Review

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Enabling plant synthetic biology through genome engineering

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Synthetic biology seeks to create new biological systems, including user-designed plants and plant cells. These systems can be employed for a variety of purposes, ranging from producing compounds of industrial or therapeutic value, to reducing crop losses by altering cellular responses to pathogens or climate change. To realize the full potential of plant synthetic biology, techniques are required that provide control over the genetic code – enabling targeted modifications to DNA sequences within living plant cells. Such control is now within reach owing to recent advances in the use of sequence-specific nucleases to precisely engineer genomes. We discuss here the enormous potential provided by genome engineering for plant synthetic biology.

The importance of genome engineering for synthetic biology

Synthetic biology (see Glossary) is often hard to define because it encompasses a broad range of methodologies for manipulating and harnessing living systems. In simplest terms, synthetic biology combines science and engineering to design and construct new biological parts, devices, and systems [1]. One area of synthetic biology, and the focus of this review, is the generation of user-designed organisms. These organisms are created for a variety of purposes, ranging from producing valuable compounds that are ultimately purified away from the host to improving the response of an organism to the environment by designing genetic circuits that respond better to external cues. To fully practice in this area of synthetic biology one requires control over DNA sequences, from the *in silico* design and *in vitro* synthesis of standardized genetic elements to the *in vivo* manipulation of host DNA and gene expression.

There are now a wide variety of tools available for *in vivo* manipulation of the genetic material, including recombinases, integrases, RNAi technology, and sequence-specific nucleases, the latter being the focus of this review. Extraordinary advances in sequence-specific nuclease technology within the past 5 years have made it possible for most labs, even those with minimal molecular biology expertise, to precisely manipulate plant genomes, including altering DNA sequences and changing patterns of gene expression. We focus here on sequence-specific nucleases and how they have been used to create genetic modifications for synthetic biology projects. We also discuss future roles for these tools in plant synthetic biology, using examples from several projects, including the ongoing C_4 rice project, where photosynthesis in rice is to be completely redesigned for higher efficiency [2], and the nitrogen-fixing cereals project, where cereals are to be modified to uptake atmospheric nitrogen [3] (Box 1).

Why practice synthetic biology in plants?

Plants have largely been unexploited for synthetic biology, but they offer great potential. Plants are the most important source of the primary metabolites that feed the world (i.e., proteins, fatty acids, and carbohydrates) and they also produce a diverse array of secondary metabolites of value for medicine and industry. Further, there is a good understanding of plant systems biology, they are sessile, they can fight off pathogens, and they are not subject to the ethical issues that sometimes limit the use of animal cells. Finally,

Glossary

Gene targeting: a process that uses the homologous recombination pathway to introduce DNA sequence changes within genomes. Instead of using homologous sequence present on the sister chromatid or homologous chromosome, it is possible to 'trick' the cell into using a user-supplied donor molecule for repair [8]. Differences in sequences within the donor molecule, compared to the chromosomal target, will be copied and stably incorporated into the host genome.

Guide RNA (gRNA): Cas9 is targeted to a specific DNA sequence using a gRNA. This gRNA consists of two RNA molecules – a CRISPR RNA (crRNA) and a transactivating CRISPR RNA (tracrRNA). To reduce the complexity of the system for genome engineering, the crRNA and tracrRNA can be fused into a single gRNA.

Homologous recombination (HR): a DNA double-strand break (DSB) repair pathway. Repair is template-directed and uses homologous sequences present on the sister chromatid, homologous chromosome, or a user-supplied donor molecule.

Non-homologous end joining (NHEJ): a major DNA DSB repair pathway. Repair is non-template directed, and involves the direct religation of the exposed DNA ends. Repair can result in deletions, substitutions, or insertions at the break site [57]. The error-prone nature of NHEJ, combined with the ability to direct DSBs with sequence-specific nucleases, provides genome engineers with an approach to mutagenize sequences within living cells.

Sequence-specific nucleases: a family of enzymes consisting of meganucleases, zinc-finger nucleases, TALENs, and CRISPR/Cas. All sequence-specific nucleases can be engineered to bind to and cleave a DNA sequence of interest.

Synthetic biology: combines science and engineering to design and construct new biological parts, devices, and systems. The definition also includes the creation and integration of new biological systems.

Trait stacking: also known as gene stacking, this refers to the process of adding two or more genes into a plant genome at the same location.

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Keywords: synthetic biology; sequence-specific nuclease; zinc-finger nuclease; TALEN; CRISPR/Cas; genome engineering.

0167-7799/
 http://dx.doi.org/10.1016/j.tibtech.2014.11.008

Box 1. A spotlight on two ambitious synthetic biology projects

Examples of plant synthetic biology projects include the nitrogen-fixing cereals project (Figure IA) and the C_4 rice project (Figure IB).

By engineering cereals to uptake atmospheric nitrogen, there will be a reduced dependency on inorganic fertilizers. There are two possible approaches for modifying cereals to uptake atmospheric nitrogen: transfer the nodulation signaling pathway from legumes to promote root nodule symbiosis with *Rhizobium* bacteria, or engineer the nitrogenase enzyme to function in plant cells.

Engineering the C_4 photosynthesis pathway into C_3 rice promises to increase yield. One approach to engineering this pathway in rice is to convert the single-cell C_3 cycle into a two-celled C_4 cycle. In this case the initial carbon fixation is catalyzed within mesophyll cells by phosphoenolpyruvate carboxylase (PEPC) forming the four-carbon oxaloacetate from bicarbonate and PEP. Oxaloacetate is then metabolized into malate, and the four-carbon acid diffuses into the bundle sheath cell. There, the four-carbon acid is decarboxylated to provide increased concentrations of carbon dioxide to RuBisCO, which is confined in bundle sheath cells.

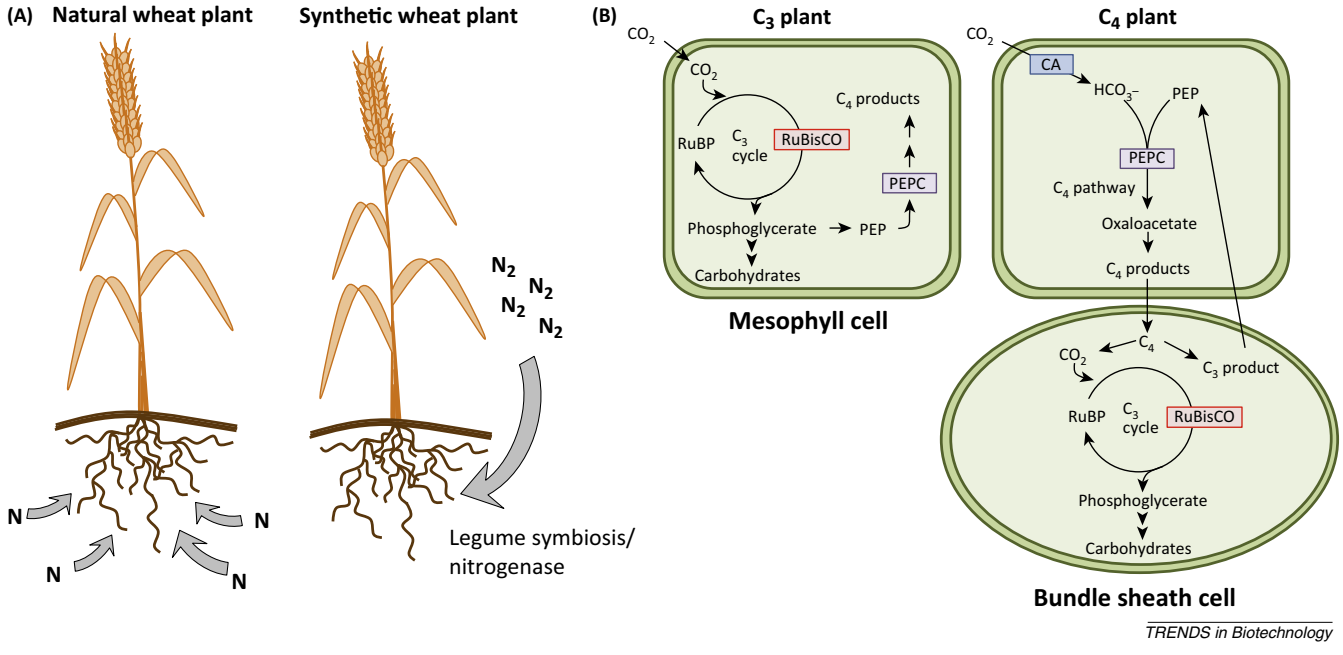

Figure I. Two examples of synthetic biology projects aimed at generating nitrogen-fixing cereals or C_4 photosynthetic rice.

plants use abundant and inexpensive nutrients (carbon dioxide and sunlight) to produce their primary and secondary metabolites, and their total biomass is enormous: approximately 210 billion tons of plant material are produced each year [4].

Approximately 30 years ago the first plants were generated with novel functions, including herbicide tolerances and insect resistances [5]. These plants were made through transgenesis, in which user-designed DNA was randomly integrated into plant genomes. While this was an important first step in designing plants with novel functions, the past few years have witnessed the emergence of more sophisticated and precise methods for engineering DNA in living cells. When these methods are used to their fullest potential, they can generate any type of modification within plant genomes, ranging from precisely introducing one or more transgenes at a desired locus, to removing unwanted or unnecessary DNA from the host, to accurately controlling expression of host or synthetic genes.

Even by focusing on user-designed plants, the breadth of projects that fall under the synthetic biology term is enormous. Examples of such projects include: (i) modifying cereals, including wheat, to fix atmospheric nitrogen, (ii) redesigning metabolic pathways to increase the yield of secondary metabolites or to generate compounds with enhanced properties, (iii) transferring the C_4 photosynthesis pathway to rice, (iv) modifying the glycosylation pathway in plants to accommodate production of therapeutic proteins, and (v) introducing synthetic signal transduction systems that respond to external cues [6]. A common ground for most synthetic biology projects is the need for standardized genetic parts (e.g., promoters, terminators, genes), and the subsequent need for tools and techniques for modifying plant genomes.

Engineering genomes with sequence-specific nucleases

One method to efficiently and precisely modify plant genomes involves introducing targeted DNA double-strand breaks (DSBs) at a locus of interest. Normally, DSBs are highly toxic lesions, and to preserve the integrity of their genomes, all living organisms have evolved pathways to repair such breaks. In general, plant cells have two main DNA repair mechanisms: non-homologous end joining and homologous recombination [7]. As described in greater detail below, repair by either pathway can be exploited to introduce sequence changes within genomes.

In the past two decades, significant effort has been invested in developing reagents that create targeted DNA DSBs. Currently, researchers have a choice between four classes of sequence-specific nucleases: meganucleases, zinc-finger nucleases, TALENs, and CRISPR/Cas (Box 2). All classes are similar in that they can be customized to bind and cleave a target DNA sequence of interest (approximately 18–40 bp in length). Specificity of 18 bp makes it possible to target a single locus in a complex genome. An 18 bp signature occurs once in 68 billion bp of DNA; the wheat genome, for example, is ~17 billion bp.

Choosing the right tool for the job

One of the first steps in engineering plant genomes is to design and construct one or multiple sequence-specific nucleases. How does one choose between the different classes of nucleases? We list here the defining characteristics of each class to help researchers make informed decisions about nuclease choice.

Meganucleases

Meganucleases were the first class of sequence-specific nucleases used in plants [8], and they continue to be deployed to achieve complex genome modifications [9]. An advantage of meganucleases is their size. They are among the smallest nucleases – comprising only ~165 amino acids (aa) – making them amenable to most delivery methods, including vectors with limited cargo capacities, such as plant RNA viruses [10]. Relative to other sequence-specific nucleases, however, meganucleases are challenging to redesign for new target specificity. Redesign is hindered by the non-modular nature of the protein. For example, within the LAGLIDADG family of meganucleases, the amino acids responsible for binding DNA overlap with those for DNA cleavage [11]; therefore, attempting to alter the DNA-binding domain can affect the enzyme's catalytic activity. As a result, the use of meganucleases in plants has been limited to naturally occurring meganucleases (e.g., I-*Sce*I, I-*Cre*I) or to redesigned nucleases made by groups with expertise in structure-based design or the capacity to carry out high-throughput *in vitro* screens to identify active nucleases from libraries of variants.

Zinc-finger nucleases

Like the meganucleases, zinc-finger nucleases are relatively small (~300 aa per monomer; ~600 aa per nuclease pair), making them amenable to most delivery methods. DNA targeting by zinc-finger nucleases is achieved by arrays of zinc fingers, each of which typically binds to a nucleotide triplet. Whereas redesigning the zinc-finger DNA-binding domain is not as difficult as for meganucleases, there are still challenges in achieving new target specificity, mostly due to the influence of context on zinc-finger function. For example, a zinc finger that recognizes GGG in one array may not recognize this sequence when positioned next to different zinc fingers. As a result, modular assembly of zinc fingers has had limited success [12]. One of the more successful methods for redirecting targeting involves screening libraries of three zinc-finger variants to identify those that best recognize and bind to their intended target sequence [13]. More recently, modular methods for constructing zinc-finger arrays have been successful that use two-finger units to minimize context effects [14]. Consequently, generating functional zinc-finger nucleases is now achievable by most research labs.

TALENs

TALENs are a recent addition to the arsenal of sequence-specific nucleases, and they quickly became adopted for plant genome engineering. One advantage of TALENs, compared to meganucleases and zinc-finger nucleases, is their modular DNA binding domain. The TALE DNA binding domain is composed of direct repeats consisting of 33–35 aa. Two amino acids within these repeats, termed repeat-variable diresidues (RVDs), recognize a target nucleotide (e.g., the most widely used RVDs and their nucleotide targets are HD, cytosine; NG, thymine; NI, adenine; and NN, guanine and adenine). This one-to-one correspondence

Box 2. Engineering plant DNA using sequence-specific nucleases

DNA DSBs can be targeted to sequences of interest using sequence-specific nucleases. There are four major classes of sequence-specific nucleases: meganucleases, zinc-finger nucleases, TALENs, and CRISPR/Cas (Figure IA). Although these enzymes are structurally different, all can be engineered to recognize and cleave different DNA sequences.

Meganucleases

Meganucleases (also referred to as homing endonucleases) were initially found to be encoded by mobile introns, and, since then, they have been repurposed for creating targeted DSBs within genomes. Their relatively small size (~165 aa) and large DNA recognition sequence (~18 bp) has made meganucleases an attractive option for genome engineering.

Zinc-finger nucleases

Zinc-finger nucleases are chimeric fusion proteins that consist of a DNA-binding domain and a DNA-cleavage domain. The DNA-binding domain is composed of a set of Cys_2His_2 zinc fingers (usually 3–6). Each zinc finger contacts typically 3 bp of DNA, and arrays of 3 or 6 fingers recognize 9 or 18 bp, respectively. The DNA-cleavage domain is derived from the *Fok*I restriction enzyme. *Fok*I activity requires dimerization; therefore, to site-specifically cleave DNA, two zinc-finger nucleases are designed to bind to DNA in a tail-to-tail orientation [58]. With their relatively small size (~300 aa per zinc-finger nuclease monomer), and the further advancements in methods for redirecting targeting [14], zinc-finger nucleases should continue to be an effective technology for editing plant DNA.

TALENs

Transcription activator-like effector nucleases (TALENs) are another class of sequence-specific nucleases, and like zinc-finger nucleases, are composed of a DNA binding domain and a *Fok*I cleavage domain. The DNA binding domain is derived from TALE proteins found in *Xanthamonas sp.* Each TALE DNA binding domain is composed of repeat sequences consisting of 33–35 aa. Within each repeat are two variable amino acids (RVDs) that facilitate binding to a single DNA base.

CRISPR/Cas and alternative CRISPR/Cas nucleases

The CRISPR (clustered, regularly interspaced, short palindromic repeats)/Cas (CRISPR-associated) system is the most recent addition to the family of sequence-specific nucleases. The CRISPR/Cas system employed for genome engineering consists of a Cas9 endonuclease and a guide RNA (gRNA). Approximately 20 nucleotides within the gRNA are responsible for directing Cas9 cleavage. A protospacer adjacent motif (PAM) is required for DNA cleavage. To increase target specificity, DSBs can be generated using dCas9 (nuclease-inactive Cas9) fusions to *Fok*I, or using Cas9 nickases (Figure IB).

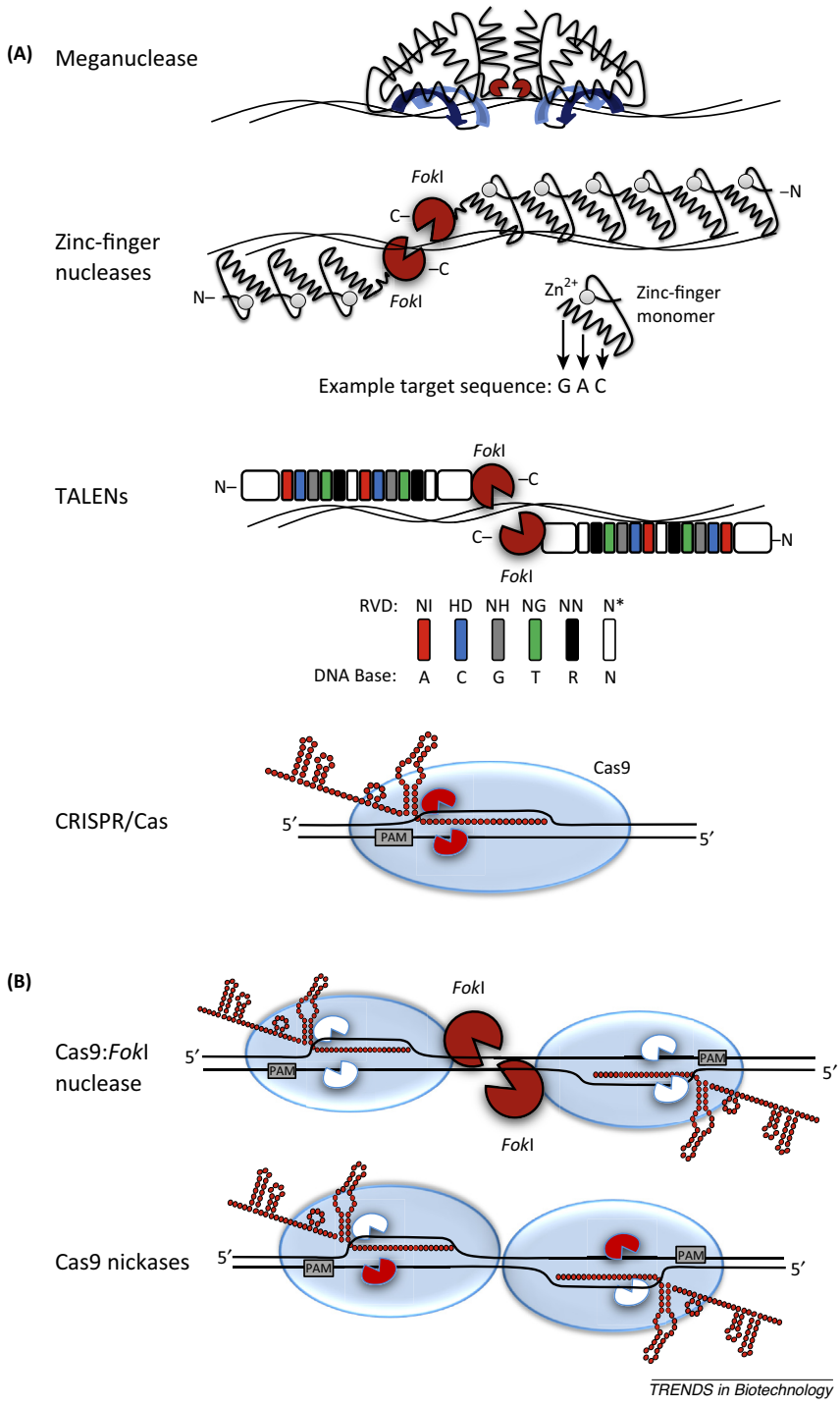

Figure I. The variety of nucleases. **(A)** Main types of sequence-specific nucleases. **(B)** Alternative CRISPR/Cas nucleases.

of a single RVD to a single DNA base, together with effective methods for cloning arrays of the DNA binding motif [15–18], have nearly eliminated the design challenges encountered with zinc-finger nucleases and meganucleases. Another advantage of TALENs is their target specificity. TALEN monomers are typically designed with 15–20 RVDs, and, as a result, a TALEN target site is frequently >30 bp. This relatively large target site makes TALENs the most specific of all the nucleases, and may contribute to reduced toxicity compared to zinc-finger nucleases [19]. The only real drawback to the use of TALENs is their large size (~950 aa; ~1900 aa per pair) and repetitive nature, making delivery to plant cells a challenge. TALENs are typically delivered to plant cells by direct delivery of DNA to protoplasts, or by stable integration of TALEN-encoding constructs into plant genomes.

CRISPR/Cas

The most recent addition to the sequence-specific nuclease family, CRISPR/Cas, is proving to be the nuclease-of-choice

for plant genome engineering. Unlike the other three nuclease classes, which target DNA through protein/DNA interactions, CRISPR/Cas uses a guide RNA molecule (gRNA) to direct an endonuclease, Cas9, to a target DNA sequence. As a result, redirecting CRISPR/Cas is extremely simple, requiring only the cloning of a 20 nt sequence (complementary to a target DNA sequence) within a gRNA expression construct.

One limitation of the CRISPR/Cas system may be off-target cleavage [20,21]. Whereas 20 nucleotides are used to direct Cas9 binding and cleavage, the system tolerates mismatches, with a higher tolerance for mismatches at the 5′ end of the targeting-RNA sequence [20]. To reduce the likelihood of off-target cleavage, alternative CRISPR/Cas reagents have been developed, including paired Cas9 nickases [22,23], fusion of catalytically-dead Cas9 to *Fok*I [24,25], and shortening of the gRNA targeting sequence [26]. Possibly the simplest approach to minimize off-target cleavage is to design gRNAs that have minimum sequence homology to other sites within the plant genome. In addition to potential off-targeting, another limitation of the CRISPR/Cas system is size. Cas9 is ~1400 aa, making it one of the largest sequence-specific nucleases. However, for vectors that are unable to deliver Cas9, it may be possible to generate plant lines that constitutively express Cas9; therefore, only the delivery of gRNA(s) is required.

The potential of genome engineering for plant synthetic biology

To help describe how genome engineering can contribute to synthetic biology, we categorize the goals of synthetic biology projects into three groups: those that require precise insertion of DNA into plants genomes, those that require elimination or adjustment of host sequence, and those that require control over transcription of host or non-host genes. We describe how these modifications can be achieved using sequence-specific nucleases, and how they relate to synthetic biology projects.

Site-specific DNA integration

Generating plants with novel function frequently requires integrating foreign DNA (e.g., promoters, genes, terminators, and other transcription regulatory elements) into the plant genome. Conventional approaches for delivering this DNA include *Agrobacterium* or biolistics, both of which result in the random integration of one or more copies of the DNA sequence. While effective, these methods have several limitations: transgene expression often varies depending on chromosomal context and, when multiple transgenes are integrated at random sites on different chromosomes, they segregate independently, presenting a challenge for breeding regimes that seek to move transgenes into new germplasm. Using sequence-specific nucleases, foreign DNA can be precisely integrated at a locus of interest either through homologous recombination or non-homologous end joining (NHEJ)-mediated insertion (Figure 1). Not only does this enable trait stacking to expedite breeding efforts, but it may reduce variability in gene expression. Because of the minimal targeting constraints for TALENs and CRISPR/Cas systems, nearly all chromosomal positions are amenable to site-specific integration. Notably, there may be sites that are inaccessible or difficult to cleave because of epigenetic factors (e.g., chromatin structure, methylation) or genetic factors (e.g., repetitive DNA that permits nuclease binding to multiple sites within a genome).

Transgene stacking was first demonstrated in maize at a preintegrated synthetic target sequence [27] (Table 1). In this case the target for integration was a transgene construct that included the herbicide-tolerance gene phosphinothricin acetyltransferase (PAT) followed by a 'trait landing pad' with zinc-finger nuclease target sites flanked by DNA sequences for recombining with an incoming donor molecule. After co-transforming immature embryos with DNA encoding the zinc-finger nucleases and donor DNA (containing a second herbicide tolerance gene, *AAD1*, flanked by sequences of homology to the trait landing pad), 5% of the transgenic events contained the targeted integration event. Using a similar approach, trait stacking was accomplished in cotton; however, instead of using pre-engineered zinc-finger nucleases to break a synthetic target sequence, a meganuclease was designed to cleave an endogenous locus [9].

Besides integrating genes through homologous recombination, targeted gene insertion can also be accomplished through NHEJ. In this case the linear donor molecules, which do not contain flanking homology arms, are captured at DSBs during repair by NHEJ. Whereas this method has not been used extensively in plants [28–30], it has been employed frequently in mammalian cells [31]. With additional improvements in the efficiency that foreign DNA is captured at DSBs [32], this method has the potential to be an effective approach for targeted integration of DNA into plant genomes.

The ability to site-specifically integrate DNA into plant genomes will be of particular value for synthetic biology projects that require the transfer of numerous genetic parts to confer a novel biological function. For example, to reduce dependency on inorganic fertilizers, there is an ambitious synthetic biology project aimed at engineering cereals with the ability to fix atmospheric nitrogen. Currently, there are two conceivable approaches: either transfer the Nod factor signaling pathway from legumes to cereals, or transfer the nitrogenase enzyme from nitrogen-fixing bacteria to plant cells. With respect to the nitrogenase approach, numerous genes need to be transferred into a host plant, and many of these genes need to be properly regulated for nitrogenase to be active [33]. Although there are several challenges related to the execution of this system in plants (e.g., how will nitrogenase be protected from oxygen exposure), most of the nitrogen fixation (*nif*) genes are known, and the relative expression levels of these genes have been characterized. Furthermore, in an effort to generate standardized genetic elements for nitrogenase activity, the *nif* genes from *Klebsiella oxytoca* were refactored by removing all non-coding DNA and regulatory elements, and the codon composition was changed to be as different as possible to the original sequence but still encode the same protein [33]. Using sequence-specific nucleases, these genetic elements can be integrated, together with their desired regulatory elements, into safe-harbor loci within plant genomes where

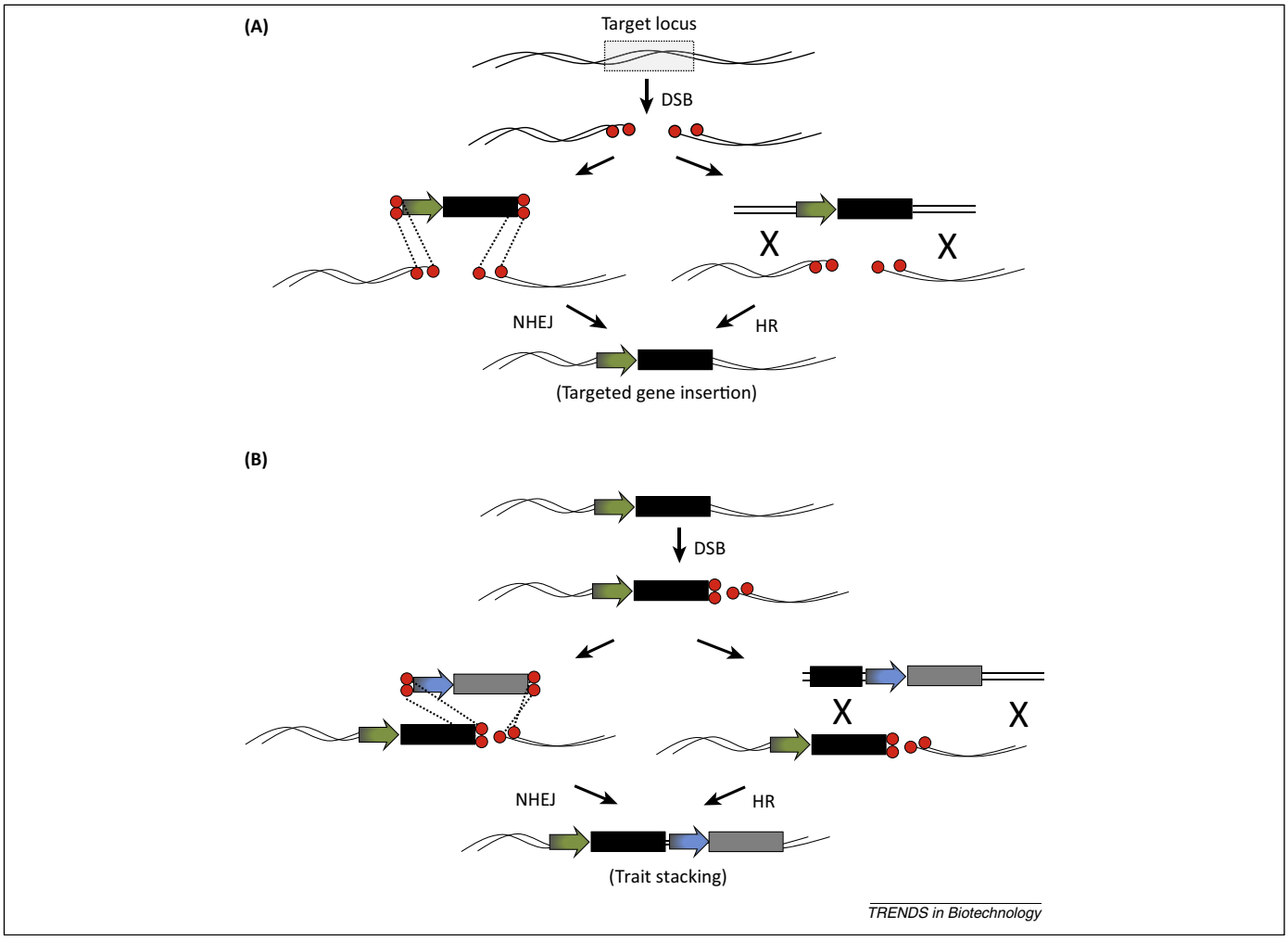

Figure 1. Approaches for the site-specific introduction of DNA into plant genomes. **(A)** Targeted insertion of DNA (e.g., promoter and coding sequence) into a desired locus can be facilitated through double-strand breaks (DSBs). A sequence-specific nuclease is delivered to plant cells together with a user-designed DNA sequence. Repair of the DSB by non-homologous end joining (NHEJ) can result in the direct ligation of the user-supplied DNA sequence into a break site. By contrast, the DSB can stimulate homologous recombination (HR) between a donor molecule and chromosomal sequence. **(B)** Trait stacking can be achieved using similar approaches. In this case a second user-designed DNA sequence is delivered to plant cells and inserted downstream of the first inserted sequence. Red circles, free DNA ends.

gene expression is dependable. Alternatively, as another approach to controlling gene expression, nitrogen fixation genes can be integrated downstream of endogenous cereal promoters that have the desired expression characteristics.

Another synthetic biology project in which targeted DNA insertion will be of value is the C_4 rice project, which requires generation of a two-celled photosynthesis cycle. In this system carbon dioxide is taken up by mesophyll cells and shuttled to bundle sheath cells where it is concentrated around ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). A crucial feature of this approach is to express RuBisCO and the enzymes of the Calvin–Benson cycle specifically within bundle sheath cells, and not mesophyll cells. Using sequence-specific nucleases, it may be possible to insert bundle sheath-specific promoters into the promoter region of RuBisCO- or Calvin–Benson cycle-related genes, thereby promoting tissue-specific function of the RuBisCO enzyme or of the Calvin–Benson cycle.

Rewriting host DNA

One of the more powerful uses of sequence-specific nucleases is for editing DNA within a plant genome. As previously noted, the advent of TALENs and CRISPR/Cas makes it possible to generate DSBs at nearly any site within a chromosome, enabling diverse types of genetic modifications. These modifications include targeted mutations, gene knockouts, deletions, gene replacements, controlled translocations and inversions, and alterations of transcriptional regulatory elements (Figure 2). The ability to achieve these modifications allows synthetic biologists not only to remove sources of unwanted or undesired DNA (e.g., inhibitory genes and pathways) but also to improve or adjust genic and transcriptional regulatory sequences.

One of the simplest ways to deploy sequence-specific nucleases is to create gene knockouts (Figure 2B). Knockouts are achieved when a broken chromosome is erroneously repaired by the NHEJ pathway. To demonstrate this approach for mutagenizing endogenous plant genes, zinc-finger nucleases were engineered to target sequences within the *Arabidopsis ADH1* or *TT4* genes [34]. Plants delivered the *ADH1* or *TT4* zinc-finger nucleases gave rise to mutant seed at a frequency of 69% and 33%, respectively. In addition to zinc-finger nucleases, TALENs and CRISPR/Cas have been successfully used to mutagenize host genes. For example, since its introduction in early

Table 1. List of genome modifications achieved in plants using sequence-specific nucleases

Type of DNA modification	Nuclease	Delivery method(s)	Donor?	Plant(s)	Target(s)	Refs
Trait stacking	Meganuclease	Bombardment	Yes	Cotton	Intergenic sequence	[9]
	Zinc-finger nuclease	Bombardment	Yes	*Zea mays*	Transgene	[27]
Rewriting host DNA: gene knockout	Meganuclease	Stable integration	No	*Zea mays*	Intergenic sequence	[59]
	Meganuclease	Stable integration; *Agrobacterium* T-DNA (transient)	No	*Zea mays*	*MS26*	[60]
	Zinc-finger nuclease	Stable integration	No	*Arabidopsis thaliana*	*ADH1, TT4*	[34]
	Zinc-finger nuclease	Stable integration	No	*Glycine max*	*DCL1a/b, DCL4a/b, RDR6a, HEN1a*, transgene	[61]
	Zinc-finger nuclease	Stable integration	No	*Arabidopsis thaliana*	*ABI4*	[62]
	Zinc-finger nuclease	Stable integration	No	*Arabidopsis thaliana*	*ADH1, TT4, MPK*	[63]
	Zinc-finger nuclease	RNA Virus	No	Tobacco; *Petunia hybrida*	Transgene	[10]
	Zinc-finger nuclease	Stable integration; *Agrobacterium* T-DNA (transient)	No	*Arabidopsis thaliana*; tobacco	Transgene	[64]
	Zinc-finger nuclease	Stable integration	No	*Arabidopsis thaliana*	Transgene	[65]
	TALEN	Stable integration	No	*Arabidopsis thaliana*	*ADH1, TT4, MAPKKK1, DSK2B, NATA2, GLL22*	[66]
	TALEN	Stable integration	No	*Glycine max*	*FAD2-1A/B*	[51]
	TALEN	Stable integration	No	*Hordeum vulgare*	*PAPhy_a*	[67]
	TALEN	*Agrobacterium* T-DNA (transient)	No	*Oryza sativa*	*SWEET14*	[68]
	TALEN	Protoplasts	No	*Arabidopsis thaliana; Tobacco*	*AtTT4, AtADH, NbSurB*	[15]
	TALEN	Protoplasts	No	*Oryza sativa; Brachypodium*	*OsDEP1, OsBADH2, SPL, SBP*	[43]
	TALEN	Bombardment	No	*Triticum aestivum*	*MLO*	[29]
	TALEN	Protoplasts; Stable integration	No	*Zea mays*	*PDS, IPK1A, IPK, MRP4*	[69]
	TALEN	Stable integration	No	*Hordeum vulgare*	Transgene	[70]
	Cas9 nickases	Stable integration	No	*Arabidopsis thaliana*	RTEL1	[89]
	CRISPR/Cas	Stable integration	No	*Arabidopsis thaliana*	*TT4, GAI, BRI1, JAZ1, CHLI, AP1*, transgene	[40]
	CRISPR/Cas	Stable integration	No	*Arabidopsis thaliana*	*ADH1; TT4*	[41]
	CRISPR/Cas	Protoplasts; *Agrobacterium* T-DNA (transient)	No	*Arabidopsis thaliana; Nicotiana benthamiana*	*AtPDS3, AtRACK1c, NbPDS3*	[37]
	CRISPR/Cas	Protoplasts; *Agrobacterium* T-DNA (transient)	No	Tobacco; *Arabidopsis thaliana*; Sorghum; *Oryza sativa*	*OsSWEET14*, transgene	[38]
	CRISPR/Cas	Protoplasts; Bombardment	No	*Oryza sativa; Triticum aestivum*	*OsPDS, OsBADH2,* Os02g23823, Os*MPK2, TaMLO*	[71]
	CRISPR/Cas	Stable integration	No	*Arabidopsis thaliana*	Transgene	[42]
	CRISPR/Cas	Stable integration	No	*Arabidopsis thaliana; Oryza sativa*	*AtBRI1, AtJAZ1, AtGAI, OsROC5, OsSPP, OsYSA*	[39]
	CRISPR/Cas	*Agrobacterium* T-DNA (transient)	No	Sweet orange	*PDS*	[72]
	CRISPR/Cas	Protoplasts	No	*Zea mays*	*IPK*	[68]
	CRISPR/Cas	Stable integration	No	*Oryza sativa*	*SWEET11/13/1a/1b*	[44]
	CRISPR/Cas	Stable integration	No	*Oryza sativa*	*PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP, YSA*	[73]
Rewriting host DNA: large deletion	Zinc-finger nuclease	Stable integration	No	Tobacco	Transgene	[47]
	Zinc-finger nuclease	Stable integration	No	*Arabidopsis thaliana*	*RPP4* gene cluster	[74]
	CRISPR/Cas	Protoplasts	No	*Arabidopsis thaliana*	*PDS3*	[37]
	CRISPR/Cas	Protoplasts; Stable integration	No	*Oryza sativa*	Labdane-related diterpenoid gene clusters on Chr 2, 4 and 6	[44]
Rewriting host DNA: gene replacement	Meganuclease	*Agrobacterium* T-DNA (transient)	Yes	Tobacco	Transgene	[8]
	Meganuclease	*Agrobacterium* T-DNA (transient)	Yes	Tobacco	Transgene	[75]

Table 1 (*Continued*)

Type of DNA modification	Nuclease	Delivery method(s)	Donor?	Plant(s)	Target(s)	Refs
	Zinc-finger nuclease	*Agrobacterium* T-DNA (transient; donor only)	Yes	*Arabidopsis thaliana*	Transgene	[76]
	Zinc-finger nuclease	*Agrobacterium* T-DNA (transient; donor only)	Yes	*Arabidopsis thaliana*	*PPO*	[77]
	Zinc-finger nuclease	DNA replicons	Yes	Tobacco	Transgene	[78]
	Zinc-finger nuclease	*Agrobacterium* T-DNA (transient)	Yes	Tobacco (suspension culture cells)	*CHN50*, transgene	[79]
	Zinc-finger nuclease	Protoplasts	Yes	*Arabidopsis thaliana*	*ADH1*	[63]
	Zinc-finger nuclease	Whiskers	Yes	*Zea mays*	*IPK1*	[80]
	Zinc-finger nuclease	Protoplasts	Yes	Tobacco	Transgene	[81]
	Zinc-finger nuclease	Protoplasts	Yes	Tobacco	*SurA/B*	[45]
	TALEN	Protoplasts	Yes	Tobacco	*SurA/B*	[46]
	CRISPR/Cas	Protoplasts	Yes	*Oryza sativa*	*PDS*	[43]
	CRISPR/Cas	Protoplasts	Yes	*Nicotiana benthamiana*	*PDS*	[37]
	CRISPR/Cas	Stable integration	Yes	*Arabidopsis thaliana*	ADH1	[89]
Controlling gene expression	TALE repressor (SRDX)	Stable integration	N/A[a]	*Arabidopsis thaliana*	*RD29A*, transgene	[52]
	TALE activator (native TALE activation domain, VP16, GAL4)	*Agrobacterium* T-DNA (transient)	N/A	*Nicotiana benthamiana*	Transgene	[82]
	Zinc-finger activator (VP16)	Stable integration	N/A	*Brassica napus*	*KasII*	[53]
	dCas9 activator (EDLL and TAL effector domain); dCas9 repressor (SRDX)	*Agrobacterium* T-DNA (transient)	N/A	*Nicotiana benthamiana*	PDS, transgene	[88]

[a]N/A, not applicable.

2013 [35,36], the CRISPR/Cas system has been used to mutagenize genes in several plant species including tobacco [37,38], *Arabidopsis* [37–42], rice [38,43,44], wheat [43], and sorghum [38].

In some cases, highly precise genetic modifications are desired. These can range from substitutions of single nucleotides within a genome to replacements of suboptimal host genes with optimized variants (Figure 2C). Such modifications are achieved using sequence-specific nucleases to stimulate homologous recombination between a user-supplied donor molecule and a chromosomal target. For example, using custom-designed zinc-finger nucleases, several nucleotide substitutions were introduced into the tobacco acetolactate synthase genes, thereby conferring resistance to imidazolinone and sulphonylurea herbicides [45]. In a similar study, TALENs were used to introduce targeted modifications to the tobacco acetolactate synthase genes, thereby demonstrating their utility of TALENs for gene editing [46]. More recently, CRISPR/Cas was used to achieve gene editing in rice and *Nicotiana benthamiana* protoplasts [37,43].

Besides introducing relatively small insertions or deletions, targeted DSBs can facilitate the creation of large chromosomal deletions, inversions (Figure 2D), or translocations (Figure 2E). These types of modifications may be useful for removing unwanted or inhibitory DNA sequences, or even entire genetic pathways. In general, this method requires two simultaneous DNA DSBs, either on the same chromosome or on different chromosomes, followed by NHEJ-directed repair. One of the first examples demonstrating gene deletion was performed in tobacco [47]. A zinc-finger nuclease pair was used to create DSBs at two sites that flank a preintegrated β-glucuronidase (GUS) transgene. As predicted, plants with a GUS-negative phenotype contained deletions of the entire 4.3 kb GUS gene. In a more recent study, large sequences of DNA (115–245 kb) containing labdane-related diterpenoid gene clusters were efficiently deleted from the rice genome using CRISPR/Cas [44]. Notably, whereas targeted chromosomal inversions have recently been demonstrated in *Arabidopsis* plant cells [48], examples of chromosomal translocations are limited to mammalian cells [49,50].

At the heart of synthetic biology is the desire to create a minimal cell, that is, to engineer a cell in which nonessential components are removed while still maintaining the ability to divide and pass down a genetic code. This minimal cell can then be used as a factory for building new biological systems. One way to achieve a minimal cell is through a top-down approach in which unnecessary components are deleted. Although a minimal plant cell is still a distant dream, the potential tools and methodologies for generating a 'simpler' plant cell are frequently being employed. For example, TALENs were used to reduce the number of components in the fatty acid metabolic pathway in soybean [51]. In this case, fatty acid desaturase 2 was inactivated such that the flow of molecules through

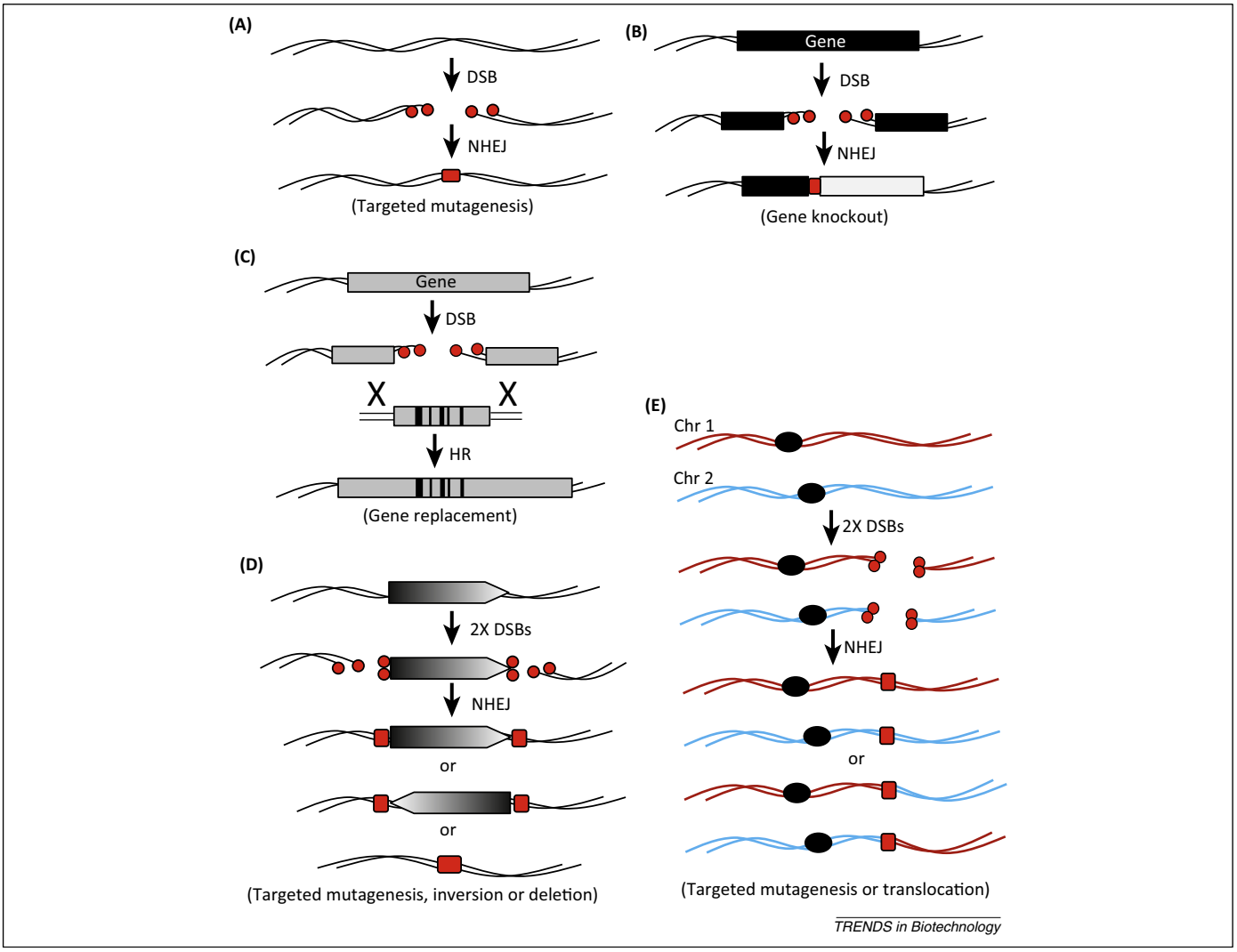

Figure 2. Approaches to rewriting plant DNA. Sequence-specific nucleases can be employed to generate double-strand breaks (DSBs) in **(A)** intergenic or **(B)** genic sequences. Repair by non-homologous end joining (NHEJ) can result in mutations, usually small insertions or deletions, at the break site (red box). DSBs occurring in coding sequences can result in frameshift mutations that destroy gene function. **(C)** Subtle sequence changes can be introduced into genes using sequence-specific nucleases to stimulate recombination with a donor molecule. **(D)** Targeting two DSBs within the same chromosome can result in either a mutation, inversion of intervening sequence, or deletion of intervening sequence. **(E)** Targeting two breaks on two different chromosomes (Chr) can facilitate two targeted mutations or translocations. Abbreviation: HR, homologous recombination.

the fatty acid metabolism pathway halted at oleic acid (mono-unsaturated) instead of stopping at linoleic acid (poly-unsaturated). Furthermore, CRISPR/Cas was used to delete kilobases of DNA in rice that were not necessary for plant growth or division [44]. In general, the ability to knock out or delete sequences from plant cells will be vital for progress towards a minimal cell. However, a strong understanding of the necessary and unnecessary genetic components would greatly facilitate this progression.

Controlling gene expression

In some synthetic biology applications it may be necessary to adjust the expression of host genes. These adjustments may range from enhancing or dampening the expression of a single gene to completely turning on or off the expression of multiple genes. The DNA binding domain used to target sequence-specific nucleases can be adopted to help control gene expression. In this case the nuclease domains (either the *Fok*I sequence from zinc-finger nucleases and TALENs, or the HNH and RuvC domains within Cas9) are removed or inactivated through mutation, and transcriptional activator or repressor domains are fused to the DNA-binding protein (Box 3). By engineering the resulting fusion protein to bind DNA near or within host promoters, expression of downstream genes can be controlled.

A recent example demonstrating control of gene expression used TALE-based repressors to reduce expression of endogenous genes in *Arabidopsis* [52]. A TALE was designed to recognize sequences at two different sites within the *Arabidopsis* genome: the promoter of a pre-integrated luciferase transgene and the promoter of the endogenous *RD29A* gene. The TALE coding sequence was then fused to the EAR-repression domain (SRDX) and stably integrated into the *Arabidopsis* genome. Luciferase expression and RT-PCR confirmed a decrease in the expression of the target genes in plants expressing the TALE repressor. Additional studies have used zinc-finger activators (zinc-finger arrays fused to the transcription-activation

Box 3. Controlling gene expression through DNA targeting

Practicing plant synthetic biology requires not only control over nucleotide sequences within plant genomes but also control over expression levels of host genes. The DNA binding domain of sequence-specific nucleases can be repurposed to help modulate the expression of endogenous genes. DNA-binding domains from zinc-finger arrays, TALEs, or catalytically-dead Cas9 (dCas9) and a gRNA are used to localize activator (Figure IA) or repressor (Figure IB) domains to genes of interest. Control of gene expression in plants has been achieved using zinc-finger activators (VP16), TALE activators (the endogenous C-terminal activation domain, VP16, GAL4) and TALE repressors (SRDX). CRISPR/Cas has also been used in plants to achieve gene activation (dCas9:ELDD and dCas9:TAD) and gene repression (dCas9:SRDX), and it has been employed in bacterial, yeast, and mammalian cells. In these cases, repressor or activator domains were directly fused to the Cas9 C-terminus or indirectly attached to the gRNA through an RNA-binding scaffold [83–86,88]. Uniquely, and in addition to these approaches, Cas9 can reduce gene expression by physically interfering with RNA polymerase progression [87].

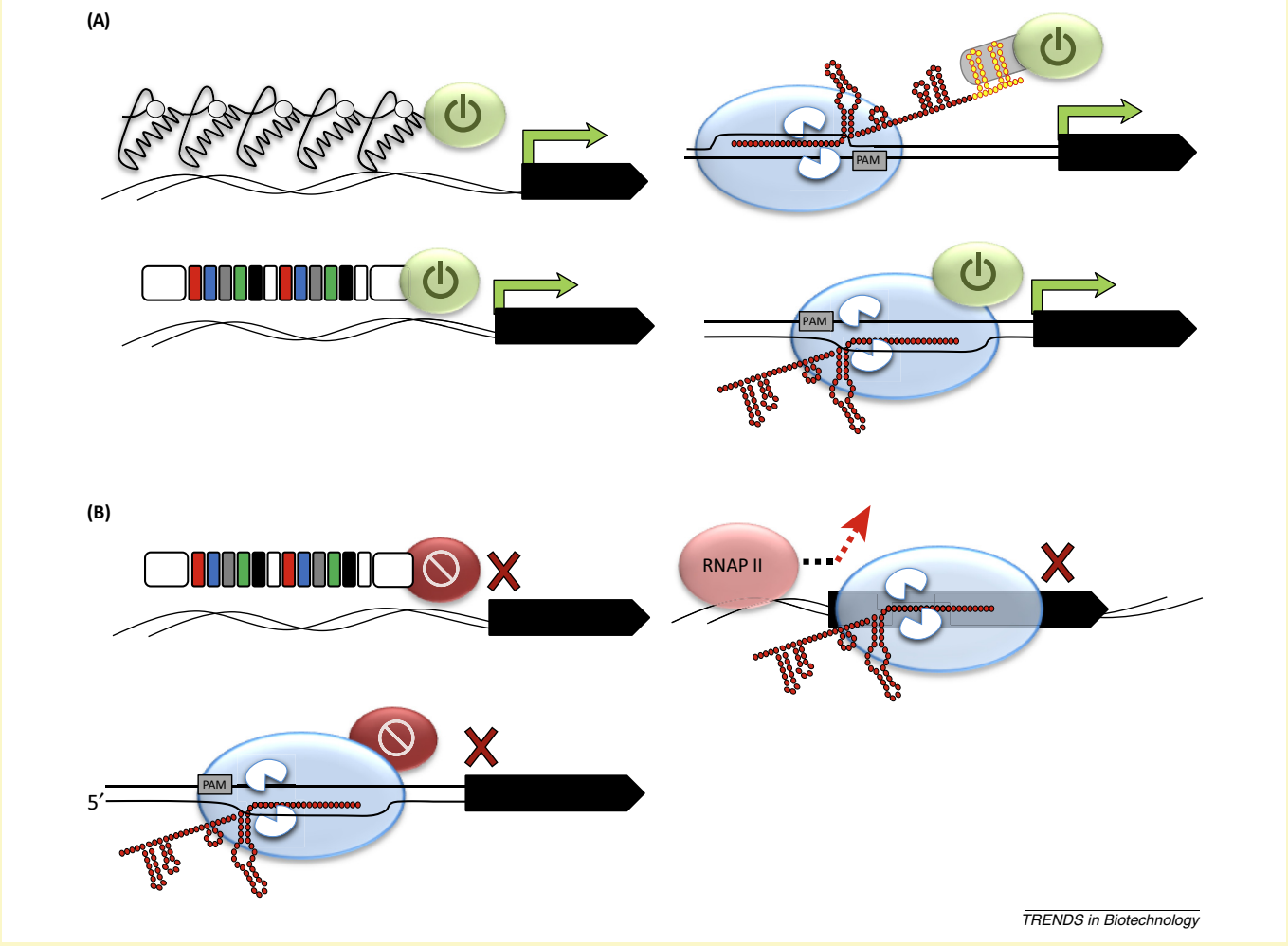

Figure I. Transcriptional control with nuclease DNA binding domains. **(A)** Programmable transcriptional activators. **(B)** Programmable transcriptional repressors. Abbreviations: PAM, protospacer adjacent motif; RNAP II, RNA polymerase II.

domain of the herpes simplex virus viral protein VP16) to enhance transcription of the endogenous gene β-ketoacyl-ACP synthase II in *Brassica napus* [53]. Notably, while CRISPR/Cas has been implemented as a transcription regulator in bacteria [54], yeast [55], and human cells [23], it has only recently been applied in plants [88]. To date, Cas-based transcriptional activators appear to be less effective than TALE-based activators in mammalian cells [56].

The ability to control gene expression will play an important role in future synthetic biology applications. For example, optimizing expression levels of genes in metabolic pathways may increase the flux of intermediates through a pathway, thereby increasing total yield of a desired compound. Furthermore, fine-tuning gene expression within complex gene circuits may help to strengthen a desired cellular change in response to a specific input. Consider, for example, the nitrogen-fixing cereal project, in which proper expression and regulation of *nif* genes is crucial for nitrogenase activity [33]. By targeting activators and repressors to promoter regions of the *nif* genes (after the *nif* genes have been inserted into the plant genome) a wide range of gene expression levels can be achieved for each individual gene. This wide range of transcription can facilitate optimization of the trait. A similar approach can be employed to optimize biological systems with more complex pathways. Consider, for example, the C_4 rice plant is genetically complete: essential C_4-related genes have been introduced into the rice genome and inhibitory endogenous genes

have been removed. Although all the genetic elements might be in place, this does not guarantee that the rice plants will efficiently fix carbon by the C_4 pathway. Instead, the rice prototype may require fine-tuning of gene expression to optimize protein levels and, consequently, the flux of molecules through the pathway. Taken together, the likelihood that the nitrogen-fixing cereals and C_4 rice project will meet with success depends on a highly coordinated effort to modify the genome of the plant – from the integration of well-defined genetic elements to the fine control of their expression levels. All of this is attainable using sequence-specific nucleases and targeted transcriptional regulators.

Concluding remarks and future perspectives

As we further understand plant systems biology, and as the availability of standardized genetic parts increases, we will better understand how to design synthetic systems in plants. Concurrent with these efforts, there will be a demand for methods to edit DNA within living plant cells using zing-finger nucleases, TALENs, and CRISPR/Cas reagents. In the past four years, sequence-specific nucleases have been used to achieve a wide range of modifications, from subtle nucleotide changes within host genes, to the deletion of megabases of DNA. The DNA-binding domains of sequence-specific nucleases have also been adopted as tools for controlling gene expression. Together with well-defined and programmable DNA parts, plant genome engineering has great potential to facilitate ambitious synthetic biology projects, including introducing nitrogen fixation into cereals and improving the photosynthetic capacity of rice.

Disclaimer statement

D.F.V. is the CSO of Cellectis Plant Sciences, a plant genome engineering company. N.J.B. works for Cellectis Plant Sciences. Both D.F.V. and N.J.B. are authors on patents pertaining to plant genome engineering tools and methods.

References

1 Arkin, A. *et al.* (2009) What's in a name? *Nat. Biotechnol.* 27, 1071–1073
2 Von Caemmerer, S. *et al.* (2012) The development of C_4 rice: current progress and future challenges. *Science* 336, 1671–1672
3 Oldroyd, G.E.D. and Dixon, R. (2014) Biotechnological solutions to the nitrogen problem. *Curr. Opin. Biotechnol.* 26, 19–24
4 Kircher, M. (2012) The transition to a bio-economy: emerging from the oil age. *Biofuels Bioprod. Biorefining* 6, 369–375
5 Vaeck, M. *et al.* (1987) Transgenic plants protected from insect attack. *Nature* 328, 33–37
6 Antunes, M.S. *et al.* (2009) Engineering key components in a synthetic eukaryotic signal transduction pathway. *Mol. Syst. Biol.* 5, 270
7 Wyman, C. and Kanaar, R. (2006) DNA double-strand break repair: all's well that ends well. *Annu. Rev. Genet.* 40, 363–383
8 Puchta, H. *et al.* (1996) Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5055–5060
9 D'Halluin, K. *et al.* (2013) Targeted molecular trait stacking in cotton through targeted double-strand break induction. *Plant Biotechnol. J.* 11, 933–941
10 Marton, I. *et al.* (2010) Nontransgenic genome modification in plant cells. *Plant Physiol.* 154, 1079–1087
11 Prieto, J. *et al.* (2007) The C-terminal loop of the homing endonuclease I-CreI is essential for site recognition, DNA binding and cleavage. *Nucleic Acids Res.* 35, 3262–3271
12 Ramirez, C.L. *et al.* (2008) Unexpected failure rates for modular assembly of engineered zinc fingers. *Nat. Methods* 5, 374–375
13 Maeder, M.L. *et al.* (2008) Rapid 'open-source' engineering of customized zinc-finger nucleases for highly efficient gene modification. *Mol. Cell* 31, 294–301
14 Sander, J.D. *et al.* (2011) Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods* 8, 67–69
15 Cermak, T. *et al.* (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* 39, e82
16 Reyon, D. *et al.* (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol.* 30, 460–465
17 Briggs, A.W. *et al.* (2012) Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. *Nucleic Acids Res.* 40, e117
18 Schmid-Burgk, J.L. *et al.* (2013) A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes. *Nat. Biotechnol.* 31, 76–81
19 Mussolino, C. *et al.* (2014) TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. *Nucleic Acids Res.* 42, 6762–6773
20 Fu, Y. *et al.* (2013) High-frequency off-target mutagenesis induced by CRISPR–Cas nucleases in human cells. *Nat. Biotechnol.* 31, 822–826
21 Cho, S.W. *et al.* (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res.* 24, 132–141
22 Ran, F.A. *et al.* (2013) Double nicking by RNA-guided CRISPR cas9 for enhanced genome editing specificity. *Cell* 154, 1380–1389
23 Mali, P. *et al.* (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* 31, 833–838
24 Guilinger, J.P. *et al.* (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat. Biotechnol.* 32, 577–582
25 Tsai, S.Q. *et al.* (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nat. Biotechnol.* 32, 569–576
26 Fu, Y. *et al.* (2014) Improving CRISPR–Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* 32, 279–284
27 Ainley, W.M. *et al.* (2013) Trait stacking via targeted genome editing. *Plant Biotechnol. J.* 11, 1126–1134
28 Chilton, M-D.M. and Que, Q. (2003) Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. *Plant Physiol.* 133, 956–965
29 Wang, Y. *et al.* (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947–951
30 Salomon, S. and Puchta, H. (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. *EMBO J.* 17, 6086–6095
31 Gabriel, R. *et al.* (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat. Biotechnol.* 29, 816–823
32 Maresca, M. *et al.* (2013) Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Res.* 23, 539–546
33 Temme, K. *et al.* (2012) Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7085–7090
34 Zhang, F. *et al.* (2010) High frequency targeted mutagenesis in *Arabidopsis thaliana* using zinc finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12028–12033
35 Mali, P. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826
36 Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823
37 Li, J-F. *et al.* (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* 31, 688–691
38 Jiang, W. *et al.* (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Res.* 41, e188
39 Feng, Z. *et al.* (2013) Efficient genome editing in plants using a CRISPR/Cas system. *Cell Res.* 23, 1229–1232

40 Feng, Z. *et al.* (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4632–4637
41 Fauser, F. *et al.* (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J.* 79, 348–359
42 Jiang, W. *et al.* (2014) Efficient CRISPR/Cas9-mediated gene editing in *Arabidopsis thaliana* and inheritance of modified genes in the T2 and T3 generations. *PLoS ONE* 9, e99225
43 Shan, Q. *et al.* (2013) Targeted genome modification of crop plants using the CRISPR–Cas system. *Nat. Biotechnol.* 31, 686–688
44 Zhou, H. *et al.* (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Res.* 42, 10903–10914
45 Townsend, J.A. *et al.* (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature* 459, 442–445
46 Zhang, Y. *et al.* (2013) Transcription activator-like effector nucleases enable efficient plant genome engineering. *Plant Physiol.* 161, 20–27
47 Petolino, J.F. *et al.* (2010) Zinc finger nuclease-mediated transgene deletion. *Plant Mol. Biol.* 73, 617–628
48 Qi, Y. *et al.* (2013) Targeted deletion and inversion of tandemly arrayed genes in *Arabidopsis thaliana* using zinc finger nucleases. *G3* 3, 1707–1715
49 Brunet, E. *et al.* (2009) Chromosomal translocations induced at specified loci in human stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10620–10625
50 Piganeau, M. *et al.* (2013) Cancer translocations in human cells induced by zinc finger and TALE nucleases. *Genome Res.* 23, 1182–1193
51 Haun, W. *et al.* (2014) Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant Biotechnol.* 12, 934–940
52 Mahfouz, M.M. *et al.* (2012) Targeted transcriptional repression using a chimeric TALE–SRDX repressor protein. *Plant Mol. Biol.* 78, 311–321
53 Gupta, M. *et al.* (2012) Transcriptional activation of *Brassica napus* β-ketoacyl-ACP synthase II with an engineered zinc finger protein transcription factor. *Plant Biotechnol. J.* 10, 783–791
54 Bikard, D. *et al.* (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR–Cas system. *Nucleic Acids Res.* 41, 7429–7437
55 Farzadfard, F. *et al.* (2013) Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth. Biol.* 2, 604–613
56 Gao, X. *et al.* (2014) Comparison of TALE designer transcription factors and the CRISPR/dCas9 in regulation of gene expression by targeting enhancers. *Nucleic Acids Res.* 42, e155
57 Gorbunova, V. and Levy, A.A. (1997) Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. *Nucleic Acids Res.* 25, 4650–5467
58 Kim, Y.G. *et al.* (1996) Hybrid restriction enzymes: zinc finger fusions to FokI cleavage domain. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1156–1160
59 Gao, H. *et al.* (2010) Heritable targeted mutagenesis in maize using a designed endonuclease. *Plant J.* 61, 176–187
60 Djukanovic, V. *et al.* (2013) Male-sterile maize plants produced by targeted mutagenesis of the cytochrome P450-like gene (MS26) using a re-designed I-CreI homing endonuclease. *Plant J.* 76, 888–899
61 Curtin, S.J. *et al.* (2011) Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. *Plant Physiol.* 156, 466–473
62 Osakabe, K. *et al.* (2010) Site-directed mutagenesis in *Arabidopsis* using custom-designed zinc finger nucleases. *Proc. Natl. Acad. Sci. U.S.A.* 107, 12034–12039
63 Qi, Y. *et al.* (2013) Increasing frequencies of site-specific mutagenesis and gene targeting in *Arabidopsis* by manipulating DNA repair pathways. *Genome Res.* 23, 547–554
64 Tovkach, A. *et al.* (2009) A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells. *Plant J.* 57, 747–757
65 Even-Faitelson, L. *et al.* (2011) Localized egg-cell expression of effector proteins for targeted modification of the *Arabidopsis* genome. *Plant J.* 68, 929–937
66 Christian, M. *et al.* (2013) Targeted mutagenesis of *Arabidopsis thaliana* using engineered TAL effector nucleases. *G3 (Bethesda)* 3, 1697–1705
67 Wendt, T. *et al.* (2013) TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants. *Plant Mol. Biol.* 83, 279–285
68 Li, T. *et al.* (2012) High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nat. Biotechnol.* 30, 390–392
69 Liang, Z. *et al.* (2014) Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. *J. Genet. Genomics* 41, 63–68
70 Gurushidze, M. *et al.* (2014) True-breeding targeted gene knock-out in barley using designer TALE-nuclease in haploid cells. *PLoS ONE* 9, e92046
71 Shan, Q. *et al.* (2013) Rapid and efficient gene modification in rice and *Brachypodium* using TALENs. *Mol. Plant* 6, 1365–1368
72 Jia, H. and Wang, N. (2014) Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS ONE* 9, e93806
73 Zhang, H. *et al.* (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12, 797–807
74 Qi, Y. *et al.* (2013) Targeted deletion and onversion of tandemly arrayed genes in Arabidopsis thaliana using zinc finger nucleases. *G3* 3, 1707–1715
75 Reiss, B. *et al.* (2000) RecA stimulates sister chromatid exchange and the fidelity of double-strand break repair, but not gene targeting, in plants transformed by *Agrobacterium*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3358–3363
76 De Pater, S. *et al.* (2009) ZFN-induced mutagenesis and gene-targeting in *Arabidopsis* through *Agrobacterium*-mediated floral dip transformation. *Plant Biotechnol. J.* 7, 821–835
77 De Pater, S. *et al.* (2013) ZFN-mediated gene targeting of the *Arabidopsis* protoporphyrinogen oxidase gene through *Agrobacterium*-mediated floral dip transformation. *Plant Biotechnol. J.* 11, 510–515
78 Baltes, N.J. *et al.* (2014) DNA replicons for plant genome engineering. *Plant Cell* 26, 151–163
79 Cai, C.Q. *et al.* (2009) Targeted transgene integration in plant cells using designed zinc finger nucleases. *Plant Mol. Biol.* 69, 699–709
80 Shukla, V.K. *et al.* (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature* 459, 437–441
81 Wright, D.A. *et al.* (2005) High-frequency homologous recombination in plants mediated by zinc-finger nucleases. *Plant J.* 44, 693–705
82 Geißler, R. *et al.* (2011) Transcriptional activators of human genes with programmable DNA-specificity. *PLoS ONE* 6, e19509
83 Nissim, L. *et al.* (2014) Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. *Mol. Cell* 54, 698–710
84 Cheng, A.W. *et al.* (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res.* 23, 1163–1171
85 Maeder, M.L. *et al.* (2013) CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods* 10, 977–979
86 Gilbert, L. *et al.* (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154, 442–451
87 Qi, L.S. *et al.* (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183
88 Piatek, A. *et al.* (2014) RNA - guided transcriptional regulation in planta via synthetic dCas9 - based transcription factors. *Plant Biotechnol. J.* Published online November 14, 2014. (http://dx.doi.org/10.1111/pbi.12284)
89 Schiml, S. *et al.* (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. *Plant J.* Published online November 11, 2014. (http://dx.doi.org/10.1111/tpj.12704)