Review

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Promises and pitfalls of synthetic chromosomes in plants

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This review summarizes synthetic chromosome construction in plants and speculates on how they might be applied to various uses in the future, as well as the possible impact for synthetic biology. The procedures to construct foundational platforms for synthetic chromosomes in plants are described, together with some challenges to be overcome in growing such chromosomes and expanding their applications.

Artificial chromosomes and genetic engineering

Several decades ago, yeast artificial chromosomes were produced by assembling a construct containing a centromere, a selectable marker, an origin of replication, and telomeres to cap the ends [1]. After introduction into cells, this synthetic construct was able to function as an independent chromosome. Many years later, the small genome of *Mycoplasma mycoides* was chemically synthesized with identifying landmarks and introduced into genetically eviscerated cells, and the synthesized molecule was able to replicate in this environment and produce daughter cells [2]. Recently, a yeast chromosome has been synthesized and shown to be functional [3]. These proof-of-concept experiments portend many interesting possibilities for generating to specification synthetic chromosomes that will be capable of any number of useful functions, particularly in bacteria and yeasts. Another taxon in which artificial chromosomes might have strong applications in the context of synthetic biology is the plant kingdom. In plants, modifications could be made that have agricultural advantages [4], as well as amenability to biopharmaceutical production of useful proteins or metabolites [5]. Some of the possible applications of synthetic chromosomes in plants and some of the obstacles that need to be overcome in moving forward are discussed below.

Plant genomes have been engineered by the addition of transgenes. In most transformation protocols the insertion sites are random in the genome, resulting in variable expression among different isolates derived from the same construct. Transgene integration into chromosomes could also potentially induce mutations which might produce undesirable consequences. Further, transfer of selected transgenes from one line to another requires several generations for background exchange, with the closely linked genes being particularly difficult to replace. Synthetic chromosome platforms, as described below, will avoid these issues and allow rapid transfer into many varieties. Producing artificial, synthetic, or engineered minichromosomes is one of the building blocks for synthetic biology in plants.

The long-range hope of engineering minichromosomes would be to combine traits that can improve nutrition, reduce fertilizer use, reduce pesticide use, increase biomass/yield, foster harvest efficiency, introduce drought tolerance, and provide resistance to viral, bacterial, or fungal pests, among other possibilities in agriculture, or the production of pharmaceuticals for medicinal purposes [4]. At the moment, most transgenic crops contain very few modifications. Stacking traits for such a wide variety of characteristics might be a distant goal but, given the challenges of an increasing world population, decreasing water availability, and static amounts of arable land, continuing the human tradition of optimizing crops to sustain an advancing civilization seems to be a reasonable idea.

Foundation of synthetic chromosomes in plants

Engineered minichromosomes have now been produced in plants, and the techniques to advance this technology are in place [6–11]. The current state of the art is worth reviewing to project the path forward in synthetic chromosome biology. Attempts have been made at producing artificial chromosomes in plants in a manner analogous to how they were produced in budding yeast – namely by transforming constructs that contain a centromere sequence and selectable marker at a minimum [4]. This approach was tried in several plant species after the initial identification of the respective centromere sequences. A publication bias against negative results has kept the concept alive, and there have been many attempts to employ this approach. Some claims of success have been published, but we have provided a detailed evaluation elsewhere [4] and this will not be re-iterated here.

The problem, which could not have been foreseen *a priori*, was the epigenetic nature of centromere function in plants [12,13]. The centromeric DNAs of budding yeast and plants behave very differently. Yeast centromeres are very small (125 bp) and induce the formation of a kinetochore whenever and wherever they are present. Plant centromeres, by contrast, are highly-repetitive, usually Mb-sized structures, and it might be the case that the DNA underlying them has no role in determining the site of an active kinetochore. Typically, the centromere DNA is associated with a specific

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variant of histone 3, CENH3. However, in a careful test of centromere DNA function in rice and maize, stable insertions of the introduced DNA were found but without any evidence of independent centromere function [14]. Subsequently, a variety of cases of inactive centromeres were found, further documenting that the sequences did not genetically determine the presence of an active centromere [12]. These inactive centromeres can be perpetuated over many generations. There is now emerging evidence that CENH3 can be incorporated into a chromosome *de novo* to produce an active centromere over unique nucleotide sequences of chromosomal fragments lacking a canonical centromere [13,15]. The association of CENH3 with maize chromosomes expanded outside the limits of the centromeric DNA when the chromosomes were introduced into oat, for example, to match the much larger centromeres in the host genome [16]. Taken together, this evidence illustrates that the centromeric DNA is neither necessary nor sufficient to organize a functional kinetochore. Instead, the pre-existing site of CENH3 in each cell cycle seems to direct the location in the next cell generation.

Engineering minichromosomes thus requires use of an endogenous centromere from which the remainder of the chromosome is cleaved away, as well as the addition of sequences that enable the rebuilding of a synthetic chromosome to specification [7]. This has been achieved using telomere-mediated chromosomal truncation [6] (Box 1). The telomeres are the specialized ends of chromosomes that prevent chromosomal fusions and allow resolution of DNA replication. The presence of telomere arrays at one end of a transgene will prevent the ligation of that side of the incoming construct to the target chromosomal site, but the other end of the transgene will be added to the double-stranded DNA opening of the recipient molecule. The end of the old chromosome will be lost and replaced by the transgene capped with a telomere array. The contents of the introduced sequences will determine the manipulations that may be conducted with the minichromosome in the future. The efficiency of telomere-mediated truncation is sufficiently high that synthetic chromosomes can be produced that need only consist of an endogenous centromere, added transgenes, and little else. Alternatively, such minichromosomes can also be engineered by using supernumerary B chromosomes [6] or telotrisomics [8] (i.e., an extra chromosome with the centromere and only one arm) as the starting target material.

A proof of concept has shown that site-specific recombination cassettes placed at the ends of minichromosomes are capable of recombination and that engineered minichromosomes can be modified *in vivo* [17]. The next step in building a synthetic plant chromosome is to develop procedures for the addition of new sequences to the chromosome. Methods exist for targeting exogenous sequences that were previously inserted into normal chromosomes, and therefore the techniques can certainly be adapted for use with minichromosomes [18–20] (Figure 1). A challenge will come when proceeding to the next step: making the synthetic chromosome grow by leaps and bounds.

Techniques such as genome editing with CRISPR/Cas9 [21–25] have yet to be applied to engineered minichromosomes. Minichromosomes represent a larger-scale genetic engineering platform than currently possible with such genetic modification systems. However, genome-editing techniques have the potential to advance significantly the applications of minichromosomes.

Box 1. Production of engineered minichromosome platforms by telomere-mediated chromosomal truncation

Maize has 10 normal chromosomes and an extra supernumerary B chromosome (Figure IA). This target genotype is transformed with a construct of transgenes and telomere repeat arrays at one end. Upon transformation, the transgene side of the construct is ligated to the break in the chromosome. The transgene cargo is thereby added to an endogenous centromere. The telomere side assembles a telomere cap that prevents ligation, thus truncating the chromosome (Figure IB). Using a B chromosome for this purpose avoids any complications of aneuploidy because the B chromosome is basically inert. Other approaches to recover truncated chromosomes in species without B chromosomes include using tetraploid derivatives as the target for transformation with truncating constructs, which will allow recovery of deleted chromosomes through the haploid gametophyte generations. Another approach is to use a telotrisomic line as the target. Such lines contain an extra chromosome that has only one arm present. When truncation occurs next to the centromere, and transgenes are added in the process, an engineered minichromosome platform would be produced.

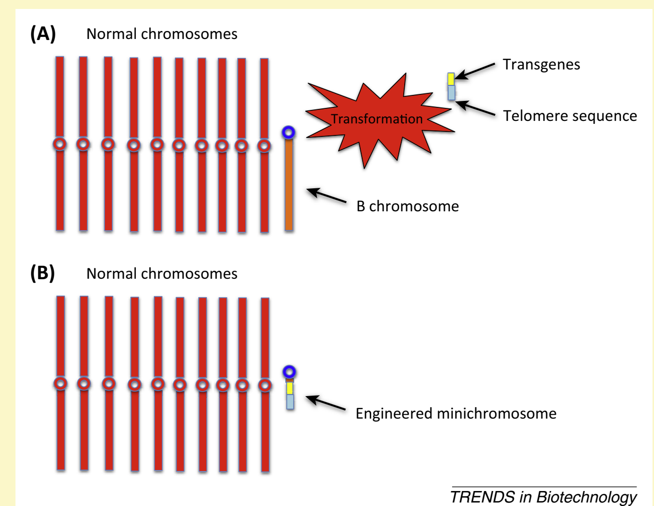

Figure I. Telomere-mediated chromosomal truncation. **(A)** Normal chromosomes (red) and the supernumerary chromosome (orange) of maize. Circles represent centromeres. **(B)** Normal chromosomes and engineered minichromosome.

Combination of synthetic chromosomes and doubled haploid breeding

Minichromosomes can be combined with doubled haploid breeding, such that any new transgenes could be transferred into new lines by introgressing the engineered minichromosome into a haploid inducer line or a line engineered to produce haploids (Box 2) [4,26]. A proof-of-concept example involved incorporation of the supernumerary B chromosome into a haploid inducer line of maize. When this line was then used to generate haploids, a workable frequency of the 1 n (haploid) plants contained the B chromosome from the inducer male parent in an otherwise maternal haploid [6]. From such lines there is reason to believe that haploids can be produced that will incorporate an engineered minichromosome in analogous fashion. Engineered haploid inducers that utilize a modified centromeric histone also transmit a lone chromosome

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