Experimental Cell Research 390 (2020) 111951

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Charting the path to fully synthetic plant chromosomes

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ARTICLE INFO

Keywords:
Plant biotechnology
Synthetic biology
Centromere
Kinetochore
CENPA
CENH3
Plant transformation
Meiosis
Chromosome segregation

ABSTRACT

The concepts of synthetic biology have the potential to transform plant genetics, both in how we analyze genetic pathways and how we transfer that knowledge into useful applications. While synthetic biology can be applied at the level of the single gene or small groups of genes, this commentary focuses on the ultimate challenge of designing fully synthetic plant chromosomes. Engineering at this scale will allow us to manipulate whole genome architecture and to modify multiple pathways and traits simultaneously. Advances in genome synthesis make it likely that the initial phases of plant chromosome construction will occur in bacteria and yeast. Here I discuss the next steps, including specific ways of overcoming technical barriers associated with plant transformation, functional centromere design, and ensuring accurate meiotic transmission.

1. Introduction

The rise of synthetic biology, which involves engineering genes to produce useful biological products [1,2], has catalyzed a creative burst in the fields of genetics and biotechnology. The early successes have been based on modifying a few genes (e.g. Refs. [3,4]), but the scale is shifting towards more complex efforts to manipulate entire genomes. Craig Venter's synthetic reengineering of the *Mycoplasma* genome [5] and subsequent minimized version [6], more recent resynthesis of the *E. coli* genome [7] and entire yeast chromosomes [8] point to a future in biotechnology that involves routine large-scale reengineering of microbial genomes for applied purposes.

There has been a recent surge of interest in applying these principles to plant improvement and biotechnology [9]. The original effort to engineer plants to express a novel pathway was golden rice [10], which involved expressing heterologous proteins from daffodil and a bacterial species to improve vitamin A content. Similar efforts have improved folate (a vitamin) content and modified glucosinolate expression (plant defense compounds) [11]. Potentially more rewarding, but considerably more challenging, are ongoing efforts to reengineer central metabolism [12]. A case in point is the major carbon fixing enzyme Rubisco, which (in a normal oxygen-rich environment) creates toxic products that must be metabolized at a net loss of carbon by photorespiration. Recent data show that photorespiration can be reengineered, resulting in a remarkable 40% increase in biomass [13]. Other more ambitious proposals include replacing the Rubisco function with several enzymes or replacing the entire Calvin cycle where Rubisco resides [14].

These bold solutions will require metabolic reengineering at a scale that cannot be accomplished using traditional methods and will soon merge into the arena of whole chromosome design. Synthetic chromosomes will create opportunities to remove unnecessary sequences and to reorganize genes into functional groupings. Most plant genomes are bloated [15] with vast transposon-rich intergenic spaces [16,17]. Much of this intergenic space is unnecessary for genome function and could be removed. The *cis*-regulatory sequences (enhancers) that often occur within intergenic spaces can be identified by chromatin signatures [18] and retained in a resynthesized plant genome. A maize genome with unnecessary intergenic regions removed could be ~80% smaller [19], and much more amenable to manipulation. Genes could then be reordered and organized into groups with similar functions as was done in *Mycoplasma* [6]. In plants, the most likely targets for manipulation will be genes involved in nutrition, photosynthesis, resilience to stress, or secondary metabolism. The ability to reorganize and express suites of genes will make it easier to test hypotheses based on multiple genes, and to understand how genes in complex pathways interact to control phenotype. Once useful metabolic pathways are identified they can be used to improve plant performance or produce valuable chemicals and bioproducts, potentially transforming the bioeconomy [9].

Two different approaches to creating synthetic chromosomes have been discussed, the so-called “top down” method that involves altering an existing chromosome and the “bottom up” method which envisions a total synthesis of the chromosome. I will briefly review the top down method and focus the bulk of the discussion on the total synthesis approach. Of course, the final solution may involve a mixture of the two

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https://doi.org/10.1016/j.yexcr.2020.111951
Received 11 November 2019; Received in revised form 3 March 2020; Accepted 5 March 2020
Available online 06 March 2020

approaches or entirely new methods that have yet to be envisioned.

2. Partially synthetic top-down minichromosomes

In an earlier commentary on the potential of plant synthetic chromosomes, Birchler highlighted his top down approach to minichromosome engineering [20]. In this and several other reviews (e.g. Ref. [21]) he argued that centromeres are not only too difficult to engineer, but that even if we could engineer them it would not be necessary. Instead, he argues, the better approach is to identify existing chromosomes that can be broken down and built back up around their already established centromeres. The maize B chromosome is perfect for this use because it is a supernumerary chromosome that has a functional centromere and no essential genes [22]. Birchler's group began by deleting most of the single long arm to make a minichromosome [23], inserted a *lox* integration site on the minichromosome, and then demonstrated that a second transgene construct could be inserted into the *lox* site using Cre recombinase [24]. Additional transgenes can be added stepwise using Cre-Lox or other recombinases [24,25] building out the chromosome arm in a manner similar to yeast chromosome engineering [26].

These were prescient first steps taken at a time when few dared to imagine engineering entire plant chromosomes. However, the top down approach is not without weaknesses. One is that the method is based on modifying an existing supernumerary chromosome. Few plants have B chromosomes to work with, and while it may be possible to construct minichromosomes from natural chromosomes in tetraploids [27], significant time and effort would be required to get to the point where engineering is possible. The second is the reliance on assembly in the plant. Since transformation is carried out by standard *Agrobacterium* T-DNA methods (see below), the most DNA that could be added at each generation would be ~150 kb [28]. A single 150 kb construct may be more than enough for many applications, but building an entire chromosome by this approach will necessarily take many years, limited by the generation time of the plant under study.

3. Fully synthetic plant chromosomes

To avoid the laborious plant transformation cycle inherent to the top-down approach, plant chromosomes in part or whole could be first built in yeast. Because yeast naturally recombines sequences with overlapping homologous regions, has a rapid generation time, and has excellent selectable markers, this method is orders of magnitude faster than any other approach. The basic strategy was originally pioneered by the Venter group, who showed that as many as 25 overlapping 17–35 kb fragments (initially built in *E. coli*, see Ref. [29]) could be accurately assembled in yeast [30], and has been steadily adapted and improved [8,31]. By these methods it is possible to assemble entire bacterial genomes as large as ~1 Mb in yeast [32] and transfer them out again [5]. The smallest natural plant chromosome is about ~2.1 Mb [33], although chromosome size is probably not dictated by physical or fitness constraints. Much smaller or larger chromosomes are likely to function similarly well. This was elegantly illustrated in recent publications showing that the entire yeast genome could be assembled onto one or two chromosomes with little phenotypic effect [34,35]. It is also important to remember that an engineered plant chromosome would be far more gene-dense than a natural plant chromosome.

Although creating a plant chromosome in yeast would itself be a remarkable achievement, that is just the beginning. From there, the chromosome will have to be: i) transformed into a plant cell, where it must ii) form a functional centromere, and iii) segregate at both mitosis and meiosis. I will consider each of these problems in turn.

3.1. The transformation problem

It will be very difficult to adapt current plant transformation methods to the task of creating a synthetic plant chromosome. The major transformation methods are *Agrobacterium*-mediated transformation [36,37] and biolistic transformation [38,39]. *Agrobacterium* is a natural plant pathogen that transforms host plants with genes that alter growth and produce metabolites for the bacterium; to do this, it normally transfers about 24 kb of sequence and can be altered to transfer as much as 150 kb [40]. The *Agrobacterium* system is designed to interface with standard *E. coli* cloning and manipulation methods, which become unwieldy beyond this ~150 kb limit. Biolistic transformation, which involves coating metal beads with DNA and driving them into plant cells by force, is in principle more versatile. It can be used to transform DNA, RNA, or protein (e.g. Ref. [41]) and has been used to create transgene arrays as long as 1100 kb [42]. However, recent data show that the brute-force nature of biolistic transformation is highly destructive to both the transgene and genome and rarely delivers intact molecules larger than a few kilobases [43]. Newer methods involving engineered nanoparticles that naturally penetrate cell walls (without force) are being developed, but at present these can only accommodate small plasmid-scale constructs [44].

Those hoping to apply synthetic biology tools to mammalian systems [45,46] face the same bottleneck of having to work within the confines of *E. coli* cloning vectors. Instead of attempting to adapt *E. coli* cloning methods to fit larger molecules, the Venter group reasoned that it may make more sense to move engineered DNA directly from yeast into animal cells [47]. In a proof of principle paper, they developed methods to fuse yeast spheroplasts with cultured cells to deliver the entire 1.1 Mb *Mycoplasma* genome into human embryonic kidney cells [47]. The literature provides no guidance on whether it is possible to fuse yeast cells with plant cells, which have the additional obstacle of cell walls. However the necessary protocols for preparing plant protoplasts (lacking cell walls) and fusing them with other protoplasts have been well developed over several decades [48]. For example, protoplast fusion was used to create a hybrid between domesticated potato and a wild relative with virus resistance [49].

Protoplasts prepared from most species are delicate and difficult to regenerate into whole plants. Nevertheless, several early studies suggest that protoplasts may be particularly versatile for synthetic biology applications. For example, tobacco and Arabidopsis protoplasts were fused to recombine chromosomes from the two species using the Cre-Lox recombinase system [50]. Another group claimed to have introduced entire wheat chromosomes into maize protoplasts by polyethylene-mediated transformation [51]. In yet another case, purified chromosomes from *Petunia alpicola* were microinjected into *Petunia hybrida* protoplasts, whole plants regenerated, and transformants expressing unique *Petunia alpicola* traits identified in progeny [52]. Although these studies provide a skeletal groundwork they are nevertheless a place to start. The reality is that synthetic plant chromosome design will not be possible without major new developments in plant transformation technologies.

3.2. The centromere problem

There is a complex, largely epigenetic relationship between centromeric DNA sequence and the location of kinetochore proteins that function to move chromosomes. The primary determinant of centromere location is Centromere protein A (CENPA/CENH3) a histone variant that replaces canonical H3 in centromeres and recruits overlying kinetochore proteins [53]. In plants like maize, CENPA binds common repeats that occur in centromeric regions, but not through a causal or functional relationship [54]. CENPA also binds to a variety of other repetitive sequences. If natural centromere sequences are deleted, the entire centromere can move to a previously non-centromeric location [55]. Finally, when natural centromere sequences are transformed into plants, centromeres do not form on those sequences [56]. These data suggest that any plan that involves engineering a centromere by simply introducing centromere repeats is unlikely to work [21].

Although human artificial chromosomes can be created by

transforming cell lines with centromeric DNA [57,58], several features of these chromosomes have made them impractical as vehicles for medical applications or synthetic biology, most notably their tendency to multimerize to sizes >1 Mb (often taking up other non-centromeric sequences in the process) [59,60]. In recent years an alternative method has been developed based on protein tethering, where a cell line is first transformed with a synthetic array of LacO repeats, and then transformed with a fusion protein containing LacI (which binds to LacO) and a key kinetochore protein such as CENPA. In these lines the LacI-CENPA fusion binds to the LacO repeats, recruits additional kinetochore proteins, and the resulting centromere can then be inherited epigenetically without the original fusion protein [61,62]. The tethering system has been extensively used to test the function of proteins involved in centromere assembly [63–67]. The major elements of a kinetochore tethering system have been established in maize, including lines with synthetic repeat arrays containing binding sites for LacI and several other common bacterial and yeast DNA binding proteins [42]. The next step will be to show that DNA binding domain-CENPA fusion proteins will bind to the plant repeat arrays and activate heritable centromere formation.

Another major issue is the size of the centromere. There is a clear relationship between genome size and centromeres size such that species with large genomes have larger centromeres [68], ranging from ~1800 kb in maize ([69] but see Ref. [70]) to 200–300 kb in smaller genomes (e.g. Ref. [71]). Within genomes, centromere sizes are similar regardless of chromosome size [72]. However, natural centromeres have a low density of CENPA-containing nucleosomes [54,73]; centromeres might be engineered to have a high density of CENPA nucleosomes and potentially be much smaller. In *Drosophila*, small circular chromosomes function (albeit imperfectly) with CENPA distributed over only ~15 kb of 256 densely packed LacO repeats and flanking plasmid DNA [61]. Similarly, a recent analysis of human artificial chromosomes showed that CENPA was densely distributed over a relatively small (~200–300 kb) region [60]. In a small genome plant it may be possible to engineer a functional centromere that is as small as ~100 kb. Early-stage plant synthetic chromosomes are likely to be circular, since most human artificial chromosomes are circular and appear to segregate normally in mitosis [60,74]. However, large ring chromosomes are unstable [75,76] suggesting that some mechanism for adding telomeres will also be needed in the long term.

3.3. The meiotic transmission problem

As highlighted by Birchler [21,77,78] a number of studies indicate that small chromosomes do not pair properly at meiosis and frequently display a phenomenon known as premature sister chromatid separation [70,77,79–83]. These failures are associated with all small chromosomes, including those engineered by the top down approach [78], and would presumably affect fully synthetic chromosomes. Cytological analyses show that unpaired chromosomes visibly lag at the midzone in meiosis I and instead of moving to one pole or another, the sister chromatids separate (this would not normally happen until meiosis II) and segregate to telophase I nuclei. Oftentimes, however, lagging chromosomes never move and never separate, and remain in the spindle midzone; other times, the sisters separate but do not make it to telophase nuclei. Even when the sisters do fully segregate, they enter meiosis II with only one chromatid, which often lags at the spindle midzone and stays there. Chromosomes that are lost in the cytoplasm usually degrade and are lost, resulting in a poor transmission frequency.

This phenotype can be largely attributed to a lack of chromosome pairing and recombination. The evidence is based on analyses of small chromosomes that either have no natural pairing partner in the cell (are monosomic for the small chromosomes (e.g. Ref. [80]), or are present as a third chromosome with partial homology to two other large homologs that will naturally pair to the exclusion of the small chromosome (e.g. Ref. [70]). The fact that natural minichromosomes are generally made up centromeric and pericentromeric DNA (depleted for single copy sequences and open chromatin that mediate homology recognition [84,85]) compounds the problem and makes it unlikely they will pair even when brought to a disomic state (as borne out observation [83]). An extensive literature demonstrates that unpaired chromosomes of any type, large or small, have the same tendencies to lag at anaphase I and display premature sister chromatid separation [86,87]. One of the earliest examples was an asynaptic mutant in *Oenothera*, where the chromosomes failed to recombine and the resulting univalents divided to single chromatids that separated at meiosis I [88]. This common phenotype can be largely attributed to the lack of chiasmata, which hold homologs in opposition and promote the separation of homologs [87]. However proper pairing (mediated by the synaptonemal complex [87]) also actively promotes sister chromatid cohesion [89] and helps to maintain the sister kinetochores in close opposition so they move to the same pole [90]. In summary, any chromosome that fails to recombine is likely to show meiotic transmission defects.

To help ensure normal meiotic transmission, synthetic chromosomes should be propagated in the disomic state and engineered to promote at least one crossover. Much is now known about the genes and pathways that regulate recombination and how they promote or suppress crossovers (e.g Ref. [91,92]), although less is known about how the structure of the genome influences these events [84,85,93,94]. In general, gene-rich synthetic chromosomes should be far more likely to recombine than chromosomes composed primarily of pericentromeric heterochromatin. Sequences that increase the likelihood of recombination could be engineered into the chromosome in cis (hotspots [95,96]) and combined with mutants to increase the frequency of recombination of small chromosomes in trans [91,92]. These or any other measures to increase recombination on synthetic chromosomes are likely to pay off in higher transmission rates.

4. Perspective

To create and deploy synthetic chromosomes we will need to harness knowledge from the entire 100-year history of plant genetics, including classical cytogenetics, transmission genetics, molecular cloning, systems biology and metabolic modeling, genomics and epigenomics, careful gene annotation, gene synthesis, and new transformation methods. It is a worthy goal for all the scientific and practical reasons outlined here and more - for the sheer magnitude of the endeavor. The intent of this commentary is to spur additional thinking and effort, as well as to encourage criticism and correction. In the process, we are likely to spark the imagination of young scientists, broaden participation, and promote cross-disciplinary collaborations that enable this goal and help to solve other yet unknown challenges in genetics and plant biology.

CRediT authorship contribution statement

R. Kelly Dawe: Writing - original draft.

Acknowledgements

I thank Jonathan Gent and Feng Zhang for critically reading this manuscript. Work on synthetic chromosomes in the Dawe laboratory is supported by a grant from the National Science Foundation (1444514).

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