completely homozygous. By this method, once a minichromosome is back-crossed into a haploid inducing line, it can be transferred to new lines with just two crosses: One to generate the haploid and one for self-pollination. The resultant progeny will be

completely homozygous which is basically not possible if the minichromosome had been back-crossed into the line using a typical introgression scheme.

Conclusions

As the population of the world continues to increase, there is a growing need to find innovative ways to utilize the static amount of arable land. Biofortification of crops through conventional breeding and fertilizer application are two strategies to make headway toward improving nutritional value, but genetic engineering offers another strategy for addressing global food security. Gene stacking could allow for complex traits and pathways to be expressed with minimal selective breeding. Minichromosomes could provide a stable and heritable platform for this gene stacking. Because a bottom up approach toward creating an artificial chromosome is not yet possible, the top–down strategy for minichromosome creation is a viable option. B chromosomes, which are present or could be introduced in some crop species, offer a platform for creation of minichromosomes with the advantage that they lack essential genes. Other options as outlined above are also available. Minichromosomes have been shown to reliably express transgenes and to be transmitted from generation to generation. There is potential to make large scale additions to minichromosomes, which could allow for the introduction of multiple genes at once instead of through several transformations. There are now a variety of recombination systems, as well as genome editing technologies, which can be utilized to edit an

existing minichromosome and make minichromosomes a truly custom platform. The ability to transfer minichromosomes through haploid breeding would allow for the rapid introduction of minichromosomes to many inbred lines, including those not amenable to transformation, without the need for generations of introgression. For these reasons, minichromosomes could provide a tool for improved biofortification of a variety of crop species.

References

Bevan M.W., Flavell R.B., Chilton M.A. (1983) Chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature, 304, 184–187.

Birchler J.A., Krishnaswamy L., Gaeta R., Masonbrink R., Zhao C. (2010) Engineered Minichromosomes in Plants. CRC Crit. Rev. Plant. Sci. 29, 135–147.

Birchler J.A., Gao Z., Sharma A., Presting G.G., Han F. (2011) Epigenetic aspects of centromere function in plants. Curr. Opin. Plant Biol. 14, 217–222.

Bruulsema T.W., Heffer P., Welch R.M., Cakmak I., Moran K. (2012) Fertilizing Crops to Improve Human Health: A Scientific Review; International Plant Nutrition Institute: Norcross, GA, USA.

Burrack L.S. and Berman J. (2012) Neocentromeres and epigenetically inherited features of centromeres. Chromosome Res. 20, 607–619.

Chase C.D. and Gabay-Laughnan S. (2004) Cytoplasmic male sterility and fertility restoration by nuclear genes. In Molecular Biology and Biotechnology of Plant Organelles; Daniell, H., Chase, C.D., Eds.; Springer: Dordrecht, The Netherlands, 22, 593–621.

Cheng Z., Yan H., Yu H., Tang S., Jiang J., Gu M., Zhu L. (2001) Development and applications of a complete set of rice telotrisomics. Genetics, 157, 361–368.

Coe, E.H. (1959) A line of maize with high haploid frequency. Am. Natl. 93, 381–382.

Fajkus J., Fulnecková J., Hulanova M., Berkova K., Říha K., Matyásek R. (1998) Plant cells express telomerase activity upon transfer to callus culture, without extensively changing telomere lengths. Mol. Gen. Genet. 260, 470–474.

FAO; IFAD; WFP (2014) Strengthening the enabling environment for food security and nutrition. In the State of Food Insecurity in the World 2014; FAO: Rome, Italy.

Fitzgerald M.S., McKnight T.D., Shippen D.E. (1996) Characterization and developmental patterns of telomerase expression in plants. Proc. Natl. Acad. Sci. USA, 93, 14422–14427.

Fraley R.T., Rogers S.G., Horsch R.B., Sanders P.R., Flick J.S., Adams S.P., Bittner M.L., Brand L.A., Fink C.L., Fry J.S., Galluppi G.R., Goldberg S.B., Hoffmann N.L., Woo S.C. (1983) Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA, 80, 4803–4807.

Fu S., Lv Z., Gao Z., Wu H., Pang J., Zhang B., Dong Q., Guo X., Wang X., Birchler, J.A., Han F. (2013) De novo centromere formation on a chromosome fragment in maize. Proc. Natl. Acad. Sci. USA. 110, 6033–6036.

Gaeta R.T., Masonbrink R.E., Krishnaswamy L., Zhao C., Birchler J.A. (2012) Synthetic chromosome platforms in plants. Annu. Rev. Plant Biol. 63, 307–330.

Gaeta R.T., Masonbrink R.E., Zhao C., Sanyal A., Krishnaswamy L., Birchler J.A. (2013) In vivo modification of a maize engineered minichromosome. Chromosoma, 122, 221–232.

Gidoni D., Srivastava V., Carmi N. (2008) Site-specific excisional recombination strategies for elimination of undesirable transgenes from crop plants. In Vitro Cell. Dev. Biol. Plant. 44, 457–467.

Gong Z., Yu H., Huang J., Yi C., Gu M. (2009) Unstable transmission of rice chromosomes without functional centromeric repeats in asexual propagation. Chromosome Res. 17, 863–872.

Grindley N.D., Whiteson K.L., Rice P.A. (2006) Mechanisms of site-specific recombination. Annu. Rev. Biochem. 75, 567–605.

Halpin C. (2005) Gene stacking in transgenic plants—The challenge for 21st century plant biotechnology. Plant Biotechnol. J. 3, 141–155.

Hamilton C.M., Frary A., Lewis C., Tanksley S.D. (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc. Natl. Acad. Sci. USA. 93, 9975–9979.

Hamilton C.M., Frary A., Xu Y., Tanksley S.D., Zhang H. (1999) Construction of tomato genomic DNA libraries in a binary-BAC (BIBAC) vector. Plant J. 18, 223–229.

Han F., Lamb J.C., Birchler J.A. (2006) High frequency of centromere inactivation resulting in stable dicentric chromosomes of maize. Proc. Natl. Acad. Sci. USA. 103, 3238–3243.

Han F., Gao Z., Birchler J.A. (2009) Reactivation of an inactive centromere reveals epigenetic and structural components for centromere specification in maize. Plant Cell. 21, 1929–1939.

Han F., Gao Z., Yu W., Birchler J.A. (2007) Minichromosome analysis of chromosome pairing, disjunction, and sister chromatid cohesion in maize. Plant Cell. 19, 3853–3863.

Hemann M.T., Strong M.A., Hao L., Greider C.W. (2001) The Shortest Telomere, Not Average Telomere Length, Is Critical for Cell Viability and Chromosome Stability. Cell. 107. 67–77.

Henikoff S. and Furuyama T. (2010) Epigenetic inheritance of centromeres. Cold Spring Harb. Symp. Quant. Biol. 75, 51–60.

Herrera-Estrella L., Depicker A., Montagu V.M., Schell J. (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature. 303, 209–213.

Hirschi K.D. (2009) Nutrient biofortification of food crops. Annu. Rev. Nutr. 29, 401–421.

Jin W., Melo J.R., Nagaki K., Talbert P.B., Henikoff S., Dawe R.K., Jiang J. (2004) Maize centromeres: Organization and functional adaptation in the genetic background of oat. Plant Cell. 16, 571–581.

Jones N. and Houben A.B. (2003) Chromosomes in plants: Escapees from the A chromosome genome. Trends Plant Sci. 8, 417–423.

Kanizay L. and Dawe R. (2009) Centromeres: Long intergenic spaces with adaptive features. Funct. Integr. Genomics. 9, 287–292.

Kapusi E., Ma L., Teo C.H., Hensel G., Himmelbach A., Schubert I., Mette M., Kumlehn J., Houben A. (2012) Telomere-mediated truncation of barley chromosomes. Chromosoma. 121, 181–190.

Kato A., Lamb J.C., Birchler J.A. (2004) Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. Proc. Natl. Acad. Sci. USA. 101, 13554–13559.

Kilian A., Heller K., Kleinhofs A. (1998) Development patterns of telomerase activity in barley and maize. Plant Mol. Biol. 37, 621–628.

Leonard A.C. and Mechali M. (2013) DNA replication origins. Cold Spring Harb. Perspect. Biol. 5, doi:10.1101/cshperspect.a010116.

Liu Z., Yue W., Li D., Wang R., Kong X., Lu K., Wang G., Dong Y., Jin W., Zhang X. (2008) Structure and dynamics of retrotransposons at wheat centromeres and pericentromeres. Chromosoma. 117, 445–456.

Liu W., Yuan J.S., Stewart C. (2013) Advanced genetic tools for plant biotechnology. Nat. Rev. Genet. 14, 781–793.

Murai N., Kemp J.D., Sutton D.W., Murray M.G., Slightom J.L., Merlo D., Reichert N.A., Sengupta-Gopalan C., Stock C.A., Barker R.F., Hall T.C. (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science. 222, 476–482.

Makarov V.L., Hirose Y., Langmore J. (2001) Long g tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell 2001, 88, 657–666.

Masonbrink R.E. and Birchler J.A. (2012) Accumulation of multiple copies of maize minichromosomes. Cytogenet. Genome Res. 137, 50–59.

Matzke A.J. and Matzke M.A. (1998) Position effects and epigenetic silencing of plant transgenes. Curr. Opin. Plant Biol. 1, 142–148.

Nasuda S., Hudakova S., Schubert I., Houben A., Endo T. (2005) Stable barley chromosomes without centromeric repeats. Proc. Natl. Acad. Sci. USA. 102, 9842–9847.

Ow, D.W. (2004) SIVB congress symposium proceeding: Transgene management via multiple site-specific recombination systems. In Vitro Cell. Dev. Biol. Plant. 41, 213–219.

Randolph L.F. (1941) Genetic characteristics of the B chromosomes in maize. Genetics. 26, 608–631.

Roman H. (1947) Mitotic nondisjunction in the case of interchanges involving the B-type chromosome in maize. Genetics. 32, 391–409.

Shakirov E.V. and Shippen D.E. (2004) Length Regulation and dynamics of individual telomere tracts in wild-type *Arabidopsis*. Plant Cell. 16, 1959–1967.

Shizuya H., Birren B., Kim U., Mancino V., Slepak T., Tachiiri Y., Simon M. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc. Natl. Acad. Sci. USA. 89, 8794–8797.

Staub R.W. (1987) Leaf striping correlated with the presence of B chromosomes in maize. J. Hered. 78, 71–74.

Teo C.H., Ma L., Kapusi E., Hensel G., Kumlehn J., Schubert I., Houben A., Mette M.F. (2011) Induction of telomere-mediated chromosomal truncation and stability of truncated chromosomes in Arabidopsis thaliana. Plant J. 68, 28–39.

Vega J.M., Yu W., Han F., Kato A., Peters E.M., Zhang Z.S., Birchler J.A. (2008) *Agrobacterium*-mediated transformation of maize (Zea mays) with Cre-lox site specific recombination cassettes in BIBAC vectors. Plant Mol. Biol. 66, 587–598.

Wang Y., Yau Y., Perkins-Balding D., Thomson J.G. (2011) Recombinase technology: Applications and possibilities. Plant Cell Rep. 30, 267–285.

Wolfgruber T.K., Sharma A., Schneider K.L., Albert P.S., Koo D., Shi J., Gao Z., Han F., Lee H., Xu R., Allison J., Birchler J.A., Jiang J., Dawe R.K., Presting G.G. (2009) Maize centromere structure and evolution: Sequence analysis of centromeres 2 and 5 reveals dynamic Loci shaped primarily by retrotransposons. PLoS Genet. 5, e1000743.

Ye F. and Signer E.R. (1996) RIGS (repeat-induced gene silencing) in Arabidopsis is transcriptional and alters chromatin configuration. Proc. Natl. Acad. Sci. USA. 93, 10881–10886.

Yu W., Han F., Birchler J.A. (2007a) Engineered minichromosomes in plants. Curr. Opin. Biotechnol. 2007, 18, 425–431.

Yu W., Han F., Gao Z., Vega J.M., Birchler J.A. (2007b) Construction and behavior of engineered minichromosomes in maize. Proc. Natl. Acad. Sci. USA 2007, 104, 8924–8929.

Zhang Z., Qiu F., Liu Y., Ma K., Li Z., Xu S. (2008) Chromosome elimination and in vivo haploid production induced by stock 6-derived inducer line in maize (Zea mays L.). Plant Cell Rep. 2008, 27, 1851–1860.

Zhao X., Xu X., Xie H., Chen S., Jin W. (2013) Fertilization and uniparental chromosome elimination during crosses with maize haploid inducers. Plant Physiol. 2013, 163, 721–731.

Zhong C.X., Marshall J.B., Topp C., Mroczek R., Kato A. Nagaki K., Birchler J.A., Jiang J., Dawe R.K. (2002) Centromeric retroelements and satellites interact with maize kinetochore protein CENH3. Plant Cell 2002, 14, 2825–2836.

Zhu C., Naqvi S., Gomez-Galera S., Pelacho A.M., Capell T., Christou P. (2007) Transgenic strategies for the nutritional enhancement of plants. Trends Plant Sci. 12, 548–555.

Chapter 2: Site-Specific Recombinase Genome Editing Toolkit in Maize

Note: The information in this chapter is prepped for publication including the following authors:

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Author Contributions

JC established FLPe and R recombinase expression lines, generated and screened recombinase lines for homozygous backgrounds, created DsRed transient expression test constructs, extracted and bombarded recombinase embryos, analyzed bombarded embryos for DsRed signal on a fluorescent stereo microscope and wrote the manuscript. **NG** conceptualized and demonstrated the strategy of using transient GUS expression to show functionality of phiC31 Integrase and phiC31 Excisionase in maize, which was later changed to DsRed for increased resolution. **CZ** provided support and performed *Agrobacterium*-mediated transformation to establish Cre, phiC31 Integrase and phiC31 Excisionase expression lines. **NS** provided support and feedback on the manuscript. **JB** provided guidance over all experiments and edited the manuscript.

Summary

Site-specific recombinase enzymes function in heterologous cellular environments to initiate strand-switching reactions between unique DNA sequences termed recombinase binding sites. Depending on binding site position and orientation, reactions result in integrations, excisions, or inversions of targeted DNA sequences in a precise and predictable manner. Here, we established five different stable recombinase expression lines in maize through *Agrobacterium*-mediated transformation of T-DNA molecules that contain coding sequences for Cre, R, FLPe, phiC31 Integrase, and phiC31 Excisionase. Through the bombardment of recombinase activated DsRed transient expression constructs, we have determined that all five recombinases are functional in maize plants. These recombinase expression lines could be utilized for a variety of genetic engineering applications, including selectable marker removal, targeted transgene integration into predetermined locations, and gene stacking.

Introduction

Successful transfer and expression of foreign DNA in plant cells through the process of transformation was achieved over 30 years ago (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983; Muraieta et al. 1983). Since then, genetic engineering has been used as a major tool in a worldwide effort to increase agricultural productivity and performance in staple crops, such as rice, soybean and maize. Future challenges of sustaining a growing population and

adapting to shifting environmental conditions create a need for advancements in crops beyond what is possible using conventional genome editing methods, which randomly integrate DNA into host genomes using *Agrobacterium tumefactions* or biolistic bombardment. The absence of targeting machinery, together with low DNA carrying capacity on commonly used plasmid vectors, limits researchers to a few genes in a single transformation experiment, a process that takes six months – one year in most plant species. This creates a bottleneck when applying known biological systems, such as biosynthetic pathways or disease resistance clusters, to create healthy and affordable food supplies (Altpeter et al. 2016). Multiple transgenes in different loci become increasingly difficult to maintain in subsequent generations due to independent segregation in meiosis, requiring a labor intensive and time-consuming process of introgression into agronomically significant cultivars. Additionally, recovered events from plant transformations exhibit differences in expression level, copy number, and can possibly interfere with endogenous gene function; therefore, it is necessary to screen each event for optimal insertion locations, eliminating upwards of 90% of T_0 plants under commercial parameters (Anand and Jones 2018). Recent developments of precise genome editing techniques, such as zinc finger nucleases (ZFN) (Bibikova et al. 2003; Carroll D. 2011), transcription activator-like effector nucleases (TALENs) (Christian et al. 2010; Bogdanove and Voytas 2011), and CRISPR Cas9 (Jinek et al. 2012), create promising avenues for targeted modifications; however, they have a low frequency of transgene

targeting. Alternatively, the use of site-specific recombinases could enable efficient strategies for targeted modifications and possibly give researchers more control over established transgenic lines.

Site-specific recombinases are a class of specialized enzymes that catalyze strand-switching reactions between two specific DNA sequences termed recombinase-binding sites (RBS). While they are most prevalent in phage, bacterial, and lower eukaryotic organisms, serving specific biological functions, recombinases are active in a variety of heterologous environments, including *in-vitro* conditions. The reactions involve: 1) recognition and binding of recombinase dimers to binding sites, 2) formation of a synaptic complex between two bound sites, 3) recombinase-mediated strand exchange and fusion events, 4) disassembly of the synaptic complex (Grindley et al, 2006). Depending on position and orientation of the sites, reactions will result in integrations, excisions, or inversions of intervening DNA sequences in precise and predictable manners (Figure 2.1). While the most widely used and described recombinases are Cre (causes recombination) and FLP (flippase), there exists a collection of enzymes that are all unique in terms of origin and sequence recognition; however, all fall into one of the two specific families, reflecting the recombinase amino acid group that catalyzes strand-switching reactions.

The Tyrosine family of recombinases include Cre (Sauer and Henderson, 1990), FLP (Golic and Lindquist, 1989), and R (Onouchi et al. 1991), which bind to identical recombinase-binding sites *lox, FRT,* and *RS*, respectively. Since

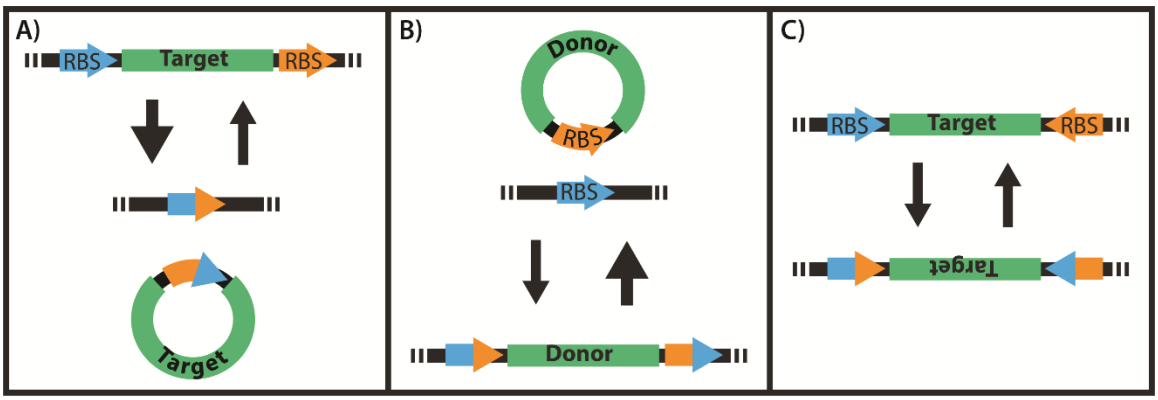


Figure 2.1 - Site-Specific Recombinase Modifications: Depending on recognition site position and orientation, recombinase reactions will result in **(A)** excisions, **(B)** integrations, or **(C)** inversions of specific sequences, such as selectable markers and genes of interest. DNA used for targeted integrations must be in a circular conformation to avoid fragmentation of engineered sequences. Arrows indicate directionality of the recombinase reaction. Large arrows indicate favorability of intramolecular reactions.

Tyrosine recombinases catalyze strand switching reactions between identical sites, the reaction is bidirectional, favoring intramolecular over intermolecular recombination (Table 2.1). The Serine family of recombinases includes phiC31 Integrase and phiC31 Excisionase (Thorpe and Smith, 1998), which binds to non-identical sites *attB/attP* and *attL/attR*, respectively. Recombination reactions between non-identical sequences create hybrid binding sites that are unrecognized by recombinases mediating the strand switching reaction. PhiC31 Integrase forms a synaptic complex between *attB* and *attP* binding sites and induces a strand switching reaction to form *attL* and *attR*, which are unrecognizable in the absence of the helper protein phiC31 Excisionase. If Excisionase helper proteins are expressed in the presence of phiC31, recombination reactions between attL and attR favor the reverse direction.

The first application using Cre to excise a stably integrated selectable marker gene from tobacco occurred in the early 90's (Dale and Ow, 1990). Since then, recombinases have been used in a number of plant systems including rice (Hoa et al. 2002; Hu et al. 2007; Radhakrishnan and Srivastava, 2005), tobacco (Dale and Ow, 1991; Albert et al. 1995), wheat (Srivastava et al. 1999), tomato (Stuurman et al. 1996), barley (Kapusi et al. 2012), soybean (Li et al. 2009;), *Arabidopsis* (Vergunst and Hooykaas 1998; Hong et al. 2001; Thomson et al. 2010), and maize (Lyznik et al. 1996; Srivastava and Ow 2001; Zhang et al. 2003; Kerbach et al. 2005; Anand et al. 2019). In these studies, recombinase enzymes have been used for the purpose of selectable marker removal,

Recombinase - Binding Site	Binding Site Sequence	Directionality
Cre - *lox*	**5′** ATAACTTCGTATAATGTATGCTATACGAAGTTAT **3′**	Bidirectional
R - *RS*	**5′** CGAGATCATATCACTGTGGACGTTGATGAAAGAATACGTTATTCTTTCATCAAATCGT **3′**	Bidirectional
FLPe - *FRT*	**5′** GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC **3′**	Bidirectional
PhiC31 Integrase	*attB* - **5′** GTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCG **3′** *attP* - **5′** CCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGGG **3′**	Unidirectional
PhiC31 Excisionase	*attL* - **5′** CGGTGCGGGTGCCAGGGCGTGCCCTTGAGTTCTCTCAGTTGGGGGCGTAG **3′** *attR* - **5′** GTAGTGCCCCAACTGGGGTAACCTTTGGGCTCCCCGGGCGCGTACTCCAC **3′**	Unidirectional

Table 2.1 - Recombinase Binding Site Sequence and Directionality: Each recombinase listed is unique in terms of the respective binding sequence, which is between 34 – 58 bp. These sequences can be incorporated into T-DNA molecules to perform site-specific modifications of DNA.

transgene targeting to predetermined locations, or resolution of multiple transgene concatemers. While most studies use bidirectional recombinases, the use of mutant binding sites or transient expression forces unidirectional activity and “traps” desired modifications, such as targeted integrations (Albert et al. 1995); however, integration stability using double mutant sites with FLP/*FRT* has not been successful, and R/*RS* has no known mutant sites (Wang et al. 2011). Alternatively, the use of a strategy referred to as recombinase mediated cassette exchange (RMCE) has been used to successfully to direct transgenes to predetermined locations using FLP/*FRT* and R/*RS* systems (Anand et al. 2019, Ebinuma et al. 2015).

While much work has been performed using recombinases in plant systems, researchers using these enzymes are required to create stable expression lines to carry out modifications; otherwise, they must rely on strategies of transient expression, a process that uses the conventional transformation procedure to deliver recombinase expression cassettes via *Agrobacterium* or biolistic bombardment. In this work, we have established and demonstrated functionality of five different recombinase systems in maize: Cre, R, FLPe, phiC31 Integrase, and phiC31 Excisionase, which can be used as tools to carry out genome modifications.

Results

Establishing Recombinase Expression Lines:

Each recombinase expression line was established through *Agrobacterium* transformation of Hi-II immature embryos (Lee and Zhang, 2014) and selected in tissue culture using a bialophos resistance selectable marker (Figure 2.2). DNA from leaf tissue of regenerated T_0 events was analyzed for the presence of the T-DNA using PCR primer sets that are specific for each recombinase (Table 2.2). Positive events were crossed to Hi-II plants to produce heterozygous T_1 recombinase lines, which were selfed to produce T_2 homozygous lines. T_2 plant roots were screened for homozygotes using a single gene detection fluorescent *in-situ* hybridization method (Lamb et al. 2007). The identified lines were selfed to maintain and perpetuate homozygous recombinase stocks. Plants from homozygous recombinase stocks were crossed to Hi-II tester lines to produce 100% heterozygous immature embryos, which were used in transient reporter assays to determine recombinase functionality in maize.

Experimental Design:

The strategy to test recombinase functionality in maize cells was adapted from a similar transient assay from Dale and Ow 1990, in which a construct containing a reporter gene is flanked by oppositely oriented recombinase-binding sites and inverted with respect to a constitutive promoter. Here, a *DsRED* gene (*Dicosoma* red fluorescent protein, Bevis and Glick, 2002) is flanked by

.

Primer (size bp)	Sequence	T_a
Cre (447 bp)	**F - 5′** GAACGTGCAAAACAGGCTCT **3′** **R - 5′** ATCCTTAGCGCCGTAAATCA **3′**	60°C
R (769 bp)	**F - 5′** CCAGCGCTCTATTTCCAAGA **3′** **R - 5′** GGCCTCCTTATCCATCTCGT **3′**	60°C
FLPe (560 bp)	**F - 5′** GTGAGGGTGAAAGCATCTGG **3′** **R - 5′** AGCACGCTTATCGCTCCAAT **3′**	60°C
PhiC31 Int (500 bp)	**F - 5′** CGTGACGATTGTTTCCACTC **3′** **R - 5′** AAGGATTCGCATAACGGTTG **3′**	60°C
PhiC31 Exc (502 bp)	**F - 5′** TTACGGCTTCGAGCTTGTTT **3′** **R - 5′** ACGCCTGAAGCTCATACCAC **3′**	60°C

Table 2.2 - Primer Sequences for Screening Recombinase Expression Lines: Each primer set listed is 20 bp in length and designed to anneal to target sequences at 60°C when performing a PCR reaction. The band size in base pairs (bp) of each recombinase is included after the name in parentheses.

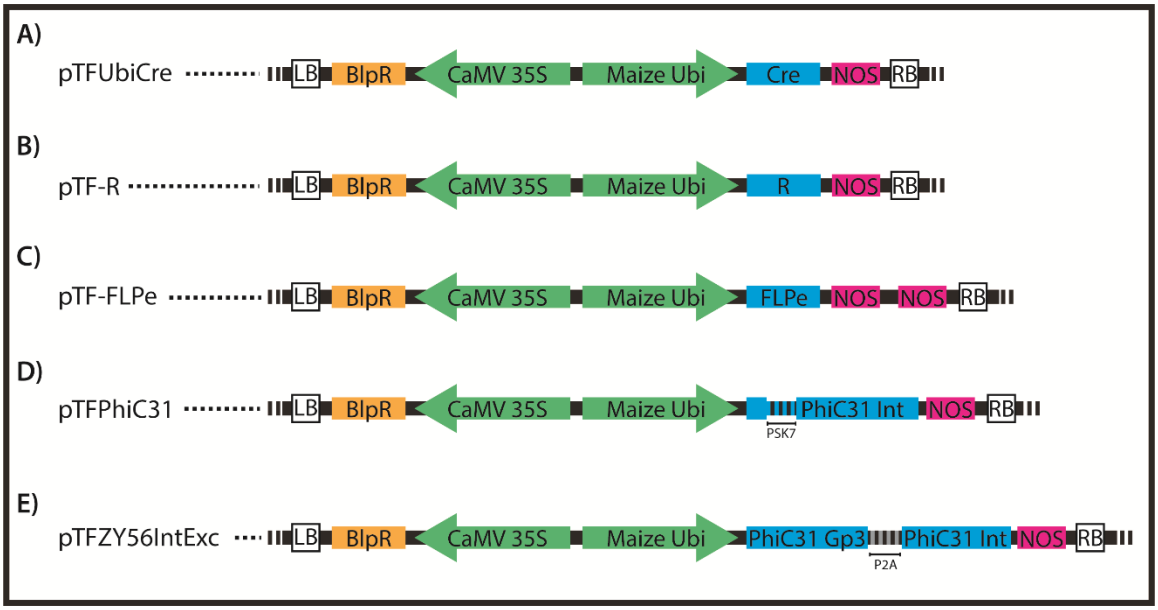


Figure 2.2 - Structure of Recombinase T-DNA Vectors: T-DNA vectors illustrated here are not drawn to scale. All transformation vectors used in this study contain a bialaphos selectable marker (BlpR) under the control of a constitutive cauliflower mosaic virus (CaMV) 35S promoter to select for positive events in plant tissue culture conditions. **(A)** Cre expression is driven by a maize ubiquitin 1 (Ubi1) promoter and terminated by a nopaline synthase termination coding sequence (Nost). **(B)** R expression is controlled by maize Ubi1 and terminated by Nost. **(C)** FLPe is expressed by a maize Ubi1 promoter and terminated by two successive Nost coding sequences. **(D)** pTFPhiC31 expression is controlled by maize Ubi1 and terminated by Nost. PhiC31 Integrase coding sequence contains an intron derived from the *Petunia hybridia Psk7* gene, which helps increase recombinase expression. **(E)** pTFZY56IntExC contains the coding sequences for the excisionase modifying protein (Gp3) and integrase, which are fused by a P2A cleaving peptide. P2A enables the coexpression of Gp3 and integrase using a single maize Ubi1 promoter.

oppositely oriented sites and inverted with respect to a maize ubiquitin promoter (Ubi) (Figure 2.3B). Each *DsRED* reporter construct was delivered into a respective recombinase expression line via biolistic bombardment of immature embryos (Figure 2.3A). If the recombinase is functional, the introduction of a *DsRED* reporter construct will result in an intramolecular inversion reaction between flanking binding sites, which will properly orient the *DsRED* gene with maize Ubi and activate expression (Figure 2.3C).

Immature Embryo Bombardment:

Recombinase embryos bombarded with *DsRED* reporter constructs were analyzed for signal 24 hours after bombardment under green light excitation (Figure 2.4-2.8). Each stable expression line exhibits red fluorescence “dot” signals on the embryos, a result of the recombinase enzymes binding to respective recombinase binding sites upon delivery of reporter constructs and reorienting *DsRED* to a position that enables expression from the maize Ubi promoter. When comparing these results to Hi-II control groups bombarded with the same reporter constructs, it is clear that all stable recombinase expression lines are functional in maize.

Discussion

Site-specific recombinases are specialized enzymes that naturally function to promote rearrangements of conserved DNA binding sites that are generally

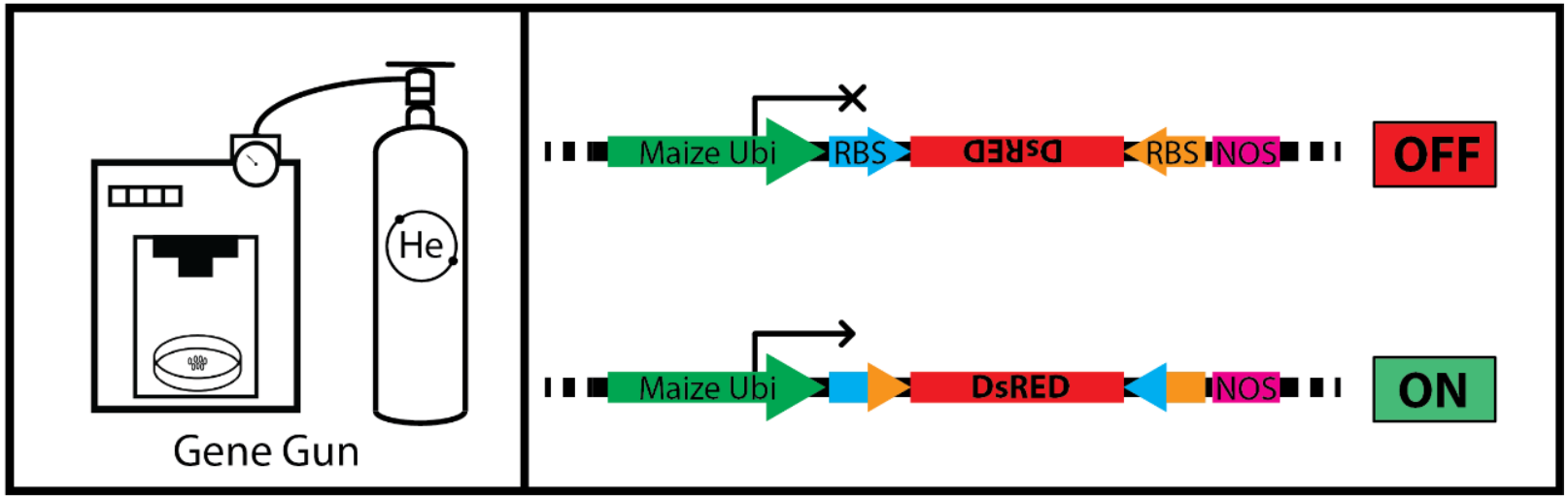


Figure 2.3 - Testing Recombinase Activity in Maize: (A) 1000/He Particle Delivery System (BioRad) was used to deliver a DsRed expression construct into immature embryos (9-12 days after pollination) of different recombinase expression lines and Hi-II controls. **(B)** Anti-DsRed expression construct was used to test recombinase activity. Constructs contain a maize ubiquitin-1 promoter (Ubi) behind an inverted DsRED gene, which is flanked by recombinase recognition sequences unique to each recombinase tested. **(C)** After bombardment of an inverted DsRed construct, recombinase enzymes bind to respective sites and catalyze an inversion reaction, activating DsRed expression.

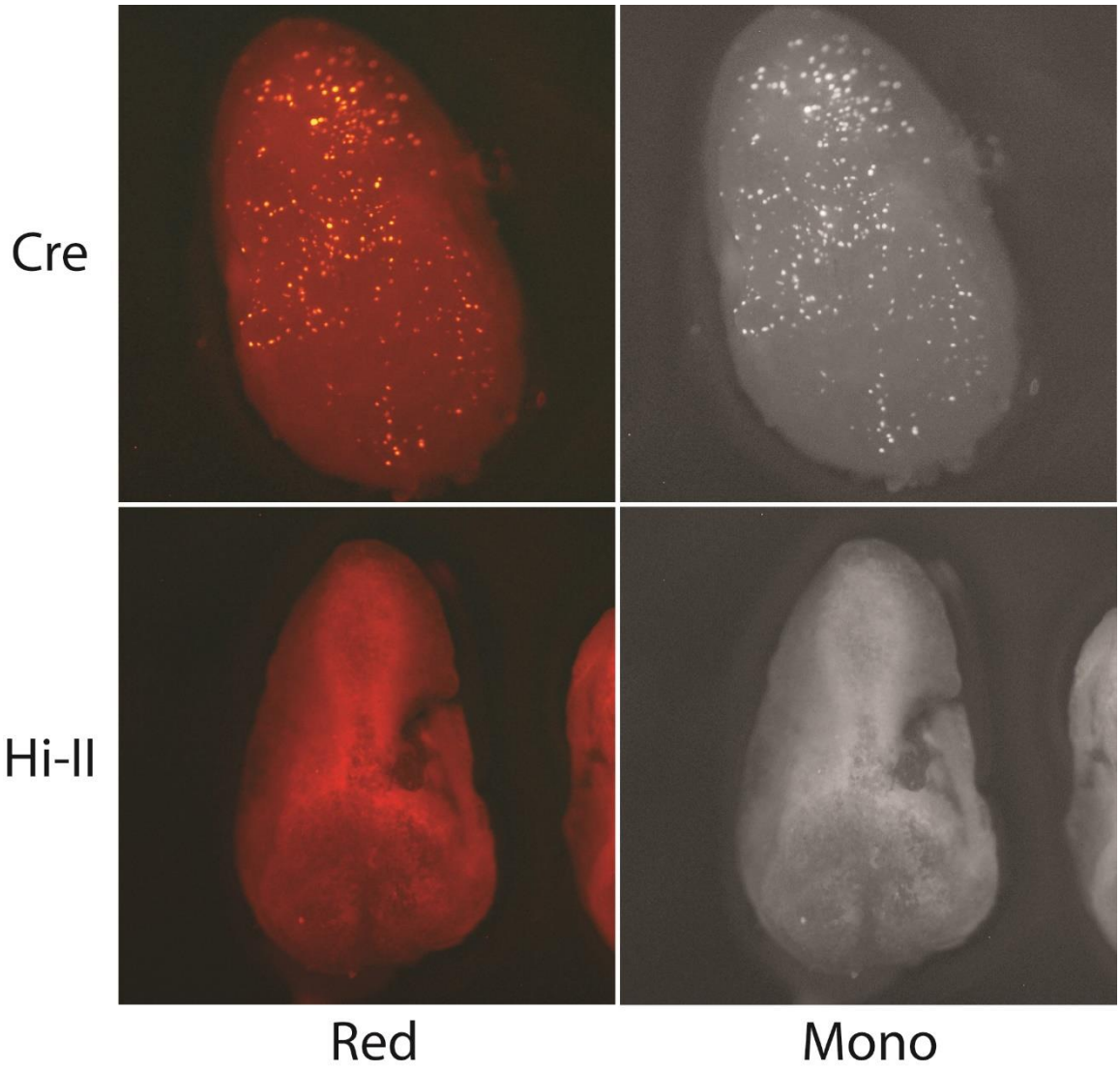


Figure 2.4 - Fluorescent Imaging of Cre and Hi-II Embryo Bombardment: Top row shows red and monochrome images of Cre – expressing line immature embryos after bombardment with a *DsRed* reporter construct (Figure 2B). Bottom row shows red and monochrome images of Hi-II control immature embryos bombarded with Cre *DsRed* reporter construct.

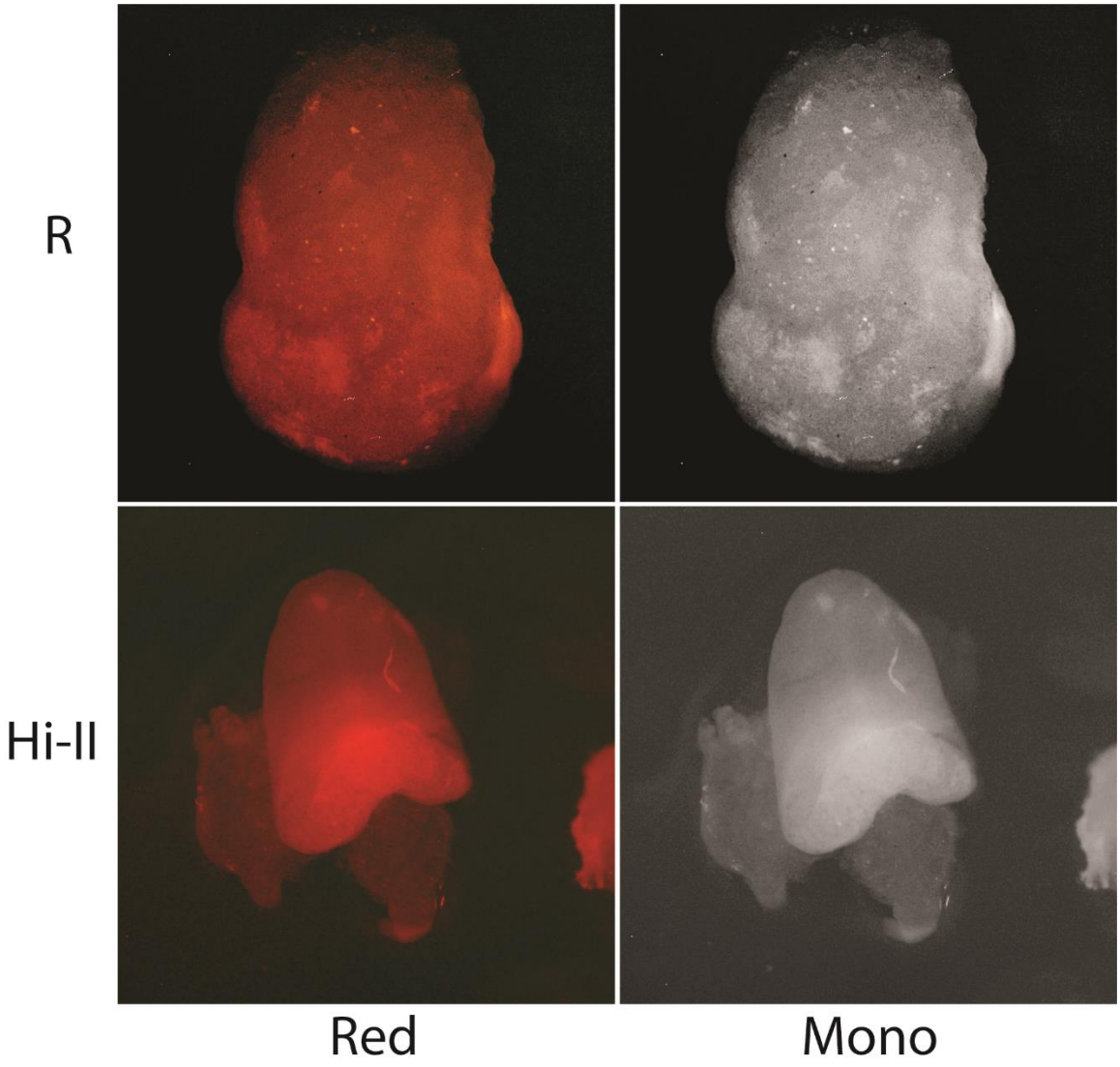


Figure 2.5 - Fluorescent Imaging of R and Hi-II Embryo Bombardment: Top row shows red and monochrome images of R – expressing line immature embryos after bombardment with a *DsRed* reporter construct (Figure 2B). Bottom row shows red and monochrome images of Hi-II control immature embryos bombarded with R *DsRed* reporter construct.

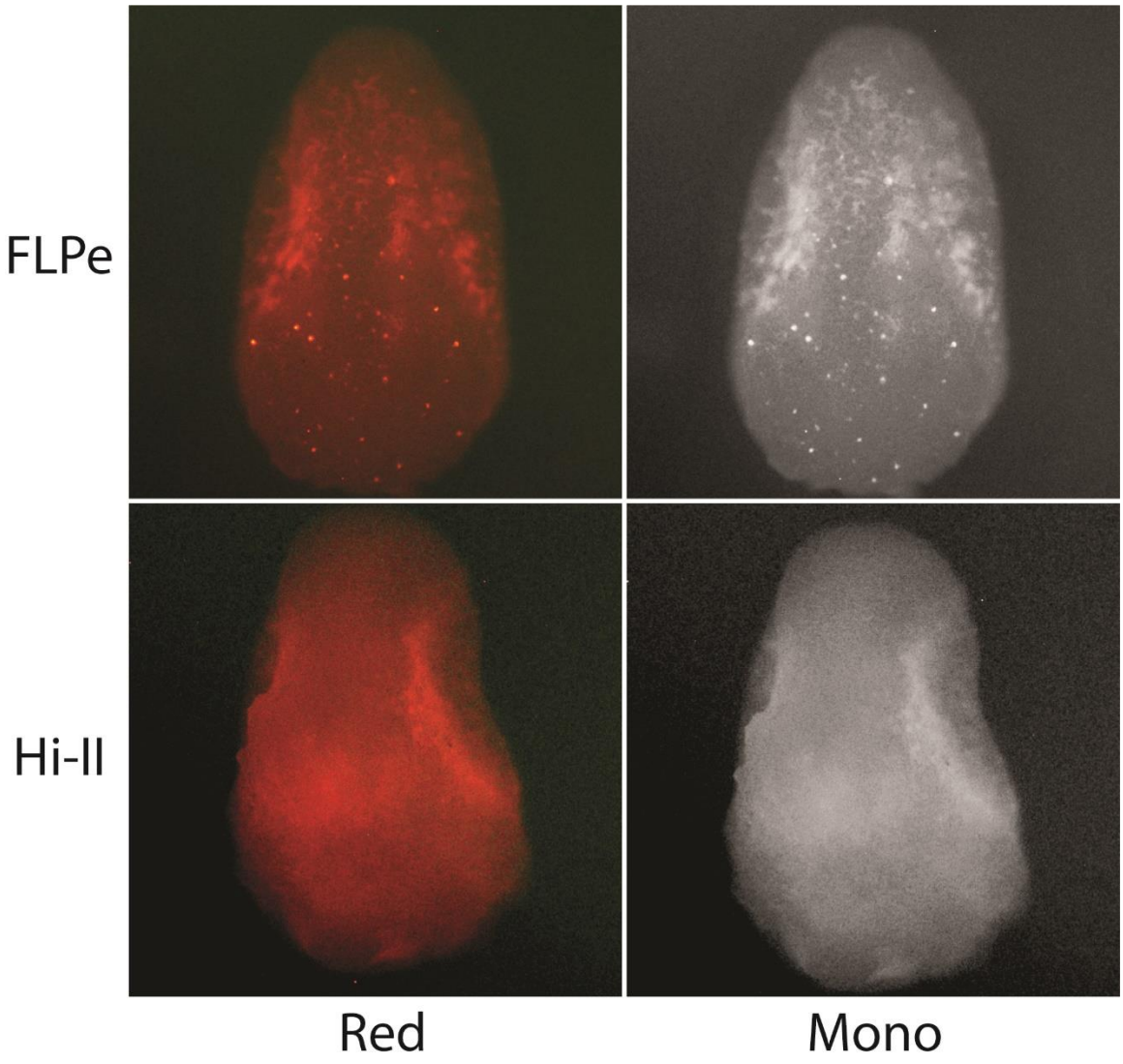


Figure 2.6 - Fluorescent Imaging of FLPe and Hi-II Embryo Bombardment: Top row shows red and monochrome images of FLPe – expressing line immature embryos after bombardment with a *DsRed* reporter construct (Figure 2B). Bottom row shows red and monochrome images of Hi-II control immature embryos bombarded with FLPe *DsRed* reporter construct.

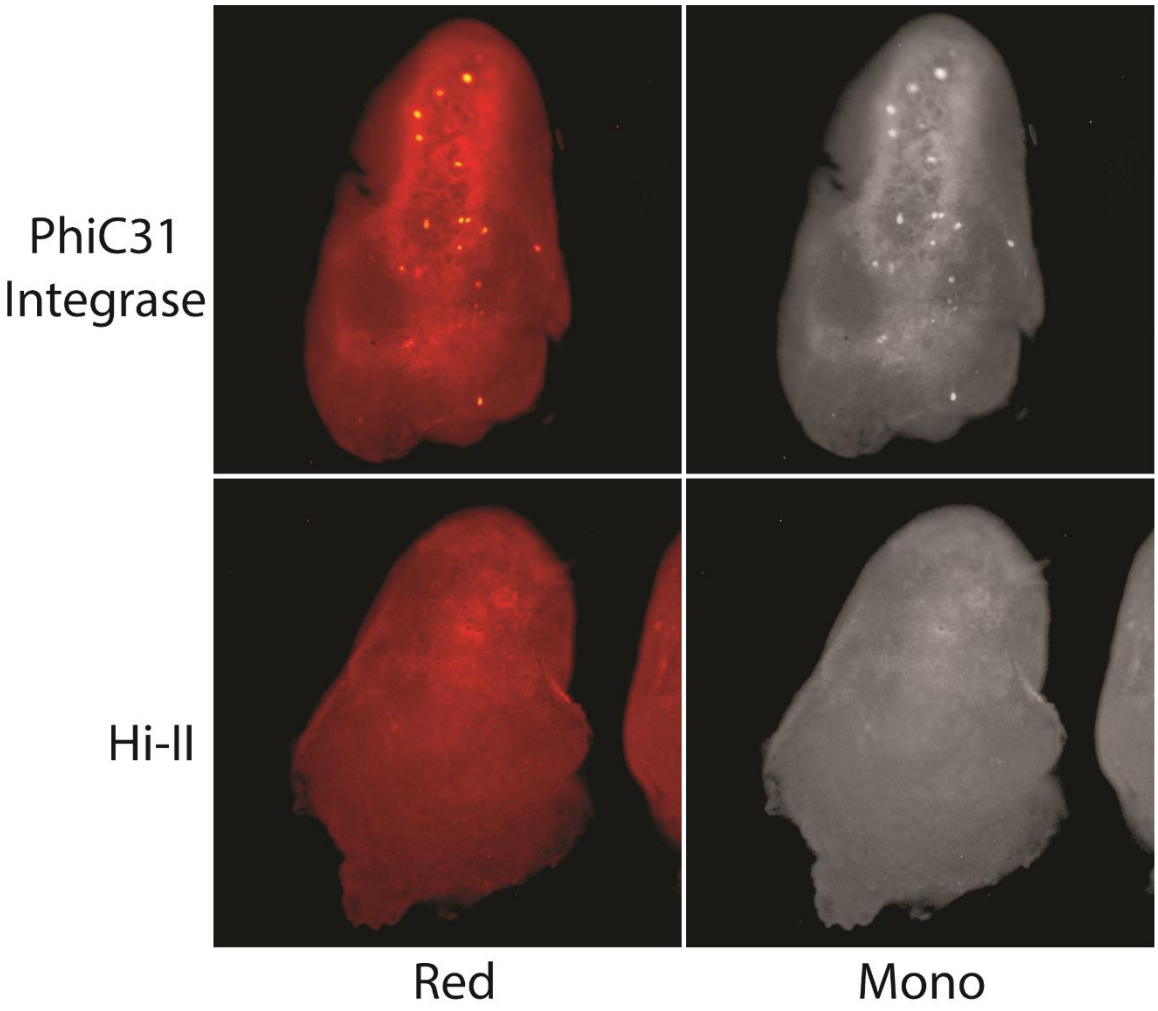


Figure 2.7 - Fluorescent Imaging of phiC31 Integrase and Hi-II Embryo Bombardment: Top row shows red and monochrome images of phiC31 Integrase – expressing line immature embryos after bombardment with a *DsRed* reporter construct (Figure 2B). Bottom row shows red and monochrome images of Hi-II control immature embryos bombarded with PhiC31 Integrase *DsRed* reporter construct.

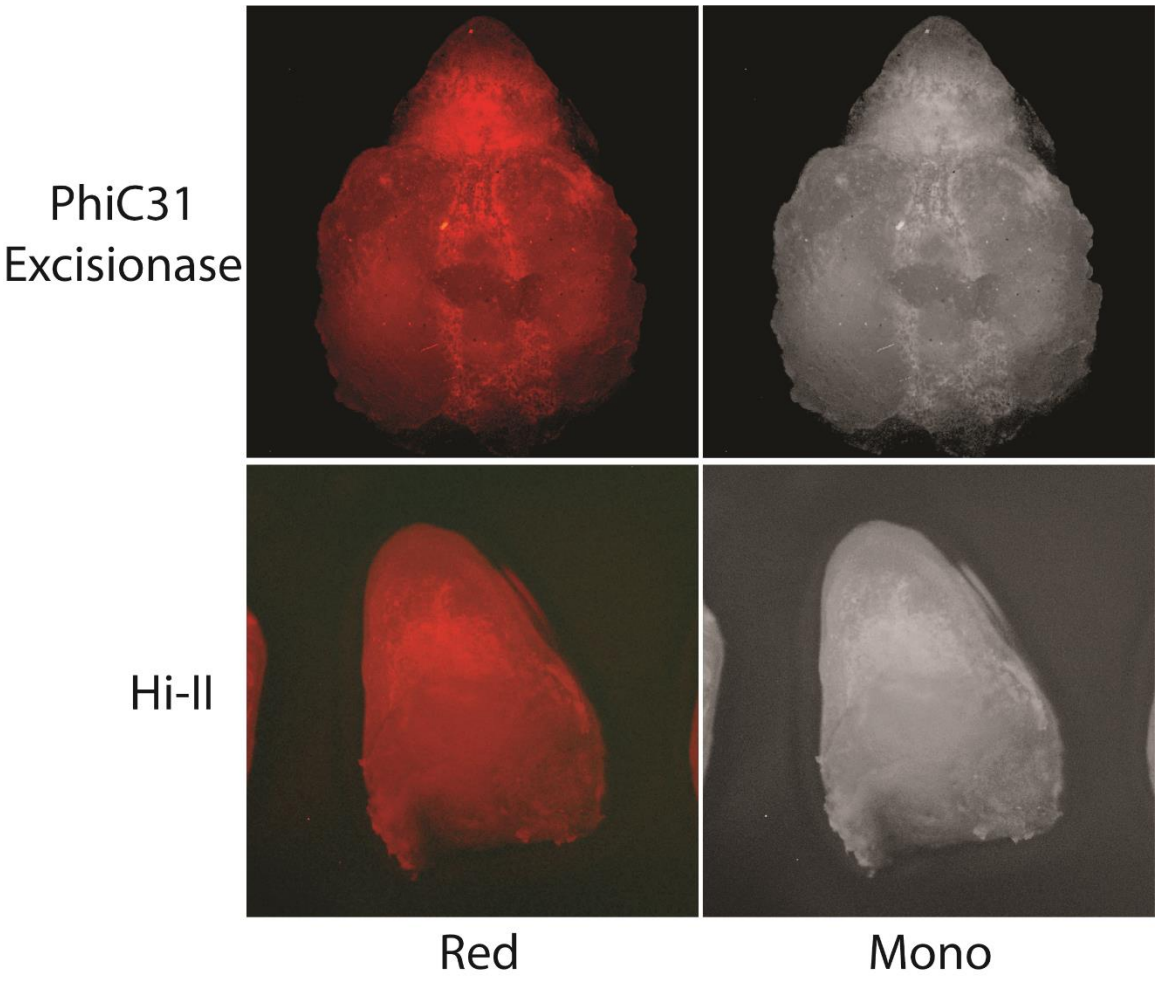


Figure 2.8 - Fluorescent Imaging of phiC31 Excisionase and Hi-II Embryo Bombardment: Top row shows red and monochrome images of phiC31 Excisionase – expressing line immature embryos after bombardment with a *DsRed* reporter construct (Figure 2B). Bottom row shows red and monochrome images of Hi-II control immature embryos bombarded with PhiC31 Excisionase *DsRed* reporter construct.

under 100 bp in length (Table 2.1). Due to the size of each sequence, binding sites can easily be incorporated into transformation vectors, enabling precise modifications through the introduction of a respective recombinase system. Strategies for enzyme delivery vary between studies and are dependent on the goals of each research project. While creative methods for expression have previously been demonstrated, such as use of inducible promoters and/or autoexcision methods (Zuo et al. 2000; Hoff et al. 2001; Zhang et al. 2003; Li et al. 2007; Éva et al. 2017), most common approaches are transient or stable expression.

Transient expression relies on the use of *Agrobacterium* or particle bombardment to deliver recombinase coding sequences into host cells containing target sites, leading to a burst of expression that is diminished over time. This enables researchers to make timely modifications of targeted sequences without having to create and maintain stable recombinase expression lines. However, transient expression strategies still utilize plant tissue culture and require a means to select for positive events in the regeneration process. Additionally, it is commonly accepted that recombinase coding sequences are degraded and eventually lost; however, Srivastava and Ow (2001) identified 40% of excision modifications contained a stably integrated Cre recombinase coding sequence after bombardment (Wang et al. 2011). Similarly, Anand et al. (2019) reported presence of FLP and/or Cre in T_0 plants when using *Agrobacterium* as the mode of delivery to induce a RMCE reaction. Taken together, transient

expression of recombinases using *Agrobacterium* or bombardment adds the benefit of timely modifications; however, it requires tissue culture and subsequent screening to obtain a desired plant background.

In the present work, five different recombinase coding sequences were stably integrated into the maize genome through *Agrobacterium*-mediated transformation. Immature embryos from these lines were subjected to particle bombardment using test constructs containing inverted and inactive *DsRed* sequences (Figure 2-B). Upon incorporation into host cells expressing the respective recombinase, *DsRed* coding sequences are reoriented through an inversion reaction and expressed by the maize ubiquitin promoter (Figure 2-C). Cells containing both recombinase and target site(s) should fluoresce red under green light excitation, which we observed for each of the five recombinases analyzed (Figure 4-8). While signal is detectable for each recombinase, it is important to note that the level of fluorescence is different for each enzyme. The easiest explanation for this observation would be enzyme efficiency; however, there are several factors that could account for this difference. These include, but are not limited to, bombardment conditions, binding site copy number, recombinase directionality, and reduced enzyme expression due to insert location in the maize genome. The presented lines could be used in a number of genetic engineering applications that involve the need to make precise and predictable site-specific modifications, including marker gene removal and targeted integrations. The only requirement with these systems is the

incorporation of respective recombinase binding sites into target transformation vectors, where the number of different sites and positioning is dependent on the specific application.

Selectable marker excision from stable transgene integrations focuses on removal of antibiotic or herbicide resistances genes used in the initial establishment of transgenic plants but which are no longer needed. Here, the binding sites are positioned in a “head-to-tail” orientation, flanking the marker coding sequence (Figure 1-A). In general, recombinase expression lines are created in parallel and used together in a breeding strategy with target lines. Zhang et al. (2003) first demonstrated this method in maize using the Cre/*lox* system and has since then been used with other recombinases, such as wild type or modified versions of FLP from *Saccharomyces cerevisiae* (Kerbach et al. 2005; Li et al. 2009). The reported efficiencies of these FLP variants in monocots is relatively low; however, Akbudak and Srivastava (2011) demonstrated a thermostable version of FLP, enhanced FLP (FLPe), is incredibly effective in rice, but has yet to be evaluated in maize. While other recombinase systems, such as R and phiC31, are presumed to be functional for excision purposes in maize, there have been no successful reports to date.

Transgene targeting to predetermined locations in host genomes has received much attention from the plant research community due to the benefits of increased control and predictability in the transformation process. While most strategies focus on using targeted nucleases to induce double stranded breaks

(DSB) in a cell with codelivered DNA to foster homologous recombination (HR) or non-homologous end joining (NHEJ), recombinase-mediated gene targeting utilizes cells previously modified to contain a recombinase coding sequence and a binding site positioned in an optimal location, exhibiting known levels of expression and distinct from endogenous genes. Upon delivery of a transgene containing a respective binding site(s), recombinase enzymes bind to both transgene and target to activate a intermolecular strand switching reaction (Figure 1-B).

The drawback to gene targeting via recombinase enzymes is the need to independently establish recombinase expression and targets lines, which are later crossed together to create cells used in transgene targeting experiments. This is a labor intensive and time-consuming process that requires the use of conventional plant transformation methods and subsequent screening of T_0 plants. To avoid the additional steps in creating a stable recombinase lines, transient expression may be utilized to carry out desired modifications. However, it is possible that limiting the enzyme expression may decrease the overall targeting efficiency. Indeed, time restrictions may be a reason for increased concentration on methods of HR or NHEJ for gene delivery, since recent advances in targeted nucleases allow quick and precise edits in any known genomic sequence. However, both HR and NHEJ have proven to be inefficient, requiring thousands of embryos for a few events. Additionally, HR seems to restrict the amount of DNA that can be incorporated into target sites. The

establishment of stable recombinases expression lines may serve as a solution to researchers looking to use recombinase enzymes in gene targeting. Here, several different approaches could facilitate efficient integrations into predetermined locations, such as the use of promoter traps in cell cultures or utilization of multiple different recombinases to initiate rearrangements of stable DNA into different locations via breeding. Regardless of the strategy, having a collection of publicly available recombinase expression lines will allow the ability to explore this alternative possibility to transgene targeting, which may become powerful tools in the future of genome engineering.

The future of genetic engineering in plants will most likely use combined editing strategies allowing researchers to efficiently stack a collection of genes in predetermined genomic locations. With current limitations in publically available selectable marker genes, this at present will require a cyclical strategy of transgene targeting followed by selection removal to prime lines for secondary modifications. This selection recycling strategy was originally presented by David Ow (2005) and utilizes the collective activity of many different recombinases and binding sites. Since then, other approaches have been proposed, which also incorporate the use of targeted nucleases to remove previous selection markers (Srivastava 2019). The recombinase toolkit that we have created may function as foundational material to enable maize researchers to stack a collection of genes in predetermined locations, which will be inherited as a single unit in each

successive generation. This toolkit will expand limits of genome modifications in maize.

Materials and Methods

Plant Transformation Constructs:

Cre recombinase expression vector, pTFUbiCre, was previously described by Gaeta et al. 2013. pTFUbiCre contains a maize Ubiquitin I promoter (Ubi) driving the expression of Cre, followed by a nopaline synthase termination sequence (NOSt). Tissue culture selection is provided by a bialaphos resistance marker, expressed by the CaMV 35S promoter from the Cauliflower mosaic virus. A *SmR* gene positioned on the vector backbone confers resistance to spectinomycin and streptomycin in *E. coli* or *Agrobacterium* cell cultures (Figure 3-A).

Coding sequences for R recombinase were provided by Jan Schaart of Wageningen University & Research in Wageningen, Netherlands (Schaart et al. 2004). The R sequence was synthesized by GenScript and modified to contain a SV40 nuclear localization signal (NLS) on the 3' end. The R gene was cloned into pTFUbiCre using restricton endonucleases *Xma*I and *Sac*I, which replaced Cre with R to create pTF-R (Figure 3-B).

Enhanced version of FLP, FLPe, was provided by Vibha Srivastava of the University of Arkansas (Akbudak and Srivastava, 2011). Ubi-FLPe-NOSt-NOSt coding sequence from pAA8 was PCR amplified using forward and reverse

primers 5’ – TGTGGAATTGTGAGCGGATA – 3’ and 5’ – TGCCACCTGACGTCTAAGAA – 3’, respectively. PCR products were digested and cloned into transformation vector pZY101 using *Hin*dIII and *Eco*RI restriction endonucleases to produce pTF-FLPe (Figure 3-C).

PhiC31 Integrase construct, pICH13130, was provided by Mario Gils of Leibniz-Institut fur Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany (Rubtsova et al. 2008). pICH13130 contains a Ubi promoter driving expression of a modified phiC31 Integrase gene that includes a 300 bp intron insert from *Petunia hybrida* and a SV40 NLS on the 3’ end. Ubi-PhiC31 Integrase-NOSt was cloned into the binary transformation vector pTF101.1 to produce pTFPhiC31 (Figure 3-D).

PhiC31 Excisionase construct, pCS-kRI, was provided by Michele Calos of Stanford University School of Medicine (Furruggio et al. 2012). Since phiC31 Excisionase is a modifying protein that binds to phiC31 Integrase, enabling the recombination of attL and attR binding sites, both Integrase and Excisionase must be present on the same vector. pCS-kRI contains phiC31 excisionase fused to phiC31 Integrase via a P2A peptide. The phiC31 Excisonase/Integrase coding sequence was cloned into plasmid pWY56c, which contains nested multiple cloning site (MCS) between a Ubi promoter and a NOSt sequences. The Ubi-phiC31 Excisionase/Integrase-NOSt fragment was subsequently cloned into the transformation vector pZY101 to form pZY56IntExc (Figure 3-E).

All constructs listed above were verified using Sanger sequencing.

DsRed Test Constructs:

The plasmid pWY56c, which contains a nested MCS between Ubi and NOSt sequences, was used in the production of all *DsRED* test constructs. As shown in Figure 2-B, each test construct contains a *DsRED* gene, flanked by oppositely oriented recombinase binding sites and inverted with respect to a maize Ubi promoter. These sequences (Table 2.1), RBS-*DsRed*-RBS, were each synthesized and cloned into pWY56c by GeneWiz to produce pCre-AntiDsReD, pR-AntiDsRed, pFLPe-AntiDsRed, pIntegrase-AntiDsRed, and pExcisionase-AntiDsRed. It is important to note that pCre-AntiDsReD contains mutant RBS sites of *lox66* and *lox*71 (Albert et al. 1995).

Plant Transformation:

Each recombinase expression vector was transformed into EHA105 *Agrobacterium* cells (provided by Stanton B. Gelvin, Purdue University) by electroporation and selected LB agar plates using rifampicin (30 ug/ml), spectinomycin (100 ug/ml), and streptinomycin (100 ug/ml) antibiotics. Single colonies were suspended in 5 ml of LB medium, containing the appropriate antibiotics, and grown for 24 - 48 hrs at 28°C, shaking at 250 rpm. Glycerol stocks of each culture were stored at -80°C and used to create *Agrobacterium* growth plates used in the transformation procedure.

Immature embryo explants were obtained from crosses of Hi-II A x Hi-II B lines (Armstrong and Green 1985). Embryos were collected 9 – 12 days after pollination at a length of ~ 1.2 – 1.5 mm. Immature embryos were sterilized and inoculated with *Agrobacterium* EHA105 infection media (OD_{600} = 0.3 – 0.5) for 3-4 hr. Embryos were transferred to cocultivation plates and placed at 25°C for 3-4 days. Infected embryos were transferred to callus induction plates and placed at 28°C for 11-12 days. Type II callus from callus induction plates were transferred to selection plates containing 3 ug/ml bialaphos and switched to new nutritional media every 2 weeks until resistant cell masses were formed, which can take up to 12 weeks. Root and shoot regeneration were carried out sequentially, taking ~3 weeks each. For a full description of the plant transformation process, including a complete list of media reagents see Lee and Zhang (2014).

PCR analysis of T_0 Transgenics

Genomic DNA from leaf tissue was extracted from T_0 transgenic plants using the method described (Leach et al. 2016). PCR on extracted genomic DNA was performed using standard conditions of 35 cycles: 30s denature at 95°C, 30s annealing at 60°C, and Xs extension at 72°C. Primer sets used for the screening process are listed in Table 2.2 and are all designed to amplify a fragment of approximately 500 bp.

Embryo Bombardment:

Particle bombardment was performed on immature embryo explants of Hi-II x homozygous recombinase expression and Hi-II control lines, harvested 10 – 12 days after pollination. Embryos length was approximately 1.5 – 2 mm in length. After extraction from ears, embryo explants were placed on N6 media for 3 – 5 days prior to bombardment (Songstad et al. 1996). 4 hours prior to bombardment, embryos were transferred to osmotic media. 0.6 µm gold particles (BioRad) were prepared following the steps outlined by Iowa State Plant Transformation Facility (Sanford et al. 1993; Frame et al. 2000). Approximately 1 µg of each of each recombinase test construct was used in the 120 µl gold mixture. 10 µl of the gold particle suspension was pipetted onto macrocarriers (BioRad) and allowed to dry in a hood. After drying, another 10 µl of the gold particle suspension was added again, equating to ~166 ng/shot. The bombardment procedure was performed with a 1000/He Particle Delivery System (BioRad) using 650 psi rupture disks. After bombardment, embryos rested on osmotic media for 1 hr before being transferred to N6 media. The embryos were analyzed under a Leica M205 FA Stereo Microscope 1 – 3 days after bombardment.

References:

Akbudak M.A. and Srivastava V. (2011) Improved FLP recombinase, FLPe, efficiently removes marker gene from transgene locus developed by Cre-lox mediated site-specific gene integration in rice. Mol Biotechnol 49(1), 82-89.

Albert H., Dale E.C., Lee E., Ow D.W. (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. Plant J. 7, 649–659.

Altpeter F., Springer N.M., Bartley L.E., Blechl A.E., Brutnell T.P., Citovsky, V., Conrad, L.J., Gelvin S.B., Jackson D.P., Kausch A.P., Lemaux, P.G., Medford, J.I., Orozco-Cárdenas, M.L., Tricoli D.M., Van Eck, J., Voytas, D.F., Walbot, V., Wang, K., Zhang Z.J. (2016) Advancing Crop Transformation in the Era of Genome Editing. Plant Cell 28 (7), 1510-1520.

Anand A. and Jones T.J. (2018) Advancing *Agrobacterium*-based crop transformation and genome modification technology for agricultural biotechnology. Curr Top Microbiol Immunol 481.

Anand A., Wu E., Li Z., TeRonde S., Arling M., Lenderts B., Mutti J.S., Gordon-Kamm W., Jones T.J., Chilcoat N.D. (2019) High efficiency *Agrobacterium*-mediated site-specific gene integration in maize utilizing the FLP-FRT recombination system. Plant Biotechnol J. doi: 10.1111/pbi.13089.

Armstrong C.L. and Green C.E., (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164, 207–214.

Bevan M.W., Flavell R.B., Chilton M.D. (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

Bibikova M., Beumer K., Trautman J.K., Carroll D. (2003) Enhancing gene targeting with designed zinc finger nucleases. Science 300. 764.

Bevis B.J. and Glick B.S. (2001) Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). Nature Biotechnol 20(1), 83-87.

Carroll D. (2011) Genome engineering with zincfinger nucleases. Genetics 188. 773–782.

Christian M.L., Cermak T., Doyle E.L., Schmidt C., Zhang F., Hummel A., Bogdanove A.J., Voytas D.F. (2010) Targeting DNA Double Stranded Breaks with TAL Effector Nucleases. Genetics 186. 757-761.

Dale E.C. and Ow D.W. (1990). Intra- and Intramolecular Site-Specific Recombination in Plant Cells Mediated by Bacteriophage P1 Recombinase. Gene 91(1), 79-85.

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

Ebinuma H., Nakahama K., Nanto K. (2015) Enrichments of gene replacement events by *Agrobacterium*-mediated recombinase-mediated cassette exchange. Mol Breeding 35(82).

Éva C., Téglás F., Zelenyánszki H., Tamás C., Juhász A., Mészáros K., Tamás L. (2017) Cold inducible promoter driven Cre-lox system proved to be highly efficient for marker gene excision in transgenic barley. J Biotechnol 265, 15-24.

Farruggio A. P., Chavez C. L., Mikell C. L. and Calos M. P. (2012). Efficient reversal of phiC31 integrase recombination in mammalian cells. Biotechnol. J 7, 1332-1336.

Fraley R.T., Rogers S.G., Horsch R.B., Sanders P.R., Flick J.S., Adams S.P., Bittner M.L., Brand L. a, Fink C.L., Fry J.S., Galluppi G.R., Goldberg S.B., Hoffmann N.L., Woo, S.C. (1983) Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. U. S. A. 80, 4803–4807.

Frame B.R., Zhang H., Cocciolone S.M., Sidorenko L.V., Dietrich C.R., Pegg S.E. (2000) Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. 36(1), 21-29.

Gaeta R.T., Masonbrink R.E., Zhao C., Sanyal A., Krishnaswamy L., Birchler J. A. 2013. In vivo modification of a maize engineered minichromosome. Chromosoma 122, 221–232.

Golic K.G. and Lindquist S. (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell 59, 499–509.

Grindley, N.D.F., Whiteson K.L., Rice P. A. (2006) Mechanisms of site-specific recombination. Annu. Rev. Biochem. 75, 567–605.

Herrera-Estrella L., Block M.D., Messens E., Hernalsteens J.P., Montagu M. V, Schell, J. (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2, 987–995.

Hoa T.T., Bong B.B., Huq E., Hodges T.K. (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 K.M., Mundy J. (2001) A recombinase-mediated transcriptional induction system in transgenic plants. Plant Mol Biol 45, 41–49.

Hong L., Lyznik L.A., Gidoni D., Hodges T.K. (2000) FLP-mediated recombination for use in hybrid plant production. Plant J 23, 423-430.

Hu Q., Kononowicz-Hodges H., Nelson-Vasilchik K., Viola D., Zeng P., Liu H., Kausch A.P., Chandlee J.M., Hodges T.K., Luo H. (2007) FLP recombinase-mediated site-specific recombination in rice. Plant Biotechnol J 6, 176-188.

Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J.A., Charpentier E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science 337. 816-821.

Kapusi E., Kempe K., Rubtsova M., Kumlehn J., Gils M., (2012) PhiC31 Integrase-Mediated Site-Specific Recombination in Barley. PLoS ONE 7(9). e45353.doi:10.1371/journal.pone.0045353.

Kerbach S., Lörz H., Becker D. (2005) Site-specific recombination in *Zea mays*. Theor Appl Genet 111, 1608-1616.

Lee H. and Zhang Z.J. (2013) *Agrobacterium*-Mediated Transformation of Maize (*Zea mays*) Immature Embryos. Methods in Molecular Biology 1099, 273–280.

Li Z., Xing A., Moon B.P., Burgoyne S.A., Guida A.D., Lee C., Caster C.S., Barton J.E., Klein T.M., Falco S.C. (2007) A Cre/loxP-mediated self-activating gene excision system to produce marker gene free transgenic soybean plants. Plant Mol Biol 65,329–341.

Li Z., Xing A., Moon B.P., Mcardell R.P., Mills K., Falco S.C., (2009) Site-Specific Integration of Transgenes in Soybean via Recombinase-Mediated DNA Cassette Exchange. Plant Physiol 151, 1087-1095.

Lyznik L.A., Rao K.V., Hodges T.K. (1996) FLP-mediated recombination of FRT sites in the maize genome. Nucl Acids Res 24. 3784–3789.

Murai N., Kemp J., Sutton D., Murray M., Slightom J., Merlo D., Reichert N., C S.G., Stock C., Barker R., Hall T. (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222, 476–482.

Onouchi H., Yokoi K., Machida C., Matsuzaki H., Oshima Y., Matsuoka K., Nakamura K., Machida Y. (1991) Operation of an efficient site-specific recombination system of *Zygosaccharomyces rouxii* in tobacco cells. Nucleic Acids Res. 19, 6373–6378.

Ow, D.W. (2005) SIVB Congress Symposium Proceeding: Transgene Management via Multiple Site-specific Recombination Systems. Vitr Cell Dev Biol - Plant 41, 213–219.

Rubtsova M., Kempe K., Gils A., Ismagul A., Weyen J., Gils M., (2008) Expression of active Streptomyces phage phiC31 integrase in transgenic wheat plants. Plant Cell Rep 27(12), 1821-1831.

Sanford J.C., Smith F.D., Russell J.A. (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol 217, 483-509.

Sauer, B., Henderson, N. (1988) Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. Nucleic Acids Res. 17, 147–161.

Schaart J.G., Krens F.A., Pelgrom K.T.B., Mendes O., Rouwendal G.J.A. (2004) Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol J 2, 233-240.

Songstad D.D., Armstrong C.L, Petersen W.L., Hairston B., Hinchee M.A.W. (1996) Production of transgenic maize plants and progeny by bombardment of hi-II immature embryos. In Vitro Cell Dev Biol Plant 32(3), 179-183.

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

Srivastava V., and Ow D.W. (2001) Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct. Plant Mol Biol 46, 561-566.

Srivastava V. (2019) Gene Stacking in Plants Through the Application of Site-Specific Recombination and Nuclease Activity. Transgenic Plants. Methods in Molecular Biology 1864, 267-277.

Stuurman J., **de Vroomen M.J., Nijkamp H.J., van Haaren M.J.** (1996) Single-site manipulation of tomato chromosomes in vitro and in vivo using Cre-lox site-specific recombination. Plant Mol Biol 32(5), 901-913.

Thorpe, H.M. and Smith M.C.M. (1998) In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc. Natl. Acad. Sci. U.S.A. 95 (10), 5505-5510.

Thomson J.G, Chan R., Thilmony R., Yau Y., Ow D.W. (2010) PhiC31 recombination system demonstrates heritable germinal transmission of site-specific excision from the Arabidopsis genome. BMC Biotechnol 10(17).

Vergunst A.C. and Hooykaas P.J.J. (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.

Wang Y., Yau Y.Y., Perkins-Balding D., Thomson J.G. (2011) Recombinase technology: Applications and possibilities. Plant Cell Rep. 30, 267–285.

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

Zuo J., Niu Q.W., Moller S.G., Chua N.H. (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. Nat Biotechnol 19, 157–16

Chapter 3: Establishing an Amendable Plant Artificial Minichromosome and B Insert Platform

Note: The information provided in this chapter is unpublished.

Contributions

The work detailed here would not have been possible without the guidance and support of Nathaniel Graham, Nathan Swyers, Patrice Albert, and Changzeng Zhao.

Summary

Artificial minichromosome engineering utilizes telomere mediated chromosomal truncation (TMCT) to produce small, autonomous chromosomes that exist in association with the native chromosomal sets. Through the incorporation of recombinase binding sites, recombinases could be used to make precise modifications to minichromosomes, such as selection removal or additions of transgenes in a targeted manner. Here, we utilize TMCT on plant backgrounds that contain high copy numbers of supernumerary B chromosomes. In doing so, we have created two B derived chromosome platforms that enable a described transgene targeting strategy to locations outside the normal sets of chromosomes using site-specific recombinase systems.

Introduction

Artificial minichromosomes are small chromosomes that exist in association with native chromosomal sets, containing all the necessary components needed for maintenance and stability in a cellular environment. The basic requirements for chromosome autonomy were discovered in the creation of the first artificial chromosome in yeast (YACs) and include a centromere, telomeres, and origins of replication (Murray and Szostak, 1983). Since this initial establishment, artificial minichromosomes have been constructed in a number of organisms including bacterial (BACs), fungal (FACs), mammalian (HACs), and plants (PACs) (Murray and Szoztak, 1983; Farr et al., 1991; Shizuya et al., 1992; Heller et al., 1996; Aleksenko and Clutterbuck, 1997; Yu et al., 2006; Bok et al., 2015). While considerable effort has focused on the utility and advancement of YAC, BAC, FAC and HAC systems, plant based artificial chromosomes have received less attention and are behind in development, despite the advantages PACs offer for the fields of genetic engineering and agriculture.

Conventional genetic engineering methods in plants rely on the use of *Agrobacterium tumefaciens* or biolistic bombardment for transgene delivery into host genomes, both of which result in random integrations. This requires researchers to assess each T_0 event for potential mutations of endogenous gene function, expression level, and transgene copy number. Additionally, maintenance of multiple transgene insertions become increasingly difficult with each added gene, making the use of traits requiring coordinated expression of

multiple DNA fragments an impossibility. With single gene traits conferring resistances to insects or herbicides revolutionizing agriculture through increased yields and decreased chemical application to fields, it is easy to imagine that a technology enabling efficient targeting of multiple gene fragments to a location outside the normal sets of chromosomes would further push the boundaries of genetic engineering and boost agricultural efficiency (Yu et al., 2016). Plant artificial chromosomes offer this possibility by creating a non-invasive platform that is stably maintained in meiosis and mitosis. If used together with site-specific recombination (SSR) technology, transgenes could be efficiently targeted to artificial chromosomes to create chromosome-based vectors that can be stably inherited in each successive generation.

Artificial chromosome production in plants can theoretically follow two approaches: the bottom-up or top-down method (Gaeta et al., 2012). The bottom up method is an *in vitro* assemblage of all the necessary chromosomal components prior to transformation. It is assumed that DNA associated with natural formation of complexes, such as centromere kinetochore assembly, will form *de novo* upon delivery into host cells, creating an autonomous chromosomal fragment. While the bottom-up method has been demonstrated in both yeast and mammalian-based systems (Harrington et al., 1997; Murray and Szostak, 1983), this approach is not yet possible in plants, despite reports of success (Carlson et al., 2007; Ananiev et al., 2009). In plants, centromere formation is strongly

epigenetic and unrelated to sequences located at the primary constriction of chromosomes (Han et al. 2006; 2009; Birchler et al. 2009).

In contrast, the bottom-up method utilizes pre-existing chromosome machinery through the process of telomere mediated chromosomal truncation (TMCT), circumventing the epigenetic aspects associated with centromere formation to successfully establish plant artificial chromosomes (Yu et al. 2006). Integrations of telomere repeats into host genomes was first observed to induce chromosomal breakages in mammalian cells using repeats of $TTAGGG_n$ (Farr et al. 1992) and has since been adapted for plant systems such as maize (Yu et al., 2006, 2007; Gaeta et al. 2013), *Arabidopsis* (Nelson et al. 2011; Teo et al., 2011), barley (Kapusi et al. 2012), wheat (Yuan et al., 2017), brassica (Yan et al., 2017) and rice (Xu et al. 2012). PAC formation via TMCT utilizes telomere sequence repeats present in most plant species, $(TTTAGGG)_{n,}$ which naturally function as protective ends preventing chromosome degradation (Watson and Riha, 2010). Transformation of these arrays together with genes of interest through *Agrobacterium* or biolistic bombardment randomly integrate these sequences into host chromosomes, inducing truncation of preexisting chromosomes and forming PACs known as artificial minichromosomes. The only requirement for stable minichromosome creation in plants via TMCT is the presence of a target where the truncation of a chromosome arm will have little effect to plant growth and development.

Ideal targets for minichromosome production are supernumerary chromosomes known as B chromosomes, which usually produce no vital products needed for the survival of the organism. First identified in the insect *Metapodius* by E.B. Wilson in 1907, and termed 'B chromosomes' by Randolph in 1928, the unique properties of these supernumerary chromosomes have been topics of scientific research for over 100 years (Jones et al., 2008). In maize, B chromosomes contain no necessary genes and are maintained in the population through mitotic drive, or a two-part, trans-acting accumulation mechanism of non-disjunction during pollen maturation followed by preferential fertilization of the egg at a rate of 65-70% (Roman, 1947, 1949; Carlson, 1978). Factors controlling the accumulation properties of maize B chromosomes act in trans and are located on the proximal and distal tip of the long arm (Ward, 1973; Lin, 1978). TMCT of B chromosomes removes this distal tip, stabilizing the chromosome where it is inherited at relatively similar frequencies as compared to A chromosomes (Yu et al., 2007). This process was first demonstrated by the Birchler lab in 2006, where a 2.6 kb *Arabidopsis* telomere array (Richards and Ausubel, 1988) was cloned together with a selectable marker and SSR systems to produce the first B-derived artificial minichromosome (Yu et al., 2006; 2007).

The most promising use of B-derived artificial minichromosomes is in the field of genetic engineering, where incorporated SSR binding sites could be used to stack genes into a location outside the normal sets of chromosomes. Targeted transgene integrations and selectable marker deletions have been demonstrated

in several different plant systems, including maize (Lyznik et al. 1996; Srivastava and Ow 2001; Zhang et al. 2003; Kerbach et al. 2005; Anand et al. 2019); therefore, a system to stack transgenes on an artificial minichromosome should be possible (Birchler, 2015). Here, we have established an artificial minichromosome and a B chromosome insert that contain the framework to enable a cyclical strategy of selection removal followed by transgene targeting through the use of site-specific recombination systems.

Results

Molecular Strategy and Construct Design:

B-derived minichromosome platforms that function as target sites for transgene integrations via site-specific recombinase systems need to be carefully designed in a way that enables selection in both the initial minichromosome establishment and primary integration steps. In maize, there are only a few reliable selectable marker genes available, requiring stacking regimes to utilize strategies that recycle selection through sequential site-specific excision followed by site-specific integration. This method was first proposed by David Ow (2005) and uses a collection of recombinases that are expressed in a timely manner to carry out targeted modifications for selection removal and subsequent integrations. The recombinase genome editing toolkit described in Chapter 2 will be used together with established minichromosome lines to enable such function through breeding or tissue culture strategies.

The pZP-Telo-JC truncation construct (Figure 3.1) was designed to enable selection of insertional events through the use of a maize Ubiquitin I promoter (Ubi) driving expression of a coding sequence that confers resistance to the herbicide bialaphos (*bar*), followed by a nopaline synthase terminator (NOSt). *bar*-NOSt is flanked by *FRT* recombinase binding sites positioned in a head-to-tail orientation, enabling removal of selection through FLPe expression. Downstream of Ubi-*FRT*-*bar*-NOSt-*FRT* is a *attP* recombinase binding site followed by NOSt, which enables phiC31-mediated targeting of DNA molecules with matching *attB* binding sites. A multiple cloning site (MCS) was integrated into pZP-Telo-JC to allow for the addition of extra cargo in future chromosome truncation experiments. A telomere array ($TTTAGGG_n$) ~2 kb was cloned near the right border (RB) of the T-DNA construct, which has been shown to induce TMCT upon stable integrations into host chromosomes (Yu et al. 2006; 2007; Gaeta et al. 2013).

Plant Material for Transformation:

To increase the chances of obtaining a B-derived artificial minichromosome, it is necessary to have plant material containing many copies of the B chromosome target. Table 3.1 outlines 27 Hi-II A+B seed packets that were screened via root tip digestion (Figure 3.2) and chromosome spread analysis on a light microscope (McCaw et al., 2016a). 2-3 plants were screened in each packet. Lines containing ≥ 5 B chromosomes were selected as

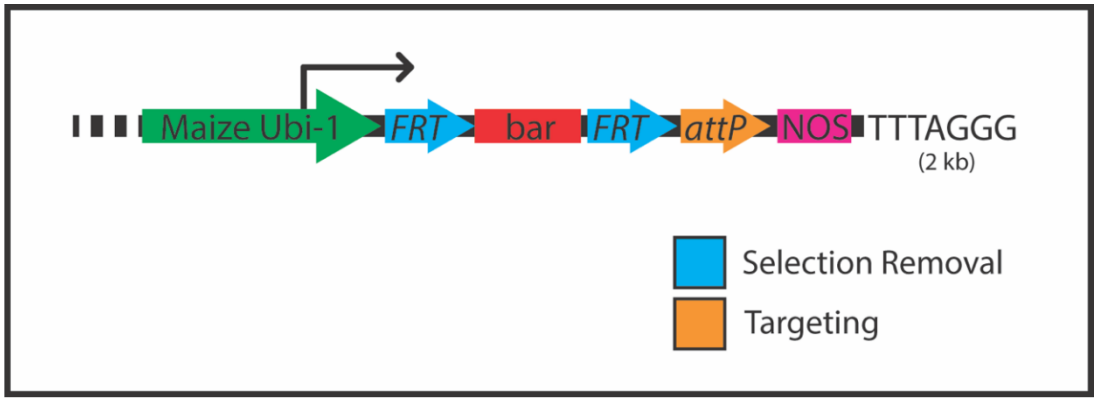


Figure 3.1 - pZP-Telo-JC Truncation Construct Map: The truncation construct used to generate artificial minichromosomes contains a maize ubiquitin-1 promoter driving the expression of a coding sequence that confers resistance to bialaphos herbicide treatments (*bar*). The *bar* gene is flanked by *FRT* recombinase binding sites, which are recognized by FLP recombinase enzymes to remove selection (blue). Downstream of *FRT*-*bar*-NOSt-*FRT* is an attachment phage (*attP*) site, which can be utilized together with phiC31 recombinase to target donor molecules containing an attachment bacteria (*attB*) binding site (orange). ~2 kb of telomere repeat sequence ($TTTAGGG_n$) was cloned near the right border of the T-DNA construct to induce telomere-mediated chromosomal truncations (TMCT) upon integration into the maize genome.

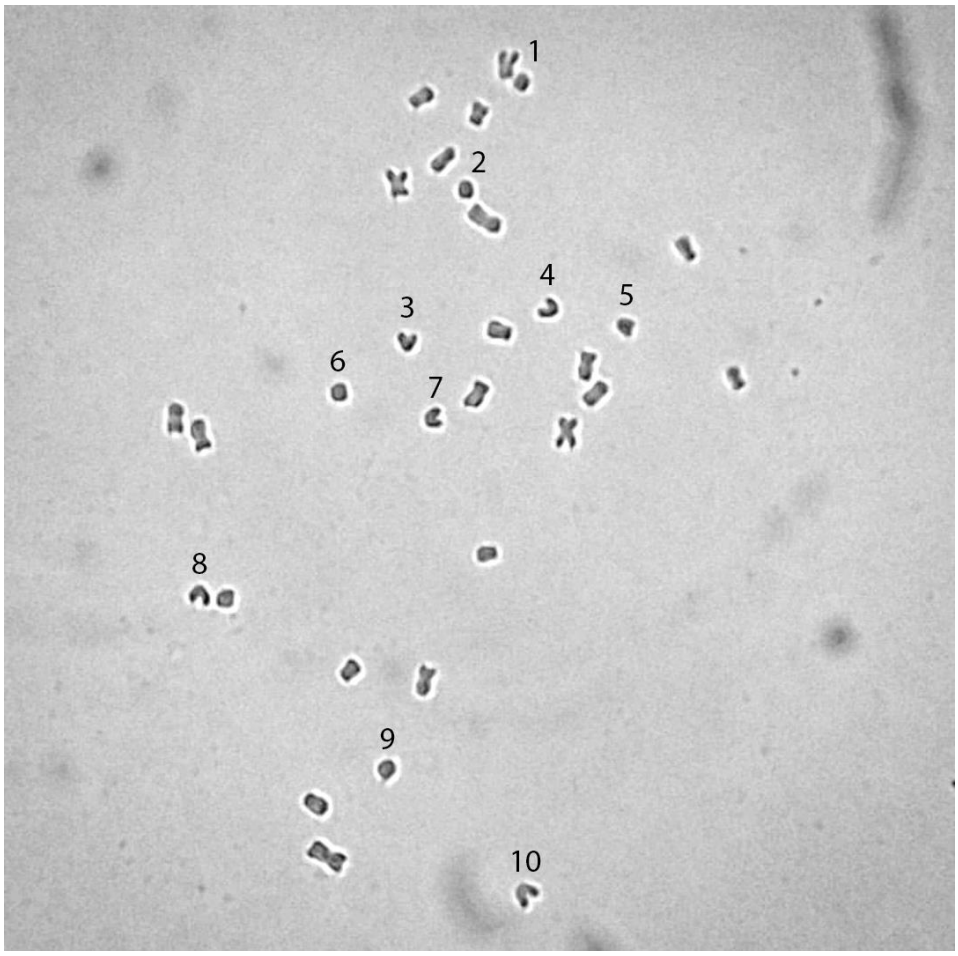


Figure 3.2 - Hi-II A+B Root Tip Chromosome Spreads to Assess B Chromosome Count: To increase the probability of obtaining a B derived minichromosome, Hi-II A+B seed packets were screened via root tip digestion followed by chromosome spread screening with an Olympus CX21 light microscope (McCaw et al., 2016a). Plants containing ≥ 5 B chromosomes were selected as plant material for *Agrobacterium*-mediated transformation using the pZP-Telo-JC truncation construct. The presented image is an example of an image obtained from a Hi-II A+B line containing 10 B chromosomes (430, see Table 3.1), which are each marked numerically. B chromosomes are telocentric and morphologically distinct from the native chromosomal set, enabling simple screening of chromosome count with a light microscope.

Packet No.	B Ch. Count
17-1-1	0
17-1-2	2
17-1-3	5
17-1-4	2
17-1-5	5
17-1-6	5
17-1-9	4
17-1-10	1
17-1-11	0
17-1-14	2
17-1-15	3
17-1-17	3
17-1-18	0
430	10

Packet No.	B Ch. Count
17-3-3	4
17-3-4	2
17-3-5	7
17-3-10	1
17-3-12	4
17-3-13	3
17-3-14	1
17-3-18	3
17-4-1	0
17-4-5	7
17-4-7	0
17-4-8	5
17-4-9	1

Table 3.1 - Hi-II A+B Seed Packets Screen for B Chromosome Copy Number: 27 seed packets were screened using root tip digestion and chromosome spread analysis (McCaw et al., 2016a). Packets containing ≥ 5 B chromosomes were used as plant material in *Agrobacterium*-mediated transformation experiments using pZP-Telo-JC.

the Hi-II A+B material used in the Hi-II B x Hi-II A+B cross to produce immature embryo explants for plant transformation.

Agrobacterium-Mediated Transformation:

Due to the random nature of *Agrobacterium*-mediated transformation, it was necessary to transform a large number of F_1 (Hi-II A+B x Hi-II B) immature embryos to obtain stable B-derived artificial minichromosomes. Additionally, the observed efficiencies of TMCT are relatively low in maize, with around 9% of events resulting in chromosome cleavage (Yu et al. 2006, 2016). With the addition of a high copy number of B chromosomes, this efficiency seems to increase (Yu et al., 2006, 2007; Gaeta et al., 2013); therefore, it was necessary to use progenitor Hi-II A+B lines with high levels of supernumerary chromosomes (Table 3.1). Table 3.2 outlines the dates and conditions (OD_{600}) of each transformation procedure using pZP-Telo-JC. A total of 5099 embryos were used in the *Agrobacterium* transformation experiment at an average OD_{600} of 0.6. Table 3.3 highlights the number of positive events recovered, the date transformed with pZP-Telo-JC, and whether T_1 seed was recovered in greenhouse conditions. A total of 40 events were recovered from 5099 immature embryos, giving a total transformation efficiency of 0.78%.

Date	Construct	Embryo No.	OD_{600}
4/26/17	pZP-Telo-JC	180	0.5
5/25/17	pZP-Telo-JC	269	0.7
5/26/17	pZP-Telo-JC	191	0.45
5/30/17	pZP-Telo-JC	373	0.45
6/2/17	pZP-Telo-JC	190	0.5
6/22/17	pZP-Telo-JC	350	0.7
7/28/17	pZP-Telo-JC	215	0.73
8/2/17	pZP-Telo-JC	529	0.7
8/7/17	pZP-Telo-JC	540	0.55
8/15/17	pZP-Telo-JC	493	0.95
8/23/17	pZP-Telo-JC	778	0.5
8/28/17	pZP-Telo-JC	459	0.47
9/1/17	pZP-Telo-JC	532	0.5

Total	5099
Average OD	0.59

Table 3.2 - *Agrobacterium*-Mediated Transformation of Hi-II Immature Embryos Using pZP-Telo-JC Truncation Construct: This table highlights embryo count and OD_{600} of each transformation carried out in the 2017 season. The total number of embryos used in the creation of 17-27 (minichromosome) and 17-13 (B insert) platforms is 5099. The average *Agrobacterium* concentration used was $OD_{600} = 0.59$.

Event	Date Transformed	PCR +	Seed
17-1	5/25/17	-	-
17-2	5/25/17	X	X
17-3	5/25/17	X	X
17-4	5/26/17	neg	-
17-5	5/25/17	X	X
17-6	5/25/17	X	-
17-7	5/25/17	X	-
17-8	5/25/17	X	X
17-9	5/30/17	X	X
17-10	8/7/17	neg	X
17-11	8/7/17	X	X
17-12	8/7/17	X	X
17-13	8/7/17	X	X
17-14	8/7/17	X	X
17-15	8/2/17	neg	X
17-16	8/7/17	X	X
17-17	8/23/17	neg	X
17-18	8/28/17	neg	X
17-19	8/22/17	X	X
17-20	8/28/17	-	X

Event	Date Transformed	PCR +	Seed
17-21	9/1/17	-	X
17-22	9/1/17	-	X
17-23	9/1/17	-	-
17-24	8/15/17	-	X
17-25	8/15/17	-	-
17-26	8/15/17	-	-
17-27	8/15/17	X	X
17-28	8/15/17	X	X
17-29	8/15/17	X	X
17-30	8/15/17	X	X
17-31	8/28/17	X	-
17-32	9/1/17	neg	X
17-33	9/1/17	X	X
17-34	8/28/17	neg	X
17-35	9/1/17	X	-
17-36	9/1/17	-	X
17-37	9/1/17	-	X
17-38	9/1/17	-	-
17-39	9/1/17	-	X
17-40	9/1/17	-	-

Table 3.3 - pZP Events Recovered from *Agrobacterium*-Mediated Transformation of Hi-II Immature Embryos: This table highlights the event number (year-number), date of transformation (Refer to Table 2 for details), PCR analysis results, and recovery of T_1 seed from a T_0 x Hi-II B cross in greenhouse conditions. "-" = not tested; "X" = positive; "neg" = negative

Fluorescent In Situ Hybridization:

T_0 plants that were PCR positive for the stable integration of pZP-Telo-JC were used in fluorescent in situ hybridization analysis to determine the point of integration and whether a TMCT event occurred. Most events observed contained stable integrations into A chromosomes and did not induce TMCT; however, event 17-27 contained a B-derived minichromosome that is maintained in association with a complete native chromosome set (Figure 3.3; Figure 3.4). Hybridization with B centromere-specific centromere repeat (B repeat) oligos labeled with Texas Red shows the 17-27 minichromosome is derived from a B chromosome, when compared to signal present on other B chromosomes in the background (Figure 3.3). Single gene hybridization with pZP-MCS plasmids labeled with Texas Red shows the insert location in on the minichromosome (Figure 3.4). Event 17-13 contains an insert of pZP-Telo-JC on a B chromosome, which did not induce a TMCT event (Figure 3.5). While 17-13 did not produce a minichromosome, it was still seen as a beneficial platform to use in gene stacking regimes. The nondisjunction property of the full B could be used to manipulate the dosage of any cargo.

Inheritance Patterns of B Inserts and Minichromosomes:

PCR analysis of the T_3 generation of 17-13 and 17-27 plants shows inheritance patterns of the pZP-Telo-JC transgenes (Table 3.4), which is in line with previous research observing small chromosome behavior in meiosis

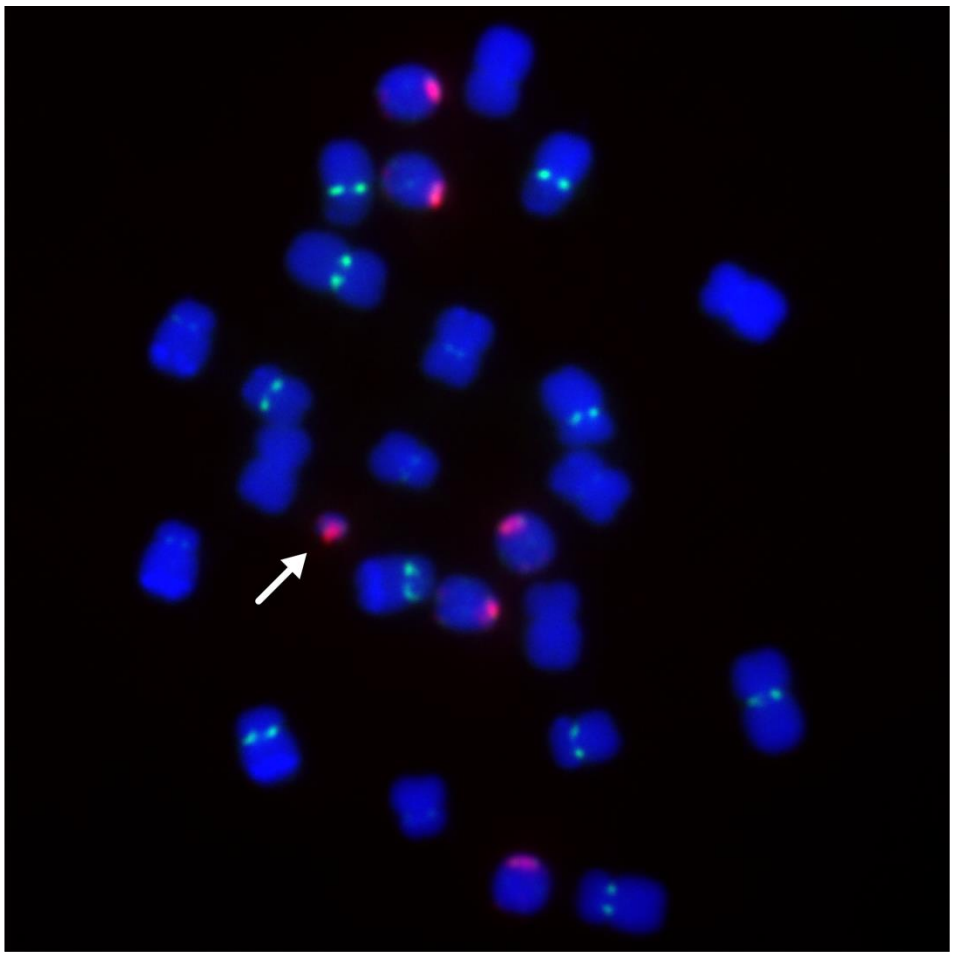


Figure 3.3 - Fluorescent In Situ Hybridization of a 17-27 T_0 Plant Using B Repeat Red Probes: A cocktail containing CentC, TAG, and B repeat red were hybridized to slides containing chromosome spreads from 17-27 T_0 plants. The white arrow points to a small chromosome that exhibits red signal from the B repeat probe. This observation indicates that the minichromosome is derived from a supernumerary B chromosome.

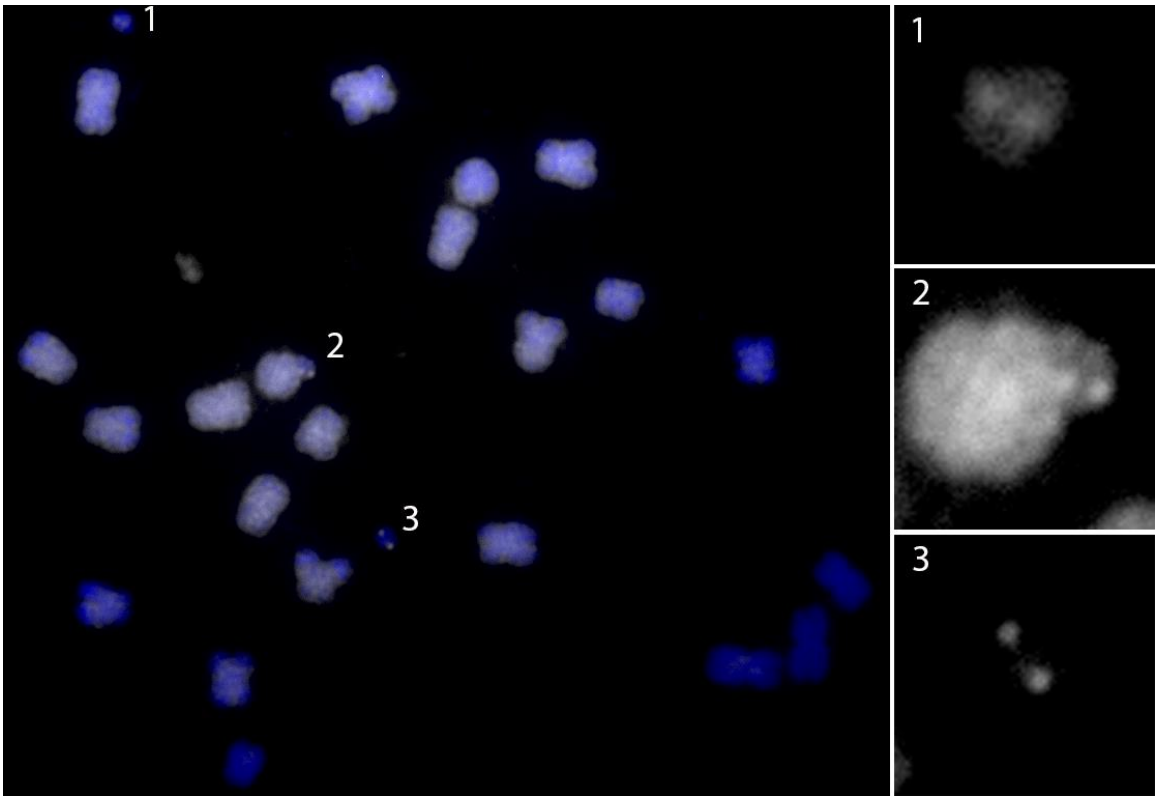


Figure 3.4 - Red and Blue Channel of Fluorescent In Situ Hybridization of a 17-27 T_1 Plant Using the pZP-MCS Red Probe: The B chromosome trans-acting accumulation mechanism in the second mitosis cycle of pollen maturation can lead to an increase of artificial minichromosome targets, despite the absence of a distal tip. The T_1 generation of selfed event 17-27 minichromosome lines exhibit variations in minichromosome copy number, which can be identified morphologically or with FISH. Here, three different minichromosomes from the same spread were labeled using pZP-MCS red probes, which show up as two white dots on each sister chromatid using a pseudocolored monochrome red filter overlay. Magnified monochrome red filter images of each minichromosome shows clear signal located near the B centromere (1,2,3).

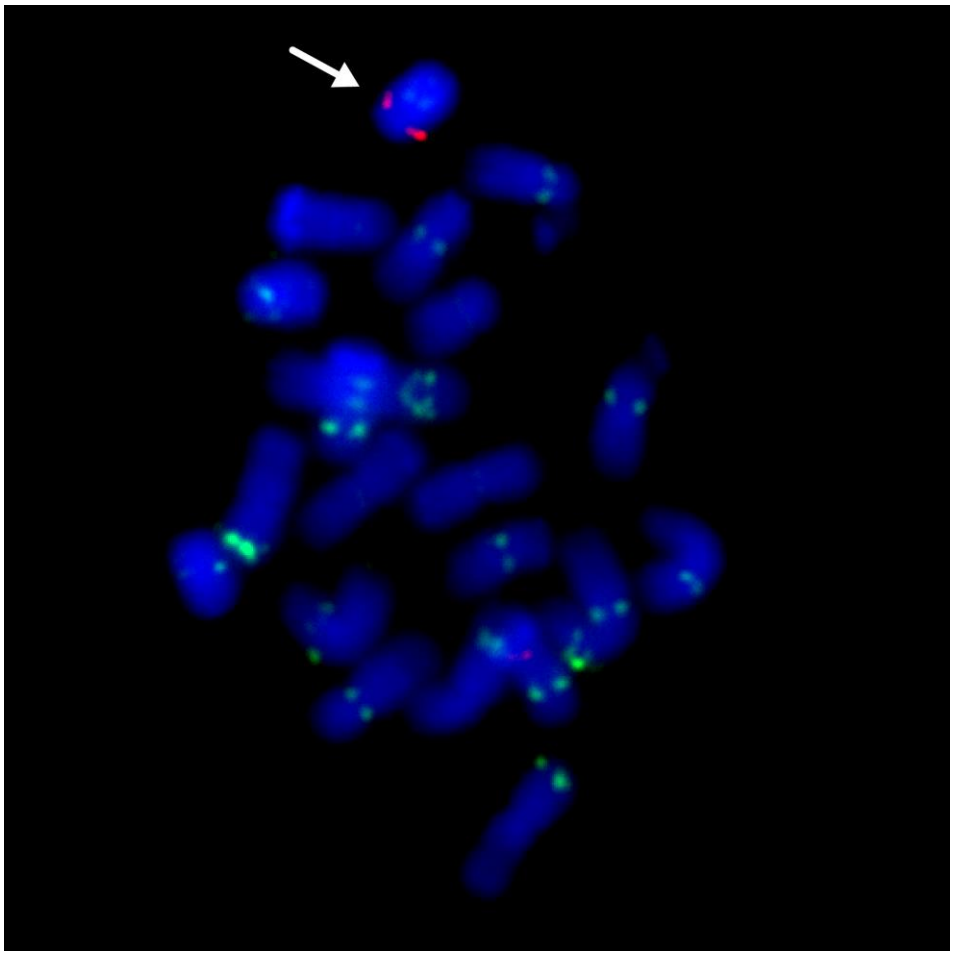


Figure 3.5 - Fluorescent In Situ Hybridization of a 17-13 T_0 Plant Using B Using the pZP-MCS Red Probe: A cocktail containing CentC, TAG, and pZP-MCS red were hybridized to slides containing chromosomes spreads from 17-13 T_0 plants. The white arrow point to a B chromosome that exhibits red signal, which indicates an insert of pZP-Telo-JC on the B chromosome.

Line	Plants Screened	Positive	Frequency %
pZP-Telo-JC 17-13 (B Insert)	149	67	45
pZP-Telo-JC 17-27 (Minichromosome)	63	10	16

Table 3.4 – Inheritance Frequency of 17-13 (B Insert) and 17-27 (Minichromosomes): 17-13 and 17-27 events display differential inheritance patterns in each generation, which is related to B chromosome size. Smaller chromosomes fail to find homologous partner in meiosis I, resulting in lower inheritance frequencies (Yu et al., 2007; Han et al., 2009). This observation is supported by the frequencies observed in the B insert (17-13) and minichromosome (17-27).

(Han et al., 2007; Birchler and Han, 2013). Intact B chromosomes successfully pair with homologous partners in meiosis I, resulting in inheritance frequencies comparable to A chromosomes. The 17-13 B insert transgene is inherited at a frequency of ~45% when crossed as a heterozygote. In contrast, minichromosome sister chromatid cohesion generally fails in anaphase I of meiosis, which results in unpredictable transmission rates that are usually dependent on minichromosome size (Han et al., 2007). The observed inheritance frequency of 17-27 is approximately ~16%, which is comparable to other minichromosomes generated by telomere mediated chromosomal truncation (Yu et al., 2007).

Discussion

Plant artificial chromosomes adapted for use in genetic engineering offer significant advantages over conventional methods of transgene delivery, which generate drastic variations in T_0 plants due to the absence of targeting machinery. The random nature of *Agrobacterium tumefaciens* and biolistic bombardment create limitations that bottleneck the utility of more complex biological systems, such as biosynthetic pathways or disease resistance clusters; a result of low DNA carrying capacity on commonly used T-DNA vectors and maintenance of multiple transgene insertions becoming increasingly difficult with each added gene. Additionally, introgression of transgenes into other lines can create conditions of linkage drag, where it is difficult to segregate negatively

impacting alleles through repeated backcrossing cycles. PAC systems circumvent these limitations by creating stable, autonomous platforms that exist in association with the native chromosomal sets. Through the use of precision genome editing technology, such as SSR systems, multiple DNA fragments could be sequentially targeted or rearranged in vivo to specific locations on artificial chromosomes. PAC vectors containing multiple transgene integrations would be inherited as a single unit in each generation without segregation or transferred to other plant lines without the risk of linkage drag.

In the present work, an artificial minichromosome (17-27) and a B insert (17-13) platform were created in maize though the process of *Agrobacterium*-mediated transformation (Figure 3.3; Figure 3.4; Figure 3.5) using truncation construct pZP-Telo-JC (Figure 3.1). FISH analysis on chromosome spreads from T_0 or T_1 plants indicates transgene insertions for both 17-27 and 17-13 occurred near the B chromosome centromeric region, with signal for pZP-MCS-Red localizing to the proximal region of the long arm (Figure 3.4; Figure 3.5). Interestingly, red signal for the 17-27 minichromosome does not appear to be located at the point of chromosome cleavage (Figure 3.4); however, it is a common observation in many FISH images that the signal is not located in the precise location on the chromosome, a product of chromosome configuration, preparation method or the “color combining” process of obtaining the image.

The pZP-Telo-JC transgene is designed to enable increased control over stable integrations though the incorporation of site-specific recombinase sites.

Two *FRT* sites flank the *bar* herbicide selectable marker, which enables selection removal upon introduction of the FLPe recombinase enzyme (Figure 3.6) (Golic and Lindquist, 1989). Downstream of *FRT*-*bar*-NOS-*FRT* is a single attachment P (*attP*) site, which is recognized by the unidirectional recombinase phiC31 Integrase (Thorpe and Smith, 1998). Together with the maize Ubiquitin 1 promoter, donor DNA molecules with an attachment bacteria (*attB*) and selectable marker could be used in a promoter trap strategy to select for targeted integrations in a cellular environment expressing phiC31 Integrase (Figure 3.6). However, this is only possible if the *FRT*-*bar*-NOSt-*FRT* fragment is removed prior to transgene targeting. Therefore, a strategy of selection removal followed by targeted integrations should be designed into the project timeline (Figure 3.6). Depending on the application, the specific DNA donor molecule is variable but should be present in a circular conformation and contain a targeting motif (*attB*-selection-NOSt). Minichromosomes or B inserts containing the recombinase machinery could be used in a breeding strategy together with recombinase expression lines to carry out a cyclical process of selection removal followed by targeted integrations of donor DNA molecules. Integrations would be in a location that exists in association with the native chromosomal set and exhibit predicable levels of expression.

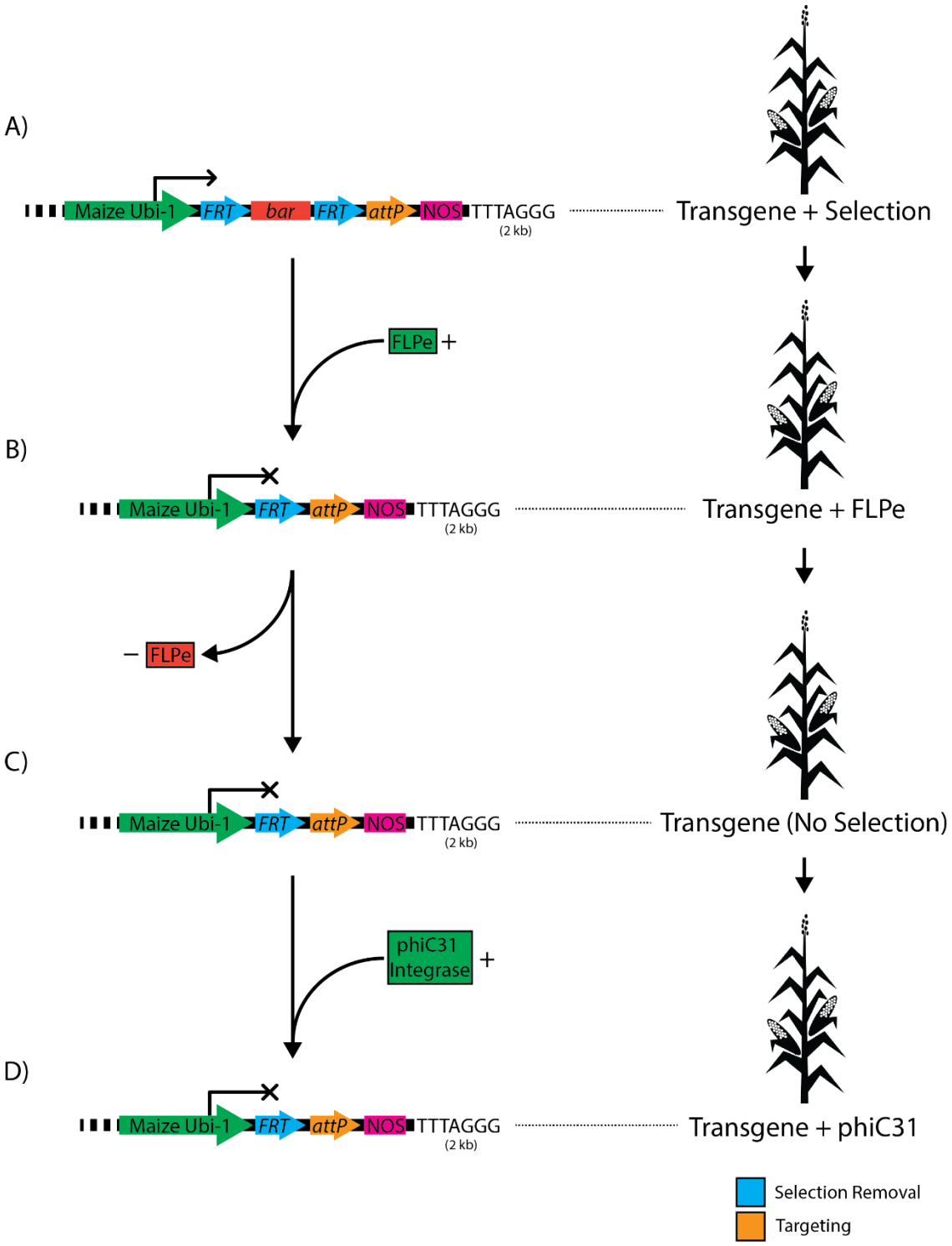

A)
Maize Ubi-1
FRT
bar
FRT
attP
NOS
TTTAGGG
(2 kb)
Transgene + Selection
FLPe
+
B)
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
Transgene + FLPe
–
FLPe
C)
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
Transgene (No Selection)
phiC31
Integrase
+
D)
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
Transgene + phiC31
Selection Removal
Targeting

Figure 3.6 - Molecular Strategy for Preparing Minichromosome Lines to Target Transgenes via phiC31 Integrase: Stable integrations of pZP-Telo-JC are selected in tissue culture using a *bar* selectable marker gene (A). T_0 pZP-Telo-JC plants are crossed to a FLPe expression line to remove the *FRT* flanked *bar* gene (B). T_1 transgenic plants containing pZP-Telo-JC + FLPe are crossed to Hi-II tester lines to segregate away FLPe (C). T_2 progeny containing pZP-Telo-JC without the *bar* gene or FLPe are selected and crossed to phiC31 stable expression lines (D). T_3 plants with pZP-Telo-JC (no *bar*) and phiC31 recombinase genes can theoretically be used in targeted integration experiments utilizing a donor molecule containing *attB* binding sites.

In addition to improving conventional plant transformation methods, the presented minichromosome and B insert give researchers the flexibility to choose an avenue that best suits the needs of the specific project. While TMCT of B chromosomes removes a site required for the accumulation mechanism located at the distal tip to stabilize the chromosome, an intact B chromosome insertion will still nondisjoin at the second pollen mitosis cycle. This will enable an accumulation of high copy numbers of B chromosome inserts, which can be used to vary levels of transgene expression in different plants (Birchler, 2014). Possible applications of B insert accumulation include biopharmaceutical production or metabolic engineering (Gaeta et al., 2012).

Engineering more complex biological systems, such as biosynthetic pathways or disease resistance clusters, will require a combination of methods that enable researchers to stack collections of genes in a targeted and efficient manner. With the use of conventional T-DNA or plasmid vectors, using a cyclical process of selection removal followed by targeted integration will be time consuming; however, the adoption of other technologies, such as binary bacterial artificial chromosomes (BiBACs) could expedite the gene transfer process while retaining any regulatory mechanisms that exists in endogenous sequences (Hamilton et al., 1996). As stated previously, this will require a BiBAC donor molecules to be in a circular conformation and contain a targeting motif, such as *attP-bar*-NOSt. If possible, the proposed strategy of stacking genes on artificial

minichromosome or B Chromosome inserts will greatly increase the rate of transgene incorporation and expand the limits of genetic engineering in plants.

Material and Methods

Truncation Construct used in Plant Transformation (pZP-Telo-JC):

DNA fragment *FRT-BAR*-NOSt-*FRT* was PCR amplified from pZPGLP1 using forward and reverse primers 5' – TCAAGGATCCAGCCAGAAGTTCC TATTCTCTAGAAAGTATAGGAACTTCGTAACCATGAGCCCAGAACG – 3' and 5' – AACTGGCGCGCCTTGAGAAGTTCCTATACTTTCTAGAGAATAGGAA CTTCTCGAATTCTCATGTTTGACAGC – 3', respectively. *FRT-BAR*-NOSt-*FRT* was cloned into pZPGLP1 using *Bam*HI and *Asc*I restriction endonucleases to produce pZP-*FRT1/2*. pZP-*FRT*1/2 was sequence verified using Sanger sequencing.

DNA fragment *attP*-NOSt was PCR amplified from pZP-*FRT*1/2 using forward and reverse primers 5' – CTTCTCAAGGCGCGCCAGTTCCCC AACTGGGGTAACCTT – 3' and 5' – TTTAAACTGAAGGCGGGAAA – 3', respectively. *attP*-NOSt was cloned into pZP-*FRT*1/2 using *Asc*I and *Zra*I restriction endonucleases to produce pZP-*attP*-NOSt. pZP-*attP*-NOSt was sequence verified using Sanger sequencing.

DNA fragment MCS was generated through an annealing process between top and bottom strands 5' – GACGTCTTCGAAGTTTAAACGAGCTCA CGCGTCACGTGCCTCAGCATTTAAATTCCGGAAGGCCTCCTGCAGGTTAAT

TAATGTACAACTAGTGATATC – 3’ and 5’ – GATATCACTAGTTGTACATTAAT TAACCTGCAGGAGGCCTTCCGGAATTTAAATGCTGAGGCACGTGACGCGTG AGCTCGTTTAAACTTCGAAGACGTC – 3’, respectively. MCS was cloned into pZP-*attP*-NOSt using *Aat*II and *Pme*I restriction endonucleases to produce pZP-MCS. pZP-MCS was sequence verified using Sanger sequencing.

A telomere array was digested from pWY82 using *Spe*I and *Stu*I restriction nucleases. pZP-MCS backbone was digested using *Spe*I and *Stu*I restriction nucleases. The telomere dropout and pZP-MCS were run on a low melting point agarose gel (Figure 3.7). The linearized pZP-MCS backbone was extracted from the gel (Figure 3.7). The telomere dropout was excised at ~2kb, with respect to the molecular size ladder. The telomere insert and pZP-MCS vector backbone were cloned together through an in-gel ligation and transformed into ElectroMAX Stbl4 cells (ThermoFisher) (Graham et al., 2015). Plasmids were extracted from spectinomycin resistant *E. coli* colonies and digested with *Spe*I and *Pac*I restriction enzymes (Figure 3.8A). A Southern blot hybridization assay using radiolabeled $TTTAGGG_n$ sequences with γ-^{32}P (Figure 3.8B)**.** Plasmid 1, or pZP-Telo-JC, contained the largest telomere insert and was selected for use in telomere-mediated chromosomal truncation experiments. The borders of pZP-Telo-JC telomere insert were sequence verified to contain $TTTAGGG_n$ repeats (Figure 3.9).

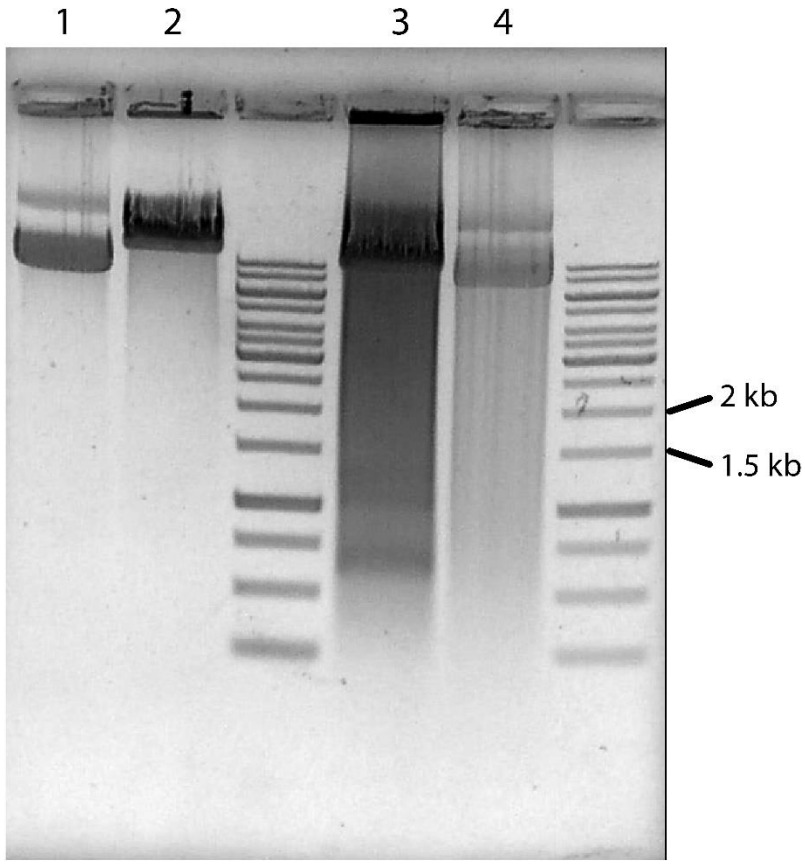


Figure 3.7 - Low Melting Point Agarose Gel of pZP-MCS and pWY82 Digests Prior to In-Gel Ligation: pZP-MCS backbone was digested using *Spe*I and *Stu*I restriction endonucleases (2). pWY82 telomere inserts were excised from backbone using *Spe*I and *Stu*I restriction endonucleases, resulting in a smear pattern of differential TTTAGGG repeat sizes (3,4). High volumes loaded into agarose gels can affect movement of DNA, which is the reason for loading high and low volumes, 3 and 4, respectively. Less movement of the pWY82 backbone can be observed in lane 3 with respect to lane 4. Since DNA concentration was more important than band size, lane 3 was used in the gel extraction procedure.

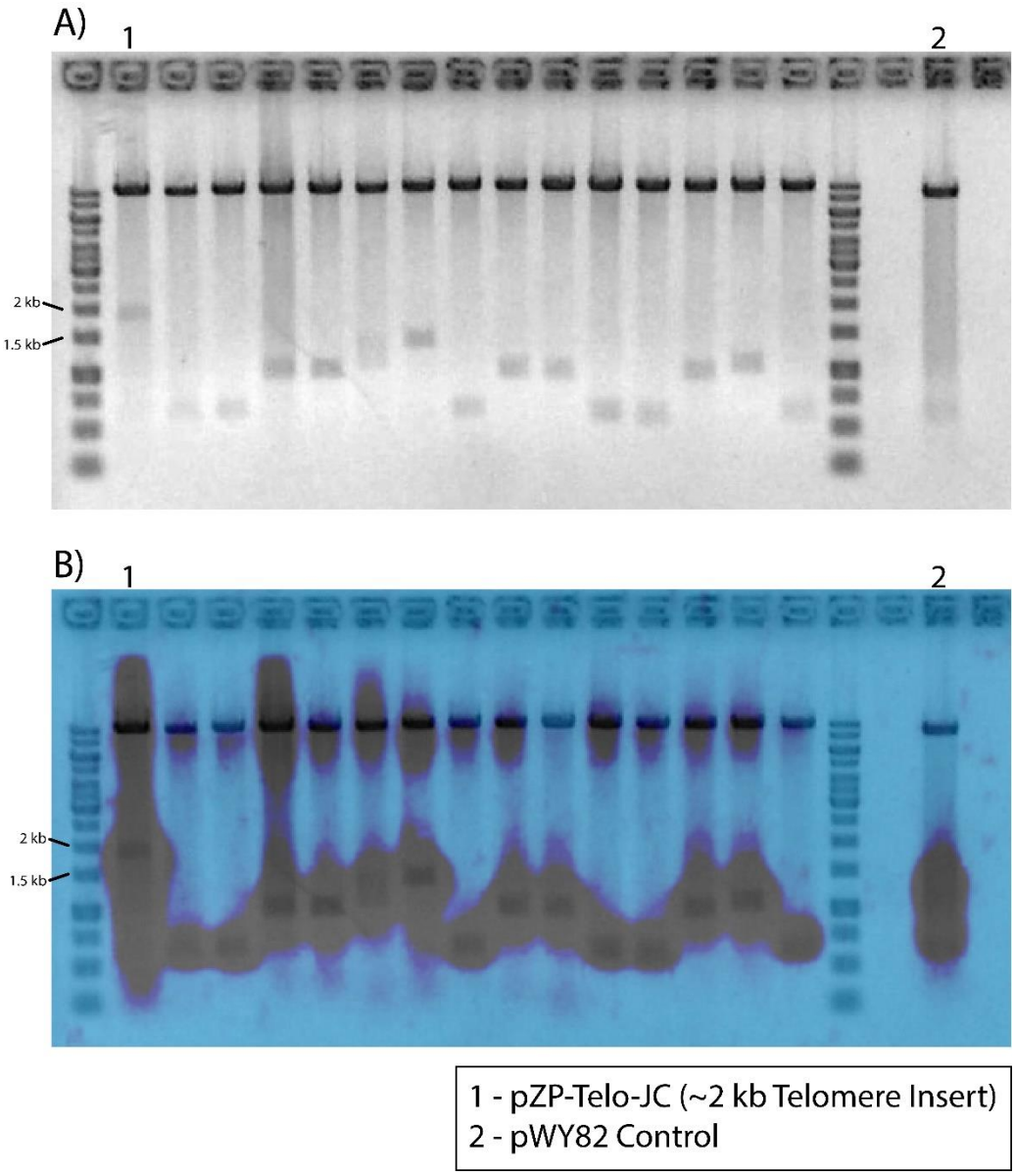


Figure 3.8 - Restriction Digest and Southern Blot Analysis of Telomere Fragment Ligations into pZP-MCS Backbone: Restriction digest analysis of candidate pZP-Telo-JC constructs shows variability of telomere size inserts (A). Southern blot analysis of dropouts using γ-^{32}P labeled $TTTAGGG_n$ oligos displays strong signal around insert dropouts (B) when compared to the pWY82 control (2). The construct containing ~2 kb telomere sequence was chosen as the pZP-Telo-JC truncation construct (Figure 3.1).

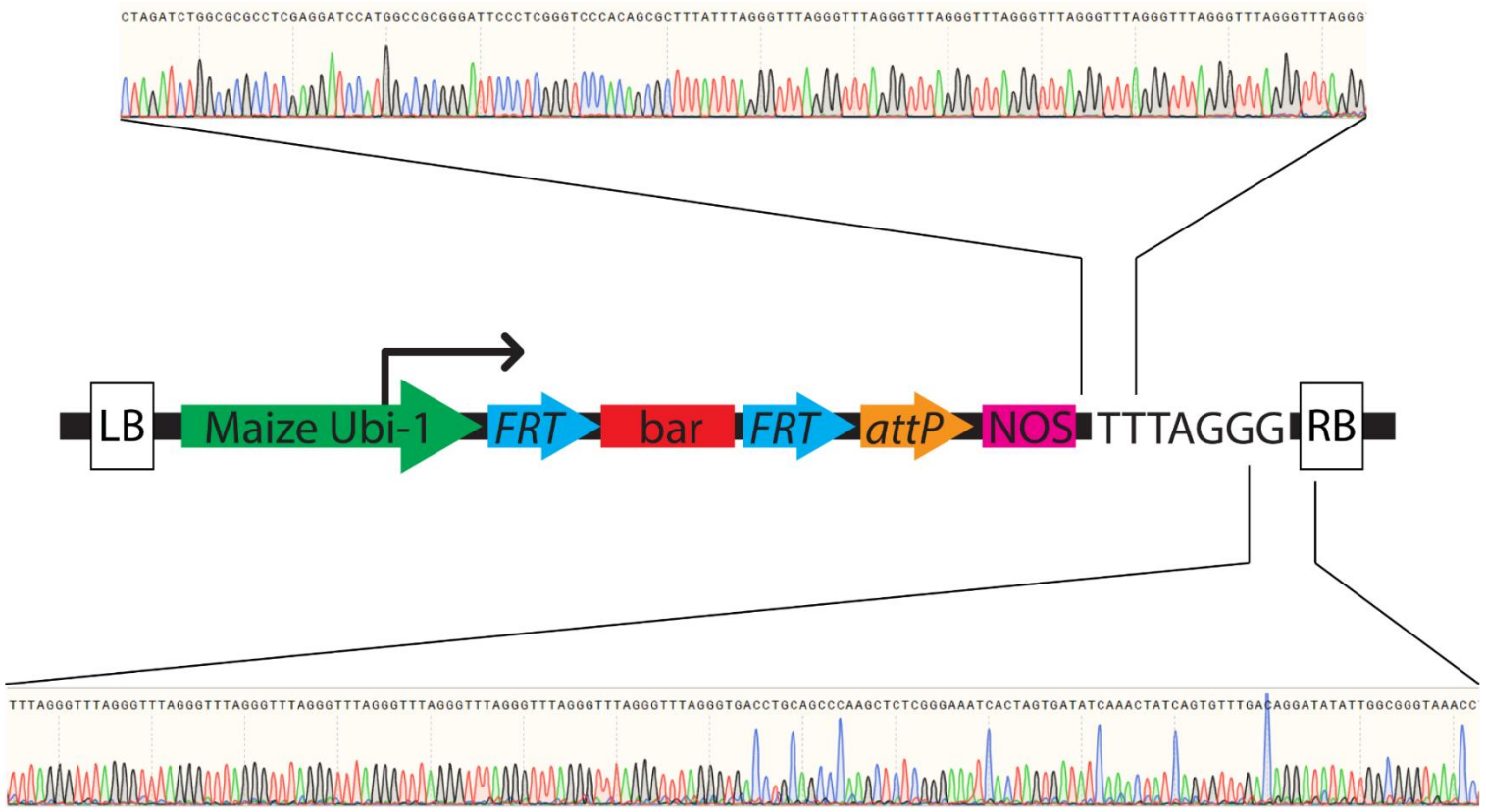


Figure 3.9 - Sequence Analysis of Telomere Insert in pZP-Telo-JC Truncation Construct. Sequence analysis confirms the insertion of a telomere array into pZP-MCS to create pZP-Telo-JC.

Plant Material for Transformation:

Immature embryo explants were harvested 9 – 12 days after pollination between Hi-II A + B and Hi-II B inbred lines (Armstrong and Green, 1985). Hi-II is the most common genotype used in transformation due it its high frequency of friable, type II embryogenic callus formation. Lines A and B are crossed together to create vigorous F_1 immature embryos used in the transformation procedure.

Plant Transformation:

pZP-Telo-JC was transformed into EHA105 *Agrobacterium* cells (provided by Stanton B. Gelvin, Purdue University) by electroporation and selected on LB agar plates using rifampicin (30 ug/ml), spectinomycin (100ug/ml), and streptinomycin (100 ug/ml) antibiotics. Positive colonies were suspended in 5 ml of LB containing the appropriate antibiotics, and grown for 24-28 hours at 28°C, shaking at 250 rpm. 500 µl of each colony was used to create glycerol stocks that were stored at -80°C. These stocks were used to generate *Agrobacterium* growth plates used in the transformation procedure.

F_1 immature embryos were obtained 9 – 12 days after pollination between Hi-II B and Hi-II A + ≥ 5 B chromosomes at a length of ~1.5 – 2 mm. Collected embryos were infected with *Agrobacterium* EHA105 + pZP-Telo-JC infection media at an average OD_{600} of 0.6 for 3 – 4 hours. Infected embryos were transferred to callus induction media and allowed to rest at 28°C for a period of 11 – 12 days. Type II callus formed from infected embryos were transferred to

bialaphos (3 ug/ml) media and switched to new selection plates every 2 weeks until resistant cells were formed. Resistant callus was sequentially transferred to shooting and rooting media, which can take up to ~3 weeks each. A detailed description of this process, including lists of media reagents can be found in Lee and Zhang (2014).

Calculation for Transformation Efficiency:

Transformation efficiency is calculated by dividing the total number of events by the total number of embryos used in the transformation of pZP-Telo-JC. While this gives a significantly lower number compared to frequencies seen in other studies, this calculation is a clear representation of efficiency with respect to the number of embryos needed to generate positive events.

PCR analysis of T_0 Transgenics:

Leaf tissue samples of T_0 transgenic plants were used to extract genomic DNA for PCR analysis (Leach et al. 2016). The forward and reverse primers, 5' – CGTGAGCTCGTTTAAACT – 3' and 5' – TCTAGACGTACGTGGCCAAT – 3', respectivly, were used to verify the presence of the pZP-Telo-JC transgene. PCR on extracted DNA was performed using standard conditions of 35 cycles: 30s denature at 95°C, 30s annealing at 60°C, and 77s extension at 72°C. Amplified fragment measures to approximately 1,267 bp on an agarose gel.

Fluorescent in situ Hybridization:

Root samples were collected from T_0 or T_1 plants that were PCR positive for the presence of pZP-Telo-JC inserts. Samples were treated with nitrous oxide (N_2O) for 2.5 hrs and subsequently transferred into 1.7 ml tubes containing 90% acetic acid. After 10 min, roots were washed with 70% ethanol to remove acetic acid and placed into new 1.7 ml tubes. Tubes were filled with fresh 70% ethanol and placed at -20°C until root digestion steps.

Both B repeat and pZP-MCS red probes were generated through a nick translation (NT) reaction using 1mM of Texas Red 5-dCTPs and non-labeled dNTPs (2mM each). NT reactions were purified via ethanol precipitation using salmon sperm (10 mg/ml), 0.1 volume of 3M acetic acid, and 2.0 volume of 100% ethanol, then stored at -20°C for at least 2 hrs before proceeding with centrifugation at 16,000 x g for 30 min. The supernatant was carefully discarded, and probe pellets were washed 2x with 70% ethanol. Red probe pellets were resuspended in 2x SSC, 1x TE buffer and stored at -20°C until use in hybridization.

Root samples were digested with 1x citric buffer containing 1% Pectolyase Y-23 and 2% Cellulase Onozuka R-10 (Karlan Research Products) for a period of 45 min. Each digestion mixture was washed with 70% ethanol to stop the reaction before resuspending in 100% acetic acid. A blunt dissecting probe was used to smash solid root tips in each tube. Inside a humid chamber, 6 µl of the root digest/cell suspension was pipetted onto a glass microscope slide and

allowed to dry for 10 min. After drying, each slide was cross-linked using a UV cross-linker at the energy of 120 – 125 mJ/cm^2. Each slide was visualized with an Olympus CX21 light microscope to assess the quality of the chromosome spreads.

8 µl karyotyping cocktails containing 1.5 µl CentC (90 ng), 1 µl TAG (20 ng), 3 µl NT red probe and 2.5 µl 2x SSC/1x TE was pipetted onto each slide and covered with a plastic cover slip before being placed into a tray and transferred into a boiling water bath for 5 min. After 5 min, the slides were placed into a humid chamber overnight (~16 hrs). After ~16 hrs, each tube was washed with warm 2x SSC and dried with a Kimwipe. Each dried slide was treated with 1 drop of Vectashield with 5% 4′,6-diamidino-2-phenylindole (DAPI) and covered with a glass cover slip. Chromosome spreads were visualized with an Olympus BX61 fluorescent microscope. For further details of the FISH procedure see McCaw et al., 2016a, 2016b.

References

Aleksenko A. and Clutterbuck AJ. (1997) Autonomous plasmid replication in *Aspergillus nidulans*: AMA1 and MATE elements. Fungal Genet Biol. 21(3), 373-387.

Anand A., Wu E., Li Z., TeRonde S., Arling M., Lenderts B., Mutti J.S., Gordon-Kamm W., Jones T.J., Chilcoat N.D. (2019) High efficiency *Agrobacterium*-mediated site-specific gene integration in maize utilizing the FLP-FRT recombination system. Plant Biotechnol J. doi: 10.1111/pbi.13089.

Ananiev E. V., Wu C., Chamberlin,M.A., Svitashev S., Schwartz C., Gordon-Kamm W., Tingey S. (2009) Artificial chromosome formation in maize (*Zea mays L.*). Chromosoma 118, 157–177.

Armstrong C.L. and Green C.E., (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164, 207–214.

Birchler J.A. (2014) Engineered minichromosomes in plants. Curr Opin Plant Biol. 19, 76-80.

Birchler J.A. (2015) Engineered minichromosomes in plants. Chromosome Res.23 (1), 77-85.

Birchler J.A., Han F. (2009) Maize Centromeres: Structure, Function, Epigenetics. Annu Rev Genet 43, 287-303.

Birchler J.A., Han F. (2013) Meiotic behavior of small chromosomes in maize. Front Plant Sci. 4, 1-4.

Bok J.W., Ye R., Clevenger K.D., Mead D., Wagner M., Krerowicz A., Albright J.C., Goering A.W., Thomas P.M., Kelleher N.L., Keller N.P., Wu C.C. (2015) Fungal artificial chromosomes for mining of secondary metabolome. BMC Genomics 16(343), DOI 10.1186/s12864-015-1561.

Carlson W.R. (1974) B chromosomes induce nondisjunction in 9B9 pollen. Maize Genet. 81–83.

Carlson S.R., Rudgers G.W., Zieler H., Mach J.M., Luo S., Grunden E., Krol C., Copenhaver G.P., Preus D. (2007) Meiotic transmission of an in vitro-assembled autonomous maize minichromosome. PLoS Genet. 3, 1965–1974.

Farr C., Fantes J., Goodfellow P., Cooke H. (1991) Functional reintroduction of human telomeres into mammalian cells. Proc. Natl. Acad. Sci. USA. 88, 7006–7010.

Gaeta R.T., Masonbrink R.E., Krishnaswamy L., Zhao C., Birchler J.A. (2012) Synthetic chromosome platforms in plants. Annu. Rev. Plant Biol. 63, 307–330.

Gaeta R.T., Masonbrink R.E., Zhao C., Sanyal A., Krishnaswamy L., Birchler, J.A, (2013) In vivo modification of a maize engineered minichromosome. Chromosoma 122, 221–232.

Golic K.G. and Lindquist S. (1989) The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. Cell 59, 499–509.

Graham N., Swyers N., Gaeta R., Zhao C., Cody J.P., Birchler J.A. (2015) Telomere-mediated chromosomal truncation via *Agrobacterium* tumefaciens or particle bombardment to produce engineered minichromosomes in plants. Bio-protocol 5(18), e1595.

Hamilton C.M., Frary A., Lewis C., Tanksley S.D. (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc. Natl. Acad. Sci. USA 93, 9975–9979.

Han F., Lamb J. C., Birchler, J. A. (2006). High frequency of centromere inactivation resulting in stable dicentric chromosomes of maize. Proc. Natl. Acad. Sci. U.S.A. 103, 3238–3243.

Han F., Gao Z., Birchler J.A. (2009) Reactivation of an inactive centromere reveals epigenetic and structural components for centromere specification in maize. Plant Cell 21(7), 1929-1939.

Harrington J. J., Van Bokkelen G., Mays R. W., Gustashaw K., Willard H.F. (1997) Formation of de novo centromeres and construction of first-generation human artificial microchromosomes. Nat. Genet. 15, 345–355.

Heller R., Brown K.E., Burgtorf C., Brown W.R. (1996) Mini-chromosomes derived from the human Y chromosome by telomere directed chromosome breakage. Proc. Natl. Acad. Sci. USA. 93, 7125–7130.

Jones R.N., Viegas W., Houben A. (2008) A century of B chromosomes in plants: So what? Ann. Bot. 101, 767–775.

Kapusi E., Ma L., Teo C.H., Hensel G., Himmelbach A., Schubert I., Mette M.F., Kumlehn J., Houben A. (2012) Telomere-mediated truncation of barley chromosomes. Chromosoma 121, 181–190.

Kerbach S., Lörz H., Becker D. (2005) Site-specific recombination in *Zea mays*. Theor Appl Genet 111, 1608-1616.

Leach K.A., McSteen P.C., Braun D.M. (2016) Genomic DNA Isolation from Maize (*Zea mays*) Leaves Using a Simple, High-Throughput Protocol. Curr Protoc Plant Biol 1,15-27.

Lee H. and Zhang Z.J. (2013) *Agrobacterium*-Mediated Transformation of Maize (*Zea mays*) Immature Embryos. Methods in Molecular Biology 1099, 273–280.

Lin BY. (1978) Regional control of nondisjunction of the B chromosome in maize. Genetics 90(3), 613-627.

Lyznik L.A., Rao K.V., Hodges T.K. (1996) FLP-mediated recombination of FRT sites in the maize genome. Nucl Acids Res 24. 3784–3789.

McCaw M., Swyers N., Graham N., Cody J., Zhao C., Birchler J. (2016a) Preparation of chromosomes from *Zea mays*. Curr Proto Plant Biol 1, 501-509.

McCaw M., Graham N., Cody J., Swyers N., Zhao C., Birchler J. (2016b) Fluorescence In Situ Hybridization to Maize (*Zea mays*) Chromosomes. Curr Proto Plant Biol 1, 530-545.

Murray A.W. and Szostak J.W. (1983) Construction of artificial chromosomes in yeast. Nature 305, 189–193.

Nelson A.D., Lamb J.C., Kobrossly P.S., Shippen D.E. (2011) Parameters affecting telomere-mediated chromosomal truncation in *Arabidopsis*. Plant Cell 23, 2263–2272.

Ow, D.W. (2005) SIVB Congress Symposium Proceeding: Transgene Management via Multiple Site-specific Recombination Systems. Vitr Cell Dev Biol - Plant 41, 213–219.

Richards E.J. and Ausubel F.M. (1988) Isolation of a higher eukaryotic telomere from Arabidopsis thaliana. Cell 53(1), 127-136.

Roman H., (1947) Mitotic nondisjunction in the case of interchanges involving the B-type chromosome in maize. Genetics 32, 391–409.

Roman H., (1949) Factors affecting mitotic nondisjunction in maize. Rec. Genet. Soc. Am 18, 112.

Shizuya H., Birren B., Kim U.J., Mancino V., Slepak T., Tachiiri Y., Simon M. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc. Natl. Acad. Sci. USA. 89, 8794–8797.

Srivastava V., and Ow D.W. (2001) Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct. Plant Mol Biol 46, 561-566.

Teo C.H., Ma L., Kapusi E., Hensel G., Kumlehn J., Schubert I., Houben A., Mette M.F. (2011) Induction of telomere-mediated chromosomal truncation and stability of truncated chromosomes in *Arabidopsis thaliana*. Plant J. 68, 28-39.

Thorpe, H.M. and Smith M.C.M. (1998) In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc. Natl. Acad. Sci. U.S.A. 95 (10), 5505-5510.

Ward E.J. (1973) Nondisjunction: localization of the controlling site in the maize B chromosome. Genetics 73, 387–391.

Watson J.M. and Riha K. (2010) Comparative biology of telomeres: Where plants stand. FEBS Lett. 584, 3752–3759.

Xu C., Cheng Z., Yu W. (2012) Construction of rice mini-chromosomes by telomere-mediated chromosomal truncation. Plant J. 70, 1070–1079.

Yan X., Li C., Yang J., Wang L., Jiang C., Wei W. (2017) Induction of telomere-mediated chromosomal truncation and behavior of truncated chromosomes in *Brassica napus*. Plant J. 91(4), 700-713.

Yu W., Lamb J.C., Han F., Birchler J.A. (2006) Telomere-mediated chromosomal truncation in maize. Proc. Natl. Acad. Sci. USA. 103, 17331–17336.

Yu W., Han F., Gao Z., Vega J.M., Birchler J.A. (2007) Construction and behavior of engineered minichromosomes in maize. PNAS 104(21), 8924-8929.

Yu W., Yau Y., Birchler J.A. (2016) Plant artificial chromosome technology and its potential application in genetic engineering. Plant Biothechnol J. 14, 1175-1182.

Yuan J., Shi Q., Guo., Liu Y., Su H., Guo X., Lv Z., Han F. (2017) Site-specific transfer of chromosomal segments and genes in wheat engineered chromosomes. J Genet Genomics 44(11), 531-539.

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

Chapter 4: Engineering Binary Bacterial Artificial Chromosome Donor Molecules

Note: The information provided in this chapter is unpublished.

Contributions

The work detailed here would not have been possible without the guidance and support of Nathaniel Graham, Patrice Albert, and Changzeng Zhao.

Summary

Binary bacterial artificial chromosomes (BiBACs) combine the DNA carrying capacity of bacterial artificial chromosomes (BAC) with the transformability of the binary T-DNA system utilizing *Agrobacterium tumefaciens*. Through the incorporation of specific recombinase binding sites on BiBAC T-DNA vectors, it may be possible to circularize and target stable inserts into predetermined locations, such as artificial minichromosomes or B inserts from Chapter 3. Here, we describe an alternating BiBAC system that could possibly enable researchers to stack collections of transgenes in a single location in the maize genome using the site-specific recombinase toolkit established in Chapter 2.

Introduction

Agrobacterium tumefaciens is a gram-negative soil bacterium that senses phenolic substances from plant wounds, such as acetosyringone, to transduce a signaling pathway initiating the processing, secretion, and trafficking of single-stranded transfer DNA (T-DNA) from tumor inducing plasmids (Ti-plasmids) into host genomes (Lee and Gelvin, 2008). While the natural function of this process is to deliver T-DNA oncogene and opine synthase coding sequences, forcing plants to create a suitable environment for *Agrobacterium* growth and development (Christie and Gordon, 2014), these genes are non-essential for DNA transfer into host cells (Lee and Gelvin, 2008). This discovery enabled researchers to create the first transgenic plants though the manipulation of Ti-plasmids using co-integration/exchange systems to replace oncogenes and opine synthases with selectable markers and genes of interest (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983; Lee and Gelvin, 2008).

Since then, *Agrobacterium*-mediated gene transfer has been streamlined through the creation of the binary vector system, which separates the T-DNA region from the Ti-plasmid signal transduction machinery to enable simple T-DNA vector modifications in *Escherichia coli* (Hoekema et al., 1983; Framond et al., 1983). This breakthrough initiated the development of a diverse number of T-DNA binary vector systems, which contain unique features that give researchers flexibility when carrying out specific genetic engineering projects (Lee and Gelvin, 2008); however, most vectors are designed for ease of handling in *E. coli* and

broad host ranges in *Agrobacterium*. For this reason, many common binary vectors contain origins of replication that are not suitable for the maintenance and transformation of large gene fragments due to decreased transformation efficiency and possible downstream complications resulting from complex insertions (Meyer and Saedler, 1996; Ye et al., 2011; Que et al., 2014). While it is assumed that conventional T-DNA vectors are capable of maintaining large inserts above 300 kb (Tao and Zhang, 1998), transformation of DNA sizes above 30 kb is difficult and uncommon. With the observation that *Agrobacterium* could naturally transfer 160 kb of the Ti-plasmid by switching the left and right borders of the T-DNA sequence (Miranda et al., 1992), it was clear that large gene transfer into plants was possible and led to the creation of binary bacterial artificial chromosomes (BiBAC) (Hamilton et al., 1996; Frary and Hamilton, 2001).

BiBAC technology combines the DNA carrying capacity of bacterial artificial chromosomes (BAC) (Shizuya et al., 1996) with the transformability of the binary T-DNA system (Hamilton et al., 1996; Hamilton, 1997), enabling the maintenance of large T-DNA inserts (>300 kb; 150 kb average) and subsequent transformation into host genomes. BiBAC vectors are maintained in low copy numbers (1-2 copies/cell) through the incorporation of F and Ri origins from *Escherichia coli* and *Agrobacterium rhizogenes*, respectively (Hamilton et al., 1996; Hamilton, 1997). While this makes modifications to the BiBAC vector more difficult, it serves in reducing the chance of complex insertions in *Agrobacterium*

transformation experiments (Ye et al., 2011). Through the use of the pCH32 helper plasmid, containing extra copies of the signal transduction machinery (*virG, virE1, and virE2*), successful transformation of 150 kb has been demonstrated in tobacco (Hamilton et al., 1996) and tomato (Frary and Hamilton, 2001). Additionally, BiBAC transformation of large fragments has been reported in other plant species, including rice (He et al., 2010; Wang et al., 2015) and maize (Vega et al., 2008). Studies have observed that maize transformation efficiency is decreased 10-fold with large T-DNA fragments when compared to smaller transgene insertions (Que et al., 2014), which is in line with other work that compared transformation efficiencies with respect to T-DNA size in tobacco, rice, and cotton (Park et al., 2000). While obtaining positive transformants with BiBACs is rare, the added benefit of stably integrating large DNA fragments with one T-DNA molecule is arguably more efficient than sequential transformation of smaller fragments, which are difficult to maintain in a single plant.

Until recently, the transformation size limitation of most T-DNA vectors hasn't been an issue, with early genetic engineering focusing on the incorporation of single gene traits conferring resistance to insects or herbicide treatments. While these traits have greatly benefited agriculture through increased yields and decreased chemical applications to fields, there is currently a limit to the number of improvements scientist can make to a single plant, which is directly related to conventional methods of genetic engineering technology. In addition to the random nature of *Agrobacterium* transgene integration**,** the

amount of DNA delivered in a single transformation experiment is limited by the utilized T-DNA binary vector system. This greatly narrows the scope of available traits, excluding more complex biological systems that require coordinated expression and regulation of many gene products. With future challenges of sustaining a growing population and adapting to shifting environmental conditions, there is a need for technology that increases the amount of DNA delivered on T-DNA molecules while enabling transgene targeting to specific areas of the genome.

Here, we developed a BiBAC vector system that is compatible with the site-specific recombinase genome editing toolkit (Chapter 2), which can be used to remove selection from stable BiBAC integrations and subsequently create circular extrachromosomal elements that can be targeted to specific locations in the maize genome, such as artificial minichromosomes (17-27) or B inserts (17-13) from Chapter 3. Using an alternating BiBAC vector system, large DNA fragments could be sequentially stacked in a predetermined location. If possible, this would expand the limits of genetic engineering by enabling researchers to stack collections of genes in a single location in the genome that can be inherited as a single unit in subsequent generations.

Results

Molecular Strategy and Construct Design:

BiBAC vectors that function as donor molecules for transgene targeting need to be designed in a way that enables selection removal and subsequent circularization through the use of a collection of site-specific recombinase expression lines (Chapter 2). This process will require multiple generations of crossing in and segregating away specific recombinase enzymes to modify sequences that will prime the BiBAC donor molecule for movement into a predetermined location, such as an artificial minichromosome. While the proposed strategy could theoretically be expedited through the direct targeting of transgenes via *Agrobacterium* transformation or biolistic bombardment, BiBAC T-DNA size creates issues of transformation efficiency and possible fragmentation, respectively. Therefore, stable integration and subsequent modifications through breeding or tissue culture may increase the probability of obtaining targeted integrations since every cell of a modified plant will contain a copy of the BiBAC T-DNA.

The pJC-YattB BiBAC donor constructs (Figure 4.1A) are designed to enable selection of insertional events through the use of a maize Ubiquitin I promoter (Ubi) driving the expression of a coding sequence that confers resistance to bialaphos herbicide treatments, followed by a nopaline synthase terminator (NOSt). Ubi-*bar*-NOSt sequences are flanked by *RS* recombinase binding sequences positioned in a head-to-tail orientation, enabling the removal

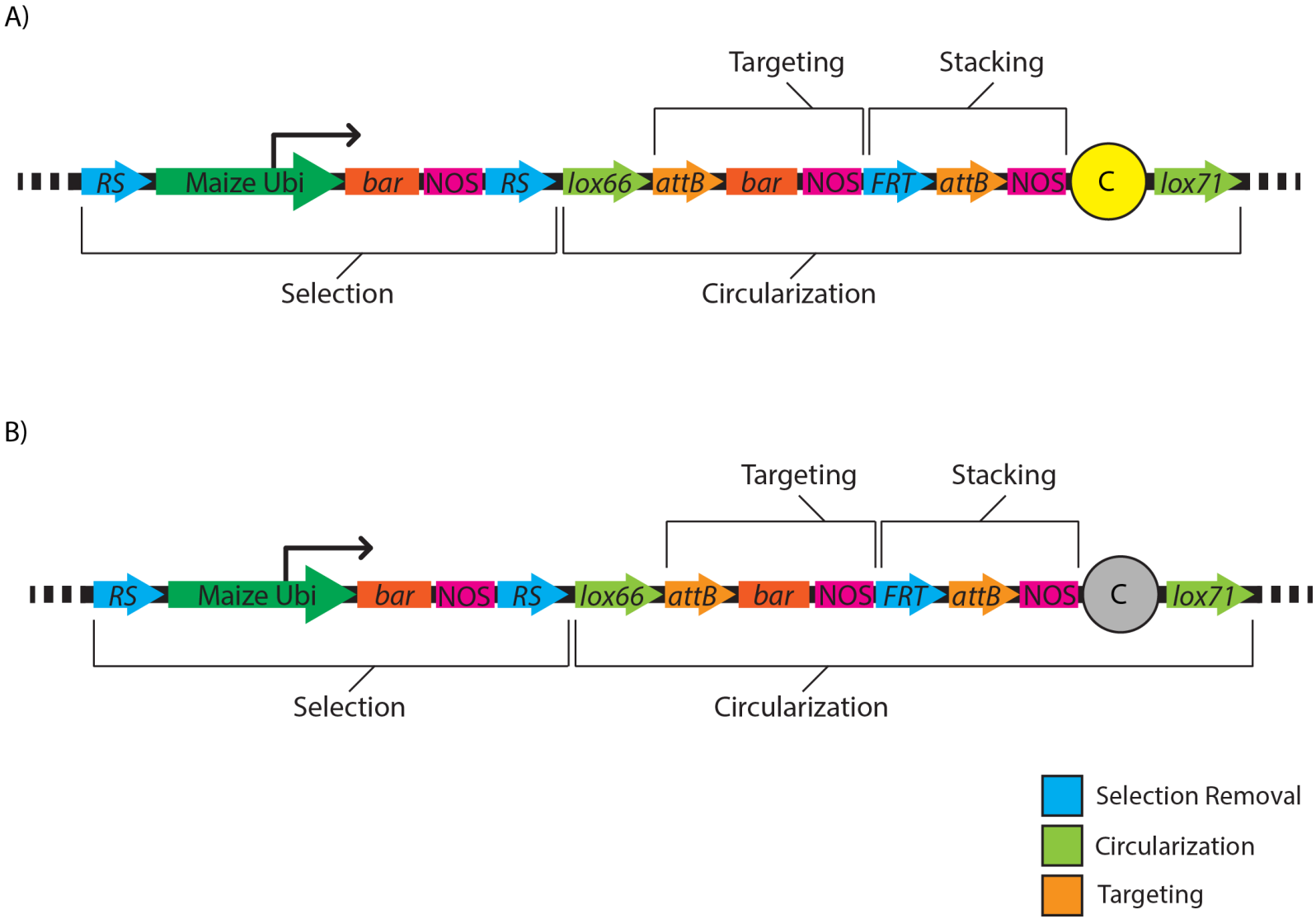
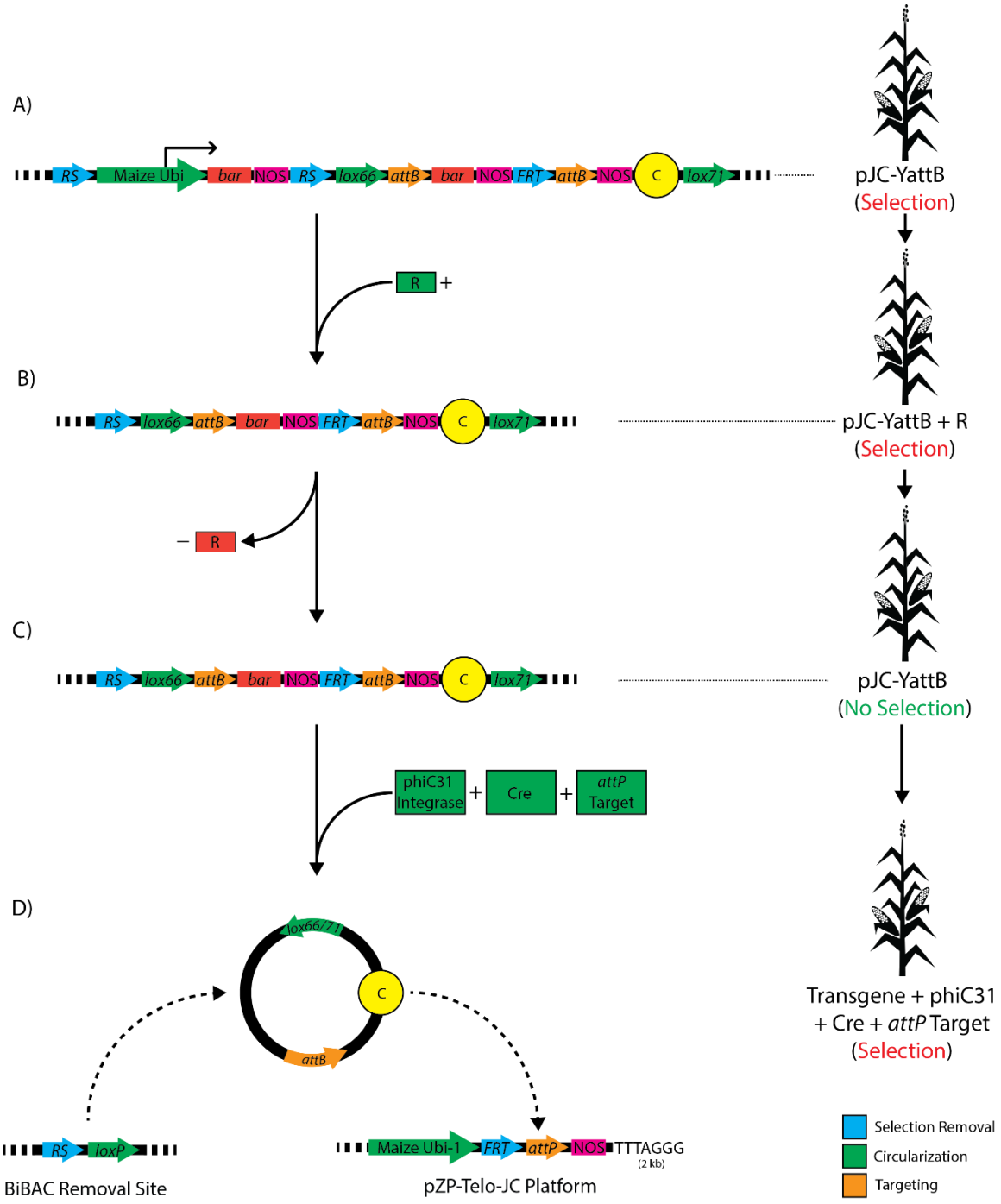

A)
Targeting
Stacking
RS
Maize Ubi
bar
NOS
RS
lox66
attB
bar
NOS
FRT
attB
NOS
C
lox71
Selection
Circularization
B)
Targeting
Stacking
RS
Maize Ubi
bar
NOS
RS
lox66
attB
bar
NOS
FRT
attB
NOS
C
lox71
Selection
Circularization
Selection Removal
Circularization
Targeting

Figure 4.1- pJC-YattB and pJC-YattP BiBAC Vector Maps: BiBAC donor molecules contain a maize ubiquitin-1 promoter driving the expression of a coding sequence (*bar)* conferring resistance to bialaphos herbicide treatments used to select for positive events in tissue culture. Ubi-*bar*-NOSt is flanked by *RS* binding sites, which are recognized by R recombinase enzymes to remove selection (selection region; blue). Downstream of *RS*-Ubi-*bar*-NOSt-*RS* is a circularization region that is flanked by mutant *loxP* sites, *lox66* and *lox71* (Albert et al., 1995), which are bound by Cre recombinase enzymes to circularize the BiBAC inserts in a unidirectional manner (Green). Within the circularization region are the Targeting (Orange) and Stacking subregions (Blue/ orange). The Targeting subregion for pJC-YattB utilizes phiC31 Integrase to target transgenes in a unidirectional manner to areas of the genome containing *attP* sites. The Stacking subregion is utilized for selection removal and subsequent integration of a secondary BiBAC (Figure 4.3). The Targeting subregion for pJC-YattP utilizes phiC31 Integrase to target transgenes in a unidirectional manner to areas of the genome containing *attB* sites. The Stacking subregion is utilized for selection removal and subsequent integration of a tertiary BiBAC (Figure 4.3). Cargo for the BiBAC vector is represented as a “C”

of selection through R expression (Figure 4.1A; Figure 4.2B). Downstream of *RS*-Ubi-*bar*-NOSt-*RS* is a region that is flanked by mutant *lox66* and *lox71* in a head-to-tail orientation, which are utilized to carry out unidirectional circularization of flanked sequences through Cre recombinase expression (Albert et al., 1995) (Figure 4.1A; Figure 4.2D). Within the circularization region are three distinct subregions that are responsible for targeting, stacking, and a multiple cloning site (MCS) for DNA cargo. The targeting subregion contains a promoterless *attB-bar*-NOSt coding sequence, which functions as a promoter trap in intermolecular recombination reactions with Ubi-*FRT-attP* targets using phiC31 Integrase (Figure 4.2D; Figure 4.3A). Upon integration, the targeting subregion forms Ubi-*FRT-attR-bar*-NOSt-*FRT* with target sequences (Figure 4.3B). Using FLPe stable expression lines, the primary BiBAC integration line stacking subregion is modified to remove the *FRT* flanked *bar* selectable marker gene (Figure 4.3B; Figure 4.3C). FLPe-mediated *bar* removal forms Ubi-*FRT-attB,* functioning as a target for secondary BiBAC vectors that contain *attP* recombinase binding sites (Figure 4.3D).

The pJC-YattP BiBAC constructs (Figure 4.1B) are essentially identical to the pJC-YattB BiBAC vectors (Figure 4.1A); however, pJC-YattB vectors contain a targeting subregion that contain a promoterless *attP-bar*-NOSt coding sequence, which functions as a promoter trap in intermolecular recombination reactions with Ubi-*FRT-attB* targets using phiC31 Integrase (Figure 4.3E). Upon integration of a pJC-YattP BiBAC, the targeting subregion forms Ubi-*FRT-attR*-


A)
RS
Maize Ubi
bar
NOS
RS
lox66
attB
bar
NOS
FRT
attB
NOS
C
lox71
pJC-YattB
(Selection)
R
+
B)
RS
lox66
attB
bar
NOS
FRT
attB
NOS
C
lox71
pJC-YattB + R
(Selection)
–
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C)
RS
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attB
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NOS
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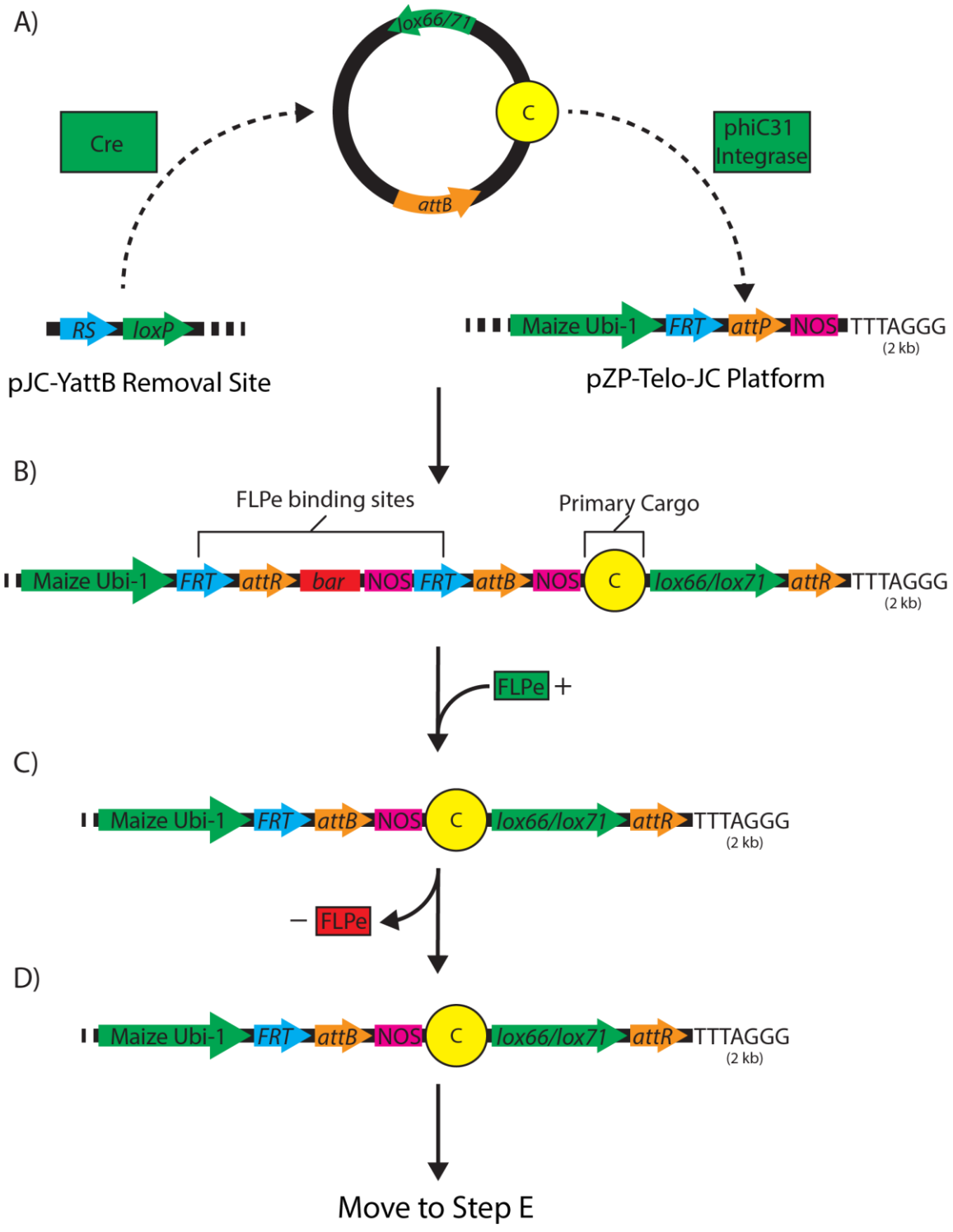
lox71
pJC-YattB
(No Selection)
phiC31
Integrase
+
Cre
+
attP
Target
D)
lox66/71
C
attB
Transgene + phiC31
+ Cre + attP Target
(Selection)
RS
loxP
BiBAC Removal Site
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC Platform
Selection Removal
Circularization
Targeting

Figure 4.2 - Molecular Strategy for Priming and Mobilizing BiBAC Inserts to Predetermined Locations: Stable integrations of pJC-YattB is selected in tissue culture using a *bar* selectable marker (A). T_0 pJC-YattB plants are crossed to R expression lines, which removes the selectable marker that is flanked by *RS* recombinase binding sites (B). T_1 transgenic plants containing pJC-YattB + R are crossed to Hi-II tester lines to segregate away R (C). T_2 progeny containing pJC-YattB without *bar* selection or R recombinase are selected and crossed to lines containing phiC31 Integrase, Cre, and *attP* target lines (D). Lines containing all the components will sequentially circularize the BiBAC donor molecule with Cre and integrate it into a *attP* target, such as the pZP-Telo-JC platform, with phiC31 Integrase. Cargo for the BiBAC vector is represented as a “C”.


A)
lox66/71
C
attB
Cre
phiC31
Integrase
RS
loxP
pJC-YattB Removal Site
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC Platform
B)
FLPe binding sites
Primary Cargo
Maize Ubi-1
FRT
attR
bar
NOS
FRT
attB
NOS
C
lox66/lox71
attR
TTTAGGG
(2 kb)
FLPe +
C)
Maize Ubi-1
FRT
attB
NOS
C
lox66/lox71
attR
TTTAGGG
(2 kb)
– FLPe
D)
Maize Ubi-1
FRT
attB
NOS
C
lox66/lox71
attR
TTTAGGG
(2 kb)
Move to Step E

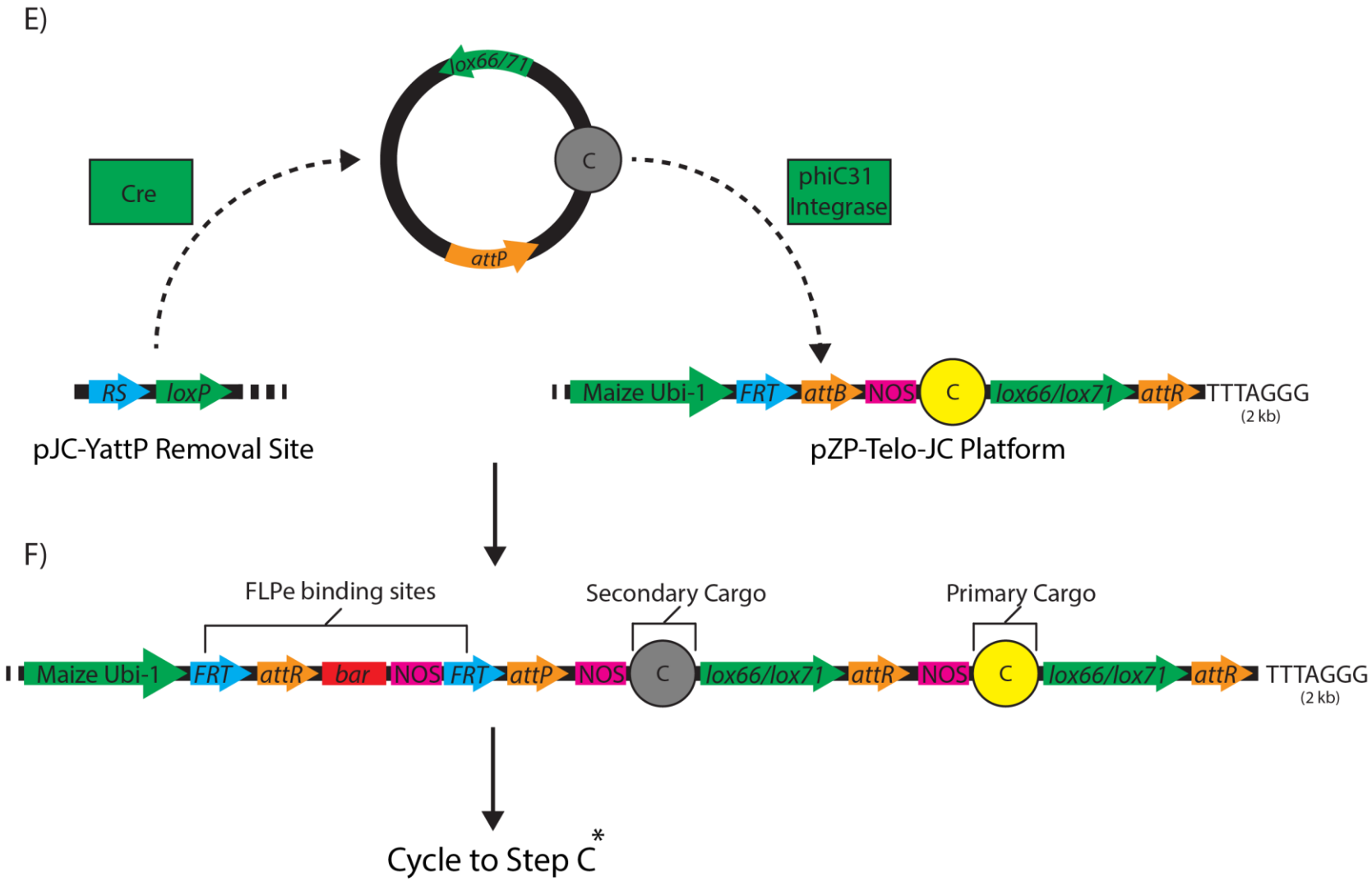

E)
lox66/71
C
attP
Cre
phiC31
Integrase
RS
loxP
pJC-YattP Removal Site
Maize Ubi-1
FRT
attB
NOS
C
lox66/lox71
attR
TTTAGGG
(2 kb)
pZP-Telo-JC Platform
F)
FLPe binding sites
Secondary Cargo
Primary Cargo
Maize Ubi-1
FRT
attR
bar
NOS
FRT
attP
NOS
C
lox66/lox71
attR
NOS
C
lox66/lox71
attR
TTTAGGG
(2 kb)
Cycle to Step C*

Figure 4.3 - Cycled Stacking of pJC-YattB and pJC-YattP Vectors to Target Platforms: Lines containing all the components will sequentially circularize the BiBAC donor molecule with Cre and integrate it into a *attP* target, such as the pZP-Telo-JC platform, with phiC31 Integrase (A). Upon integration, the targeting subregion forms Ubi-*FRT-attR-bar*-NOSt-*FRT* with target sequences (B). Using FLPe stable expression lines, the primary BiBAC integration line stacking subregion is modified to remove the *FRT* flanked *bar* selectable marker gene (C). FLPe-mediated *bar* removal forms Ubi-*FRT-attB* (D), functioning as a target for secondary BiBAC vectors that contain *attP* recombinase binding sites (E). Upon integration of a pJC-YattP BiBAC, the targeting subregion forms Ubi-*FRT-attR-bar*-NOSt-*FRT* with target sequences (F). The Target platform contains both primary and secondary cargo inserts (F). The stacking process can be cycled to step C; however, targeting will be utilizing an *attP* target site.

bar-NOSt-*FRT* with target sequences (Figure 4.3F). Using FLPe stable expression lines, secondary BiBAC integration line stacking subregion is modified to remove the *FRT* flanked *bar* selectable marker gene, forming Ubi-*FRT*-*attP* (Figure 4.3C). The Ubi-*FRT*-attP site is used as a target for tertiary BiBAC vectors that contain *attB* recombinase binding sites.

The pJC-YattB and pJC-YattP BiBAC vectors function as an alternating system with platform target sequences that contain complementary *attB* or *attP* recombinase binding sites. This enables researchers to indefinitely amend large BiBAC gene fragments into a predetermined location in the maize genome. In this work, we target platform locations outside the native chromosomal set created in Chapter 3.

Genomic Yeast DNA Integration into pJC BiBAC MCS:

To demonstrate the transfer of a large amount of DNA into a predetermined location in the maize genome, such as an artificial minichromosome, pJC-YattB and pJC-YattP constructs were used in a cloning reaction together with genomic DNA fragments from *Saccharomyces cerevisiae* (yeast). Table 4.1 shows the number of clones on hand with insert sizes varying from ~35 to 230 kb. Pulse field gel analysis of 4 out of 11 pJC-YattB (Figure 4.4) and 10 out of 10 pJC-YattP (Figure 4.5) constructs show varying size of the yeast DNA inserts. Since pJC-YattB is the primary BiBAC targeted to *attP* platforms, the 4 constructs from the pulse field were used to create ~550 bp

BiBAC Vector	Yeast Insert (kb)
pJC-YattB-35	~35
pJC-YattB-40	~40
pJC-YattB-45A	~45
pJC-YattB-45B	~45
pJC-YattB-45C	~45
pJC-YattB-95	~95
pJC-YattB-100A	~100
pJC-YattB-100B	~100
pJC-YattB-100C	~100
pJC-YattB-230A	~230
pJC-YattB-230B	~230

BiBAC Vector	Yeast Insert (kb)
pJC-YattP-35A	~35
pJC-YattP-35B	~35
pJC-YattP-40	~40
pJC-YattP-45A	~45
pJC-YattP-45B	~45
pJC-YattP-97	~97
pJC-YattP-100A	~100
pJC-YattP-100B	~100
pJC-YattP-110	~110
pJC-YattP-200	~200

Table 4.1 - The pJC-YattB and pJC-YattP Vector Insert Sizes: This table highlights the pJC-YattB BiBAC vectors with respect to the genomic yeast insert size.

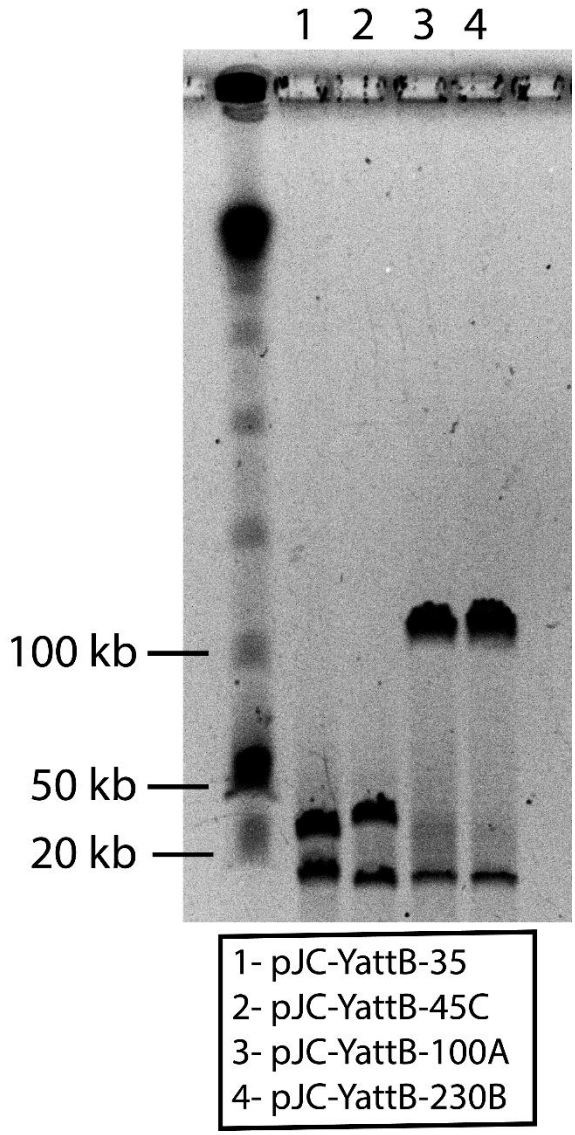


Figure 4.4 - Pulse Field Gel Analysis of pJC-YattB Genomic Yeast Inserts: The pJC-YattB clones 35(1), 45C(2), 100A(3), and 230(4) were digested with *Asc*I and *Not*I restriction endonucleases, which excised the genomic yeast DNA from the BiBAC vector backbone. Pulse field gel analysis of the digested vectors exhibited varying insert sizes. Top band of each lane represents the genomic yeast fragment, while the bottom is the BiBAC vector backbone.

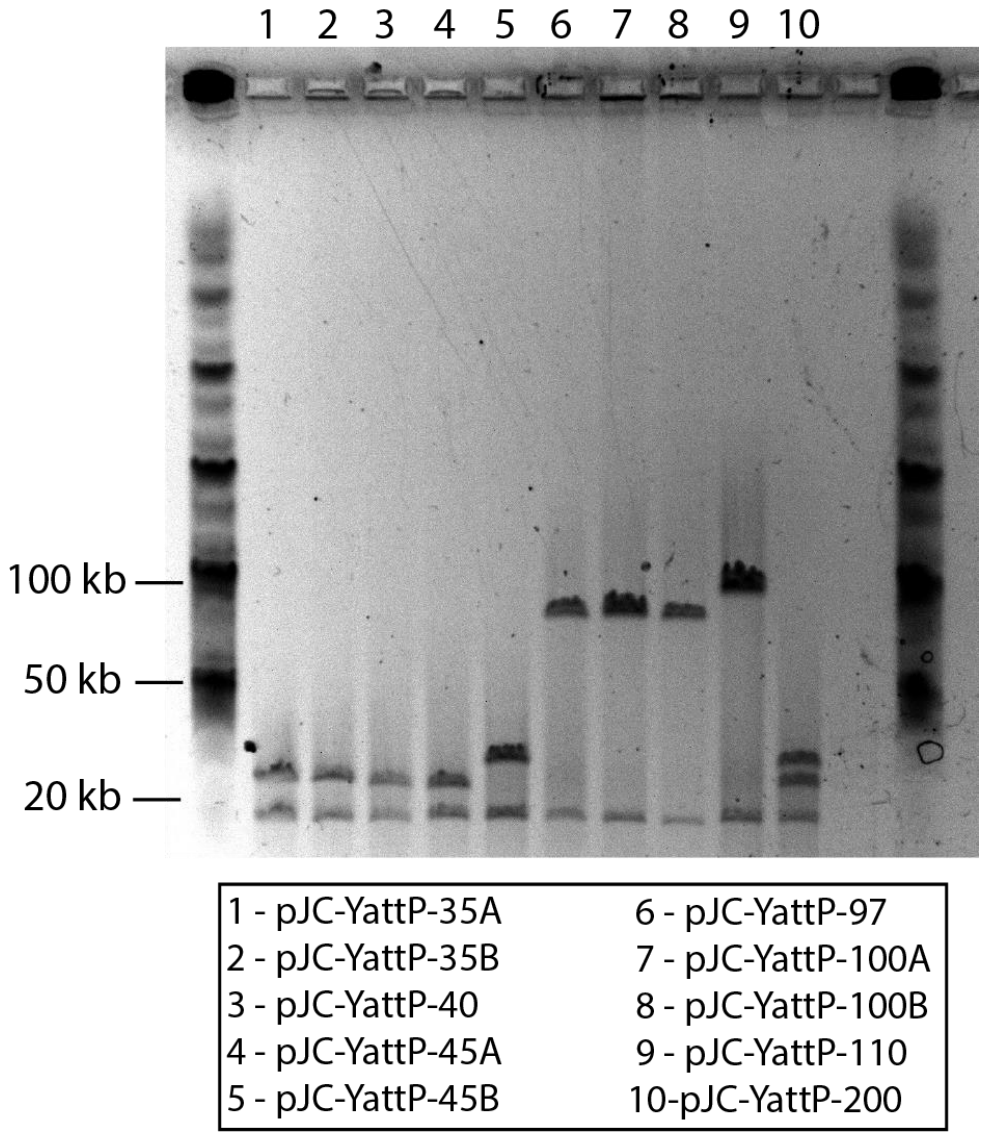


Figure 4.5 - **Pulse Field Gel Analysis of pJC-YattP Genomic Yeast Inserts:** The pJC-YattB clones 35A(1), 35B(2), 40(3), 45A(4), 45B(5), 97(6), 100A(7), 100B(8), 110(9), and 200(10) were digested with *Asc*I and *Not*I restriction endonucleases, which excised the genomic yeast DNA from the BiBAC vector backbone. Pulse field gel analysis of the digested vectors exhibited varying insert sizes. Top band of each lane represents the genomic yeast fragment, while the bottom is the BiBAC vector backbone.

insert TruSeq libraries for Illumina sequencing using a NextSeq 500 system. The construct pJC-YattB-35 was chosen for processing analysis using Geneious bioinformatics software for *de novo* assembly of the Illumina reads (Figure 4.6). By mapping the assembled yeast fragment to a *Saccharomyces cerevisiae* reference genome, the yeast insert aligns to chromosome 11 and contains a collection of endogenous genes that are highlighted in Table 4.2**.**

Agrobacterium Transformation of pJC BiBACs:

Hi-II immature embryo explants were harvested 9-12 days after pollination between Hi-II A and Hi-II B inbred lines and utilized in the transformation experiments using the pJC-YattB-35 BiBAC T-DNA molecule (Figure 4.1A). Table 4.3 outlines the dates, embryo count, and conditions (OD_{600}) of each transformation procedure. A total of 2497 embryos was used in the *Agrobacterium* transformation experiment at an average OD_{600} of 0.52. Table 4.4 highlights the number of positive events recovered, date of transformation, and whether T_1 seed was recovered in a cross with a Hi-II tester plant. A total of 6 events was recovered from 2497 embryos, giving a total transformation efficiency of 0.24%.

Hi-II immature embryo explants were harvested 9-12 days after pollination between Hi-II A and Hi-II B inbred lines and utilized in the transformation experiments using the pJC-YattP-40 BiBAC T-DNA molecule (Figure 4.1B). Table 4.5 outlines the dates, embryo count, and conditions (OD_{600}) of each

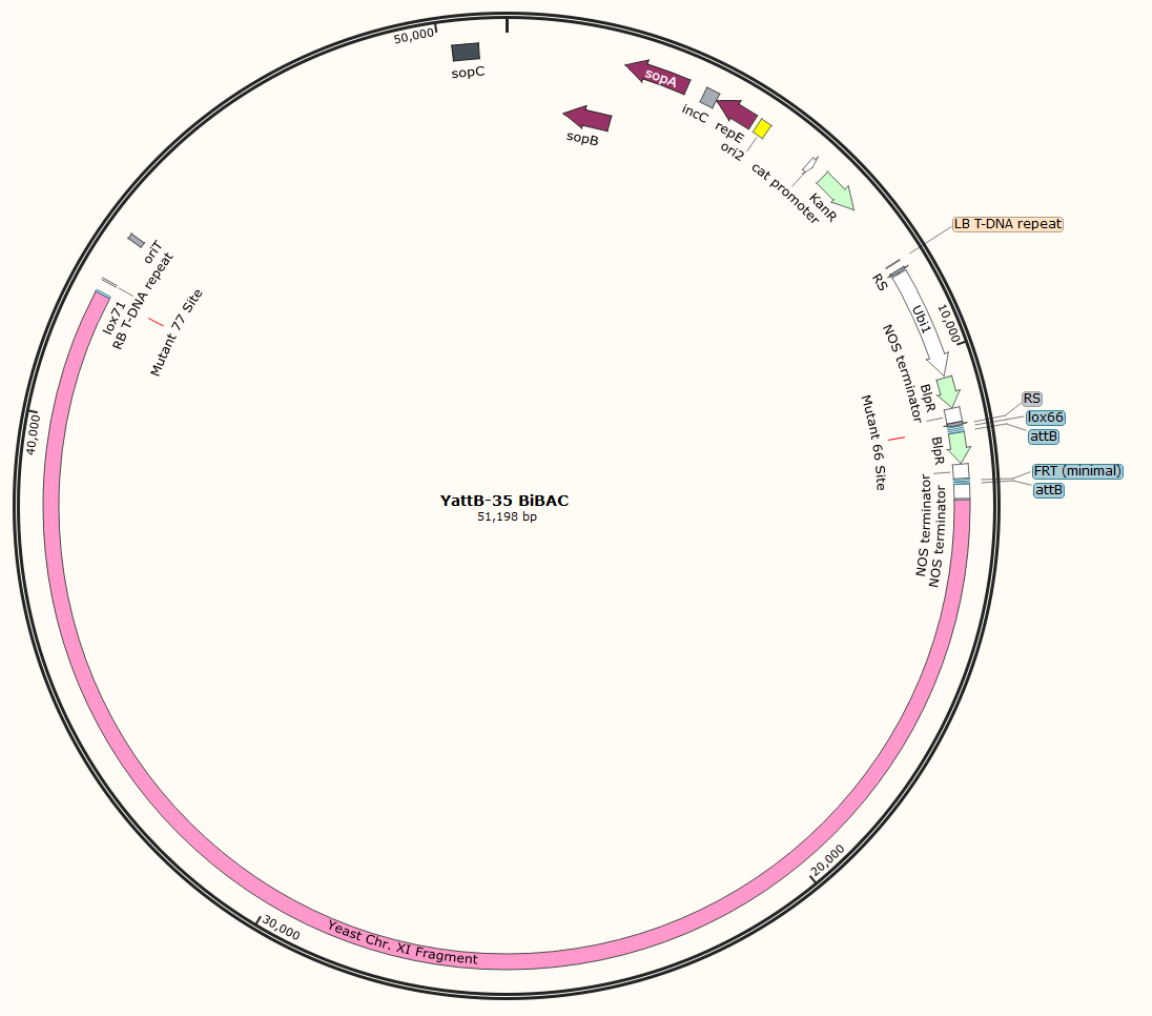


Figure 4.6 - Vector Map Derived from De Novo Assembly of pJC-YattB-35 Illumina Reads: Illumina reads from a pJC-YattB-35 TruSeq library and processed with Genious bioinformatics software assembled a circular map containing all the components outlined in Figure 4.1. The genomic yeast fragment is derived from chromosome 11 and contains a collection of 16 genes of varying function (Table 4.2).

Gene	Description
DCW1	Putative mannosidase; GPI-anchored membrane proteinrequired for cell wall biosynthesis in budformation
PRI2	Subunit of DNA primase; DNA primase is required forDNA synthesis and double-strand break repair
MMO1	hypothetical protein; MMO1 is a non-essential gene
PHD1	Transcriptional activator that enhances pseudohyphal growth; physically interacts with the Tup1-Cyc8 complex and recruits Tup1p to its targets; regulates expression of FLO11, an adhesin required for pseudohyphal filament formation; similar to StuA, an A. nidulans developmental regulator; potential Cdc28p substrate; PHD1 has a paralog, SOK2, that arose from the whole genome duplication
SPC42	Central plaque component of spindle pole body (SPB); involved in SPB duplication, may facilitate attachment of the SPB to the nuclear membrane
VPS24	One of four subunits of the ESCRT-III complex; forms an endosomal sorting complex required for transport III (ESCRT-III) subcomplex with Did4p; involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway
NFU1	Protein involved in Fe-S cluster transfer to mitochondrial clients; protects [4Fe-4S] clusters from damage due to oxidative stress; acts along with Bol3 at a late step in the transfer of [4Fe-4S] clusters from the ISA complex to client proteins; Fe-S loaded homodimer at steady state; similar to NifU, a bacterial protein required for Fe/S cluster maturation; ortholog of the human NFU1, mutations of which are associated with Multiple Mitochondria Dysfunctions Syndrome (MMDS1)
PTM1	hypothetical protein; copurifies with late Golgi vesicles containing the v-SNARE Tlg2p
SNR69	C/D box small nucleolar RNA (snoRNA); guides 2'-O-methylation of large subunit (LSU) rRNA at position C2948
RGT1	Glucose-responsive transcription factor; regulates expression of several glucose transporter (HXT) genes in response to glucose; binds to promoters and acts both as a transcriptional activator and repressor; recruits Tup1p/Cyc8p to target gene promoters; RGT1 has a paralog, EDS1, that arose from the whole genome duplication
AIM26	hypothetical protein; null mutant is viable
UGP1	UDP-glucose pyrophosphorylase (UGPase); catalyses the reversible formation of UDP-Glc from glucose 1-phosphate and UTP, involved in a wide variety of metabolic pathways, expression modulated by Pho85p through Pho4p; involved in PKA-mediated oxidative stress resistance and long-term survival in stationary phase; UGP1 has a paralog, YHL012W, that arose from the whole genome duplication
TUL1	Subunit of the DSC ubiquitin ligase complex; golgi-localized RING-finger ubiquitin ligase (E3) involved in sorting polar transmembrane domain containing membrane proteins to multivesicular bodies for delivery to the vacuole; proposed involvement in the quality control of misfolded TMD containing proteins; ortholog of fission yeast dsc1
Putative hydrolase	hypothetical protein
TTI1	Subunit of the ASTRA complex, involved in chromatinremodeling; detected in highly purified mitochondriain high-throughput studies; similar toS. pombe Tti1p; telomere length regulator involved in thestability or biogenesis of PIKKs such as TORC1
tRNA-Val	Valine tRNA (tRNA-Val), predicted by tRNAscan-SE analysis

Table 4.2 - The Genes in the pJC-YattB-35 Yeast Insert: De novo assembly of Illumina sequencing reads from pJC-YattB-35 were mapped to a *Saccharomyces cerevisiae* reference genome using Genious bioinformatics software. 16 genes of varying function are outlined in this table, all of which are stably integrated into maize without interference of endogenous gene function.

Date	Construct	Embryo No.	OD_{600}
4/24/17	pJC-YattB-35	431	0.6
6/22/17	pJC-YattB-35	278	0.7
7/28/17	pJC-YattB-35	226	0.62
8/4/17	pJC-YattB-35	540	0.33
8/17/17	pJC-YattB-35	594	0.33
8/28/17	pJC-YattB-35	428	0.55

Total	2497
Average OD	0.52

Table 4.3 - *Agrobacterium*-Mediated Transformation of Hi-II Immature Embryos Using pJC-YattB-35 BiBAC Vectors. This table highlights the embryo count and *Agrobacterium* concentration (OD_{600}) of each transformation carried out in the 2017 season. The total number of embryos used in the creation of the 6 pJC-YattB-35 events is 2497. The average OD_{600} = 0.52.

Event	Date Transformed	PCR +	T_1 Seed
1	6/22/17	X	X
2	8/4/17	-	-
3	8/28/17	X	X
4	8/28/17	X	X
5	8/28/17	X	X
6	8/4/17	-	-

Table 4.4 - pJC-YattB-35 Events Recovered from *Agrobacterium*-Mediated Transformation of Hi-II Immature Embryos. This table highlights the event number, date of transformation, PCR analysis results, and recovery of T_1 seed in a cross with a Hi-II B tester line in greenhouse conditions. "-" = not tested; "X" = positive.

Date	Construct	Embryo No.	OD_{600}
8/15/18	pJC-YattP-40	314	0.55
8/20/18	pJC-YattP-40	215	0.53

Total	529
Average OD	0.54

Table 4.5 - *Agrobacterium*-Mediated Transformation of Hi-II Immature Embryos Using pJC-YattP-40 BiBAC Vectors. This table highlights the embryo count and *Agrobacterium* concentration (OD_{600}) of each transformation carried out in the 2018 season. The total number of embryos used in the creation of the 6 pJC-YattP-40 events is 529. The average OD_{600} = 0.54.

Event	Date Transformed	PCR +	T_1 Seed
1	8/15/18	-	X
2	8/15/18	-	-
3	8/15/18	-	-
4	8/20/18	-	-
5	8/20/18	-	-

Table 4.6 - pJC-YattP-40 Events Recovered from *Agrobacterium*-Mediated Transformation of Hi-II Immature Embryos. This table highlights the event number, date of transformation, PCR analysis results, and recovery of T_1 seed in a cross with a Hi-II B tester line in greenhouse conditions. Events 2, 4, and 5 died in tissue culture conditions, while event 3 died in the greenhouse. Event 1 seed was recovered; however, has not been PCR screened to verify the integrity of the BiBAC insert. "-" = not tested; "X" = positive.

transformation procedure. A total of 529 embryos was used in the *Agrobacterium* transformation experiment at an average OD_{600} of 0.54. Table 4.6 highlights the number of positive events recovered, date of transformation, and whether T_1 seed was recovered in a cross with a Hi-II tester plant. A total of 5 events was recovered from 529 embryos, giving a total transformation efficiency of ~0.9%; however, only 1 event was regenerated and crossed to Hi-II tester plants in greenhouse conditions.

PCR analysis of pJC-YattB-35 BiBAC Inserts:

Figure 4.7 and Table 4.7 outline PCR primer sequences and DNA binding sites, respectively, used to screen for BiBAC integrity. Of the recovered T_1 seed, pJC-YattB-35 event 4 lost a portion of the right border sequence containing the *lox71* site required for BiBAC circularization, while events 1 and 3 were completely intact. Figure 4.8 highlights the PCR fragments amplified from different regions of the transgene insert, indicating that event 1 is completely intact. pJC-YattB-35 #1 was chosen as the insert to use in future gene targeting experiments.

Fluorescent in situ Hybridization of pJC-YattB-35 Inserts:

T_0 plants PCR positive for stable integrations of pJC-YattB-35-1 were used in single gene fluorescent in situ hybridization analysis to determine the point of

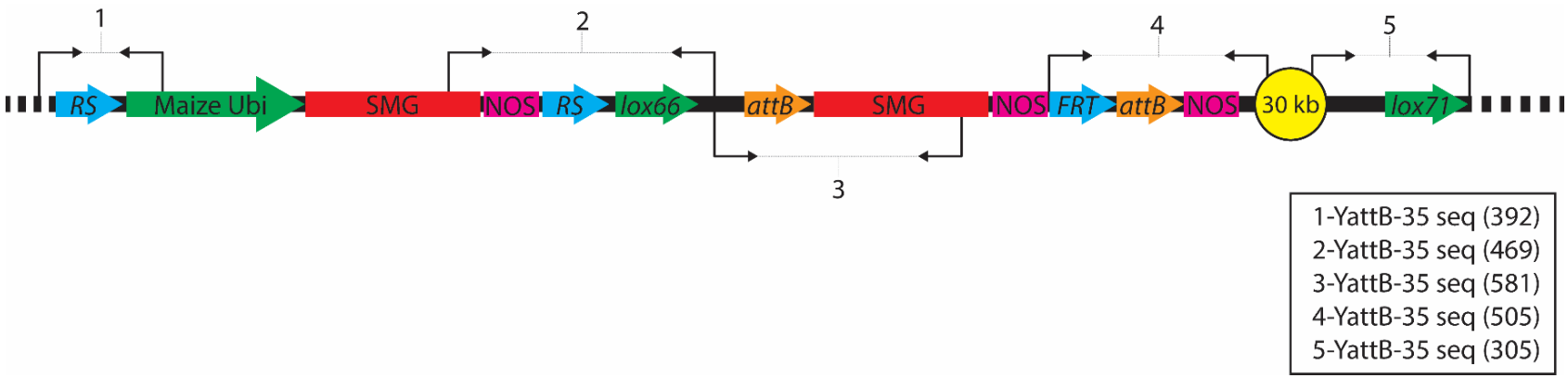


Figure 4.7 – Binding Locations of Primers Used to Screen pJC-YattB-35 Events: Arrows on vector map indicate the relative binding location of primer sets used to analyze the integrity of BiBAC events. The number between each amplified region is with respect to the primer outlined in the key. Further information of primer sets can be found in Table 4.7.

Primer (size bp)	Sequence	T_a
1-YattB-35 seq (392)	F - 5′ TGTAGATGTCCGCGGTTAAT 3′ R - 5′ AAAAAGGAGAACACATGCACA 3′	60°C
2-YattB-35 seq (469)	F - 5′ GTTTCTGGCAGCTGGACTTC 3′ R - 5′ GGTACCCAATTCGCCCTAT 3′	60°C
3-YattB-35 seq (581)	F - 5′ CACTATAGGGCGAATTGGGTA 3′ R - 5′ GAAGTCCAGCTGCCAGAAAC 3′	60°C
4-YattB-35 seq (505)	F - 5′ ATCCGATGATAAGCTGTCAAAG 3′ R - 5′ TTGCCCGTTTCTTAGGTGTC 3′	60°C
5-YattB-35 seq (305)	F - 5′ CATTTCCTGCAGCATTTCCT 3′ R - 5′ CAGGCCAAATTCGCTCTTAG 3′	60°C

Table 4.7 – Primer Sequences for Screening pJC-YattB-35 Events: Each primer set listed is designed to anneal to target sequences at 60°C when running a PCR reaction. The banding size of each recombinase is included after the name in parentheses. For information regarding binding location of each primer set, refer to Figure 4.7.

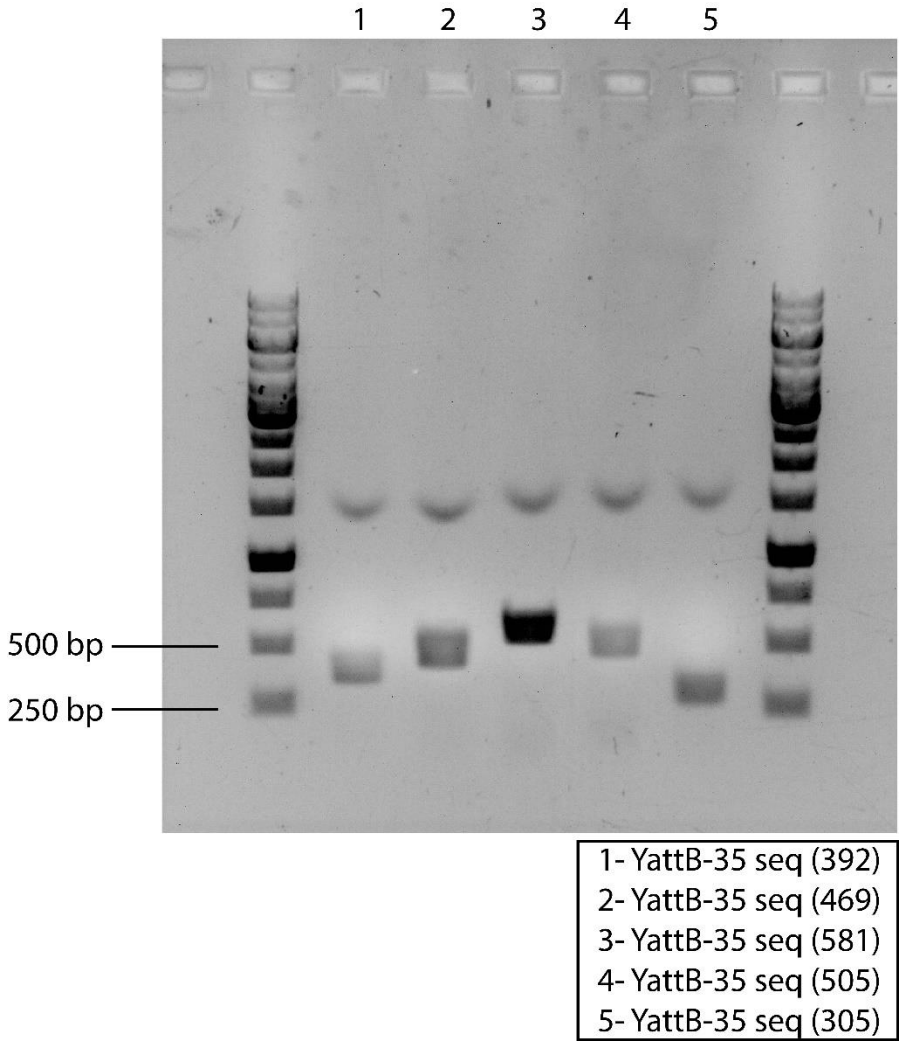


Figure 4.8 – Agarose Gel Image of PCR Fragments Amplified from Different Regions of the pJC-YattB-35-1 Event: Genomic DNA isolated from leaf tissue of T_0 pJC-YattB-35-1 plants was used as a template in PCR reactions utilizing the primer sets outlined in Table 4.7 and Figure 4.7.

integration. Hybridization with pJC-YattB-35 BiBACs labeled with Texas Red shows the insert location on chromosome 6 (Figure 4.9).

Additional Information on pJC-YattP-40 Inserts:

The pJC-YattP-40 event was chosen for *Agrobacterium*-mediated transformation. However, this vector has not yet been Illumina sequenced because it will be used at a later time for a second addition to a landing target site. Stable integrations of pJC-YattP-40 have not yet been PCR analyzed to determine integrity of the insert. Additionally, pJC-YattP-40 have not yet been screened using fluorescent in situ hybridization to determine the insert location.

Discussion

While commonly used T-DNA vectors are assumed to be capable of maintaining DNA above 300 kb (Tao and Zhang, 1998), the utility of these systems with large gene inserts in genetic engineering is difficult and uncommon. Conventional T-DNA vectors were originally designed for ease of handling, enabling high concentrations of DNA to be obtained using common plasmid extractions methods in *Escherichia coli*. This increases the efficiency of T-DNA vector modifications but limits the amount of DNA that can be effectively transformed onto host plant organisms utilizing *Agrobacterium*-mediated transformation. If taken together with the random nature of transgene integrations in conventional genetic engineering methods, the use of common T-DNA vectors

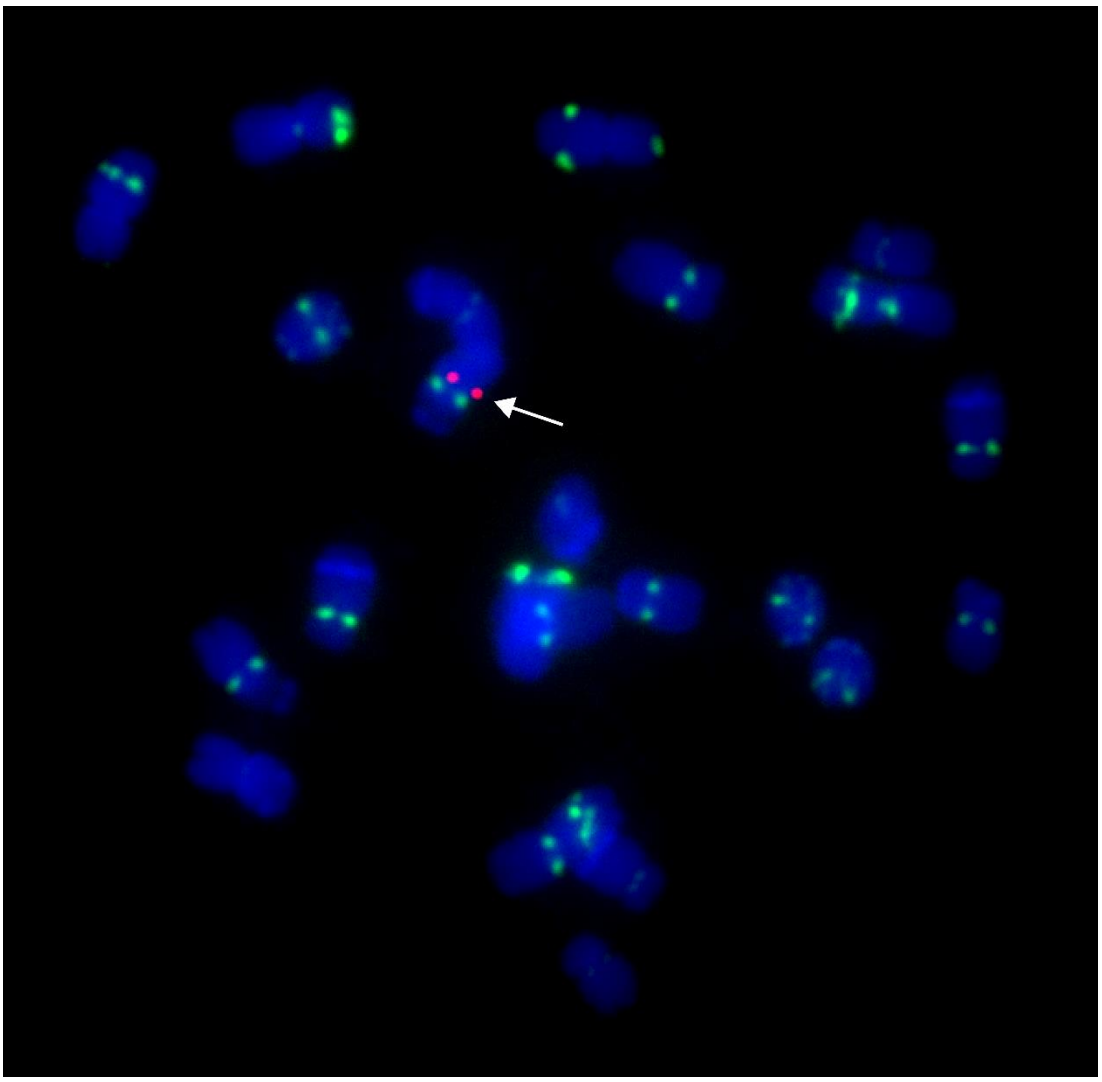


Figure 4.9 - Fluorescent In Situ Hybridization of pJC-YattB-35-1 T_1 Plants Using pJC-YattB-35 Red Probes: A cocktail containing CentC, TAG, and pJC-YattB35 red were hybridized to slides containing chromosomes spreads from pJC-YattB-35-1 plants. The white arrow points to red signal on chromosome 6, which indicates the insertion point of pJC-YattB-35-1.

further reduces the number of traits that can be utilized to improve plants due to the difficulty of maintaining multiple transgene inserts in successive generations. With many useful traits requiring coordinated expression and regulation of several genes, there is a need for an improved method that increases the amount of DNA delivered into plants and enables researchers to target genes to a single location in the genome.

Though the use of binary bacterial artificial chromosomes (BiBAC) and site-specific recombinase (SSR) systems, this work focuses on creating a BiBAC donor vector that enables researchers to integrate large T-DNA fragments into host cells and subsequently mobilize the insert for transfer into predetermined locations in the maize genome (Figure 4.2D). While most gene targeting studies focus on direct transfer of the donor molecule to predetermined sites (Srivastava and Ow, 2002; Srivastava and Thomson, 2016; Anand et al. 2019), pJC-YattB BiBACs were designed for stable integrations prior to targeting, which avoids possible T-DNA fragmentation and may increase the probability of obtaining a downstream targeting event since every cell of a modified plant will contain a copy of the BiBAC transgene. However, this strategy requires the timely expression of a collection of SSR systems at different points of the transfer process, which will take several generations of processing to prime BiBAC inserts for transfer (Figure 4.2).

The pJC-YattB-35-1 event is an intact T-DNA molecule (Figure 4.8) that contains ~30 kb of genomic DNA from *Saccharomyces cerevisiae* (Figure 4.4)

and is stably integrated on chromosome 6 in the maize genome (Figure 4.9). Plants containing pJC-YattB-35-1 inserts were designed to be crossed to a R recombinase stable expression line (Chapter 2) to remove the selectable marker gene flanked by *RS* recombinase binding sites (Figure 4.1A; Figure 4.2B). This produces progeny that contain both modified pJC-YattB-35-1 inserts and R, which are crossed to Hi-II tester plants to segregate away the recombinase enzyme (Figure 4.2C). Plants that contain only the modified pJC-YattB-35-1 insert are used as the background in the BiBAC transfer process, requiring the coordinated expression of both Cre and phiC31 integrase recombinase enzymes (Figure 4.2D). A line containing an *attP* target site and stably expressing Cre and phiC31 integrase is crossed to the modified pJC-YattB-35-1 insert line to initiate the transfer process (Figure 4.2D). Cre recombinase binds to *lox66* and *lox71* binding sites, inducing a circularization of the T-DNA insert (Figure 4.2D). These extrachromosomal molecules contain *attB* binding sites, which are bound by phiC31 integrase to induce a unidirectional recombination reaction that integrates the BiBAC into a site that contains an *attP* sequence (Figure 4.2D).

In Chapter 3, we engineered *attP* sites behind a maize ubiquitin promoter on the pZP-Telo-JC truncation construct, which was used to create minichromosome (17-27) and B insert (17-13) lines. If these lines are used together with pJC-YattB-35-1 inserts, it may be possible to transfer a large amount of DNA into an artificial minichromosome or B chromosome using the coordinated expression of Cre and phiC31 recombinases (Figure 4.2D), which

were demonstrated to be functional in maize in Chapter 2. While the efficiency of such transfer is unknown, it is assumed that the primary BiBAC transfer will be possible through either breeding or tissue culture strategy. Once stable integration is identified, primary BiBAC integration lines can be crossed to FLPe stable expression lines to prime minichromosomes or B inserts for the secondary BiBAC integration with pJC-YattP inserts (Figure 4.3). If possible, this cyclical system will create an avenue for researchers to stack genes in a single location in the maize genome, which could be used to create lines expressing traits that require the coordinated expression and regulation of many genes.

Material and Methods

pJC-YattB BiBAC Vector Construction:

DNA fragment *RS*-Ubi-*bar*-NOSt-*RS*-*lox66* (Fragment 1) was synthesized by Genscript and cloned into pBluscript II KS(+) using *Bam*HI and *Hin*dIII restriction endonucleases to produce pBluSK-FragmentNo1. DNA fragment *attB*-*bar*-NOSt-*FRT*-*attB*-NOSt-MCS-*lox71* (Fragment 2) was synthesized by Genscript and cloned into pBluscript II KS(+) using *Xho*I and *Sac*I restriction endonucleases to produce pBluSK-FragmentNo2(*attB*). Fragment 1 from pBluSK-FragmentNo1 was subcloned into pBluSK-FragmentNo2(*attB*) using *Bam*HI and *Hin*dIII restriction endonucleases to produce pBluSK-FragmentNo1/2(*attB*), which contained the sequence *RS*-Ubi-*bar*-NOSt-*RS*-*lox66*-*attB*-*bar*-NOSt-*FRT*-*attB*-NOSt-MCS-*lox71*. Restriction digest analysis of

pBluSK-FragmentNo1/2 using *Swa*I and *Pac*I enzymes was carried out to verify integrity of the plasmid prior to BiBAC cloning steps.

DNA fragment *RS*-Ubi-*bar*-NOSt-*RS*-lox66-*attB*-*bar*-NOSt-*FRT*-*attB*-NOSt-MCS-*lox71* from pBluSK-FragmentNo1/2(*attB*) was gel extracted and subcloned into BiBAC vector pNG6 using *Swa*I and *Pac*I restriction endonucleases to produce pJC-attB. Sequence integrity of pJC-attB fragment 1 and fragment 2 inserts were verified by PCR TOPO TA cloning and subsequent Sanger sequencing reactions (Figure 4.10). Fragment 1 was amplified from pJC-attB using forward and reverse primers 5' – GCACATGGCTCAGTTC TCAA – 3' and 5' – GGTACCCAATTCGCCCTAT – 3', respectively, and cloned into TOPOXL Invitrogen vectors. Fragment 2 was amplified from pJC-attB using forward and reverse primers 5' – CACTATAGGGCGAATTGGGTA – 3' and 5' – GCTGCACTCCAACAGAAACA – 3', respectively, and cloned into 4-TOPO Invitrogen vectors.

Genomic DNA from *Saccharomyces cerevisiae* was cloned into pJC-attB by Intact Genomics using *Asc*I and *Not*I restriction endonucleases to produce pJC-YattB BiBACs with yeast insertions of varying sizes (Table 4.1). Insert sizes of 4 out of 11 pJC-YattB BiBACs were verified on a pulse field gel using *Asc*I and *Not*I restriction endonucleases (Figure 4.4). Sequence analysis of 4 pJC-YattB vectors was carried out using Illumina sequencing on a NextSeq 500 system followed by de novo assembly of pJC-YattB-35 utilizing Geneious software

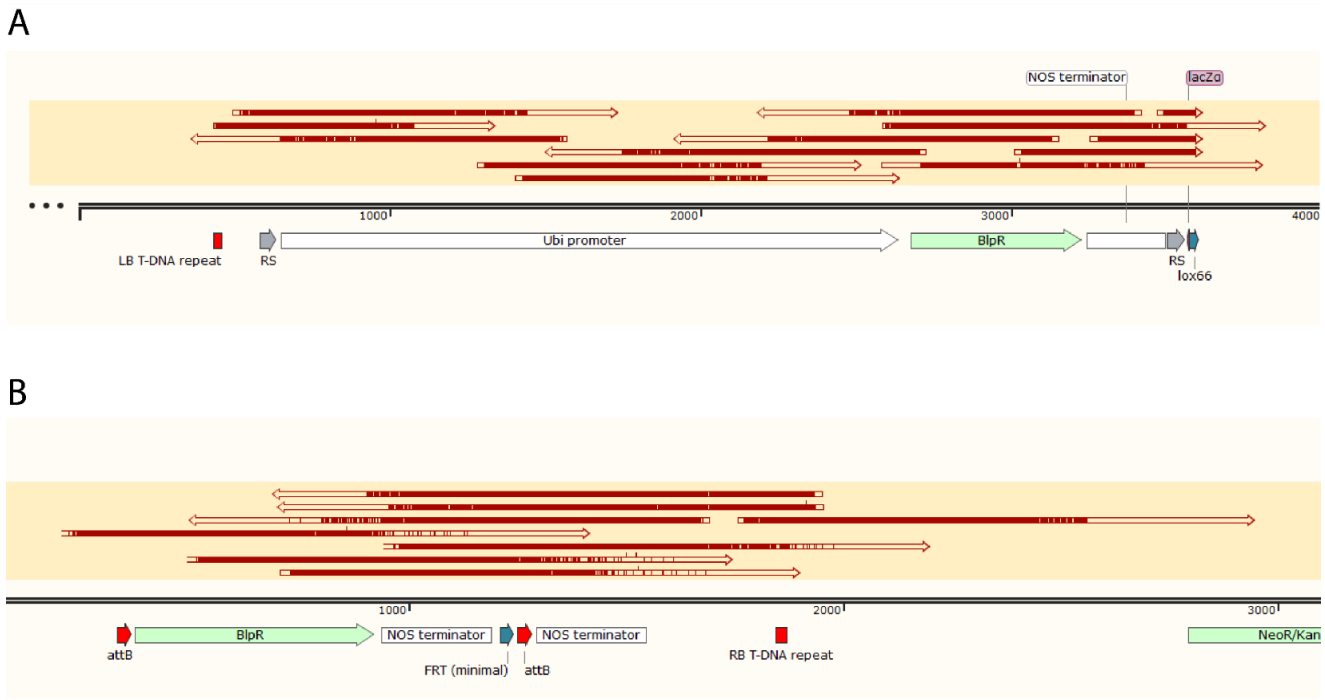


Figure 4.10 - Sanger Sequencing of pJC-attB Fragment 1 and Fragment 2 Prior to Cloning Genomic Yeast Inserts: Fragment 1 (A) and Fragment (2) were PCR amplified and cloned into TOPO vectors to verify integrity via Sanger sequencing. Each red arrow above the construct shows the relative primer binding location and running track of the read. Breaks within the read indicate “no calls” in the sequencing. The collective sequencing data from all the reads of fragment 1 (A) and fragment 2 (B) indicate that the sequence is correct prior to cloning yeast DNA.

(Figure 4.6). A detailed description of the steps required to handle and manipulate BiBAC constructs can be found in Cody et al., 2018 (Appendix 2).

pJC-YattP BiBAC Vector Construction:

DNA fragment *RS*-Ubi-*bar*-NOSt-*RS-lox66* (Fragment 1) was synthesized by Genscript and cloned into pBluscript II KS(+) using *Bam*HI and *Hin*dIII restriction endonucleases to produce pBluSK-FragmentNo1. DNA fragment *attP*-*bar*-NOSt-*FRT-attP*-NOSt-MCS-*lox71* (Fragment 2) was synthesized by Genscript and cloned into pBluscript II KS(+) using *Xho*I and *Sac*I restriction endonucleases to produce pBluSK-FragmentNo2(*attP*). Fragment 1 from pBluSK-FragmentNo1 was subcloned into pBluSK-FragmentNo2(*attP*) using *Bam*HI and *Hin*dIII restriction endonucleases to produce pBluSK-FragmentNo1/2(*attP*), which contained the sequence *RS*-Ubi-*bar*-NOSt-*RS-lox66-attP-bar*-NOSt-*FRT-attP*-NOSt-MCS-*lox71*. Restriction digest analysis of pBluSK-FragmentNo1/2 using *Swa*I and *Pac*I enzymes was carried out to verify integrity of the plasmid prior to BiBAC cloning steps.

DNA fragment *RS*-Ubi-*bar*-NOSt-*RS-lox66-attP-bar*-NOSt-*FRT-attP*-NOSt-MCS-*lox71* from pBluSK-FragmentNo1/2(*attP*) was gel extracted and subcloned into BiBAC vector pNG6 using *Swa*I and *Pac*I restriction endonucleases to produce pJC-attP. Sequence integrity of pJC-attP fragment 1 and fragment 2 inserts were verified by PCR TOPO TA cloning and subsequent Sanger sequencing reactions (Figure 4.11)**.** Fragment 1 was amplified from pJC-

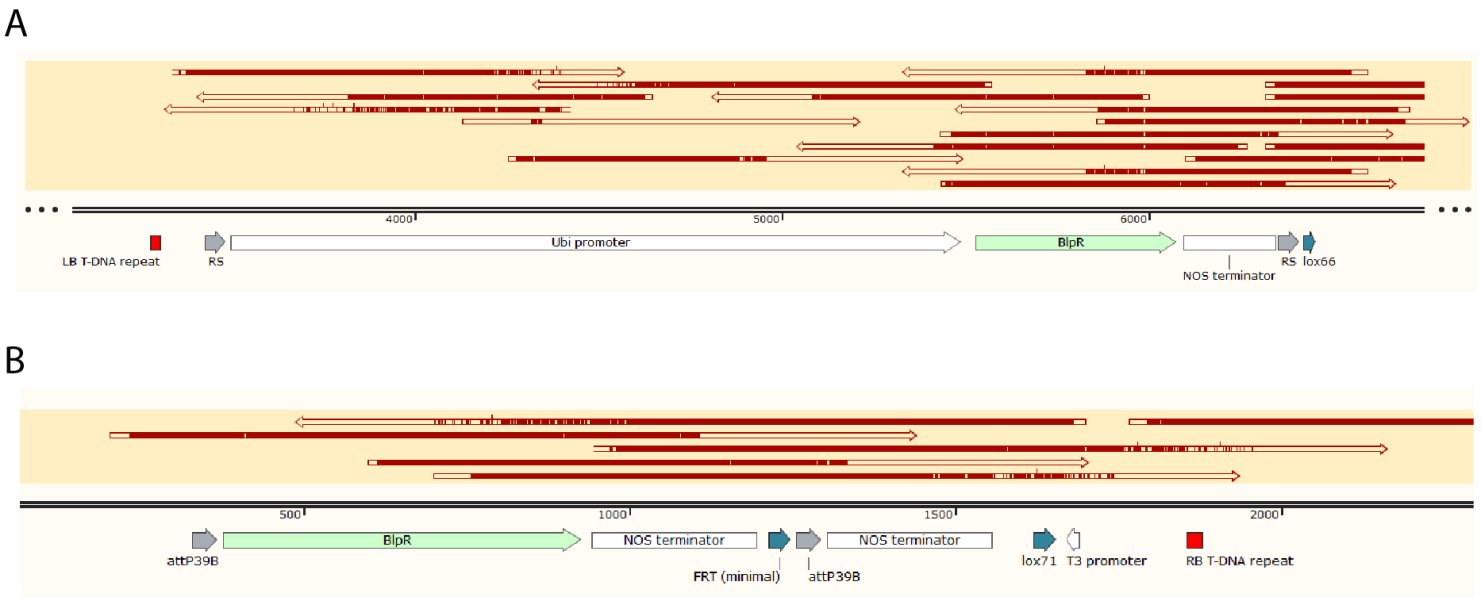


Figure 4.11 - Sanger Sequencing of pJC-attP Fragment 1 and Fragment 2 Prior to Cloning Genomic Yeast Inserts: Fragment 1 (A) and Fragment (2) were PCR amplified and cloned into TOPO vectors to verify integrity via Sanger sequencing. Each red arrow above the construct shows the relative primer binding location and running track of the read. Breaks within the read indicate “no calls” in the sequencing. The collective sequencing data from all the reads of fragment 1 (A) and fragment 2 (B) indicate that the sequence is correct prior to cloning yeast DNA.

attP using forward and reverse primers 5’ – GCACATGGCTCAGTTC TCAA – 3’ and 5’ – GGTACCCAATTCGCCCTAT – 3’, respectively, and cloned into TOPOXL Invitrogen vectors. Fragment 2 was amplified from pJC-attP using forward and reverse primers 5’ – CACTATAGGGCGAATTGGGTA – 3’ and 5’ – GCTGCACTCCAACAGAAACA – 3’, respectively, and cloned into 4-TOPO Invitrogen vectors.

Genomic DNA from *Saccharomyces cerevisiae* was cloned into pJC-attP by Intact Genomics using *Asc*I and *Not*I restriction endonucleases to produce pJC-YattP BiBACs with yeast insertions of varying sizes (Table 4.1). Insert sizes of 10 out of 10 pJC-YattP BiBACs were verified on a pulse field gel using *Asc*I and *Not*I restriction endonucleases (Figure 4.5). A detailed description of the steps required to handle and manipulate BiBAC constructs can be found in Cody et al., 2018 (Appendix 2).

Pulse Field Gel Analysis of BiBAC Constructs:

Pulse field gel electrophoresis on pJC-YattB and pJC-YattP BiBAC vectors using the CHEF-DR III system from BioRad. Vectors were digested with *Asc*I and *Not*I restriction endonucleases to dropout yeast insert fragment. Agarose gels were casted using 1% SeaKem Gold Agarose. Refer to BioRad’s website for detailed instructions on running and operating the CHEF-DR III equipment.

Plant Transformation of pJC-YattB-35 BiBACs:

BiBAC vector pJC-YattB-35 was transformed into EHA105 *Agrobacterium* cells containing pCH32 helper plasmids (See Appendix 2) by electroporation and selected on LB agar plates containing rifampicin (30 ug/ml), tetracycline (3 ug/ml) and kanamycin (50 ug/ml) antibiotics. Positive colonies were suspended in 5 ml of LB medium, containing the appropriate antibiotics, and grown for 24 – 48 hrs at 28°C shaking at 250 rpm. Glycerol stocks of each culture were generated and stored at -80°C. 2 days prior to the transformation experiment, -80°C stocks were used to create *Agrobacterium* growth plates used to create the infection media.

Immature embryo explants were collected 9-12 days after pollination between Hi-II A x Hi-II B lines (Armstrong and Green, 1985) and measured to be ~1.2 – 1.5 mm in length. Embryos were sterilized and inoculated with pJC-YattB-35 infection media (OD_{600} = 0.5) for 3-4 hrs. Infected embryos were transferred to cocultivation plates and placed at 25°C for 4 days. After 4 days, embryos were transferred to callus induction plates and placed at 28°C for ~11-12 days. Once friable type-II callus was visible, tissue was transferred to selection plates containing 3 ug/ml bialaphos and switched to new selection media plates every 2 weeks until resistant cell masses were formed. This process can take up to 12 weeks; however, positive callus should be visible at ~4-6 weeks. Root and shoot regeneration of positive callus was carried out sequentially, taking ~3 weeks each. For a full description of the preparation and process of *Agrobacterium*-

mediated transformation using BiBAC constructs, including a full list of media reagents, see Cody et al., 2018 (Appendix 2).

Plant Transformation of pJC-YattB-35 BiBACs:

BiBAC vector pJC-YattP-40 was transformed into AGL1 *Agrobacterium* cells containing pCH32 helper plasmids (See Appendix 2) by electroporation and selected on LB agar plates containing rifampicin (30 ug/ml), tetracycline (3 ug/ml) and kanamycin (50 ug/ml) antibiotics. Positive colonies were suspended in 5 ml of LB medium, containing the appropriate antibiotics, and grown for 24 – 48 hrs at 28°C shaking at 250 rpm. Glycerol stocks of each culture were generated and stored at -80°C. Two days prior to the transformation experiment, -80°C stocks were used to create *Agrobacterium* growth plates used to create the infection media.

Immature embryo explants were collected 9-12 days after pollination between Hi-II A x Hi-II B lines (Armstrong and Green, 1985) and measured to be ~1.2 – 1.5 mm in length. Embryos were sterilized and inoculated with pJC-YattP-40 infection media (OD_{600} = 0.5) for 3-4 hrs. Infected embryos were transferred to cocultivation plates and placed at 25°C for 4 days. After 4 days, embryos were transferred to callus induction plates and placed at 28°C for ~11-12 days. Once friable type-II callus was visible, tissue was transferred to selection plates containing 3 ug/ml bialaphos and switched to new selection media plates every 2 weeks until resistant cell masses were formed. This process can take up to 12

weeks; however, positive callus should be visible ~4-6 weeks. Root and shoot regeneration of positive callus was carried out sequentially, taking ~3 weeks each. For a full description of the preparation and process of *Agrobacterium*-mediated transformation using BiBAC constructs, including a full list of media reagents, see Cody et al., 2018 (Appendix 2).

PCR analysis of pJC-YattB T_0 Transgenics:

Genomic DNA from T_0 plant leaf tissue was extracted using the method described (Leach et al., 2016), and amplified using primers described in table 4.5. Standard PCR conditions of 35 cycles: 30s denature at 95°C, 30s annealing at 60°C, and Xs extension (X depends on the product size, where 1 kb = 1 min) were used to amplify DNA fragments ~250 - 500 bp in length on an agarose gel (Figure 4.6).

Fluorescent in situ hybridization:

Root samples were collected from T_0 or T_1 pJC-YattB-35 plants that were positive for all PCR fragments. Samples were treated with nitrous oxide (N_2O) for 2.5 hrs and subsequently transferred into 1.7 ml tubes containing 90% acetic acid. After 10 min, roots were washed with 70% ethanol to remove acetic acid and placed into new 1.7 ml tubes. Tubes were filled with fresh 70% ethanol and placed at -20°C until root digestion steps.

pJC-YattB-35 red probes were generated through a nick translation (NT) reaction using 1mM of Texas Red 5-dCTPs and non-labeled dNTPs (2mM each). NT reactions were purified via ethanol precipitation using salmon sperm (10 mg/ml), 0.1 volume of 3M acetic acid, and 2.0 volume of 100% ethanol, then stored at -20°C for at least 2 hrs before proceeding with centrifugation at 16,000 x g for 30 min. The supernatant was carefully discarded, and probe pellets were washed 2x with 70% ethanol. Red probe pellets were resuspended in 2x SSC, 1x TE buffer and stored at -20°C until use in hybridization.

Root samples were digested with 1x citric buffer containing 1% Pectolyase Y-23 and 2% Cellulase Onozuka R-10 (Karlan Research Products) for a period of 45 min. Each digestion mixture was washed with 70% ethanol to stop the reaction before resuspending in 100% acetic acid. A blunt dissecting probe was used to smash solid root tips in each tube. Inside a humid chamber, 6 µl of the root digest/cell suspension was pipetted onto a glass microscope slide and allowed to dry for 10 min. After drying each slide was cross-linked using a UV cross-linker at the energy of 120 – 125 mJ/cm^2. Each slide was visualized with an Olympus CX21 light microscope to assess the quality of the chromosome spreads.

8 µl karyotyping cocktails containing 1.5 µl CentC (90 ng), 1 µl TAG (20 ng), 3 µl NT red probe and 2.5 µl 2x SSC/1x TE was pipetted onto each slide and covered with a plastic cover slip before being placed into a tray and transferred into a boiling water bath for 5 min. After 5 min, the slides were placed into a

humid chamber overnight (~16 hrs). After ~16 hrs, each tube was washed with warm 2x SSC and dried with a Kimwipe. Each dried slide was treated with 1 drop of Vectashield with 5% 4′,6-diamidino-2-phenylindole (DAPI) and covered with a glass cover slip. Chromosome spreads were visualized with an Olympus BX61 fluorescent microscope. For further details of the FISH procedure see McCaw et al., 2016a, 2016b. (Appendix 4; Appendix 5).

References

Albert H., Dale E.C., Lee E., Ow D.W., (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. Plant J. 7, 649–659.

Anand A., Wu E., Li Z., TeRonde S., Arling M., Lenderts B., Mutti J.S., Gordon-Kamm W., Jones T.J., Chilcoat N.D. (2019) High efficiency *Agrobacterium*-mediated site-specific gene integration in maize utilizing the FLP-FRT recombination system. Plant Biotechnol J. doi: 10.1111/pbi.13089.

Armstrong C.L. and Green C.E., (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164, 207–214.

Bevan M.W., Flavell R.B., Chilton M.D. (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

Cody J.C., Nathaniel G.D., Birchler J.A. (2018) BiBAC Modification and Stable Transfer into Maize (Zea mays) Hi-II Immature Embryos via *Agrobacterium*-Mediated Transformation. Curr Protoc Plant Biol 2(4), doi.org/10.1002/cppb.20061.

Fraley R.T., Rogers S.G., Horsch R.B., Sanders P.R., Flick J.S., Adams S.P., Bittner M.L., Brand L. a, Fink C.L., Fry J.S., Galluppi G.R., Goldberg S.B., Hoffmann N.L., Woo, S.C. (1983) Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. U.S.A. 80, 4803–4807.

Framond A.J., Barton K.A., Chilton MD. (1983) Mini–Ti: A New Vector Strategy for Plant Genetic Engineering. Bio/Technology 1, 626-269.

Frary A. and Hamilton C. (2001) Efficiency and Stability of High Molecular Weight DNA Transformation: an Analysis in Tomato. Transgenic Research 10(2). 121-132.

Gordon J.E. and Christie P.J. (2018) The *Agrobcterium* Ti plasmid (2014) Microbiol Spectr 2(6), doi: 10.1128/microbiolspec.PLAS-0010-2013.

Hamilton C.M., Frary A., Lewis C., Tanksley S.D., (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc. Natl. Acad. Sci. U.S.A. 93, 9975–9979.

Hamilton C.M. (1997) A binary-BAC system for plant transformation with high-molecular-weight DNA. Gene 200(1-2) 107-116.

He R., Pan J., Zhu L., He G. (2010) *Agrobacterium*-Mediated Transformation of Large DNA Fragments Using a BIBAC Vector System in Rice. Plant Molecular Biology Reporter 28(4), 613-619.

Herrera-Estrella L., Block M.D., Messens E., Hernalsteens J.P., Montagu M. V, Schell, J. (1983) Chimeric genes as dominant selectable markers in plant cells. EMBO J. 2, 987–995.

Hoekema A., Hirsch P.R., Hooykaas P.J.J., Schilperoort R.A. (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium* tumefaciens Ti-plasmid. Nature 303, 179-180.

Leach K.A., McSteen P.C., Braun D.M. (2016) Genomic DNA Isolation from Maize (*Zea mays*) Leaves Using a Simple, High-Throughput Protocol. Curr Protoc Plant Biol 1,15-27.

Lee LY. and Gelvin S.B. (2008) T-DNA binary vector and systems, plant physiol 146, 325-332.

McCaw M., Swyers N., Graham N., Cody J., Zhao C., Birchler J. (2016a) Preparation of chromosomes from *Zea mays*. Curr Proto Plant Biol 1, 501-509.

McCaw M., Graham N., Cody J., Swyers N., Zhao C., Birchler J. (2016b) Fluorescence In Situ Hybridization to Maize (*Zea mays*) Chromosomes. Curr Proto Plant Biol 1, 530-545.

Meyer P., Saedler H. (1996) Homology-dependent gene silencing in plants. Annu Rev Plant Physiol Plant Mol Biol 47, 23-48.

Miranda A., Janssen G., Hodges L., Peralta E.G., Ream W. (1992) *Agro-bacterium* tumefaciens transfers extremely long T-DNAs by a unidirec- tional mechanism. J Bacteriol 174: 2288–2297.

Park S.H., Lee BM, Salas M.G., Srivatanakul M., Smith R.H. (2000) Shorter T-DNA or additional virulence genes improve *Agrobactrium*-mediated transformation. Theor Appl Genet 101(7), 1015-1020.

Que Q., Elumalai S., Li X., Zhong H., Nalapalli S., Schweiner M., Fei X., Nuccio M., Kelliher T., Gu W., Chen Z., Chilton MD.M (2014) Maize transformation technology development for commercial event generation. Front Plant Sci 5(379). doi: 10.3389/fpls.2014.00379

Srivastava V. and Ow D.W. (2001) Biolistic mediated site-specific integration in rice. Molecular Breeding 8, 345-350.

Srivastava V. and Thomson J. (2016) Gene stacking by recombinases. Plant Biotechnol J 14, 471-482.

Tao Q. and Zhang H.B. (1998) Cloning and stable maintenance of DNA fragments over 300 kb in Escherichia coli with conventional plasmid-based vectors. Nucleic Acids Res. 26, 4901–4909.

Vega J.M., Yu W., Han F., Kato A., Peters E.M., Zhang Z.J., Birchler J.A. (2008) *Agrobacterium*-mediated transformation of maize (Zea mays) with Cre-lox site specific recombination cassettes in BIBAC vectors. Plant Mol Biol 66(6), 587-598.

Wang Y., Zeng H., Zhou X., Huang F., Peng W., Liu L., Xiong W., Shi X., Luo M. (2015) Transformation of rice with large maize genomic DNA fragments containing high content repetitive sequences. Plant Cell Rep 34(6), 1049-1061.

Ye X., Williams E.J., Shen J., Johnson S., Lowe B., Radke S., Strickland S., Esser J.A., Petersen M.W., Gilbertson L.A. (2011) Enhanced production of single copy backbone-free transgenic plants in multiple crop species using binary vectors with a pRi replication origin in *Agrobacterium tumefaciens*. Transgenic Res 20(4), 773-786.

Chapter 5: Recombinase-Mediated BiBAC Circularization and Gene Transfer to Predetermined Genomic Locations

Note: The information provided in this chapter is unpublished.

Contributions

The work detailed here would not have been possible without the guidance and support of Nathaniel Graham, Nathan Swyers, Patrice Albert, and Changzeng Zhao.

Summary

This chapter explores the utility of the site-specific recombinase genome editing toolkit (Chapter 2) in both breeding and tissue culture conditions to initiate the circularization and targeting of a stably integrated pJC-YattB BiBAC vector (Chapter 4) to predetermined locations, such as artificial minichromosomes or B chromosome inserts (Chapter 3).

Note: Table 5.1 is located at the end of the chapter.

Introduction

Targeted integrations of transgenes into predetermined genomic locations has the potential to circumvent limitations observed in conventional genetic engineering methods in plants that utilize *Agrobacterium tumefaciens* or biolistic bombardment to deliver DNA into host cells. As stated in previous chapters, the transformation process is random and can create downstream complications that require extensive screening efforts to select for optimal T_0 plant backgrounds. While notable advancements such as optimization of tissue culture conditions and the use of morphogenic regulators have increased transformation efficiencies and expanded susceptible germplasms, respectively (Shrawat and Lörz, 2006; Lowe et al., 2016; Mookkan et al., 2017), progress is lacking in areas focusing on creating a reliable gene targeting system. Considerable efforts have focused on the utility of nucleases, such as CRISPR Cas9, to induce double stranded breaks and subsequently knock-in transgenes through either homology directed repair (HR) or nonhomologous end joining (NHEJ); however, DSB knock-ins currently exhibit frequencies that are far too low for practical application (Srivastava, 2019). Alternatively, the use of site-specific recombinases has shown great promise with high efficiencies in a number of plant systems, including *Arabidopsis* (Vergunst and Hooykaas, 1998), tobacco (Albert et al., 1995; Day et al., 2000) rice (Srivastava and Ow, 2002; Srivastava, 2004), soybean (Li et al., 2009), and maize (Srivastava and Ow, 2001; Anand et al., 2019).

In Chapter 2, we described a functional recombinase genome editing toolkit in maize that contains a collection of enzymes that all recognize unique recombinase binding-site sequences to catalyze strand switching reactions. Depending on the binding-site location and orientation, reactions will result in precise and predictable rearrangements of DNA. While mechanistically similar, recombinases exhibit differences in directionality and efficiency, which certainly affects their specific utility in genetic engineering. As previously stated in Chapter 2, the most widely used and described recombinase enzymes are Cre (causes recombination) and FLP (flippase) (Srivastava and Thomson, 2016). Cre and FLP are routinely used to remove selectable markers and/or target genes to predetermined genomic locations at a high efficiency (Albert et al., 1995; Li et al., 2009; Akbudak and Srivastava, 2011; Nandy and Srivastava, 2012). While Cre and FLP reactions are completely reversible, strategies to force unidirectional activity, such as the use of mutant binding sites or transient enzyme expression, have been shown to be successful (Srivastava and Ow, 2001; Albert et al., 1995). Other recombinase enzymes, such as phiC31 Integrase, catalyze reactions between non-identical binding sites, which naturally results in unidirectional reactions (Thorpe and Smith, 1998). PhiC31 Integrase-mediated transgene targeting has been demonstrated in a number of organisms, including zebrafish (Roberts et al., 2014), *Drosophila* (Bateman et al., 2005), human cells (Groth et al., 2000), and mice (Thyagarajan et al., 2001). Additionally, phiC31 Integrase was shown to be functional in plant models (Kapusi et al., 2012);

therefore, it is expected phiC31 Integrase will function in maize. While most studies focus on the direct incorporation of transgenes into predetermined locations, direct targeting of large gene fragments presents several possible complications.

In creating a system that enables targeting large gene fragments to specific locations, it is uncertain that direct incorporation of DNA through biolistic bombardment or *Agrobacterium* would be possible due to fragmentation and the nature of the T-DNA transfer process, respectively. From Chapter 4, *Agrobacterium*-mediated transformation to obtain stable integrations of pJC-YattB-35 BiBAC (~30 kb T-DNA) resulted in a transformation efficiency of 0.2% (Table 4.3; Table 4.4). If these T-DNA molecules were used together with site-specific recombinases to direct a targeted integration upon delivery, it would require the combined activity of two different recombinase systems to circularize and subsequently target DNA into a predetermined location. While this strategy may be possible through co-infection, there is still uncertainty surrounding the specific mechanism of the T-DNA transfer process (Gelvin, 2017). For recombinases to bind to target sites and induce strand-switching reactions, DNA must be double stranded (Grindley et al., 2006). The feasibility of BiBAC targeting through co-infection depends the stage the T-DNA is converted from a single-stranded to a double stranded molecule, which is currently unknown (Gelvin, 2017). If T-DNA is converted to double-stranded DNA prior to integration into a host genome, then the utilization of a coinfection strategy may increase

BiBAC transformation and targeting efficiencies; however, due to the uncertainty of the T-DNA transfer process, this work focused on stable integration of BiBAC T-DNAs prior to targeting to predetermined locations.

Stable integration of a BiBAC T-DNA molecule containing recombinase binding-site sequences for circularization and subsequent targeting could be used together with a plant containing a landing site target. From here, two strategies are conceivable for introducing recombinases into cells that contain both components: breeding or transient expression. In this chapter, we explore both avenues of targeting BiBAC insert (Chapter 4) to artificial minichromosome or B inserts (Chapter 3) using the site-specific recombinase genome editing toolkit (Chapter 2) in a breeding strategy and tissue culture conditions.

Results

Molecular Strategy:

To create a system that would enable researchers to transfer large genetic cargo to predetermined locations, such as artificial minichromosomes or B chromosome inserts, selectable marker genes used to identify stable insertions of the donor and target molecules will need to be removed in each line prior to further modifications (Figure 5.1; Figure 5.2). The pZP-Telo-JC and pJC-YattB-35 constructs have the capacity to be modified with FLPe and R recombinases, respectively, to remove *bar* herbicide resistance (Figure 5.1A; Figure 5.2A). This process was carried out in both pZP-Telo-JC (17-27 and 17-13) and pJC-

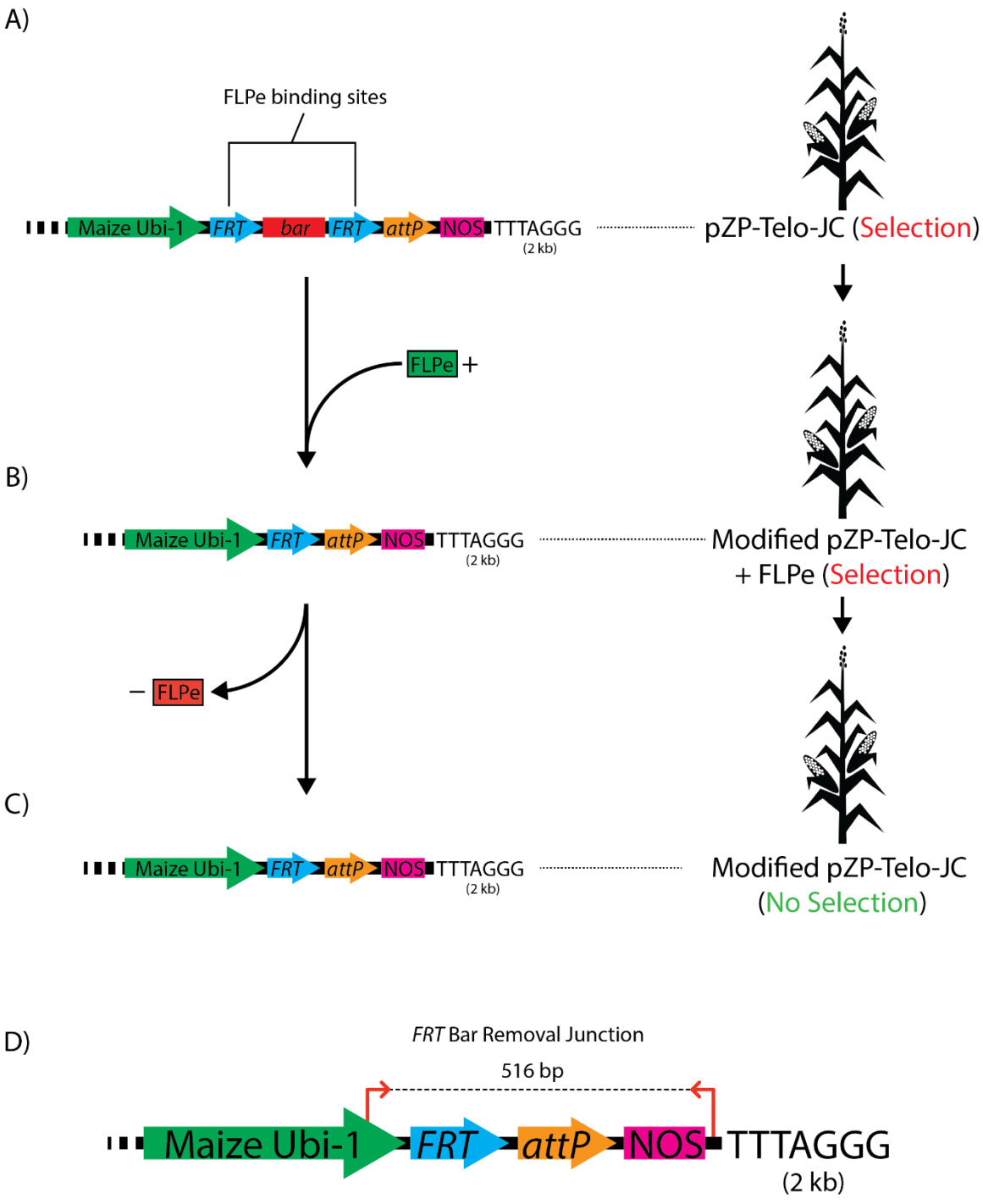

A)
FLPe binding sites
Maize Ubi-1
FRT
bar
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC (Selection)
FLPe +
B)
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
Modified pZP-Telo-JC
+ FLPe (Selection)
– FLPe
C)
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
Modified pZP-Telo-JC
(No Selection)
D)
FRT Bar Removal Junction
516 bp
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)

Figure 5.1 - FLPe-Mediated Selection Removal from Stable pZP-Telo-JC Minichromosome (17-27) and B Insert (17-13) Platforms. Stable integrations of pZP-Telo-JC are selected in tissue culture using a *bar* selectable marker gene, which is flanked by *FRT* recombinase binding sites (A). The pZP-Telo-JC transgenic plants are crossed to a FLPe expression line to remove the bialaphos resistance coding sequence (B). Progeny that contain both pZP-Telo-JC + FLPe are crossed to a Hi-II tester line to segregate away the FLPe recombinase coding sequence (C). *Bar* gene marker removal is identified through PCR analysis utilizing primer sets that flank the *FRT bar* removal junction (D).

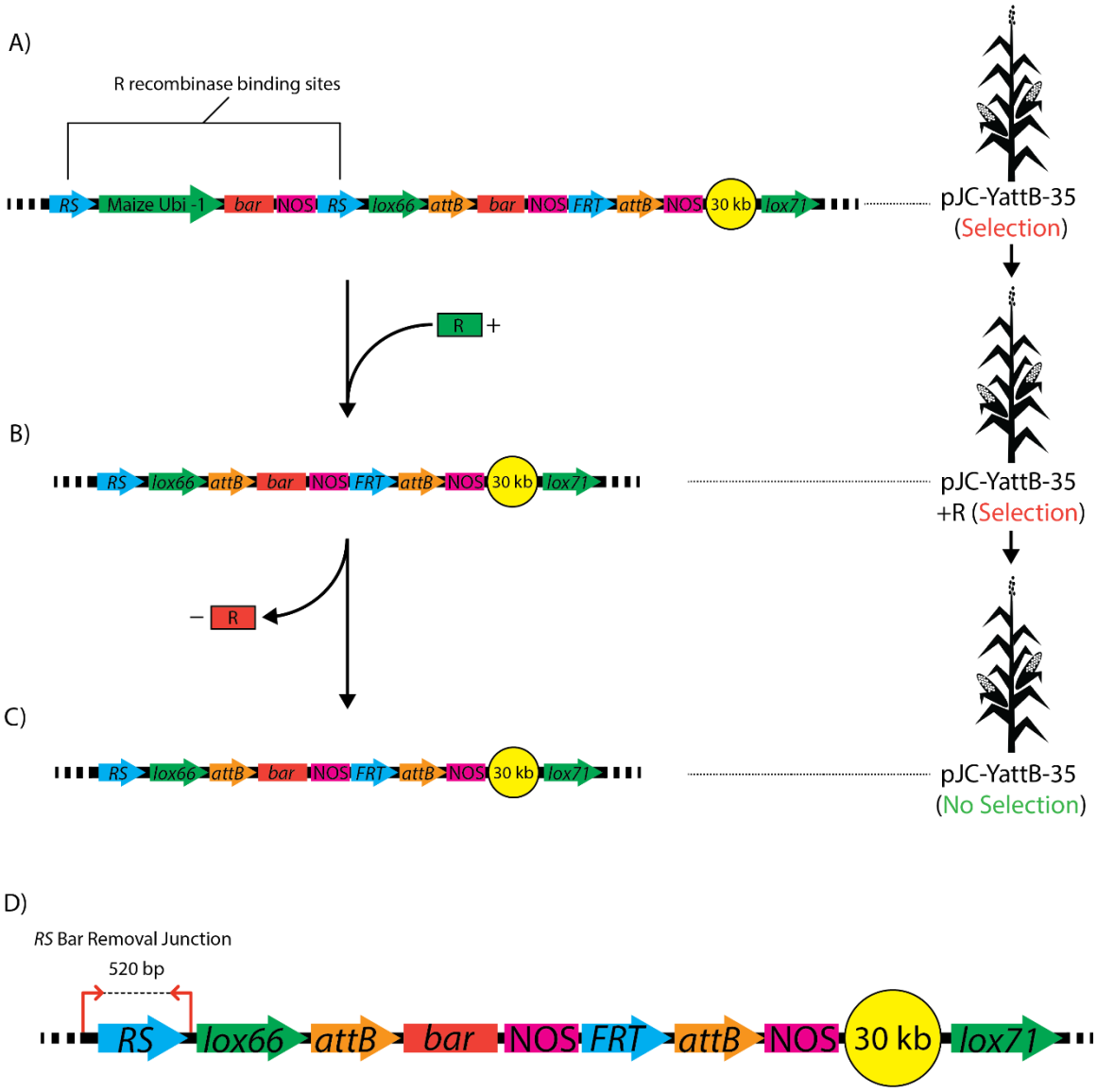

A)
R recombinase binding sites
RS
Maize Ubi -1
bar
NOS
RS
lox66
attB
bar
NOS
FRT
attB
NOS
30 kb
lox71
pJC-YattB-35
(Selection)
R
+
B)
RS
lox66
attB
bar
NOS
FRT
attB
NOS
30 kb
lox71
pJC-YattB-35
+R (Selection)
–
R
C)
RS
lox66
attB
bar
NOS
FRT
attB
NOS
30 kb
lox71
pJC-YattB-35
(No Selection)
D)
RS Bar Removal Junction
520 bp
RS
lox66
attB
bar
NOS
FRT
attB
NOS
30 kb
lox71

Figure 5.2 - R Recombinase-Mediated Selection Removal from Stable pJC-YattB-35-1 BiBAC Integrations: Stable integrations of pJC-YattB-35 are selected in tissue culture using a *bar* selectable marker gene, which is expressed by a maize ubiquitin-1 (Ubi) promoter and confers resistance to bialaphos herbicide treatments (A). The Ubi-*bar*-NOSt gene fragment is flanked by *RS* binding sites, enabling selection removal through expression of R recombinase enzymes (B). Transgenic plants that contain pJC-YattB-35 + R are crossed to a Hi-II tester line to segregate away the R recombinase coding sequence (C). Ubi-*bar*-NOSt removal is identified through PCR analysis utilizing primer sets that flank the *RS bar* removal junction (D).

YattB-35-1 transgenic events through a breeding strategy with recombinase expression lines FLPe and R, respectively, to create separate modified lines that contain an *attP* target platform (pZP-Telo-JC) and a donor BiBAC molecule (pJC-YattB-35) (Figure 5.1B; Figure 5.2B).

In Chapter 4, we described a strategy for circularization and targeting of the pJC-YattB-35 BiBAC donor molecule, which requires the combined expression of Cre and phiC31 integrase in the presence of a stably integrated *attP* site (Figure 5.3). In this process, Cre recombinase binds to mutant *loxP* sites on the BiBAC molecule, *lox66* and *lox71*, to induce a unidirectional recombination reaction that results in the circularization of flanked sequences (Figure 5.3). If this circular molecule is in the same cell as phiC31 Integrase and a minichromosome or B insert *attP* platform, PhiC31 will bind to non-identical *attB* and *attP* sites on the BiBAC circle and platform, respectively, to induce a unidirectional reaction that results in the integration of the BiBAC molecule into the target site (Figure 5.3). A promoter trap strategy was designed into the integration model between pZP-Telo-JC platforms and circular pJC-YattB-35 BiBAC molecules, where integrations fuse a maize ubiquitin promoter with the coding sequence for a promoterless *bar* selectable marker (Figure 5.4). This gives researchers the flexibility to utilize breeding and tissue culture strategies to target BiBACs to specific locations in the genome.

Depending on the specific strategy utilized for BiBAC targeting, researchers will need to obtain plant backgrounds that contain specific

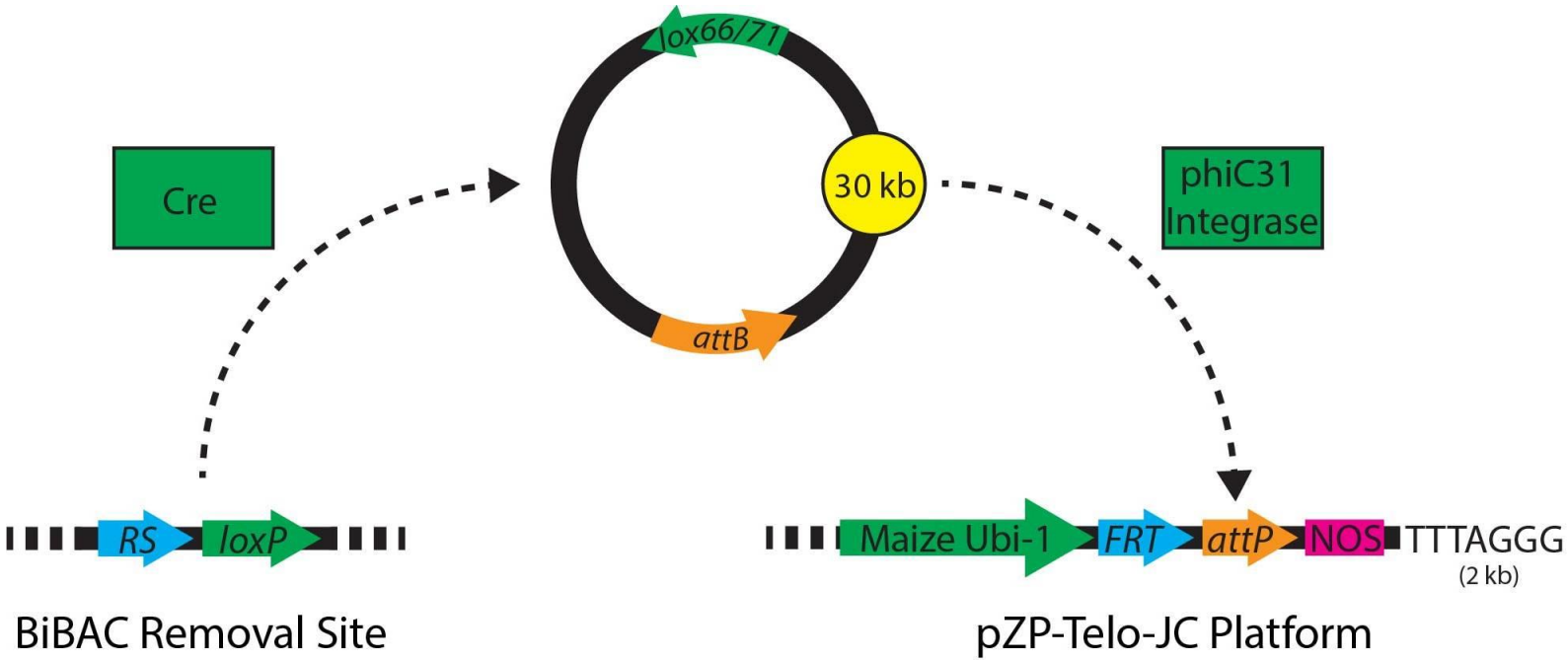


Figure 5.3 - BiBAC Circularization and Subsequent Targeting: Through the use of Cre recombinase, pJC-YattB-35-1 BiBAC inserts will be circularized from the BiBAC Removal Site. BiBAC circular molecules in the presence of a pZP-Telo-JC platform will be targeted through phiC31-mediated intermolecular recombination between *attB* and *attP* binding sites on the BiBAC circle and platform, respectively.

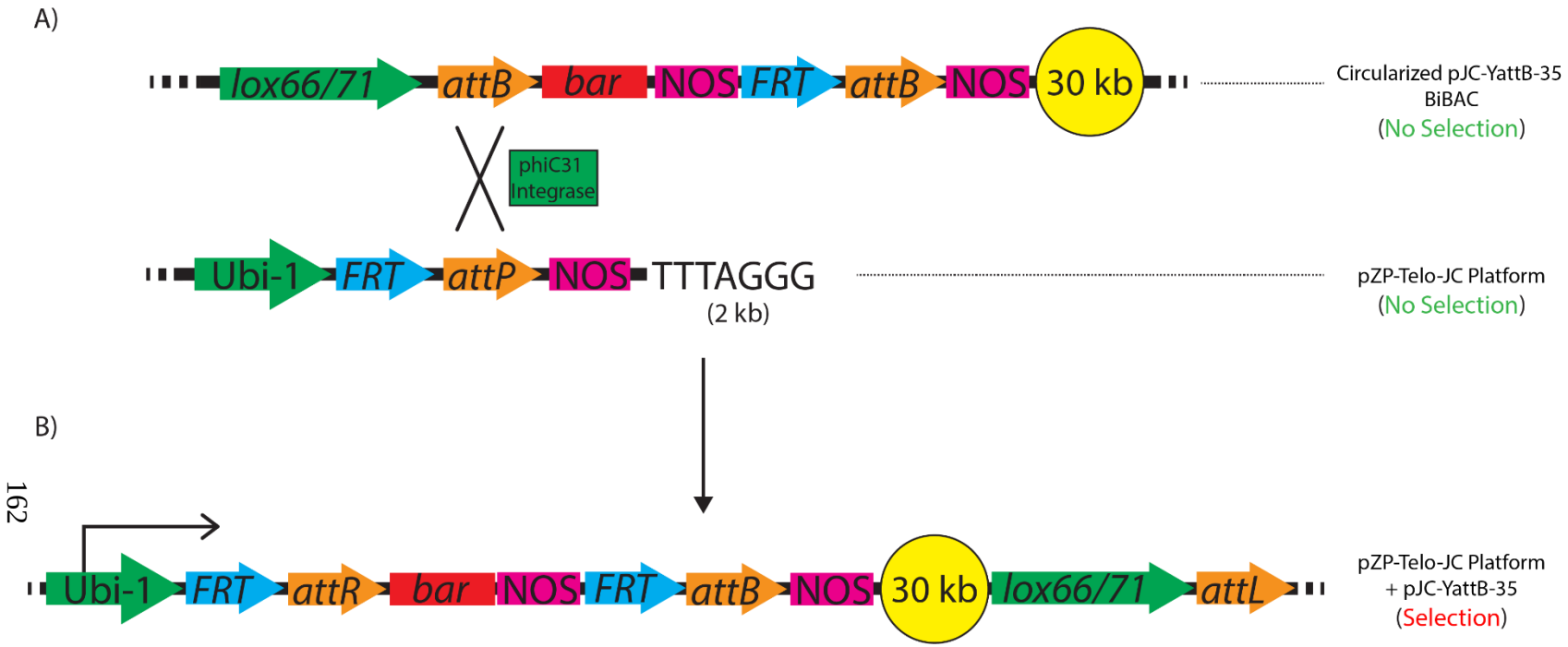


Figure 5.4 - Promoter Trap Strategy for BiBAC Integration Selection: Circularized BiBAC molecules contain an *attB* binding site followed by a promoterless *bar* sequence. Through phiC31-mediated intermolecular recombination between *attB* and *attP* binding sites, the promoterless *bar* gene is fused to the empty maize ubiquitin-1 promoter on the pZP-Telo-JC platform. This will activate expression of the *bar* coding sequence, which will enable positive selection utilizing bialaphos herbicide treatments.

components needed to carry out each reaction. A breeding model requires the Cre recombinase enzyme to be kept separate from the pJC-YattB-35 BiBAC to avoid premature circularization. The phiC31 coding sequence can be expressed together with either pJC-YattB-35 or pZP-Telo-JC because it requires both *attB* and *attP* recombinase binding sequences to be present for a strand switching reaction to occur. While there are a number of configurations that can theoretically be used to accomplish the end plant background containing all components to carry out BiBAC targeting reactions, we created a stacked plant containing Cre, phiC31 and modified pZP-Telo-JC and crossed it together with modified pJC-YattB-35 plants (Figure 5.5).

Tissue culture strategies do not require the use of recombinase expression lines to carry out circularization and subsequent BiBAC targeting. In contrast to the described breeding strategies, recombinase expression constructs were transiently expressed from a construct that contains both Cre and phiC31 coding sequences, pTFCreInt-Kan (Figure 5.6). This is accomplished through the bombardment of immature embryos obtained from a plant background that contains both modified versions of pZP-Telo-JC platforms and pJC-YattB-35 BiBAC donors (Figure 5.7). The pZP-Telo-JC + pJC-YattB-35 embryos bombarded with pTFCreInt-Kan are placed on bialaphos selection media, where integration events can be selected through the previously described promoter trap strategy (Figure 5.4). From here, resistant callus is moved through plant regeneration steps to obtain plants that contain a BiBAC targeted integration.

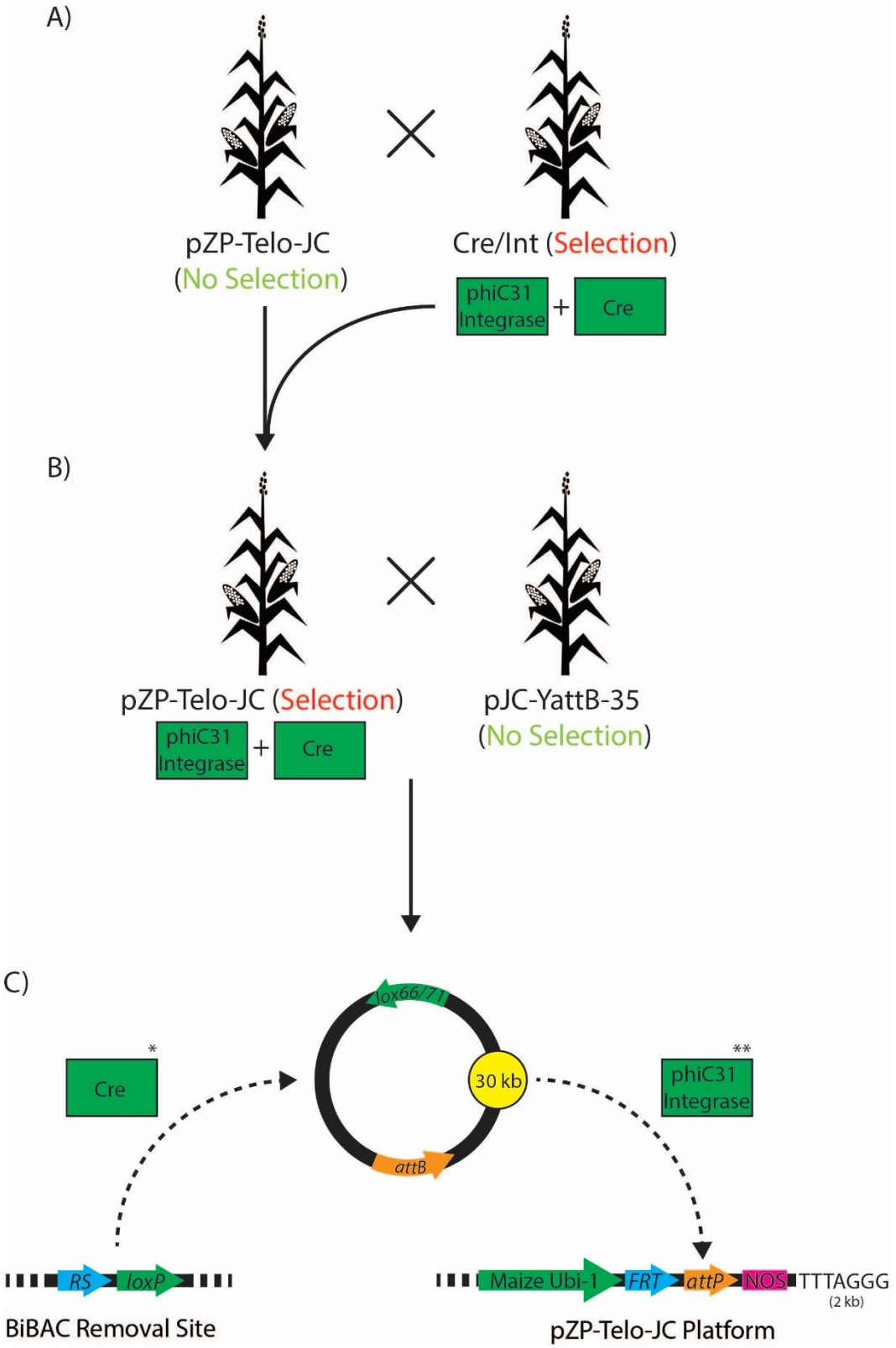

A)
pZP-Telo-JC
(No Selection)
Cre/Int (Selection)
phiC31 Integrase
+
Cre
B)
pZP-Telo-JC (Selection)
phiC31 Integrase
+
Cre
pJC-YattB-35
(No Selection)
C)
lox66/71
30 kb
attB
*
Cre
**
phiC31 Integrase
RS
loxP
BiBAC Removal Site
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC Platform

Figure 5.5 - Molecular Strategy Outlining BiBAC Targeting Through Breeding: Plants that are PCR positive for modified pZP-Telo-JC (17-13) or pZP-Telo-JC (17-27) inserts were crossed to stacked Cre and phiC31 Integrase expression lines to create progeny that contain modified pZP-Telo-JC (17-13 or 17-27), Cre and phiC31 Integrase without FLPe (A). The pZP-Telo-JC (17-13 or 17-27), Cre and phiC31 Integrase lines are crossed to modified pJC-YattB-35-1 plants without R to create progeny that contain pZP-Telo-JC (17-13 or 17-27), pJC-YattB-35-1, Cre and phiC31 Integrase transgenes (B), which contains all the necessary components that enable Cre-mediated BiBAC circularization* and subsequent targeting to minichromosomes (17-27) or B Inserts (17-13) utilizing phiC31 Integrase** (C).

Figure 5.6 - Construct Map of pTFCreInt-Kan Transient Expression Construct: The vector illustrated in this figure is not drawn to scale. The pTFCreInt-Kan construct was designed for bombardment experiments to initiate pJC-YattB-35-1 BiBAC circularization and subsequent targeting to pZP-Telo-JC platforms through the transient expression of Cre and phiC31 Integrase recombinase enzymes (Figure 5.7). Cre expression is driven by a maize Ubi-1 promoter and terminated by a nopaline synthase termination coding sequence (NOSt). PhiC31 Integrase expression is controlled by maize Ubi-1 and terminated by NOSt. The phiC31 Integrase coding sequence contains an intron derived from the *Petunia hybrida Psk7* gene, which helps increase recombinase expression. The pTFCreInt-Kan construct contains a kanamycin selectable marker under the control of a constitutive cauliflower mosaic virus (CaMV) 35S promoter; however, CaMV 35S is not used for selection purposes in the bombardment experiments used in this study.

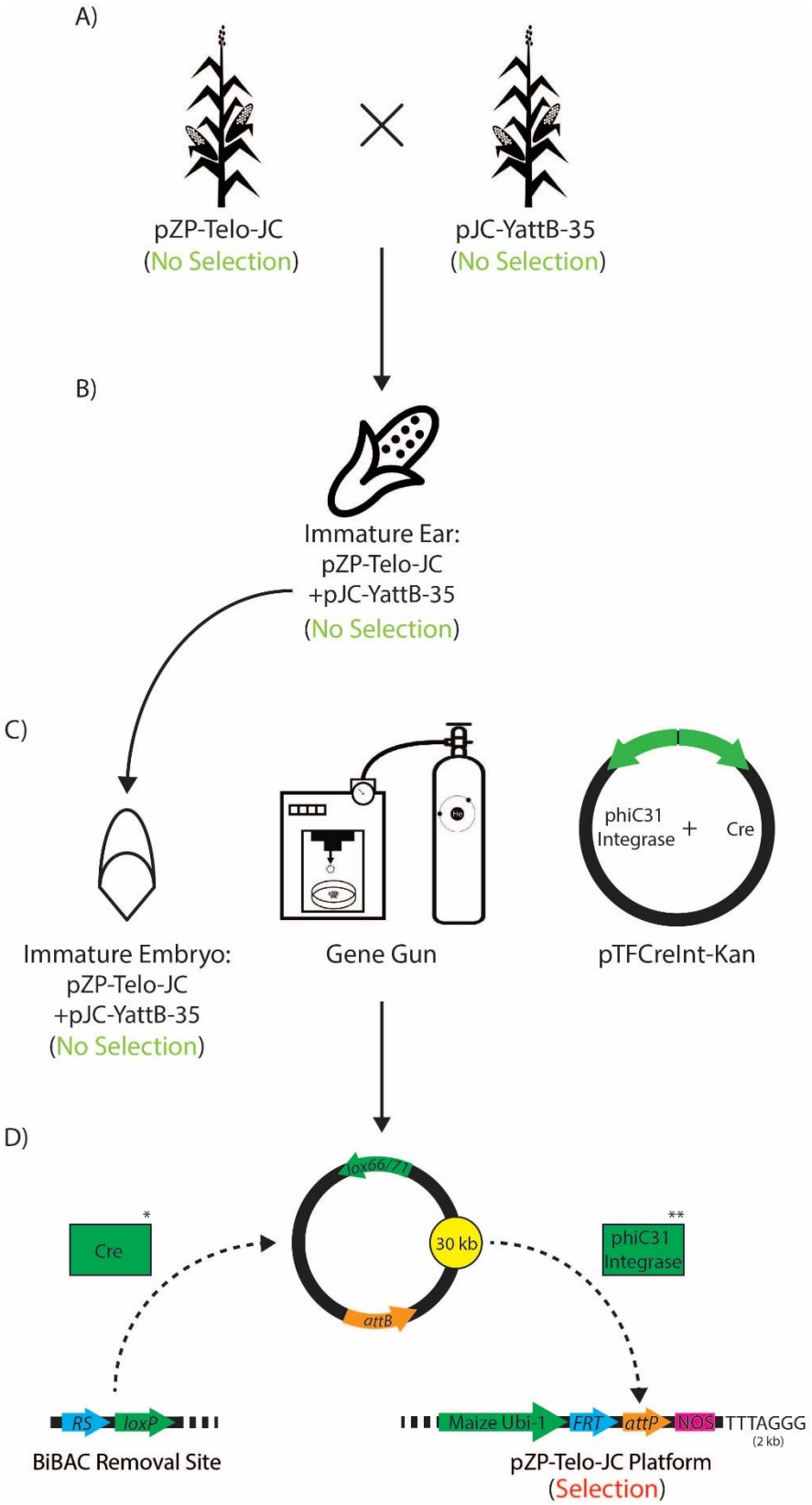

A)
pZP-Telo-JC
(No Selection)
pJC-YattB-35
(No Selection)
B)
Immature Ear:
pZP-Telo-JC
+pJC-YattB-35
(No Selection)
C)
Immature Embryo:
pZP-Telo-JC
+pJC-YattB-35
(No Selection)
Gene Gun
phiC31
Integrase
+
Cre
pTFCreInt-Kan
D)
lox66/71
30 kb
attB
*
Cre
**
phiC31
Integrase
RS
loxP
BiBAC Removal Site
Maize Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC Platform
(Selection)

Figure 5.7 - Molecular Strategy outlining BiBAC Targeting Through Bombardment: Separate plants PCR positive for stable integrations of modified pZP-Telo-JC B Insert (17-13) or pJC-YattB-35-1 were crossed together to create a line that contains both transgenes (A). Immature embryos from this cross were extracted 9 – 12 days after pollination (B). These immature embryos were bombarded with the pTFCreInt-Kan construct (Figure 5.6), which transiently expresses the Cre and phiC31 Integrase recombinase enzymes once delivered into the host cell (C). Cells that contain a pZP-Telo-JC platform, pJC-YattB-35-1 BiBAC and pTFCreInt-Kan construct will transiently express Cre* to circularize the BiBAC and transiently express phiC31 Integrase** to target the circular molecule to minichromosomes or B Inserts (D).

Recombinase-Mediated Selectable Marker Gene Removal from pZP-Telo-JC Platforms and pJC-YattB-35 BiBAC Integrations

FLPe Modification of pZP-Telo-JC Platforms:

Plants that were PCR positive for pZP-Telo-JC events 17-27 (minichromosome) and 17-13 (B insert) were crossed together with the FLPe stable expression line to remove a *bar* selectable marker gene flanked by *FRT* recombinase binding sites (Figure 5.1A; Figure 5.1B). Progeny of this cross were screened for the presence of FLPe and absence of *bar* through the use of primer sets that bound to the FLPe coding sequence and *FRT bar* removal junction on the pZP-Telo-JC transgene, respectively (Figure 5.1D; Table 5.1). Plants containing both FLPe and the pZP-Telo-JC transgene exhibited banding patterns on an agarose gel indicative of FLPe-mediated selection removal (Figure 5.8). Plants containing only the pZP-Telo-JC transgene were unreduced, which could be PCR amplified using the same primer set that flanks the *FRT* junction; however, the banding pattern is ~1 kb larger in size compared to FLPe modified sequences (Figure 5.8). Sanger sequence analysis of the amplified *FRT* junction shows successful removal of the *bar* selectable marker and reformation of a single *FRT* recombinase binding site (Figure 5.9).

R Recombinase Modification of pJC-YattB-35-1 BiBAC Donor:

Plants that were PCR positive for pJC-YattB-35-1 were crossed together with the R recombinase stable expression line to remove a Ubi-*bar*-NOSt

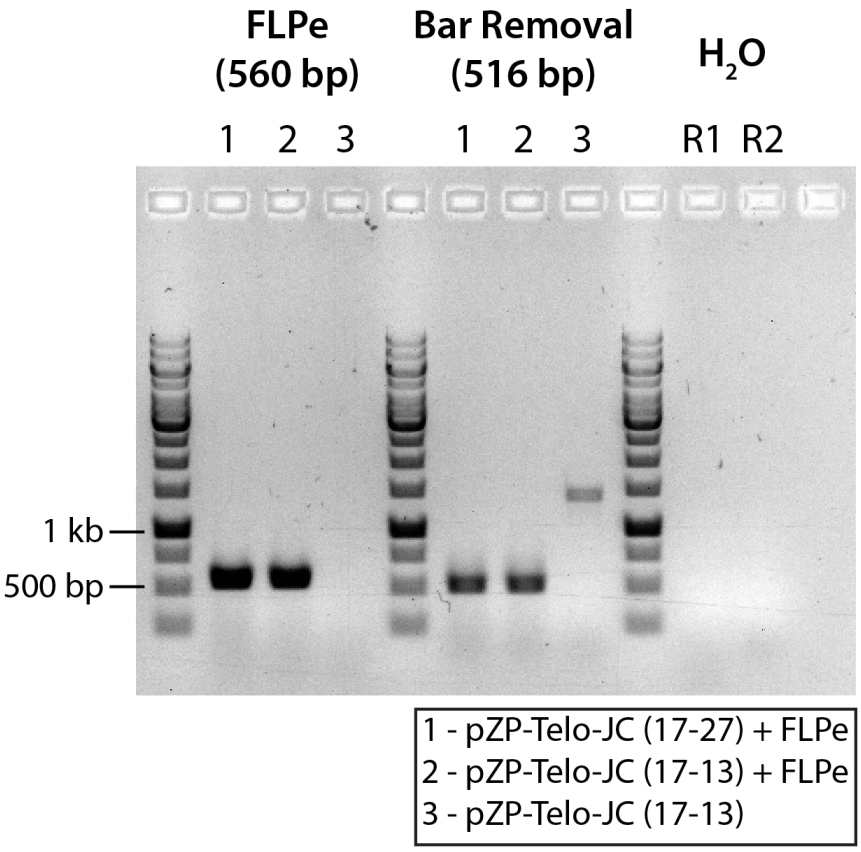


Figure 5.8 - Agarose Gel Image Highlighting FLPe-Mediated Removal of the *Bar* Selectable Marker from pZP-Telo-JC Minichromosomes (17-27) and B Inserts (17-13): Progeny from a cross between pZP-Telo-JC minichromosomes (17-27) or B Inserts (17-13) and FLPe recombinase expression lines (Figure 5.1A; Figure 5.1B) were analyzed for the removal of the *bar* selectable marker coding sequence utilizing PCR analysis on genomic DNA from leaf tissue extractions. Any plant that displayed banding patterns indicative of FLPe (560 bp) and pZP-Telo-JC transgene presence would result in a DNA fragment of 515 bp when using pZP *Bar* Removal (516) primer sets (Lane 1; Lane 2). If FLPe is absent in a plant background that contains a pZP-Telo-JC transgene, the pZP *Bar* Removal (516) primer sets generate a DNA fragment of ~950 bp, which indicates the presence of the *bar* selectable marker gene (Lane 3). Primer set data used in this analysis can be found in Table 5.1.

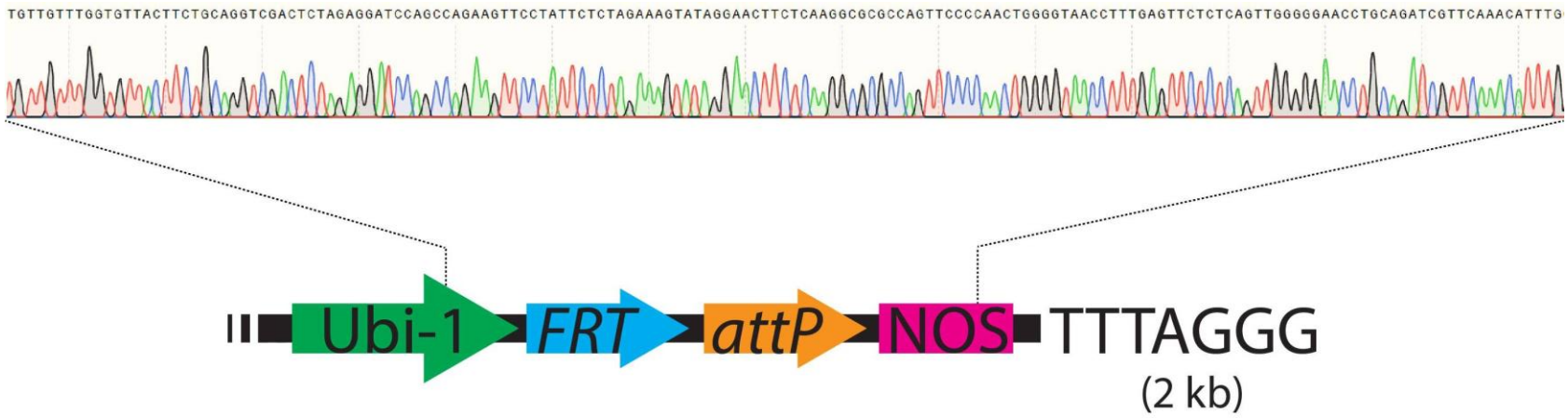


Figure 5.9 - Sanger Sequence Analysis of FLPe-Mediated Selection Removal from pZP-Telo-JC Transgenes: Stable integrations of pZP-Telo-JC minichromosomes (17-27) and B Inserts (17-13) were crossed to FLPe expression lines to remove the *FRT* flanked bialophos resistance marker gene (Figure 5.1A; Figure 5.1B). Genomic DNA was extracted from leaf tissue of the progeny and used in PCR analysis utilizing primer sets that flank the *FRT bar* removal junction (Figure 5.1D; Table 5.1). Sanger sequence analysis of this PCR fragment verifies the removal of the bialaphos herbicide resistance marker and formation of a single *FRT* binding site.

selection fragment flanked by *RS* binding sites (Figure 5.2A; Figure 5.2B). Progeny of this cross were screened for presence of R and absence of Ubi-*bar*-NOSt through the use of primer sets that bound to the R coding sequence and the *RS bar* removal junction, respectively (Figure 5.2D; Table 5.1). Plants containing both R and the pJC-YattB-35-1 transgene exhibited PCR banding patterns on an agarose gel indicative of R-mediated removal of the Ubi-bar-NOSt fragment (Figure 5.10). Plants containing only the pJC-YattB-35-1 BiBAC insert did not amplify a *RS bar* junction removal fragment in multiple samples, which indicates that R is required for the formation of the *RS bar* removal junction (Figure 5.10). Sanger sequence analysis of the amplified *RS* junction shows successful removal of the Ubi-*bar*-NOSt fragment and reformation of a single *RS* recombinase binding site (Figure 5.11A). Additionally, Sanger sequence analysis of the modified pJC-YattB-35-1 BiBAC confirms the sequence integrity of other recombinase binding sites, which function in the circularization and targeting of the BiBAC donor molecule (Figure 5.11A; Figure 5.11B). Modified pJC-YattB-35-1 lines were crossed to Hi-II tester plants to segregate BiBAC inserts from R recombinase. Progeny of the modified pJC-YattB-35-1 x Hi-II cross was PCR screened for the presence of the BiBAC transgene and absence of R (Figure 5.12).

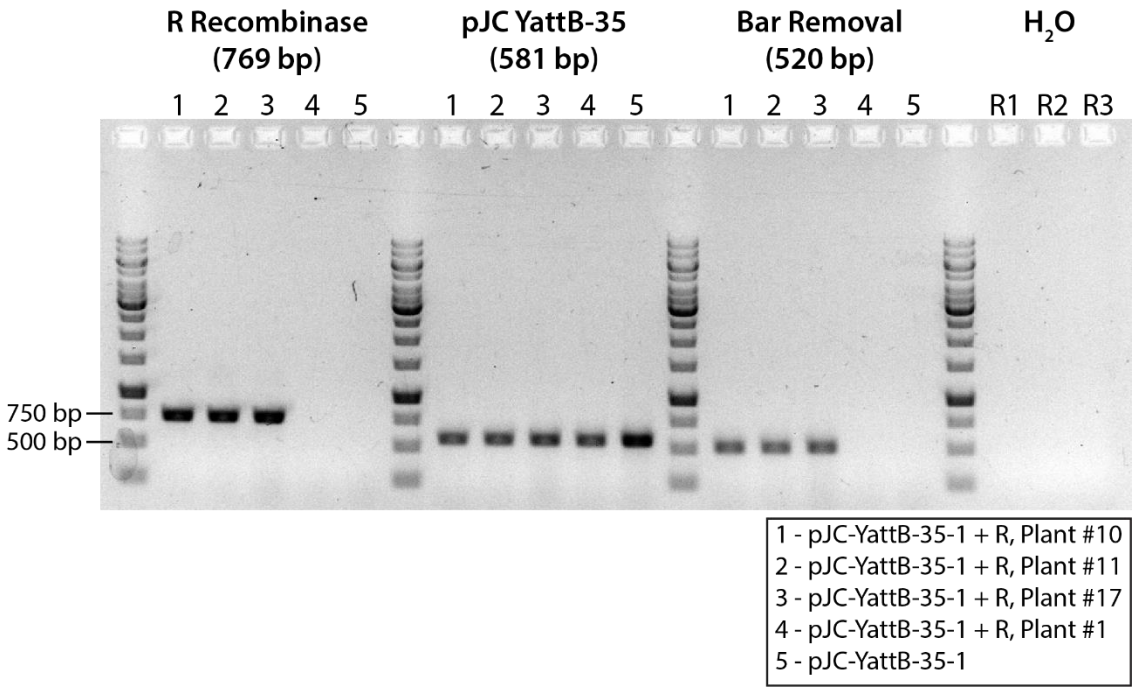


Figure 5.10 - Agarose Gel Image Highlighting R Recombinase-Mediated Removal of the *Bar* Selectable Marker Gene from pJC-YattB-35-1 BiBAC Transgenes: Progeny from a cross between pJC-YattB-35-1 BiBAC inserts and the R recombinase expression line (Figure 5.2A) were analyzed for the removal of the *bar* selectable marker gene (Figure 5.2B) utilizing PCR on genomic DNA from leaf tissue extractions. Any plant that displays banding patterns indicating presence of R recombinase and the pJC-YattB-35-1 BiBAC would also be positive for removal of the *bar* selectable marker gene (Lane 1; Lane 2; Lane 3). Any plant positive for pJC-YattB-35-1, but negative for R recombinase was also negative for the removal of the *bar* selectable marker gene (Lane 4; Lane 5). Primer set data used in this analysis can be found in Table 5.1.

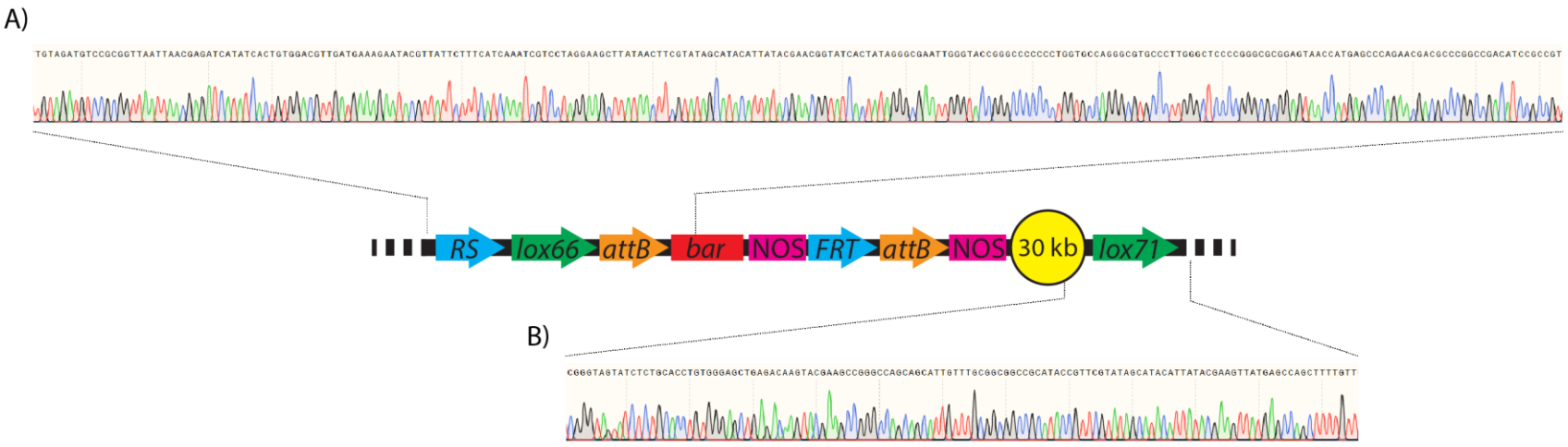


Figure 5.11 - Sanger Sequence Analysis of R Recombinase-Mediated Selection Removal from pJC-YattB-35 Transgenes: Stable integrations of pJC-YattB-35-1 BiBAC integrations (Figure 5.2A) were crossed to R recombinase expression lines to remove the Ubi-*bar*-NOSt DNA fragment that confers resistance to bialaphos herbicide treatments (Figure 5.2B). Genomic DNA was extracted from leaf tissue of the progeny and used in PCR analysis utilizing primer sets that flank the *RS bar* removal junction (A) and the *lox71* junction (B) (Table 5.1). Sanger sequence analysis of the verifies the removal of the bialaphos resistance marker gene and integrity of the *lox66* mutant binding site used in Cre-mediated circularization (A). Sanger sequence analysis verifies the integrity of the that flank the *lox71* mutant binding site used in Cre-mediated circularization (B).

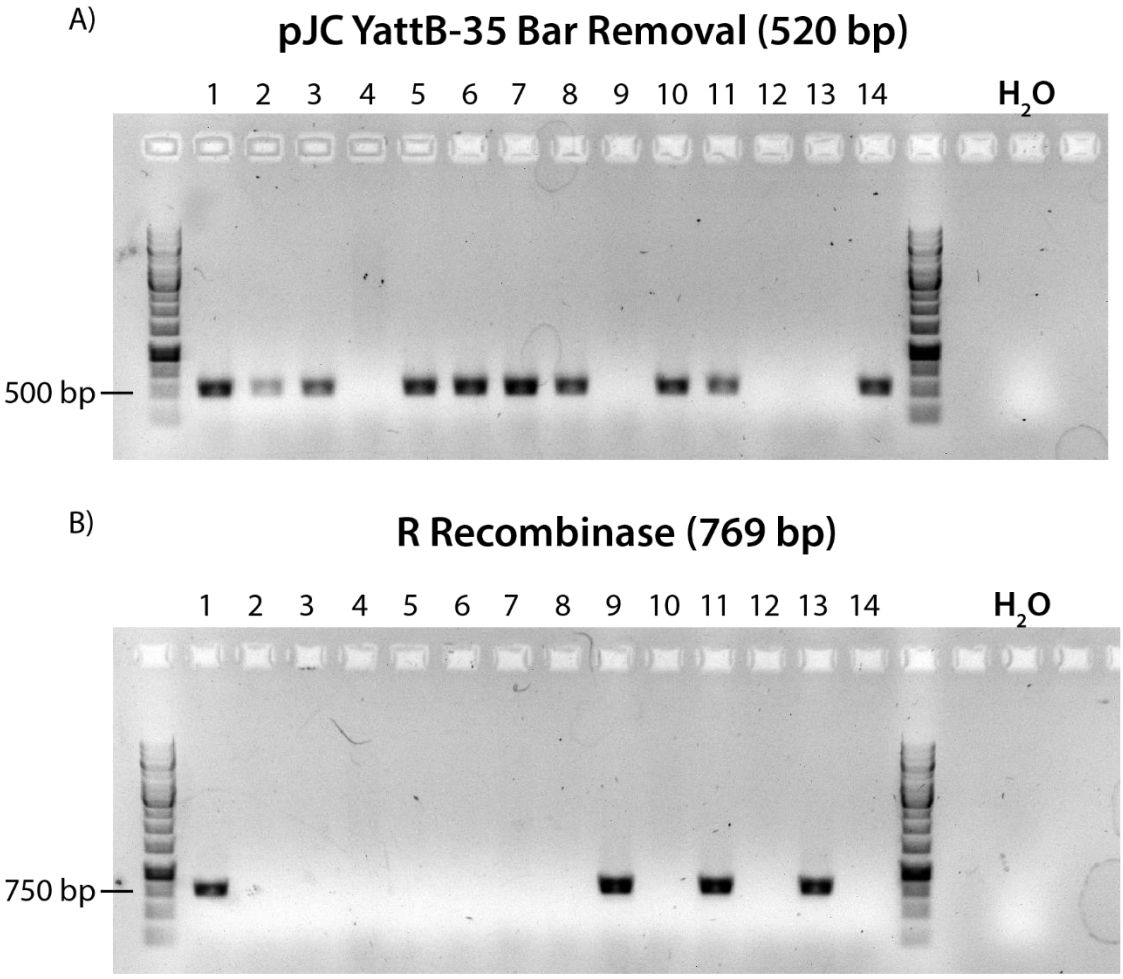


Figure 5.12 - Agarose Gel Image of Modified pJC-YattB-35-1 Segregation from R Recombinase Enzymes: Plants that were positive for the presence of modified pJC-YattB-35-1 BiBAC inserts were crossed to Hi-II tester plants to segregate away R recombinase enzymes. 8 out of 14 plants analyzed contained modified pJC-YattB-35-1 Transgenes (A) without the presence of the R recombinase coding sequences (B). Primer set data used in this analysis can be found in Table 5.1.

Testing Components of BiBAC Circularization and Targeting

Cre-Mediated BiBAC Circularization:

Unmodified pJC-YattB-35-1 plants were crossed to a Cre expression line to determine if the *lox66* and *lox71* sites on the BiBAC could be used to create a circular molecule (Figure 5.13). Genomic DNA was extracted from leaf tissue of the progeny and used as template in PCR reactions that utilized primer sets that amplify the removal and circle junctions (Table 5.1). DNA fragment size on an agarose gel indicate Cre recombinase is efficient at recombining mutant *loxP* sites to form BiBAC circular molecules (Figure 5.14). Sequence analysis of both *loxP* site from the BiBAC removal junction (Figure 5.15B; Figure 5.16B) and the BiBAC circularization junction (Figure 5.15A; Figure 5.16A) indicate the correct configurations of mutant *loxP* sites, which form a *lox66/71* site on the BiBAC circle. As previously stated, this reaction is unidirectional and cannot be reversed due to decreased affinity of Cre to *loxP* sites containing two mutations (Albert et al., 1995).

Persistence of BiBAC Circular Molecules:

Genomic DNA was extracted from leaf 2 and leaf 13 tissue samples from unmodified pJC-YattB-35-1 + Cre plants and used as template in a PCR reaction using a primer set that amplifies the BiBAC circle junction (Figure 5.17). In a few cases, the BiBAC circle junction showed persistence in mitotically active leaf tissues extracted in early and late stages of plant development (Figure 5.17).

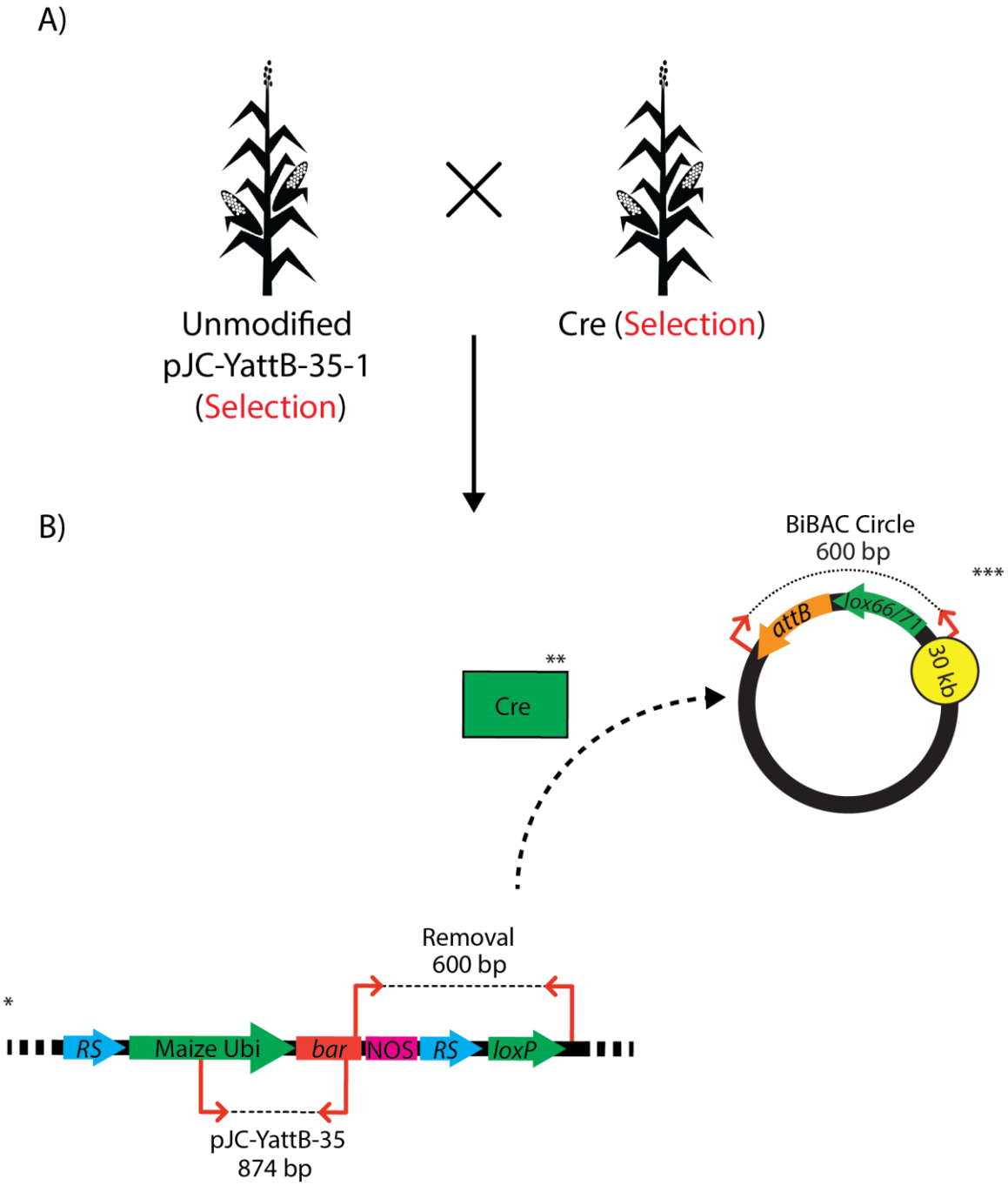

A)
Unmodified
pJC-YattB-35-1
(Selection)
Cre (Selection)
B)
BiBAC Circle
600 bp
***
attB
lox66/71
30 kb
**
Cre
Removal
600 bp
*
RS
Maize Ubi
bar
NOS
RS
loxP
pJC-YattB-35
874 bp

Figure 5.13 - Molecular Strategy to Test Cre Functionality to Circularize Stably Integrated pJC-YattB-35-1 BiBACs: Unmodified pJC-YattB-35-1 BiBAC inserts were crossed to Cre recombinase stable expression lines (A). Progeny that contains both pJC-YattB-35-1 BiBAC inserts and Cre recombinase** transgenes will result in the formation of the 30 kb BiBAC circle*** (B) This Cre-mediated circularization yields a single *loxP* site and double mutant *lox66/71* site on the removal site* and BiBAC circle***, respectively (B). PCR analysis utilizing specific primer sets, outlined in Table 5.1 and highlighted by red arrows, are used to determine the presence of the BiBAC removal site* and the BiBAC circle (B).

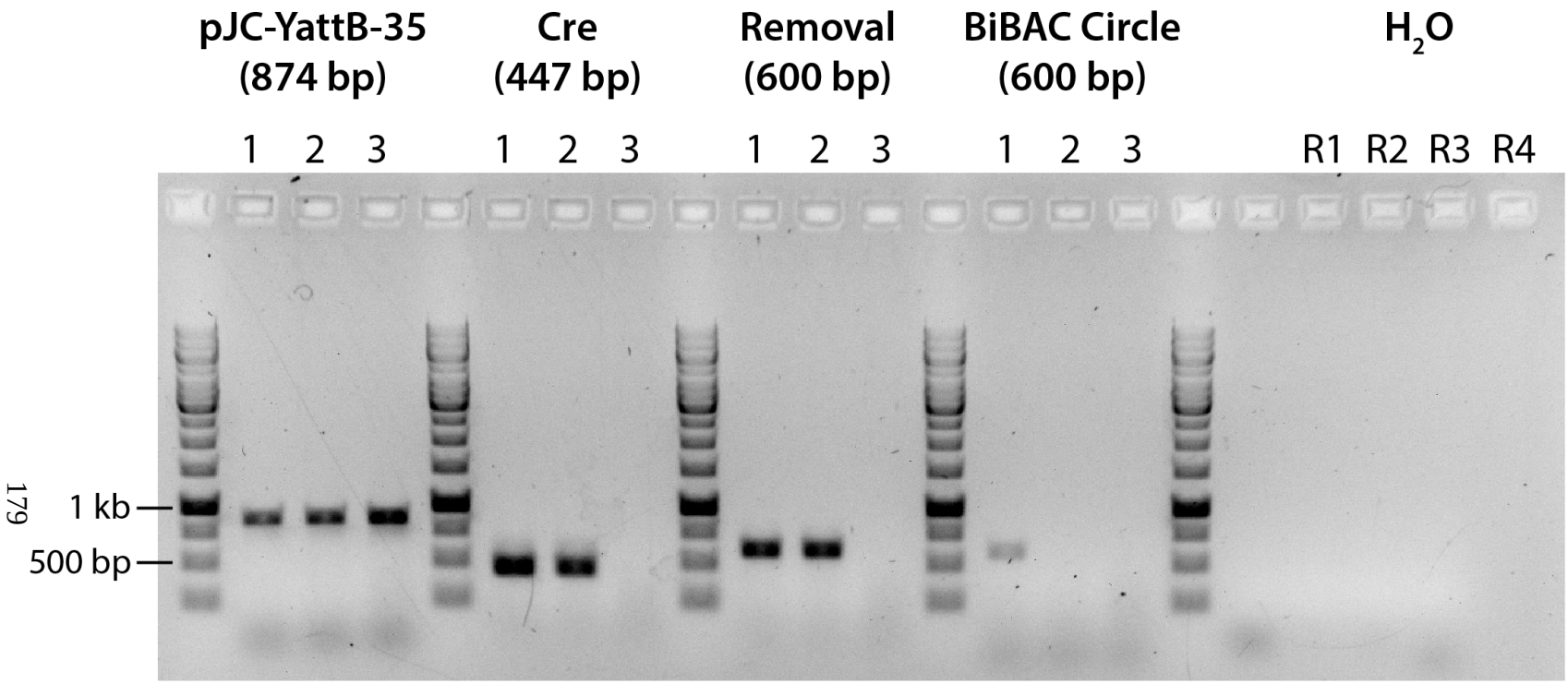

pJC-YattB-35
(874 bp)
Cre
(447 bp)
Removal
(600 bp)
BiBAC Circle
(600 bp)
H2O
1 2 3 1 2 3 1 2 3 1 2 3 R1 R2 R3 R4
1 kb
500 bp
1 - Unmod pJC-YattB-35-1 + Cre, Plant 6
2 - Unmod pJC-YattB-35-1 + Cre, Plant 9
3 - Unmod pJC-YattB-35-1 + Cre, Plant 5

Figure 5.14 - Agarose Gel Image Highlighting Cre-Mediated BiBAC Circularization: Unmodified pJC-YattB-35-1 plants crossed to Cre recombinase expression lines were used to test the circularization of the 30 kb BiBAC fragment (Figure 5.13), an essential step in the BiBAC transfer process for both breeding (Figure 5.5) and bombardment strategies (Figure 5.7). PCR analysis of plant 6 (Lane 1) and plant 9 (Lane 2), which contain an unmodified pJC-YattB-35-1 transgene and Cre recombinase, were positive for the removal of the 30 kb fragment and formation of the BiBAC removal site; however; PCR analysis of the resulting BiBAC circle is not always detectable. Plant 6 (Lane 1) displayed a faint positive band, while plant 9 (Lane 2) was negative for BiBAC circle formation. Previous (data not shown) and subsequent analysis (Figure 5.17) of the BiBAC circle in plant 9 (Lane 2) indicate the presence of the circular molecule in this background. This result indicates that BiBAC circles are at lower concentrations than stably integrated DNA.

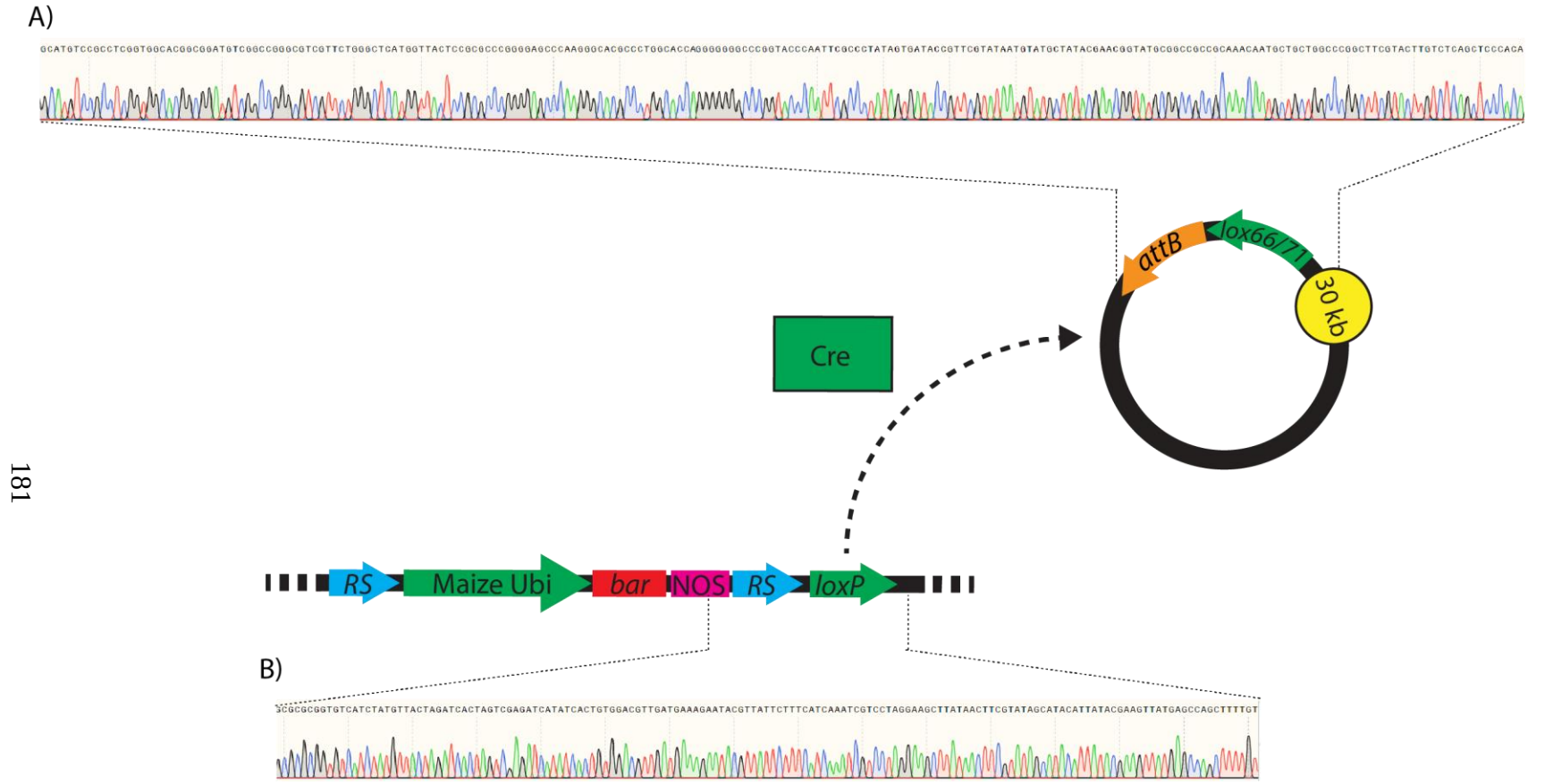

A)
attB
lox66/71
30 kb
Cre
RS
Maize Ubi
bar
NOS
RS
loxP
B)

Figure 5.15 - Sanger Sequence Analysis of Cre-Mediated BiBAC Circularization from Unmodified pJC-YattB-35-1 Inserts: Stable integrations of unmodified pJC-YattB-35-1 transgenes were crossed to Cre recombinase expression lines to test BiBAC circularization (Figure 5.14). Sanger sequence analysis of the BiBAC circle (A) and BiBAC removal site (B) verify the integrity of all resulting recombinase binding sites after Cre-mediated circularization, which forms a *loxP* on the BiBAC removal site and a double mutant *lox66/71* on the BiBAC circle.

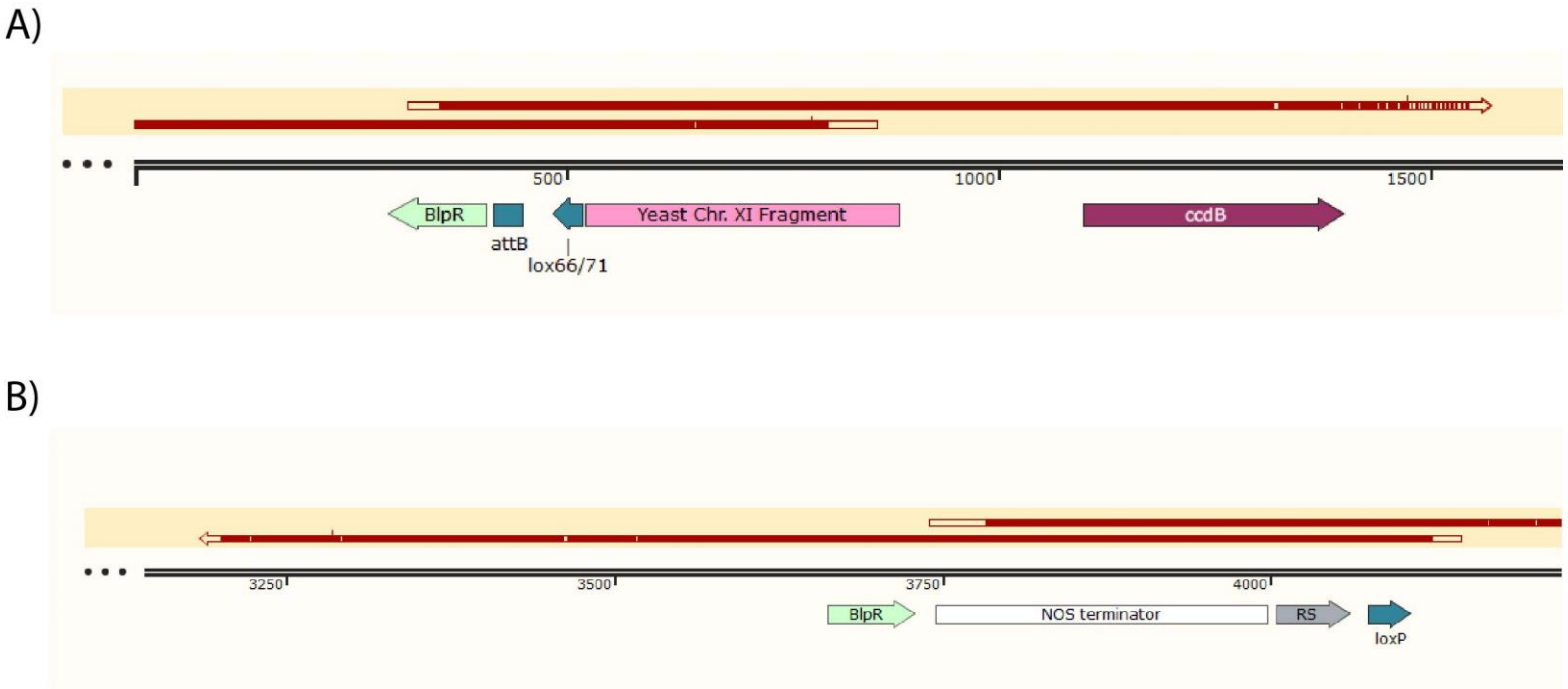


Figure 5.16 - Alignment of Sanger Sequence Data to the BiBAC Circle and BiBAC Removal Site Using SnapGene Software: Red bars/arrows above the construct shows the relative binding site and running track of each Sanger sequencing read on the BiBAC circle (A) and BiBAC removal site (B). Breaks in the red bars/arrows represent "no calls" in the sequencing. The collective sequence data derived from the BiBAC circle (A) and BiBAC removal site (B) indicate successful circularization and verify the integrity of each resulting recombinase binding site.

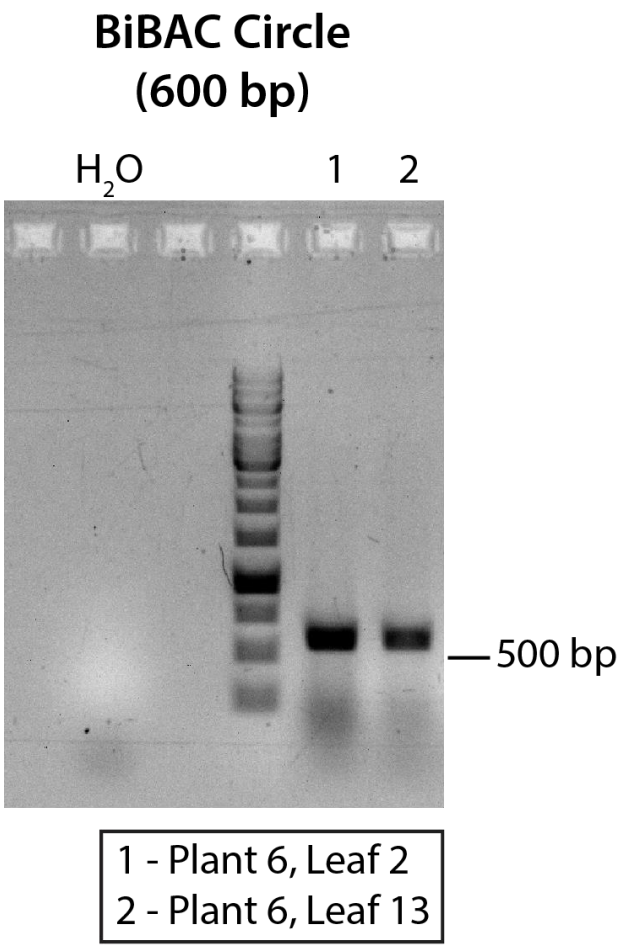


Figure 5.17 - BiBAC Circle Persistence in Mitotically Active Leaf Tissues: PCR analysis on genomic DNA extractions from leaf tissue at different stages of development indicate the persistence of Cre-mediated circularized BiBAC molecules. Leaf 2 (Lane 1) and Leaf 13 (Lane 2) both display banding patterns that indicate the presence of the BiBAC circular molecule. Primer set data used in this analysis can be found in Table 5.1.

phiC31 Integrase-Mediated Targeting to GLP Sites:

Immature embryo explants containing a GLP Ubi-*attP* platform and stably expressing phiC31 recombinase were used as material for a bombardment experiment with *attB*-DsRed donor molecules (Figure 5.18; Figure 5.19; Figure 5.20). Genomic DNA from bombarded embryos were extracted ~1-2 days after bombardment and used as template in a PCR analysis utilizing primer sets that amplify the integration junction site (Table 5.1). These embryos exhibited a PCR fragment size of 400 bp, indicating that transgene targeting through the use of phiC31 Integrase was successful (Figure 5.21-Lane 1). Embryos derived from the same ear that were not subject to bombardment with *attB*-DsRed did not amplify a DNA fragment through the same PCR analysis (Figure 5.21-Lane2). Additionally, use of the *attB*-DsRed donor molecule as template DNA did not amplify a PCR product (Figure 5.21-Lane 3).

Recombinase-Mediated BiBAC Gene Transfer Through Breeding

Creation of Stacked Lines Containing Cre, phiC31 Integrase and pZP-Telo-JC Platform:

Heterozygous Cre and phiC31 Integrase expression lines were crossed together to produce stacked Cre + phiC31 recombinase stocks, which were identified by PCR screening the progeny for the presence of both enzyme encoding sequences (Figure 5.22). These lines were crossed together with the modified pJC-Telo-JC lines (17-27 and 17-13) to produce progeny that contained

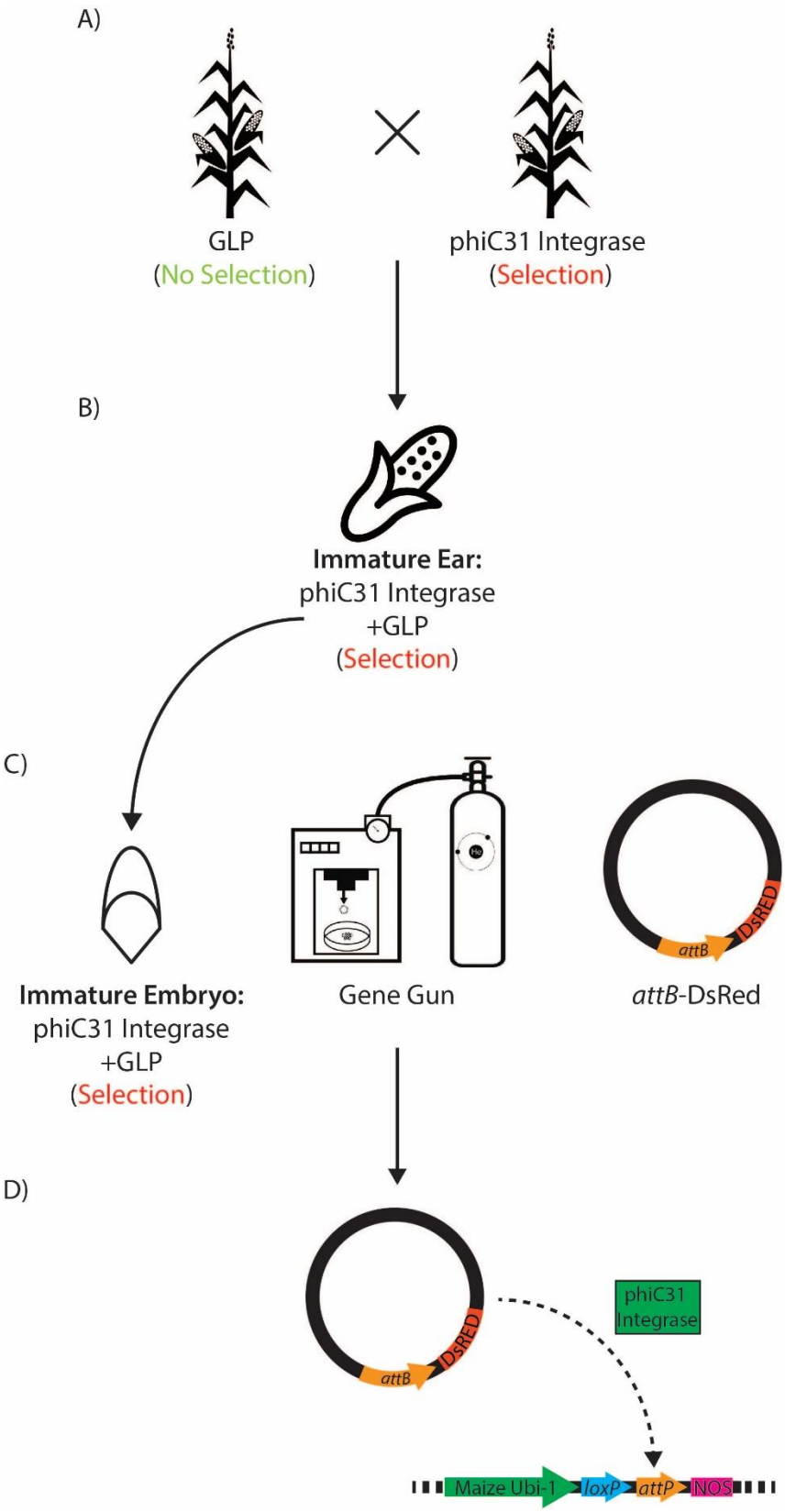

A)
GLP
(No Selection)
phiC31 Integrase
(Selection)
B)
Immature Ear:
phiC31 Integrase
+GLP
(Selection)
C)
Immature Embryo:
phiC31 Integrase
+GLP
(Selection)
Gene Gun
attB
DsRED
attB-DsRed
D)
attB
DsRED
phiC31
Integrase
Maize Ubi-1
loxP
attP
NOS

Figure 5.18 - Molecular Strategy Outlining Transgene Targeting of DsRed Donor Molecules to Predetermined GLP Target Locations Utilizing Stable Expression of PhiC31 Integrase: Stable GLP integration lines (Figure 5.20) were crossed to phiC31 integrase expression lines to generate a background that contained both GLP and phiC31 Integrase (A). Immature embryos derived from a self-cross of this plant background were extracted 9 – 12 days after pollination (B). These immature embryos were used in a bombardment experiment utilizing an attB-DsRed donor construct detailed in Figure 5.19 (C). In a cell that contains both GLP and is stably expressing phiC31 Integrase, attB-DsRed donor molecules will be targeted to GLP target sites through a phiC31-mediated intramolecular recombination reaction between attB and attP binding sites on the DsRed donor molecule and GLP target, respectively (D).

Figure 5.19 - Construct Map of the *attB*-DsRed Donor Molecule: The *attB*-DsRed donor molecule used in GLP bombardment contains a phiC31 Integrase binding site, *attB,* followed by a DsRed coding sequence.

Figure 5.20 - Construct Map of Stable GLP Target Sites: GLP integrations are designed for use in experiments that target donor molecules utilizing phiC31-Integrase enzymes. GLP contains an empty maize Ubiquitin-1 (Ubi-1) promoter that does not drive expression of any coding sequence. Downstream of Ubi-1 is a *loxP* recombinase binding site, which was previously used to remove a *loxP* flanked *bar* selectable marker gene. Downstream of *loxP* is an *attP* binding site, which is used together with phiC31 integrase to target donor molecules that contain *attB.*

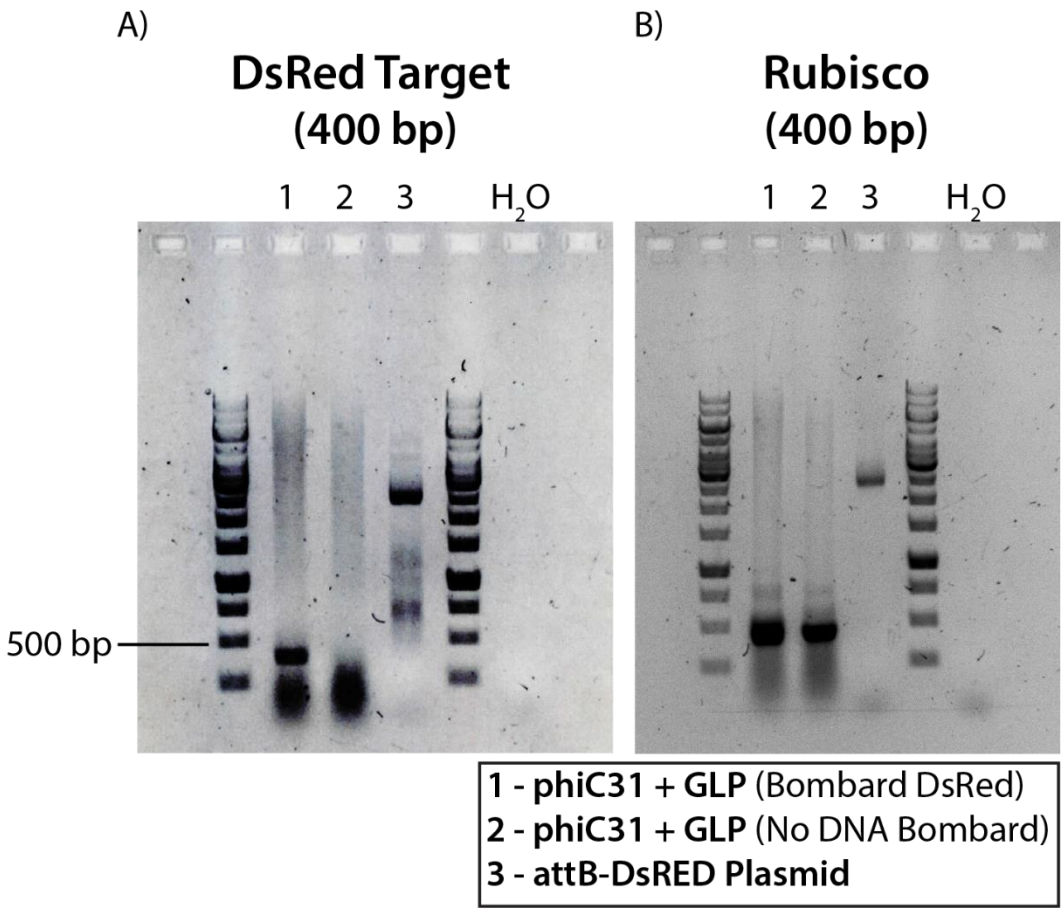


Figure 5.21 - Agarose Gel Image Highlighting Transgene Targeting of attB-DsRed Donor Molecules to GLP Target Sites Using Stable PhiC31 Integrase Expression: To test phiC31 Integrase functionality in transgene targeting donor DNA molecules to predetermined locations, bombardment was carried out on maize immature embryos derived from a cross between GLP and phiC31 Integrase transgenic plants (Figure 5.18). The phiC31 Integrase + GLP embryos bombarded with the attB-DsRed donor molecule (Figure 5.19) exhibit banding patterns in PCR analysis that indicate a targeted integration (Lane 1). Immature embryos that contain phiC31-Integrase + GLP that are not bombarded with DNA do not display DsRed targeting bands (Lane 2). PCR analysis on attB-DsRed constructs used in the bombardment also do not show banding patterns that are indicative of transgene targeting (Lane 3). Rubisco primer sets were used to verify the integrity of the DNA extractions.

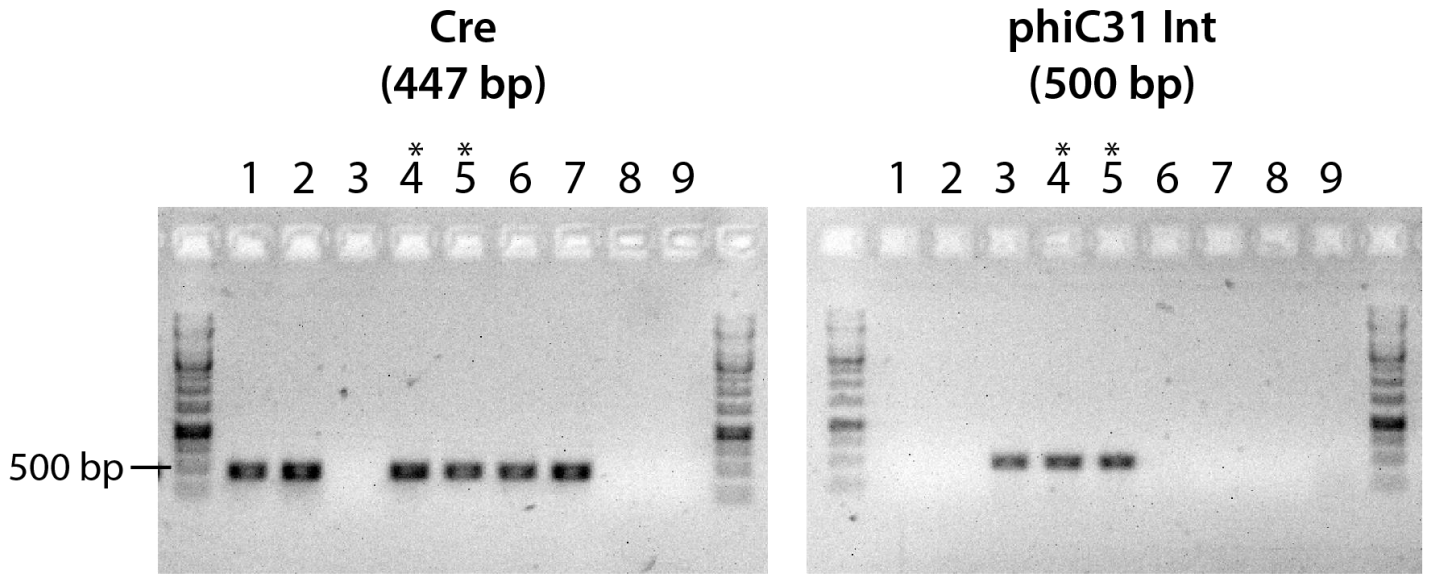


Figure 5.22 - Agarose Gel Image of Cre and phiC31 Integrase Recombinase Stacked Plants: Cre and phiC31 Integrase expression lines described in Chapter 2 were crossed together to create a Cre + phiC31 Integrase stacked plants. PCR analysis indicates the presence of both Cre and phiC31 Integrase coding sequences in plant 4 (Lane 4) and plant 5 (lane 5). These plants were self-crossed to perpetuate the Cre + phiC31 Integrase stock. Primer set data used in this analysis can be found in Table 5.1.

Cre, phiC31 Integrase, and the minichromosome or B insert platform without FLPe (Figure 5.23).

BiBAC Gene Transfer into Artificial Minichromosomes or B Chromosome Platforms Through Breeding:

Plants that were PCR positive for Cre, phiC31 Integrase, and the pZP-Telo-JC minichromosome (17-27) or B insert (17-13) platform were crossed together with modified pJC-YattB-35-1 plants to produce progeny that contained all the necessary components needed for the BiBAC transfer process (Figure 5.24A; Figure 5.24B). However, BiBAC integration could not be detected using a primer set designed to amplify the integration site junction (Figure 5.24C; Table 5.1).

Recombinase-Mediated BiBAC Transfer Through Bombardment

Bombardment of Immature Embryos Containing BiBAC Donor Molecules and Platform (17-13):

Plants that were PCR positive for modified pZP-Telo-JC (17-13) and pJC-YattB-35-1 inserts were selfed as heterozygotes to produce immature embryos that were used in bombardment experiments with the Cre and phiC31 Integrase transient expression construct, pTFCreInt-Kan (Figure 5.6; Figure 5.7). Figure 5.25 shows the background of each plant screened prior to the bombardment process, while Table 5.2 highlights the number of embryos used in the

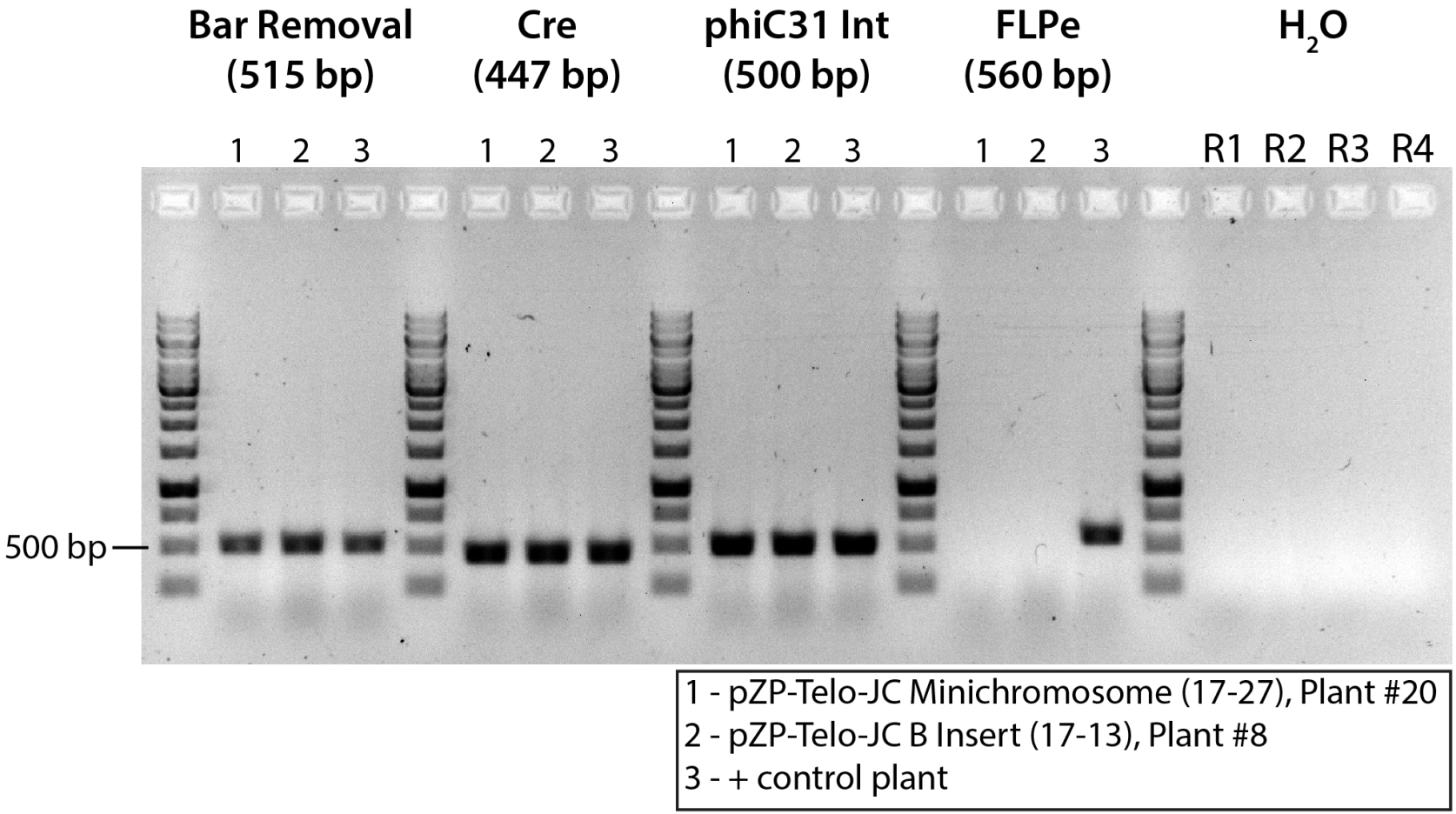

Bar Removal
(515 bp)
Cre
(447 bp)
phiC31 Int
(500 bp)
FLPe
(560 bp)
H2O
1 2 3 1 2 3 1 2 3 1 2 3 R1 R2 R3 R4
500 bp
1 - pZP-Telo-JC Minichromosome (17-27), Plant #20
2 - pZP-Telo-JC B Insert (17-13), Plant #8
3 - + control plant

Figure 5.23 - Agarose Gel Image Highlighting the Background of Modified pZP-Telo-JC + Cre + phiC31 Integrase Plants Used in a Cross to Modified pJC-YattB-35-1 BiBAC Lines: Progeny from a cross between modified pZP-Telo-JC minichromosomes (17-27) or B inserts (17-13) plants (Figure 5.1B) and stacked Cre + phiC31 Integrase expression lines (Figure 5.22) were analyzed using PCR on genomic DNA extracted from leaf tissue. Modified pZP-Telo-JC Minichromosome Plant #20 (Lane 1) and pZP-Telo-JC B insert Plant #8 (Lane 2) contain modified pZP-Telo-JC, Cre and phiC31 Integrase transgenes in the absence of FLPe Recombinase. These plants were selected to use in a cross to modified pJC-YattB-35-1 plants to create plants that contained all the components required for BiBAC transfer through a breeding strategy (Figure 5.5).

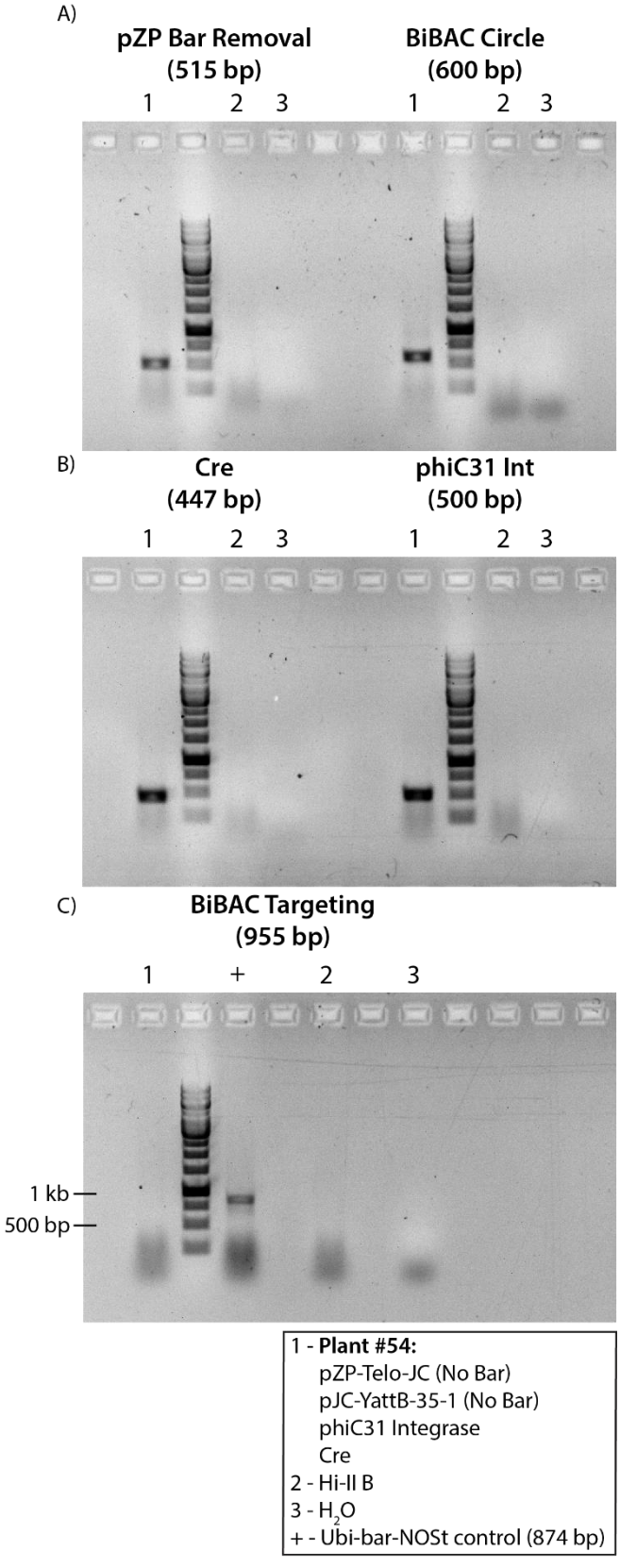

A)
pZP Bar Removal
(515 bp)
BiBAC Circle
(600 bp)
1 2 3
1 2 3
B)
Cre
(447 bp)
phiC31 Int
(500 bp)
1 2 3
1 2 3
C)
BiBAC Targeting
(955 bp)
1 + 2 3
1 kb
500 bp
1 - Plant #54:
pZP-Telo-JC (No Bar)
pJC-YattB-35-1 (No Bar)
phiC31 Integrase
Cre
2 - Hi-II B
3 - H2O
+ - Ubi-bar-NOSt control (874 bp)

Figure 5.24 - Agarose Gel Image Highlighting the Background of a Plant that Contains All the Necessary Components Required for pJC-YattB-35 BiBAC Transfer into pZP-Telo-JC B inserts (17-13): Plant #54 is progeny from a cross between modified pZP-Telo-JC B insert (17-13) + Cre + phiC31 Integrase without FLPe recombinase (Figure 5.23) and a modified pJC-YattB-35-1 BiBAC plant without R recombinase (Figure 5.12). Utilizing primer sets highlighted in Table 5.1, PCR analysis on genomic DNA confirms the presence of modified pZP-Telo-JC (A), BiBAC circular molecules (A), Cre recombinase (B), and phiC31 Integrase (B), but is negative for BiBAC targeting to the pZP-Telo-JC (17-13) B insert platform (C).

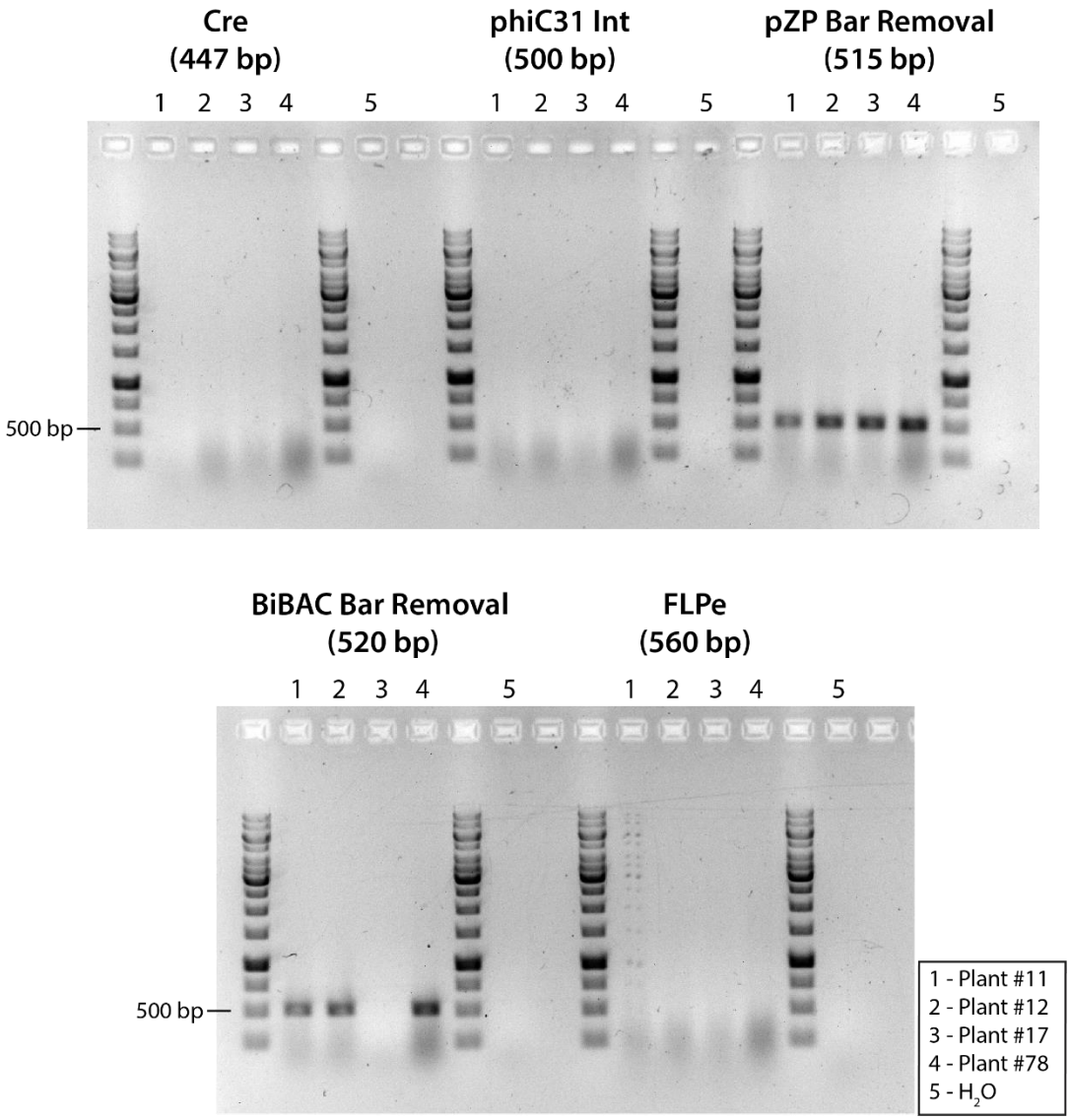

Cre
(447 bp)
phiC31 Int
(500 bp)
pZP Bar Removal
(515 bp)
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
500 bp
BiBAC Bar Removal
(520 bp)
FLPe
(560 bp)
1 2 3 4 5
1 2 3 4 5
500 bp
1 - Plant #11
2 - Plant #12
3 - Plant #17
4 - Plant #78
5 - H_2O

Figure 5.25 - PCR analysis on Genomic DNA from Candidate Plants for BiBAC Transfer Experiments Utilizing Biolistic Bombardment Strategies: Genomic DNA was extracted from leaf tissue and used as template in PCR reactions utilizing primer sets highlighted in Table 5.1. Plant #11 contains both modified pZP-Telo-JC B inserts (17-13) and pJC-YattB-35-1 BiBAC stable integrations without Cre or phiC31 Integrase coding sequences (Lane 1), therefore, was selected as the experimental group. Plant #12 (Lane 2) and Plant #78 (Lane 4) also displayed an appropriate background to be selected as experimental groups, however, were self-crossed to perpetuate the line for future experiments. Plant #17 only contained a stable integration of pZP-Telo-JC (17-13), so it was selected as a negative control group (Lane 3).

Date	Line	Construct	Embryo Count
3/22/19	Plant #11	pTF-CreInt-Kan	62
3/22/19	Plant #17	pTF-CreInt-Kan	44

Table 5.2 - Embryo Count of Biolistic Bombardment Experiments to Induce BiBAC Transfer into pZP-Telo-JC B Inserts (17-13): This table highlights the embryo count of each bombardment carried out on immature embryos of Plant #11 and Plant #17 using pTFCreInt-Kan transient expression constructs (Figure 5.6). For the background of each plant refer to Figure 5.25. Plant #11 contains both modified pZP-Telo-JC B insert (17-13) and pJC-YattB-35-1 transgenes, therefore was used as the experimental group. Plant #17 contains only the modified pZP-Telo-JC B insert (17-13) and was used as a negative control group. It is important to note that due to segregation in meiosis not all embryos contain all the components required for BiBAC transfer.

experiment. Plant 11 and plant 17 were both selected to use in the bombardment as the experimental and control groups, respectively. Since Plant 17 did not contain pJC-YattB-35-1, BiBAC targeting would not be possible with transient expression utilizing pTFCreInt-Kan; therefore, Plant 17 could be used to compare to the experimental plate containing embryos from plant 11, which contained both pZP-Telo-JC (17-13) and pJC-YattB-35-1 inserts (Figure 5.25). Plants 12 and 78 were selfed to perpetuate the line for future use. After 4 weeks on bialaphos selection media, no resistant callus was found (Figure 5.26). This indicates that the BiBAC transfer process did not occur. It is important to note that due to segregation in meiosis not all embryos in the experiment outlined (Table 5.2) contain all the components required for BiBAC transfer. This lowered inheritance frequency could decrease the chances of obtaining a successful targeting event.

Analysis of phiC31 Integrase and FLPe Activity Using ddPCR

To assess phiC31 Integrase function to induce intermolecular recombination reactions between single copy *attB* and *attP* binding sites in different genomic locations, we created plant lines that contained transgene inserts on chromosome 6 (modified pJC-YattB-35-1) and a B chromosome (modified pZP-Telo-JC 17-13) (Figure 5.27). These plants also contained the coding sequence for the phiC31 Integrase enzyme (Figure 5.27). If all components are within the same plant, phiC31 Integrase should bind to the *attB*

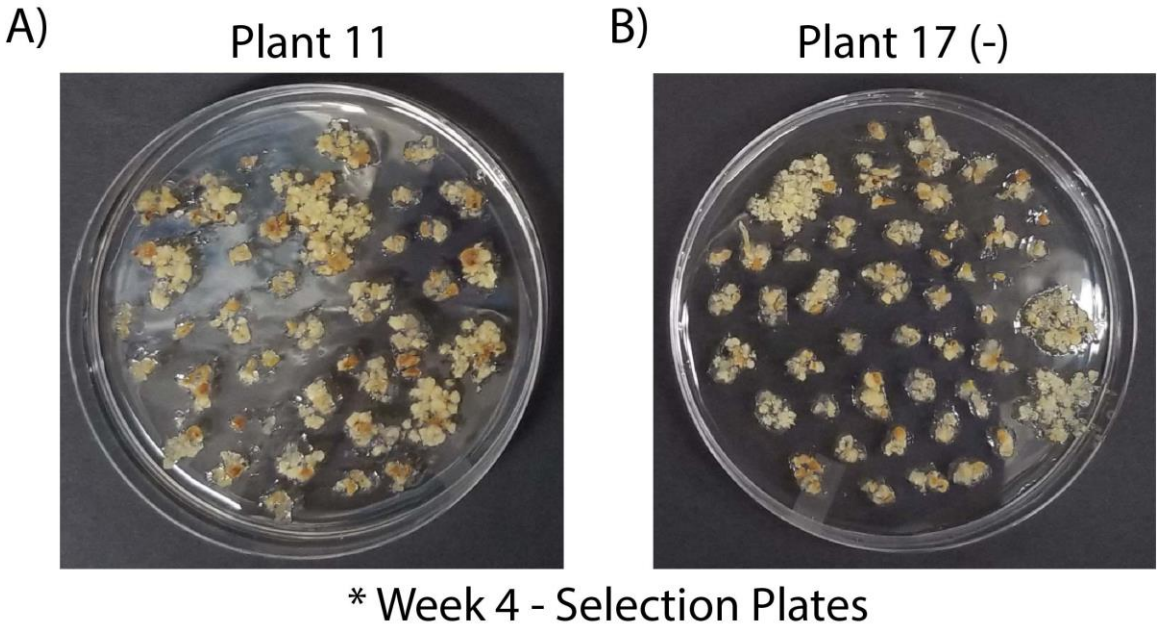


Figure 5.26 - Week 4 Selection Plates from Bombardment Strategy Experiments to Select for BiBAC Gene Transfer in Tissue Culture: Figure 5.25 outlines the plant background of Plant #11 and Plant #17 used as the experimental group and negative control group, respectively. Plant #11 contains both modified pZP-Telo-JC B insert (17-13) and pJC-YattB-35-1 Transgenes, therefore, should result in the circularization and subsequent targeting of the BiBAC molecule upon delivery of the pTFCreInt-Kan transient expression construct (Figure 5.6) into host cells. BiBAC transfer into the pZP-Telo-JC platform is designed to have a promoter trap that “turns on” expression of a bialaphos resistance selectable marker upon successful integration (Figure 5.4); however, callus derived from bombarded embryos of Plant #11 are not resistant to bialaphos selection media at week 4 (A) when compared to the Plant #17 control group (B).

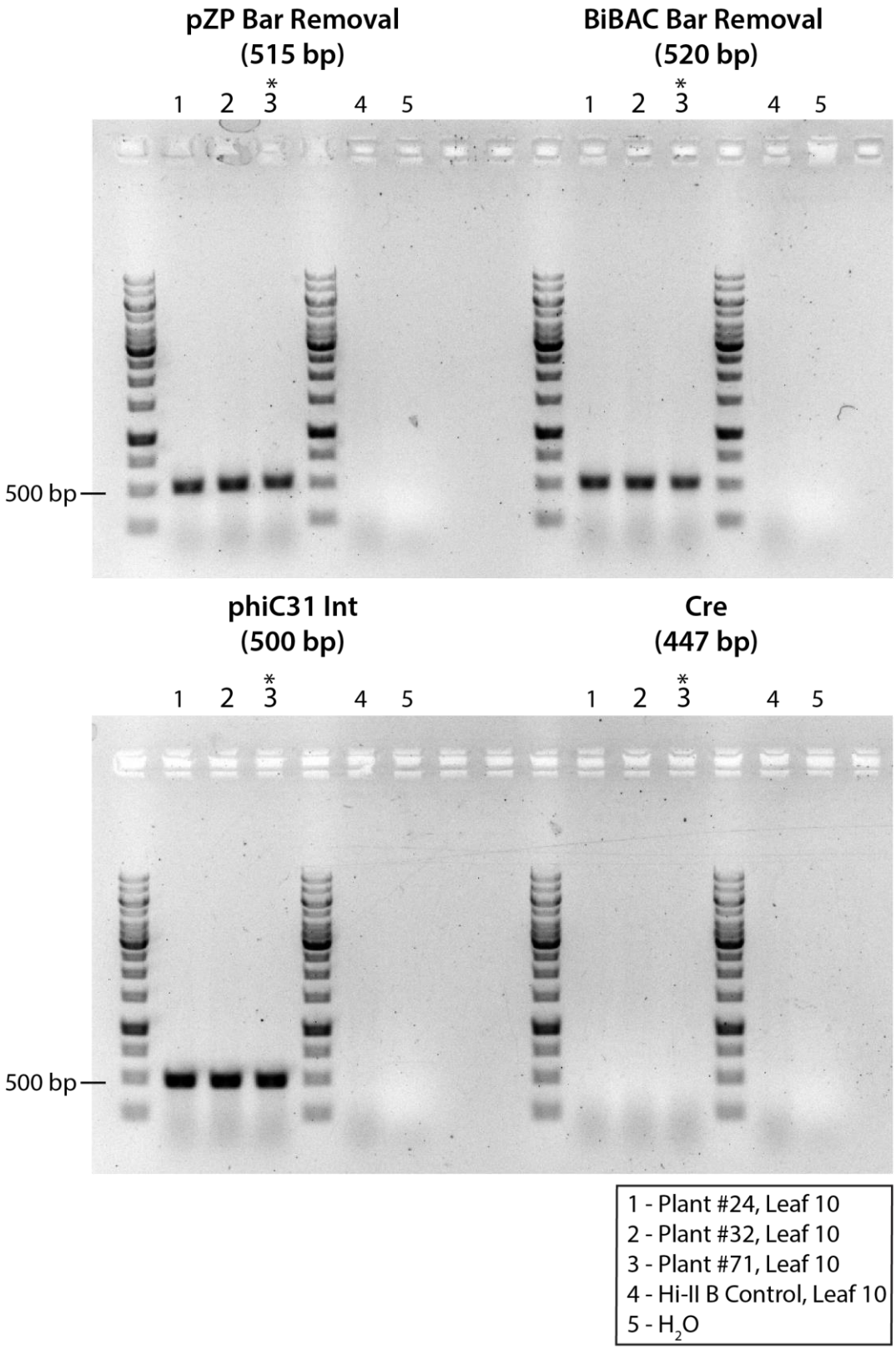

pZP Bar Removal
(515 bp)
BiBAC Bar Removal
(520 bp)
1 2 3* 4 5
1 2 3* 4 5
500 bp
phiC31 Int
(500 bp)
Cre
(447 bp)
1 2 3* 4 5
1 2 3* 4 5
500 bp
1 - Plant #24, Leaf 10
2 - Plant #32, Leaf 10
3 - Plant #71, Leaf 10
4 - Hi-II B Control, Leaf 10
5 - H2O

Figure 5.27 - Agarose Gel Image Displaying PCR Profiles of Candidate Plants Used in Digital Droplet PCR PhiC31 Integrase Translocation Analysis: Genomic DNA isolated from leaf tissue of candidate plant that previously tested positive in PCR analysis for modified pZP-Telo-JC (17-13), modified pJC-YattB-35-1, and phiC31 Integrase transgenes were re-evaluated prior to ddPCR analysis. Late leaf samples (Leaf 10) were used as template in PCR reactions utilizing primer sets highlighted in Table 5.1. Plant #24, Plant #32, and Plant #71 all tested positive for the presence of modified pZP-Telo-JC (17-13), modified pJC-YattB-35-1, and phiC31 Integrase in the absence of Cre recombinase. Plant #71* (Lane 3) was picked as the experimental group for the ddPCR analysis.

and *attP* site on pJC-YattB-35-1 and pZP-Telo-JC 17-13, respectively, to induce a unidirectional intermolecular recombination reaction that results in a translocation between chromosome 6 and the B chromosome (Figure 5.28). Digital droplet PCR analysis on 100 ng/µl of genomic DNA utilizing primer sets that bind to one of the phiC31 translocation junction sites (Table 5.1) quantified the occurrence of this modification as 0.167 copies/µl (Figure 5.31).

To compare the results obtained from the phiC31 Integrase ddPCR translocation analysis, we created plant lines that contained transgene inserts on chromosome 6 (modified pJC-YattB-35-1) and a B chromosome (modified pZP-Telo-JC 17-13) that also expressed FLPe recombinase enzymes (Figure 5.29). If FLPe is expressed in the same plant that contains both pJC-YattB-35-1 and pZP-Telo-JC 17-13 transgene inserts, FLPe will bind to the single copy *FRT* sites in each location to induce a translocation between chromosome 6 and the B chromosome (Figure 5.30). Digital droplet PCR analysis on 100 ng/µl of genomic DNA utilizing primer sets that bind to one of the FLPe translocation junction sites (Table 5.1) quantified the occurrence of this modification as 44.1 copies/µl (Figure 5.31)

Discussion

To circumvent possible complications associated with direct targeting of large gene fragments, this work focuses on exploring the possible strategy of stable insertion of BiBAC transgenes and subsequent rearrangement of this

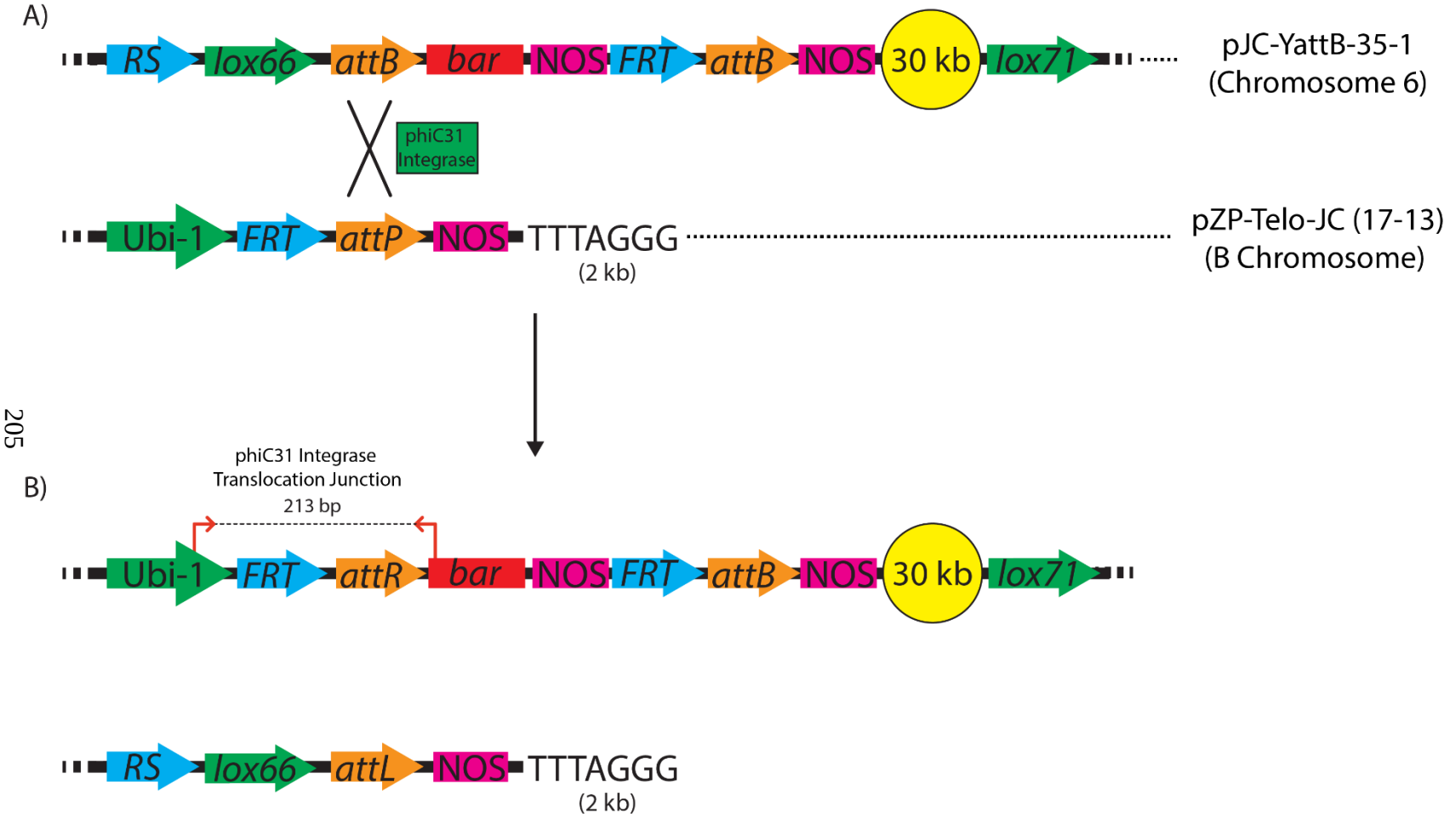

A)
RS
lox66
attB
bar
NOS
FRT
attB
NOS
30 kb
lox71
pJC-YattB-35-1
(Chromosome 6)
phiC31
Integrase
Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC (17-13)
(B Chromosome)
B)
phiC31 Integrase
Translocation Junction
213 bp
Ubi-1
FRT
attR
bar
NOS
FRT
attB
NOS
30 kb
lox71
RS
lox66
attL
NOS
TTTAGGG
(2 kb)

Figure 5.28 - PhiC31 Integrase-Mediated Intermolecular Recombination Reactions to Induce a Translocations Between pJC-YattB-35 BiBACs (Chromosome 6) and pZP-Telo-JC B Inserts (B Chromosome): Theoretically, plants that contain pZP-Telo-JC platforms, pJC-YattB-35 BiBAC inserts and phiC31 transgenes have the necessary components to induce a translocation reaction between *attB* and *attP* sites in different chromosomal locations (A). The resulting translocation reaction can be detected through ddPCR analysis utilizing a primer set that binds to unique features that are arranged into a position that enable DNA amplification and subsequent detection (B).

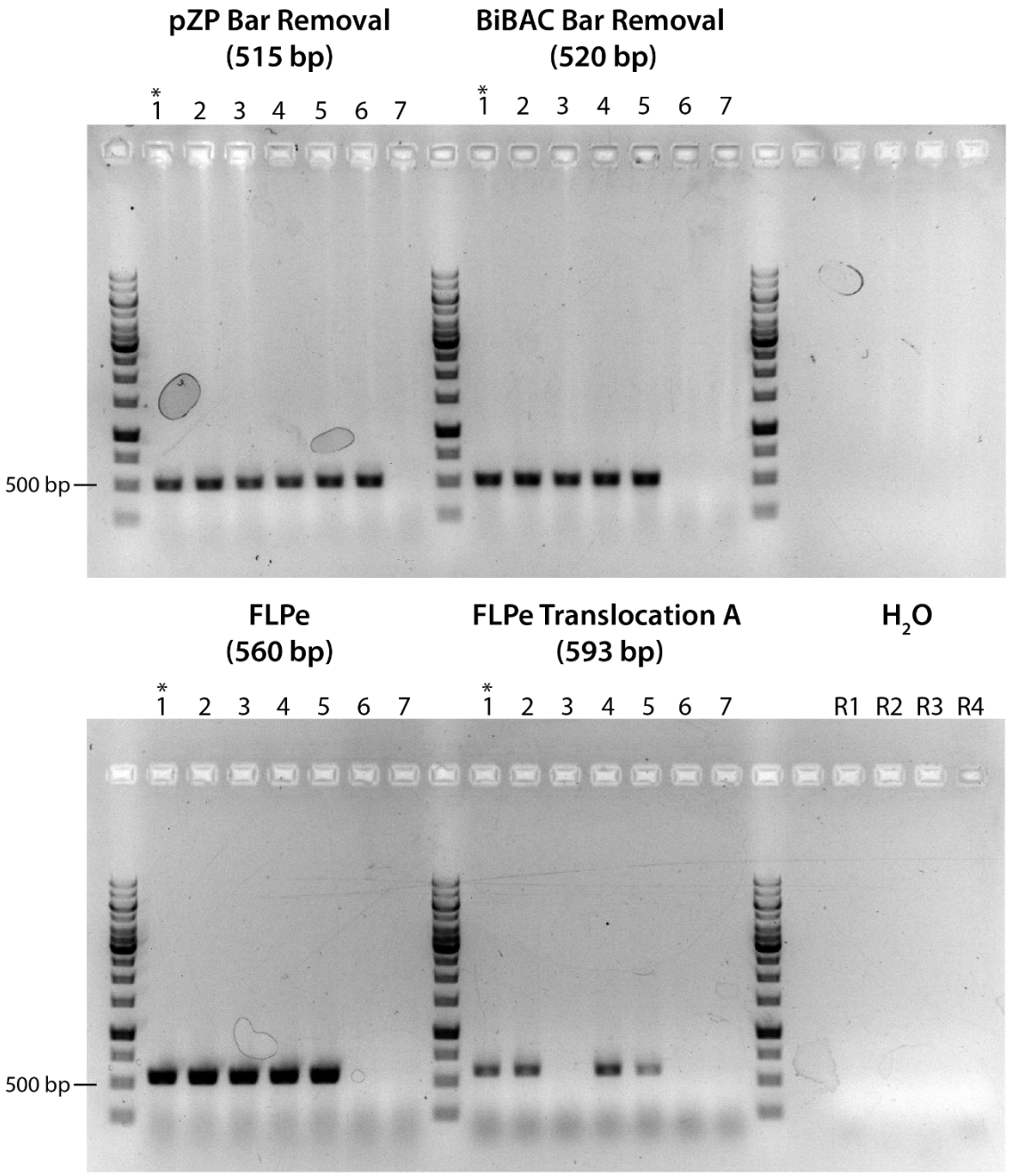

pZP Bar Removal
(515 bp)
BiBAC Bar Removal
(520 bp)
* 1 2 3 4 5 6 7
* 1 2 3 4 5 6 7
500 bp
FLPe
(560 bp)
FLPe Translocation A
(593 bp)
H2O
* 1 2 3 4 5 6 7
* 1 2 3 4 5 6 7
R1 R2 R3 R4
500 bp
1 - Plant #19, Leaf 10
2 - Plant #19, Leaf 3
3 - Plant #6, Leaf 10
4 - Plant #6, Leaf 3
5 - Plant #32, Leaf 3
6 - BiBAC Target Plant 54
7 - Hi-II B, Leaf 10

Figure 29 - Agarose Gel Image Displaying PCR Profiles of Candidate Plants Used in Digital Droplet PCR FLPe Translocation Analysis: Genomic DNA isolated from leaf tissue of candidate plant that previously tested positive in PCR analysis for pZP-Telo-JC (17-13), pJC-YattB-35-1, and FLPe transgenes were re-evaluated prior to ddPCR analysis. Early (Leaf 3) and late (Leaf 10) samples were tested on Plant #19 and Plant #6 (Lanes 1-4) to assess possible differences in modifications. BiBAC target Plant #54 was used as a control group, since the background was previously tested as shown in Figure 5.24. Plant #19 (Leaf 10)* was selected for ddPCR analysis because it displayed PCR banding patterns for each reaction (Lane 1), which indicates the presence of all the necessary components for FLPe-mediated translocations.

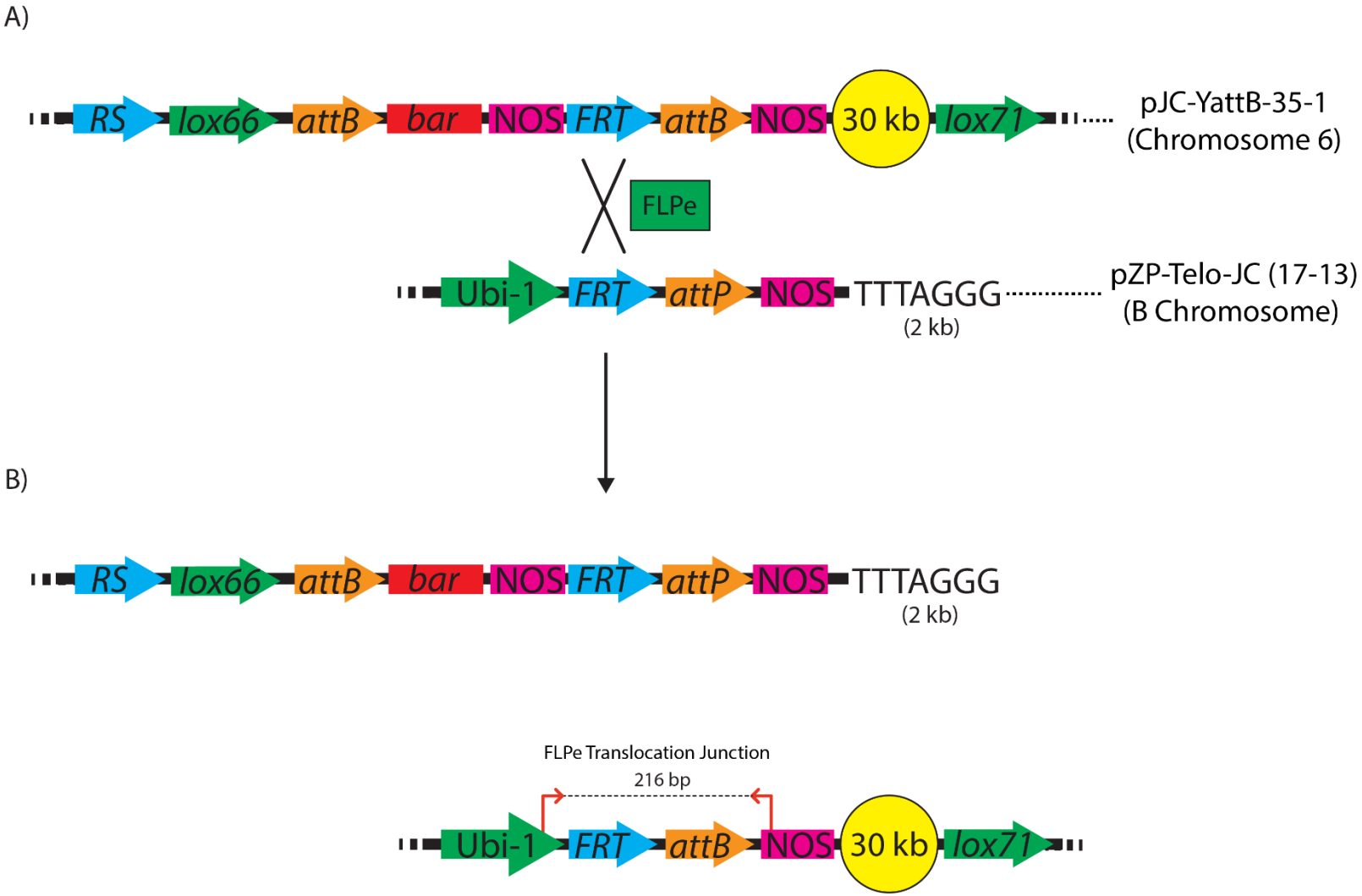

A)
RS
lox66
attB
bar
NOS
FRT
attB
NOS
30 kb
lox71
pJC-YattB-35-1
(Chromosome 6)
FLPe
Ubi-1
FRT
attP
NOS
TTTAGGG
(2 kb)
pZP-Telo-JC (17-13)
(B Chromosome)
B)
RS
lox66
attB
bar
NOS
FRT
attP
NOS
TTTAGGG
(2 kb)
FLPe Translocation Junction
216 bp
Ubi-1
FRT
attB
NOS
30 kb
lox71

Figure 5.30 - FLPe-Mediated Intermolecular Recombination Reactions to Induce a Translocations Between pJC-YattB-35 BiBACs (Chromosome 6) and pZP-Telo-JC B Inserts (B Chromosome): Theoretically, plants that contain pZP-Telo-JC platforms, pJC-YattB-35 BiBAC inserts and phiC31 transgenes have the necessary components to induce a translocation reaction between *FRT* sites in different chromosomal locations (A). The resulting translocation reaction can be detected through ddPCR analysis utilizing a primer set that binds to unique features that are arranged into a position that enable DNA amplification and subsequent detection (B).

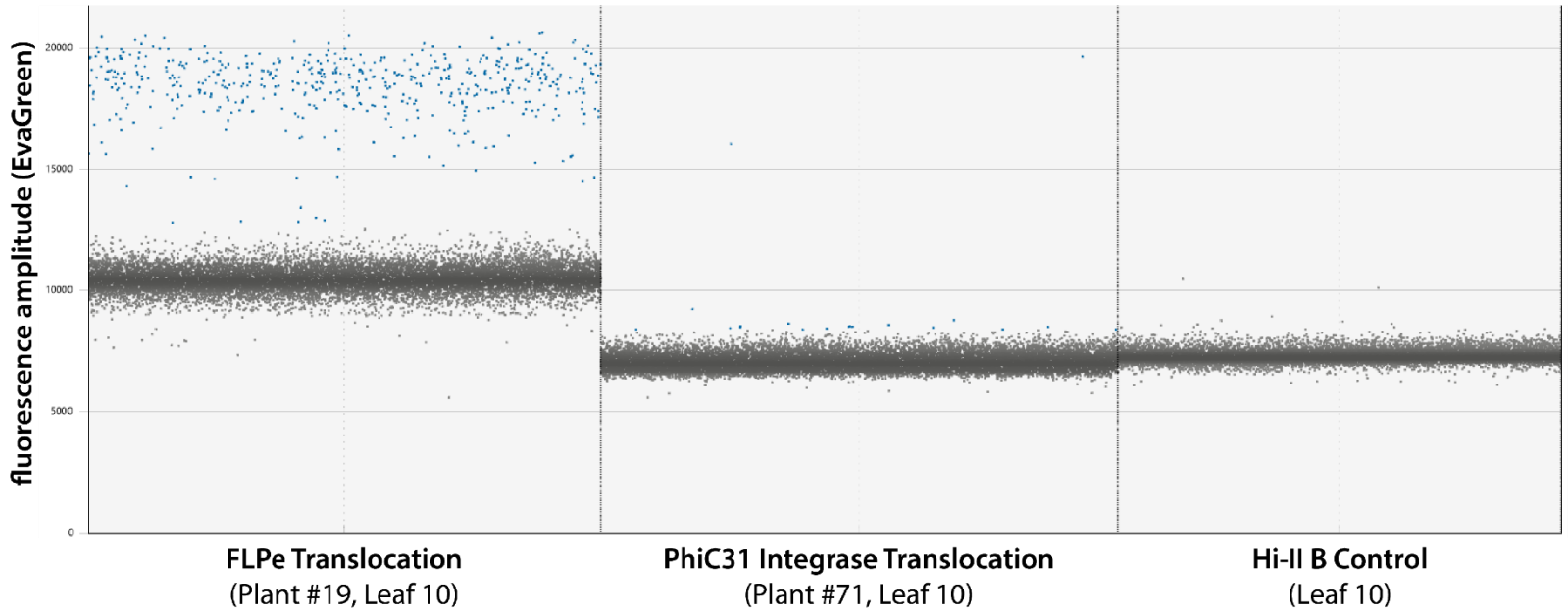

fluorescence amplitude (EvaGreen)
20000
15000
10000
5000
0
FLPe Translocation
(Plant #19, Leaf 10)
PhiC31 Integrase Translocation
(Plant #71, Leaf 10)
Hi-II B Control
(Leaf 10)

Figure 5.31 - Digital Droplet PCR Analysis to Assess Functionality of FLPe and PhiC31 Integrase to Induce Intermolecular Recombination Reactions on Single Copy Binding Sites: Plants that contain all the components for recombinase-mediated translocation reactions between stable transgene insertions located on Chromosome 6 (pJC-YattB-35-1) and a B chromosome (pZP-Telo-JC 17-13) were used in digital droplet PCR analysis to quantify the frequency of these modifications. A stringent threshold for positive events was set at a fluorescent amplitude of 15,000, where dots above this line are colored blue and represent positive events. The FLPe recombinase efficiently induced translocation reactions between chromosome 6 and a B chromosome, producing a reading of 44.1 copies/µl of the targeted modification. The phiC31 Integrase recombinase induced recombination between *attB* and *attP* sites at a 264 fold decrease when compared to FLPe enzymes, 0.167 copies/µl of the targeted modification.

integrated DNA to predetermined locations in the maize genome through the use of the site-specific recombinase systems established in Chapter 2. In Chapter 3, we created targeting platforms pZP-Telo-JC (17-13) and pZP-Telo-JC (17-27), which are located on full sized B chromosomes and B derived artificial minichromosomes, respectively. As previously stated in Chapter 3, these target locations not only give researchers the flexibility to choose an avenue that best suits the needs of the specific project (see page 86), but also avoids possible downstream complications associated with conventional plant genetic engineering methods. The 17-13 B insert and 17-27 minichromosome are designed to accept cargo that has been engineered into the BiBAC T-DNA vector pJC-YattB from Chapter 4, which theoretically can maintain large gene inserts >300 kb. To carry out this transfer process, the site-specific recombinase enzymes Cre and phiC31 Integrase must be present in the same cellular environment as both the pZP-Telo-JC platform and the pJC-YattB BiBAC insert, which can be accomplished through either breeding (Figure 5.5) or bombardment strategies (Figure 5.7). If all components are in the same cell, Cre recombinase will bind to mutant *loxP* sites, *lox66* and *lox71*, on the pJC-YattB molecule to induce a unidirectional recombination reaction that results in BiBAC circularization (Figure 5.3). Subsequently, phiC31 Integrase will bind to non-identical *attB and attP* recombinase binding sites on the BiBAC circle and pZP-Telo-JC platform, respectively, to induce a unidirectional reaction that results in the integration of the BiBAC into the pZP-Telo-JC target site (Figure 5.3). This

process creates a promoter trap, fusing a maize ubiquitin-1 promoter with the coding sequence for a bialaphos resistance selectable marker gene (Figure 5.4), enabling selection for positive transfer events in tissue culture conditions. This promoter trap method, however, is only required for bombardment strategies (Figure 5.7). BiBAC transfer to artificial minichromosomes or B inserts through breeding (Figure 5.5) can be identified through PCR analysis.

Prior to carrying out the BiBAC transfer process through breeding or bombardment, it was necessary to remove the *bar* selectable marker coding sequences from both pZP-Telo-JC platforms (Figure 5.1) and pJC-YattB-35-1 BiBAC inserts (Figure 5.2), which were originally used to select for transgenic plants in tissue culture (Chapter 3; Chapter 4). By crossing both pZP-Telo-JC (17-13) B insert and pZP-Telo-JC (17-27) Minichromosome lines to the FLPe stable expression line, we were able to successfully remove the *bar* selectable marker coding sequence (Figure 5.8). Sanger sequence analysis of the *FRT bar* removal junction verifies the excision of the *bar* and validates the integrity of the *attP* binding site used in future targeting experiments (Figure 5.9). Similarly, the pJC-YattB-35-1 BiBAC insert line was crossed to the R recombinase stable expression line to remove the *bar* selectable marker coding sequence (Figure 5.10). Sanger sequence analysis of the *RS bar* removal junction verifies the excision of the *bar* and validates the integrity of all recombinase binding sites used in BiBAC circularization and subsequent targeting (Figure 5.11).

To verify the functionality of Cre-mediated circularization of stable pJC-YattB-35-1 BiBAC inserts, unmodified pJC-YattB-35-1 plants were crossed to a Cre recombinase stable expression line (Figure 5.13). With this, it was possible to monitor the progeny for presence of the pJC-YattB-35-1 insert and BiBAC removal site through PCR analysis on genomic DNA extracted from leaf tissue (Figure 5.13; Figure 5.14). In all progeny that contained the unmodified pJC-YattB-35-1 BiBAC insert and Cre recombinase, the BiBAC removal site was detectable; however, in progeny that only contained the unmodified pJC-YattB-35-1 BiBAC insert, the BiBAC removal site was not detectable (Figure 5.14). This indicates that Cre recombinase is necessary for the removal of the *lox66* and *lox71* flanked BiBAC DNA fragment. Sanger sequence analysis was utilized to verify the removal of the BiBAC insert and formation of a single *loxP* site without mutations (Figure 5.15B; Figure 5.16B). While we could always detect the BiBAC removal site if both the pJC-YattB-35-1 and Cre were present in the same plant, this does not hold true for the detection of the BiBAC circular molecule (Figure 5.14).

Based on numerous studies that utilize Cre recombinase for the removal of undesirable sequences, it was assumed that BiBAC circularization would occur early in embryo development and observation of these circular molecules would not be possible due to degradation or dilution though cellular divisions. This assumption was true for some plants that were analyzed (Figure 5.14-Lane 2); however, in other plants that contained both the unmodified pJC-YattB-35-1

transgene and Cre recombinase, detection the of BiBAC circular molecules was possible through PCR analysis on genomic DNA extracted from leaf tissue utilizing a primer set that amplifies the *lox66/71* BiBAC circle junction upon Cre-mediated circularization (Figure 5.13; Figure 5.14-Lane 1). This primer set will only yield a PCR product if the removed BiBAC DNA is circularized, orienting the primer binding sites into a position that amplifies a 600 bp product (Figure 13B). Sanger sequence analysis of this PCR product confirms circularization and formation of a single *lox66/71* site that contains two mutations (Figure 5.15A; Figure 5.16A).

The persistence of extrachromosomal DNA elements excised through the use of site-specific recombinase enzymes has been a subject of debate among researchers in the field of plant genetic engineering since the first observation from Srivastava and Ow (2003). While persistence of circular molecules created with recombinases have been observed in tomato (Coppoolse et al., 2005), wheat (Srivastava and Ow, 2003), barley (Kapusi et al., 2012) and maize (Yang et al., 2009), other studies that utilize recombinases for targeted gene removal have not observed maintenance of excised DNA. This could be due to the extrachromosomal circles not existing in some instances, or that researchers assumed that excised DNA would eventually degrade and did not conduct an analysis that would detect this persistence. Nevertheless, our analysis clearly detects the maintenance of circularized ~30 kb BiBAC molecules in the 13th leaf stage of plant development (Figure 5.17). While the specific mechanism

controlling the persistence of the BiBAC circle in mitotically active cells is unknown, it is reasonable to believe that these circles could be replicating at some undetermined level. Based on comparative banding intensity between the Removal Site and BiBAC Circle PCR reactions, which contained equimolar amounts of template, we assume the level of replication would have to be low (Figure 5.14). Indeed, Cre excised DNA circles are not supercoiled like other excised circular molecules (Kinoshita et al., 1985) due to the type I topoisomerase activity of Cre, which "relaxes" DNA with *loxP* binding sites (Abremski et al., 1986; Srivastava and Ow, 2003). This could give the necessary enzyme complexes for DNA replication access to the circular molecule. In the context of BiBAC targeting, this could give phiC31 Integrase enzymes an increased window of time to find the *attB* and *attP* in different areas of the genome to initiate the integration process into target sites through breeding or bombardment.

To assess the feasibility of utilizing PhiC31 Integrase for transgene targeting to predetermined locations, we carried out a bombardment experiment on immature embryos that expressed phiC31 Integrase and contained a GLP insert using *attB*-DsRed donor molecules (Figure 5.18; Figure 5.19). GLP inserts contain an empty maize ubiquitin-1 promoter followed by an *attP* recombinase binding site (Figure 5.20). If embryos contain a GLP insert and stably express phiC31 Integrase enzymes, *attB*-DsRed molecules delivered into cells via biolistic bombardment should be targeted to predetermined locations through

phiC31 Integrase-mediated intermolecular recombination reactions between the *attB* and *attP* binding sites on the *attB*-DsRed donor and GLP target, respectively (Figure 5.18D). 1-2 days after bombardment, genomic DNA was extracted from bombarded embryos and used as template in a PCR analysis utilizing a primer set that flanks the integration junction (Table 5.1; Figure 5.18D). Embryos bombarded with the *attB*-DsRed donor molecule exhibited banding patterns that indicated successful targeting to the GLP platform (Figure 5.21).

The GLP bombardment results (Figure 5.21) indicate that recombinase-mediated transgene targeting through the use of phiC31 is possible; however, it is important to note that both breeding (Figure 5.5) and bombardment (Figure 5.7) BiBAC targeting strategies only utilizes a single copy of each attachment site, *attB* and *attP*. In contrast, the bombardment of the *attB*-DsRed molecule delivers many copies of the *attB* site into cells that contain a single *attP* site. It is possible that the stoichiometry between enzyme and binding site is important in obtaining a successful targeted product. While the GLP bombardment experiment does not perfectly mirror the conditions that are used in the designed BiBAC targeting experiments, it demonstrates that DNA can be targeted to predetermined locations utilizing phiC31 Integrase under the right conditions.

To create conditions to test BiBAC targeting to minichromosomes or B inserts through breeding (Figure 5.5), modified pZP-Telo-JC minichromosomes (17-27) and modified pZP-Telo-JC B inserts (17-13) from Figure 5.8 were crossed to stacked Cre + phiC31 Integrase lines from Figure 5.22. Progeny from

this cross were screened using PCR analysis for the presence of modified pZP-Telo-JC, Cre and phiC31 Integrase transgenes and absence of FLPe Recombinase (Figure 5.23). We obtained two plants, Plant #20 and Plant #8, which exhibited the correct backgrounds for both the minichromosome (Figure 5.23-Lane 1) and the B insert (Figure 5.23-Lane 2), respectively. These plants were crossed to modified pJC-YattB-35-1 lines segregated from R recombinase (Figure 5.12). Progeny from these crosses were screened using PCR analysis for the presence of modified pZP-Telo-JC, modified pJC-YattB-35-1, Cre and phiC31 Integrase transgenes. While we did not obtain a plant background for the artificial minichromosome aspect of this project due to low frequency of inheritance (see Table 3.4), we did obtain a pZP-Telo-JC B insert plant, Plant #54, that contained all the necessary components for BiBAC transfer through breeding (Figure 5.24A; Figure 5.24B). However, BiBAC targeting in Plant #54 was unsuccessful (Figure 5.24C).

To create conditions to test BiBAC targeting to predetermined locations through the use of bombardment and subsequent tissue culture conditions (Figure 5.7), plants that contained modified pZP-Telo-JC B inserts and modified pJC-YattB-35-1 without Cre or phiC31 Integrase (Figure 5.25) were self-crossed to generate immature embryos that were used in the bombardment experiment. These embryos were bombarded with the pTFCreInt-Kan construct described in Figure 5.6. Once delivered into cells that contain both modified pZP-Telo-JC B inserts and modified pJC-YattB-35-1 BiBAC transgenes, the Cre and phiC31

Integrase recombinases are transiently expressed, which should result in the circularization and subsequent targeting of the BiBAC molecule to the pZP-Telo-JC platform. As previously described, this process was designed to create a fusion between the maize ubiquitin-1 promoter on the pZP-Telo-JC platform with the coding sequence for a bialaphos resistance selectable marker on the BiBAC circular molecule (Figure 5.4). In all cells that BiBAC transfer occurs, this will initiate expression of the coding sequence that confers resistance to bialaphos treatment in tissue culture conditions. Table 5.2 describes in further detail the number of embryos used in this bombardment process. Through the bombardment of 62 embryos with pTFCreInt-Kan transient expression construct (Table 5.2-Plant #11), we did not obtain a positive event after 4 weeks of selection (Figure 5.26).

The results derived from the various parts of this study indicate to us that BiBAC transfer to predetermined locations in the genome, such as artificial minichromosomes or B inserts, should be possible; however, the specific approach would need to be reworked to ensure success in the future. It is clear that the utilization of Cre recombinase enzymes to induce the circularization of a large extrachromosomal DNA fragment, such as a BiBAC insert, is a feasible approach to mobilizing genetic elements prior to transfer (Figure 5.14; Figure 5.15; Figure 5.16). Additionally, the observation of BiBAC circle persistence in mitotically active leaf tissues suggests that targeting could happen later in plant development (Figure 5.17). While we did observe phiC31 Integrase activity in

maize cells through the use of a DsRed reporter construct (Figure 2.7) and utilized this enzyme to direct targeted integrations to GLP targets through the bombardment of *attB*-DsRed Donor constructs (Figure 5.19; Figure 5.20; Figure 5.21), both of these analyses do not perfectly represent phiC31 Integrase utility in the same context as the BiBAC targeting experiments through breeding or bombardment.

The breeding and bombardment approaches to BiBAC transfer utilize a single copy of both *attB* and *attP* in different genomic locations, which are used by phiC31 integrase to induce an intermolecular recombination reaction. While evidence provided in this study suggests that phiC31 integrase is functional in maize, we do not provide support that phiC31 can carry out recombination reactions between single copy binding sites in different locations at high efficiency. To assess the functionality of phiC31 integrase in inducing intermolecular reactions, we generated a plant line that contains a modified pZP-Telo-JC (17-13) B insert, modified pJC-YattB-35-1 BiBAC integration and the phiC31 Integrase coding sequence (Figure 5.27). If functional, phiC31 Integrase will induce a intermolecular recombination reaction that creates a translocation between the *attP* site on pZP-Telo-JC (17-13) and the *attB* site on pJC-YattB-35-1 BiBAC integrations in chromosome 6, respectively (Figure 28). Based on the previous BiBAC targeting results (Figure 5.24; Figure 5.26), it was assumed that if this translocation reaction was occurring, it would be at a rate that was too low to detect with conventional PCR methods; therefore, we utilized digital droplet

PCR (ddPCR), a technique that has been reported to detect rare edits at frequencies < 0.5% of the extracted cells while using low concentrations of DNA (Berman et al., 2015). For comparative purposes, we created plants that contain a modified pZP-Telo-JC (17-13) B insert, modified pJC-YattB-35-1 BiBAC integration and the FLPe coding sequence (Figure 5.29). This genotype should also exhibit translocations between the B insert and chromosome 6; however, it utilizes the FLPe-*FRT* recombinase system (Figure 5.30). Figure 5.31 displays the results derived from the comparative ddPCR analysis on FLPe and phiC31 Integrase-mediated translocations. This result indicates that phiC31 integrase is capable of inducing recombination reactions between *attB* and *attP* binding sites in different locations in the genome; however, this reaction occurs at an incredibly low rate (0.167 copies/µl). When compared to the frequency observed in FLPe-mediated translocations, which is at a 264 fold increase (44.1 copies/ µl), it is safe to assume that phiC31 is the component that needs to be reworked to ensure success of BiBAC transgene targeting in the future.

Material and Methods

Transgenic Plant Material:

Minichromosome (17-27) and B insert (17-13) platforms were generated through *Agrobacterium*-mediated transformation with the pZP-Telo-JC truncation construct. Vector description and steps outlining the establishment of stable integrations can be found in Chapter 3.

High molecular weight donor molecule pJC-YattB-35-1 was generated through *Agrobacterium*-mediated transformation with the pJC-YattB-35 BiBAC vector. Construct description and steps outlining the transformation process used to establish BiBAC integrations can be found in Chapter 4.

The FLPe recombinase stable expression line was generated through *Agrobacterium*-mediated transformation of Hi-II immature embryos using the pTF-FLPe T-DNA vector. Details outlining the steps used to clone pTF-FLPe and transform the coding sequences into Hi-II can be found in Chapter 2.

The R recombinase stable expression line was generated through *Agrobacterium*-mediated transformation of Hi-II immature embryos using the pTF-R T-DNA vector. Details outlining the steps used to clone pTF-R and transform the coding sequences into Hi-II can be found in Chapter 2.

The Cre recombinase stable expression line was generated through *Agrobacterium*-mediated transformation of Hi-II immature embryos using the pTFUbiCre T-DNA vector. Details outlining the steps used to clone pTFUbiCre and transform the coding sequences into Hi-II can be found in Chapter 2.

The phiC31 Integrase stable expression line was generated through *Agrobacterium*-mediated transformation of Hi-II immature embryos using the pTFPhiC31 T-DNA vector. Details outlining the steps used to clone pTFUbiCre and transform the coding sequences into Hi-II can be found in Chapter 2.

The attB-DsRed Bombardment Construct:

The *attB*-DsRed coding sequence was synthesized by GeneWiz and subloned into pWY56c using restriction endonucleases *Pac*I and *Bam*HI. The official name for the *attB*-DsRed construct on file is pWYBDsReDIINoUbi.

Cloning pTFCreInt-Kan Transient Expression Construct:

Cre recombinase coding sequence was PCR amplified from pTFUbiCre and cloned into pTFPhiC31 using *Avr*II and *Sbf*I restriction endonucleases to produce pTFCreInt-*Bar*. The coding sequence that confers resistance to kanamycin antibiotic selection was PCR amplified from BiBAC vector pNG6 and cloned into pTFCreInt-*Bar* using *Saw*I and *Aat*II to produce pTFCreInt-Kan.

PCR analysis of Transgenic Plant Material:

Genomic DNA from transgenic plant leaf tissue was extracted using the method described (Leach et al., 2016) and amplified using primers described in Table 5.1. Standard PCR conditions of 35 cycles: 30s denature at 95°C, 30s annealing at 60°C, and Xs extension (X depends on the product size, where 1 kb = 1 min).

BiBAC circle junction analysis utilized primer sets described in Table 5.1. Since it was assumed that the extrachromosomal circles existed at a lower concentration in comparison to stably integrated material, the number of PCR cycles was extended to 40-45. This enabled DNA fragment detection on conventional agarose gels (Figure 5.14).

Preparation of PCR amplified DNA for Sanger Sequence Analysis:

DNA fragments amplified from target sequences using primer sets in Table 5.1 were prepared for Sanger Sequencing utilizing TOPO TA cloning kits from Invitrogen. In this, 2 - 3 µl of PCR product was used in a reaction mixture with pCR 4-TOPO vectors, under manufacturer's conditions, to clone DNA fragments into vectors that were used as the templates for Sanger sequencing reactions. Sequence data was processed using SnapGene software. For further information on TA cloning using Invitrogen products, refer to the manufacturer's instructions.

Biolistic Bombardment:

Particle bombardment was performed on Hi-II immature embryo explants, harvested 10 – 12 days after pollination. Embryo length was approximately 1.5 – 2 mm in length. After extraction from ears, embryo explants were placed on N6 media for 3 – 5 days prior to bombardment (Songstad et al. 1996). 4 hours prior to bombardment, embryos were transferred to osmotic media. 0.6 µm gold particles (BioRad) were prepared following the steps outlined by Iowa State Plant Transformation Facility (Sanford et al. 1993; Frame et al. 2000). Approximately 1 µg of each of each recombinase test construct was used in the 120 µl gold mixture. 10 µl of the gold particle suspension was pipetted onto macrocarriers (BioRad) and allowed to dry in a hood. After drying, another 10 µl of the gold

particle suspension was added again, equating to ~166 ng/shot. The bombardment procedure was performed with a 1000/He Particle Delivery System (BioRad) using 650 psi rupture disks. After bombardment, embryos rested on osmotic media for 1 hr before being transferred to N6 media. Once friable type-II callus was visible, tissue was transferred to selection plates containing 3 ug/ml bialaphos and monitored for resistant cells.

Digital Droplet PCR:

Genomic DNA from transgenic plant leaf tissue was extracted using the method described (Leach et al., 2016) and diluted to 100 ng/µl. Each reaction mixture contains ~100 ng of genomic template that is partitioned into ~15,000 – 20,000 spherical droplets, resulting in 0, 1, 2, or more genomic targets in each bubble (Mazaika and Homsy, 2014). Each droplet also contains EvaGreen dye, which binds to amplified PCR products to emit a fluorescence signal. When complete reactions are passed through a ddPCR reader, such as the QX200 from BioRad, the fluorescent amplitude is measured in real time for each spherical droplet and recorded in a graphical form (Figure 5.31). Comparative analysis of phiC31 and FLPe activity was carried out using primer sets that amplified recombination site junctions (Table 5.1). For further information regarding the experimental procedure, supplies, and thermocycler conditions refer to the BioRad instructions.

Recombinase Primer Sets:

Primer (size bp)	Sequence	T_a
Cre (447 bp)	**F - 5′** GAACGTGCAAAACAGGCTCT **3′** **R - 5′** ATCCTTAGCGCCGTAAATCA **3′**	60°C
R (769 bp)	**F - 5′** CCAGCGCTCTATTTCCAAGA **3′** **R - 5′** GGCCTCCTTATCCATCTCGT **3′**	60°C
FLPe (560 bp)	**F - 5′** GTGAGGGTGAAAGCATCTGG **3′** **R - 5′** AGCACGCTTATCGCTCCAAT **3′**	60°C
PhiC31 Int (500 bp)	**F - 5′** CGTGACGATTGTTTCCACTC **3′** **R - 5′** AAGGATTCGCATAACGGTTG **3′**	60°C

Modification Primer Sets:

Primer (size bp)	Sequence	T_a
FRT Bar Removal Junction (516 bp)	**F - 5′** TTTAGCCCTGCCTTCATACG **3′** **R - 5′** CGTGAGCTCGTTTAAACTTCG **3′**	60°C
RS Bar Removal Junction (520 bp)	**F - 5′** TGTAGATGTCCGCGGTTAAT **3′** **R - 5′** GTAGAGCGTGGAGCCCAGT **3′**	60°C
BiBAC Circle (600 bp)	**F - 5′** TTCTTCACCACTGGATGCTG **3′** **R - 5′** GTACGGAAGTTGACCGTGCT **3′**	60°C
BiBAC Circle Removal Site (600 bp)	**F - 5′** GTTTCTGGCAGCTGGACTTC **3′** **R - 5′** AGAAAGCTCGCAATTGAGGT **3′**	60°C
BiBAC Circle Removal Site (600 bp)	**F - 5′** GTTTCTGGCAGCTGGACTTC **3′** **R - 5′** AGAAAGCTCGCAATTGAGGT **3′**	60°C
DsRed Target (400 bp)	**F - 5′** TTAGCCCTGCCTTCATACGC **3′** **R - 5′** TACACCTTGGAGCCGTACTG **3′**	60°C
BiBAC Targeting (955 bp)	**F - 5′** GGAACTGTATGTGTGTGTCATACATC **3′** **R - 5′** GAAGTCCAGCTGCCAGAAAC **3′**	60°C

Common Feature Primer Sets:

Primer (size bp)	Sequence	T_a
pJC-YattB-35 (874 bp)	**F - 5′** GGAACTGTATGTGTGTGTCATACATC **3′** **R - 5′** GAAGTCCAGCTGCCAGAAAC **3′**	60°C
pJC-YattB-35 (581 bp)	**F - 5′** CACTATAGGGCGAATTGGGTA **3′** **R - 5′** GAAGTCCAGCTGCCAGAAAC **3′**	60°C

Digital Droplet PCR Primer Sets:

Primer (size bp)	Sequence
phiC31 Integrase Translocation Junction (213 bp)	**F - 5′** CCCTGCCTTCATACGCTATT **3′** **R - 5′** CGTTCTGGGCTCATGGTTAC **3′**
FLPe Translocation Junction (216 bp)	**F - 5′** TTTAGCCCTGCCTTCATACG **3′** **R - 5′** ATTGCCAAATGTTTGAACGA **3′**

Table 5.1 – Primer Set Data Used in PCR Analysis of Transgenic Material: The following table outlines the primer sets used in PCR analysis of transgenic material. The primer set name, product size and sequence are provided.

References

Abremski, K., Wierzbicki, A., Frommer, B. and Hoess R.H. (1986) Bacteriophage P1 Cre-loxP site-specific recombination: sitespecific DNA topoisomerase activity of the Cre recombination protein. J. Biol. Chem. 261, 391–396.

Akbudak M.A. and Srivastava V. (2011) Improved FLP recombinase, FLPe, efficiently removes marker gene from transgene locus developed by Cre-lox mediated site-specific gene integration in rice. Mol Biotechnol 49(1), 82-89.

Albert H., Dale E.C., Lee E., Ow D.W. (1995) Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. Plant J. 7, 649–659.

Anand A., Wu E., Li Z., TeRonde S., Arling M., Lenderts B., Mutti J.S., Gordon-Kamm W., Jones T.J., Chilcoat N.D. (2019) High efficiency *Agrobacterium*-mediated site-specific gene integration in maize utilizing the FLP-FRT recombination system. Plant Biotechnol J. doi: 10.1111/pbi.13089.

Bateman J.R., Lee A.M., Wu C. (2006) Site-Specific Transformation of Drosophila via φC31 Integrase-Mediated Cassette Exchange. Genetics. 173, 769-777.

Berman J.R., Cooper S., Zhang B., Karlin-Neumann G., Litterst C., Jouvenot Y., Hefner E., Miyaoka Y., Conklin B. (2015) Ultra-sensitive quantification of genome editing events using droplet digital PCR. Bio-Read Laboratories, Inc. Bulletin 6712.

Coppoolse E.R., de Vroomen M..J, van Gennip F., Hersmus B.J., van Haaren M.J. (2005) Size does matter: cre-mediated somatic deletion efficiency depends on the distance between the target lox-sites. Plant Mol Biol. 58, 687–698.

Day C.D., Lee E., Kobayashi J., Holappa L.D., Albert H., Ow D.W. (2000) Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. Genes Dev. 14, 2869-2880.

Frame B.R., Zhang H., Cocciolone S.M., Sidorenko L.V., Dietrich C.R., Pegg S.E. (2000) Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. 36(1), 21-29.

Groth A.C., Olivares E.C., Thyagarajan B., Calos M.P. (2000) A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci USA. 23, 5995-6000.

Kapusi E., Kempe K., Rubtsova M., Kumlehn J., Gils M., (2012) PhiC31 Integrase-Mediated Site-Specific Recombination in Barley. PLoS ONE 7(9). e45353.doi:10.1371/journal.pone.0045353.

Kinoshita Y., Ohnishi N., Yamada Y., Kunisada T., Yamagishi H. (1985) Extrachromosomal circular DNA from nuclear fraction of higher plants. Plant and Cell Physiology. 26, 1401-1409.

Li Z., Xing A., Moon B.P., Mcardell R.P., Mills K., Falco S.C., (2009) Site-Specific Integration of Transgenes in Soybean via Recombinase-Mediated DNA Cassette Exchange. Plant Physiol 151, 1087-1095.

Lowe K., Wu E., Wang N., Hoerster G., Hastings C., Cho M., Scelonge C., Lenderts B., Chamberlin M., Cushatt J., Wang L., Ryan L., Khan T., Chow-Yiu J., Hua W., Yu M., Banh J., Bao Z., Brink K., Igo E., Rudrappa B., Shamseer P., Bruce W., Newman L., Shen B., Zheng P., Bidney D., Falco C., Register J., Zhao Z., Xu D., Jones T., Gordon-Kamm W. (2016) Morphogenic regulators baby boom and wuschel improve monocot transformation. Plant Cell. 28, 1998-2015.

Mazaika E. and Homsy J. (2014) Digital droplet PCR: CNV analysis and other applications. Curr Protoc Hum Genet. doi:10.1002/0471142905.hg0724s82.

Mookkan M., Nelson-Vasilchik K., Hague J., Zhang Z.J., Kausch A.P. (2017) Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators *BABY BOOM* and *WUSCHEL2*. Plant Cell Reports. 36, 1477-1491.

Nandy S. and Srivastava V. (2012) Marker-free site-specific gene integration in rice based on the use of two recombination systems. Plant Biotechnol J. 10, 904-912.

Roberts J.A., Miguel-Escalada I., Slovik K.J., Walsh K.T., Hadzhiev Y., Sanges R., Stupka E., Marsh E.K., Balciuniene J., Balciunas D., Müller F. (2014) Targeted transgene integration overcomes variability of position effects in zebrafish. Development. 141, 715-724.

Sanford J.C., Smith F.D., Russell J.A. (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol 217, 483-509.

Shrawat A.K. and Lörz H. (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. Plant Biotechnol J. 4, 575-603.

Songstad D.D., Armstrong C.L, Petersen W.L., Hairston B., Hinchee M.A.W. (1996) Production of transgenic maize plants and progeny by bombardment of hi-II immature embryos. In Vitro Cell Dev Biol Plant 32(3), 179-183.

Srivastava V. (2019) Gene stacking in plants through the application of site-specific recombination and nuclease activity. Methods Mol. Biol. 1864, 267-277.

Srivastava V. and Ow D. (2001) Single-copy primary transformants of maize obtained through the co-introduction of a recombinase-expressing construct. Plant Molecular Biology. 46, 561-566.

Srivastava V. and Ow D. (2002) Biolistic mediated site-specific integration in rice. Mol Breed. 8, 345-350.

Srivastava V. and Ow D. (2003) Rare instances of Cre-mediated deletion product maintained in transgenic wheat. Plant Mol Biol. 52, 661–668.

Srivastava V. and Thomson J. (2016) Gene Stacking by recombinases. Plant Biotechnology J. 14, 471-482.

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

Thorpe, H.M. and Smith M.C.M. (1998) In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc. Natl. Acad. Sci. U.S.A. 95 (10), 5505-5510.

Thyagarajan B., Olivares E.C., Hollis R.P., Ginsburg D.S., Calos M.P. (2001) Site-specific genomic integration in mammalian cells mediated by phage phiC31 integrase. Mol Cell Biol. 21, 3926-3934.

Vergunst A.C. and Hooykaas P.J.J. (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.

Yang M., Djukanovic V., Stagg J., Lenderts B., Bidney D., Falco S.C., Lyznik L.A. (2009) Targeted mutagenesis in the progeny of maize transgenic plants. Plant Mol Biol. 70, 669–679.

Chapter 6: Conclusion

Recombinase-mediated BiBAC gene transfer and stable integration into predetermined locations in the maize genome requires the utility of 4 different site-specific recombinase systems, establishment of a large DNA donor molecule and creation of a target platform. In this work, we have demonstrated the functionality of 5 different recombinase systems in maize (Cre, FLPe, R, PhiC31 Integrase and PhiC31 Excisionase), which enable precise and predictable modifications to DNA depending on the location and orientation of the respective recombinase binding site (Chapter 2). We also have generated an alternating BiBAC vector system that utilizes Cre and phiC31 Integrase systems to sequentially circularize and target large DNA fragments to predetermined locations (Chapter 4). BiBAC targeting with the pJC vectors orients recombinase binding sites in a way that enables a cyclical strategy of phiC31 Integrase-mediated targeting and FLPe-mediated selection removal, creating a method for gene stacking large DNA fragments (Figure 4.3). We proposed that the ideal location for BiBAC gene stacking is on a supernumerary B chromosome, which is present in variable numbers and contains no necessary genes (Chapter 1). Further, removal of the distal tip of a B chromosome through telomere-mediated truncation stabilizes inheritance patterns of B chromosomes by removing the distal factor that is required for accumulation; this aspect is a possible benefit in creating a transgene accumulation system. Therefore, we created both a

minichromosome (17-27) and a B insert (17-13) B chromosome derived platforms (Chapter 3).

As previously stated in Chapter 5, pJC-YattB BiBAC gene transfer into B chromosome platforms theoretically could be accomplished through either breeding (Figure 5.5) or bombardment (Figure 5.7) strategies. Through breeding, we created a plant background that contained all the necessary components for BiBAC circularization and targeting to the B insert (17-13) platform; however, PCR analysis utilizing primer sets that flank the BiBAC targeting junction did not yield a product when compared to a control (Figure 5.24). Similarly, bombardment of a transient expression construct into embryos derived from a self-crossed plant that contained both BiBAC and platform did not generate bialaphos resistant callus after 4 weeks of selection (Figure 5.26). The combined negative results from breeding and bombardment approaches indicated to us that a component in the transfer process was not working at a high enough efficiency to obtain a targeted product.

Prior experiments examining components of Cre-mediated circularization and phiC31-mediated targeting were both positive (Figure 5.14; Figure 5.21). Additionally, analysis of Cre and phiC31 Integrase recombinase functionality on binding site sequences in maize cells was analyzed in Chapter 2 (Figure 2.4; Figure 2.7). While we are certain that Cre-mediated BiBAC circularization was occurring, the parameters required for efficient targeting with phiC31 Integrase are unknown. The PhiC31 Integrase GLP targeting experiment in Chapter 5

utilized bombardment, which introduces high copy numbers of binding sites into maize cells. This approach was successful; however, BiBAC breeding and bombardment strategies targeting single copy *attB* and *attP* binding sites was unsuccessful. From this data, we concluded that it is possible that the stoichiometry between phiC31 Integrase enzyme and binding site is important in obtaining a targeted product. Additionally, we decided to explore the possibility of using a different recombinase to accomplish BiBAC targeting.

Upon analysis of the binding site sequences between the pJC BiBAC donor and pZP-Telo-JC platforms, we identified single *FRT* sites in both locations, which could be bound by FLPe to induce intramolecular recombination reactions. The initial analysis of this reaction was not provided in Chapter 5 but will be included here. In a plant background containing pJC-YattB-35, pZP-Telo-JC (17-13) and FLPe, PCR analysis indicated FLPe is functional in inducing intermolecular recombination reactions between non-homologous chromosomes, resulting in a translocation between the donor and platform transgenes (Figure 6.1). Sanger sequence analysis confirms the integrity of this FLPe-mediated translocation reaction, which forms Ubi-*FRT-attB*-NOSt-Yeast (Figure 6.2). Since FLPe was capable of inducing recombination reactions between single binding sites in different genomic locations, we decided to run a comparative analysis of FLPe and phiC31 Integrase utilizing digital droplet PCR (ddPCR). The ddPCR results indicated that phiC31 Integrase is capable of inducing intermolecular recombination reactions at a low rate when compared to FLPe (Figure 5.31);

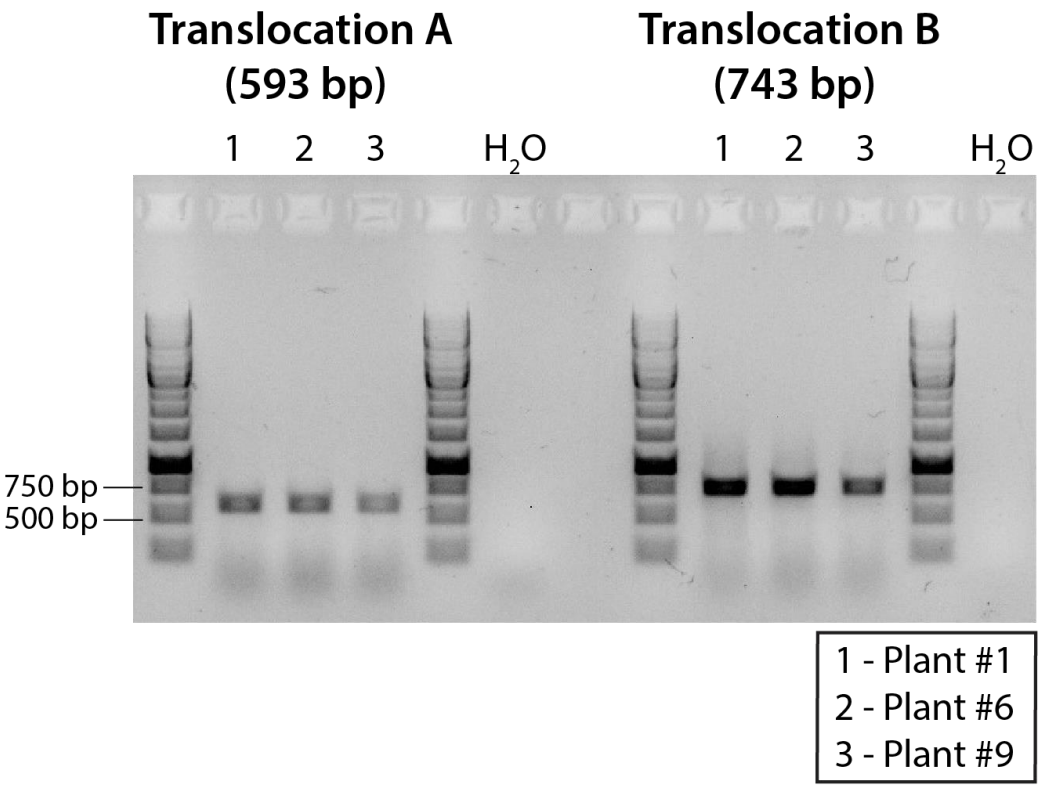
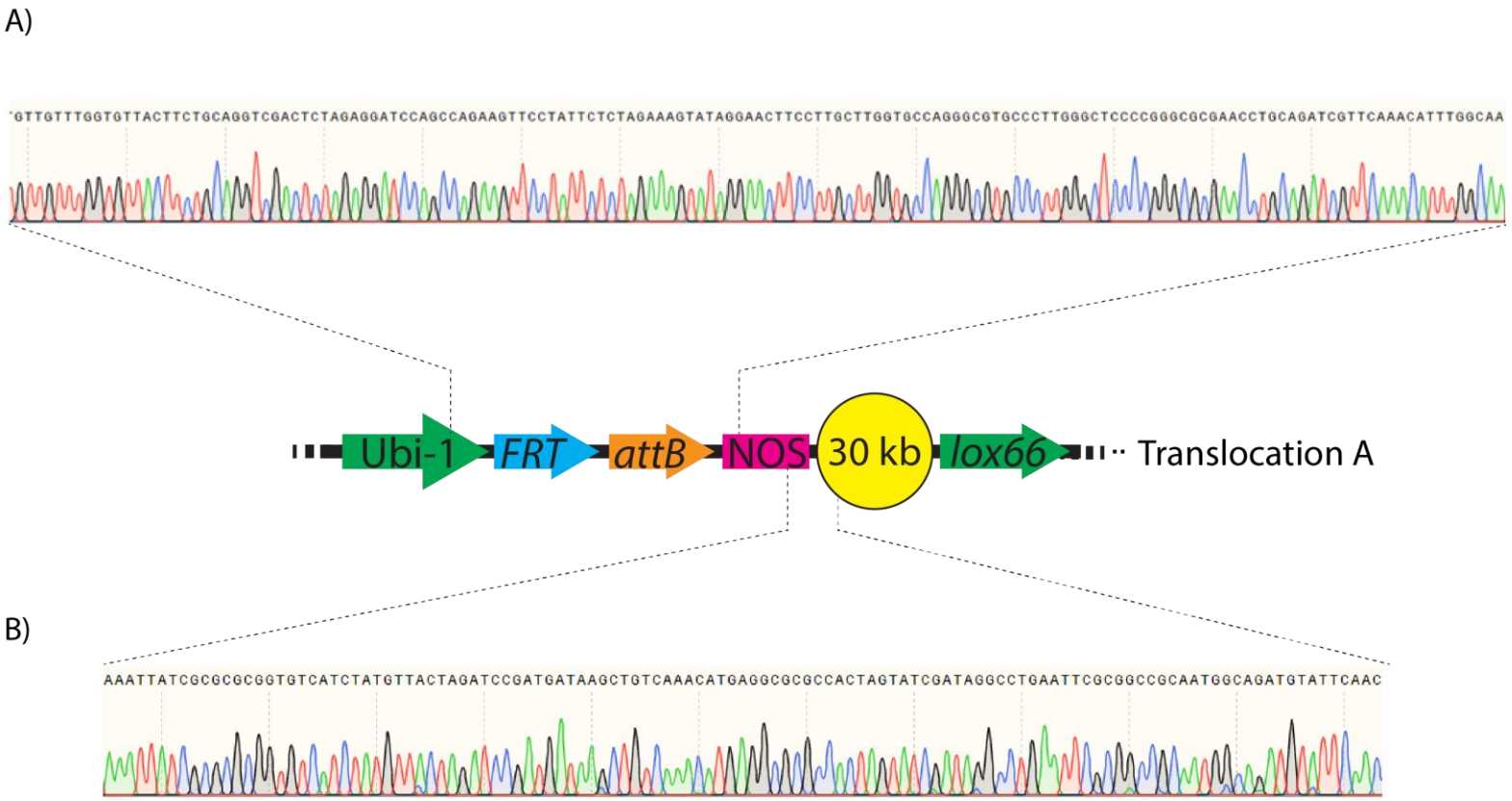


Figure 6.1 – Agarose Gel Image Displaying Translocation Between *FRT* Binding Sites on pJC-YattB-35-1 and pZP-Telo-JC (17-13) B Insert Transgenes: PCR primer sets flanking the *FRT* Translocation junctions in two different sites in the genome were used to analyze FLPe-mediated translocations between pJC-YattB-35-1 and pZP-Telo-JC (17-13) inserts. Plant #1 (lane 1), Plant #6 (lane 2) and Plant #9 (lane 3) all contain the components required for FLPe-mediated translocations and displayed the DNA banding patterns that indicated a successful translocation between pJC-YattB-35-1 and pZP-Telo-JC (17-13).



Sanger Sequence Analysis of FLPe-Mediated Translocations Between *FRT* Binding Sites on pJC-YattB-35-1 and pZP-Telo-JC (17-13) B Insert Transgenes: Sequence analysis of Translocation A PCR product from primer sets used in Figure 6.1 verifies a translocation between pJC-YattB-35-1 and pZP-Telo-JC (17-13) transgenes, which indicated the presence of an *attB* binding site (A) and genomic yeast DNA (B)

therefore, it is potentially possible but uncertain if phiC31 Integrase mediated BiBAC targeting is possible. It is important to note that the ddPCR analysis was carried out on stably integrated transgenes. Once Cre is introduced into a background will all components, the BiBAC molecule will be circularized (Figure 5.14). While we did observe maintenance of the BiBAC circles in mitotically active leaf tissues (Figure 5.17), we assume these circles are present at a low copy number.

Moving forward, there are a number of different avenues that were uncovered during the course of this study, which could possibly lead to the development of an efficient BiBAC targeting system. For instance, modulating the stoichiometry between the phiC31 Integrase enzyme and binding site may be the key to obtaining a targeted product. Since the target platform is located on a B chromosome, there is the added benefit of increasing the copy number above a point that would be possible for inserts in the native chromosome sets. Additionally, there is the possibility of creating a breeding strategy that would result in a final plant that contains homozygous phiC31 Integrase. Since phiC31 requires both *attP* and *attB* sites to carry out a recombination reaction, both BiBAC donor and platform could be crossed to phiC31 and selfed to create homozygous backgrounds before crossing to obtain the final plant, which would also be homozygous for phiC31 Integrase. Unfortunately, this is not

possible with Cre recombinase; however, the data obtained from this study suggests a homozygous Cre background would be unnecessary.

To create an efficient bombardment strategy, we will need to create a plant background that is homozygous for both BiBAC donor and target platform. In this, all extracted embryos from a cross to a tester line will contain both components, and a self-cross will increase the binding site copy number. The results obtained from the bombardment in Chapter 5 were not analyzed for homozygosity; therefore, we are unsure of the precise background of each of the embryos used in the experiment. Once created, homozygous lines could be used in a large-scale analysis utilizing bombardment strategies for BiBAC targeting, which may yield a positive result in the near future.

An additional possible approach would be to create large numbers of immature embryos of a genotype containing the target chromosome, the donor transgene and Cre + phiC31 Integrase without *bar* selection (currently unavailable). The system is designed for integrations of the donor to activate *bar* selection. The embryos could be subjected to such selection on callus induction medium. Any rare recombination events could potentially be selected and regenerated into plants.

Appendix 1: Vector Maps of Constructs Used in This Study

Summary

The constructs outlined in the following figures highlight important features that are important for growth and modification of specific DNA vectors. Each figure was generated using SnapGene software, which scales each feature with respect to DNA length. Further information detailing the contents of the vectors can be found in the respective chapter it was utilized, which is noted at the bottom of each figure.

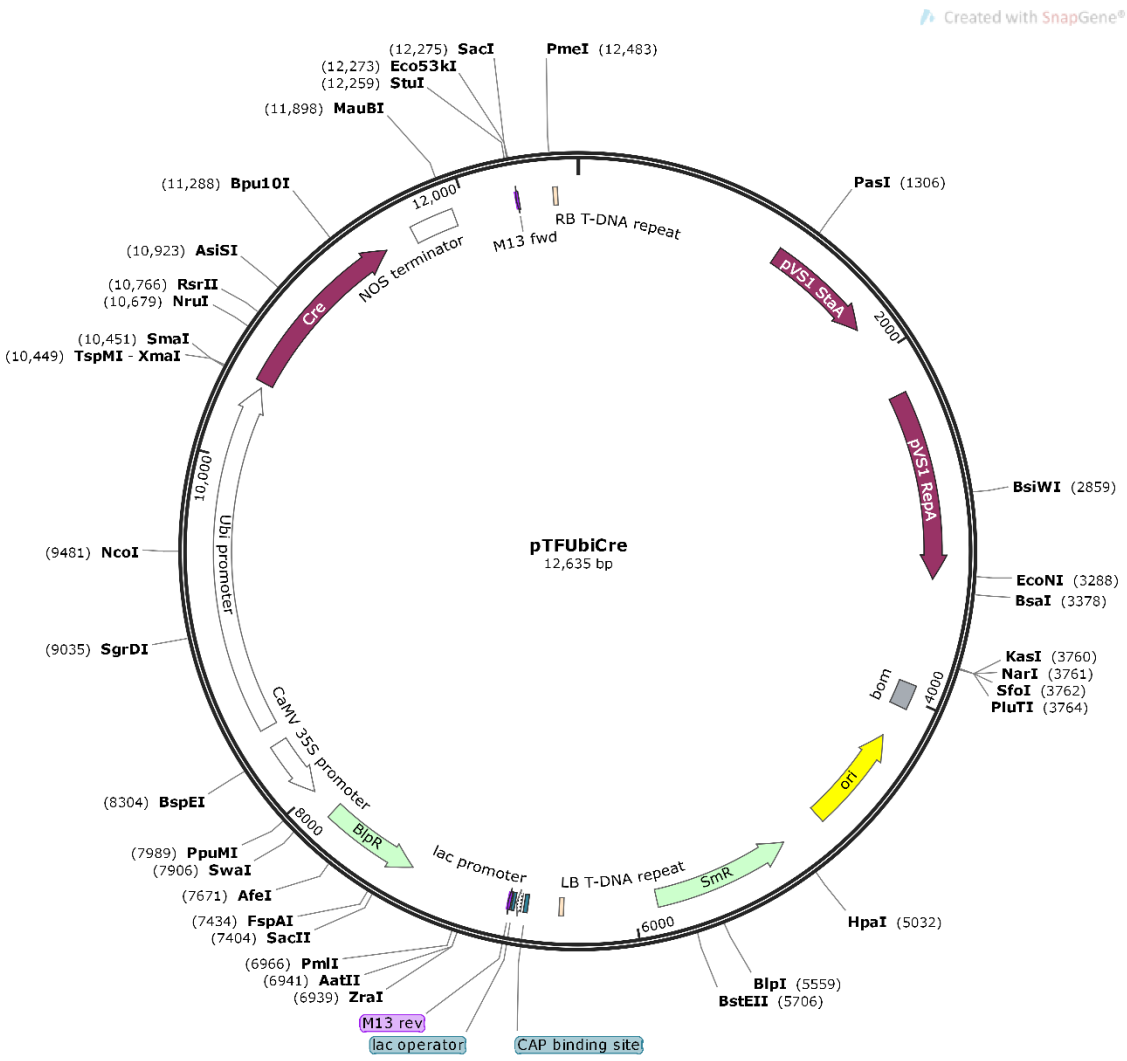


Figure A1.1 - pTFUbiCre Vector Map: Cre recombinase T-DNA construct was transformed into maize Hi-II immature embryos in Chapter 2. Cre was also used to circularize BiBAC inserts in Chapter 5.

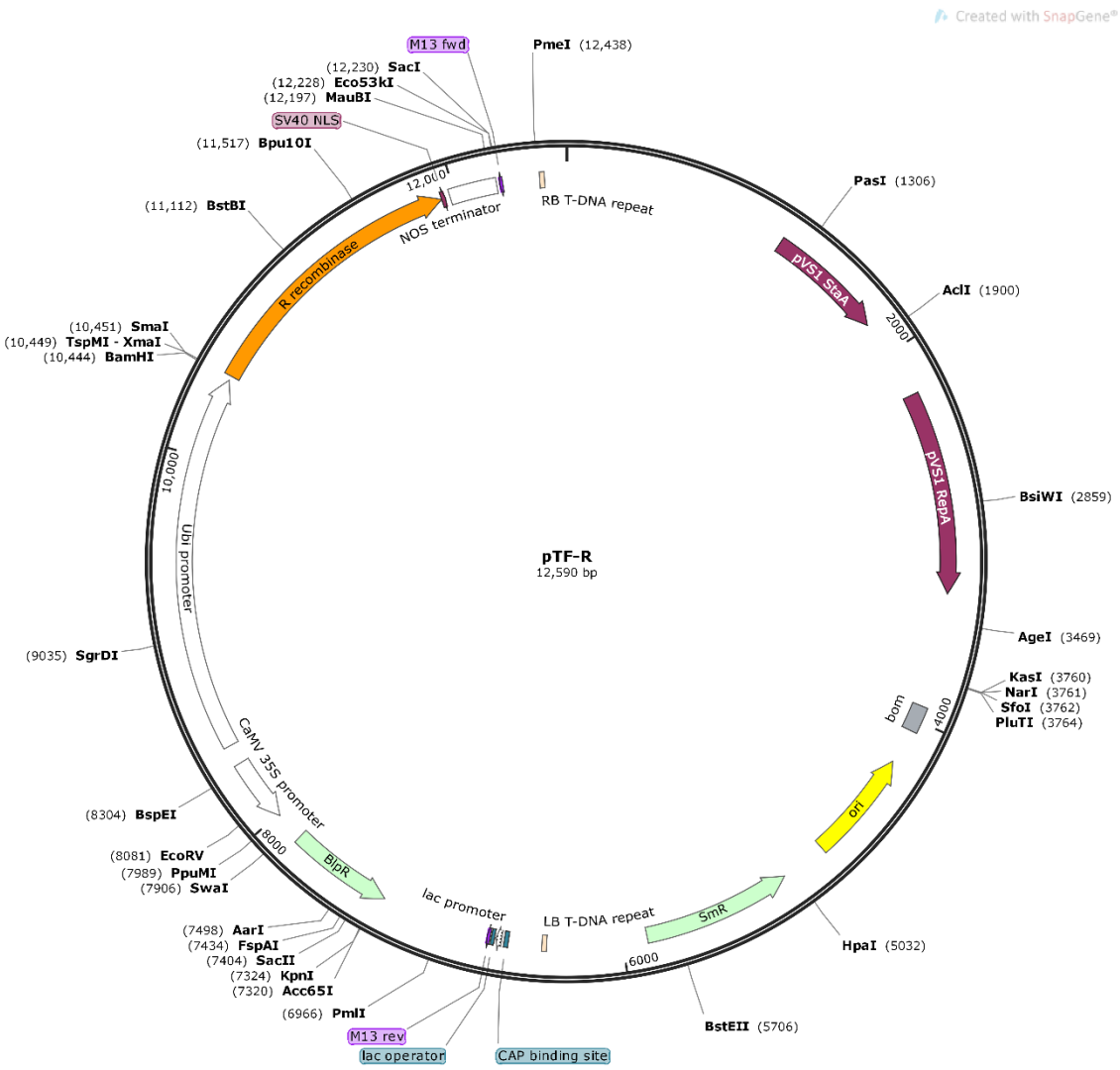


Figure A1.2 - pTF-R Vector Map: R recombinase T-DNA construct was transformed into maize Hi-II immature embryos in Chapter 2. R was also used to remove *bar* selection from BiBAC inserts in Chapter 5.

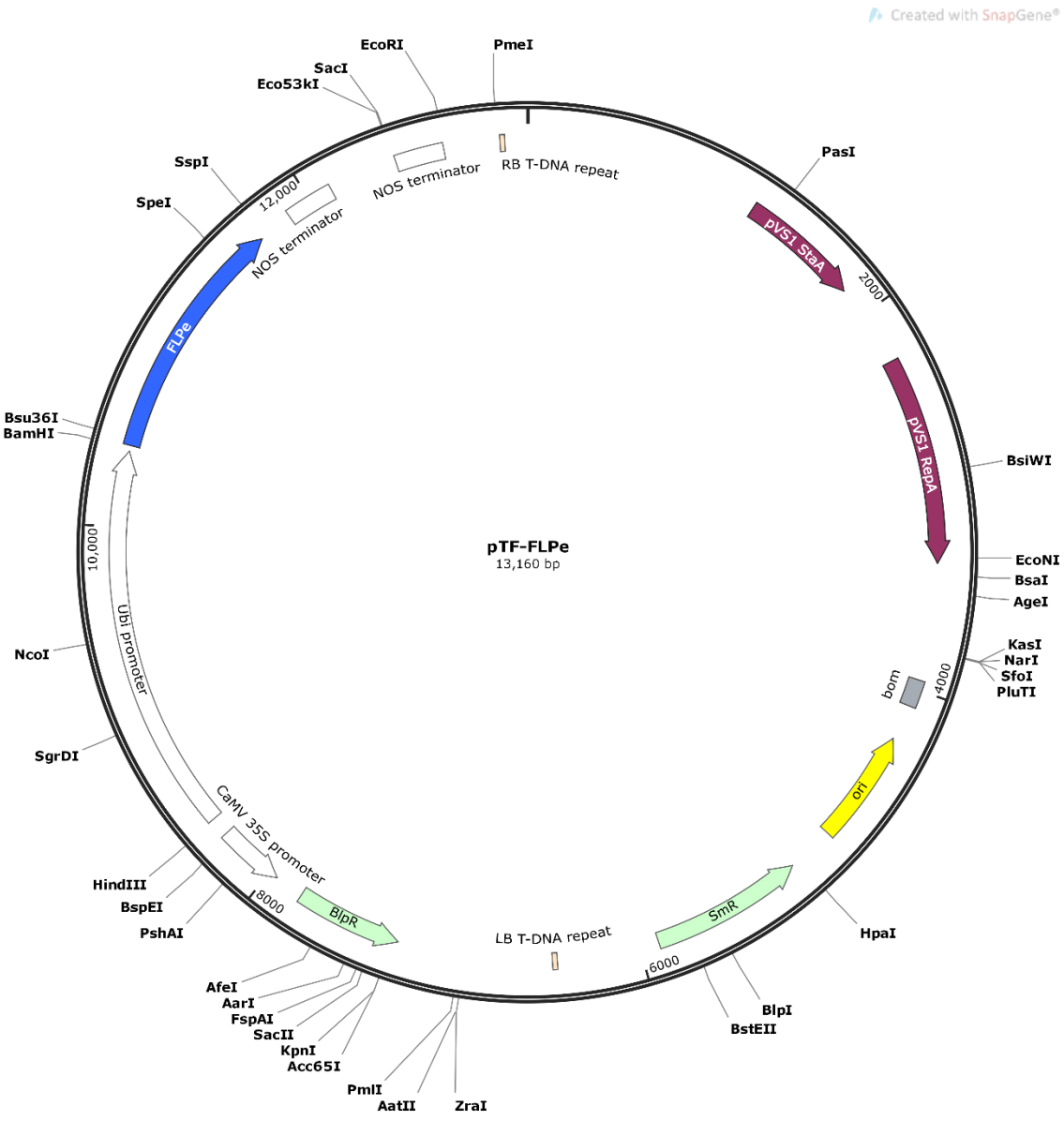


Figure A1.3 - pTF-FLPe Vector Map: FLPe T-DNA construct was transformed into maize Hi-II immature embryos in Chapter 2. FLPe was also used to remove *bar* selection from Minichromosomes and B inserts in Chapter 5.

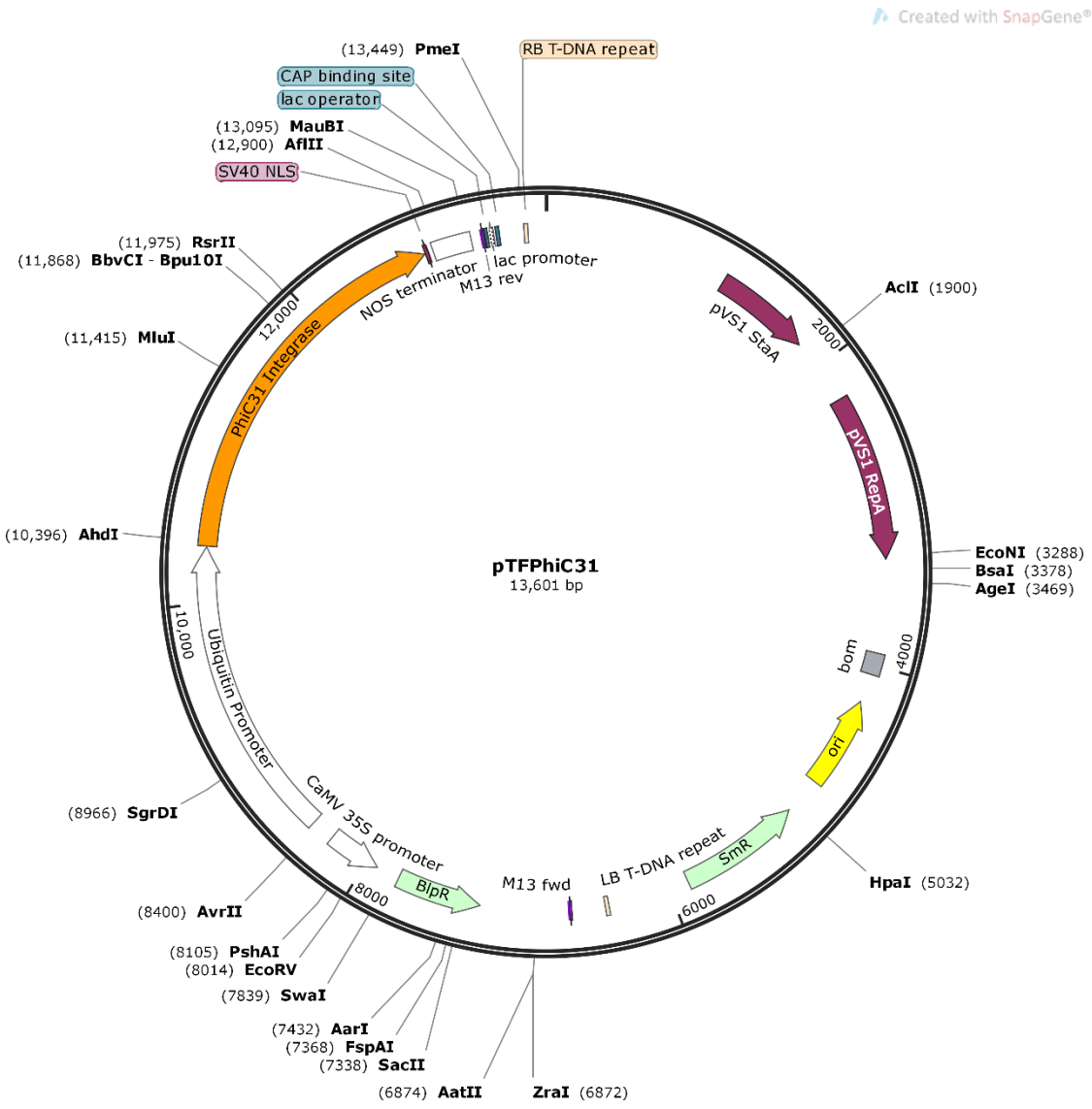


Figure A1.4 - pTFPhiC31 Vector Map: PhiC31 Integrase T-DNA construct was transformed into maize Hi-II immature embryos in Chapter 2. PhiC31 Integrase was also used to target BiBAC molecules to Minichromosomes and B inserts in Chapter 5.

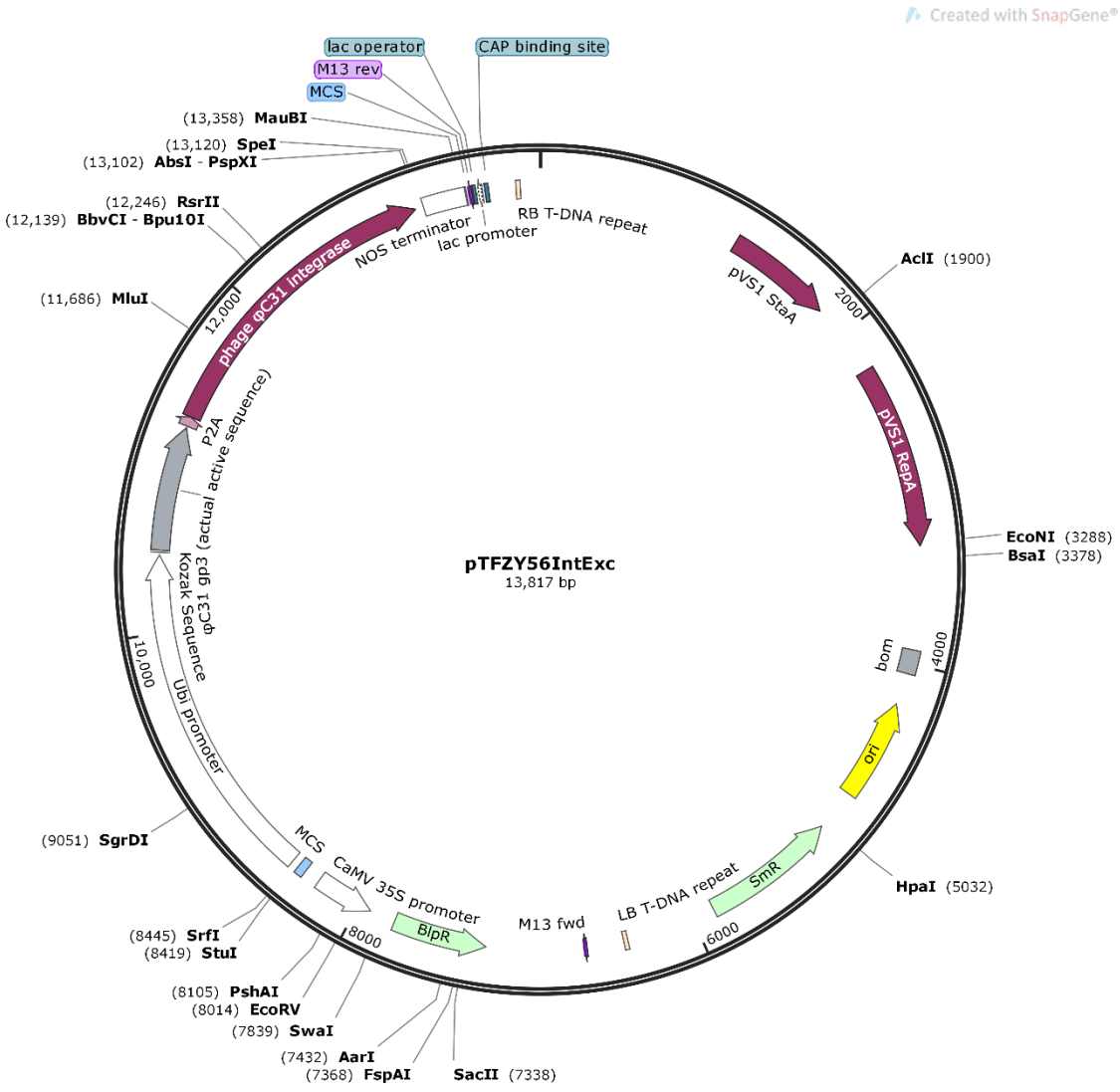


Figure 5 - pTFZY56IntExc Vector Map: PhiC31 Excisionase T-DNA construct was transformed into maize Hi-II immature embryos in Chapter 2.

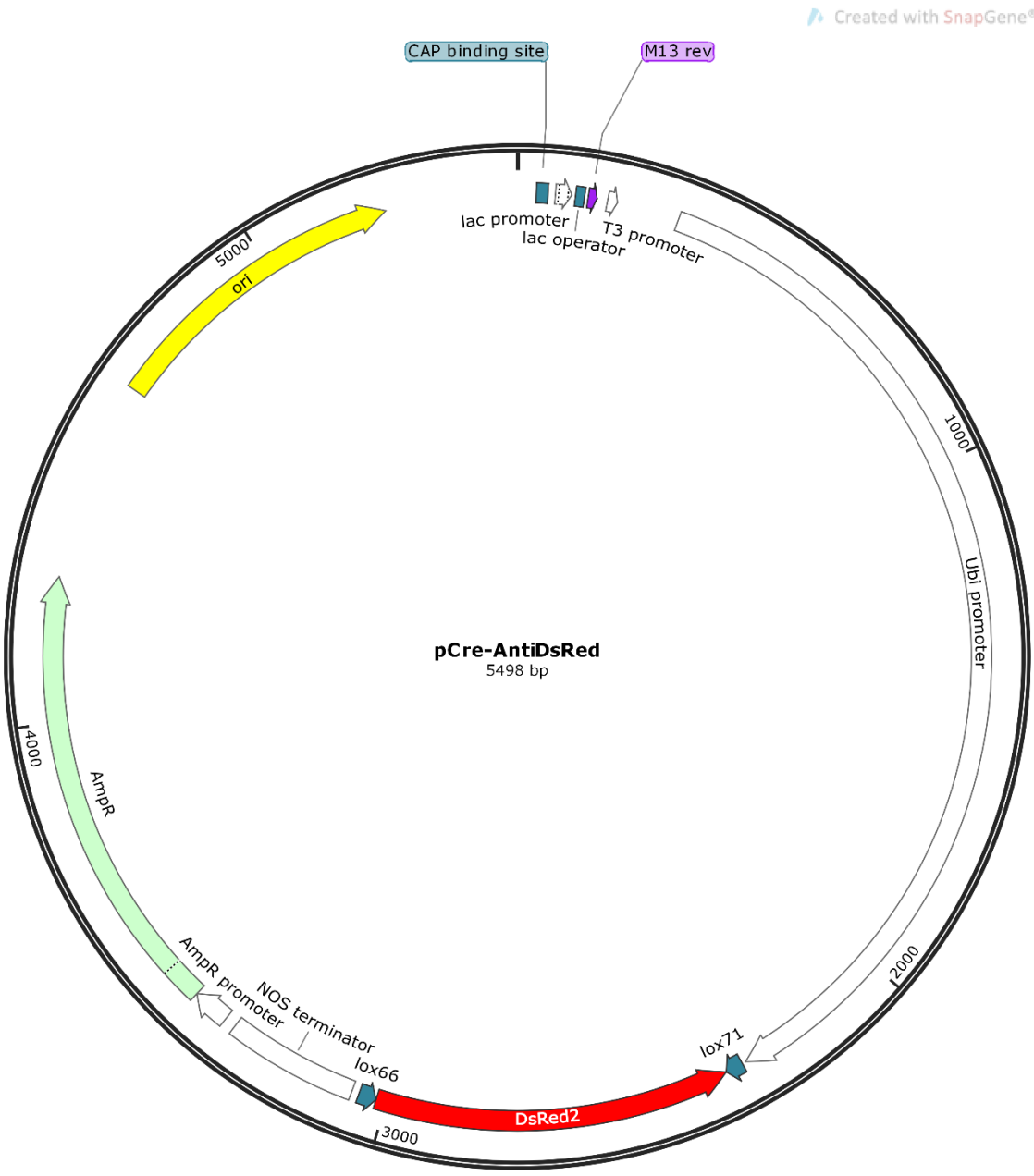


Figure A1.6 - pCre-AntiDsRed Vector Map: Transient DsRed construct used to test functionality of Cre recombinase in maize cells.

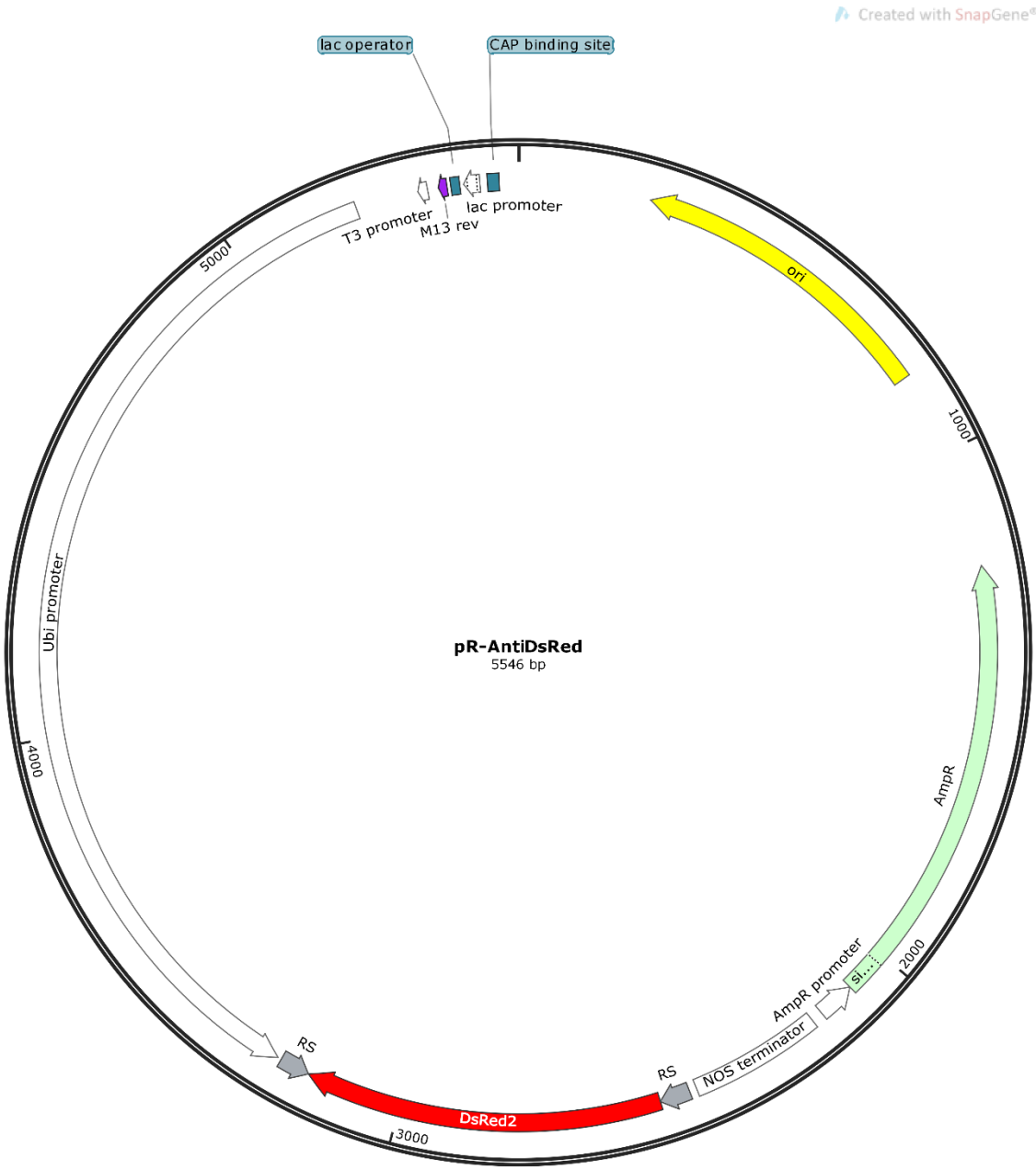


Figure A1.7 - pR-AntiDsRed Vector Map: Transient DsRed construct used to test functionality of R recombinase in maize cells.

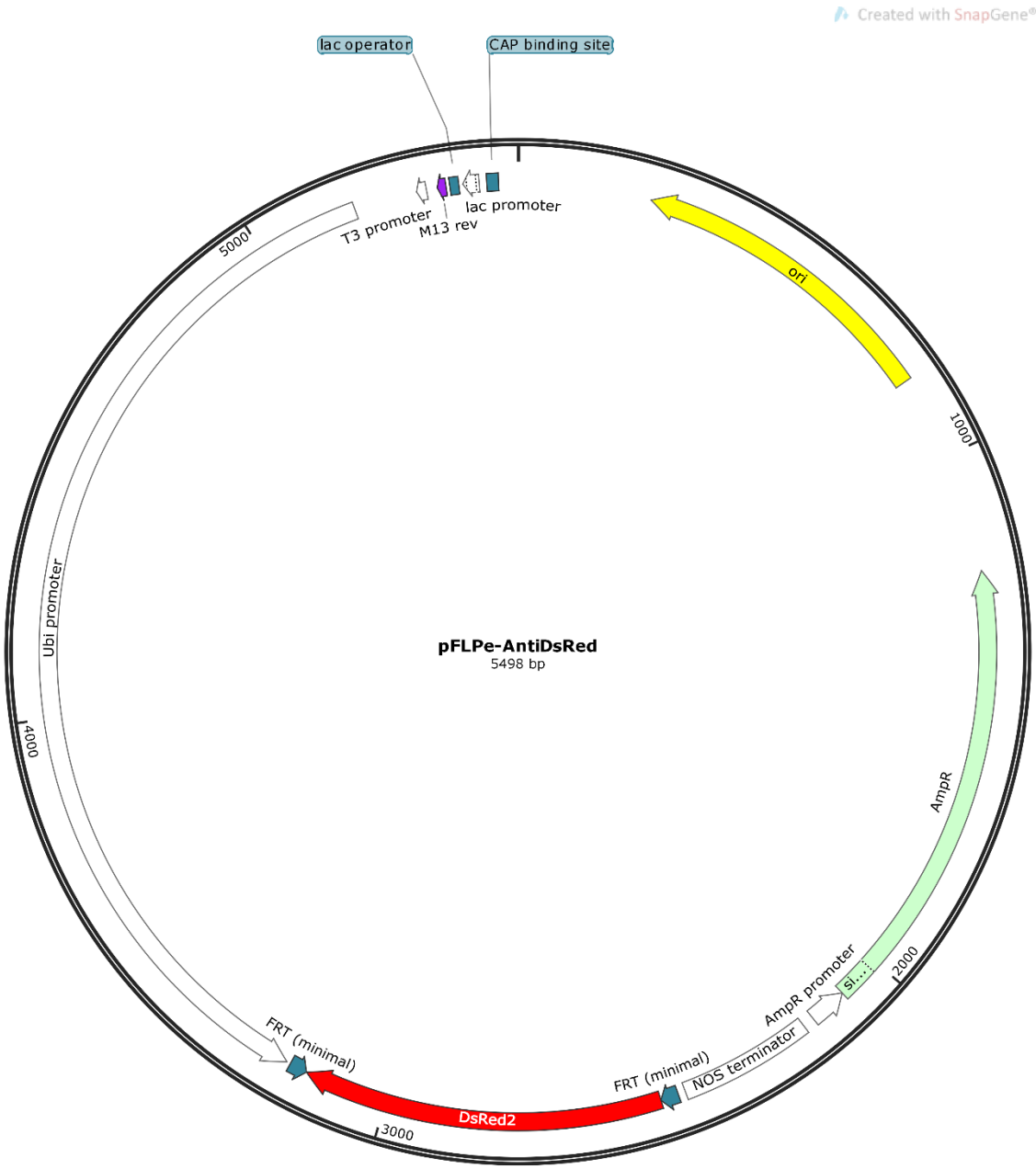


Figure A1.8 - pFLPe-AntiDsRed Vector Map: Transient DsRed construct used to test functionality of FLPe recombinase in maize cells.

Figure A1.9 - pIntegrase-AntiDsRed Vector Map: Transient DsRed construct used to test functionality of PhiC31 Integrase recombinase in maize cells.

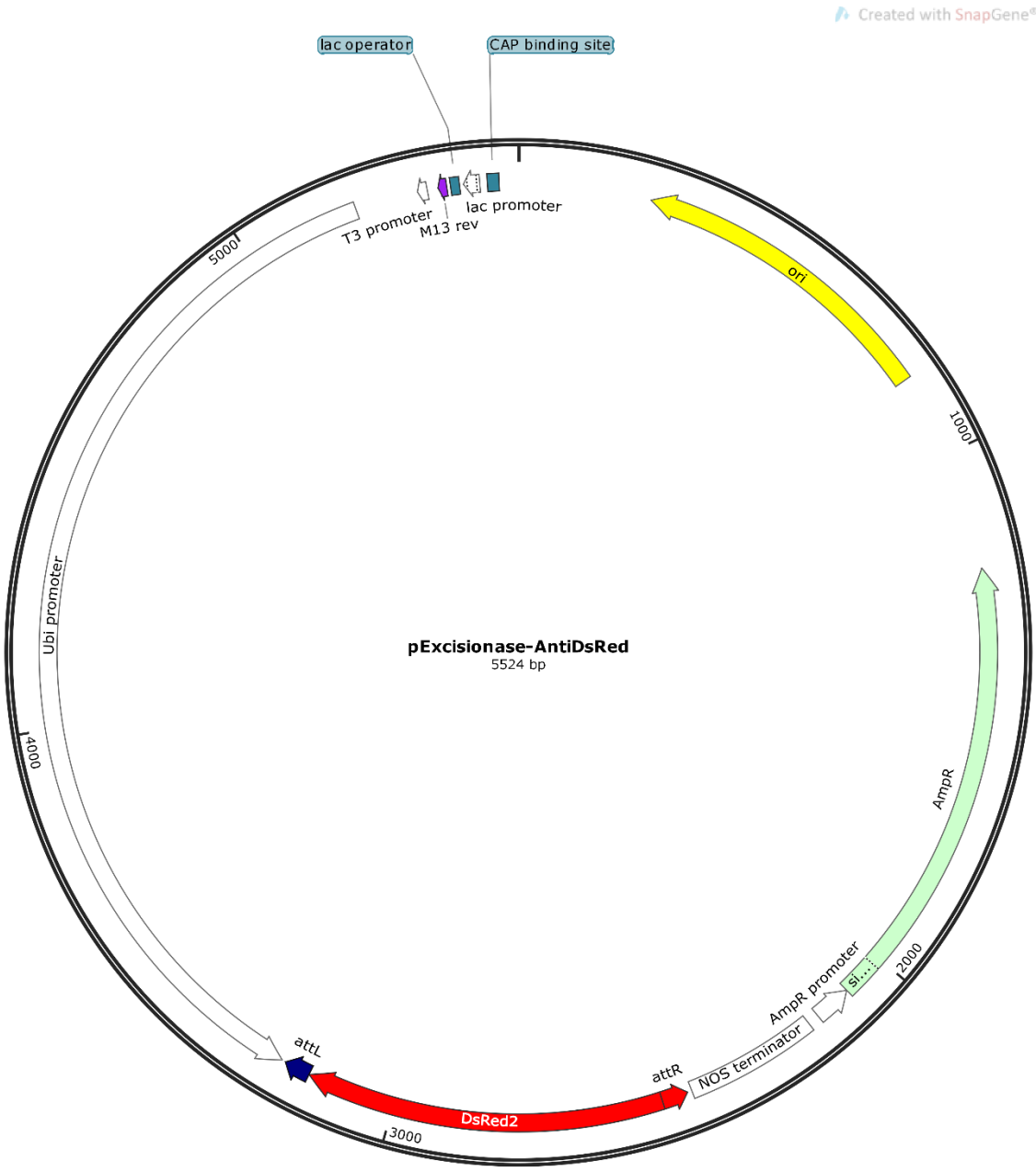


Figure A1.10 - pExcisionase-AntiDsRed Vector Map: Transient DsRed construct used to test functionality of PhiC31 Excisionase recombinase in maize cells.

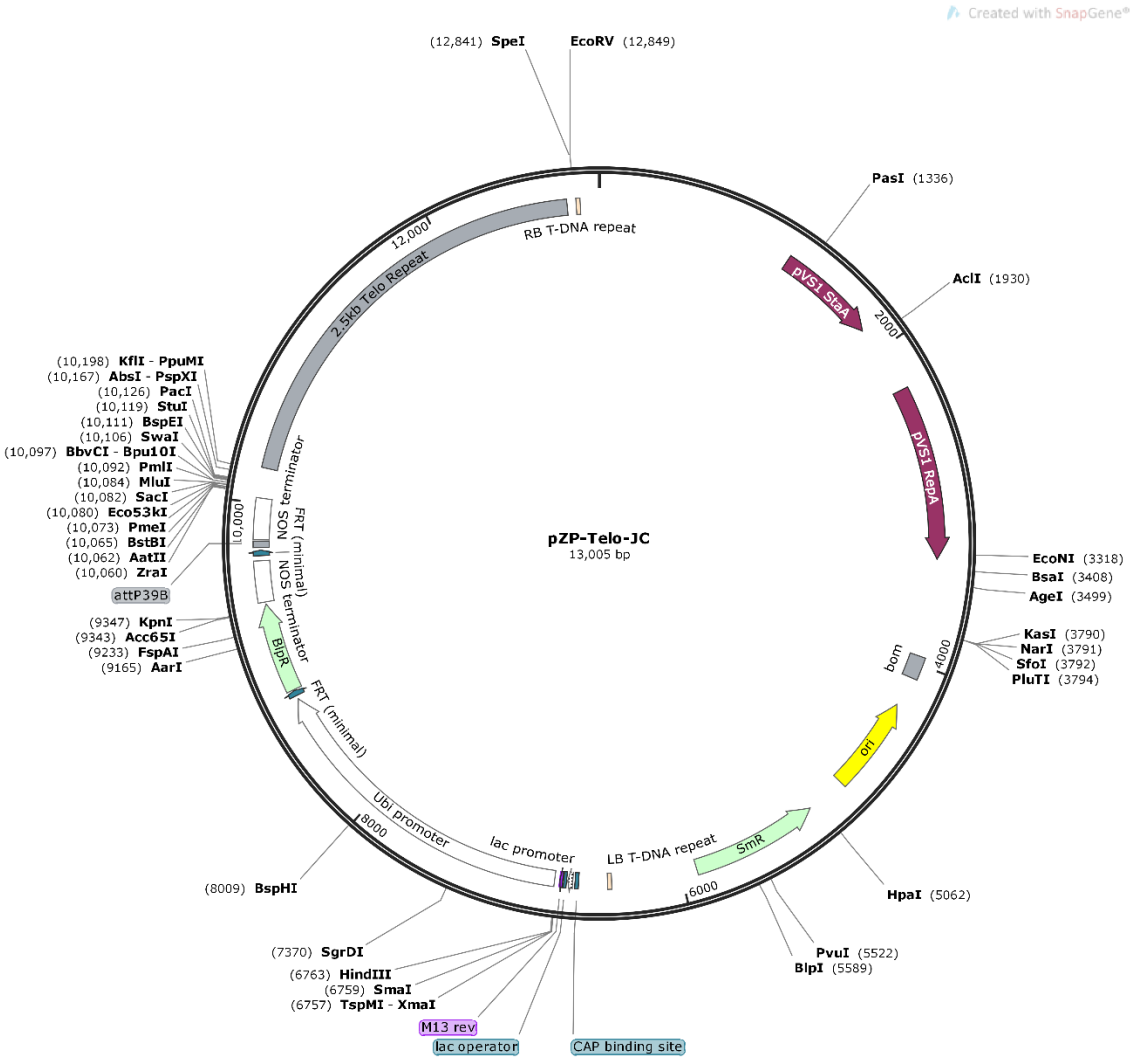


Figure A1.11 - pZP-Telo-JC Vector Map: Truncation construct used to create the minichromosome (17-27) and B insert (17-13) platforms in Chapter 3. These platforms were used as targets for BiBAC transfer in Chapter 5.

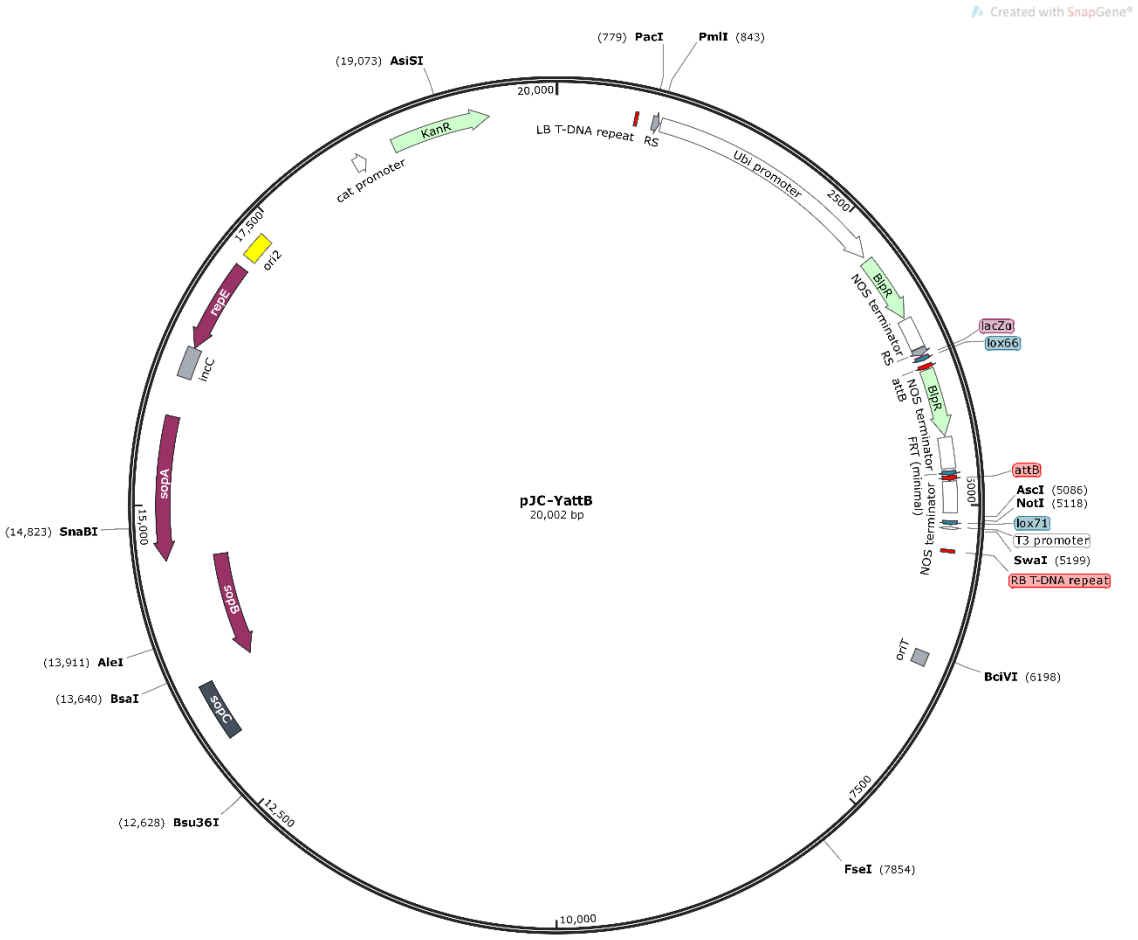


Figure A1.12 - pJC-YattB Vector Map: BiBAC construct used to create yeast insertion lines containing *attB* recombinase binding sequences in Chapter 4.

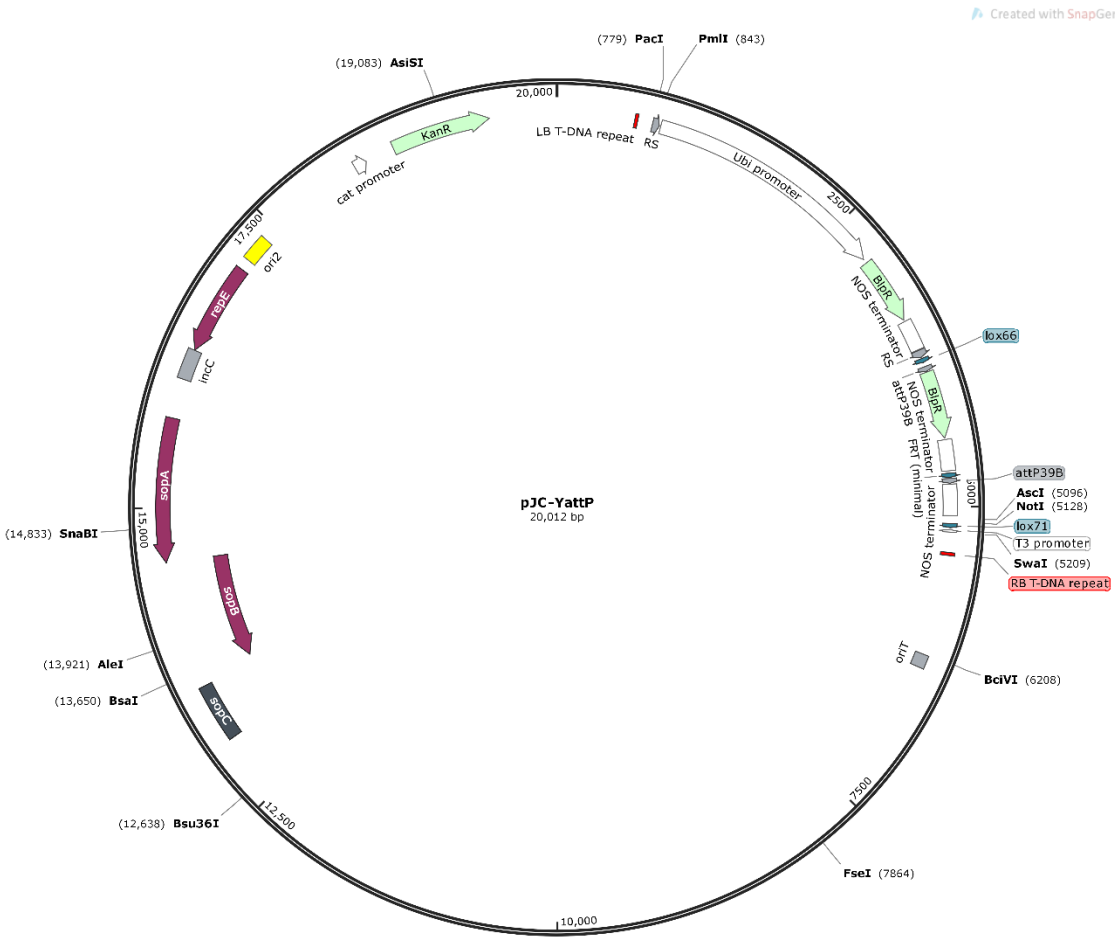


Figure A1.13 – pJC-YattP Vector Map: BiBAC construct used to create yeast insertion lines containing *attP* recombinase binding sequences in Chapter 4.

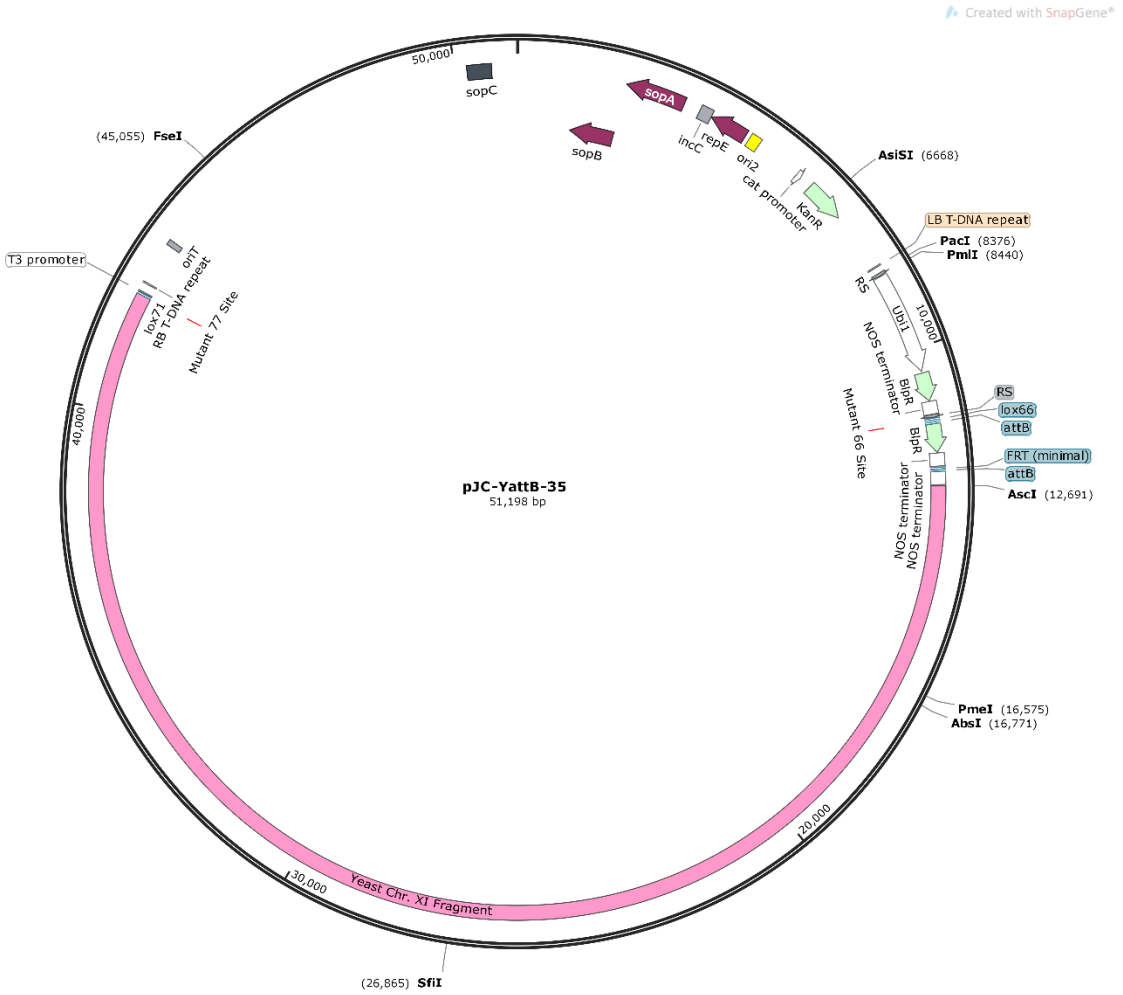


Figure A1.14 - pJC-YattB-35 Vector Map: Truncation construct used to create the minichromosome (17-27) and B insert (17-13) platforms in Chapter 3. These platforms were used as targets for BiBAC transfer in Chapter 5.

Appendix 2: BiBAC Modification and Stable Transfer into Maize (*Zea mays*) Hi-II Immature Embryos via *Agrobacterium*-Mediated Transformation

Note: The information in this chapter was published under the title:

Cody J.P., Graham N.D., Birchler J.A. (2018) BiBAC Modification and Stable Transfer into Maize (*Zea mays*) Hi-II Immature Embryos via *Agrobacterium*-Mediated Transformation. Current Protocols in Plant Biology. 2, 350-369.

Summary

Binary Bacterial Artificial Chromosomes (BiBAC) are large insert cloning vectors that contain the necessary features required for *Agrobacterium*-mediated transformation. However, the large size of BiBACs and low-copy number in *Escherichia coli* (DH10B) and *Agrobacterium tumefaciens* make cloning experiments more difficult than other available binary vector systems. Therefore, a protocol that outlines preparation, modification, and transformation of high-molecular weight (HMW) constructs is advantageous for researchers looking to use BiBACs in plant genomics research. This unit does not cover the cloning of HMW DNA into BiBAC vectors. Researchers looking to clone HMW DNA into BiBACs can refer to Zhang et al. (2012; doi: 10.1038/nprot.2011.456).

Introduction

HMW DNA vectors, such as bacterial artificial chromosomes (BAC), large-insert plasmid-based clones (PBC), and P1-derived artificial chromosomes (PAC), have been used in many studies to construct genome-wide physical maps to elucidate structural and functional properties of DNA encoding agronomically favorable traits. These include, but are not limited to, salt-tolerance, nitrogen fixation, and disease resistance gene clusters. The temporal and spatial properties of multiple genes and regulatory factors required for precise expression can span thousands of base pairs, making plant transformation and complementation experiments laborious due to subsequent subcloning steps required by BAC, PBC, and PAC systems.

To circumvent this limitation, Hamilton and colleges developed binary bacterial artificial chromosomes (BiBAC) vectors, HMW constructs capable of maintaining up to 300 kb inserts and containing the necessary machinery required for *Agrobacterium*-mediated transformation (Hamilton, 1997). This enables researchers to transfer large gene fragments directly into host organisms, eliminating the need for subsequent cloning steps and maintaining temporal and spatial properties needed for proper expression. In comparison to other HMW vector systems, BiBACs are much larger, with a backbone of ~23 kb. Together with low copy number F and Ri origins, this makes BiBAC handlingand modification particularly difficult.

Here, we outline the steps necessary to successfully manipulate and transform BiBAC vectors into maize (Figure A2.1).

High-Yield Purification of BiBAC DNA from *Escherichia coli* (Protocol 1)

Obtaining high yields of DNA from E. coli is required for subsequent modification and transformation experiments of BiBAC vectors; however, the makeup of these vectors makes this process much more difficult than with standard cloning vectors. BiBAC vectors contain the F origin of replication (Shizuya et al., 1992), which makes them single copy within a cell, limiting the amount of plasmid DNA that can be obtained from a single culture. Additionally, these vectors are also slow growing, especially once a large amount of DNA has been inserted (Figure A2.2A). To overcome these problems, it is necessary to alter the traditional method of plasmid extraction and purification. To compensate for the single-copy nature of the plasmids, large (250 ml to 1 liter) cultures are grown, instead of the typical 5 ml cultures used with typical cloning vectors.

Materials:

- −80°C E. coli BiBAC freezer stock (CCTEC; http://www.ctl.cornell.edu)
- 150 × 15–cm LB agar plates with appropriate antibiotics
- Appropriate antibiotics in sterile ddH2O LB medium (see recipe)
- 500 ml of autoclaved LB medium in 1000-ml Erlenmeyer flask

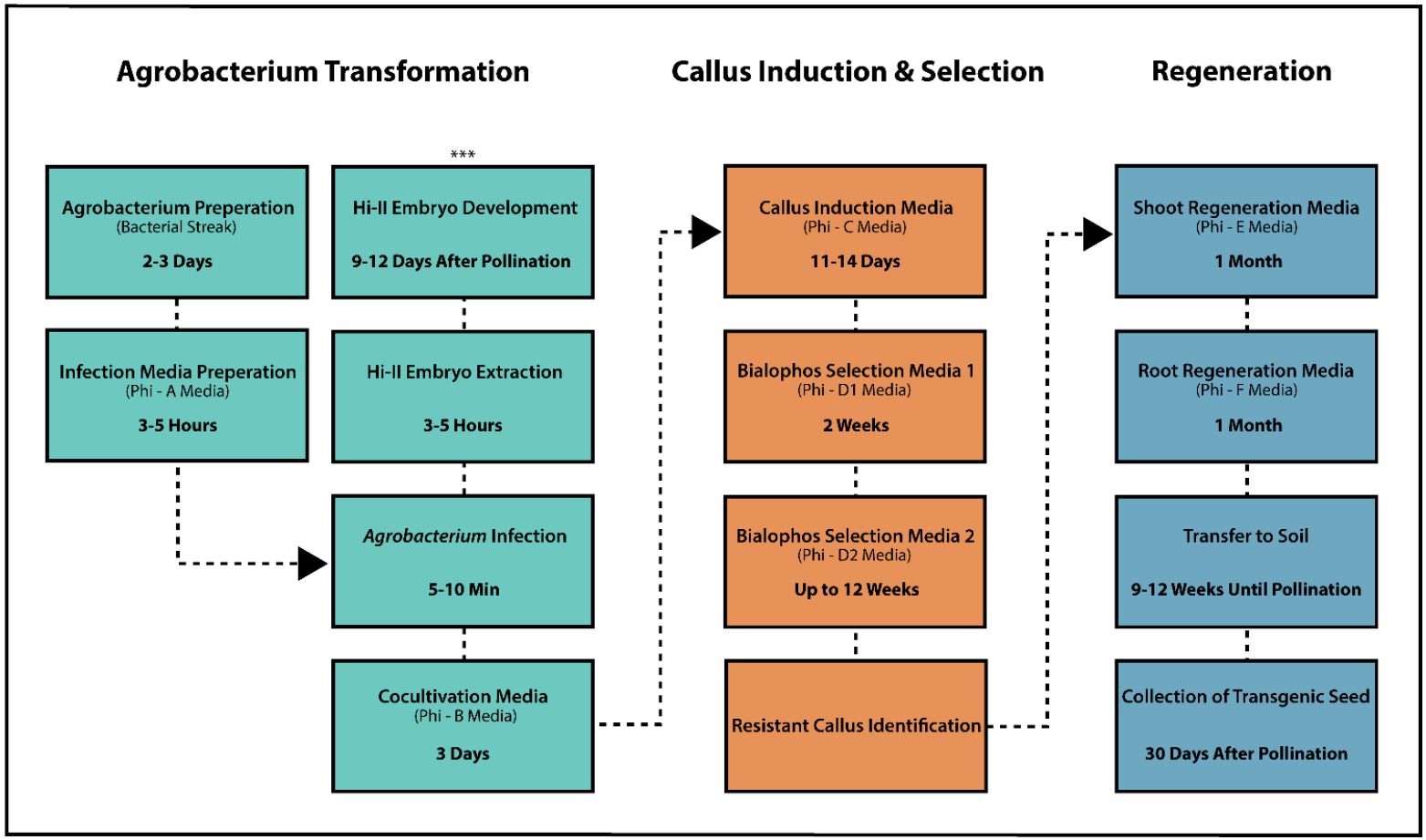

Agrobacterium Transformation
Callus Induction & Selection
Regeneration
***
Agrobacterium Preperation
(Bacterial Streak)
2-3 Days
Infection Media Preperation
(Phi - A Media)
3-5 Hours
Hi-II Embryo Development
9-12 Days After Pollination
Hi-II Embryo Extraction
3-5 Hours
Agrobacterium Infection
5-10 Min
Cocultivation Media
(Phi - B Media)
3 Days
Callus Induction Media
(Phi - C Media)
11-14 Days
Bialophos Selection Media 1
(Phi - D1 Media)
2 Weeks
Bialophos Selection Media 2
(Phi - D2 Media)
Up to 12 Weeks
Resistant Callus Identification
Shoot Regeneration Media
(Phi - E Media)
1 Month
Root Regeneration Media
(Phi - F Media)
1 Month
Transfer to Soil
9-12 Weeks Until Pollination
Collection of Transgenic Seed
30 Days After Pollination

Figure A2.1 - Flow Chart Highlighting Steps in the Maize Transformation Process: Each step is represented as a box, containing the step name and approximate time required to complete. Colors represent the three overarching stages of the transformation process, *Agrobacterium* Transformation (aqua), Callus Induction & Selection (light brown), and Regeneration (blue). The process follows along the dotted line, from the beginning of *Agrobacterium*Transformation until the end of Regeneration. The *Agrobacterium* transformation stage contains two starting steps, *Agrobacterium* Preparation and Hi-II Embryo Development. These steps need to be carried out in parallel until *Agrobacterium* infection. The total time required to complete the entire transformation process is ~1 year. ***Hi-II A and Hi-II B seeds must be planted and grown 9 to 12 weeks before the Embryo Development step.

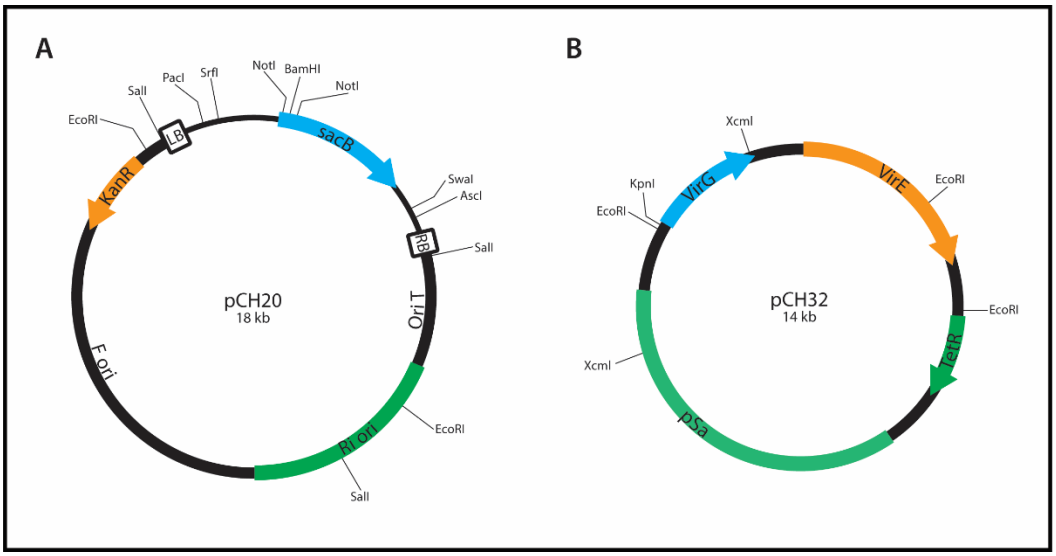


Figure A2.2 - **Vector Map of pCH2O BiBAC Vector (A) and pCH32 Helper Plasmid (B):** These constructs and other BiBACs or helper plasmids are available from ctl.cornell.edu.

- Nucleobond Xtra BAC Kit (Macherey-Nagel, cat. no. 740436.10)
- Centrifuge
- Inoculation loops (Fisherbrand, cat. no. 22363602)
- Shaking Incubator: setting 37°C; 250 rpm/min
- 17 × 100–mm polystyrene culture test tubes (Thermo Fisher Scientific, cat. no. 14-956-6D)
- Electronic pipette Incubator: setting 37°C Aluminum foil

Preparation of E. coli inoculation:

1. Using an inoculation loop, take −80°C E. coli BiBAC freezer stock and streak onto a 150 × 15–cm LB agar plate containing appropriate antibiotics.
2. Place BiBAC growth plate overnight in a 37°C incubator. BiBAC E. coli cultures typically grow slowly, so it may take 2 days until colonies are visible. After growth, the BiBAC growth plate may be stored at 4°C and used for several months.
3. Prepare a starter culture by aseptically transferring 5 ml of LB medium containing the appropriate antibiotic with an electronic pipette into a 17 × 100–mm polystyrene culture test tube.
4. Using an inoculation loop, pick a single colony on the BiBAC growth plate and transfer into the 5 ml starter culture tube.
5. Place the tube in a 37°C shaking incubator for 6 hr set to 250 rpm.

6. After 6 hr incubation, transfer 5 ml starter culture into 500 ml of autoclaved LB medium containing the appropriate antibiotic in a 1-liter Erlenmeyer flask using an electronic pipette.

7. Cover the flask with aluminum foil and place in a 37° C shaking incubator overnight set to 250 rpm.

Column purification of BiBAC DNA:

Extracting BiBAC plasmid DNA is complicated due to the large plasmid size. Traditional alkaline lysis plasmid extractions differentiate plasmid DNA from genomic DNA by relying on the large genomic DNA tangling during the lysis process. Due to the large size, BiBAC DNA can also become tangled during the alkaline lysis procedure, decreasing the overall yield. There are protocols designed specifically for large plasmids (Sinnett & Montpetit, 2003), however in our experience these protocols are much less efficient that commercial kits. These kits use specifically designed columns to differentiate plasmid DNA from genomic DNA, usually with a higher yield than traditional methods. There are many manufacturers that make extraction kits for BiBAC plasmid DNA, each with their own advantages and disadvantages. In our experience with BiBAC vectors, the Nucleobond Xtra BAC kit results in high-quality plasmid DNA.

8. Extract BiBAC DNA following the manufacturer's instructions.

9. Store BiBAC DNA up to 1 month at 4°C.

Due to the large size of BiBAC DNA, it is best to store the plasmid at 4°C to avoid freeze thaw cycles that may shear the DNA. The higher storage temperature may allow for nucleases to become active during storage, so care must be taken to perform the extraction in a sterile environment.

MODIFICATION OF BiBAC CONSTRUCTS (Protocol 2)

This protocol outlines the process of modifying BiBAC vectors through traditional restriction enzyme digestion and ligation experiments. The provided steps are comparable to other commonly practiced cloning experiments; however, one must factor in adjustments to account for the high molecular weight of the BiBAC vector. The main challenge researchers face with BiBAC modifications is the limited number of sites available for digestion. Careful planning is crucial for success. It is often best to perform the majority of cloning work in a traditional vector, before moving the transfer DNA into the BiBAC vector.

Materials:

- Purified BiBAC vector (Basic Protocol 1)
- Sterile ddH2O Antarctic Phosphatase (New England Biolabs, cat no. M0289S)

- Restriction enzymes to generate complementary overhangs between vector and insert (New England Biolabs, Sigma Aldrich, Thermo Fisher Scientific, etc.)
- Insert DNA suspended in sterile H2O T4 DNA ligase (New England Biolabs, cat no. M0202S) Electrocompetent DH10B cells (Thermo Fisher Scientific, cat. no 18297010)
- Insulated bucket filled with ice
- SOC medium (Thermo Fisher Scientific, cat. no. 15544034)
- 150 × 15–cm LB agar plates with appropriate antibiotics
- 0.6-ml microcentrifuge tubes
- Micropipettes
- Incubator: set to 37°C 80°C
- heat block
- Electroporator
- Electroporation cuvettes
- 17 × 100–mm polystyrene culture tubes (Thermo Fisher Scientific, cat. no. 14-956-6D)
- Shaking incubator: set to 37°C
- Additional reagents and equipment for ethanol precipitation (Green and Sambrook, 2012)

Preparation of BiBAC vector:

1. Add the following components into a 0.6-ml microcentrifuge tube using a micropipette:

BiBAC vector DNA:	10 µg
Restriction enzyme buffer:	10% reaction volume

Restriction enzyme 1:	2% reaction volume
Restriction enzyme 2:	2% reaction volume
Sterile ddH2O:	Fill to reaction volume.

Example reaction:

BiBAC vector DNA:	137.5 µl
Restriction enzyme Buffer (10×):	16 µl
Restriction enzyme 1:	3 µl
Restriction enzyme 2:	3 µl
Sterile ddH2O:	0.5 µl
Total volume:	160 µl.

2. Place for to 1 to 3 hr in a 37°C incubator.

   The reaction should be allowed to run longer than recommended enzyme digestion time to completely linearize BiBAC vectors. This will decrease the number of false positive colonies recovered in later screening steps.

   The improved fidelity of some restriction enzymes will allow overnight digestion without risk of star activity.

3. Heat-inactivate the enzyme for 20 min in an 80°C heat block. Heat inactivation of the restriction enzyme will guarantee no interference with the ligation process.

4. Add the following components into the original reaction mixture:

Phosphatase buffer (10×)	10% reaction volume

Digest mixture (steps 1 to 3):	X µl
Phosphatase enzyme:	1 % reaction volume
H2O:	Fill to reaction volume

Example reaction:

Digest mixture:	160 µl
Phosphatase buffer (10×)	16 µl
Phosphatase enzyme:	2 µl

H2O: 2 µl

Total volume: 180 µl

5. Place the reaction for 30 min to 1 hr in a 37°C incubator.

   If the restriction digest produces a 3 overhang, a longer incubation time will be required to remove the 5 phosphate.

6. Heat-inactivate the enzyme for 20 min in an 80°C heat block.

7. Clean DNA via ethanol precipitation (Green and Sambrook, 2012).

Preparation of insert:

8. Add the following components into a 0.6-ml microcentrifuge tube using a micropipette:

Insert DNA:	2 µg
Restriction enzyme buffer (10×):	10% reaction volume
Restriction enzyme 1:	~2 % reaction volume
Restriction enzyme 2:	~2 % reaction volume
H2O:	Fill to reaction volume

Example reaction:

Insert DNA:	*7 µl*
Enzyme buffer (10×):	*2 µl*
Enzyme 1:	*0.5 µl*
Enzyme 2:	*0.5 µl*
H2O:	*10 µl*
Total volume:	*20 µl*

9. Place the reaction for 15 min to 1 hr in a 37°C incubator.For some constructs, a longer incubation time will yield better digestions.

10. Heat-inactivate the enzyme for 20 min in an 80°C heat block.

11. Clean DNA via ethanol precipitation (Green and Sambrook, 2012).

Ligation of insert into BiBAC:

12. Calculate the amount of insert to use in the ligation reaction with the following equation:

$$\textit{Insert Mass (ng)} = R \times \frac{Insert\ Length\ (bp)}{Vector\ Length\ (bp)} \times \textit{Vector Mass (ng)}$$

The value R is the molar ratio of insert to vector. If the ratio is 3:1, R=3. The insert tovector ratio will impact ligation efficiency; therefore, it is suggested to try different ratiosin parallel.

13. Follow the manufacturer's instructions for use of T4 DNA ligase.

***Transformation into DH10B cells*:**

DH10B electrocompetent cells enable efficient transformation and maintenance of HMW DNA vectors, such as BiBACs.

14. Preset an electroporator to the settings recommended by the manufacturer for standard E. coli transformation.

15. Remove electrocompetent DH10B cells from −80°C and thaw on ice.

16. Pipet 2 µl of ligation mixture into thawed DH10B cells and gently mix by tapping side of tube. Successful transformation can be achieved using 25 to 50 µl of DH10B cells.

17. Pipet total amount of DH10B cells into a cuvette, avoiding the formation of bubbles between electrodes. Place the cuvette on ice until next step. Tapping the cuvette on a hard surface will remove bubbles if they have formed.

18. Electroporate cells following manufacturer's instructions.

19. Add 500 µl of SOC medium to the electroporated cells and transfer into a 17 × 100–mm polystyrene culture tube.

20. Shake the transformed cells for 1 hr at 250 rpm in a 37°C shaker.

21. Plate 100 µl cells on prewarmed LB plates containing appropriate antibiotics.

22. Place the plates in 37°C incubator overnight to 2 days.

23. After colonies are visible, screen for positive transformants. Since BiBAC extraction is time consuming and expensive, it is recommended to use colony PCR as a screening method.

24. After a transformant colony has been identified, inoculate a 3 to 5 ml overnight culture. This will be used to create a permanent glycerol stock to store at −80°C.

To further modify the BiBAC vector (Basic Protocol 2) or transform into Agrobacterium cells (Basic Protocol 3), Basic Protocol 1 must be repeated to isolate the BiBAC vector.

Transformation into *Agrobacterium Cells* (Protocol 3)

When transforming BiBAC vectors into maize cells, it is best to also include a helper plasmid during transformation. The helper plasmid pCH32, (Frary & Hamilton, 2001), increases the copies of the virulence genes virG, virE1, and virE2, which is thought to assist with the transfer of HMW DNA into plant cells (Figure A2.2B). Prior to inserting BiBAC DNA into *Agrobacterium* cells, it is best to insert the plasmid pCH32 into DH10B cells. The BiBAC vector is then inserted into these cells for transformation.

The line of *Agrobacterium* tumefaciens used for transformation in our laboratory is EHA105. Other *Agrobacterium* lines, such as LBA4404, could also

be used. If the EHA105 cells on hand are not electrocompetent, prepare electrocompetent cells by following steps 9 to 21.

Materials:

- Electrocompetent *Agrobacterium* tumefaciens EHA105 cells (Hood, Gelvin, Melchers, & Hoekema, 1993)
- Ice
- Plasmid pCH32 (Cornell CCTEC: ctl.cornell.edu)
- SOC medium (Thermo Fisher Scientific, cat. no. 15544034)
- LB plates containing 5 mg/ml tetracycline and 25 mg/ml rifampicin LB growth medium (Thermo Fisher Scientific, cat. no. BP9723-2)
- Sterile autoclaved water
- BiBAC construct DNA (Basic Protocol 2)
- Sterile inoculation loops
- Electroporation cuvettes
- Pipettes
- Electroporator
- 17 × 100–mm polystyrene culture tubes (Thermo Fisher Scientific, cat. no. 14-956-6D)
- 28°C shaker
- 28°C incubator
- 2-ml tubes
- Centrifuge

Transformation of pCH32 into EHA105 cells:

1. Thaw a 50 µl aliquot of EHA105 cells in ice.

2. Chill an electroporation cuvette on ice.

3. Add 10 to 15 ng of pCH32 to thawed EHA105 cells, gently pipette to mix.

4. Pipette cells into the cuvette, tap on the bench to remove any air bubbles.

5. Electroporate following manufacturer's instructions.

6. Add 1 ml of SOC medium to electroporated cells, and transfer into a 17 × 100–mm polystyrene culture tube.

7. Shake for 90 min at 250 rpm at 28°C.

8. Spread 20 to 50 µl of cells onto prewarmed LB plate containing 5 mg/ml tetracycline and 25 mg/ml rifampicin.

9. Place the plates for 2 to 3 days in 28°C incubator, until colonies have formed.

Prepare electrocompetent EHA105 containing pCH32 helper plasmid:

10. Pick 3 to 5 single colonies and transfer into 2 ml LB medium in 17 × 100–mm polystyrene culture tubes containing 5 mg/ml tetracycline and 25 mg/ml rifampicin.

11. Place the tubes for 24 to 48 hr in 28°C shaker at 250 rpm, until growth is observed.

Cells may take longer to grow when in the presence of tetracycline.

12. Transfer 200 µl of starter culture into fresh 25 ml LB medium containing 5 mg/ml tetracycline and 25 mg/ml rifampicin.
13. Place in the shaker for 24 to 48 hr until strong growth is observed.
14. Divide the culture into separate 2-ml tubes.
15. Centrifuge the culture for 2 min at 16000 × g, 25°C, to pellet the cells.
16. Remove liquid medium with a pipette and discard.
17. Place the tubes on ice and add 1 ml of autoclaved ddH2O and resuspend by pipetting up and down gently.
18. Centrifuge for 2 min at 16000 × g, 25°C, to pellet the cells.
19. Without disrupting the pellet, remove the liquid medium with a pipette and discard.
20. Repeat steps 17 to 19 twice, for a total of three washes.
21. Following the last wash, add 100 µl autoclaved ddH2O into each tube, and resuspend by pipetting up and down.
22. Divide into 50-µl aliquots in separate 1.7-ml tubes.
23. Freeze the cells at −80°C to create permanent, electrocompetent EHA105 *Agrobacterium* stocks containing pCH32 helper plasmid.

Transformation of BiBAC into EHA105 cells containing helper plasmid:

24. Thaw a 50-µl aliquot of EHA105 cells containing helper plasmid pCH32 for each BiBAC construct to be transformed.

25. Chill an electroporation cuvette on ice.

26. Add 100 to 200 ng of BiBAC DNA to thawed cells.

    Due to the large size of BiBAC constructs, a larger amount of DNA must be added to the cells for transformation. If transformation into EHA105 cells is low, consider altering the amount of DNA added at this step.

27. Tap the cuvette on the bench to remove air bubbles.

28. Electroporate following the manufacturer's instructions.

29. Add 1 ml of SOC medium to the electroporated cells and transfer into a 17 × 100–mm polystyrene culture tube.

30. Shake the transformed cells for 2 hr at 250 rpm in a 28°C shaker.

31. Plate 100 to 500 µl cells on prewarmed LB plates containing appropriate antibiotics.

32. Place the plates in 28°C incubator overnight to 2 days.

    Larger constructs may take longer to grow, especially when in the presence of tetracycline.

33. Once colonies are visible, use a sterile loop to transfer a single colony into a 3 to 5 ml LB culture containing the appropriate antibiotics.

34. Incubate the culture for 1 to 2 days at 28°C.

35. Use 500 to 1000 µl of culture to create a permanent glycerol stock to be stored at −80°C.

    This stock will be used in Support Protocol 2 to create Agrobacterium infection medium.

Transformation of BiBAC Constructs into Immature Maize Embryos (Protocol 4):

Transformation into maize embryos is a lengthy process, and one that should be familiar before attempting to transform BiBAC constructs. The overall process is similar to that described in (Vega, Yu, Kennon, Chen, & Zhang, 2008), with modifications made to better accommodate BiBAC vectors. The majority of alterations take place before the selection step, as following the integration of DNA into the plant cells the growth of callus should behave the same as traditional methods.

NOTE: Support Protocols 1, 2, and 3 should be completed prior to Protocol 4.

Materials:

- Hi-II A × B ear(s) (Support Protocol 1 should be started 9 to 12 days prior to infection)
- Bleach
- 2 × 1.8 liters of autoclaved H2O in 2-liter Erlenmeyer flask
- TWEEN 20 Phi–A infection medium (see recipe)
- *Agrobacterium* infection medium (see Support Protocol 2)
- Phi–B cocultivation medium plates (see Support Protocol 3 and recipe)
- Phi–C callus induction medium plates (see recipe)
- Phi–D callus selection medium plates (see recipe)
- Phi–F root regeneration medium tubes (see recipe)
- Soil
- Phi–E shoot regeneration medium plates (see recipe)
- Sterile laminar flow hood
- Forceps, sterile
- Spectrophotometer
- 1000- to 2000-ml glass beakers #11 scalpel blade (Feather, cat. no. 504170) Spatula, sterile
- 1.7-ml microcentrifuge tubes
- Micropipettes
- Parafilm
- Incubator

Immature embryo extraction from maize ears:

1. Collect Hi-II A X B ear from the greenhouse when embryos are approximately 1.5 to 2 mm in length, which occurs about 9 to 12 days after pollination (see Support Protocol 1).

    Maize immature embryo development is temperature dependent. High greenhouse temperatures increase the rate of development, while cooler temperatures reduce growth rates.

2. Dehusk and remove silks to expose the immature kernels (Figure A2.3A).
3. Inside a laminar flow hood, insert sterile forceps into the bottom of the ear and place in a clean 1000- to 2000-ml glass beaker.
4. Sterilize ear in a 1:1 mixture of bleach and autoclaved ddH2O with 2 to 3 drops of TWEEN 20 for 25 min at 25°C (Figure A2.3B).
5. Carefully dump bleach mixture into a secondary container.
6. To remove residual bleach, wash ear with autoclaved ddH2O for 5 min.
7. Carefully dump water into a secondary container.
8. Repeat steps 6 and 7 for a total of three washes.
9. Remove the top-half of Hi-II kernels with a #11 scalpel blade (Figure A2.3C).
10. Using a sterile spatula, remove each 1.5- to 2-mm embryo and place into a 1.7-ml microcentrifuge tube filled with Phi–A medium (Figure A2.2D).

    Use 60 to 80 embryos/tube.

    Immature embryos are positioned at the top of the kernel. For easy removal, insert spatula in the bottom of the kernel and gently rotate

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Figure A2.3 - *Agrobacterium* Transformation of Maize: (A) Maize Hi-II A/B ear 10 days after pollination. (B) Sterilization of Hi-II ears with 1:1 water bleach mixture. (C) Removal of the top half of Hi-II kernels with #11 scalpel blade. (D) Embryo extraction. Arrow points to the embryo on the bottom side of the removed endosperm. (E) Suspension of embryos in Phi–A infection medium before and after wash. (F) Placement of embryos on Phi–B cocultivation medium with the scutellum facing down. This image was taken 3 days after infection to show *Agrobacterium* formation around embryos before transfer to Phi–C callus induction medium.

counter-clockwise. This will separate the endosperm from the pericarp, exposing the embryo.

11. After all embryos have been removed from Hi-II ears, wash with 1 ml of clean Phi–A infection medium. Repeat this step 2 to 3 times.
12. Suspend in Phi–A infection medium until infection.

Agrobacterium infection of embryos:

13. Using a micropipette, remove Phi–A infection medium from embryo tubes and dispose in a secondary container.
14. Pipet 1 ml of *Agrobacterium* infection medium (see Support Protocol 2) into each 1.7-ml tube, close the cap, and invert until embryos are suspended in solution.
15. Wait 5 to 10 min.

Periodically invert tubes to prevent the *Agrobacterium* from settling.

16. Carefully place the infection medium and embryos onto Phi–B cocultivation medium plates (see Support Protocol 3).

    If embryos are stuck at the bottom of the 1.7-ml tube, a sterile spatula can be used to recover them.

17. Using a micropipette, remove the *Agrobacterium* infection media from Phi–B cocultivation medium plates and dispose in a secondary container.

    Tipping the plates to the side and allowing the medium to pool is the most efficient way of removal.

18. Using a spatula, position each embryo on the plate with the scutellum facing down (Figure A2.3F).
19. Wrap the plate in Parafilm and place for 3 days in a 25°C incubator.

    The time used for infection should be altered to compensate for the growth rate of the Agrobacterium used for transformation. The typical Agrobacterium strain will take 2 days to form healthy colonies; however, some strains containing large BiBAC plasmids can take 3 to 4 days. This extended growth time should be compensated for during the infection step. This extension will be construct specific, and can vary widely.

Callus induction:

Phi–C callus induction medium must be prepared before proceeding to steps 20 to 22 (see recipe).

20. Remove embryo plate from the 25°C incubator and place inside a laminar flow hood before removing the Parafilm.
21. Using sterile forceps, transfer each embryo onto a Phi–C callus induction medium plate.

    It is important to leave enough space for each embryo to form callus. 5 mm of space should be sufficient.
22. Wrap each plate with Parafilm, and place for 10 to 14 days in a 28°C incubator.

The rate of callus formation will depend on the health and developmental stage of the immature embryo upon extraction. Selection of bialaphos-resistant callus

23. Remove the callus plate from the 28°C incubator and place inside a laminar flow hood before removing Parafilm.
24. Using sterile forceps, transfer each individual calli to Phi–D1 callus selection medium plates.

    It is important to maximize callus contact with the medium, so each calli must be flattened with forceps.

    After flattening, there is a risk of transferring cells between calli. To avoid this, wipe forceps by dragging it through medium on the original plate.
25. Wrap each plate with Parafilm, and place for 2 weeks in a 28°C incubator.
26. Remove callus selection plate from the 28°C incubator and place inside a laminar flow hood before removing Parafilm.
27. Using sterile forceps, transfer each individual calli to Phi–D2 callus selection medium plates.

    Some sections of calli at this point may appear brown. This is a visual indication that the tissue does not contain a resistant transgene and may be discarded.
28. Wrap each plate with Parafilm, and place for 2 weeks in a 28°C incubator.
29. Repeat steps 26 to 28 every 2 weeks until all remaining calli are resistant to the bialaphos medium.

Each callus represents a single transformation event and may proceed to regeneration steps once identified. This process will take anywhere from 2 to 4 months.

Regeneration of transgenic plants:

Phi–E shoot regeneration medium plates must be prepared before proceeding to steps 30 to 33 (see recipe).

Phi–F root regeneration medium tubes must be prepared before proceeding to steps 34 to 36 (see recipe).

30. Remove the callus selection plate from the 28°C incubator and place inside a laminar flow hood before removing Parafilm.
31. Using sterile forceps, transfer each individual calli to Phi–E shoot regeneration medium plates.
32. Wrap each plate with Parafilm, and place for 2 weeks in a 28°C incubator.
33. Repeat steps 30 to 32 every 2 weeks until shoots are visible (Figure A2.4D). Shoot regeneration will take 4 to 6 weeks.
34. Remove the shoot plate from the 28°C incubator and place inside a laminar flow hood before removing Parafilm.
35. Using sterile forceps, carefully transfer shoot into Phi–F root regeneration medium tubes.
36. Cap the tube with a polypropylene cap and transfer into plant growth chamber no. 1 for 1 month.

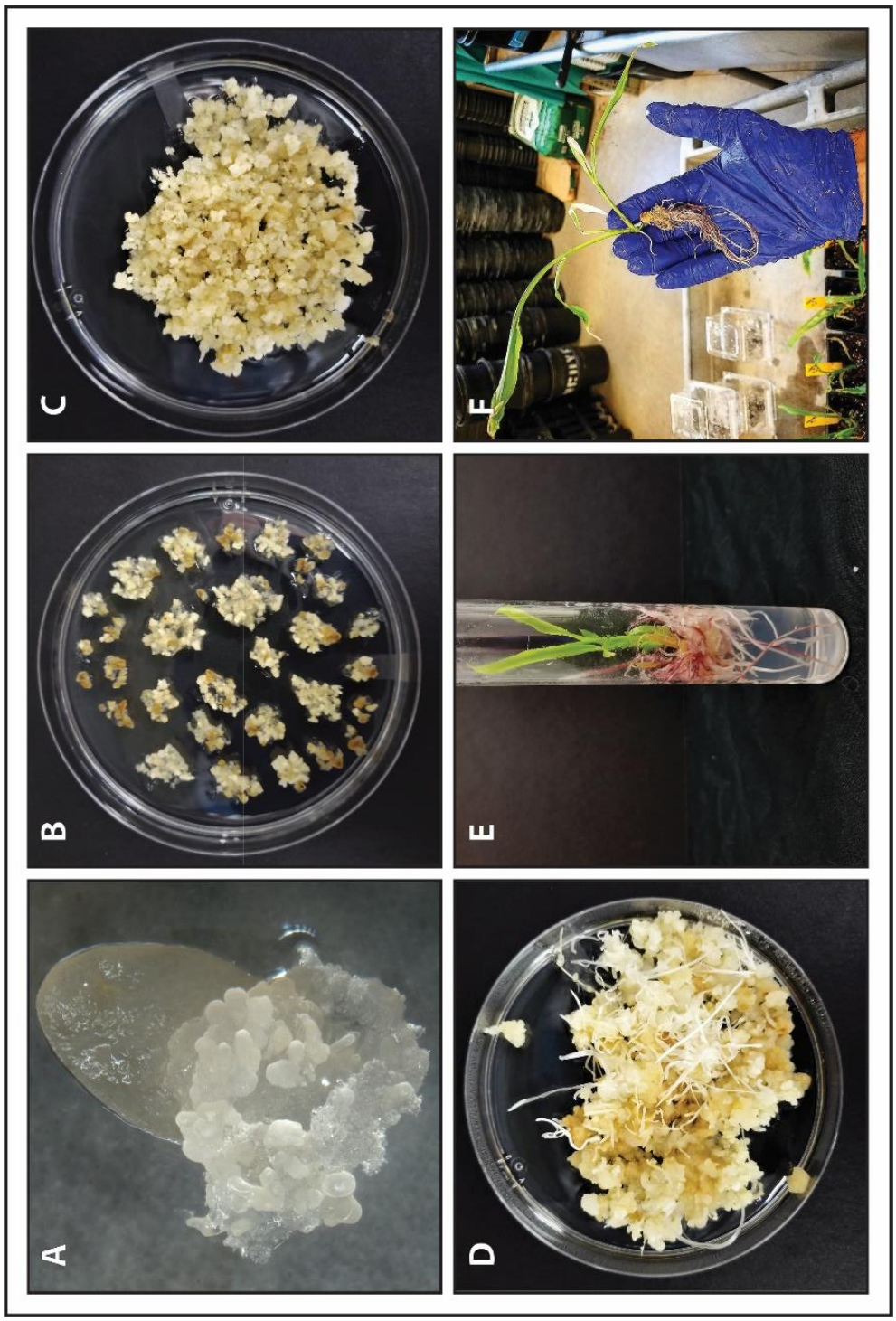
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Figure A2.4 - Callus Induction, Selection, and Regeneration: (A) Callus formation from immature embryo 12 days after transfer to Phi–C callus induction medium. (B) Callus on second round of Phi–D2 callus selection media (~8 weeks post-infection). (C) Resistant, mature calli on Phi–D2 callus selection medium. (D) Shoot development in Phi–E shoot regeneration medium. (E) Root formation and shoot development in Phi–F root regeneration medium. (F) Transfer of regenerated plant from Phi–F root regeneration medium to soil.

The rate of root and shoot development varies among calli. In general, researchers canexpect to see a T0plant ready for soil transfer in 1 month; however, it may take up to2 months. There is a chance that multiple plants regenerate from the same callus. These plants should be treated no differently than other transgenic materials. If separation is attempted at this stage, there is a risk of the plant dying.

Soil transfer of T_0 transgenic plants

After the plant has grown to the 2ndleaf stage, it is of appropriate size to transfer into soil.

37. Using forceps, remove plants from root regeneration medium tubes. The plant will remain rooted in the media upon removal.
38. Rinse roots under running water to remove remaining root regeneration medium.
39. Carefully place plants in soil with the base ~1 in. underneath the surface.
40. TransferT0plants to growth chamber no. 2 for 1 to 2 weeks. The purpose of this step is to acclimate the transgenic plants to greenhouse conditions.
41. OnceT0Plants have reached the 3rd leaf stage, transplant into 2-gallon pots and place in greenhouse.

Flowering time for T0plants is unpredictable and should be considered when regenerating plants. It is advised to plant seeds in the greenhouse each week once T0are transferred to Phi–F root regeneration medium.

Producing Hi-II Immature Maize Embryos (Support Protocol 1)

The maize genotype primarily used in *Agrobacterium* mediated transformation is Hi-II A and Hi-II B inbred lines, formed through initially crossing A188 and B73 (Armstrong, Green, & Phillips, 1991). Hi-II A/B F1 immature embryos have a high frequency of type-II callus formation, a friable and embryogenic callus enabling efficient cell selection and regeneration. Type-II callus formation is highly dependent on the health and developmental stage of the embryo; therefore, a section outlining production of Hi-II immature embryos is necessary.

Materials:

- Hi-II A seed Hi-II B seed
- Osmocote Plus 16-9-12 slow release fertilizer (ICL-SF, product code A903206)
- Peter's Professional 20-20-20 fertilizer (ICL-SF, product code G99290)
- Iron chelate fertilizer (Carl Pool)
- Small germination pots
- 2-gallon pots

1. Germinate Hi-II A and Hi-II B seed in small germination pots, allowing growth to the 2nd leaf stage.
2. Transplant Hi-II seedlings into 2-gallon pots filled with soil.
3. Cover the surface of the soil with a tablespoon of Osmocote 16-9-12 slow release fertilizer.

In general, Hi-II pollen sheds before proper ear development; therefore, planting must be staggered. Repeat steps 1 – 3 on a weekly basis.

4. Water and fertilize plants, as necessary.

   In our experience, Hi-II grows the best if allowed to completely dry before adding additional water.

   It is suggested to water in Peter's Professional 20-20-20 on a weekly or biweekly basis. Iron chelate should be added following the manufacturer's instructions.

5. When plants are in the reproductive growth stages, pollinate Hi-II B ears with Hi-II A pollen.

   Hi-II B is generally crossed as a female due to its larger ear size.

   Hi-II B and Hi-II B inbred lines are crossed together to increase plant vigor, enhancing the quality of obtained embryos.

6. Collect ear when embryos are approximately 1.5-2 mm in length, which occurs about 9 to 12 days after pollination.

7. Proceed with BiBAC transformation (see Basic Protocol 4, step 1).

Preparation of *Agrobacterium* (Support Protocol 2)

The following protocol outlines the steps required for preparing the Phi–A infection medium used in the BiBAC transformation. This process must be started 2 to 3 days before the date of the experiment.

Additional Materials (also see Basic Protocol 4):

- BiBAC *Agrobacterium* stock
- 150 × 15–cm LB agar plates with appropriate antibiotics
- Phi–A infection medium (see recipe)
- Acetosyringone stock (see recipe)
- N6 vitamin stock (see recipe)
- Inoculation loop
- Spectrophotometer
- Shaker set to 120 rpm

Initiation of BiBAC Agrobacterium inoculation:

1. Remove BiBAC *Agrobacterium* stock from a −80°C freezer and place in a sterile laminar flow hood.
2. Using an inoculation loop, make a streak on a 150 × 15 cm LB agar plate containing appropriate antibiotics.
3. Place the *Agrobacterium* plate for 2 to 3 days in a 28° to 30°C incubator. *Agrobacterium* harboring constructs with tetracycline resistance typically grow slower.
4. On the day of the transformation experiment, check the plate for adequate growth and proceed to the infection medium inoculation steps.

Inoculation of Agrobacterium in Phi–A infection medium:

5. Remove prepared Phi–A infection medium from a −20°C freezer and thaw on laboratory bench or in a water bath.

6. In a sterile laminar flow hood, add 0.1% volume of acetosyringone (100 mM) and N6 vitamin (1000×) stock solutions. For example, a 40 ml aliquot would receive 40 µl of acetosyringone and N6 vitamin.
7. Mix well by inverting 5 times.
8. Remove *Agrobacterium* plate from the 28° to 30°C incubator and place in sterile laminar flow hood.
9. Using an inoculation loop, transfer a sample of BiBAC harboring *Agrobacterium* into the Phi–A infection medium.
10. Check ocular density at λ = 600 (OD600) using a spectrophotometer. This value should be between 0.3 to 0.5.
11. Place *Agrobacterium* infection suspension on a shaker set to 120 rpm for 3 to 4 hr.
12. Proceed with infection of immature embryos (see Basic Protocol 4, step 13).

Preparation of Phi-B Cocultivation Medium (Support Protocol 3):

Cocultivation medium must be prepared prior to *Agrobacterium*-mediated transformation of immature embryos. Below are instructions on the preparation and storage of Phi–B cocultivation medium.

Materials:

- 250 ml aliquot of Phi–B cocultivation medium (see recipe)
- N6 vitamin stock (see recipe)

- Silver nitrate stock (see recipe)
- Acetosyringone stock (see recipe)
- Microwave
- Sterile laminar flow hood
- Micropipettes
- 100 × 15–cm polystyrene plates

1. Remove 250 ml aliquot of Phi–B cocultivation medium from 4°C and microwave on high until solidified agar turns to liquid.
2. In a sterile laminar flow hood, add the following reagents using a micropipette:

N6 vitamin stock (1000×):	250 µl
Silver nitrate stock (8.5 mg/ml):	25 µl
Acetosyringone stock (100 mM):	250 µl.

3. Mix well by swirling.
4. Pour into 100 × 15–cm polystyrene plates and allow agar to solidify.
5. Proceed with cocultivation steps (see Basic Protocol 4, step 16) or store up to 3 days at 4°C.

Reagents and Solutions

Use ddH2O for all reagents and solution recipes:

2,4 dichlorophenoxyacetic acid (2,4-D) stock solution:

- 100 mg of 2,4-D (Sigma-Aldrich, cat no. D7299) in 2 ml of NaOH (1 N)
- Adjust to 100 ml with ddH2O

- Store up to 6 months at 4°C

Acetosyringone stock solution (100 mM):

- 0.196 g acetosyringone (Sigma, cat no. D134406) into 5 ml methanol
- Fill to 10 ml with 5 ml ddH2O
- Filter sterilize with a 0.22-µm filter
- Divide into 1-ml aliquots
- Store up to 6 months at −20°C

Bialaphos stock solution (3 mg/ml):

- 30 mg of bialaphos (Gold Biotechnology, cat no. B0178) in 10 ml of ddH2O
- Filter sterilize with a 0.22-µm filter
- Store up to 2 months at −20°C

Glycine stock solution (2 mg/ml):

- 100 mg of glycine (Sigma-Aldrich, cat no. G7126) in 50 ml of ddH2O
- Filter sterilize with a 0.22-µm filter
- Store up to 6 months at 4°C

Liquid LB medium:

- 12.5 g LB granulated powder into 500 ml of ddH2O
- Autoclave on liquid cycle for 30 min
- Store indefinitely at room temperature

MS vitamin stock solution (1000×):

Add the following reagents into 50 ml of ddH2O:

- 5 g myo-inositol
- 0.005 g thiamine
- 0.025 g nicotinic acid HCl
- 0.025 g pyridoxine HCl
- Filter sterilize with a 0.22 μm filter
- Store at 4°C for up to 6 months

N6 vitamin stock solution (1000×):

Add the following reagents into 50 ml of ddH2O:

- 0.1 g glycine
- 0.025 nicotinic acid
- 0.05 g thiamin HCl
- 0.025 g pyridoxine HCl
- Filter sterilize with a 0.22 μm filter
- Store at 4°C for up to 6 months

Phi–A infection medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 2 g N6 salts
- 68.5 g sucrose
- 36 mg glucose
- 0.7 g L-proline
- 0.5 g MES
- 1.5 ml 2,4–D (1 mg/ml; see recipe)
- Fill to 1000 ml with H2O

- Adjust pH to 5.2 with 1 M KOH
- Sterilize using a 0.22-μm filter vacuum filtration system
- Divide into 40-ml aliquots
- Store up to 6 months at −20°C

Phi–B cocultivation medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 2 g N6 salts
- 30 g sucrose
- 0.7 g L-proline
- 0.5 g MES
- 1.5 ml 2,4–D (1 mg/ml; see recipe)
- Fill to 1000 ml with H2O
- Adjust pH to 5.8 with 1 M KOH
- Divide into 250-ml aliquots
- Add 2 g of granulated agar into each bottle
- Autoclave on liquid cycle for 30 min
- Store up to 1 year at 4°C

Phi–C callus induction medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 4 g N6 salts
- 30 g sucrose
- 0.7 g L-proline
- 0.5 g MES
- 1.5 ml 2,4–D (1 mg/ml; see recipe)

- Fill to 1000 ml with H2O
- Adjust pH to 5.8 with 1 M KOH
- Add 3 g Gelrite
- Autoclave on liquid cycle for 30 min
- Allow solution to cool to 55°C in a water bath
- In a sterile laminar flow hood, add the following reagents:
- 1000 µl N6 vitamin stock (1000×; see recipe)
- 100 µl silver nitrate stock (8.5 mg/ml; see recipe)
- 250 mg of cefotaxime: freshly dissolved and filter sterilized with a 0.22-µm filter Pour into 100 × 15–cm polystyrene plates
- Store up to 1 month at 4°C

Phi–D1 callus selection medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 4 g N6 salts
- 30 g sucrose
- 0.7 g L-proline
- 0.5 g MES
- 1.5 ml 2,4–D (1 mg/ml; see recipe)
- Fill to 1000 ml with H2O
- Adjust pH to 5.8 with 1 M KOH
- Add 3 g Gelrite
- Autoclave on liquid cycle for 30 min
- Allow solution to cool to 55°C in a water bath
- In a sterile laminar flow hood, add the following reagents:
- 1000 µl N6 vitamin stock (1000×; see recipe)
- 100 µl silver nitrate stock (8.5 mg/ml; see recipe)

- 250 mg of cefotaxime: freshly dissolved and filter sterilized with a 0.22-µm filter 500 µl bialophos stock (3 mg/ml; see recipe)
- Pour into 100 × 15–cm polystyrene plates
- Store up to 1 month at 4°C

Phi–D2 callus selection medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 4 g N6 salts
- 30 g sucrose
- 0.7 g L-proline
- 0.5 g MES
- 1.5 ml 2,4–D (1 mg/ml; see recipe)
- Fill to 1000 ml with H2O
- Adjust pH to 5.8 with 1 M KOH
- Add 3 g Gelrite Autoclave on liquid cycle for 30 min
- Allow solution to cool to 55°C in a water bath
- In a sterile laminar flow hood, add the following reagents:
- 1000 µl N6 vitamin stock (1000×; see recipe)
- 100 µl silver nitrate stock (8.5 mg/ml; see recipe)
- 250 mg of cefotaxime: freshly dissolved and filter sterilized with a 0.22-µm filter 1000 µl of bialophos stock (3 mg/ml)
- Pour into 100 × 15–cm polystyrene plates
- Store up to 1 month at 4°C

Phi–E shoot regeneration medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 4.3 g MS salts
- 60 g sucrose
- Fill to 1000 ml with H2O
- Adjust pH to 5.6 with 1 M KOH
- Add 3g Gelrite
- Autoclave on liquid cycle for 30 min
- Allow solution to cool to 55°C in a water bath
- In a sterile laminar flow hood, add the following reagents:
- 1000 µl MS vitamin stock (1000×; see recipe)
- 100 µl glycine stock (2 mg/ml; see recipe)
- 250 mg of cefotaxime: freshly dissolved and filter sterilized with a 0.22-µm filter
- 1000 µl of bialophos stock (3 mg/ml)
- Pour into 100 × 15–cm polystyrene plates
- Store up to 1 month at 4°C

Phi–F root regeneration medium (1 liter; Lee and Zhang, 2014):

Add the following reagents into 600 ml of ddH2O:

- 2.9 g MS salts
- 30 g sucrose
- Fill to 1000 ml with H2O
- Adjust pH to 5.6 with 1 M KOH (see recipe)
- Add 3g Gelrite
- Autoclave on liquid cycle for 30 min
- Allow solution to cool to 55°C in a water bath
- In a sterile laminar flow hood, add the following reagents:
- 1000 µl MS vitamin stock (1000×; see recipe)

- 100 µl glycine stock (2 mg/ml; see recipe)
- Using an electronic pipette, divide into 15-ml aliquots into 25 × 150–mm flat-bottom culture tubes (Pyrex, cat no. 9850-25XX)
- Store up to 6 months at 4°C

Potassium hydroxide (KOH), 1 M:

- 5.6 g KOH pellets in 100 ml of ddH2O

Silver nitrate stock solution (8.5 mg/ml):

- 340 mg of silver nitrate (Sigma-Aldrich, cat. no. S7276) in 40 ml of ddH2O
- Filter sterilize with a 0.22-µm filter
- Store up to 6 months at 4°C

Commentary

Background Information Binary bacterial artificial chromosomes (BiBACs), are vectors based on the F origin of replication, which allows maintenance of at least 300 kb of DNA (Shizuya et al., 1992). Additionally, the necessary maintenance machinery for *Agrobacterium tumefaciens* has been introduced, allowing for the transfer of the large genetic cargo to plant cells. 150 kb of DNA has been successfully transferred in tobacco plants (Hamilton, Frary, Lewis, & Tanksley, 1996), demonstrating the utility of the system. The ability to insert such

large genetic cargo into the BiBAC system allows researchers to insert multiple genes of interest into a single genetic locus, alleviating the complex crossing schemes that would result from multiple transformation events required with standard transformation vectors.

Critical Parameters

If bacteria inoculation harboring BiBAC vectors is below OD600 of >2, allow culture to shake longer. The low copy number in combination with the specific antibiotic used for selection may decrease the growth rate. The helper plasmid for BiBAC transformation, pCH32, contains a tetracycline selectable marker that decreases the growth rate of *E.coli* and *Agrobacterium* harboring cells. To improve BiBAC ligation efficiency, it is suggested to proceed from digestion to ligation steps as soon as possible. There is a possibility that vector and insert suspension contain nucleases that could chew back sticky ends created in the digestion process.

In the development of Hi-II immature embryos for transformation, ambient temperatures will affect the rate of development. As temperature increases, so does the growth rate of embryos. Since size of the immature embryo affects transformation efficiency, it is highly important to start checking embryos 9 days after pollination.

Troubleshooting

If a large number of *E. coli* colonies screened are negative for modified BiBAC vectors, check the quality of restriction enzymes used in the digestion experiment. Low cutting efficiency will leave circularized BiBAC vectors in the background of the ligation mixture, resulting in transformation of unmodified BiBACs. Background can be further decreased by using high fidelity restriction enzymes and/or increasing digestion times.

If bacterial contamination occurs after *Agrobacterium* transformation, there is the possibility that the *Agrobacterium* is contaminated. Alternatively, check to make certain the laminar flow hood is functioning properly.

There is a risk of contamination with each reagent and at each stage of the transformation process. Be certain to take the necessary steps that no contamination occurs during the experimental process. If several plates become contaminated over a short period of time, check the laminar flow hood filter.

Anticipated Results

Expect to obtain a low transformation efficiency when using BiBAC vectors (<1%).

Time Considerations

The total time required to complete the transformation process, from Hi-II seed to T0 seed, will take approximately 1 year. Callus selection, shoot regeneration, and root regeneration times are highly variable, increasing or decreasing the total time to complete the process. This estimation, however, does not include time required for purification, modification, and preparation of BiBAC vectors.

Acknowledgements

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Citations

Armstrong C.L., Green C.E., & Phillips R.L. (1991) Development and availability of germplasm with high Type II culture formation response. Maize Genetics Cooperation Newsletter, 92–93.

Frary A. & Hamilton C.M. (2001) Efficiency and stability of high molecular weight DNA transformation: An analysis in tomato. Transgenic Research, 10, 121–132. doi: 10.1023/A:1008924726270.

Green, M.R. & Sambrook J. (2012) Molecular cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Hamilton C.M., Frary A, Lewis C., & Tanksley S.D. (1996) Stable transfer of intact high molecular weightDNAinto plant chromosomes. Proceedings of the National Academy of Sciences of the United States of America, 93, 9975–9979. doi: 10.1073/pnas.93.18.9975.

Hamilton C.M. (1997) A binary-BAC system for plant transformation with high-molecularweight DNA. Gene, 200, 107–116. doi: 10.1016/S0378-1119(97)00388-0.

Hood E., Gelvin S., Melchers L., & Hoekema A. (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Research, 2, 208–218. doi: 10.1007/BF01977351.

Shizuya H., Birren B., Kim U.J., Mancino V., Slepak T., Tachiiri Y., & Simon M. (1992) Cloning and stable maintenance of 300- kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proceedings of the National Academy of Sciences of the United States of America, 89, 8794– 8797. doi: 10.1073/pnas.89.18.8794.

Sinnett D., & Montpetit A. (2003) Isolation of Cosmid and BAC DNA from E. coli. Methods in Molecular Biology, 235, 99–102. doi: 10.1385/1-59259-409-3:99.

Vega J.M., Yu W., Kennon A.R., Chen X., & Zhang Z.J. (2008). Improvement of *Agrobacterium*-mediated transformation in Hi- II maize (Zea mays) using standard binary vectors. Plant Cell Reports, 27, 297–305. doi: 10.1007/s00299-007-0463-z.

Zhang H., Scheuring C.F., Zhang M., Zhang Y., Wu C., Dong J.J., & Li Y. (2012) Construction of BiBAC and BAC Libraries form a Variety of Organisms for Advanced Genomics Research. Nature Protocols, 7, 479–499. doi: 10.1038/nprot.2011.456.

Appendix 3: Production of Engineered Minichromosome Vectors via the Introduction of Telomere Sequences

Note: The information in this chapter was published under the title:

Graham N.D., Swyers N.C., **Cody J.P.**, McCaw M.E., Zhao C., Birchler J.A. (2016) Production of engineered minichromosome vectors via the introduction of telomere sequences. Chromosome and Genomic Engineering in Plants. Methods in Molecular Biology, vol 1469. Humana Press, New York, NY

Summary

Artificial minichromosomes are non-integrating vectors capable of stably maintaining transgenes outside of the main chromosome set. The production of minichromosomes relies on telomere-mediated chromosomal truncation, which involves introducing transgenes and telomere sequences concurrently to the cell to truncate an endogenous chromosomal target. Two methods can be utilized; either the telomere sequences can be incorporated into a binary vector for transformation with *Agrobacterium tumefaciens*, or the telomere sequences can be co-introduced with transgenes during particle bombardment. In this protocol, the methods required to isolate and introduce telomere sequences are presented. Following the methods presented, standard transformation procedures can be followed to produce minichromosome containing plants.

Introduction

Artificial minichromosomes are any non-integrating vectors with large DNA carrying capacities that can be stably maintained in sequential generations (Gaeta et al., 2012). Production of minichromosomes is a result of telomere truncation, where telomere sequences are introduced into a chromosome during transformation resulting in the loss of genetic material distal to the insertion point. Engineered minichromosomes must be generated in this way to utilize an endogenous centromere because isolating centromere sequences and re-introducing them into a cell will not work due to the epigenetic nature of centromeres (Gaeta et al., 2012). Telomere-mediated truncation is accomplished through transformation of a plant with a plasmid containing a telomere repeat after the selectable marker and other desired genetic cargo, or by particle bombardment with a plasmid and separate telomere repeat. It is presumed that the non-homologous end joining pathway for double strand break repair attaches the plasmid to a double-stranded break in an endogenous chromosome. The presence of the telomere sequence then recruits telomerase, which adds telomere repeats to the end of the plasmid, creating a functional end of the chromosome. B chromosomes in maize and other plants are good targets for creating minichromosomes because they contain no genes essential to the survival of the plant. If an A chromosome is truncated, the event will generally result in the loss of genes, which are essential to the survival of the plant, making recovery of truncation events less likely. Truncating a B chromosome will have

little to no effect on the survival of the plant. The use of a B chromosome has the added benefit of having no linkage between the transgene and endogenous genes in the transformed line. Transgenes on an A chromosome may be linked to alleles from the transformable line, which are undesirable in a high yielding commercial line. A suite of transgenes can be carried on a single minichromosome and introgressed into new lines as a single unit, reducing the complexity of breeding programs to stack multiple different transgenes in a single plant. The complexity and duration of introgressing multiple transgenes into a line may possibly be further reduced by transferring minichromosomes through haploid induction and doubling the ploidy of a resultant haploid containing a minichromosome would create a completely homozygous line with minichromosomes (Gaeta et al., 2012).

Minichromosomes exist in association with the normal chromosome set and are subject to modification via site-specific recombination technology. Modifications add or remove gene fragments in a targeted manner, enabling continuous concatenation of sequences while recycling a single selection marker. As minichromosome technology develops, this strategy could allow researchers to stack multiple genes or whole biosynthetic pathways on a single location within the genome. This would circumvent limitations associated with current popular genetic engineering methods, such as disruption of endogenous gene function, transgene silencing, linkage drag, and inefficient recovery of multiple transgenes (Yu et al., 2007).

This system relies on the development of a minichromosome platform, which is used as a target for subsequent modification events. In plants, platforms are produced through telomere-mediated truncation of pre-existing genetic material via *Agrobacterium* tumefaciens transformation or particle bombardment. Each method requires the utilization of a telomere array; however, the mode of delivery and materials used in these processes are slightly different. *Agrobacterium* transformation requires an advanced cloning strategy to position a telomere fragment near the right border of a T-DNA vector, while particle bombardment simply requires an isolation of the telomere sequence. Preparation of telomere for both cloning and bombardment can be carried out in two different ways, through gel extraction or telomere repeat concatenation via PCR.

Due to the difficulty in manipulating the repetitive sequences of the telomere, it must be moved into a transformation vector via in-gel ligation. After positive clones have been identified they must be screened and sequenced to ensure that the insert is intact and in the correct orientation. Following sequencing of positive colonies, those in the correct orientation should be screened for insert size. The minimum size required for telomere truncation has not yet been determined, but it is thought that the greatest chance of success will come from the use of the largest telomere sequence possible. Interestingly, telomere sequences often form a secondary structure within agarose gels making it difficult to get an accurate size estimate. Performing a Southern hybridization (Southern, 1975) on these agarose gels will show evidence of the

full size of the telomere repeat that is present in a clone. For particle bombardment, telomere DNA conglomerates can be produced using polymerase chain reaction. Differing lengths of telomere DNA are created by the annealing of specific primers to each other resulting in the creation of telomere repeats of varying sizes. The resulting fragments of telomere can be visualized by gel electrophoresis and particular sizes of telomere can be obtained by DNA gel extraction from an agarose gel. The obtained telomere DNA can then be used in particle bombardment with a construct of interest to create a minichromosome. The protocol described in the following section has been adapted from a protocol that labels telomere to make fluorescent probes (Ijdo et al., 1991).

Materials

Materials for In-Gel Ligation into Agrobacterium Competent Vector:

1. *Agrobacterium* tumefaciens competent transformation vector (see Note 1).
2. Plasmid pWY82 (see Note 2).
3. Oligonucleotide (TTTAGGG) 10 can be synthesized in either the 5′ or 3′ direction.
4. Luria broth.
5. Spectinomycin.
6. 2xYT Medium.
7. QIAprep Spin Miniprep Kit (Qiagen).

8. Restriction Enzymes compatible with pWY82 and target plasmid.
9. Agarose.
10. DNA Gel Loading Dye (6×).
11. GeneRuler 1 kb DNA Ladder (Life Technologies).
12. Ethidium Bromide.
13. Low Melting Point Agarose.
14. Antarctic phosphatase.
15. T4 DNA Ligase and Ligase Buffer.
16. ElectroMax Stbl4 Cells (Life Technologies).
17. S.O.C Media (Super Optimal Broth with Catabolic Repressor).
18. Agar.
19. Petri Dishes.

Equipment for In-Gel Ligation into Agrobacterium Competent Vector:

1. 30 °C Incubator.
2. 30 °C Shaker.
3. 250 mL baffl ed culture fl asks.
4. Vacuum Concentrator.
5. Nanodrop Spectrophotometer.
6. Gel electrophoresis system.
7. Ultraviolet Transilluminator.

Recipes for In-Gel Ligation into Agrobacterium Competent Vector:

1. **Luria Broth**: For 500 mL dissolve 12.5 g of LB media in 400 ml water. Bring to 500 mL and autoclave for 20 min.
2. **Luria Broth Plates**: For 500 mL, dissolve 12.5 g of LB media and 6 g of agar is 400 mL water. Bring to 500 mL and autoclave for 20 min. Place in 50 °C water bath until completely cooled then add appropriate antibiotics. Gently mix and pour thin layer into petri dishes. Store at 4 °C for up to 1 month.
3. **2xYT Broth**: For 500 mL of culture dissolve 15.5 g of 2× YT in 400 mL water. Bring final volume to 500 mL and autoclave for 20 min.
4. **TAE**: To prepare 1 L of 50× TAE add 242 g trizma base, 14.6 g EDTA, and 57.1 mL of acetic acid to 500 mL of water and dissolve. Bring total volume to 1 L with water.

Materials for Preparation of Telomere for Particle Bombardment:

1. Telomere Primers (see Note 3):
    i. Forward Primer-5′ $(TTTAGGG)_{10}$ 3′.
    ii. Reverse Primer-5′ $(CCCTAAA)_{10}$ 3′.
2. LongAmp ® Taq DNA Polymerase (New England BioLabs).
3. DNA Gel Loading Dye (6×).
4. GeneRuler 1 kb DNA Ladder (Life Technologies).

5. Ethidium Bromide.
6. Wizard ® SV Gel and PCR Clean-Up System (Promega).
7. Nuclease-Free Water.

Equipment for Preparation of Telomere for Particle Bombardment:

1. Gel electrophoresis system.
2. Ultraviolet Transilluminator.

Methods

Insertion of Telomere Through In-Gel Ligation:

1. Streak pWY82 onto LB plates containing 100 mg/mL spectinomycin.
2. Place plates into 30 °C incubator for 48 h (see Note 4).
3. Begin a starter culture by picking a single colony into 3 mL of 2xYT liquid media containing 100 mg/mL spectinomycin and shaking for 48 h at 30 °C.
4. Add 500 µL of starter culture to 125 mL of 2xYT in a baffl ed culture fl ask and shake at 250 rpm at 30 °C until the culture reaches an OD600 ~2 (~48 h).
5. Extract culture 4 mL at a time with the QIAprep Spin Miniprep kit (Qiagen) eluting with 50 µL of 50 °C nuclease-free water.
6. Combine each miniprep into one 1.7 mL tube and reduce the volume in a vacuum concentrator until the concentration is ~1 µg/µL when measured with a Nanodrop spectrophotometer.

7. Individually test each restriction enzyme to be used by digesting 1 µg of plasmid following manufacturer's instructions.
8. Add 6× loading dye to each digest after digestion is complete.
9. Load each digest into a 1 % (w/v) TAE agarose gel flanked by GeneRuler 1 kb DNA ladder.
10. Run gel until loading dye approaches bottom of gel.
11. Stain gel with 0.5 µg/mL ethidium bromide for 30 min.
12. Visualize gel under UV light to check integrity of plasmid and efficiency of restriction enzymes (see Note 5) (Figure A3.1).
13. Digest 10 µg of pWY82 and 5 µg of the target plasmid with 10 units of each restriction enzyme according to the manufacturer's instructions in a 50 µL total volume.
14. Prepare a 1 % (w/v) TAE low melting point agarose gel and allow to solidify for 30 min in a 4 °C cold room (see Note 6).
15. Pre-chill the 1× TAE for electrophoresis by filling the electrophoresis chamber and allowing to chill in 4 °C cold room.
16. After restriction digest has completed, treat the target plasmid with 5 units of Antarctic phosphatase for 15 min at 37 °C (see Note 7).
17. Add 15 µL of 6× loading dye to each restriction digest and gently mix.
18. Carefully lower low melting point agarose gel into the electrophoresis chamber (see Note 8).
19. Load the full restriction digest into gel.

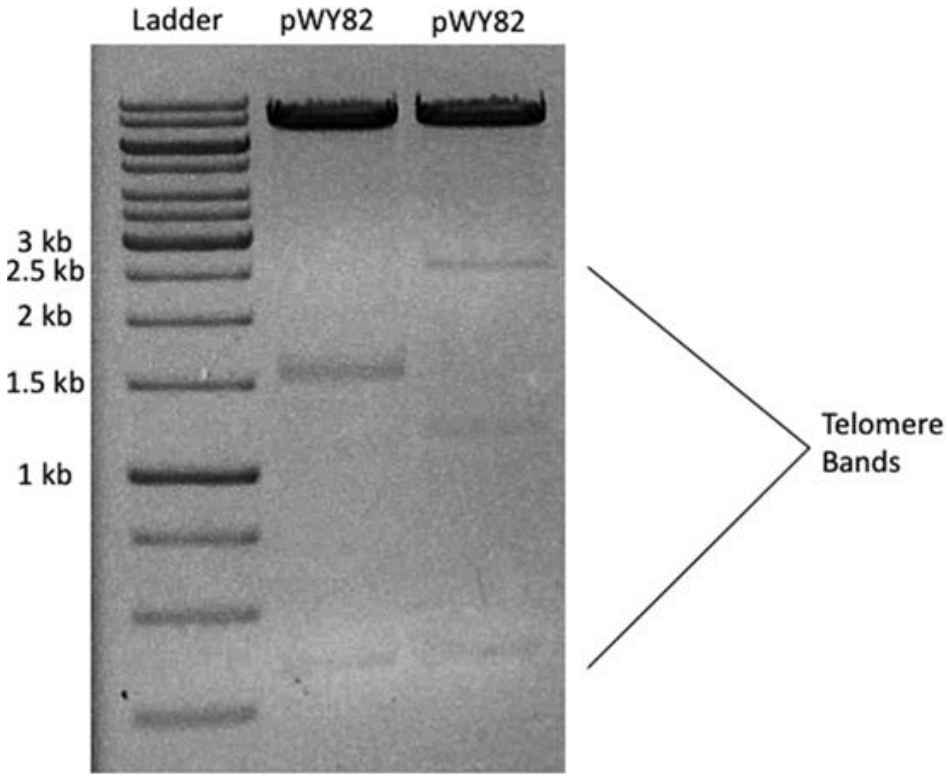

Figure A3.1 - Example Digest of pWY82: pWY82 in both lanes was cut with Eco RV and Hin dIII though different sized bands can be seen throughout the gel

20. Load 6 µL of GeneRule 1 kb DNA ladder into the flanking wells of the gel.
21. Run the gel at 100 V in the cold room until the lower band of the loading dye is at the bottom of the gel.
22. Carefully move the gel to a glass dish and stain with 0.5 µg/ mL ethidium bromide for 30 min.
23. Visualize under UV light and estimate the DNA concentration by comparing the intensity of the ladder to sample bands according to manufacturer's instructions.
24. Excise the uppermost telomere band with a fresh scalpel and place into a 1.7 mL tube (Figure A3.2).
25. Excise the target plasmid backbone with a fresh scalpel and place into a 1.7 mL tube (Figure A3.2).
26. In order to remove salts from the agarose slice add 1 mL of nuclease-free water to each tube and place at 4 °C overnight.
27. Completely remove water from each tube and place in a 70 °C water bath.
28. Flick tubes every minute until gel has completely melted.
29. Once agarose has melted (~5 min), move to a 37 °C water bath.
30. Allow gel fragments to cool to 37 °C, about 5 min.
31. Prepare ligation mixture in a 1.7 mL tube as shown in Table 1.
32. Once each component has been added, quickly mix by pipetting before ligation re-solidifies. (a) Flick tube until gel is floating in water.
33. Incubate the solidified ligation overnight at room temperature.

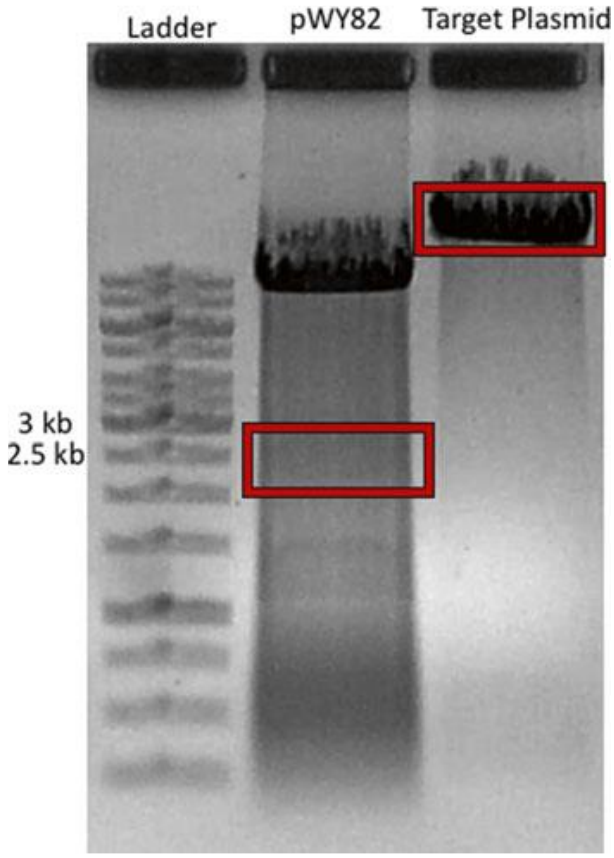

Figure A3.2 - Example Gel Used for Telomere Ligation: The boxed sections were removed and used for ligation

Ligase buffer	5 µL
Target plasmid gel fragment	100 ng
Ligase	1 µL (400 units)
Telomere gel fragment	To 50 µL

Table A3.1 - **Ligation reaction mixture components.**

34. Remove salts by incubating ligation in 1 mL of nuclease-free water for 15 min.
35. While ligation is dialyzing, begin to thaw Stbl4 cells on ice.
36. Replace water with 50 µL of fresh nuclease-free water and place in a 70 °C water bath.
37. Flick tube every minute until completely melted.
38. Add 2 µL of ligation to 40 µL of Stbl4 cells and electroporate according to manufacturer's instructions.
39. Resuspend cells in 900 µL of S.O.C media and shake in 15 mL culture tubes at 30 °C for 1.5 h at 250 rpm.
40. Plate 100 µL of transformation onto LB plates with appropriate antibiotics.
41. Incubate plates at 30 °C (see Note 9).
42. Screen colonies via colony PCR or colony hybridization (see Note 10).
43. Confirm telomere orientation using standard sequencing methods (see Note 11).

Screening Potential Colonies for Telomere Size:

1. Prepare a starter culture by inoculating 3 mL of 2xYT liquid media cultures with the appropriate antibiotics of each colony to be tested, pWY82 for a positive control, and empty target vector as a negative control (see Note 12).
2. Shake at 250 rpm for 8 h at 30 °C.

3. Inoculate fresh 5 mL 2xYT liquid cultures with appropriate antibiotics and 100 µL of starter cultures.
4. Shake cultures at 250 rpm for 12–18 h at 30 °C.
5. Extract 4 mL of each culture with the QIAprep Spin Miniprep Kit (Qiagen) and elute with 40 µL of 50 °C nuclease-free water.
6. Estimate concentration of each extraction using a nanodrop spectrophotometer.
7. Digest 1 µg of each plasmid extraction with restriction enzymes that flank the telomere insert as closely as possible in a 50 µL reaction volume.
8. Pour a 1 % (w/v) 1× TAE agarose gel and insert a comb large enough to contain a 60 µL volume and allow to solidify at room temperature.
9. Add 10 µL of 6× loading dye to each restriction digest once completed.
10. Mix thoroughly by pipetting.
11. Load each digest into agarose gel and run at 100 V in 1× TAE.
12. Stain gel in 0.5 µg/mL ethidium bromide solution for 30 min.
13. Visualize with UV light to confirm successful digest.
14. Transfer to a nitrocellulose membrane by Southern transfer (Green et al., 1989).
15. Follow Southern hybridization protocol (Green et al., 1989) using the radiolabeled oligonucleotide $(TTTAGGG)_{10}$.
16. Compare the signal to the size standard to estimate the insert size.

Production of Telomere Fragments via Polymerase Chain Reaction:

1. PCR Reaction Assembly (see Note 13):
    a. The following PCR reaction (Table 2) has been assembled using LongAmp Taq DNA polymerase from New England Biolabs (volumes listed are per reaction volumes) (see Note 14):
2. Use the following thermocycler protocol to perform PCR with the assembled reaction mixtures (Table 3).
3. After completion of thermocycler protocol, gel electrophoresis should be performed. Load the entire volume of each sample into the gel, as DNA gel extraction will be performed following visualization by gel electrophoresis. Example protocol for gel electrophoresis can be found in Sambrook and Russell 2001. Telomere DNA will appear as “smears,” such as those visible in Figure A3.3.
4. DNA gel extraction is performed using a kit such as Wizard ® SV Gel and PCR Clean-Up System. Excise the gel band corresponding to the size of telomere DNA desired. Follow manufacturer’s instructions for extraction of DNA from the gel piece (see Note 15).
5. The resulting DNA from the gel extraction can be used in a cobombardment with a transgene.

Notes

1. This vector should have cloning sites that are compatible with those bordering the telomere sequence in plasmid pWY82.

Nuclease-free water	12 µl	24 µl
5x LongAmp Taq buffer	4 µl	8 µl
Forward telomere primer	0.5 µl	1 µl
Reverse telomere primer	0.5 µl	1 µl
10 mM DNTPs	2 µl	4 µl
LongAmp Taq polymerase	1 µl	2 µl

Table 2 - **PCR Reaction Components**.

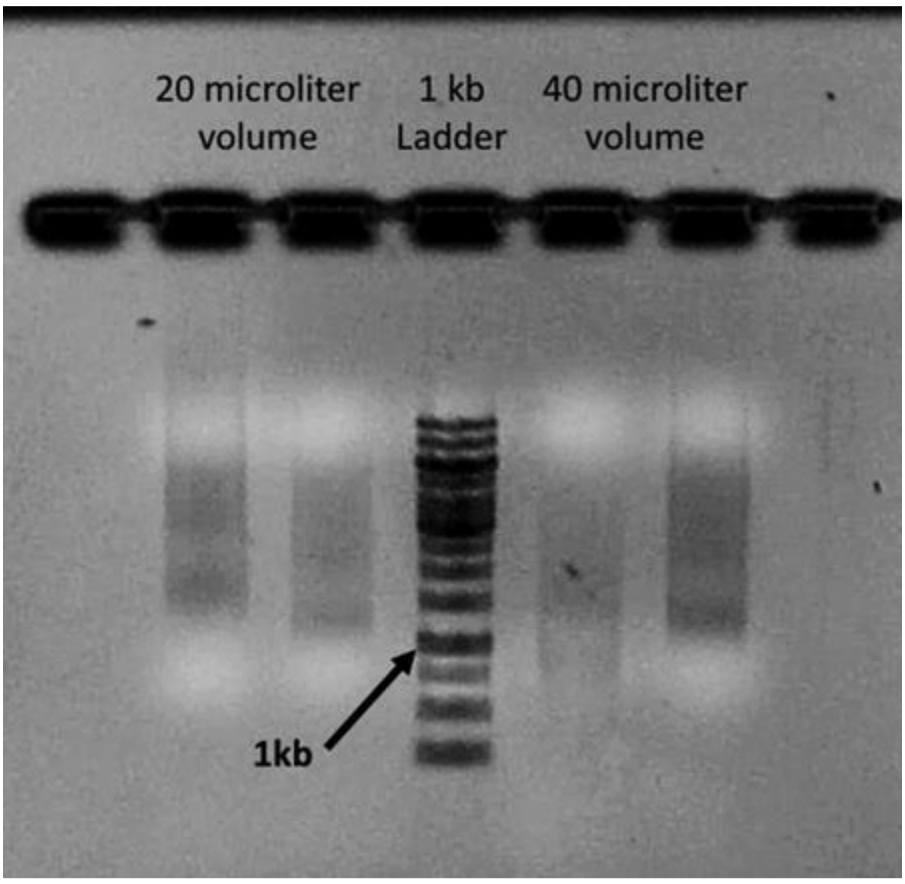

Figure A3.3 - Telomere PCR example using 20 and 40 µL reaction volumes: The “smears” in the sample lanes are indicative of telomere DNA conglomerates formed of various sizes

2. This plasmid contains ~2.6 kb of telomere sequence and can be obtained through AddGene (addgene.org Plasmid# 65721).
3. A working stock of these primers should be prepared at a 1.25 µM/µL concentration.
4. pWY82 is maintained in STBL2 cells to stabilize the repeated sequences. These cells are slow growing and often will not be visible on plates or in culture for 24 h.
5. Telomere sequences are unstable, and often will form secondary structures when run in an agarose gel. In most cases, the telomere will appear as multiple bands, or a smear, in addition to the 2.6 kb full telomere band (Figure 3A.1).
6. A comb must be used that is large enough to fi t 65 µL.
7. If restriction enzymes and Antarctic phosphatase are both purchased from the same manufacturer, it is not usually necessary to clean up the restriction digest between reactions.
8. Caution: low melting point gels must be handled extremely carefully as they are fragile.
9. As cells must be grown at 30 °C, it often takes 48 h for colonies to appear.
10. Though the technique requires radiation safety training, Southern Hybridization (Sambrook et al., 1989) is the recommended procedure for screening colonies for telomere. In our experience, hundreds of colonies can be screened at once, and using a labeled oligonucleotide probe of

$(TTTAGGG)_{10}$ in either the 5′ or 3′ direction is extremely sensitive. Screening via colony PCR has been used in our lab; however, it is not as effective as Southern Hybridization. While most colony PCR procedures will suggest choosing primers that will cause amplification across the inserted DNA, this is not possible with telomere as the complex repeats will disrupt amplification. As a result, the PCR will fail and give false negative results. Consequently, there are two options for colony PCR to detect telomere repeat insertion. First, reliable primers can be used flanking the insertion site and a blank band can be considered a positive insertion. While this method has been successful, it relies on the polymerase failing during amplification. The second option relies on a primer in the target plasmid, and another on the sequence adjacent to the telomere repeats that will also be inserted into the target plasmid during ligation. This method has also been successful; however, because the primers originate in different plasmids, the user is not able to have a positive control.

11. Due to the complexity of the telomere repeats, sequencing will often fail after a few hundred base pairs. While this procedure cannot determine the complete length of inserted telomere, it is helpful to ensure that the ligation was completed in the correct orientation.
12. Prepare in the morning so that full cultures can be started in the evening and allowed to grow overnight.

13. A proofreading Taq polymerase should be used, for example, LongAmp Taq DNA Polymerase from New England Biolabs.
14. Make several reactions so that plenty of DNA can be obtained from DNA gel extraction. If using a different proofreading Taq, follow manufacturer's instructions for volume of buffer, Taq, and DNTPs if required.
15. Performing DNA gel extraction will greatly reduce the amount of DNA in each sample. For this reason, it is recommended that multiple thermocycler reactions are prepared to ensure enough DNA is obtained. Nuclease-free water may be used for elution of DNA from the kit's column to ensure no interference with ligation reactions.
16. *Agrobacterium* transformation and particle bombardment are the two methods that have been successfully used to induce telomere mediated truncation in maize (Gaeta et al., 2013; Yu et al., 2006). Particle bombardment can accomplish telomere-mediated truncation by bombarding in a transgene with attached telomere or separately by cobombardment of the transgene with free telomere arrays (Yu et al., 2006). The standard protocols for both particle bombardment and *Agrobacterium* transformation are unchanged by inclusion of telomere arrays and should be followed for the organism of interest.
17. Visualization of the genome using fluorescence in situ hybridization (FISH) is useful for finding the general location of chromosomal insertions or truncations (Gaeta et al., 2013; Yu et al., 2006). If the inserted transgene is

small, a protocol has been established for finding small targets in the maize genome using FISH (Lamb et al., 2007), and should be applicable to other organisms. Minichromosomes can be distinguished from standard transgene inserts as the transgene will be located on the tip of a chromosome arm, and the chromosome is usually distinctly shorter when compared to its homologue.

Acknowledgment

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References

Gaeta R.T., Masonbrink R.E., Krishnaswamy L., Zhao C., Birchler J.A. (2012) Synthetic chromosome platforms in plants. Annu. Rev. Plant Biol. 63, 307–330

Gaeta R.T., Masonbrink R.E., Zhao C., Sanyal A., Krishnaswamy L., Birchler J. A. 2013. In vivo modification of a maize engineered minichromosome. Chromosoma 122, 221–232.

Green M.R., Sambrook J. (2012) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Ijdo J.W., Wells R.A., Baldini A., Reeders S.T. (1991) Improved telomere detection using a telomere repeat probe (TTAGGG)n generated by PCR. Nucleic Acids Res 19:4780

Lamb J.C., Danilova T., Bauer M.J., Meyer U.M., Holland J.J., Jensen M.D., Birchler J.A. (2007) Single-gene detection and karyotyping using small-target fl uorescence in situ hybridization on maize somatic chromosomes. Genetics 175:1047–1058

Sambrook J., Fritsch E.F., Maniatis T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Southern E.M. (1975) Detection of specifi c sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Yu W., Han F., Gao Z., Vega J.M., Birchler J.A. (2007) Construction and behavior of engineered minichromosomes in maize. PNAS 104(21), 8924-8929

Yu W., Lamb J.C., Han F., Birchler J.A. (2006) Telomere-mediated chromosomal truncation in maize. Proc. Natl. Acad. Sci. USA. 103, 17331–17336.

Appendix 4: Preparation of Chromosomes from *Zea mays*

Note: The information in this chapter was published under the title:

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Summary

High-quality preparations of chromosomes are useful for many purposes. To prepare metaphase chromosome spreads in maize, root tips are harvested and treated with nitrous oxide to stop cell division at metaphase before being fixed in acetic acid. This process allows a high number of condensed chromosome spreads to be obtained at the end of the procedure. To prepare chromosome spreads from various stages of meiosis, anthers are first fixed before being examined for developmental stage. Cells are digested with a mixture of enzymes before the chromosomes are dropped onto glass sides and fixed under UV light.

Introduction

These protocols describe how mitotic metaphase and meiotic nuclei can be maximized for visualization. The resulting samples are ideal for labeling with fluorescence in situ hybridization or can be viewed with a compound microscope. These protocols describe the collection and preparation of maize tissue samples as well as glass slide preparation of those tissues. The metaphase protocol (see Basic Protocol 1) describes how maize root tips are harvested and prepared so that a large number of metaphase nuclei are present for chromosome visualization. The meiosis protocol (see Basic Protocol 2) describes how maize tassels are harvested and anthers prepared on slides for visualization.

Basic Protocol 1: Preparation of Metaphase Chromosomes

Distinguishing individual maize chromosomes and specific regions on those chromosomes can be difficult unless nuclei are visualized at specific times during mitosis. Capturing nuclei in metaphase facilitates chromosome identification. Preparing slides that have the maximum number of nuclei in metaphase also allows for further analyses using methods such as fluorescence in situ hybridization (FISH) easier. This protocol will describe how to prepare root tips of maize plants for visualization of metaphase chromosomes.

Materials:

- Maize seeds

- Nitrous oxide gas (local medical gas supply company)
- 90% glacial acetic acid, store at 4°C
- 70% ethanol 1× citric buffer (see recipe)
- Digestive enzyme solution (see recipe)
- Methanol Ice bucket containing ice
- Paper towels or Kimwipes
- 30°C incubator
- Razor blade or scalpel, tweezers, blunt-ended stainless steel dissecting probe 0.6- or 1.5-ml tubes
- Pressure chamber for nitrous oxide treatment (custom manufactured; https://birchler.biology.missouri.edu/wp-content/uploads/2015/07/Maize-Karyotyping-and-FISH-Manual_2015.pdf, page 54) Filter paper (Whatman no. 1 or thicker)
- 37°C water or dry bath Humid chamber Glass microscope slides (1 × 3–in.) Compound microcope UV cross-linker (120 to 125 mJ/cm2)

Prepare root tips:

1. To acquire root tips for slide preparation, germinate maize seeds on wet paper towels or Kimwipes and then take the roots. Roots can also be harvested directly from young plants or even adult plants before flowering. Primary roots should be preferentially harvested as they have an abundance

of mitotic cells, though roots which appear similar to primary roots from older plants will also work. If germinating seedlings on paper towels, incubate seedlings for 2 to 3 days at 30°C before harvesting.

2. Cut ~1.5 cm off of the tip of the root and place into a 0.6- or 1.5-ml tube. Pierce top of tube with dissecting probe to allow gas to enter. Lightly mist the inside of the tube with water to keep the root tip moist. Be careful to not saturate the roots in water, as this may prevent the roots from being exposed to the gas.
3. Treat tubes containing root tips with N2O in a chamber for 1 to 3 hr (Kato, 1999).

   The longer the root tip is exposed to N2O, the more compact the chromosomes are in the nuclei.

   Kato (1999) compared nitrous oxide treatment to other methods of arresting metaphase. Nitrous oxide is the most effective method, but other treatments can be used.
4. After N2O treatment, immediately remove tubes and place on ice, then treat root tips with 90% acetic acid for 10 min.

   This step achieves fixation of the root tips.

Wash root tips:

5. Remove acetic acid solution and wash root tips with 70% ethanol and then place root tips into new tubes containing fresh 70% ethanol.

Root tips can be stored for months to 1 year or more in 70% ethanol at −20°C.

6. Remove ethanol and replace with 1× citric buffer, then allow roots to soak ~15 min.

   Distilled water can be used in place of 1× citric buffer.

7. Lay root tips onto filter paper and gently wipe in the direction of the root cap to remove the substance on the cap. Wiping away this substance allows for a better digestion of the root tip.

Digest root tips:

8. Cut off 0.7 to 1.0 mm of the distal tip of the root tip, where the actively dividing meristem is located, and transfer to a 0.6-ml tube containing 20 µl of enzyme solution on ice.

   Attempt to cut root tips the same size so as to keep digestion time for each root tip consistent.

   The meristem has an opaque, whitish appearance at the tip of the root.

9. Incubate tubes 45 to 60 min in a 37°C water bath to allow for digestion.

   Digestion time varies depending on size of root tip, age of enzyme, as well as other variables. Average digestion time is ~50 min.

10. Remove tubes from incubation and place on ice.
11. Rinse tubes two times with 70% ethanol, taking care not to destroy or throw away digested root tip. Carefully dripping the ethanol along the inside of the

tube at an angle helps prevent destruction of the digested root tip. If the root tip is destroyed, it is possible to centrifuge the tube of digested material and recover enough for a slide. Removing the ethanol after washes can be achieved by careful pouring or by careful pipetting, being sure to not destroy the fragile root tip.

12. Add 30 µl of 90% acetic acid 10% methanol mixture, or 100% acetic acid, to the tubes replacing the ethanol.

Break up root tip and place onto slides:

13. Use a blunt-ended dissecting probe to break up the root tip by smashing the root tip against side of tube. Wipe probe clean between samples to prevent contamination. Stir or tap tube to resuspend cells. Keep cells on ice.
14. Prepare a humid chamber and place labeled slides into the chamber. Humid chamber can be a simple cardboard box layered with wet paper towels. Place slides on a dry surface elevated above the wet paper towels. Square wooden dowels work well for this purpose. Cover box with damp Kimwipe to create a humid environment for the cell suspension to spread.
15. On each slide, drop 5.5 to 9.0 µl of the cell suspensions.

    The drop of cell suspension should spread in an even circle from where it was dropped. If the drop does not spread, then most likely it is not humid enough. Breathe gently on the drop to help it spread. Not removing enough of the 70% ethanol can also cause spreading problems. A dirty

slide usually causes erratic spreading but could also potentially prevent spreading.

16. After the slides have dried, view slides under a compound microscope to determine their quality based on the nature of the chromosome spreads.

    Sufficient separation of chromosomes is desired, as well as a lack of cytoplasm around the chromosome spreads. This makes for a clearer hybridization with fluorescent probes.

17. Once slides have been checked for chromosome spreads, cross-link them using a UV cross-linker at optimum setting for a total energy of 120 to 125 mJ/cm2.

    The prepared slides can now be labeled with fluorescent probes if desired, stored at 4°C, or stored for several months at −20°C.

Basic Protocol 2: Preparation of Meiotic Chromosomes

Maize chromosomes may be visualized throughout meiosis. For a complete description of the stages of meiosis with pictures, refer to Neuffer et al. (1997). Microspores are obtained from the anthers of developing tassels. To determine whether a tassel is at the desired level of maturity, florets can be obtained and preliminarily staged before removing and fixing a whole tassel as per the Support Protocol. The same method can also be used to obtain florets for chromosome preparation if a subsequent self-cross or male out-crossing of the plant is desired. The most mature anthers are found ~1/3 of the way down from

the tip of the main spike of the tassel. Anthers proximal to this point will be more mature than those distal. The tassel branches mature later than the main spike and follow this same general pattern of development. This is also the pattern observed in the timing of pollen shedding.

Materials:

- Plant with developing tassel
- 3:1 fixative: 3 parts 95% ethanol/1 part 100% acetic acid
- 70% ethanol
- Acetocarmine stain (see recipe)
- Alcohol lamp
- Deionized water or 1× citric buffer (see recipe)
- Digestive enzyme solution (see recipe)
- 100% acetic acid or 90% acetic acid
- 50-ml tubes
- Scalpel and forceps
- Plastic petri dishes
- Glass slides and 22 × 22–mm glass coverslips
- Iron dissecting probe
- Compound light microscope
- 2-ml tubes
- 37°C water bath

- Lint-free tissues
- Blunt-end, stainless-steel dissecting probe
- Humid chamber (plastic chamber with wet paper towels on the bottom and sides with square wooden dowels)
- UV cross-linker (total energy of 120 to 125 mJ/cm2)

Fix tassel:

1. Remove developing tassel from plant and place into an empty 50-ml tube, fill the remaining volume of the tube with 3:1 fixative, seal tube, and fix 24 hr at 4°C.
2. Decant fixative and replace with 70% ethanol; keep on ice for 15 min. Decant ethanol and replace with fresh 70% ethanol.

   The tassel may now be stored up to 1 year or more at −20°C.

Stage anthers:

3. Remove tassel or tassel branch and place in a petri dish. Fill dish with 70% ethanol.
4. Remove a floret from the tassel using forceps being careful not to crush the anthers inside.

   It is helpful to mark the position from which the anther is harvested as others around it will be at a similar developmental stage and this can help

infer relative development of anthers proximal or distal to the most mature point mentioned above.

5. Dissect floret in the lid of a plastic petri dish partially filled with 70% ethanol; be careful not to cut into anthers. There should be two sets of three anthers. All three anthers in each set will be at the same developmental stage and the longer group will be more developed than the smaller group. Separate the two groups then remove one anther from a group of three and place it onto a glass slide and add a drop of acetocarmine.
6. In the drop of acetocarmine, cut the anther widthwise with a sharp scalpel. Use the side of an iron dissecting probe to roll from the tip of each half of the anther towards the cut end. Remove the husk of the anther with forceps.

   The contents of the anther should extrude out of the cut end like toothpaste out of a tube.
7. Apply a glass coverslip to the drop of acetocarmine and anther contents, then heat the slide over an ethanol lamp but not to the boiling point of the acetocarmine.
8. Use a compound light microscope to determine the stage of meiosis of the anther by comparing it to the diagrams in Neuffer et al. (1997).
9. Label a 2-ml tube with the identified stage of meiosis and fill with 70% ethanol. Use this tube to store the two remaining anthers that match the identified one.

   These anthers can be stored for 1 year or more at −20°C.

Digest anthers:

10. Soak anthers in 1.5 ml deionized water or 1× citric buffer for 10 min.

    Residual ethanol can inhibit the enzyme in the next step; therefore, washing a second time with deionized water is recommended.

11. Separate anthers from each other and any somatic floret tissue on a plastic petri dish lid in deionized water or 1× citric buffer, or without water or buffer if performed quickly enough that the anthers do not begin to dry out.
12. Place each individual anther into a tube containing 20 µl enzyme solution and keep on ice.

    Ensure that the anther is at the bottom of the enzyme solution to ensure digestion from all sides.

13. Incubate tubes 30 to 60 min in a 37°C water bath.

    The digestion time depends on a number of factors including genotype, age, manufacturer of enzyme, and stage of meiosis. A digestion time of 50 min is a starting point, but the digestion time may need to be adjusted if microspores are over or under digested. Under digested microspores will have a cell membrane surrounding them.

14. Wash digested anther two times with 70% ethanol.

    The digested anther is fragile. Run the ethanol down the side of the tube to avoid breaking the anther apart and losing the microspores. If the

anther is disrupted, centrifuging the ethanol-filled tube in a microcentrifuge will help recover the microspores.

The ethanol can be carefully poured out without losing the digested anther. If the anther is stuck to the bottom of the tube, pouring is relatively easy. If the anther is moving freely in the tube, attempt to remove most of the ethanol, then twist the tube as it is gently tilted to pour. This will eventually cause the surface of the ethanol to pin the anther against the side of the tube and it should stick and allow the ethanol to be rotated away from it and poured off.

Digested anthers can be stored in the second 70% ethanol wash overnight at −20°C.

Prepare slides:

15. Pour off the 70% ethanol and set the tube upside down on a lint-free tissue to remove as much ethanol as possible. Tap the tube gently onto the lint-free tissue to remove residual ethanol.
16. Add 30 µl of 100% acetic acid or 90% acetic acid/10% methanol to the digested anther and crush the anther against the side of the tube with a blunt-end, stainless-steel dissecting probe with a rolling motion. Break apart the digested anther as well as possible.

    Wipe probe thoroughly with a lint-free tissue between samples to avoid contamination.

17. Flick the tube to further separate and suspend cells, then drop~8 µl of the suspended cell solution onto a glass slide in a humid chamber and allow it to dry.

    The drop of cell suspension should spread in an even circle from where it was dropped. If the drop does not spread, then the chamber is not humid enough and breathing gently on the drop will help it spread. Not removing enough of the 70% ethanol can also cause problems for spreading. A dirty slide usually causes erratic spreading but could also potentially prevent spreading.

18. Check slides with a light microscope to ensure proper digestion and correct meiotic staging.

    Depending on the stage of meiosis, there are different criteria for proper digestion. In every stage before telophase I, the entire cell wall of the pollen grains should be digested and very little to no cytoplasm should be visible. The internal cell walls formed during telophase I and II cannot be digested before the rest of the cell becomes over-digested. In these cases, a good digestion will be one in which the nucleus or chromosomes are relatively free of cytoplasm, yet still paired with their sister cells across a barrier of cytoplasm and cell wall.

19. Irradiate slides in a UV cross-linker at Optimal Crosslink setting.

    Slides can now be viewed for the respective purpose including probed fluorescently as per McCaw et all., (2016)

Support Protocol 2: Determining When to Harvest a Tassel

It is generally preferable to determine the stages of meiosis present in a tassel before harvesting it. If the tassel does not yet have the meiotic stage desired, the incision created to harvest a portion of the tassel can be taped shut and the plant can be checked at a later time. As the plant continues to grow, new incisions may need to be made to access the tassel. The tassel will continue to mature until removed and fixed, and if only a portion of the tassel is removed, the remainder can generally continue to maturity and shed pollen. Portions of leaves and tassel may become tangled in the incision as the plant continues to grow and may need to be manually resolved to free the tassel for crossing.

Additional Materials (also see Basic Protocol 2):

- Plant with developing tassel
- 3:1 fixative: 3 parts 95% ethanol: 1 part 100% acetic acid
- Scalpel and forceps

Locate tassel:

1. Locate the developing tassel in the whorl of the plant (it should feel like a slightly spongy bulge).

2. Using a sharp scalpel, cut a slit vertically along the side of plant taking care not to slice into the tassel. Gently pull the two edges of the cut apart to expose the developing tassel.
3. Use forceps to pluck florets from the tassel. Try to grip the base of the spikelet pairs or the tip of the glume to avoid damaging the anthers.
4. Place florets in 3:1 fixative; keep on ice.
5. Perform Basic Protocol 2, steps 5 to 8, with these not fully fixed anthers (it does not matter that they are not fully fixed when determining the stage of meiosis with acetocarmine).

   If the anthers are found to be at the desired stage, use the two anthers identical to the one checked for chromosome preparation. One can dissect the florets in 70% ethanol, then place the two unused anthers back into 3:1 fixative in a 2-ml tube labeled with their stage of meiosis and fix 24 hr at 4°C. Afterwards, the anthers can be rinsed two times in 70% ethanol and used to prepare meiotic chromosomes by starting at Basic Protocol 2, step 10.

Reagents and Solutions

Use deionized, distilled water in all recipes and protocol steps.

Acetocarmine stain

For 100 ml:

- 45 ml glacial acetic acid
- 55 ml deionized water
- Heat on stir plate inside fume hood until boiling
- Add 0.5 g carmine and stir 20 min with continued heat
- Allow solution to cool, then filter to remove any precipitate
- Store for years at room temperature (if evaporation is prevented)

Citric buffer, 1×

Prepare a 5× working stock:

- 50 mM sodium citrate 50 mM EDTA, adjusted to pH 5.5 by adding citric acid
- Autoclave and store at room temperature
- Dilute to 1× with sterile water
- Use chilled on ice

Digestive enzyme solution

For ~10 ml, mix thoroughly in tube or weigh boat on ice:

- 0.1 g (1% w/w) Pectolyase Y-23 (Karlan Research Products)
- 0.2 g (2% w/w) Cellulase Onozuka R-10 (Karlan Research Products)
- 9.7 g 1× citric buffer (see recipe)

- Quickly dispense into 20-ml aliquots in thin-walled, 0.5-ml PCR tubes (ensure caps are closed tightly)
- Quick freeze on dry ice
- Store ~1 year at −20°C

Commentary

Background Information:

Meiotic and mitotic fixation of chromosomes is a useful tool in determining structure and function of genetic material in a cellular environment. In maize, the fixation process is accomplished through acetic acid treatment of developing tassel or root tissues. Utilization of these techniques has enabled maize researchers to determine many aspects of chromosome biology. If coupled with the more recent fluorescent in situ hybridization technology, higher resolution can be accomplished though the detection of specific nucleic acids with DNA or RNA probes.

Critical Parameters and Troubleshooting:

To obtain high-quality spreads, a few parameters need to be taken into consideration before beginning preparation of mitotic tissues. When choosing which roots to harvest, primary roots are generally the best. Longer treatment of roots in N_2O gas results in more condensed chromosomes, while less time results in less-condensed chromosomes. Consistency in size of root tip slice is

essential to keeping digestion times the same across samples. Placing a ruler by the root for measuring how much to slice results in more consistent root-tip lengths. Digestion time for root tips will vary depending on the length of the root tip as well as its diameter.

Anticipated Results

Metaphase chromosome preparation:

Resulting slides should have visible nuclei with visible chromosomes. At 600× magnification using a compound microscope, approximately three spreads can be found per field of view in a good preparation. The chromosome spreads should be relatively free of cytoplasm and other cellular matter, as well as having condensed chromatin so that individual chromosomes are easily identified (Figure A4.1).

Meiotic chromosome preparation:

Depending on the stage of meiosis, the qualities of finished slides will vary. Finished slides of any stage of meiosis before Telophase I can be relatively cytoplasm free and cell walls of meiotic pollen mother cells should not be present. Cell preparations after telophase I will have an internal cell wall between the sister cells as well as some associated cytoplasm that the digestive enzyme cannot remove without over-digesting the cells. The chromosomes or chromatin

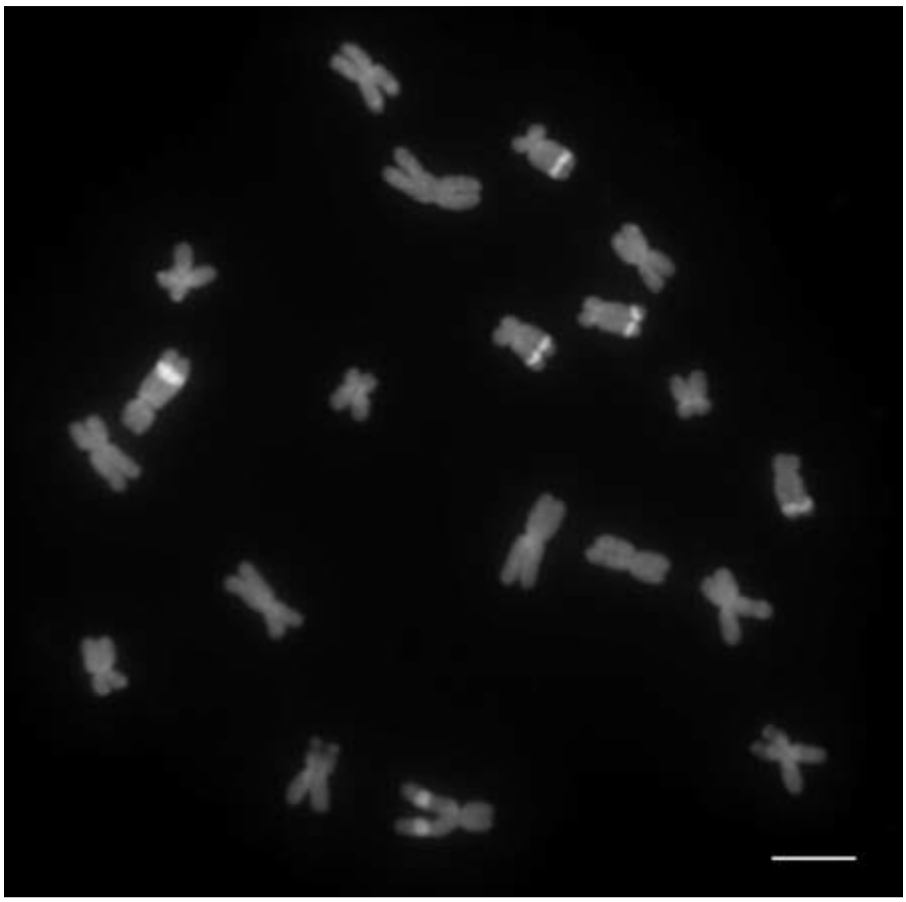

Figure A4.1 - Metaphase Chromosome Spread: A metaphase chromosome spread stained with DAPI (4',6-diamidino-2-phenylindole) shown in greyscale. The scale bar in the lower right is 5 μm. Photo credit: Patrice S. Albert.

of the sister cells should be mostly free of cytoplasm but will still have a visible barrier of cell wall and cytoplasm between them.

Time Considerations

Preparation of metaphase chromosomes:

Basic Protocol 1, steps 2 to 5 will require 4 hr. Roots can be stored in 70% ethanol up to 1 year at 20°C. Basic Protocol 1, steps 6 through 17 takes 3 hr. In general, 10 to 20 roots are processed at one time, and can generally be processed in 3 hr. Slides can be stored for several months at 4°C, although if being used for FISH painting, better signal is found with fresh slides.

Maize male meiosis chromosome preparation:

Basic Protocol 2, steps 1 and 2 requires 1 day. The tassel can be stored up to 1 year at −20°C. Steps 3 to 9 take 15 min to 3 hr. The time required to find anthers at the appropriate stage is dependent on the skill level of the researcher. Steps 10 to 14 require 1.5 hr. Anthers can be stored overnight in 70% ethanol at −20°C. Steps 15 to 19 take 1 hr. Slides can be stored for months at 4°C; however, better FISH signal is found with fresh slides.

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References

Kato A., (1999) Air drying method using nitrous oxide for chromosome counting in maize. Biotech. Histochem. 74:160-166.

McCaw M., Graham N., Cody J., Swyers N., Zhao C., and Birchler J. (2016) Fluorescence in situ hybridization to maize (Zea mays) chromosomes. Curr. Protoc. Plant Biol. 1:529-544.

Neuffer G.M., Coe E.H., and Wessler S.R. (1997) Mutants of Maize. Cold Spring Harbor Laboratories Press, Plainview, N.Y.

Appendix 5: Fluorescent In Situ Hybridization of Maize (*Zea mays*) Chromosomes

Note: The information in this chapter was published under the title:

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Summary

Fluorescence In Situ Hybridization (FISH) is the annealing of fluorescent DNA probes to their complementary sequences on prepared chromosomes and subsequent visualization with a fluorescent microscope. In maize, FISH is useful for distinguishing each of the ten chromosomes in different accessions (karyotyping), roughly mapping single genes, transposable elements, transgene insertions, and identifying various chromosomal alterations. FISH can also be used to distinguish chromosomes between different Zea species in interspecific hybrids by use of retroelement painting.

Introduction

This protocol is an accompaniment to Preparation of Chromosomes from Zea mays (McCaw et al., 2016), which describes how maize chromosomes are isolated from cells and cross-linked to a glass slide. This protocol involves the preparation of DNA from a plasmid, the incorporation of fluorescent nucleotides into that DNA, and then the hybridization of that DNA to the prepared chromosomes.

Target Selection and DNA Preparation

FISH probes can be prepared for a wide variety of sequences, allowing for the technique to be used for many applications. However, it is important that the target is chosen extremely carefully before the procedure is begun. Many of the commonly used large target probes, with a total size >4 kb, consist of high copy number repeat sequences such as knob heterochromatin or centromeric CentC. For these sequences, fluorescent oligo probes can often be purchased that require little processing and are often more cost effective than the following methods. For small targets and single genes (targets as small as 1.4 kb have been identified using the Texas red flourochrome in our laboratory), it is suggested that the sequence be analyzed before being used for a probe. These probes for small targets must be processed differently than larger probes and the procedure is more time consuming. In addition, the signal is often weaker and harder to identify when compared to the background. As such, it is important to

choose a sequence target that will give the largest chance of success. As a result, it is suggested that a sequence target be chosen that is larger than the minimum target size possible. In our experience, choosing a target greater than 3.5 kb for red probes and 6 kb for green probes produces the best images. When designing a small target probe, it must be ensured that the probe sequence is unique to the target location. Tools such as RepeatMasker (repeatmasker.org) should be utilized to ensure that any undesired repeats are removed from the probe sequence so the probe will not bind to any other homologous sites in the genome. If necessary, noncontiguous sequences can be used and pooled into a single probe and still appear as a single signal provided they are within 1 to 3 Mb of each other.

The following protocol assumes that the DNA sequences have already been chosen and cloned into a plasmid. It is possible to use noncloned sequences in much the same way; however, it is suggested that these products are first gel purified if amplified from genomic DNA. As FISH probes can sometimes require over 100 ng of DNA be added to each slide, it is suggested that at least 10 μg of DNA be amplified for processing into a probe for hybridization.

Materials:

- Forward and reverse primers

- Target plasmids: many plasmids contain common primer binding sites, which can be used to amplify inserted DNA; if a unique primer is desired, it is suggested that a program such as Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) be used to ensure proper primer design
- JumpStart REDTaq ReadyMix Reaction Mix (Sigma-Aldrich, cat. no. P0982)
- Deionized, distilled (dd) H_2O
- 3 M sodium acetate (pH 5.2)
- 100% ethanol
- 70% ethanol
- 1 × TE or nuclease-free water
- 1 × TAE electrophoresis buffer
- Agarose (Sigma-Aldrich, cat. no. A9539)
- DNA Gel Loading Dye (6×) (Life Technologies, cat. no. R0611)
- GeneRuler 1-kb DNA Ladder (Life Technologies, cat. no. SM0311)
- Ethidium bromide (Sigma-Aldrich, cat. no. E7637)
- Target DNA cloned into a plasmid
- 0.6-µl PCR tubes
- Pipets
- Microcentrifuge
- Thermal cycler
- 1.7-ml microcentrifuge tubes

- Vortex mixer
- Nanodrop spectrophotometer
- Gel electrophoresis equipment
- UV transilluminator

DNA amplification:

1. Assemble four of the following reactions (see Table A5.1) in separate 0.6-µl PCR tubes (may be assembled at room temperature).

   While one large reaction can be used, we have found a larger yield can be obtained from four smaller reactions.
2. Mix gently by pipetting and spin down the reaction in a microcentrifuge.
3. Use the following PCR conditions to amplify DNA. See Table A5.2 for PCR reaction conditions.

DNA precipitation:

4. Combine PCR reactions into a single 1.7-ml tube.
5. Add 40 µl of 3 M sodium acetate (pH 5.2) and 1100 µl of 100% ethanol.
6. Vortex thoroughly for 2 sec.
7. Incubate for 30 min to overnight at −20°C.
8. Centrifuge for 30 min at 16,000 × g, 25°C.
9. Carefully discard the supernatant.
10. Add 1 ml of 70% ethanol.

Jumpstart RedTaq ReadyMix	50 µl
Primer 1 (100 µM)	0.5 µl
Primer 2 (100 µM)	0.5 µl
Target Plasmid (50 ng)	1 µl
ddH_2O	48 µl

Table A5.1 – PCR Reaction Mixture.

1.	94°C (initial denaturation)	5 min
2.	94°C (denaturation)	30 Sec
3.	58°C (dependent on primer melting temperature; annealing)	30 Sec
4.	72°C (extension)	1 Min/kb of product
Repeat steps 2-4 × 35		
5.	72°C (final extension)	5 Min
6.	4°C (hold)	Indefinite

Table A5.2 - PCR Reaction Conditions.

11. Centrifuge for 3 min at 16,000 × g, 25°C.
12. Carefully discard supernatant and repeat steps 10 and 11.
13. Completely remove the supernatant with a pipet.
14. Allow the pellet to dry 2 to 3 min.

    It is not necessary to completely dry the pellet, and overly dry pellets are difficult to redissolve.
15. Resuspend the pellet in 50 to 200 µl 1 × TE or nuclease-free water.
16. Once completely resuspended, measure concentration with a Nanodrop spectrophotometer.

Check DNA integrity:

17. Pour a 1% (w/v) agarose gel in 1× TAE.
18. Load 5 µl of PCR product mixed with 1 µl of 6× loading dye next to a lane containing 6 µl of premixed GeneRuler 1-kb DNA Ladder.
19. Run the gel at 100 V for 30 min, or until marker dye has traversed most of the gel.
20. Shake the gel for 15 to 30 min in 0.5 µg/ml ethidium bromide.
21. Visualize with a UV transilluminator.
22. Check integrity of DNA.

    PCR product should be of the correct size and similar in concentration to that estimated by a Nanodrop or similar spectrophotometer. The PCR product should be visible as a single band, and free of any background in

the lane. If PCR product is not as expected, consider adjusting primer sequences or annealing temperature.

Labeling Probe DNA by Nick Translation

Fluorescent probes for the FISH procedure are generated through a nick translation reaction of purified PCR products or plasmid DNA. In this reaction, the enzymatic activity of two specialized proteins, DNase and DNA polymerase I, are utilized to incorporate fluorochrome-labeled dNTPs into template DNA. DNase enzymes randomly cleave the phosphodiester bonds in the DNA backbone, creating several free 5'-phosphoryl and 3'-hydroxyl ends. In these positions, DNA polymerase I performs both 5'-3' exonuclease and polymerase activity to remove nucleotides from the DNA backbone and replace them with free dNTPs from solution, respectively. Only a percentage of the incorporated dNTPs are labeled with specific fluorochromes.

Components of the nick translation reaction can be modulated to fit the requirements for specific targets. These requirements are determined by target size, which is defined by probe length and copy number on the chromosome. The length of the probe should be approximately 50 to 300 bp after the nick translation reaction. To ensure this parameter is met, the DNase concentration can be decreased or increased for small and large templates, respectively. If copy number on the chromosome is low (these targets are usually transgenes or single-copy endogenous genes), a brighter probe will be desired for detection. To

do this, DNA polymerase concentration can be increased to incorporate greater amounts of the fluorescent probe into the template DNA.

The specific fluorochromes used in the nick translation reaction are also variable. Commonly used markers include Texas Red (red), fluorescein or Alexa Fluor 488 (green), coumarin (blue), and cyanine 5 (far red). For small targets, the use of Texas Red (red) is recommended for its increased brightness. Texas Red can also be used with medium or large targets. Alexa Fluor 488 (green) can be used for all targets, while coumarin (blue) and cyanine 5 (far red) should only be used for large targets. It should be noted that all fluorochromes are light sensitive and should be stored in a dark environment to maintain quality.

Materials:

- Ice
- Template DNA (PCR product or plasmid)
- 10× nick translation buffer (see recipe)
- Labeled dNTP mixture (1 nM)
- Nonlabeled dNTPs (2 mM each/ mixed)
- DNA polymerase I
- DNase I (100 mU/µl)
- 1× TE-saturated Bio-Gel P-60 (see recipe)
- 1× TE (see recipe)
- Autoclaved salmon sperm (10 mg/ml; see recipe)

- 3 M sodium acetate (pH = 5.2; see recipe)
- 100% ethanol
- 70% ethanol
- 2× SSC
- Thermal cycler or a lidded Styrofoam container filled with cold water
- Silane-treated glass wool
- Forceps
- Pasteur pipets
- Inoculating loops
- 1.6-ml collection tubes
- Vortex mixer
- Microcentrifuge (>16,000 × g max)
- Lint-free tissues
- Heat block or water bath set to 65°C

Nick translation:

For small targets (less than 30 kb):

1a. Assemble the following reagents in a thermal cycler tube on ice:

10 µl template DNA (2 µg; 200 ng/nl)
2 µl of 10× nick translation buffer
0.5 µl labeled dNTPS (1 mM)
2 µl nonlabeled dNTP (2 mM each, mixed)

Total volume: 14.5 µl.

2a. Upon addition of each of the following enzymes to the reaction, mix the solution thoroughly by pipetting.

8 µl DNA polymerase I (10 U/µl)
0.4 µl DNase (100 mU/µl)
Total volume: 22.9 µl

3a. Incubate the reaction mixture for 2 hr at 15°C in a thermal cycler or water bath in a Styrofoam container. Overnight reactions can be carried out in a thermal cycler, but hold the temperature at 1° to 4°C after the 2 hr time mark.

It is optional to use 2 µl stop buffer (EDTA pH = 8.0) to terminate the reaction.

Optional stopping point: Store overnight at −20°C in the dark.

If probe contains Texas Red (red) or cyanine 5 (far red)-labeled sequences, move to column purification. Our laboratory has found that this will produce a cleaner probe mixture, which reduces possible nonspecific binding. Probes containing fluorescein or Alexa Fluor 488 (green) or coumarin (blue) may proceed to ethanol precipitation of small target steps. Purification of these probes will negligibly affect binding efficiency.

Procedure for large targets (larger than 100 kb):

1b. Assemble the following reagents on ice:

25 µl DNA (5 µg; 200 ng/µl)
5 µl of 10× nick translation buffer
1 µl labeled dNTPs (1 mM)
5 µl nonlabeled dNTP
7 µl sterile ddH2O
Total volume: 43 µl.

2b. Upon addition of each of the following enzymes to the reaction, mix the solution thoroughly by pipetting.

6.25 µl DNA polymerase I (10 U/µl)
1 µl DNase (100 mU/µl)
Total volume: 50.25 µl.

3b. Incubate the reaction mixture for 2 hr at 15°C in a thermal cycler or water bath in a Styrofoam container. Overnight reactions can be carried out in a thermal cycler, but hold the temperature at 1° to 4°C after the 2 hr time mark.

It is optional to use 2 µl of stop buffer (EDTA pH = 8.0) to stop the reaction.

Optional stopping point: Store overnight at −20°C in the dark.

If probe contains Texas Red (red) or cyanine-5 (far red) labeled sequences, move to column purification. All other probe sequences may proceed to ethanol precipitation of large target steps.

Probe purification:

If the generated probe contains Texas Red (red) or cyanine-5 (far red) labeled sequences, the probe must be column purified. Probes containing fluorescein or Alexa Fluor 488 (green) or coumarin (blue) may proceed to ethanol precipitation steps.

Column assembly:

4. Using forceps, insert a piece of silane-treated glass wool into a glass Pasteur pipet.
5. With an inoculating loop, push the glass wool towards the tip of the Pasteur pipet.
6. Holding the pipet at an angle, fill with 1× TE-saturated Bio-Gel P-60 and place into a 1.6-ml collection tube. As the beads settle, TE will flow from the column. Replace the displaced TE with more TE-saturated Bio-Gel P-60 until beads are level with the constriction in the pipet.
7. Ensure the quality of the column by washing with 500 µl TE. Allow all TE to run through the column into the collection tube before using in probe purification.

Column purification:

All fluorochromes used to generate probes are light sensitive, so it is important to reduce light exposure when following the column purification steps below. It is suggested to carry out the experiment in a dimly lit room or place the column under a cardboard box after adding each TE volume.

8. Add probe mixture to the prepared column.
9. Successively add the following amounts of TE to the column. Allow the designated volume to elute before moving to the next amount.

Amounts	Directions
50 µl of 1× TE	Discard eluate.
350 µl of 1× TE	Discard eluate. Move column to new 1.6 ml tube.
350 µl of 1× TE	Save eluate. Move column to new 1.6 ml tube.
350 µl of 1× TE	Save eluate. Move column to new 1.6 ml tube. Large
Probes: 350 µl of 1× TE	Save eluate.

For small probes, two tubes should be recovered from column purification. For large probes, three tubes should be recovered.

All purified probes may proceed to ethanol precipitation steps.

Ethanol precipitation:

For small targets (less than 30 kb):

10a. Add the following components to each probe sample:

3 µl salmon sperm (10 mg/ml)

0.1 vol (35 µl) of 3 M sodium acetate (pH 5.2)

2.5 vol (875 µl) of 100% ethanol

11a. Vortex for 2 sec to mix components.

12a. Incubate for +2 hr at −20°C.

Samples may be stored overnight at −20°C.

13a. Centrifuge in a microcentrifuge for 30 min at 16,000 × g, 25°C.

14a. Carefully pour off supernatant so as not to disturb pellet.

15a. Rinse the pellet with 500 µl of 70% ethanol. Repeat rinse.

16a. Carefully pour off ethanol without disturbing the pellet, then invert the tube and blot the rim on a lint-free tissue to remove as much ethanol as possible.

Brief centrifugation for 1 min at 16,000 × g, 25°C, before decanting may help secure the pellet.

17a. Add 10 µl of 2× SSC/TE mixture to tubes.

18a. Incubate on heat block or in water bath set at 65°C to dissolve pellet.

Vortexing may also help with this process.

19a. Store clean probes up to 5 years at −20°C. For large targets (less than 30 kb)

Procedure for large targets (larger than 100 kb):

10b. Add the following components to each probe sample:

5 µl salmon sperm (10 mg/ml)
0.1 vol of 3 M sodium acetate (pH 5.2)
2.5 vol of 100% ethanol.

11b. Vortex for 2 sec to mix the components.

12b. Incubate for +2 hr at −20°C.

Samples may be stored overnight at −20°C.

13b. Centrifuge in a microcentrifuge for 30 min at 16,000 × g, 25°C.

14b. Carefully pour off supernatant to not disturb pellet.

15b. Rinse pellet with 500 µl 70% ethanol. Repeat rinse.

16b. Carefully pour off ethanol without disturbing the pellet, then invert the tube and blot the rim on a lint-free tissue to remove as much ethanol as possible.

Brief centrifugation for 1 min at 16,000 × g, 25°C, before decanting may help secure the pellet.

17b. Add 10 µl of 2× SSC/TE mixture to tubes.

18b. Incubate on heat block or in water bath set to 65°C to dissolve pellet.

Vortexing may also help with this process.

19b. Store the clean probes up to 5 years at −20°C.

Fluorescence In Situ Hybridization

Probes produced in the previous sections will nowbe combined and added to the prepared slides from the previous protocol. Once the probe is added to the slide both the probe DNA and chromosomes are subjected to temperatures just below boiling to form single stranded DNA. The slides are then incubated at 55°C to allow the DNA to reanneal, thus hybridizing the fluorescent probes to the chromosomes. Slides are then finished by removing the temporary coverslip, adding a mounting medium, and affixing an optically clear glass coverslip.

The Karyotyping Cocktail:

To distinguish chromosomes from each other, a “Karyotyping cocktail” has been developed. This cocktail uses blue, green, red, and far red (pseudo-colored as white during image processing) probes to label large chromosomal targets. In addition to the basic colors, teal, orange, and yellow are also produced by labeling a target with two different colors in the correct proportions. Table A5.3 provides the common sequences used for karyotyping and their relevant vector information. Table A5.4 lists the primer sequences. Table A5.5 provides the standardized colors of each target and the concentrations in which they are commonly used. Any sequences labeled in both green and red should appear

Target (clone name)	Vector	Bacteria	Alternate primers	PCR product size	Genbank accession no.
Cent4	pBluescript	DH5α		800 bp	AF242891
CentC	pBluescript	DH5α		540 bp	AF078918 - AF078923
TR-1 (M77)	pGem-T	DH5α	MR77 F/R	400 bp	AF020266
5S Ribosomal RNA (2-3-3)	pGem-T	DH5α	BEH 2 F	700 bp	CL569181
NOR 173	pBluescript	DH10B		2 kb	CL569243
Knob 3-Copy	pGem-T	Stbl4	Knob F/R	540 bp	M32521 - M32532
TAG (1-26-2)	pGem-T	Stbl4	EBH 1 F	500 bp smear	
4-12-1	pGem-T	DH5α	BEH 2 F	1 kb	CL569186
1.1 (pMTY9ER)	pBluescript	DH5α	M13 F/R	1.1 kb	U39642

Table A5.3 - Plasmids for Amplifying Maize Probes: M13 primers can be used to amplify targets in all plasmids; however, the listed alternate primers have been used and found to give stronger signal. In the case of EBH and BEH primers, only forward primers are required as the reverse primer is the same sequence. Plasmids are available from the corresponding author.

M13 Forward	CCC AGT CAC GAC GTT GTA AAA CG
M13 Reverse	AGT GGA TAA CAA TTT CAC ACA GG
EBH1 F	AGA ATT CGG ATC CAA GCT TCT GGT TTG
BEH2 F	AGG ATC CGA ATT CAA CGT TGT CTT TG
Knob F	GGC CAC ACA ACC CCC ATT TTT G
Knob R	GGC CAT TGA TCA TCG ACC AGA
MR77 F	CCT CAA ATG CCG TTT CCT AT
MR77 R	CAC TCA CGC AAT TTG GCT AA

[a]All primers are listed 5′ to 3′.

Table A5.4 – Primer Sequences for Amplifying Maize Probes.

Color	Target sequence (clone name)	Stock concentration	Final concentration	Amount per slide
Blue	Knob	200 ng/µl	40 ng/µl	1.0 µl
Green	NOR 173	10 ng/µl	0.2 ng/µl	0.1 µl
Green	CentC	200 ng/µl	2 ng/µl	0.1 µl
Green	5S Ribosomal RNA (2-3-3)	200 ng/µl	18 ng/µl	0.45 µl
Green	4-12-1	200 ng/µl	40 ng/µl	1.0 µl
Red	TAG (1-26-2)	200 ng/µl	6 ng/µl	0.15 µl
Red	Cent4	200 ng/µl	10 ng/µl	0.25 µl
Red	5S Ribosomal RNA (2-3-3)	200 ng/µl	18 ng/µl	0.45 µl
Red	1.1 (pMTY9ER)	200 ng/µl	40 ng/µl	1.0 µl
Far Red	TR-1 (M77)	200 ng/µl	20 ng/µl	0.5 µl
Optional:				
Blue	NOR 173	200 ng/µl	200 ng/µl	0.74 µl

Table A5.5 - A List of the Primers Used and What Concentration to Produce a Karyotype Similar to Those Described by Kato et al., 2004: Karyotypes of common inbred lines are available through the link in the Internet Resources section. Not using the optional blue NOR 173 will produce a karyotype that has a green NOR instead of teal like the karyotypes shown, but will not affect identification of chromosome 6 because the NOR is not likely to be confused with any other signal.

yellow or orange once the color channels are merged. Similarly, any sequences labeled with both blue and green will appear as some variation of teal once merged. The concentrations provided in Table A5.5 should be considered a starting point, and the relative concentrations of each probe in the cocktail may need to be adjusted in order to produce the desired colors. Differences in exposure time and intensity when photographing each color channel may also result in colors which do not match the expected colors. Changing the intensity of channels during image processing can also correct the merged colors. More detailed information on image processing is outlined in the supplementary YouTube video.

Maize inbred lines are highly variable. Most of the probes in this cocktail target repetitive sequences, which will vary in size and location among different inbred lines (Albert et al., 2010).

Along with chromosome size and shape, several sequences frequently appear in the same locations and can aid in chromosome identification in a line for which a karyotype is not readily available. The signal color and location, and relative chromosome shape are shown in Figure A5.1. These sequences will not always be present, and their copy number and thus signal strength may vary. A Knob near the distal tip of 9 S is present in all analyzed maize lines but may not be detectable in roughly 50% of karyotyped lines due to low copy number and the presence of much larger Knobs on other chromosomes with stronger signal.

The presence of a Knob at the distal tip of 10 S has not been reported (there is a pericentromeric knob on 10 S in the line OH7B.)

Using these amounts of each probe should result in a final volume of 5 µl of probe per slide. 3-5 µl additional 2× SSC 1× TE can be added to the probe to raise the final volume to 8-10 µl per slide, which helps prevent bubbles of air between the coverslip and slide. These probe concentrations serve as a starting point.

Basic Green Probe Cocktail with Single Gene Target Red:

When attempting to localize a single gene such as a transgene insertion, a cocktail of a few distinctive chromosomal markers labeled in green, along with chromosome morphology is generally enough to identify most chromosomes in most lines. Figure A5.2 provides a representation of the basic size and shape of the chromosomes of the transformable lines HiII A and B. Other lines can be compared to the karyotypes provided in the link in the Internet Resources section. The copy number of CentC, and thus intensity of signal, is an important characteristic for differentiating between similarly sized chromosomes like chromosomes 7 and 8 of HiII.

The basic green cocktail is CentC, TAG, and NOR 173 diluted in 2× SSC 1× TE for a volume of 8-10 µl per slide. It is easier to use 6-FAM labeled oligo for CentC (CCT AAA GTA GTG GAT TGG GCA TGT TCG) and TAG (simply called TAG because it is an oligo; 1-26-2 is the name of the original plasmid clone)

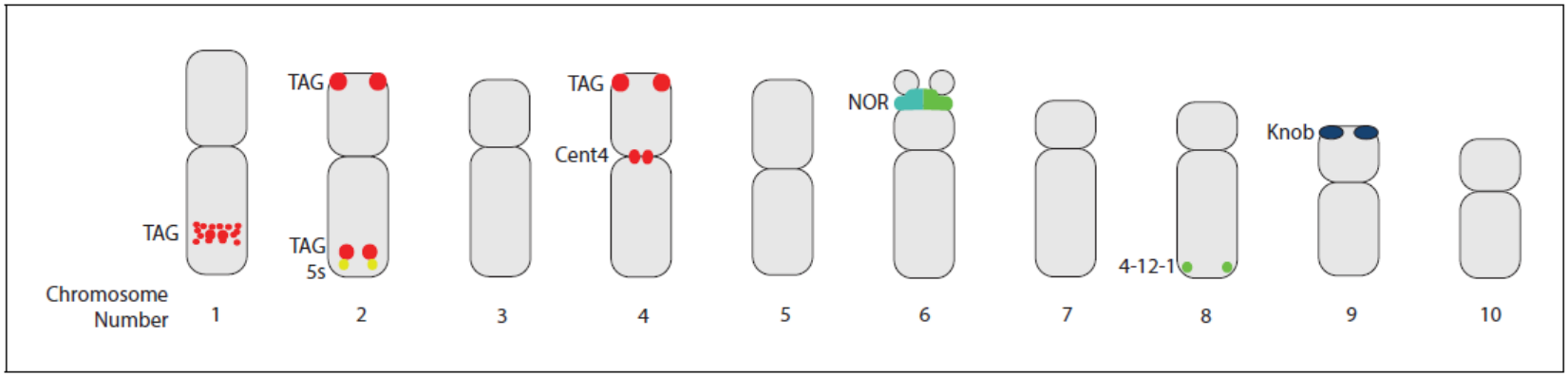

Figure A5.1 - Common Signals Used to Identify Chromosomes: A representation of the relative shape of chromosomes and the positions of repetitive elements that tend to be conserved across many lines. The size and shape of the chromosome arms can vary. Importantly, 2 to 4 can be very similar in size and shape, as can 7 and 8, and 9 and 10. The characteristic elements shown are not the only relatively conserved locations of signals; they are simply those most useful for distinguishing between chromosomes that may be of similar size and shape. Some elements may not be present in some lines, and the copy number of repeats may vary. NOR = NOR 173 and is labeled as both teal and green because the traditional maize karyotyping protocol used NOR 173 labeled in both green and blue to produce teal, though this is not necessary, and it is cheaper and easier to only label NOR as green. 5 S = 5 S Ribosomal RNA

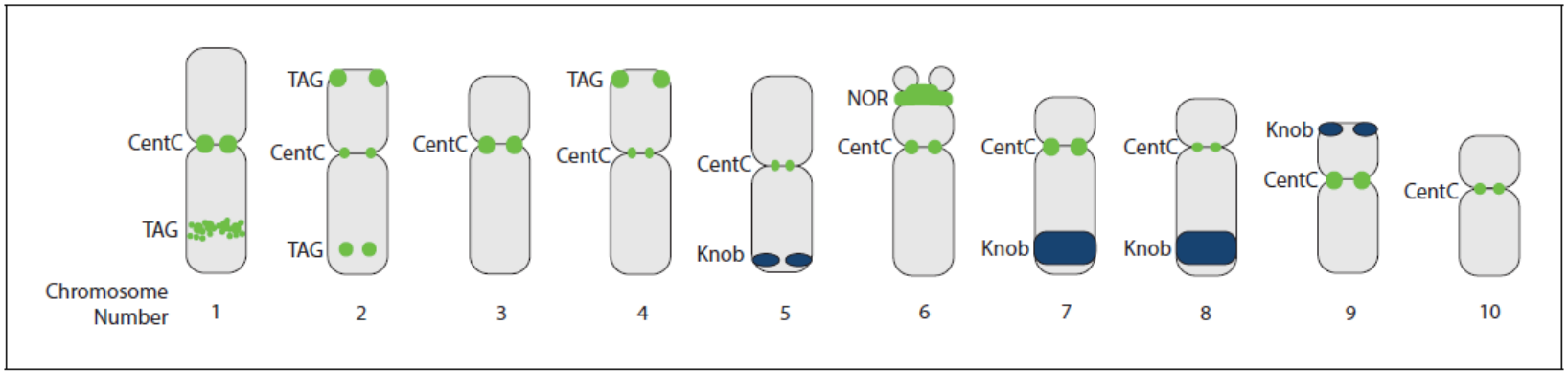

Figure A5.2 - Basic Green Karyotype of Hi-II: Representation of the signal locations and relative intensities of signals produced by using a probe cocktail consisting of CentC, TAG (1-26-2), and NOR. This cocktail is combined with DAPI, which stains chromatin blue and stains the heterochromatic Knob repeats a darker blue. Comparing chromosome shape, signal locations and intensities, and Knob locations is sufficient to determine the identity of each individual chromosome of the transformable lines HiII A and B, which do not differ appreciably from one another in these aspects. Relative signal intensity of CentC at each centromere is dependent on copy number variation, but exposure time of the green channel can alter the overall signal intensity. It is important to use an exposure time that allows for differentiation between chromosomes 7 and 8 based on the intensity of the CentC signal at the centromere. The absence of a red probe allows a visualization of a small gene or transgene, and can be useful to locate and determine homozygosity of a transgen

Color	Target Sequence	Source	Working/stock Concentration	Amount per Slide
Green	CentC	Oligo	20 ng/µl	1.5 µl
Green	TAG	Oligo	20 ng/µl	0.8 µl
Green	NOR 173	Nick translated PCR product	10 ng/µl	0.3 µl
Red	Small target of interest	Nick translated PCR product	200 ng/µl	1 µl

Table A5.6 - Basic Green Cocktail Targets and Concentrations.

(AG[TAG]18) from Integrated DNA Technologies. The concentrations used for this cocktail are listed in Table A5.6. The single gene probe can then be labeled with red.

With oligo probes, 2× SSC 1× TE is used to dilute probes from stock concentrations of 500-200 ng/µl to less concentrated working stocks. Less concentrated working stocks are prepared to provide more accurate measurement of each probe and reduce the number of freeze-thaw cycles that the probe must endure.

1.0 µl of 200 ng/µl single gene probe in red should be sufficient to visualize even 2.0 to 2.5 kb targets. Once hybridization of the single gene probe is confirmed, it may be possible to reduce the concentration to optimize the amount of probe used while still maintaining visible hybridization.

Because no probes labeled with blue are used in this cocktail, Vectashield with DAPI is added to these slides, see the Troubleshooting section in the Commentary for information about dilution.

Materials:

- Probe Prepared cells (see McCaw et al., 2016)
- 2× SSC: diluted from 20× SSC
- Vectashield (with or without DAPI)
- 22-mm × 22-mm plastic coverslips
- Square wooden dowel rods

- Metal tray
- Lint-free tissues
- Boiling water bath
- Airtight box Paper towels
- 55°C incubator
- Coplin jar
- 24-mm × 50-mm glass coverslips

1. Add 8 to 10 µl of probe to the center of the prepared cells (see McCaw et al., 2016) and cover with a 22-mm × 22-mm plastic coverslip. If there are any air bubbles under the coverslip, try to press them out to the edge, but do not slide the coverslip.

   It may be helpful to place the slides on two square wooden dowels aligned parallel to each other about 3.0-cm apart with the cell spread centered between the dowels. This placement will facilitate centering the probe and coverslip and the height will allow the tips of your fingers to go past the slide.

2. Place the slides face up in a metal tray with three to six layers of wet (with water) lint-free tissue between the slides and the tray and then place the tray in a boiling water bath for 5 min.

If the water bath has a lid or foil over it, make certain to use a cover over the slides to prevent condensed water from dripping onto them; a 1+ cm deep pipet box lid works well for this purpose.

Tissues should be wet enough to transfer heat, but not wet enough to pool water over slides. Soaking the tissues in the tray thoroughly and holding the tray vertically until water stops pouring and only infrequently drips out should give approximately the proper moisture level.

3. After 5 min, move the slides to an airtight box with moistened paper towels or tissues for humidity. The slides should be elevated from the paper towels in a metal slide rack or on a pipet tip box lid. Firmly seal the box and place overnight at 55°C.

   It has been found that this overnight hybridization is not necessary for abundant repeats probed with only end labeled oligos such as telomere or CentC. If the slide is being prepared with only oligo probes, step 3 can be skipped and one can proceed directly to step 4.

4. Submerge slides vertically into a Coplin jar filled with room temperature 2× SSC to remove coverslips.

The coverslips should fall off the slides almost immediately when placed in the 2× SSC. They will frequently fail to fall off if the slides cool too much before submersion.

5. Move slides to a second Coplin jar filled with 2× SSC that is at 55°C and place in 55°C incubator for 35 min to remove unbound probe.
6. Remove the slide from 2× SSC and shake dry by hand. Wipe the back side of the slide dry with a lint-free tissue and place on parallel square dowels from step 1.

   The position of the coverslip should be visible as a square where the 2× SSC adheres more readily. Center this square between the dowels to accurately add Vectashield in next step.

7. Add a drop of Vectashield (with or without DAPI) to the center of the probed cells and place a 22-mm × 50-mm glass coverslip on the slide.

   To reduce bubbles of air under the coverslip lower the coverslip down until it just touches the Vectashield then let it go gently. In addition, make certain the slide is almost completely dry before adding Vectashield and the coverslip or the extra 2× SSC will seal in air bubbles.

   As the Vectashield spreads, the coverslip may shift off center. Keep the coverslip aligned as it adheres to the slide because shifting the coverslip later will smear the cells and ruin parts of the slide.

If Vectashield without DAPI is applied to the slides, searching for chromosome spreads under fluorescence is slightly more difficult. It is recommended to search for spreads on the green channel and look for clearly defined, paired dots of signal. Once zoomed in to 1000×, chromosomes can be visualized by their slight background illumination. When producing karyotypes without DAPI, it is useful to keep some level of background during image processing to allow visualization of chromosome shape.

The slide is now ready to image on a fluorescence microscope or can be stored up to 3 months in the dark at 4°C without appreciable loss of quality. Storage for periods of two years or more are possible but may result in reduced signal strength.

After storage at 4°C it is recommended that slides be allowed to reach room temperature and ensure the slides are free from condensation before applying oil for visualization with a fluorescence microscope to avoid creating an emulsion of oil and water, which can blur images and requires cleaning the lenses of the microscope.

Reagents and Solutions

For culture recipes and steps, use sterile tissue–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps.

Loading dye, 6×:

For 10 ml of solution, add 25 mg of 0.25% bromphenol blue, 25 mg of 0.25% xylene cyanol FF, and 4 g of 40% sucrose to TE buffer. Fill to 10 ml with TE buffer. Store up to 5 years at 4°C.

Nick translation buffer, 10× stock:

Dissolve 6.05 g Tris base (500 mM) and 476.0 mg MgCl2.(50 mM) in water. Adjust pH to 7.8 with hydrochloric acid (HCl). Add 701 µl of 2-mercaptoethanol (100 mM) to water and bring volume to 100 ml. Autoclave to sterilize. Solution should be stored in ~ 993-µl aliquots. Store aliquots up to 5 years at −20°C. Upon thawing, add 7 µl of 2-mercaptoethanol. Replace stocks when odor is gone.

Addition of bovine serum albumin fraction V, Sigma-Aldrich A-9647 (+ 100 µg/ml) is optional.

Salmon sperm DNA:

Product used is Sigma-Aldrich D1626. Dissolve DNA in 1× TE buffer (pH = 7.8) to a concentration of 10 µg/µl, shake or stir if necessary. Once completely dissolved, autoclave 30 min. To determine the size of the DNA fragments, run the sample on an agarose gel. The fragments should be 100 to 300 bp. Store as 1-ml aliquots up to 10+ years at −20°C; a working stock can be left at room temperature for years.

Sodium acetate, 3 M:

For 200 ml of solution, add 49.2 g (anhydrous) of 3.0Msodium acetate to a container and fill to 200 ml with water. Add glacial acetic acid to solution until pH reaches 5.2. Autoclave to sterilize. Store up to 10 years at room temperature.

SSC, 20×:

For 1 liter of 20× SSC, add 175.3 g NaCl and 88.2 g sodium citrate in 800 ml of water. Adjust the pH to 7.0 using HCl. Bring volume to 1 liter and autoclave to sterilize. Store up to 10 years at room temperature.

SSC, 2×/ TE, 1×:

For 100 ml, dilute 10 ml 20× SSC and 10 ml 10× TE in 80 ml of autoclaved deionized water. Store in 50-ml aliquots up to 5 years at room temperature.

Stop buffer (EDTA):

For 500 ml of solution, add 93.1 g (dehydrate) of 0.5Mdisodium EDTA to container and bring volume to 500 ml with water. Adjust pH to 8.0 with NaOH (~20 g). Autoclave to sterilize. Store up to 10 years at room temperature.

TAE, 50×:

Dissolve 242 g trizma base, 14.6 g EDTA, and 57.1 ml of acetic acid in 500 ml of water and fill to 1000 ml. Store up to 5 years at room temperature. For a 1× stock, add 1 volumes of TAE (50×) to 49 volumes of water.

TE, 10×:

For 1000 ml of solution, add 12.1 g of 100 mM Tris base and 3.7 g (dehydrate) of 10 mM disodium EDTA to a container. Fill to 1000 ml with water and adjust the pH to 7.6 using HCl. Autoclave to sterilize. Store up to 10 years at room temperature.

TE saturated Bio-Gel P-60, 1× stock:

Product used is Bio-Rad 150-4160. Mix polyacrylamide beads and 1× TE, pH 7.6. Allow solution to sit at 4°C overnight before use. Stable up to 3 years at 4°C.

Vectashield and DAPI:

Dilute Vectashield with DAPI in plain Vectashield. Proper dilution varies based on sensitivity of the microscope being used; see Troubleshooting for more details.

Commentary

Background Information:

FISH is a useful tool for the analysis and visualization of chromosomes. It has been used to identify single genes (J. C. Lamb et al., 2007), transgene insertions (Yu, Lamb et al., 2006; Gaeta et al., 2011), repetitive elements (Kato et al., 2004; Lamb et al., 2007), and transposable elements (Yu et al., 2007). FISH has also been used to study interspecies hybrids and allopolyploids by using retroelements to develop probes that paint genomes (Lamb and Birchler, 2006). This variety of uses has made FISH an important tool for understanding the makeup of the maize genome as well as in genetic engineering for the ability to locate transgene insertions easily.

FISH follows the basic idea of performing a Southern blot analysis in that it exploits the ability of single-stranded DNA to bind to its complement (Bishop, 2010). Desired cells are fixed to microscope slides and are hybridized to a nucleic acid probe, which anneals to its complementary sequence in the DNA of the specimen. This nucleic acid probe is labeled with a reporter molecule that is, in the case of the FISH protocol described here, a fluorochrome. This enables

the detection of the probe, which is hybridized to the target DNA, by fluorescence microscopy, which visualizes the fluorescent signature of the probe (Bishop, 2010).

Using FISH as a tool has some disadvantages that are worth considering before beginning. The size of the probe target needs to be carefully considered as targets of smaller size than 2.5 kb are not easily detectible, though targets as small as 1.4 kb can be detected. Small targets that are not immediately adjacent but residing in proximity and adding to more than 3 kb can be detected well. Detection of duplications of probed DNA can be hard to visualize using FISH because of the limitations of fluorescence microscopes to identify individual probe binding sites in close proximity. Photobleaching of probes on slides is possible as well as nonspecific binding if probe sequences are not carefully considered. False-positives and false-negative results are possible because of the previously mentioned nonspecific binding of improperly designed probes, errors in processing of images, and imperfect hybridizations.

Critical Parameters:

Probe sequence must be carefully considered in regard to both size of sequence as well as its constitution. The target size is also important for deciding what fluorochrome with which the probe should be labeled.

Troubleshooting:

DNA Preparation:

Steps 1 to 3: It is suggested that a small (10 µl) PCR test reaction be carried out prior to performing the full PCR reaction and examined on an agarose gel if primers and plasmid DNA have not been used previously. Conditions, such as annealing temperature and cycle number, should be adjusted until PCR results in a single strong band before proceeding with large-scale PCR for probe DNA.

Fluorescence In Situ Hybridization:

Vectashield and DAPI: DAPI (4’,6- diamidino-2-phenylindole) is a blue fluorescent dye, which stains chromatin. Vectashield is a mounting medium for fluorescence microscopy that is available in multiple formulations, notably with DAPI and without DAPI. For probe mixtures that do not use a blue probe DAPI is useful for distinguishing the chromosomes as a whole. Depending on the microscope, the concentration of DAPI in the Vectashield may need to be adjusted. For some microscopes the factory formulation of Vectashield with DAPI works well but with others it is too intense. Using a 1:10, 1:20, or 1:40 dilution of Vectashield with DAPI to standard Vectashieldmay be necessary.With lower concentrations of DAPI the more condensed regions of chromatin

such as knobs can often be distinguished from less condensed chromatin and aid in distinguishing chromosomes.

Anticipated Results:

The production of a fluorescent probe that binds to the DNA sequence of interest and is able to be visualized by fluorescent microscopy. A minimum target size of 2.5 kb is recommended.

With the karyotyping cocktail, the final product should resemble Figure A5.3 once the image is merged and the chromosomes are identified and labeled.

Time Considerations:

DNA amplification:

- Steps 1 to 3: Assembly of PCR reaction until completion of thermocycler protocol will take 3-4 hr.

DNA precipitation:

- Steps 1 to 3: Assembly of ethanol precipitation reagents will take approximately 15 min.
- Step 4: Incubation of ethanol precipitation will take from 30 min to overnight.
- Step 5: 30 min of centrifugation is required to pellet the DNA.

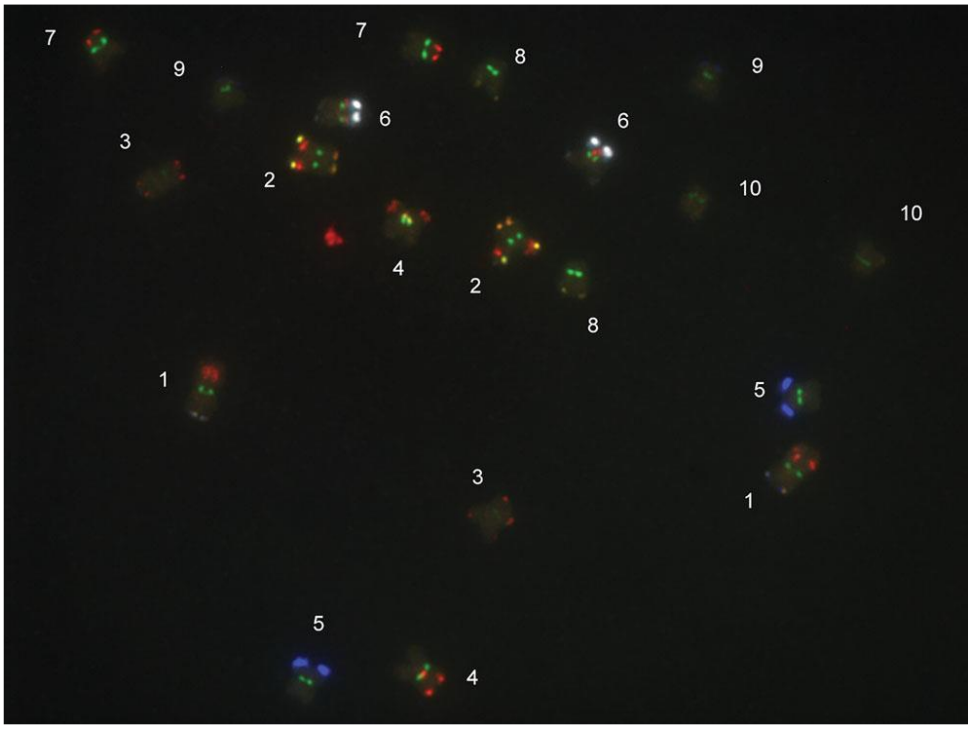

Figure A5.3 - Karyotype of Fast-Flowering Mini-Maize B: A karyotype produced using the methods described in this protocol. The white pseudocoloring of the far-red channel is produced by making a second RGB image with the same far-red channel capture representing the red, green, and blue channels. The RGB image of the real red, green, and blue channels is then merged with the image of the far-red channel in Photoshop. Each chromosome was then identified by its size and characteristic signals and labeled with its chromosome number.

- Steps 6 to13: Wash steps and elution of the DNA pellet take about 20 min.

Checking DNA integrity:

- Steps 1 to 2: The casting and assembly of the agarose gel takes about 30 min.
- Step 3: Running of the agarose gel for 30 min will allow for good resolution of DNA.
- Step 4: 15 to 30 min are required to stain agarose gel in ethidium bromide.
- Steps 5 to 6: Visualization of the gel takes about 5 min.

Nick translation:

Small targets:

- Steps 1 to 3: Assembly of nick translation reaction, as well as incubation, takes approximately 2.5 hr to complete.

Large targets:

- Steps 1 to 3: 2.5 hr is required to assemble and incubate the nick translation reaction.

Probe purification:

Column assembly:

- Steps 1 to 4: Assembly of column for probe purification takes 25 min.

Column purification:

- Steps 1 to 2: 30 to 45 min should be set aside for actually running the probe through the column.

Ethanol precipitation:

Small targets:

- Steps 1 to 2: Assembly of reagents for ethanol precipitation takes 15 min.
- Step 3: Incubation of mixture takes from 2 hr to overnight.
- Step 4: Centrifugation of ethanol precipitation mixture will take 30 min.
- Steps 5 to 9: Washing of pelleted probe DNA will take approximately 15 min.

Large targets:

- Steps 1 to 2: Assembly of reagents for ethanol precipitation takes 15 min.
- Step 3: Incubation of mixture takes from 2 hr to overnight.
- Step 4: Centrifugation of ethanol precipitation mixture will take 30 min.

- Steps 5 to 9: Washing of pelleted probe DNA will take approximately 15 min.

Karyotyping cocktail:

Hybridization and slide finishing:

- Step 1: Adding probe to slides takes about 5-10 min depending on number of slides prepared.
- Step 2: Adding prepared slides to boiling water takes approximately 5 min.
- Step 3: Prepared slides are then incubated overnight.
- Steps 4 to 6: Washing of probed slides in 2×SSC and drying takes approximately 50 min.
- Step 7: Adding DAPI to slides takes approximately 5-10 min depending on number of prepared slides.

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References

Albert P.S., Gao Z., Danilova T.V., and Birchler J.A. (2010) Diversity of chromosomal karyotypes in maize and its relatives. Cytogenet. Genome Res. 129:6-16.

Bishop R. (2010) Applications of fluorescence in situ hybridization (FISH) in detecting genetic aberrations of medical significance. Bioscience Horizons 3:85-95.

Gaeta R.T., Danilova T.V., Zhao C., Masonbrink, R.E., McCaw,M.E., and Birchler, J.A. (2011) Recovery of a telomere-truncated chromosome via a compensating translocation in maize. Genome 54:184-195.

Kato A., Lamb J.C., and Birchler J.A. (2004) Chromosome painting using repetitive DNA sequences as probes for somatic chromosome identification in maize. Proc. Natl. Acad Sci. U.S.A. 101:13554-13559.

Lamb J. and Birchler J. (2006) Retroelement genome painting: Cytological visualization of retroelement expansions in the genera Zea and Tripsacum. Genetics 173:1007-1021.

Lamb J., Meyer J., Corcoran B., Kato A., Han F., and Birchler J. (2007) Distinct chromosomal distributions of highly repetitive sequences in maize. Chromosome Res. 15:33-49.

Lamb J.C., Danilova T., Bauer M.J., Meyer J.M. Holland J.J., Jensen M.D., and Birchler J.A. (2007) Single-gene detection and karyotyping using small-target fluorescence in situ hybridization on maize somatic chromosomes. Genetics 175:1047-1058.

McCaw M., Swyers N., Graham N., Cody J., Zhao C., and Birchler J. (2016) Preparation of chromosomes from Zea mays. Curr. Protoc. Plant Biol. 1:501-508.

Yu W., Lamb J.C., Han F., and Birchler J.A. (2006) Telomere-mediated chromosomal truncation in maize. Proc. Natl. Acad Sci. U.S.A. 103:17331-17336.

Yu W., Lamb J.C., Han F., and Birchler J.A. (2007) Cytological visualization of DNA transposons and their transposition pattern in somatic cells of maize. Genetics 175:31-39.

Vita

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