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COMMENTARY

Engineered Plant Minichromosomes: A Bottom-Up Success?

Engineered minichromosomes offer an enormous opportunity to improve crop performance, as recently discussed (Houben and Schubert, 2007). Unlike conventional gene transformation technologies, minichromosomes can be used simultaneously to transfer and to express stably (multiple) sets of genes. Because minichromosomes segregate independently of host chromosomes, they provide a platform for accelerating plant breeding and for studying the specific chromatin domains (e.g., centromeric regions) inserted into them.

GENERATION OF ARTIFICIAL PLANT CHROMOSOMES

Strategies for producing artificial chromosomes follow a top-down (engineering of endogenous chromosomes) or bottom-up (de novo assembly from chromosomal constituents) approach. One example of a top-down approach involves telomere-mediated truncation of chromosomes. Farr et al. (1991) showed that cloned telomeric repeats introduced into cells may truncate the distal portion of a chromosome by the formation of a new telomere at the integration site. Using this method, a vector for gene transfer can be prepared by trimming off the arms of a natural plant chromosome and adding an insertion site for large inserts. Birchler and colleagues have recently described a platform for exploiting this strategy in maize (Yu et al., 2006, 2007; reviewed in Houben and Schubert, 2007).

A second report of chromosome engineering in plants was recently published by Preuss and colleagues (Carlson et al., 2007), who have claimed the first in vivo assembly of autonomous plant minichromosomes using a bottom-up strategy. Following a protocol similar to that used for pioneering work in human cells (Harrington et al., 1997; Ikeno et al., 1998), the authors transformed maize cells with centromeric sequences and screened for plants that assembled autonomous chromosomes de novo. The constructs combined a selectable marker gene with between 7 and 190 kb of genomic maize DNA fragments containing centromeric satellite and retroelement sequences and/or other repeats. After particle bombardment into embryonic maize tissue and subsequent transgene selection, microscopic analysis revealed some cases of fluorescent signals at chromatin fragments independent of the regular maize chromosomes. Such fragments were reported to be transmitted mitotically and meiotically (Carlson et al., 2007). As in nonplant organisms (reviewed in Grimes and Monaco, 2005), telomeres were claimed to be unnecessary for minichromosome formation because the artificial chromosomes were presumed to be circular.

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CRITICAL POINTS

The observations and conclusions of Carlson et al. (2007) are surprising in a number of respects. Hitherto the requirement(s) for de novo centromere formation are not clearly defined and appear to be strongly epigenetic (Karpen and Allshire, 1997; Dawe and Henikoff, 2006; Houben and Schubert, 2007). Indeed, a centromeric region translocated from a barley addition chromosome to a wheat chromosome did not confer centromere function (Nasuda et al., 2005). Similarly, centromeric BACs transformed into rice were stably inserted into chromosomes but appeared to lack kinetochore proteins or centromere activity (Phan et al., 2007). BAC insertion into maize chromosome arms was also reported by Carlson et al. (2007), although in most cases, the BAC DNA was detectable as small fluorescent spot-like features on chromosome spreads.

A key claim of Carlson et al. (2007) is minichromosome stability through meiosis. Although the construct from which the minichromosome should have derived is reported to be only 35 kb in size, transmission as followed by the phenotypic marker often reached 50% as a hemizygote (one copy) and 93% as a homozygote (two copies). This is novel in the artificial chromosome literature and unparalleled in plant cytogenetics. Several nonplant artificial minichromosomes, which were of much larger size, showed a lower meiotic transmission frequency (for examples, see Schubert, 2001). In plants, unpaired single or monosomic chromosomes are usually lost at high frequencies during meiosis (Dawe, 1998). An exception is the maize B chromosome, which is specialized for function as a monosomic and can be transmitted at high frequencies (Carlson and Roseman, 1992). However, the maize B chromosome centromere is ~700 kb (Jin et al., 2005), and when it is reduced in size to ~110 kb, its transmission falls to 5% (Phelps-Durr and Birchler, 2004). In the relatively simple yeast system, artificial chromosomes smaller than 50 kb are not transmitted at Mendelian levels (Murray and Szostak, 1983). According to the new maize strategy, the transmission was reported to exceed expectations from other systems. Nevertheless, 93% is still below what would be necessary from a commercial perspective. Optimizing transmission to commercially relevant levels remains a challenge that has yet to be addressed by any of the current artificial chromosome systems.

The ~19 kb of centromeric DNA on the construct described by Carlson et al. (2007) is roughly one to two orders of magnitude smaller than expected based on the literature from natural chromosomes. For instance, *Arabidopsis thaliana* centromeres contain 180-base satellite tandem repeat arrays that range from ~0.4 to ~1.4 Mb between different chromosomes. Nucleosomes that anchor the kinetochore contain a specialized H3 histone referred to as CENH3. The CENH3 binding domains of rice chromosomes range from ~750 to ~1800 kb (Yan et al., 2006). It is possible that natural and artificial chromosomes differ in this regard, as noted in humans (e.g., Okamoto et al., 2007), but currently it is difficult to envision which mechanism

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could ensure accurate transmission of the putative minichromosomes.

Because Carlson et al. (2007) did not test for the presence of CENH3 on independent chromatin fragments that showed fluorescent signals, it remains unclear whether the transmission of the phenotypic marker is based on such structures with a bona fide centromere or, as they suggest, with a neocentromere-like activity as described for terminal heterochromatin of some meiotic rye and maize chromosomes (Yu et al., 1997; Manzanero and Puertas, 2003; Dawe and Hiatt, 2004). A mechanism of transmission based entirely on neocentromere activity of this type seems unlikely, since the maize/rye neocentromeres require linked CENH3-containing centromeres for transmission (Yu et al., 1997). Alternatively, the proposed minichromosomes reported by Carlson et al. (2007) might be transmitted by a heretofore unknown mechanism of chromosome motility, which would be extraordinary.

Immunostaining of CENH3 would provide evidence that the independent fluorescence in situ hybridization signals represent minichromosomes with functional centromeres. Indeed, the fluorescence in situ hybridization signals corresponding to the 19-kb putative minichromosomal target are comparable in size to those on the natural maize chromosomes that possess megabase-sized arrays of the same centromeric DNA (Jin et al., 2005). Thus, an in vivo amplification of the centromeric construct might have occurred, as reported for a number of mammalian artificial chromosomes (e.g., Mejia et al., 2002). Immunostaining of mitotic and meiotic configurations in monosomic and disomic material with antitubulin antibodies would be helpful to test whether one or two minichromosomes properly attach to spindle fibers.

LINEAR OR CIRCULAR: DOES IT MATTER?

Ring chromosomes of a size comparable to their natural linear counterparts proceed through mitosis stably only when none or an even number of sister chromatid exchanges (SCEs) in the same direction occur during S-phase. An odd number of SCEs (or an even number of SCEs in different direction) lead to the formation of interlocked rings or to double-sized dicentric rings (McClintock, 1938). Such configurations trigger a series of breakage-fusion-bridge events, causing continuous DNA breakage and rejoining of the chromosomes concerned (McClintock, 1940). In meiosis, recombination between ring chromosomes would be predicted to produce a double bridge during anaphase I, which would also trigger chromosomal breakage and lead to elimination or rearrangement of the chromatids present at the beginning of meiosis. Thus, pairing and recombination of ring chromosomes should dramatically reduce transmission. However, if ring chromosome homologs fail to pair and segregate in a random manner during anaphase I, the maximum transmission should not exceed 75%. Thus, ring chromosomes are not expected to exhibit the high transmission frequencies reported by Carlson et al. (2007). For example, the transmission frequency of an *Antirrhinum majus* ring chromosome to the progeny from self-pollination was only ~17% (Michaelis, 1959).

Telomerase-deficient *Schizosaccharomyces pombe* mutants with ring-shaped chromosomes show reduced numbers of spores, possibly due to formation of dicentric chromosomes after meiotic recombination (Nakamura et al., 1998). The proposed circular artificial minichromosomes of maize (Carlson et al., 2007) and those reported from mammals (Ebersole et al., 2000) are characterized by a relatively high transmission frequency, despite their ring-shaped structure. A similar degree of stability has been found for a circular minichromosome of *Arabidopsis* (Murata et al., 2007). Thus, the following questions arise: Does the minute size of these artificial ring-shaped chromosomes ensure (more) stable transmission because of the lower probability of being involved in an SCE compared with larger rings? Do two potential disadvantages for normal chromosomes, circularity and smallness, somehow compensate for each other? In addition, it is still uncertain whether the circularity is actually maintained in planta. It is possible that the circular constructs were linearized or reshuffled during bombardment (e.g., Song et al., 2004) or even became integrated into a normal chromosome. Alternatively, amplification of the centromere repeats might have occurred during regeneration and plant growth. Pulsed field gel electrophoresis should be able to separate a minichromosome of ~35 kb to confirm its circularity and size, and probing with telomeric sequences might be used to check for de novo gain of telomeres. Finally, segregation analyses could help to exclude integration of reporter genes into the natural chromosomes as a potential reason for a high transmission frequency.

In summary, Carlson et al. (2007) present a potentially exciting new tool with significant biotechnological applications. The usefulness of the methodology will depend on the support provided by the further investigation still required in this area.

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