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Synthetic genetic circuits in crop plants

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The love affair between crop breeding and genetics began over a century ago and has continued unabated, from mass selection programs to targeted genome modifications. Synthetic genetic circuits, a recent development, are combinations of regulatory and coding DNA introduced into a crop plant to achieve a desired function. Genetic circuits could accelerate crop improvement, allowing complex traits to be rationally designed and requisite DNA parts delivered directly into a genome of interest. However, there is not yet a standardized pipeline from exploratory laboratory testing to crop trials, and bringing transgenic products to market remains a considerable barrier. We highlight successes so far and future developments necessary to make genetic circuits a viable crop improvement technology over this century.

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Introduction

For the purposes of this review, a synthetic genetic circuit is a set of genetic parts, including both coding and regulatory DNA, that are delivered into an organism and together carry out a desired function. The first functional genetic circuits were described in publications almost two decades ago, and comprised a genetic toggle switch and a repression based oscillator, both in *Escherichia coli* [1,2]. Since then synthetic genetic circuits have been delivered into a variety of organisms, including eukaryotes. For example, recently a 10 enzyme metabolic pathway for the production of alkaloids with pharmaceutical applications was described in yeast [3]. In human T-cells, genetic circuits have been constructed that integrate multiple cellular inputs to induce a killing response [4]. In crop plants, genetic circuits may, in the future, allow traits to be designed to order.

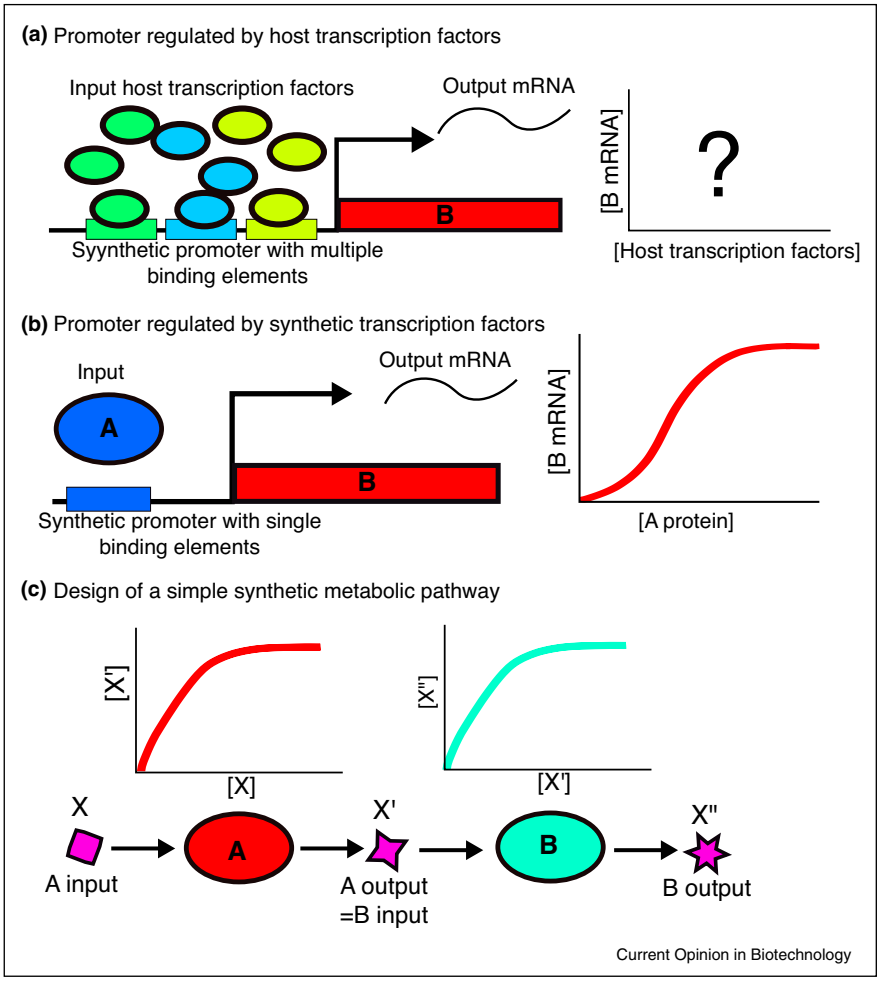
The crop genetic circuit pipeline involves several sequential stages: circuit design, DNA assembly, *in planta* laboratory prototyping, delivery into a crop plant for field trials, and ultimately delivery of the final agricultural product. Of these stages only DNA assembly has been convincingly addressed, with cheap DNA synthesis [5] and reliable methods for scarless multipart assembly of plant gene delivery vectors [6]. In contrast to DNA assembly, there are significant questions remaining at all other stages. How should circuits be designed? How can model plants be effectively used to prototype circuits? How can genetic circuits be efficiently delivered into crop plants? Finally, how can crops containing synthetic genetic circuits be brought to market without resistance from regulators and consumers? This review explores each of those issues in turn.

Approaches to genetic circuit design

Genetic circuit design begins with trait specifications such as increased nutrient level, flower color or pathogen resistance, and translates these into a DNA-based solution. A spectrum of design approaches could in theory be applied to arrive at this solution, from completely random screening of DNA designs through to rational design in a single-step. Random screening is time and labor intensive. Predictive models of plant systems, and accompanying algorithms to reliably generate functional genetic circuit designs, do not yet exist. Even in model microbes, where genetic circuit design is most advanced, software design tools are unable to reliably predict circuit function *in vivo*, although they can help reduce brute-force screening required to build a synthetic genetic circuit by suggesting designs to match user specifications [7]. For now genetic circuit design is likely to combine elements of rational selection of genetic parts with screening to select for desired function. Even without software assistance there are simple design approaches that have proven successful for genetic circuits constructed in plants.

Firstly, circuits should be constructed from *composable parts*. This means that each part should receive a defined input and predictably convert it into a defined output (Figure 1). Selecting parts with compatible outputs and inputs then allows circuits to be built up to achieve complex functions. Plant synthetic promoters composed of cis regulatory elements for endogenous plant transcription factors are commonly used for genetic engineering projects [8] but do not fit the requirements for composable parts (Figure 1a). Since plant transcription factors are often large families with similar binding profiles [9] inputs are often ill defined and the production of outputs, in this case transcriptional units, is not easily predictable,

Figure 1



Composable parts in genetic circuit design. Synthetic promoters containing cis-elements for host transcription factors **(a)** will be regulated by whatever members of that transcription family are expressed in a given cell. Inputs are thus not well defined and the conversion from these inputs to a transcriptional output is hard to predict. By contrast a minimal promoter controlled by a synthetic transcription factor **(b)** has a defined input and the relationship between input transcription factor and output gene expression can be predicted based on initial characterizations. Enzymes are composable **(c)**, converting substrates into products, and are thus often used to create synthetic metabolic pathways. The metabolic pathway here illustrates the principle of composability but is unrealistically simple, excluding, for example, enzyme cofactors. The functions depicted in this figure are illustrative only, but are inspired by Hill equations for transcriptional activation (b), and Michaelis–Menten equations for enzyme kinetics (c).

with behaviour varying according to developmental context [10] among other factors. In contrast, minimal promoters controlled by synthetic transcription factors have defined inputs, and *in vivo* promoter function can, at least broadly, be predicted from earlier characterization (Figure 1b; [11••]). Assuming substrate and product specificity, enzymes fit the requirements of composable genetic parts. They convert substrate X into product X'. Indeed synthetic metabolic pathways make up the largest group of synthetic genetic circuits developed so far in crop plants [12–16] (Figure 1c). While assembly from composable parts may facilitate successful design it is important to note that screening remains a necessary step. Composable parts actually facilitate screening since equivalent input/output conversions can be swapped out until circuit performance matches specifications.

A second enabling design approach is to use *heterologous* genetic parts, meaning those drawn from other organisms or *de novo* designs. As in the comparison of promoters controlled by host or synthetic transcription factors (Figure 1a,b) the use of heterologous parts helps to define inputs and predict outputs. In addition, heterologous parts are more likely to exhibit *orthogonality*, meaning that they do not interact with host components in unexpected ways that may interfere with circuit function. The advantage of heterologous parts and accompanying orthogonality was demonstrated in a comparison of

synthetic genetic circuits encoding ligand sensing systems in plants, constructed from either plant or bacterial components [17]. In addition, creating a new orthogonal pathway can avoid issues of incompatibility that may arise in trying to create hybrid semi-synthetic pathways. For instance several attempts have been made to replace the land plant RuBisCO with cyanobacterial versions having a higher catalytic rate and the ability to form carbon-concentrating protein complexes. Replacing all subunits of native tobacco RuBisCO [18] proved more successful than attempts to create hybrids [19]. A disadvantage of heterologous parts is that each new transgene introduced into crops for human or animal consumption must be tested for toxicity or environmental impact. Whether genetic parts are useable in a final agricultural product is of course an important design specification to be considered alongside ease and cost of circuit development.

Prototyping in model plants

Screening circuit design variants *in vivo* is an important step in the design cycle. Model plants such as *Arabidopsis*, with well annotated genomes and low-cost gene delivery, can be excellent platforms for testing design ideas and screening genetic circuit variants [20]. However, moving a genetic circuit from a model plant to a crop will expose the genetic components to an array of new parts not accounted for in prototyping. There has been little direct research into this issue, though some cite the conservation of core cellular processes as reason to be optimistic [21]. A detailed characterization of a simple transcriptional circuit in *Arabidopsis* and *Sorghum* protoplasts showed that some, though not all, circuit performance parameters correlated well between species [11••]. One solution might be to use heterologous genetic parts as described above. An alternative might be to use model plants that are close relatives to the target crop plants, though the extent to which this facilitates circuit transfer has not been tested systematically.

Delivery of genetic circuits into crop plants

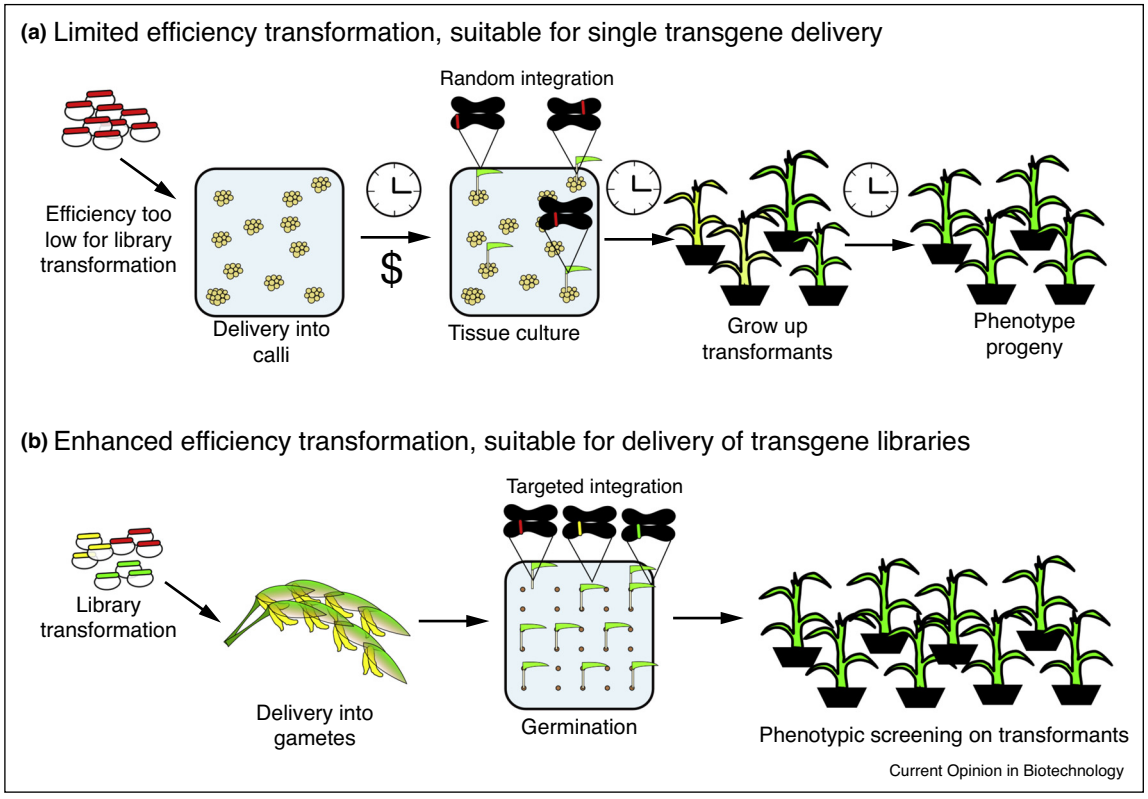
Designs prototyped in model plants must ultimately be delivered into crop plants, and where possible crop plants can themselves be used for prototyping designs. Simultaneous delivery of multiple enzyme-encoding transgenes into Maize yielded a range of transgene assortments, allowing both dissection of the metabolic pathway of interest and the recovery of lines with high titres of the desired end product [13]. However, such screening approaches are currently highly resource intensive because crop transformation is slow, expensive and low throughput (Figure 2). Delivery is largely limited to tissue culture or biolistic delivery [22]. Recovery of transgenics is time and labor-intensive [23]. By contrast, the *Agrobacterium tumefaciens* floral dip method, largely restricted to *Arabidopsis*, and more recently *Camelina* [24], produces hundreds of transgenic progeny ready from germination to be screened for a phenotype of interest. The success or failure of floral dip apparently often lies in the degree of access to developing ovules inside the flower [25], an issue that might be addressed with protocol optimizations or the use of plant varieties with altered floral structure. Indeed successful floral dip has been reported for a few crop species [26,27]. In addition, non-*Agrobacterium* plant-associated bacteria are being explored as gene delivery vectors for crop transgenesis [28].

Gene delivery can be random or targeted, and may be single or variable copy number. Transgene position effects and copy number variation are important considerations for crop engineering pipelines [29]. Whereas variability in gene expression may be surmountable when working with single transgenes, it could dramatically complicate or even preclude analysis of genetic circuits that are reliant on co-ordinated gene expression. A major contributor to variability is differential interference from neighboring genetic loci resulting from random genomic insertion [29]. Two main alternatives have been developed for targeted gene delivery: endonucleases catalyzing homologous recombination and site-specific recombinases. Homologous sequences flanking transgenes can be used to insert into the corresponding genomic locus after cutting with a targeted endonuclease. Concurrently mutating or repressing expression of DNA ligase 4 reduces activity of the competing repair pathway, and this approach has been used to promote gene targeting in *Arabidopsis* and rice [30–32]. Advances in construct design for homologous recombination using programmable endonucleases are steadily improving both reliability and efficiency of this approach [33•]. Site-specific recombinases are another means to achieve targeted gene insertion. For instance the bacterial Cre-Lox and FLP-FRT systems have been successfully demonstrated in rice [34,35]. Targeted gene delivery is an important enabling technology for crop genetic circuits, reducing the unpredictable effects of random genomic insertion (Figure 2).

Transfer of genetic circuits between varieties

Perhaps in the future, minimal genome versions of various crop plants will be available to act as chassis for new genetic circuits, analogous to the development of the liverwort *Marchantia polymorpha* as a chassis for non-agricultural applications [36]. In the meantime, genetic circuits will be integrated into existing varieties as part of breeding programs. We will need to be able to move circuits between crop varieties, a process known as introgression. This conventionally involves crossing plant lines and then multiple rounds of backcrossing, guided by DNA markers, to move just the target allele and as little as possible from flanking genomic regions [37]. If a genetic circuit has been built over time from several transgenic events, and is scattered over the crop genome, introgression would be slow if not impossible. In addition

Figure 2



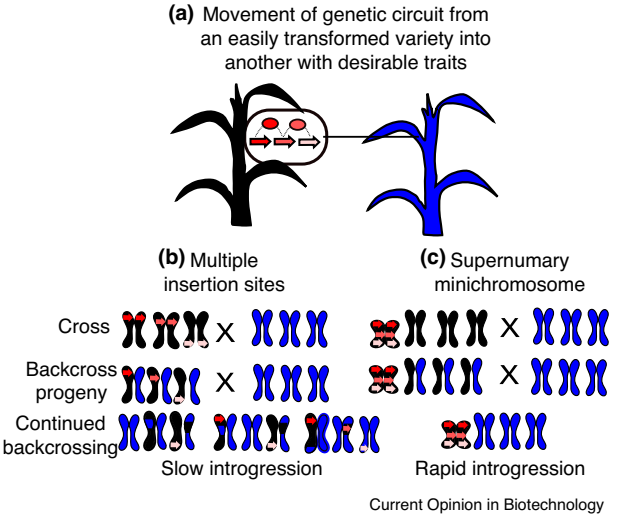
Comparison of current methods for crop plant transformation with an idealized workflow. **(a)** Currently most crop plants are transformed by delivery of DNA into calli (clumps of undifferentiated cells). Insertion occurs randomly in the genome. Tissue culture and marker selection on plates containing plant hormones and a selection agent allows for transformants to be recovered, though the stress of this process is such that reliable phenotyping is delayed until the next generation. **(b)** In the future, crop plants would ideally be transformed through a high efficiency, targeted gamete transformation to enable immediate screening.

the process of introgression is not just slow but often incomplete. Undesired alleles that are physically close to a desired allele are difficult to exclude. This problem, known as linkage drag, is a persistent problem for breeding programs [38]. Ensuring that transgenes are stacked will greatly limit this. One solution is to create a defined landing pad in the genome with endonuclease or recombinase target sites and to stack genes at this locus (Figure 3). This approach has been demonstrated in crop plants using both zinc-finger nuclease [39] and site specific recombinase approaches [40].

An even more powerful approach is to designate an entire chromosome as a landing pad for synthetic genetic circuits. This chromosome can then be easily tracked and moved between cultivars [41•] (Figure 3). Minichromosomes have been created in maize [42] and rice [43] through insertion of telomere sequences into an existing chromosome, truncating it. Recombinase target sites could be introduced along with the telomere sequences to create a landing pad for targeted transgene insertion [42]. A suggested alternative is to create Plant Artificial Chromosomes bearing centromere sequences, and use these as vectors to deliver large circuits into plants. This method has been reported in Maize [44], though these results have been contested [45].

Plastid genomes, naturally protected from chromosomal recombination, are an attractive alternative to artificial minichromosomes. In a recent study several variants of a four gene circuit for artemisinin production were transformed into tobacco plastids and assessed for performance before adding additional genes via nuclear transformation [46]. Plastid genomes are multicopy in each plant cell, which could be advantageous for applications requiring high protein production [47], though this could prove disadvantageous for circuits whose function is sensitive to copy number. Unlike nuclear genomes, plastid genomes are highly amenable to homologous recombination, facilitating genetic circuit design, as described above. Barriers remain, plastid transformation of crop plants is only commonplace for tobacco [47], and the regulation of gene expression, particularly in non-green plastids requires further research [48]. Plastids and nuclear

Figure 3



Minichromosomes could facilitate the movement of large gene circuits between cultivars. On the left a plant carrying a circuit distributed across multiple chromosomes is to be introgressed into a desirable cultivar. Recovery of individuals carrying all circuit genes and no alleles from the original cultivar would be complex and laborious. A minichromosome, carried by the delivery cultivar but not the target cultivar, could be used to more easily introgress a whole circuit [45].

minichromosomes will likely become increasingly commonplace genomic containers of synthetic genetic circuits in crop plants as the technology for their manipulation matures.

Responsible innovation

There is little purpose developing a pipeline that brings genetic circuits into crops if these crops cannot be brought to market. Considering the persistent negative attitudes and strict regulation of current genetically modified crops [49•], this may prove the most difficult barrier to overcome for application of the technology. Indeed, the Golden Rice project, hailed as the flagship genetic circuit crop, has yet to be distributed in the Philippines, with the delays largely due to negative responses from the public [50]. Some solutions may themselves be biotechnological, such as use of cytoplasmic male sterility or genetic use restriction circuits [51] to prevent transgene release [52]. Yet, there also has to be an appreciation of the broader social implications of technology. For instance, genetic use restriction circuits also prevent seed saving and replanting, requiring farmers to purchase seed each season, which could prove prohibitively expensive to small-scale farmers in less affluent regions [53].

Participatory breeding programs directly link together breeders, farmers and consumers of agricultural products into the crop development pipeline [54•]. Participatory breeding aims to develop crops appropriate for a specific region or use, as a deliberate counterpoint to centralized crop development, which aims to achieve maximal end use to improve the financial return on R&D investment [54•]. In the USA the registration of new crops has become more concentrated among a limited number of large companies with a lesser role for university-led crop development [55]. However, while plant breeding as a whole has shifted from public to private, the development of genetic circuit technology remains largely in the public domain. This provides an opportunity for members of the research community to set responsible innovation standards that address legitimate public concerns. In addition, initiatives like the Public Intellectual Property Resource for Agriculture (www.pipra.org) can help ensure that innovation is directed to clear public benefit.

Outlook

Moving from shuffling and editing natural genetic circuits to constructing new ones seems like a logical progression. Further work is needed to shape a reliable pipeline for engineering diverse crop varieties, starting from the generation of genetic circuit designs and including the process of transferring knowledge from lab to field. Broad stakeholder engagement needs to happen alongside this technology development to ensure that innovations are applied for the public good and are acceptable to regulators, growers and consumers.

Conflict of interest

The authors have no conflict of interest to report.

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