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Plant minichromosomes

James A. Birchler, Nathaniel D. Graham, Nathan C. Swyers, Jon P. Cody,
Morgan E. McCaw

Division of Biological Sciences
University of Missouri
Columbia, MO 65211

Corresponding Author:

James A. Birchler
Email: BirchlerJ@Missouri.edu
Phone: 573-882-4905
FAX: 573-882-0123

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Plant minichromosomes have the potential for stacking multiple traits on a separate entity from the remainder of the genome. Transgenes carried on an independent chromosome would facilitate conferring many new properties to plants and using minichromosomes as genetic tools. The favored method for producing plant minichromosomes is telomere-mediated chromosomal truncation because the epigenetic nature of centromere function prevents using centromere sequences to confer the ability to organize a kinetochore when reintroduced into plant cells. Because haploid induction procedures are not always complete in eliminating one parental genome, chromosomes from the inducer lines are often present in plants that are otherwise haploid. This fact suggests that minichromosomes could be combined with doubled haploid breeding to transfer stacked traits more easily to multiple lines and to use minichromosomes for massive scale genome editing.

Introduction

Engineered minichromosomes, artificial chromosomes, or synthetic chromosomes have been proposed as a next generation vehicle for genetic engineering in plants. They have the potential advantage of having a targeted site of integration of new transgenes for faithful expression, as opposed to random insertion of transgenes that often lead to variable expression or silencing as well as being potentially mutagenic. Further, they might provide the ability to stack numerous desirable genes together on an independent linkage group that will not exhibit linkage drag with endogenous genes during introgression schemes to other lines. The presence of the transgenes on a separate chromosomal entity is of particular interest because this property will allow the transgene cargo to be combined with doubled haploid (DH) breeding technology for the transfer of the genes to many new lines in a few generations, rather than an otherwise extended backcrossing regime that is seldom absolute. Not only will engineered minichromosomes provide new properties to plants that carry them but they can also be used as genetic tools to facilitate genome editing and the evaluation of transgenes in general.

Artificial chromosomes were first produced in yeast by the assembly of centromere sequences, an origin of replication, a selectable marker and telomere repeats placed on both ends [1]. When these constructs were introduced back into yeast, they functioned as a separate chromosome. This initial success served as a paradigm for producing artificial chromosomes in other species.

This example was used as a guide for plant biologists to attempt to construct artificial chromosomes. Although there are a few claims of success, which will be evaluated below, many attempts in several species resulted in failure and, of course, there has been a publication bias against negative results—the consequence of which has been that the idea has been kept alive. The probable reason for the failure of this approach to construct artificial chromosomes in plants is that there is an epigenetic basis to centromere function, which is not the case in yeast [2]. In other words, the underlying DNA at plant centromeres is apparently irrelevant to the establishment of a kinetochore, the protein complex built up at the centromere involved with attaching a chromosome to spindle microtubules for movement. The cloning of centromere sequences in bacteria and then re-introducing them into plant cells will not result in function because the chromatin features needed for function have been stripped from the DNA.

Epigenetic nature of plant centromeres

Because of the re-occurring fascination with the yeast approach to producing plant artificial chromosomes, it is worth noting the evidence for the epigenetic nature of plant centromeres, the sites on chromosomes on which the kinetochore is formed. One of the first lines of evidence was the recognition of a high frequency of centromere inactivation on chromosomes that were produced to include two functional sets of centromere DNA [3]. After this realization, numerous other instances of the presence of centromere DNA without function were recognized [3]. Further, the cloning of bacterial artificial chromosomes

containing maize centromere DNA and then transforming them back into maize and rice did not produce independent function as an artificial chromosome but rather stable integration into a normal chromosome [4]. Indeed, centromere inactivation could be directed by establishing a tug of war between large and small centromeres on the same chromosome [5,6] indicating that the failure of a kinetochore to be used in a cell cycle is irrevocable in subsequent cell cycles and indeed organismal generations.

On the other hand, de novo centromere formation was documented on chromosomal fragments that contained no canonical centromere sequences as first found in barley [7]. Subsequently, de novo centromeres were found on several chromosomal fragments in oat-maize addition lines [8] or on chromosomal segments that have no canonical centromere in maize itself [9*, 10**]. They are present over unique DNA that contains no known or detectable copies of the canonical centromere repeats. It seems apparent that de novo centromere formation is quite common on chromosomal fragments, at least in maize. If they arise on a normal chromosome, because they are typically not as stable as an endogenous centromere, they are likely cleared via inactivation from the chromosome arm in a tug of war with the endogenous copy leaving no trace of their fleeting existence.

These lines of evidence indicate that centromere sequences are not needed to form a kinetochore nor will their presence produce one. Active centromeres seem to be perpetuated from one cell cycle to the next based on the chromatin features and not the underlying DNA. The homogenization of like

sequences at centromeres across the genome on nonhomologous chromosomes is potentially a byproduct of targeted transposition of the satellite and retrotransposons that are characteristic of these regions [11]. The consequence of this activity is that as centromeres evolve, any genes in the region will be pushed out and away from the regions of the chromosome that lack recombination.

Claims of build up success

As noted above, there are two reports of success of constructing artificial chromosomes in maize using the yeast paradigm of relying on centromere sequence function. In the first case, a BAC containing 36 kb of maize DNA including 9.5 kb of centromere satellite, CentC, was introduced into maize cells via biolistics [12]. The phenotypic fluorescent marker was followed genetically in subsequent generations with statement of near perfect transmission. Fluorescent in situ hybridization (FISH) was conducted and revealed signals separate from the chromosomes.

There are several reasons to suggest that these assertions are unlikely. First, for all cases of small chromosomes (but much larger than the constructs used in this case) examined in meiosis in maize, starting with Barbara McClintock [13,14], there has been a failure of sister chromatid cohesion in meiosis I [15-17]. Therefore, it is not possible to obtain a perfect transmission under the circumstance described. The FISH technique utilized is not capable of detecting targets much below 30 kb [18]; therefore the phenotypic marker followed was probably inserted into an endogenous chromosome and not

revealed by this procedure. Furthermore, the intensity of the FISH signal on the presumed minichromosomes were much stronger than those from the endogenous centromeres, despite the fact that the introduced fragment was 9.5 kb but endogenous centromeres range from ~700 kb to 2.7 Mb [2]. Thus, these signals are probably either FISH artifacts of probe aggregates, a common occurrence, or they represent extensive conglomerates of the input DNA [see further discussion: 19, 20]. If the latter, then their activity might have resulted from de novo centromere formation.

In the second case [21], centromere BACs were introduced into maize cells with the result that only stable integrations were found. However, when telomere sequences were included in the biolistic transformations, minichromosomes were realized. This was also the case when telomere sequences were added to the centromere containing BACs. Most of the small chromosomes found were obviously rearrangements of the normal set but a few cases were very small and present in a karyotype that contained 20 normal maize chromosomes. Because of this latter result, it was claimed that these cases must represent autonomous function of the introduced BACs. However, it was acknowledged that the size of these chromosomes was much larger than the input DNA and when probed with retrotransposons, there were several types that were present on the minichromosome that were not included in the input sequences.

Because the transformation of centromere sequences alone did not produce autonomous function and minichromosomes were only generated with

the inclusion of telomere sequences, it is likely that telomere mediated chromosomal truncation is operating. The centromeres of the minichromosomes observed are probably all endogenous, which would explain the presence of different retrotransposons when compared to the input. Indeed, others have generated a minichromosome consisting of little more than an endogenous centromere, which was recovered in a karyotype with 20 normal chromosomes, using telomere mediated truncation but in the absence of centromere input [22**]. The basis of how a cleaved chromosome reduced to only the centromere could be recovered with 20 normal chromosomes is not known but potentially arises during the transformation selection for doubling of the remaining intact homologue to provide a growth advantage over an otherwise monosomic condition.

Telomere mediated truncation

Another method to generate minichromosomes involves the transformation of telomere sequences, the specialized termini, into cells. The sites of potential insertion cannot be repaired and the distal part of the chromosome arm is released and lost. This procedure was first shown in mammalian cells and succeeded in producing small chromosomes consisting of only the centromere [23,24]. These centromeres were then modified using homologous recombination.

Telomere mediated truncation was first demonstrated for plants in maize [25] but in this case a selectable marker and other sequences that could be used for subsequent minichromosome modification were included on the same

construct (Figure 1). The inclusion of the telomere sequences inside the right border of *Agrobacterium* T-DNA was found to cleave the chromosome at the potential insertion site. Apparently, the left border is ligated to the chromosome but the presence of the telomere DNA organizes a chromosomal terminus that prevents ligation of the right border. In addition to maize, telomere mediated chromosomal truncation has been documented in *Arabidopsis* [26,27], barley [28] and rice [29].

In addition to the placement of telomeres inside a transformation cassette, it is also possible to produce minichromosomes by mixing free telomere repeats with the desired transgenes and performing a co-bombardment. Co-bombardments are known to result in co-integration of the combined DNA sequences and therefore with the right orientation, a transgene will be ligated to a normal chromosome break and the presence of telomeres appended to the other end will cleave the chromosome. This approach bypasses the difficult procedure of cloning the repetitive telomere repeats into plasmid constructs and has been demonstrated in rice [29] and maize [22**].

Supernumerary B chromosomes

In addition to the normal set of chromosomes, maize (and other plant species) possesses a supernumerary chromosome called a B chromosome that is basically inert with only one arm on a near terminal centromere. It is therefore a favorite vehicle with which to generate engineered minichromosomes.

Telomere mediated truncation of B chromosomes was found to be quite frequent with either *Agrobacterium* or biolistic transformation [30]. A range of sizes of

truncated B chromosomes was recovered from near terminal cuts to those consisting basically of only the centromere all with the added sequence visualized at the severed end of the chromosome.

While basically inert, the B chromosome is maintained in populations that contain them via an accumulation mechanism. This mechanism consists of nondisjunction at the second pollen mitosis that produces the two maize sperm with a subsequent preferential fertilization of the egg as opposed to the polar nuclei by the sperm carrying the B chromosomes in the process of double fertilization [31,32]. The nondisjunction property requires the centromere but also some trans-acting factors located elsewhere on the B chromosome. One of the required factors resides at the very tip of the long arm [33]. Therefore, truncated B chromosomes do not exhibit nondisjunction on their own but rather show stabilized transmission. However, the presence of a full sized B chromosome together with a truncated minichromosome can confer onto the mini the nondisjunction property, which allows one to conduct a dosage accumulation of engineered truncated mini B chromosomes [34,35].

Engineered minichromosomes derived from the B chromosome are capable of dosage manipulation [35]. In a proof of concept experiment, various engineered mini B chromosomes in maize were combined with a normal B chromosome and a regime of self-pollination and selection was conducted. It was possible to increase the copy number of these engineered minichromosomes in this way. In addition to amplifying the expression output with strong promoters,

the gene products from the mini B could potentially be increased in expression with increased copies assuming that gene silencing can be avoided.

Transgene stacking

One potential application of engineered minichromosome is as a means to achieve extensive stacking of traits. With current procedures, transgenes are randomly inserted into the genome. To combine several such transgenes requires crossing together the various insertions followed by a subsequent self-crossing regime. It is obvious that as the number of such insertions increases, this task will become overwhelming and the introgression into new lines essentially impossible; thus, the interest in having a single entity to which transgenes could be added in significant number and for which future modifications could be conducted.

Targeted transgene insertion, deletion and stacking have been achieved at positions in normal chromosomes [36-39,40**] and therefore there is no reason to believe that these procedures cannot be applied to minichromosomes. Indeed, an in vivo deletion of a selectable marker was demonstrated for a maize minichromosome [22**]. These procedures rely on site-specific recombination systems that have been introduced into plants. A stacking system proposed by Ow [41] (or modifications thereof) and demonstrated with a proof of concept [40**] has the potential to stack transgenes in continuous fashion by using multiple recombinases and their target sequence strategically placed in the transgene stack. If applied to minichromosomes, the ability to grow them in perpetuity should be possible. Of course, genome editing procedures, in

particular CRISPR-Cas9 [42-45], could be used to modify minichromosomes as well as using the editing machinery itself carried on minichromosomes to facilitate multiple genomic changes as described below.

Stabilizing transmission of minichromosomes

As noted above, minichromosomes do not exhibit the same behavior as normal chromosomes. First, small chromosomes in maize all fail to exhibit the normal sister chromatid cohesion typical of meiosis I (MI) [17]. Thus, the sisters usually proceed to the MI poles from each other, rather than together, and as a consequence they proceed at random to the poles in meiosis II. Secondly, all studies of small chromosomes in maize indicate that when two copies are present, they seldom can find their pairing partners in prophase of MI [17]. The members of the pair therefore assort independently of each other rather than segregate from each other as normally. Thus, some measures are needed to have high transmission frequency of minichromosomes.

One solution to this issue for species with B chromosomes is to use a truncated B chromosome that is cleaved near the end of the chromosome such that because of its length, its behavior is the same as a normal chromosome. Other potential solutions might involve placing a nuclear restorer gene for gametophytic cytoplasmic male sterility (cms) [46] onto the minichromosome. In a background of cms, the restorer will only allow pollen grains carrying the minichromosome to be viable in the male and thus when crossed onto a female parent, full transmission would be expected in the progeny. A single

minichromosome carrying such a restorer with the appropriate cms, would be expected to be transmitted faithfully from one generation to the next when used continuously as a male parent onto the respective cms female parent. The cms-S cytoplasm of maize [46] and the boro II cytoplasm of rice [47] qualify to be used in this manner. Other methods involving complementation of nuclear male steriles or transgenic male steriles are possible solutions [20].

Combining minichromosomes with doubled haploid breeding

The placement of multiple transgenes onto a minichromosome has the potential to transfer the whole aggregate to many different other lines, including any that are less amenable or recalcitrant to transformation, by combining them with doubled haploid (DH) breeding to bypass a lengthy and often incomplete introgression procedure involving multiple backcrosses to a recurrent parent. Doubled haploid breeding refers to the procedure of generating haploid plants [48] of target lines or novel genotypes by various means, and then doubling the chromosome number of the recovered plants in developmental lineages leading to the flowers to make self pollination possible. The end-product is a progeny that is completely homozygous in only two generations of manipulation [49].

The inspiration for combining minichromosomes with doubled haploid breeding comes from the observation that B chromosomes incorporated into maize haploid inducer lines could be recovered in the haploid derivatives in the progeny [50**]. The haploid inducer is used as a male parent for target female lines with the chromosomes from the inducer being lost shortly after fertilization.

It has long been observed that genetic markers from the inducer lines are sometimes incorporated into doubled haploids [48] suggesting that the elimination was not always complete. If an extra normal chromosome is present as a disomic haploid, these plants are highly defective or lethal [50]. However, the inclusion of basically inert B chromosomes in the inducer lines allowed their recovery. Minichromosomes with a transgene cargo would be expected to behave in an analogous manner. Our laboratory has repeated this experiment and estimate a frequency of recovery of B chromosomes in haploids at about 1/25 [M. McCaw and J. Birchler, unpublished]. With efficient haploid production and a selection means for the minichromosome, this frequency is in a workable range.

Haploid induction systems involving altering the centromeric specific histone, CENH3, have attracted attention [52,53**]. In this system as well, there is a low frequency of inclusion of chromosomes from the inducer lines present in otherwise haploid progeny. Thus, it is likely that minichromosome transfer to haploids could occur in this system as well.

The combination of minichromosomes with DH breeding would complement modern approaches to development of new plant varieties, which relies heavily on DH technology. It would supply the ability to transfer the collection of transgenes to any number of other lines without a lengthy introgression process and without any linkage drag. The target lines need not be capable of being transformed but still the ability to introduce multiple transgenes would be conferred with this technology. The minichromosome transfer system

could be used as an evaluation tool for testing the reaction of various transgenes in different genetic backgrounds even if it is not used ultimately in seed production.

Minichromosomes for genome editing

Minichromosomes might also be used as a tool in genome editing (Figure 2). The CRISPR-Cas9 system appears to be a facile method to inactivate genes or to perform replacements. Yet, the products of the reactions at the two alleles of target genes are not always the same [54], which would complicate large scale genome editing. If CRISPR-Cas9 and the necessary guide RNAs were introduced onto a minichromosome and combined with DH breeding, the edits for multiple genes in a haploid can be doubled to capture the full spectrum across the genome without any problems of linkage with the editing transgenes or of independent assortment of multiple edits that would occur in a diploid. If the CRISPR-Cas9 machinery is present on a small engineered minichromosome, the pair of such chromosomes after doubling are not capable of homologous pairing in meiosis, as described above, and therefore a fraction of the progeny will be missing the editing minichromosome due to their failure to segregate from each other. Thus, the homozygous non-transgenic genotype of the normal set of chromosomes with multiple edits intact would be retained but the editing minichromosome would be removed.

Concluding remarks

Here we summarize the evidence for an epigenetic basis for centromere function in plants and note that this fact suggests that the most efficient means of producing minichromosomes in plants is via telomere-mediated chromosomal truncation so that an endogenous centromere that never leaves a cell can be used on these constructs. Recent developments in gene stacking and genome editing can be applied to minichromosome technology not only for modifying them but also using minichromosomes in these procedures as a genetic tool.

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Figure Legends

Figure 1: **Telomere-mediated Chromosomal Truncation**

Top: Truncation cassette is introduced into the cell via Agrobacterium infiltration or particle bombardment. The truncation cassette, once in the nucleus, may be inserted into a double-strand break. The telomere arrays on the end of the truncation construct may then be used as a template for the telomere capping machinery, signaling the end of the chromosome, and resulting in truncation of the chromosome.

Below: Insertion of the truncation cassette into an A chromosome resulting in a truncation event (B1) or an insertion but no truncation (B2). Insertion of the truncation cassette into an A chromosome resulting in an insertion but no truncation (B3) or an insertion resulting in a truncation event (B4).

Figure 2. **Combining engineered minichromosomes with the haploid transfer system to facilitate massive scale genome editing**

A. A cross is made between a target female line and a male haploid inducer line carrying a minichromosome with the editing machinery (small chromosome with a small red marker indicating the editing transgene). **B.** The haploid gametes join together but the normal chromosomes from the inducer line become eliminated (depicted by the faint chromosomes) but the editing minichromosome persists in some of the haploid generated. The editing machinery could then modify the

haploid maternal genome as indicated by horizontal bars on the chromosomes.

C. With chromosome doubling the various edits in the haploid are retained together in the duplicated genome eliminating the need to assemble them from individual events. If the minichromosome is one that does not undergo homologous pairing in meiosis because of its small size, then the editing machinery will be removed from the newly generated genotype in a portion of the progeny of the doubled haploid.

Figure 1

Figure 2
A.

Graphical Abstract