# Advanced genetic tools for plant biotechnology

*Wusheng Liu[1], Joshua S. Yuan[2] and C. Neal Stewart Jr[1,3]*

Abstract | Basic research has provided a much better understanding of the genetic networks and regulatory hierarchies in plants. To meet the challenges of agriculture, we must be able to rapidly translate this knowledge into generating improved plants. Therefore, in this Review, we discuss advanced tools that are currently available for use in plant biotechnology to produce new products in plants and to generate plants with new functions. These tools include synthetic promoters, 'tunable' transcription factors, genome-editing tools and site-specific recombinases. We also review some tools with the potential to enable crop improvement, such as methods for the assembly and synthesis of large DNA molecules, plant transformation with linked multigenes and plant artificial chromosomes. These genetic technologies should be integrated to realize their potential for applications to pressing agricultural and environmental problems.

*Agrobacterium tumefaciens*-mediated transformation
The most common plant transformation method. It involves the transfer of genes of interest from *A. tumefaciens* vectors and the subsequent integration of these genes into plant nuclear genomes.

[1]*Department of Plant Sciences, University of Tennessee, Knoxville, Tennessee 37996, USA.*
[2]*Department of Plant Pathology and Microbiology, Texas A&M AgriLife Synthetic and Systems Biology Innovation Hub, Institute of Plant Genomics and Microbiology, Texas A&M University, College Station, Texas 77843, USA.*
[3]*BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA.*
*Correspondence to C.N.S.Jr*
*e-mail: nealstewart@utk.edu*
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There are numerous complex challenges in translational and applied plant biology, which include feeding and clothing a rapidly growing global population while maintaining environmental quality. At one end of the spectrum, there is a need to improve existing plant characteristics for better crop performance, particularly with respect to improving yield and stress tolerance in crops to adapt to changing environments[1,2]. At the other end of the spectrum, there are many new functions and tasks that we might wish for plants to carry out, such as biosensing and producing valuable compounds. In the middle of this continuum is the alteration of plant metabolism, development and growth, which could improve existing functions or make new products. Biotechnology is required to help to meet these needs and expectations in plant sciences and agriculture.

Genetic engineering in plants is not a new technology; it is now more than 30 years old. The main tools for introducing heterologous DNA into plants, *Agrobacterium tumefaciens*-mediated transformation and biolistics, were invented in the 1980s. All transgenic crops that are currently commercially grown were produced using these methods. Genetic engineering directly manipulates the genome of an organism either by the introduction of one or several new genes and regulatory elements, or by decreasing the expression of endogenous genes. For either of these end points, a DNA construct is inserted into one or more chromosomes in a random manner and into one or more loci. This approach has been effective in cases in which simple traits, such as herbicide tolerance and insect resistance, have been added to plants. However, the random nature of gene insertions can have undesirable effects, and these methods are not favourable for making large concerted changes, such as adding an entire metabolic pathway into a plant[3].

Multigene transfer, site-specific integration and specifically regulated gene expression are crucial advanced approaches in plant biotechnology. In this Review, we begin by discussing recent advances that allow a more precise regulation of gene expression in plants, including synthetic promoters, transcriptional activators and repressors. Then, we address advances in genetic tools for the assembly, synthesis and transformation of large DNA inserts and multigene engineering, targeted genome modification and transgene bioconfinement. The site-specific integration of multiple or stacked transgenes could be achieved with the help of plastid transformation or engineered nucleases for precise genome editing. Artificial chromosomes could also have a key role in next-generation transgenic technologies. Even though advances in these genetic tools are also applicable to basic research in plant biology, the focus of our Review is the applications of these tools to plant biotechnology.

## Regulation of gene expression

Transcriptional regulation of endogenous genes and the precise control of transgene expression are major challenges in plant biotechnology. Synthetic promoters, and

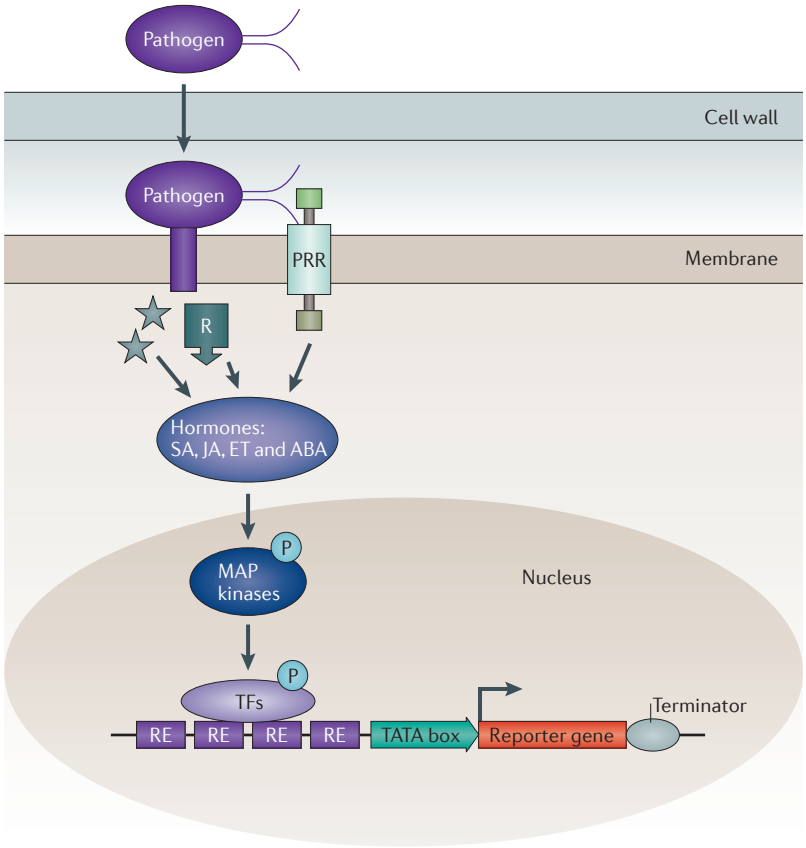


Figure 1 | **Synthetic promoters for the phytosensing of plant pathogenic bacteria.** Regulatory element (RE) tetramers (four head-to-tail copies) of one *cis*-regulatory element that is inducible by a hormone signal can be used in each bacterial pathogen-inducible promoter variant. Such regulatory elements can include a salicylic acid (SA) regulatory element, a jasmonic acid (JA) regulatory element or an ethylene (ET) regulatory element. On pathogen attack, transgenic plants activate hormone defence pathways via pattern-recognition receptors (PRRs), pathogen-secreted effectors (shown as stars) or acquired resistance (R) proteins. The mitogen-activated protein (MAP) kinase pathway is then activated to phosphorylate (P) transcription factors (TFs), which, in turn, activate the expression of pathogenesis-related genes. On binding of these transcription factors to the synthetic promoters, the fluorescent protein reporter is expressed[8,9], indicating a sensing event. ABA, abscisic acid.

transcriptional activators and repressors are the most important tools for the precise regulation of transgene expression in space and time.

***Synthetic promoters.*** Endogenous plant promoters are typically long and weak — they are generally more than 1 kb in size and are much weaker than virus-based constitutive promoters that have been commonly used in plant biotechnology. By contrast, synthetic promoters can be designed to be short and strong, and they can be used as regulatory devices for constitutive, inducible, spatial (tissue-specific) or temporal (developmental stage-specific) gene expression[4]. Synthetic promoters typically use computationally designed and empirically tested *cis*-regulatory elements (or motifs), which act as sites for transcription factor binding. The *cis*-regulatory architecture (motif sequence, position, copy number and specific combination) is the principal target of synthetic promoter engineering[4]. The typical synthetic promoter contains DNA sequences that are found in native plant promoters, but these sequences are usually rearranged and condensed in a form that does not exist in nature. Thus, synthetic chimeric promoters can be designed using varied formats and sequences to fine-tune gene expression and to avoid homology-dependent gene silencing when plants are engineered to express multiple coordinated transgenes[5–7].

In one example, multimerized *cis*-regulatory elements and signal transduction pathways were used to create phytosensors that detect plant pathogenic bacteria[8,9]. A reporter gene encoding a fluorescent protein was driven by synthetic promoters that contain plant regulatory elements which are inducible by plant signal-defence compounds (that is, salicylic acid, ethylene and jasmonic acid). These synthetic promoters were specifically inducible by different bacterial pathogens in transgenic tobacco and *Arabidopsis thaliana* (FIG. 1). These early studies illustrate the potential of using synthetic promoters to develop a range of phytosensors that will be useful in commercial agriculture and other applications.

Synthetic promoter engineering in plants is currently limited by the availability of known functional *cis*-regulatory elements and by computational modelling software. Throughout the history of plant biotechnology, deconstructive analyses by creating 5′ deletions were used to infer the functions of promoter elements. Synthetic promoter design relies more heavily on reconstructive analyses that involve adding *cis*-regulatory motifs, which are often identified by database-assisted motif analyses[10]. We are also limited in our experience regarding the design strategy for the assembly of different units to generate functional promoters. The accurate dissection and functional interpretation of the complex *cis*-regulatory architecture in plants have a key role in synthetic promoter design and provide valuable sources for both 'bottom-up' and systematic design of synthetic promoters. Such studies use both bioinformatics-based *de novo* motif discovery[11,12] and experimental approaches.

One study[13] integrated bioinformatics, using the binding site estimation suite of tools (BEST) of five bioinformatic tools, with the use of synthetic promoters for the discovery of novel elicitor-responsive *cis*-regulatory elements in *A. thaliana*. Our laboratory also applied a set of seven bioinformatic tools for the *de novo* discovery of 5–7 bp-long soybean cyst nematode (SCN)-inducible motifs that were discovered in the soybean genome. This was followed by a functional analysis using synthetic promoters in a transgenic soybean hairy root system (W.L. and C.N.S.Jr, unpublished observations). Taken together, these approaches allow the discovery and refinement of *cis*-regulatory architectures for synthetic promoter design, which can be used to markedly refine transgene expression. In addition, the construction of combinatorial promoter libraries has the potential to greatly aid the engineering of synthetic promoters[14].

Biolistics
A commonly used transformation method in which high velocity microprojectiles coated with gene constructs are used to deliver genes into cells and tissues.

Targeted genome modification
The fusion of engineered DNA-binding proteins or domains with sequence specificities to effector domains that modify genetic sequences and/or gene expression.

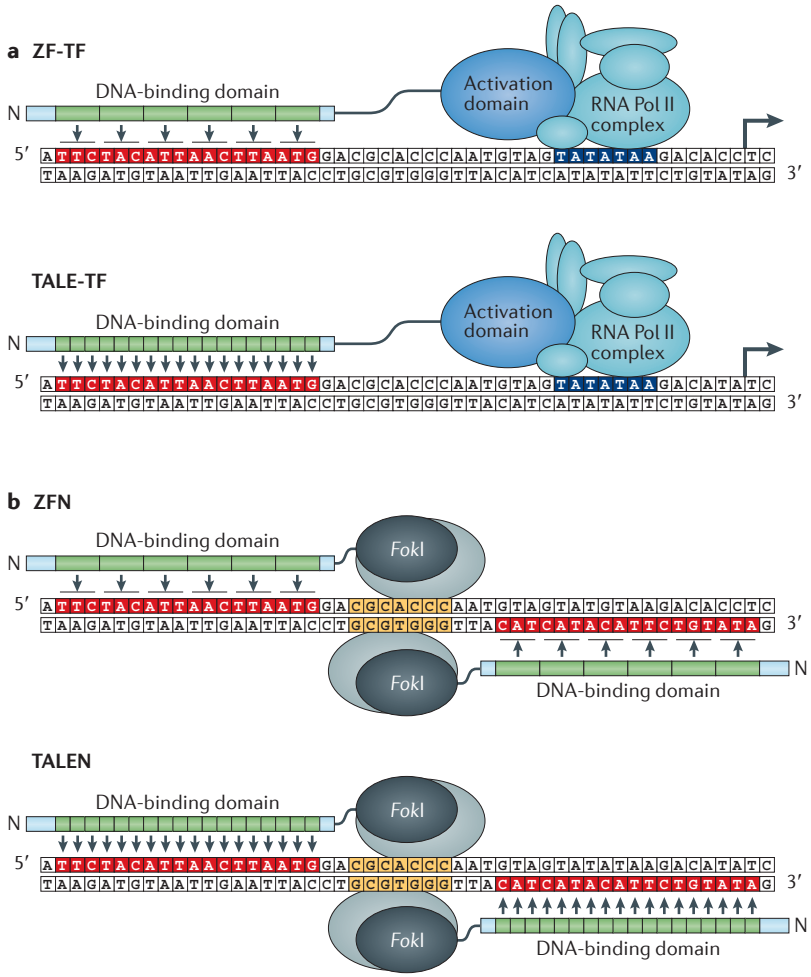


Figure 2 | **Synthetic transcription factors for targeted gene activation or targeted genome modification.** **a** | Targeted gene activation is shown. Synthetic transcription activators consist of activation domains fused to the DNA-binding domains of zinc-finger transcription factors (ZF-TFs)[16–26] or transcription activator-like effector transcription factors (TALE-TFs)[28,31]. TATA boxes are indicated in dark blue (note that TALE-TFs also work on some TATA-less genes) and the transcription initiation sites are indicated by horizontal arrows. **b** | Targeted genome modification is shown. Engineered nucleases such as zinc-finger nucleases (ZFNs)[77–79] or TALE nucleases (TALENs)[80,81] contain the non-specific DNA-cleavage domain of the *Fok*I endonuclease. They can be used to generate double-strand breaks (DSBs) at any specific genomic location and provide favourable characteristics for genome editing. The distance from the transcription initiation sites to the DNA-binding sites of ZF-TFs and TALE-TFs is not fixed and might vary with different specificities, whereas the spacer regions between the monomers of ZFNs and TALENs are 5–7 bp and 6–40 bp in length, respectively. Synthesis of zinc-fingers requires optimization of the designer tandem arrays, which are sometimes context-dependent arrays, whereas the tandem repeats of TALE DNA-binding domains are highly independent of neighbouring repeats. N, amino terminus; RNA Pol II, RNA polymerase II.

Genome editing
Genome modification achieved by the induction of a double-strand break in a specific genome site, followed by DNA-break repair and the generation of desired modifications (gene disruption, addition or correction).

Even though some plant synthetic promoters have been generated (see Supplementary information S1 (table)), synthetic promoter design for plants is still in its infancy. Most so-called synthetic promoters have been created by inserting functional promoter regions or motifs into native plant promoters without computational modelling. For example, the synthetic constitutive promoters *Pcec*[15] and *Mac*[16] were constructed by inserting transcriptional enhancer domains upstream of native constitutive promoters, which conferred much higher levels of reporter-gene expression in transgenic plants than those conferred by native promoters. As technology develops, we anticipate that even better results will be gained by using an integrative approach that combines computational modelling, large 'omics' data sets, increased knowledge about promoter–transcription factor interactions, and improved screening technologies. Such integration will result in synthetic promoters that bear little resemblance to those found in nature.

***Synthetic transcriptional activators and repressors.*** Even though synthetic promoters might be considered the first priority for fine-tuning transgene expression in plants, synthetic transcriptional activators or repressors could be used to regulate the expression of either endogenous genes or transgenes in plant genomes[17]. Fusion proteins that consist of engineered DNA-binding domains and catalytic effector domains hold great promise for targeted gene expression under the control of plant constitutive promoters[18] and for precise genome editing (FIG. 2) (see below).

Engineered zinc-finger proteins (ZFPs) have been used for gene activation or repression in plants by fusing them to transcriptional activation or repression domains, respectively (FIG. 2a). ZFPs bind to DNA targets as monomers, each of which consists of a tandem array of 3–6 or more C2H2 fingers, and target specific DNA sequences that are 9–18 bases long[19]. Synthetic zinc-finger-transcription factors (ZF-TFs) that contains activation domains have been used for the targeted activation of reporter genes[20,21] and endogenous genes in *A. thaliana*[19,22–25] and canola[26]. They have also been used in transgenic *A. thaliana* to downregulate gene expression by competing with other transcription factors for the same binding sites on the rice tungro bacilliform virus promoter[27].

Engineered transcription activator-like effectors (TALEs) can also be used for targeted gene expression (FIG. 2a). Each of these artificial regulators contains an amino-terminal translocation domain, a central DNA-binding domain and a carboxy-terminal domain, which includes nuclear localization signals and an activation domain. Synthetic TALEs might be simpler to design than ZFPs, as they do not have to be screened against expression libraries, which is a requirement of ZFP design[28]. The DNA-binding domains of *Xanthomonas* spp. TALEs are composed of 1.5–33.5 (mostly 15.5–19.5) tandem repeats that are nearly identical. Each repeat is 30–42 (typically 34) amino acids long[29]. The specificity of individual repeats is encoded in a repeat–variable diresidue at positions 12 and 13 of each repeat[28,30]. The distance from the transcription initiation sites to the DNA-binding sites of TALE-TFs and ZF-TFs is not fixed and might vary with different specificities. In one study, *Xanthomonas* TALEs that contained their own activation domains and were driven by the constitutive CaMV 35S promoter were used to specifically induce the expression of the tomato *Bs4* gene and the *A. thaliana EGL3* and *KNAT1* genes[31]. The target sites of these TALEs were 46–108 bp upstream of the transcription

initiation sites. This induction resulted in an increase in the number of trichomes in tomato and in a greatly altered leaf morphology in *A. thaliana*. In another study, targeted repression was confirmed for both transgenic and endogenous *RD29A* expression in *A. thaliana* after cold, salt or abscisic acid treatments when TALE DNA-binding domains were fused to an ERF-associated repression domain SRDX[32].

In addition, transcriptional activation, recombination and other genetic or epigenetic effects (such as methylation, acetylation or deacetylation, and amination or deamination of transgenes or endogenous genes) in plants could potentially be achieved using synthetic TALEs. For example, synthetic TALE-TFs with chimeric activation domains have been designed to target various 19–25 bp sequences of the 35S promoter, resulting in a twofold to threefold induction in reporter-gene expression (W.L., C.N.S.Jr, J. D. Chesnut, M. Mazarei, R. J. Millwood, Y. Peng, M. R. Rudis, W. Xu and J.-P. Yang, unpublished observations).

ZF-TFs and TALE-TFs are promising tools for the regulated expression of endogenous genes in their native context, and they have the potential to be applied to crop improvement. They could be used to activate key regulatory proteins that are master switches for entire metabolic and developmental pathways[26]. However, generating ZF-TFs and TALE-TFs with suitable specificity and efficiency will require careful design and testing. Important considerations include the location, nucleotide sequence and uniqueness of the DNA-binding site within the target promoter; choice of activation domain; and off-target effects. Synthetic inducible promoters could be paired with synthetic transcription factors for specific and high levels of bioproduct synthesis in space and time.

## Advanced DNA assembly and synthesis

Engineering plants for a greater range of applications requires the introduction of multiple genes or entire pathways into the plant genome. Our ability to meet this goal is highly limited by traditional approaches that rely on multiple rounds of transformation, traditional breeding methods and transgenes that are located on multiple loci. So far, multigene transfer, through the co-transformation of multiple linked or unlinked genes, has been successfully achieved in some plant species. This approach has allowed the integration of 4–9 genes into a single transgenic line, either with or without the help of plant breeding, for metabolic engineering or for the production of multimeric protein complexes[3].

The assembly and synthesis of large DNA molecules or multigenes in a single transgene vector provide an alternative approach for multigene transfer. Seamless assembly of long DNA modules, either with or without the *de novo* DNA synthesis of smaller pieces, is needed for efficient construction in this context. The commercial synthesis of ~1–3 kb-long DNA fragments is routine. Seamless assembly of these fragments (see below) can already be used to generate molecules of more than 10 kb, and, in theory, there are no boundaries to the size of DNA construct that can be produced. Using current DNA synthesis technologies, *de novo* synthesis of entire plant genomes is out of reach, but it is feasible to synthesize small artificial chromosomes and to by-pass cloning steps. There are also new tools that can be applied for more efficient DNA assembly, or for both DNA assembly and transformation (such as multiple-round *in vivo* site-specific assembly (MISSA) and the use of binary bacterial artificial chromosomes (BIBACs) (discussed below).

Recently developed DNA assembly methods have been applied to bacterial systems and could be used in plants in the future. These methods rely on either standardized restriction enzyme assembly methods or sequence-independent overlap techniques (TABLE 1). The standardized restriction enzyme assembly methods use type II restriction enzymes and produce a set of interchangeable DNA parts. Among these methods, the BioBrick[33] and BglBrick[34] methods leave 6 bp- or 8 bp-long 'scars', respectively, which might be problematic in some constructs because they could result in out-of-frame translation or in the addition of unnecessary or undesirable amino acids. By contrast, the GoldenGate assembly method generates scarless assembly of neighbouring fragments using homing endonucleases[35]. The overlap assembly techniques use either specific recombinases or the 'chew-back-and-anneal' method, which involves digesting one strand of DNA back to produce overhangs at the ends of fragments for annealing without ligation. Such methods include Gateway[36], uracil-specific excision reagent (USER)[37], InFusion[38], sequence- and ligase-independent cloning (SLIC)[39] and Gibson assembly[40]. Among these approaches, the Gibson assembly method significantly increases the efficiency of assembling large DNA molecules and was used, for example, in the assembly of the 1.08 Mb genome of *Mycoplasma mycoides* JCVI-syn1.0, which started with ~5–7-kb synthesized overlapping fragments[40]. In contrast to the restriction-enzyme assembly methods, these overlap assembly methods are not suitable for the assembly of DNA fragments with repetitive sequences, which would be targets for recombination and could result in deletion or rearrangement.

In addition to these assembly methods, several automation and assembly software packages have been published to greatly help gene assembly. For example, when a list of initial sequences is given, the j5 (REF. 41) software is useful for designing assembly strategies using the SLIC, Gibson and GoldenGate methods. Additionally, a standardized assembly system, GoldenBraid, was recently developed specifically for plant synthetic biology[42]; it extends the capabilities of the GoldenGate cloning system by converting the single-use multipartite assemblies into reusable composite parts.

## Transformation with large constructs

Multigene transfer into plants enables researchers to import entire metabolic pathways, to express multimeric protein complexes and to engineer genetic elements and regulatory hierarchies[3]. Large DNA constructs and multigenes can be integrated into a suitable plant host by either organelle or nuclear transformation. However, in our opinion, artificial chromosomes provide the most promising transgenic technology for integrating long

Homology-dependent gene silencing
A gene silencing phenomenon induced by homologous sequences at the transcriptional or post-transcriptional levels.

Zinc-finger proteins
(ZFPs). DNA-binding protein domains that consist of a tandem array of 2–9 zinc-fingers, each of which recognizes approximately three bases of DNA sequence.

Transcription activator-like effectors
(TALEs). Major virulence factors (containing an amino terminus, a unique type of central DNA-binding domain and a carboxyl terminus with the activation domain) that are secreted by the pathogenic *Xanthomonas* spp. bacterium when it infects plants. Their DNA-binding domains can be custom-designed to specifically bind to any DNA sequences.

Seamless assembly
The precise joining of DNA fragments without the addition of intervening or unwanted nucleotides at the junctions.

*De novo* DNA synthesis
The synthesis of continuous strands of DNA molecules using a laboratory instrument without the presence of pre-existing templates.

Table 1 | **Current DNA assembly methods for the synthesis of large DNA molecules**

| Method | Mechanism | Overhang (bp) | Scar (bp) | Comments | Examples of application | Refs |
|---|---|---|---|---|---|---|
| BioBricks | Type IIP restriction endonuclease | 8 | 8 | Sequentially assembles small numbers of sequences | Construction of a functional gene expressing enhanced cyan fluorescent protein | 33 |
| BglBricks | Type IIP restriction endonuclease | 6 | 6 | Uses a highly efficient and commonly used restriction endonuclease, the recognition sequences of which are not blocked by the most common DNA methylases | Construction of constitutively active gene-expression devices and chimeric, multidomain protein fusions | 34 |
| Pairwise selection | Type IIS restriction endonuclease | 65 | 4 | Requires attachment tags at each end of fragments to act as promoters for antibiotic resistance markers; rapid, as a liquid culture system is used | Assembly of a 91 kb fragment from 1–2 kb fragments | 135 |
| GoldenGate | Type IIS restriction endonuclease | 4 | 0 | Allows large-scale assembly; ligations are done in parallel | One-step assembly of 2–3 fragments | 35 |
| Overlapping PCR | Overlap | 0 | 0 | Uses overlapping primers for the PCR amplification of 1–3 kb-long fragments | Usually used for 1–3 kb-long fragments, for example, for gene cassette construction | 136 |
| CPEC | Overlap | 20–25 | 0 | Uses a single polymerase for the assembly of multiple inserts into any vector in a one-step reaction *in vitro* | One-step assembly of four 0.17–3.2 kb-long PCR fragments | 137 |
| Gateway | Overlap | 20 | 0 | Uses a specific recombinase for small-scale assembly | One-step assembly of three 0.8–2.3 kb-long fragments | 36 |
| USER | Overlap | Up to 708 | 0 | Replaces a thymidine with a uracil in the PCR primers, which leaves 3′ overhangs for cloning after cleaving by a uracil exonuclease | One-step assembly of three 0.6–1.5 kb-long fragments | 37 |
| InFusion | Overlap | 15 | 0 | Uses an enzyme mix for parallel assembly through a 'chew-back-and-anneal' method | One-step assembly of three 0.2–3.8 kb-long fragments | 38 |
| SLIC | Overlap | >30 | 0 | • Uses a T4 DNA polymerase through a chew-back method in the absence of dNTPs<br>• Uses Recombinase A* to stabilize the annealed fragments and avoid *in vitro* ligation<br>• Allows the parallel assembly of several hundred base-long fragments | Generation of a ten-way assembly of 300–400 bp-long PCR fragments | 39 |
| Gibson | Overlap | 40–400 | 0 | Uses enzymatic 'cocktails' to chew back and anneal for the parallel assembly of several kilobase-long fragments | Assembly of the 1.08 Mb *Mycoplasma mycoides* JCVI-syn1.0 genome | 40 |

*Recombinase A is essential for the repair and maintenance of DNA in *Escherichia coli*. CPEC, circular polymerase extension cloning; dNTPs, deoxynucleoside triphosphates; PCR, polymerase chain reaction; SLIC, sequence and ligation-independent cloning; USER, uracil-specific excision reagent cloning.

exogenous DNA into plants, although the proposed potential of this technology has not yet been realized.

***Organelle genome transformation.*** Homologous recombination-mediated organelle transformation makes it feasible to transfer gene clusters or unlinked genes into a single preselected locus. In this approach, the construct is flanked by two plastid sequences in the transformation vectors, which can be delivered into plastid genomes by biolistics or by polyethylene glycol treatment of protoplasts[43]. Such an integrated 'megalocus' in a plastid genome permits non-Mendelian (that is, maternal in most species) inheritance of co-delivered genes, prevents pollen-mediated spread of transgenes in most species and avoids many potential unwanted epigenetic effects[44]. Transplastomic plants have been shown to stably express transgenes at high levels owing to the numerous plastid copies (that is, transgene copies) in plant cells and owing to the absence of gene silencing. Plastid transformation has been promoted as a particularly powerful approach for the production of synthetic recombinant proteins and pharmaceuticals, and for the engineering of metabolic pathways or pest resistance[45]. However, plastid transformation has not yet delivered one of its main promises — multigene engineering of metabolic pathways through homologous recombination.

Furthermore, because plastid transformation technologies are not available for many species, this approach has only been successfully applied to agronomic-trait engineering in a few crops, such as tobacco[44,46], soybean[47], potato[48], tomato[49], lettuce[50,51], sugar beet[52], eggplant[53], carrot[54], oilseed rape[55] and cabbage[56]. There has been a recent expansion of the use of this approach beyond the Solanaceae, in which most pioneering plastid transformation was carried out. However, host specificity for plastid transformation remains a technical challenge, and this approach remains infeasible for the transfer of large constructs.

***Nuclear genome transformation.*** Nuclear genome transformation is now widely carried out in most economically important plant species. Multiple genes are also routinely stacked in transgenic plants by iterative processes; that is, successive rounds of crossing or sequential transformation of transgenic plants with additional genes. These iterative processes, however, are laborious and prone to the segregation of transgenes in subsequent generations[4]. Recent advances in large insert transformation and multigene transfer into single loci in plant nuclear genomes have been successfully achieved in a few plant species. These methods include virus-mediated transfer[57–59], the use of transformation-competent artificial chromosomes (TACs)[60,61] and BIBACs[62], MISSA-assisted transfer[63] and plant artificial chromosome-assisted transformation[64–70] (discussed below).

Viruses naturally invade and replicate in their hosts, and they can naturally express many viral genes in the host. Viral vectors can travel between cells and have the attractive feature of not integrating into the host plant genome. Currently, most plant biotechnology work using viral vectors consists of the non-transgenic overproduction of one or two recombinant proteins in tobacco or petunia[57,58]. However, an entire bacterial operon was expressed in tomato using the IL-60 platform[59].

Each TAC vector contains a bacteriophage P1 origin of replication, a Cre–*loxP* recombination system and two rare-cutter endonucleases, allowing multiple rounds of recombination in bacteria for the transfer of up to 80 kb of DNA[61]. When used in *A. thaliana*, each round of recombination resulted in the integration of the backbone of the donor vector as well as the redundant *loxP* sites, which had to be removed by digestion with a homing endonuclease, rendering this method inefficient. BIBAC vectors, which combine the features of *A. tumefaciens* binary plasmids and bacterial artificial chromosomes (BACs) that contain the assembled DNA inserts, have permitted the transfer of 150 kb of DNA into tobacco[62]. BIBAC-assisted transformation often results in single copy-insertion events that are stably inherited and expressed[71]. One downside of BIBACs is the potential for the transfer of plasmid backbone sequences[71].

MISSA uses bacterial conjugational transfer and two sets of *in vivo* site-specific lambda phage recombination events for multigene transfer. This system is composed of bacterial donor and recipient strains, and corresponding donor and recipient vectors that are based on either TAC or BIBAC vectors. It allows completely *in vivo* multigene transfer and assembly within the donor strains before transformation into rice[61]. One downside is the potential for plasmid instability in *A. tumefaciens*[63]. However, these methods (that is, TAC, BIBAC and MISSA) have not yet been widely used in plant biotechnology; thus, we cannot currently address issues about their efficiencies and relevance to particular crops.

Indeed, the downside of any *A. tumefaciens*-mediated transformation system is the inherent nature of random insertion (insertional position-effects), whereby endogenous host gene cassettes could be disrupted when transgenes integrate. Random insertions could also cause variability or even silencing of the transgene, making the co-integration of complex traits in a coordinated manner unlikely. The use of genome editing and artificial chromosomes might overcome many of the problems mentioned above.

***Plant artificial chromosomes.*** Artificial chromosomes as alternative transformation and expression vectors could have a key role in next-generation transgenic technologies. The use of minichromosomes for genetic transformation removes the problems of position-effects, the disruption of endogenous genes and the linkage to undesirable loci. Thus, plant artificial chromosomes have the potential to allow gene stacking, engineering of complete metabolic pathways into plants, coordinated transformation of complex traits, and multiple site-specific recombination and integration for crop-trait engineering and breeding.

A functional minichromosome requires three types of elements: a centromere, telomeres and an origin of replication. Among these, the centromere is the most intriguing and poorly understood genetic element in plants, considering its large size (owing to the presence of various arrays of repetitive DNA sequences), complexity and epigenetic components. Nonetheless, two approaches — bottom-up and 'top-down' approaches — have been developed for the generation of minichromosomes[72] (FIG. 3).

The bottom-up approach involves the *de novo* assembly of cloned chromosomal components, such as centromeric and telomeric sequences, a selective marker gene and genomic DNA that contains a replication origin. Even though the bottom-up approach is promising[73], extensive improvements in *de novo* minichromosome assembly in plants are needed. These include defining the minimal length that is required for a functional chromosome, understanding the effects of epigenetic signatures and inverted arrays of centromere repeats on chromosome function, and being able to predict the meiotic transmission of synthetic minichromosomes[74].

Existing chromosomes can be modified to generate chromosome-based vectors for gene transformation and transmission, and such modification provides another promising approach to generate artificial chromosomes — the top-down approach[64,69,70,75]. In one example[69], an array of 2.6 kb of *A. thaliana*-type telomeric repeats allowed the truncation of maize chromosome arms, and the resulting chromosome was transformed into maize embryos (FIG. 3). The transgenic telomere sequences might mediate chromosome 'healing' by recruiting telomerase and telomere-binding proteins. In addition, the telomeric sequence could be flanked by sites for future site-specific recombination events, such as those mediated by the Cre–*loxP* system or the FLP–*FRT* system[66,68,70,76].

As telomere-mediated truncation of A chromosomes potentially leads to massive gene loss and genome instability, naturally occurring or newly created polyploids might provide ideal platforms for chromosome engineering and plant breeding through telomere truncation of A chromosomes[68]. Alternatively, B chromosomes

Plant artificial chromosome
An engineered non-integrating vector that harbours large amounts of DNA (including telomeres, origins of replication, a centromere and genes of interest) and is transmissible in cell division after transformation into plant cells.

Cre–*loxP* recombination system
A site-specific recombination system mediated by the Cre recombinase in a genome that contains pre-existing or pre-engineered *loxP* sites which are recognized by the Cre recombinase.

Gene stacking
The accumulation of multiple trangenes of interest into the same plant genome for stacked traits.

FLP–*FRT* systems
A recombination system in which the FLP recombinase specifically recognizes the *FRT* site and mediates excision of any sequence that is flanked by the *FRT* sites.

B chromosomes
Supernumerary or accessory chromosomes that are heterochromatic. They do not contain functional genes and do not to pair with A chromosomes at meiosis.

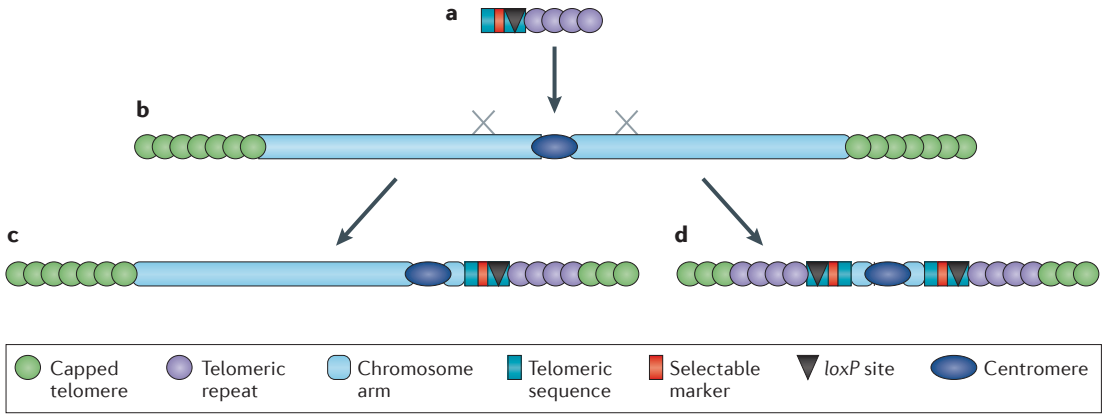


Figure 3 | **An example of the 'top-down' approach for the generation of plant artificial chromosomes.** The truncation cassette contains a telomeric repeat sequence 5′-(TTTAGGG)$_n$-3′, a selectable marker and a *loxP* site (the specific sequence target of the Cre recombinase) placed before a reporter gene that encodes a fluorescent protein (not shown) (part **a**). The truncation cassette was transformed into maize embryos by *Argobacterium tumefaciens*-mediated or biolistic transformation (part **b**), and most of the DNA was excised from one (part **c**) or both (part **d**) arms of the endogenous chromosome[132]. If both arms of the endogenous chromosome are excised, a minichromosome is formed. The truncated ends (which contain the leftover parts of the original chromosome arms, the telomeric repeat sequence, the selectable marker, the *loxP* site and a reporter gene encoding a fluorescent protein) could be recognized by telomerase and telomere-binding proteins, resulting in chromosome 'healing', and the elongation and capping of telomere ends. The integrated *loxP* site could be used for further site-specific recombination in the resulting minichromosomes.

could become favourable targets for telomere truncation because they are dispensable. Engineered mini-B chromosomes have been faithfully transmitted from generation to generation, with transmission rates of 12–39% in maize[70], a rate that would have to increase for this approach to be useful in the field. Hence, although minichromosomes are promising, they have not yet had a sizeable effect in agricultural biotechnology.

## Precise genome editing

Of all the tools discussed here, genome editing by site-specific integration, deletion and/or mutation of genes of interest is poised to have, perhaps, the greatest effect on plant biotechnology. Site-specific recombinases and/or various newer tools are useful for creating DNA double-strand breaks (DSBs) within plant genomes. DSBs are repaired by homologous recombination or by error-prone non-homologous end-joining (NHEJ). The target genomic site can be mutated by using donor DNA. Engineered nucleases such as zinc-finger nucleases (ZFNs)[77–80] or TALE nucleases (TALENs)[81,82] (FIG. 2b) could be designed and used to generate DSBs at almost any specific genomic location to facilitate genome editing. The non-specific DNA-cleavage domain of the *Fok*I endonuclease can be fused to the DNA-binding domain of both types of nucleases. The nuclease pair must dimerize after DNA binding to allow DNA cleavage within the spacer region between the two binding sites. The spacer regions between the monomers of TALENs and ZFNs are 6–40 bp and 5–7 bp in length, respectively.

ZFNs have been used in *A. thaliana* to create ABA- and glucose-insensitive plants by targeting the *ABI4* gene[83], allyl alcohol-resistant plants by targeting the *ADH1* gene and mutants lacking anthocyanins in the seed coat by targeting the *TT4* gene[84]. In tobacco, ZFNs have enabled herbicide-resistant plants to be produced, by targeting the acetolactate synthase genes (*ALS SuRA* and *SuRB*)[85]. Maize seed inositol phosphate profiles were altered by the insertional disruption of the *IPK1* gene using a ZFN[86]. As a final example, the soybean genome has also been edited using ZFNs to mutate *DCL* genes that are involved in RNA silencing, which resulted in efficient, heritable and targeted mutagenesis in duplicate genes in the subsequent generation[87].

The development of TALENs lags behind that of ZFNs by nearly a decade, but TALENs have rapidly been shown to be useful in plant biotechnology. In model plants, TALENs have been used to alter reporter-gene expression in tobacco[88,89] and to introduce insertions and deletions (indels) in the *ADH* gene in *A. thaliana*[90]. TALENs have also been used for multiple gene knockouts of four genes in rice and eight genes in *Brachypodium* spp.[91]. Another interesting application in rice was the generation of disease-resistant plants by engineering indels in the promoter of the rice *11N3* gene, the expression of which is usually exploited by a bacterial pathogen[92].

Gene targeting, gene correction and even gene disruption, through targeted gene addition by homologous recombination, have been accomplished in maize with the help of donor plasmids[86]. The efficiency of site-directed mutagenesis is typically ~0.26–3%[83,88,93] depending on the plasmid delivery methods, the genomic insertion loci and the targeted plant species and tissues. ZFNs and TALENs provide stringent specificity for plant gene targeting and genome editing[31,83,86]. Both nucleases can discriminate differences of 2–3 nucleotides between binding sites of two paralogous genes[85,87,89]. Although we now have more than a decade of experience with ZFNs, TALENs are only beginning to have an effect on plant biotechnology.

Zinc-finger nucleases
(ZFNs). Fusions of engineered zinc-finger arrays (that consist of 3–6 C2H2 fingers) to a non-specific DNA-cleavage domain of the *Fok*I endonuclease.

TALE nucleases
(TALENs). Fusions of truncated TALEs (containing an amino terminus, a custom-designed DNA-binding domain and a carboxyl terminus with the activation domain being removed) to a non-specific DNA-cleavage domain of the *Fok*I endonuclease.

Gene targeting
The incorporation of a transgene (or transgenes) of interest into one or more desired specific genomic loci for the permanent modification of plant genomes using homologous recombination (or another method) followed by the selection for a rare recombination event.

In addition, homing endonucleases (or meganucleases) provide alternative tools for site-specific recognition and DSB generation for targeted genome modification[94]. Such meganucleases are homodimers but can also be developed to form heterodimers of differently engineered monomers with large recognition sites (typically 20–30 bp). So far, the engineered meganucleases I-*Cre*I and I-*Sce*I have been successfully used for targeted genome modification in maize[95–97]. In these studies, the targeted mutagenesis efficiency was between 1% and 3% in analysed $F_1$ plants[95,97]. Unlike the modular structure of ZFNs and TALENs, the DNA-binding domains in most meganucleases are not clearly separated from their catalytic domains, which is a challenge for protein engineering[98]. Moreover, the use of existing meganucleases is highly limited to loci with pre-engineered recognition sites.

These three types of nucleases can be used for the targeted generation of mutations that can confer resistance traits[85,92,96], or for the specific and permanent removal of undesired genes or selectable markers in transgenic plants[86,91,97]. They could also be used for gene stacking, gene replacement and even the recovery of double mutants for tightly linked genes. With the help of a donor vector, they could be used for the targeted genomic integration of synthetic devices (for example, novel gene 'circuits') into plants in the near future. Engineered meganucleases, ZFNs and TALENs are tools with great potential for genome editing with transformation.

The latest tools in the genome-editing toolbox are clustered regularly interspaced short palindromic repeats (CRISPRs), which provide an alternative mechanism for genome editing. CRISPRs are loci that contain multiple short direct repeats that are incorporated with short segments of foreign DNA (called spacers) in some bacteria and archaea. When spacers are expressed as precursor RNAs and subsequently truncated to short CRISPR RNAs (crRNAs), they direct CRISPR-associated (Cas) proteins for the subsequent attack at matching protospacer sequences of invading viruses or plasmids. Among the three types of CRISPR–Cas systems, Cas9 belongs to the best studied type II CRISPR–Cas system. Cas9 is believed to be the sole protein responsible for crRNA-guided silencing of foreign DNA, with the help of a transactivating crRNA (tracrRNA) that is complementary to the repeat sequences in the pre-crRNA[99,100]. The fusion of tracrRNA with pre-crRNA, which is called single guide RNA (sgRNA), has been demonstrated to be sufficient to guide Cas9 for *in vitro* sequence-specific cleavage of target DNA[101]. So far, co-expression of Cas9 and sgRNA has been used to target the *PDS3*, *RACK1b* and *RACK1c* genes in *A. thaliana* protoplasts or leaves using agroinfiltration[102], the *PDS* gene in *Nicotiana benthamiana* protoplasts or leaves using agroinfiltration[103], and the *PDS* and *MPK2* genes in rice protoplasts[104]. The targeted mutagenesis rate was about 2.1–4.8% for agroinfiltration and 5.6–38.5% for protoplast assays[102–104], which suggests that it might be the most efficient of the four genome editing systems that are discussed here. CRISPR–Cas9 was shown to differentiate differences of two nucleotides in sequences between homologous genes for pre-crRNA recognition[102].

## Transgene removal and confinement

The potential for the transfer of transgenes into related plant species through pollen- or seed-mediated gene flow is of concern to governmental regulators because of potential environmental and human health risks[105]. Thus, transgene bioconfinement strategies, such as male or female sterility, maternal inheritance, transgenic mitigation and excision, apomixes, cleistogamy and genome incompatibility, have been proposed to limit or eliminate transgene escape from target fields and crops[105]. Selectable marker genes (usually antibiotic- or herbicide-resistant genes) might also be removed from the final products, as they are not needed after plant transformation and this removal also allows subsequent rounds of retransformation using the same selection agent[106].

Transgene removal or selectable marker-gene excision can be achieved using site-specific meganucleases, ZFNs or recombinases which break DNA on two directly orientated, engineered recognition sites that flank transgenes[106]. Some tyrosine recombinases, such as Cre, FLP and R, use a catalytic tyrosine residue to mediate cleavage and have been widely used for transgenic removal[107–114]; they are bidirectional recombinases with *loxP*, *FRT* and *RS* being their respective identical DNA recognition sites (FIG. 4a). These tyrosine recombinases do not need modification or host-specific factors to function in plants. Moreover, some serine recombinases — CinH, ParA, Bxb1 and PhiC31 — confer irreversible excision in the absence of the helper protein excisionase. CinH and ParA recombinases use *RS2* and *MRS* as their respective identical recognition sites[115], whereas Bxb1 and PhiC31 act on the recognition sites *attB* and *attP*, which differ in sequence[116,117] (FIG. 4b,c) and yield the hybrid product sites *attL* and *attR* after excision. The recognition sites of these serine recombinases are much longer than those of their well-known tyrosine counterparts, which greatly decreases the possibility of off-target recombination with the host genome. Transgenic plants that contain two specific recognition sites flanking a transgene can be crossed with the respective recombinase-expressing plant, or can be stably or transiently re-transformed with the respective recombinase construct (FIG. 4d). Alternatively, transgene removal can be achieved by expressing the recombinases under the control of an inducible or tissue-specific promoter in the construct containing the transgene that is flanked by two specific recognition sites (FIG. 4e).

Recent applications of these newly developed site-specific recombination systems for transgene removal have been reported *in planta*, for example, in tobacco[118,119], tomato[120], *A. thaliana*[121–124] and wheat[125,126]. DNA removal efficiency depends on the binding specificity of the recombinase to the recognition sites, the uniqueness of the sites within the plant genome and excision efficacy. For example, the CinH–*RS2* recombinase system is desirable owing to its high efficiency and long (119 bp) recognition site[118]. Transgenic bioconfinement should be a great help in crop improvement if the excision of transgenic traits can take place at ~100% efficiency for the production of transgene-free pollen and seed[127].

Agroinfiltration
The most common transient transformation method in plants which uses injection or vacuum infiltration to transform genes into cells and tissues using *Agrobacterium tumefaciens*.

Apomixes
The replacement of normal sexual reproduction by asexual reproduction without fertilization.

Cleistogamy
A phenomenon in which certain plants propagate using non-opening, self-pollinating flowers.

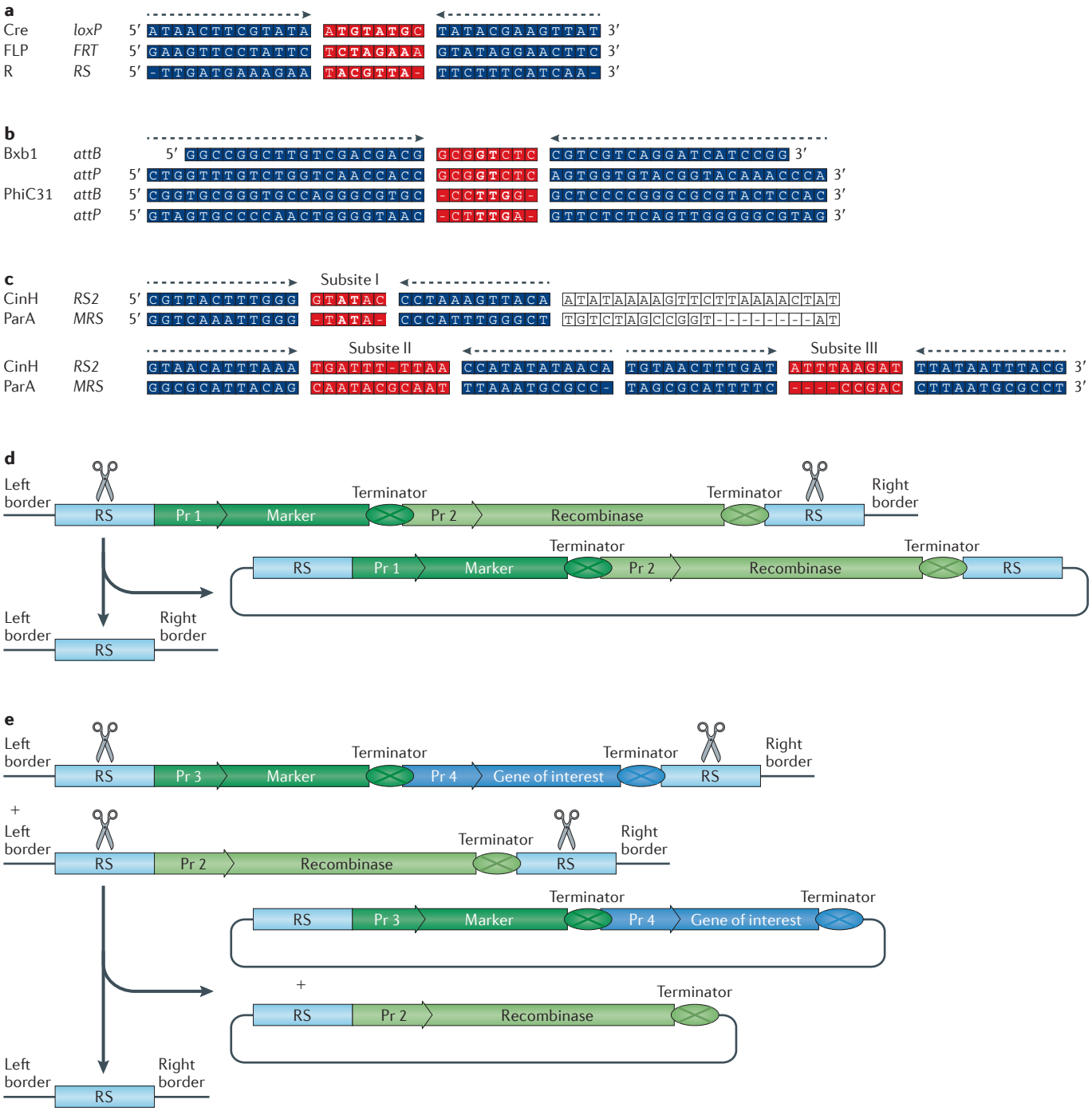


Figure 4 | **Bioconfinement methods.** Site-specific recombinase recognition sites (RSs) are depicted in red in parts **a**–**c**. The core nucleotides where strand exchange occurs in each recognition site are shown in bold except for subsites II and III in part **c**. The recognition sites of selected tyrosine recombinases are shown (part **a**). For Cre, the recognition site is *loxP*; for FLP and R, the recognition sites are *FRT* and *RS*, respectively. Each of these sites contains two oppositely oriented repeats that are almost identical in sequence and give rise to reversible recombination[133]. The two respective recognition sites of serine recombinases Bxb1 and PhiC31 are *attB* and *attP*, which differ in sequence from each other[134] (part **b**). The respective recognition sites of CinH and ParA are *RS2* and *MRS*, each of which consists of three subsites — I, II and III — with subsite I being the recombination site, whereas the other two subsites are accessory sites[112] (part **c**). Each subsite consists of two half-sites that differ in sequence, show dyad symmetry and are bound by a specific recombinase dimer. The spacer sequences flanked by two repeats are shown by dashed arrows and in blue. Bioconfinement by transgene excision or marker gene removal from plant genomes are shown in parts **d**,**e**. The recombinases can be incorporated into the same construct as the transgene or selectable marker gene (part **d**), or they can be expressed in another construct (part **e**). The promoters (Pr) driving recombinase expression can be chemically or developmentally inducible promoters, or tissue-specific promoters.

## Future perspectives

A primary goal of plant biotechnology is to continue human-driven crop evolution for higher and more sustainable food yields. Genetic engineering has enabled incremental crop improvement, including pest resistance and other input traits, to improve farming and to produce simple output traits, such as single pharmaceutical proteins. However, great advances in plant biotechnology will require more precise tools than those that have been widely used so far. As discussed above, the routine methods of transformation are beset with the problems of random transgene integration, copy-number variability and imprecise gene expression. We are unsure about the upper limits of DNA length that can be integrated into plants, but given their large genomes, it is apparent that plants can tolerate copious DNA additions. Nonetheless, when several genes are introduced into plant genomes, metabolic loads and their management might become an issue. Moreover, targeted genome modification, and plant artificial chromosomes and genomes are still far from being routinely used in most academic or industrial laboratories. For both the 'easy' and the 'hard' problems facing plant biotechnology, there are many reasons to believe that the scientific hurdles will be cleared. Some easy problems include 'tuning' transgene expression and genome editing, for which various recent breakthrough technologies are helping to solve. The hard problem of incorporating large amounts of DNA will probably take time to solve, but there are various biomimetic-based tools that are beginning to provide solutions. If nature can move hundreds of genes around in a 'package', then maybe scientists will be able to find a way to do so too.

The development of systems biology has allowed the modelling of biological systems and processes, and provided a systems-level view of these processes. Advances in genome-wide analyses and computational studies will allow a better understanding of complex regulatory and metabolic pathways. The next iteration of technological advancement will depend on how we use systems biology for the discovery and better understanding of genes and pathways that can be modified using the new genetic tools.

In addition, we are on the cusp of realizing synthetic biology for plants. Synthetic biology strives to replace or reconfigure genetic components that are found in nature using synthetic tools. Some of these synthetic tools are incremental improvements to old tools — such as synthetic promoters that replace native promoters in plant biotechnology. Other tools, such as synthetic TALENs, are completely new to plant biotechnology and are only now becoming implemented. Moreover, other synthetic biology systems, such as synthetic circuits and synthetic genomes, have been designed and implemented in simpler microbial systems and have not been applied to plants. However, the concepts of synthetic biology and many of its components can be transferred to any organism. We can apply to plants the engineering principles that are used to design and alter microorganisms, and those used to construct artificial biological systems and devices that exhibit predictable behaviours[128]. Indeed, many of the advanced tools reviewed here should probably be thought of as first-generation plant synthetic biology components, such as synthetic promoters and synthetic transcription factors for the targeted regulation of gene expression. One alternative to the use of synthetic promoters, such as in the phytosensing example described above, would involve designing *de novo* circuits, which would then be engineered into plants — a more complicated task than simply implementing a synthetic inducible promoter. The best example of this approach so far is the designer circuit developed for the phytosensing of explosives[129]. In this circuit, extracellular ligands in the form of 2,4,6-trinitrotoluene (TNT) molecules were sensed by periplasmic binding proteins that had been computationally redesigned for this purpose and were linked to gene expression through a synthetic histidine kinase-mediated signal transduction pathway in transgenic *A. thaliana* (FIG. 5). We are now realizing an increase in the component tool kit for genome modification and transgene bioconfinement. One such example that is currently being applied to plants is the use of the

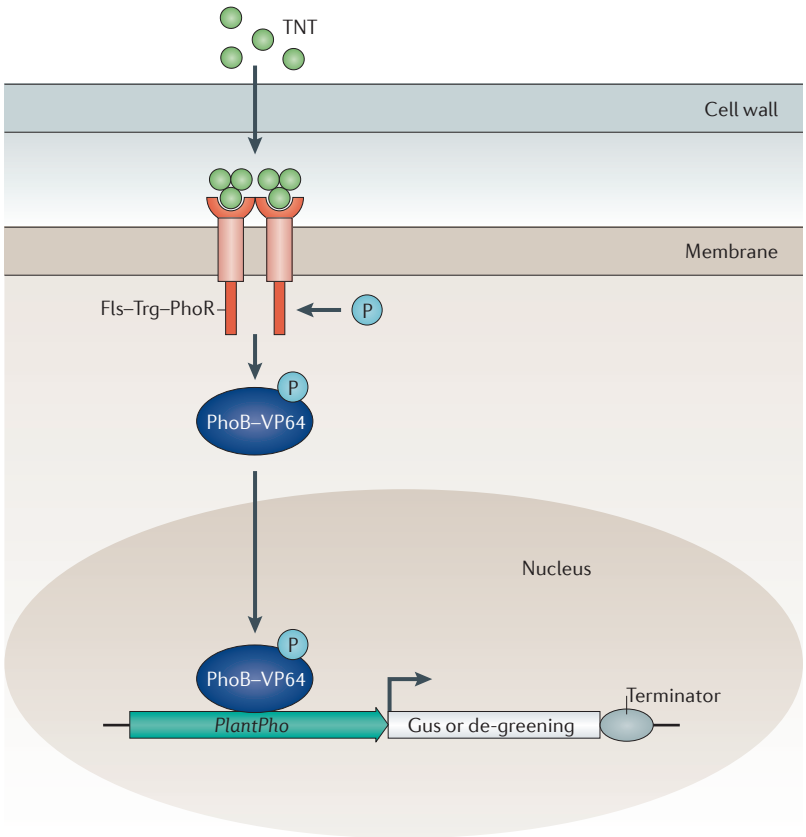


Figure 5 | **Signalling components used for phytosensing of the explosive TNT.** The 2,4,6-trinitrotoluene (TNT)-inducible device[129] contains a bacterial response regulator, PhoB. On TNT contamination, the TNT-bound designer TNT receptors induce phosphorylation (P) of the cell membrane-localized Fls–Trg–PhoR fusion protein, which, in turn, induces phosphorylation of PhoB–VP64. The phosphorylated PhoB–VP64 can translocate into the plant nucleus and activate a synthetic promoter (*PlantPho*) that drives either Gus expression or a de-greening circuit, which produces white plants when the expression of genes that inhibit chlorophyll biosynthesis and induce chlorophyll breakdown are transcriptionally induced with an oestrogen-like hormone. Gus, β-glucuronidase; Ter, terminator. Figure is modified from REF. 129.

CRISPR–Cas system for RNA-programmable genome editing[103–105]. The generation of the first synthetic plant genome is probably decades away, but plant artificial chromosomes are a step in that direction.

Encouragingly, substantial amounts of private and public funding are being targeted to advance plant synthetic biology. For example, the US Department of Energy Advanced Research Projects Agency-Energy (ARPA-E) has recently funded ten projects for their Plants Engineered to Replace Oil (PETRO) programme[130]. A total of US$36 million has been provided for projects to modify bioenergy feedstocks to produce novel compounds that can be used as drop-in fuels. One example is a grant given to the University of Illinois of more than $3.2 million to engineer sugarcane (*Saccharum* spp.) and *Sorghum bicolor* with increased photosynthesis and oil production efficiencies.

However, such projects have raised other important challenges, particularly in biosafety and governmental regulation. Plant synthetic biology and biotechnology can possibly address some biosafety concerns, but they might also amplify existing concerns, as increasingly greater amounts of DNA and proteins are being manipulated in crops. It might be the case that governmental biosafety regulations, which are considered by some people to be already outdated and overly onerous[131], will need to be modernized in order not to quash the innovation that advanced tools and synthetic biology might bring to plant agriculture. Targeted genome modification using engineered nucleases together with bioconfinement technologies is expected to gradually relieve public concerns about foreign DNA and random integration in crops, and to facilitate the deregulation of transgenic crops.


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**Competing interests statement**
The authors declare competing financial interests: see Web version for details.

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