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Chromosome Engineering in Tropical Cash Crops

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Abstract: Tropical and subtropical crops such as coffee, cacao, and papaya are valuable commodities, and their consumption is a seemingly indispensable part of the daily lives of billions of people worldwide. Conventional breeding of these crops is long, and yields are threatened by global warming. Chromosome engineering and synthetic biology principles could be used to enhance the synthesis of key metabolites and transmission of wild traits to improve resistance to stress and disease in these crops. This review gives an overview of these approaches. The adoption of these approaches may enhance the resilience of agricultural communities, lead to economic growth and secure the availability of key resources for generations to come.

Keywords: tropical cash crops; coffee; cacao; papaya; chromosome engineering; synthetic biology

Introduction

The global demand for food is hypothesized to grow by at least 70% as the population increases to 9 billion people by 2050 [1]. Unfortunately, climate change has far-reaching implications for global food security, including increased frequency of drought and intense precipitation events as well as elevated temperatures, which may exacerbate the instability caused by low diversity and the high intensity of agricultural inputs [1]. For instance, current estimates indicate that an increase of 1°C might cause a 10% to 20% reduction in the world's production of maize [2]. In fact, meta-analyses of climate change and its impact suggest that by 2030, decreases in crop yields may amount to approximately 50% [3].

The livelihood of millions of smallholder farmers and the survival of several national economies in Africa, Latin America and Asia depends on crops usually considered commodities, or minor or orphan crops; examples are coffee (*Coffea* sp.) and cacao (*Theobroma cacao*) [4–6]. These crops are also believed to be at risk due to climate change [4,6]. For a tropical crop such as cacao, which is mostly grown in West Africa, the maximum temperature tolerated (38 °C) could be exceeded during hot and dry *El Niño* years, and the dry season may extend for one additional month[6]. In Colombia, rising temperatures, longer droughts, and excessive rainfall have reduced coffee yields by 30% since 2008[7]. Also for coffee, rapid deforestation and climate change may lead to the extinction of many wild African species and the permanent loss of diversity for future plant breeding [4].

Papaya is considered an important tropical crop because of its high nutritional value[8], especially its high content of vitamin A, vitamin C, potassium, folate, niacin, thiamine, riboflavin, iron, and calcium [8]. Papaya is also cultivated for papain, an important proteolytic enzyme found in the latex of the unripe fruit [9]. This enzyme improves digestion and can cure ulcers [9]. Papain is also used in softening wool, preparing concentrated fish protein for animal feed, preparing cosmetics (toilet soap, toothpaste, and shampoo), tenderizing meat, and brewing beer [9]. Unfortunately, damage to photosynthetic carbon assimilation due to environmental stress, including water deficits, can reduce the biomass production and net carbon assimilation in papaya [9]. Therefore, a better understanding of its physiology and reproductive biology may facilitate its adaptation to climate change[9].

Unlike in major crops, minor tropical minor crops do not benefit from large public and private breeding programs that stress genomic and marker-assisted recurrent selection but rather focus on simple methods that prioritize backcrossing[5]. Hence, new approaches are urgently needed [5]. The following outlines potential new approaches.

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Fertility and enhanced meiotic pairing

The development of new plant varieties by conventional plant breeding is based on the selection of traits already present in plant species. It relies heavily on sexual reproduction to accumulate favorable alleles for stress tolerance/resistance, nutritional quality, or other agronomic and horticultural traits in a plant genome. Genes that contribute to stress tolerance/resistance or other traits can be obtained from local germplasm resources or via introduced landraces or breeding lines from other breeding programs, wild species, or genera [10]. In *T. cacao* (2*n*=2=20 chromosomes, 430-445 Mbp) [11], the improvement of and selection for desirable traits has also caused accelerated accumulation of deleterious mutations because of population bottlenecks that started 3600 years ago [12,13]. Some of the mutations are suspected to be due to the process of fertilization itself [12]. However, as compared with non-domesticated *T. cacao* varieties such as *Marañón* ($1.68x10^{-5}$) and *Purús* ($1.23x10^{-4}$), in the varieties *Amelonado*, *Contamana*, *Criollo* and *Guianna*, the same domestication process may account for differentially high recombination rates (in cM/Mb, $4.04x10^{-6}$ to $3.91x10^{-3}$) [14]. Thus, selecting for high meiotic recombination rates during conventional breeding might help ameliorate the low individual fitness of modern varieties.

Relatively little is known about the chromosomes of the 22 *Theobroma* species (*Malvaceae*)[15]. All *Theobroma* species have the same diploid number (2*n* = 20) and chromosomes with similar morphology, ranging in size from 0.5 and 2.0 mm[15]. Positive staining with Chromomycin A3 revealed that the prophase chromosomes of *T. cacao* and *T. grandiflorum* exhibit only one pair of terminal heterochromatic bands, which co-localize with a single 45S rDNA site. Each chromosomal complement displays a single 5S rDNA site in the proximal region of another chromosome pair[15]. Despite this apparent chromosome uniformity within the genus, meiotic analyses in some cultivars of *T. cacao* have revealed the occurrence of univalents and several multivalent associations, thereby suggesting structural rearrangements[15].

In terms of breeding strategies, cacao has a long juvenile period of up to 5 years, so selecting for fruit-related traits is time-consuming and expensive because the trees must be maintained for at least 3 more years to visually evaluate the pods [11]. Moreover, cacao mostly involves outbreeding and is highly heterozygous; thus, generating inbred lines from crosses is laborious, and doubled haploid lines are notoriously difficult to develop [11]. Nonetheless, there are a few self-compatible cacao clones, such as the Ecuadorean CCN51 [11] and Costa Rican CATIE-R1 (Figure 1A). Cacao tree plantations also require a large area of land and labor [11]. Each year, the infection of cacao by a variety of pathogens severely cripples global production, with up to of 30% of all pods destroyed before harvest [16]. In West Africa, severe *Phytophthora* spp. outbreaks can destroy all cacao fruit on a single farm [16]. Witches' broom disease caused by the fungal pathogen *Moniliophthora perniciosa* is also known to reduce cacao yields in many cultivation areas in Ecuador and Brazil [11]. Because diseases are a persistent problem for cacao, improved disease resistance through breeding is imperative [16].

Cultivated coffee mostly represents the species *Coffea canephora* Pierre (2*n*=2*x*=22 chromosomes, 364 Mb), and *C. arabica* L. (2*n*=4*x*=44 chromosomes), which was likely derived from hybridization of *C. canephora* and *C. eugenioides* [17]. The basic chromosome number for the genus *Coffea* is considered *n*=11, which is common for most genera of the family *Rubiaceae* [18]. Most *Coffea* species are diploid (2*n*=2x=22) and self-incompatible; however, *C. arabica* L. is the only polyploid species (2*n*=4x=44) of the genus, and it is self-compatible [18]. Although morphologically distinct, *Coffea* species present some common meiotic features, such as a small variation in chiasmata number and a high frequency of bivalents in interspecific hybrids [18]. *In situ* hybridization with total genomic DNA from *C. arabica* and *C. canephora* as probes showed considerable cross hybridization to chromosome preparations from *C. arabica*, *C. canephora*, and a *C. liberica*–introgressed *C. arabica* genotype [19]. All chromosomes can be moderately to highly labeled with probes after GISH analysis, which indicates a close genetic relation between *C. arabica* and *C. canephora* and between the *C* genome of *C. canephora* and the *Ea* subgenome (from *C. eugenioides*) present in *C. arabica* [19].

C. arabica accounts for 60% of the world's total output. The species is cultivated in Latin America and parts of Africa, including Ethiopia, Tanzania, and Kenya (see Figure 1B), whereas *C. canephora* is cultivated in central Africa, eastern Asia, and southern Brazil [20]. One of the key limitations of coffee

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breeding programs is the low genetic variability present among the cultivars of *C. arabica*. This bottleneck is due to a founder effect resulting from the small number of cv. Típica and cv. Bourbon individuals that were introduced in the American tropics to initiate commercial plantations at the beginning of the 17th century [20]. It typically takes 25 years to develop a new coffee variety [20]. Caffeine is a purine alkaloid synthesized by several eudicot plants, including coffee and cacao. Caffeine is synthesized in coffee leaves, where it has insecticidal properties, and in fruits and seeds, where it inhibits seed germination of competing species [17]. In the Arabica variety Caturra, famous for its good beverage quality, the caffeine content is approximately 1.15% (which is high); unfortunately, Caturra is susceptible to infection with *Hemileia vastatrix* (coffee leaf rust) and coffee berry disease (*Colletotrichum kahawae* infection) [20].

Papaya is a diploid organism (2*n*=18) with a small genome, 372 Mb, and has a sex chromosome system[21]. Papaya pachytene chromosomal domains, brightly stained with DAPI, account for approximately 17% of the total papaya genome, which suggests that it is largely euchromatic [22]. Papaya is an herbaceous plant with a very short juvenile period, about 4 months [8]. Once it reaches the reproductive stage, flowers are produced continuously at each leaf axil [8]. Papaya is also a trioecious species with three sex forms: female (X), male (Y or MSY) and hermaphrodite (Yh or HSY)[23]. Wild papaya populations are dioecious — one-half male and one-half female plants — whereas cultivated papaya is predominantly gynodioecious, with two-thirds hermaphrodite and one-third female plants [23]. Hermaphrodite flowers develop into fruits that tend to be elongated and have a small fruit cavity as compared with female plants (see Figure 1C), and these traits are commercially desirable [24,25]; thus, great effort has been placed on determining the sex of plants grown in the field. The X region is only 3.5 Mb, whereas the HSY and MSY regions are about 8.1 Mb and are located within chromosome 1[23]. Notably, any combination of the MSY and HSY chromosomes is inviable [23], which indicates that the MSY and HSY chromosomes lack genes that are essential for embryo development [26]. MSY and HSY are extremely methylated and heterochromatic as compared with regions located on the X chromosome [26].

Figure 1. Tropical crops that may be amenable to genome editing and chromosome engineering. A) Cacao field plot of variety CATIE-R1 at the CATIE Research Center (Limón province, Costa Rica). B) Arabica coffee plants, Catuai cultivar (León Cortés county, Costa Rica). C) Papaya, Pococí hybrid (Alajuela province, Costa Rica). Credits, A-C: Allan Mata-Quirós (CATIE, The Tropical Agricultural Research and Higher Learning Center, Costa Rica), Emmanuel López (Passiflora Coffee Farm, Costa Rica), Eric Mora-Newcomer (University of Costa Rica).

Transcriptome and whole-genome sequencing analyses of papaya have revealed the existence of a putative gene on the sex chromosome, called *Short Vegetative Phase (SVP)-like*, which might be involved in male-hermaphrodite flower differentiation and is present in naturally all-hermaphrodite populations grown in Pingtung, Taiwan [25]. The *SVP-like* gene is expressed specifically in papaya with the male form (Y or MSY chromosome) and hermaphrodite form (HSY chromosome) but is absent in papaya with the X chromosome [25]. The Y chromosome appears to code for an intact SVP-like protein with both MADS- and K-box domains. The HSY chromosome codes for a partial K-box domain, and the X chromosome codes for an even shorter fragment [25]. Close analysis suggests that in the HSY

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chromosome, the *SVP-like* gene has two insertions of *copia*-like retrotransposons [25], and a single polymorphism allows for identifying each insertion [25]. Other genes located on the HSY chromosome, such as the chromatin assembly factor 1 subunit A-like (*CpCAF1AL*), and the somatic embryogenesis receptor kinase (*CpSERK*) are also believed to mediate development of hermaphrodite flowers [26].

Mechanisms of meiosis and fertility

The process of meiosis has a dual role: to generate the genetic diversity transmitted by the gametes but also to ensure proper segregation of chromosomes into the gametes [27]. Defects in recombination and meiosis are a major source of sterility [27]. In *Arabidopsis*, meiotic recombination is initiated by the programmed formation of DNA double-strand breaks (DSBs), catalyzed by the topoisomerase-like protein, SPO11-1, and several other associated proteins such as MTOPVIB, PRD1-3, and DFO [28]. These breaks are processed by several proteins including MRE11, RAD50, NBS1 (the MRX complex) and Exo1 for endonucleolytic cleavage and removal of SPO11, COM1 and CtIP for end processing, for RPA1A-D and RPA2-3 for binding of single-strand DNA breaks at 3′ ends, and for loading recombinases [28]. Meiotic recombination results from the activity of RAD51, RAD51B-D, XRCC2, XRCC3, DMC1 and BRCA2 [28], among others.

Meiotic crossovers shuffle homologous chromosomes to produce unique combinations of alleles that are transmitted to offspring [29]. The formation of meiotic crossovers is crucial for plant breeding and genetic analyses such as the detection of quantitative trait loci or gene mapping [29]. In the absence of crossovers, the homologs segregate randomly, which is a source of infertility [27]. Thus, the formation of balanced and viable gametes requires an "obligate crossover" per chromosome pair [27].

Two major crossover formation pathways exist in plants. The major one depends on the ZMM protein complex (MSH4, MSH5, MER3, HEI10, ZIP4, SHOC1, PTD) plus MLH1/3 and produces interfering crossovers, whereas the minor pathway relies on MUS81 and produces non-interfering crossovers [28]. Crossovers are relatively rare events because active mechanisms limit their formation [29]. In the model plant *Arabidopsis*, 3 anti-crossover pathways operate and rely on the activity of RECQ4, FANCM, and FIGL1, respectively [29]. RECQ4 is a DNA helicase homologue of yeast *Sgs*; *FANCM* encodes another conserved DNA helicase (which requires cofactors MHF1-2); and *FIGL1* encodes an AAA-ATPase [29]. In *Arabidopsis*, the *recq4a/recq4b* mutant showed a four-fold increase in recombination, whereas in *fancm*$^{-/-}$ the increase was three-fold [29]. The *recq4a/recq4b/figl* mutant showed an impressive 8-fold increase in recombination [29]. Work in peas, tomato and rice has shown that the identification of allele mutants for meiotic anti-helicases may boost recombination in crops to facilitate introgression of agriculturally valuable alleles [29].

Plants have two, and possibly three, homologous recombination pathways: 1) the canonical DSB repair (DSBR) pathway and 2) synthesis-dependent strand annealing (SDSA) [30]. DSBR and SDSA pathways share common steps in the induction of breaks but differ in how the resulting displacement loop (D-loop) is resolved [30]. In the DSBR pathway, strand exchange results in double Holliday junction (dHJ) formation, and resolution of the dHJ leads to crossover creation between homologous chromosomes in meiotic recombination [30]. In the SDSA pathway, the HJ is dissolved, which results in non-crossover gene conversion [30]. In plant somatic cells, all homologous recombination of DSBs is believed to proceed through the non-crossover SDSA pathway [30]. Plants have another HDR pathway, single-strand annealing (SSA), which uses homologous sequences within the same DNA sequence for the repair of DSBs [30]. In the SSA pathway, the two free ends of the break anneal at the adjoining region of complementarity, and the non-complementary ends are trimmed [30]. Thus, the SSA pathway results in loss of sequences located between the two repeat regions [30]. The FANCM helicase is believed to be involved in both the SDSA and SSA pathways [30], and its cofactors MHF1-2 are also considered to have a similar role in limiting crossovers and to act genetically in the same pathway [28].

Besides using homologous repair, plants may repair DNA DSBs by non-homologous end joining (NHEJ), via the Ku-dependent, "canonical" NHEJ pathway or the highly error-prone Ku-independent backup-EJ pathway, especially in somatic tissues or cells, which are often used for genetic engineering [30]. The backup-EJ pathway is also called microhomology-mediated end joining[30]. In canonical NHEJ, DSBs are recognized and bound by the Ku70-Ku80 heterodimer and the XRCC4

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ligase, with minimal end processing, which results in minimal DNA loss (1 to 4 nt)[30]. In backup EJ, DSBs are bound by poly (ADP-ribose) polymerase proteins and the MRX complex to promote end-resection and generation of homology between the two DNA strands; however, the broken DNA ends are extensively resected and then extended by the error-prone DNA polymerase θ, which results in large deletions, inversions and translocations [30]. Downregulation of NHEJ genes is believed to shift most DNA repair toward homologous recombination and thus increase the efficiency of CRISPR/Cas9 editing [31,32].

Possibilities for genome editing in coffee, cacao and papaya

Genome editing, an unprecedented technological breakthrough, has provided a means of creating targeted mutations and sequence replacement in plant genomes with high specificity [33]. The CRISPR/Cas9 editing technology is based on bacterial *clustered regularly interspaced, short palindromic repeats* (CRISPR)-associated protein (Cas) adaptive immune systems that protect against invading foreign DNA via RNA-guided DNA cleavage [33]. The system is simple, versatile, cheap and efficient [33].

The structure-based physical mechanism of the CRISPR gene editing process relies on the formation of DSBs and appears to have 2 stages [34]. The first stage is palindrome (PAM) recognition [34,35]. This stage is determined by the PAM sequence and PAM–Cas9 interactions and chromatin accessibility [34]. The second stage is the formation of the R-loop, also known as the target DNA/signal guide RNA (sgRNA)-bound structure [34]. A full-length (20 base pairs) RNA–DNA hybrid helix may not be mandatory for sgRNA-target recognition and Cas9 cleavage, and shorter protospacers may also ensure high targeting efficiency. The Cas9 endonuclease may tolerate mismatches in the PAM-distal (non-seed) region, although perfect base-pairing in the PAM-proximal (seed) region is preferred [34].

In contrast to animal cells, plant zygotic cells are difficult to transform with CRISPR/Cas9 directly because of technological limitations. Currently, tissue culture is required for the transformation of most plant species, preferentially mediated by *Agrobacterium*. Thus, for most plants, callus cells are exposed to gene editing reagents for initial targeting [35]. The efficiency of transformation may depend on the codons used for Cas9 translation and the promoters used for both the sgRNAs and Cas9 gene expression [35].

For cacao, cells isolated from somatic embryo cotyledons and young leaves (Scavina 6 variety) can be transiently transformed by using *Agrobacterium* carrying a CRISPR/Cas9 construct [16]. Apparently stable expression was achieved with the vector pGSh16.1010, which carries the CaMV *35S* promoter [16]. For the *C. canephora* clone 197, the *C. canephora U6* promoter was used to transform 6-month-old embryogenic calli derived from leaf segments for 10 min with a solution containing *A. tumefaciens* strain EHA105 that were selected for tolerance to cefotaxime and hygromycin and grown into plants[33]. A pCAMBIA 5300 binary vector carrying the Cas9 sequence was successfully used for these transformants [33]. Approximately 7% of all transformants tested homozygous for mutations of the phytoene desaturase gene (*CcPDS*, *Cc04_ g00540*) [33].

For papaya, genetic engineering has mostly involved biolistic-mediated transformation related to tolerance to the papaya ringspot virus, a single-strand, positive-sense RNA Potyvirus and quite possibly the most serious viral disease of papaya worldwide [36,37]. Nonetheless, *Agrobacterium*-mediated transformation of somatic and zygotic embryos has also been reported [37]. CRISPR/Cas9-mediated genome editing against several viruses simultaneously in papaya may be technically feasible and within reach [37].

Chromosome structure editing in crops

Besides the targeted editing of key genes, the targeted modification of the chromosome structure is also a possibility in genome engineering [32]. Theoretically, if several DSBs are introduced into the genome, chromosomes might be modified in a directed manner, to create new combinations of chromosomal fragments [32]. For instance, if two DSBs are induced on the same chromosome, intrachromosomal rearrangements may occur by deleting or inverting the area between the 2 breaks.

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However, interchromosomal rearrangements may be obtained by inducing 2 or more DSBs on different chromosomes [32]. Depending on the type of tissue, a somatic or meiotic crossover may be produced by inducing breaks on one or both homologues [32].

In *Arabidopsis*, inversions up to 18 kb were successfully transmitted to the next generation by using an egg cell-specific promoter (*EC1.1*) for Cas9 (SaCas9) expression, and inversion and deletion frequencies were greater in a *ku70-1* mutant than the wild type, which suggests increased inversion formation by mutagenic microhomology-mediated backup EJ [38]. This process has been observed in mice as well [39]. In agriculture, this approach may help reverse natural inversions between related species to facilitate outcrossing and transmission of beneficial alleles [38,39]. For example, in papaya, crosses of species from the related genus *Vasconcella* might promote transmission of tolerance to ringspot virus and the fungus *Phytophthora palmivora*, which destroys the plant root meristem and also causes fruit rot [40]. However, meiosis is usually defective in these wild species[40], and lagging chromosomes and polyads are observed[40], which suggests the existence of chromosomal barriers to the formation of chiasmata.

Another yet theoretical possibility for crops would be to induce an inversion to create new linkage groups and purposely suppress recombination [32]. Suppression of recombination is also observed in chromosomal blocks that control apomixis, a form of reproduction characterized by deregulation of meiosis and embryo development [41]. Apomixis is an effective strategy to retain genome-wide parental heterozygosity; recently, apomixis has been successfully engineered in rice to facilitate clonal propagation of hybrids by seed [42].

Metabolite harvesting

Industrial biotechnology relies on methods of foreign gene introduction to engineer new pathways for the biosynthesis of products [43]. Gene deletion and introduction strategies have been successfully demonstrated in an increasing number of industrial microbes, including *Escherichia coli*, *Saccharomyces cerevisiae*, *Clostridium beijerinckii* for the synthesis of acetone and butanol, and *Corynebacterium glutamicum* for the synthesis of aminoacids [43,44]. In contrast to the traditional focus on optimizing endogenous pathways or reconstituting natural pathways to produce metabolites [44], advances in synthetic biology have enabled the design of new and highly specific metabolic pathways for desired chemicals [44].

Indeed, the *E. coli* strain BL21(DE3) is able to synthesize caffeine (and possibly theobromine) with glucose as the only carbon source [45]. To achieve this synthesis mechanism, several genes including the tea caffeine synthase gene *TCS1* from *Camellia sinensis*, the coffee xanthosine methyltransferase gene *CaXMT* from *C. arabica*, the methionine adenosyltransferase gene *SAM2* from *S. cerevisiae*, the *Vitreoscilla* hemoglobin gene *vgb*, and the guanine deaminase gene *GUD1* from *S. cerevisiae* have been codon-optimized, commercially synthesized, introduced into plasmids and then transferred into competent *E. coli* cells by chemical transformation [45]. Genetic engineering has also been suggested in *C. canephora* and *C. arabica* to create new metabolic pathways for the synthesis of caffeine and vitamin B3 [46]. In addition, the expression of N1-demethylase genes from the *Pseudomonas putida* strain CBB5 in *E. coli* allowed for the recovery of theobromine from caffeine [47]. The synthesis of soluble papain has been achieved in cells of *S. cerevisiae* and the *Pichia pastoris* strain KM71H, with yields comparable to those of pure papaya latex in the latter [48].

However, the full replication of sensorial traits for complex products (e.g., cacao paste, an arabica espresso, or papaya concentrate) may involve extensive synthetic genome engineering in the organism of choice. For example, analysis of the genome of *T. cacao* revealed a high number of genes involved in terpenoid synthesis (for aroma) (55 genes); most are clustered in chromosomes 6, 7 and 10[13]. The number of genes involved in flavonoid biosynthesis (for cancer effects and neuroprotective activities) is approximately 96, whereas that for lipid biosynthesis is 84 [13]. Moreover, the genomes of coffee and cacao are highly heterochromatic and rich in transposable elements [13,49], so genome editing approaches may need to consider that transcriptionally repressed chromatin reduces the rate at which mutations form by seven-fold, particularly when the intracellular concentration of Cas9 is low [50]. Also, CpG methylation is negatively associated with the binding of Cas9 [51].

Taken together, these results suggest that the demand for simple metabolites found in cacao, coffee or papaya may be met by engineering synthetic microbial strains, but a full product may require agricultural genome editing or engineering of very complex synthetic systems.

Concluding remarks

Advances in understanding agricultural genomes in the tropical crops coffee, cacao and papaya may allow for adaptation to climate change and increased output. Nonetheless, industrial engineering of microbial organisms may also meet the demand, either partially or completely. In any case, the economic impact may exceed current expectations and facilitate adaptation to climate change and ecological disturbance.

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