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Use of designer nucleases for targeted gene and genome editing in plants

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Abstract

The ability to efficiently inactivate or replace genes in model organisms allowed a rapid expansion of our understanding of many of the genetic, biochemical, molecular and cellular mechanisms that support life. With the advent of new techniques for manipulating genes and genomes that are applicable not only to single-celled organisms, but also to more complex organisms such as animals and plants, the speed with which scientists and biotechnologists can expand fundamental knowledge and apply that knowledge to improvements in medicine, industry and agriculture is set to expand in an exponential fashion. At the heart of these advancements will be the use of gene editing tools such as zinc finger nucleases, modified meganucleases, hybrid DNA/ RNA oligonucleotides, TAL effector nucleases and modified CRISPR/Cas9. Each of these tools has the ability to precisely target one specific DNA sequence within a genome and (except for DNA/ RNA oligonucleotides) to create a double-stranded DNA break. DNA repair to such breaks sometimes leads to gene knockouts or gene replacement by homologous recombination if exogenously supplied homologous DNA fragments are made available. Genome rearrangements are also possible to engineer. Creation and use of such genome rearrangements, gene knockouts and gene replacements by the plant science community is gaining significant momentum. To document some of this progress and to explore the technology's longer term potential, this review highlights present and future uses of designer nucleases to greatly expedite research with model plant systems and to engineer genes and genomes in major and minor crop species for enhanced food production.

Keywords

designer nucleases, CRISPR/Cas9, TAL effector nuclease, zinc finger nuclease, site-directed mutagenesis, genome editing

Disciplines

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Review article

Use of designer nucleases for targeted gene and genome editing in plants

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Summary

The ability to efficiently inactivate or replace genes in model organisms allowed a rapid expansion of our understanding of many of the genetic, biochemical, molecular and cellular mechanisms that support life. With the advent of new techniques for manipulating genes and genomes that are applicable not only to single-celled organisms, but also to more complex organisms such as animals and plants, the speed with which scientists and biotechnologists can expand fundamental knowledge and apply that knowledge to improvements in medicine, industry and agriculture is set to expand in an exponential fashion. At the heart of these advancements will be the use of gene editing tools such as zinc finger nucleases, modified meganucleases, hybrid DNA/RNA oligonucleotides, TAL effector nucleases and modified CRISPR/Cas9. Each of these tools has the ability to precisely target one specific DNA sequence within a genome and (except for DNA/RNA oligonucleotides) to create a double-stranded DNA break. DNA repair to such breaks sometimes leads to gene knockouts or gene replacement by homologous recombination if exogenously supplied homologous DNA fragments are made available. Genome rearrangements are also possible to engineer. Creation and use of such genome rearrangements, gene knockouts and gene replacements by the plant science community is gaining significant momentum. To document some of this progress and to explore the technology's longer term potential, this review highlights present and future uses of designer nucleases to greatly expedite research with model plant systems and to engineer genes and genomes in major and minor crop species for enhanced food production.

Keywords: designer nucleases, CRISPR/Cas9, TAL effector nuclease, zinc finger nuclease, site-directed mutagenesis, genome editing.

Introduction

Research with bacteria and yeast using transposon insertion to inactivate genes or using homologous recombination to allow gene DNA sequence replacement started more than 50 years ago and led to an explosive expansion of knowledge regarding the basic mechanisms needed for cell survival and growth (Eisenstark, 1977; Hastings, 1975; Kleckner, 1981; Orr-Weaver and Szostak, 1985; Radding, 1982; Roeder and Fink, 1982; Rudolph *et al.*, 1985; Williamson, 1983). Likewise, the more recent advent of techniques for producing mice and rats with targeted gene knockouts resulted in similar advances in our understanding of mechanism controlling normal and abnormal growth and development in organisms as complex as mammals (Capecchi, 1989; Searle *et al.*, 1994). Results from such pioneering research not only contributed enormously to our store of biological knowledge but also spurred growth of the field of biotechnology with its attendant contributions to rapid progress in industry, medicine and agriculture. Unfortunately, until just recently, the ability to inactivate genes or promote gene replacement by homologous recombination has been lacking in most plants and animals. This impediment is now being pushed aside with the development of facile methods for targeting specific genes for double-stranded DNA breaks (DSBs) and attendant gene knockout or gene replacement (if an appropriate donor DNA is present). In this review, we briefly describe the development and use of the designer nucleases, zinc finger nucleases (ZFNs), TAL effector

nucleases (TALENs) and CRISPR/Cas9, for gene editing with prime focus on their application and potential impact in expediting research with higher plants and to improving the productivity of important crop plants.

The burst of research stimulated by the availability of ZFNs (e.g. Ainley *et al.*, 2013; Lloyd *et al.*, 2005; Qi *et al.*, 2014), TALENs (e.g. Christian *et al.*, 2010; Li *et al.*, 2011a) and CRISPR/Cas9 (e.g. Cong *et al.*, 2013; Jinek *et al.*, 2012; Mali *et al.*, 2013; Shalem *et al.*, 2015) gene editing technologies has created a flood of publications in the last 3 years. Thus, a comprehensive review of even those articles dealing only with the use of ZFNs, meganucleases, DNA/RNA oligonucleotides, TALENs and CRISPR/Cas9 technologies in plant research is not possible. We refer readers to a number of excellent reviews regarding the use of gene editing tools in plants for a more complete overview of ZFNs (Petolino, 2015), TALENs (Doyle *et al.*, 2013; Wright *et al.*, 2014), CRISPR/Cas9 (Belhaj *et al.*, 2014, 2015; Bortesi and Fischer, 2015) and all gene editing platforms (Baltes and Voytas, 2015; Osakabe and Osakabe, 2015; Small and Puchta, 2014; Voytas, 2013). Likewise, several excellent reviews regarding the use of gene editing tools in the broad scope of biology and medicine are available (Carroll, 2014; Doudna, 2015; Doudna and Charpentier, 2014; Hsu *et al.*, 2014). The use of T-DNA insertional lines in *Arabidopsis* (Azpiroz-Leehan and Feldmann, 1997) and crops such as rice (An *et al.*, 2005) can be acknowledged as a form of gene editing (or mutagenesis) that has contributed significantly to advances in our basic understanding of plant biology. Reviews

and applications of gene editing techniques in animal agriculture are also available (Petersen and Niemann, 2015; Proudfoot *et al.*, 2015; Wu *et al.*, 2015). Other articles in this special issue of the *Plant Biotechnology Journal* provide overviews of the development of designer nucleases, the use of recombinases and RNA/DNA oligonucleotides for plant genome modification and the use of genome editing tools for aiding commercial plant breeding. The present review will highlight key contributions related to the development of gene editing techniques in model plants and to initial efforts to apply these techniques to crop improvement.

New discoveries and proof-of-concept research with *Arabidopsis*, tobacco and other model plant systems

Because of their short life cycles and ease of genetic transformation, *Arabidopsis* and tobacco have become important research tools in plant biology. Here, we will discuss the use of these plants as vehicles for testing ZFNs, TALENs and CRISPR/Cas9 as new mechanisms for gene editing and for allowing proof-of-concept experiments to explore the kinds of gene and genome modifications that are possible with plants (Table 1).

Zinc finger nucleases

Zinc finger nucleases (Figure 1a) are composed of proteins bearing multiple zinc finger domains that, together, are capable of recognizing a specific sequence of six to nine consecutive nucleotides within the genome of a particular organism (Carroll, 2014). To the C-terminal end of this DNA recognition molecule is added a nonspecific nuclease domain from the restriction enzyme *FokI* to create one-half of a ZFN pair. The second half of the pair has a similar structure but is designed to recognize and bind to a DNA sequence on the opposite DNA strand approximately 6 nucleotides away from the first ZFN. This spacing allows the two inactive *FokI* nuclease domains to dimerize, become active as a nuclease and create a double-stranded DNA break (DSB) in the middle of the spacer region between the two ZFNs. The DSB is often repaired by the nonhomologous end joining (NHEJ) DNA repair mechanism that is error-prone. That is, during the repair process, usually small number of nucleotides can be deleted or added at the cleavage site. If this faulty repair is in the coding region of a gene, it can disrupt the reading frame and create an inactive (knockout) gene. Alternatively, if a DNA fragment with strong homology to the disrupted gene (but not the exact same sequence) is present, the new DNA fragment can bind and displace the original gene sequence by a process called homologous recombination and result in 'gene replacement'. Sophisticated programs to aid in the design of ZFNs to match DNA sequence in a target gene have been developed (e.g. Hansen *et al.*, 2012; Osborn *et al.*, 2011). Custom-designed synthetic ZFNs are also available commercially from Sigma-Aldrich (St. Louis, MO) using a design program developed by Sangamo, Inc. (Urnov *et al.*, 2010). Successful use of ZFNs for gene editing in *Arabidopsis* was first reported in 2005 (Lloyd *et al.*, 2005) and demonstrated not only for targeted gene disruptions, but also for inheritance of the some of the mutant genes in the T1 generation. Two follow-up articles in 2009 (Shukla *et al.*, 2009; Townsend *et al.*, 2009) and two back-to-back articles (Osakabe *et al.*, 2010; Zhang *et al.*, 2010) in 2010 reported high efficiency in targeting specific genes in tobacco, maize and *Arabidopsis* with custom-designed ZFNs. Successful use of ZFNs for gene editing in tobacco was first reported in 2005 (Wright *et al.*, 2005)

Table 1 Major model and crop plants targeted for gene editing with engineered nucleases

Species	Nuclease	References
<i>Arabidopsis</i>	Meganuclease	Roth <i>et al.</i> (2012)
	ZFN	Lloyd <i>et al.</i> (2005); Osakabe <i>et al.</i> (2010); Zhang <i>et al.</i> (2010); Petolino <i>et al.</i> (2010); Even-Faitelson <i>et al.</i> (2011); Qi <i>et al.</i> (2013a); de Pater <i>et al.</i> (2013); Chen <i>et al.</i> (2014)
	TAL effector nucleases (TALEN)	Cermak <i>et al.</i> (2011); Christian <i>et al.</i> (2013)
	Cas9/sgRNA	Li <i>et al.</i> (2013); Jiang <i>et al.</i> (2013); Feng <i>et al.</i> (2013); Mao <i>et al.</i> (2013); Feng <i>et al.</i> (2014); Schiml <i>et al.</i> (2014); Jiang <i>et al.</i> (2014a,b); Haun <i>et al.</i> (2014); Johnson <i>et al.</i> (2015); Gao <i>et al.</i> (2015a,b,c); Liu <i>et al.</i> (2015); Ning <i>et al.</i> (2015)
Calona	Zinc finger nucleases (ZFN)	Gupta <i>et al.</i> (2012)
Cotton	Meganuclease	D'Halluin <i>et al.</i> (2013)
Potato	TALEN	Nicolia <i>et al.</i> (2015); Clasen <i>et al.</i> (2015)
	Cas9/sgRNA	Wang <i>et al.</i> (2015b)
Soya bean	ZFN	Curtin <i>et al.</i> (2011, 2013)
	TALEN	Haun <i>et al.</i> (2014)
	Cas9/sgRNA	Jacobs <i>et al.</i> (2015)
Tobacco	ZFN	Wright <i>et al.</i> (2005); Maeder <i>et al.</i> (2008); Townsend <i>et al.</i> (2009); Cai <i>et al.</i> (2009); Petolino <i>et al.</i> (2010); Baltés <i>et al.</i> (2014)
	TALEN	Cermak <i>et al.</i> (2011); Mahfouz <i>et al.</i> (2011); Zhang <i>et al.</i> (2013)
	Cas9/sgRNA	Li <i>et al.</i> (2013); Jiang <i>et al.</i> (2013); Nekrasov <i>et al.</i> (2013); Gao <i>et al.</i> (2015a,b,c); Johnson <i>et al.</i> (2015)
Tomato	TALEN	Lor <i>et al.</i> (2014)
	Cas9/sgRNA	Brooks <i>et al.</i> (2014); Ron <i>et al.</i> (2014)
Barley	TALEN	Wendt <i>et al.</i> (2013); Gurushidze <i>et al.</i> (2014)
Maize	Meganuclease	Gao <i>et al.</i> (2010); Djukanovic <i>et al.</i> (2013); Martin-Ortigosa <i>et al.</i> (2014)
	ZFN	Shukla <i>et al.</i> (2009); Ainley <i>et al.</i> (2013)
	TALEN	Liang <i>et al.</i> (2014); Char <i>et al.</i> (2015)
	Cas9/sgRNA	Liang <i>et al.</i> (2014); Cigan <i>et al.</i> pers. commun.
Rice	TALEN	Li <i>et al.</i> (2012); Shan <i>et al.</i> (2013a, 2015)
	Cas9/sgRNA	Jiang <i>et al.</i> (2013); Shan <i>et al.</i> (2013b); Feng <i>et al.</i> (2013); Miao <i>et al.</i> (2013); Zhang <i>et al.</i> (2014); Zhou <i>et al.</i> (2014); Xu <i>et al.</i> (2014); Endo <i>et al.</i> (2015); Xie <i>et al.</i> (2015); Shimatani <i>et al.</i> (2015); Zhang <i>et al.</i> (2015); Ma <i>et al.</i> (2015)
Sorghum	Cas9/sgRNA	Jiang <i>et al.</i> (2013)
Wheat	TALEN	Wang <i>et al.</i> (2014)
	Cas9/sgRNA	Shan <i>et al.</i> (2013b); Upadhyay <i>et al.</i> (2013); Wang <i>et al.</i> (2014)

including demonstration of efficient ZFN-stimulated gene replacement. In 2008, improved methods for ZFN design became available for use in plants and other eukaryotes (Maeder *et al.*,

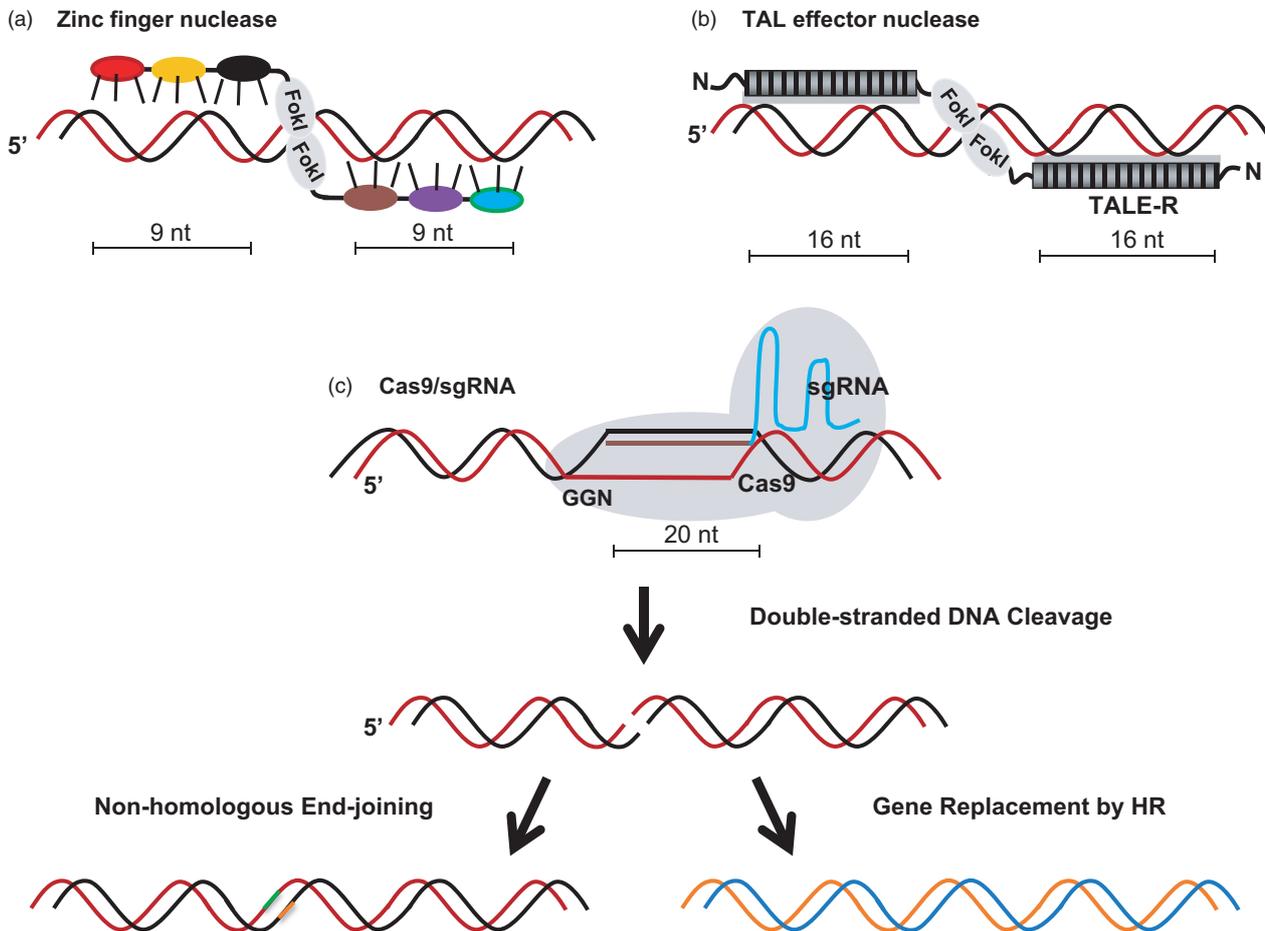


Figure 1 Designer nucleases for gene editing/gene replacement. (a) Zinc finger nucleases (ZFNs) recognize target DNA sequences by virtue of three custom-designed zinc finger proteins each recognizing three nucleotides and, together, providing 9-nucleotide specificity. ZFNs are used in pairs to allow recognition of two closely spaced DNA sequences with combined 18-nucleotide recognition specificity. The nuclease domain of the restriction enzyme FokI is attached to the C-terminus of each ZFN, and two ZFNs bind to opposite strands of DNA. Spacing between the two ZFNs allows the FokI nuclease domains to dimerize and create a double-stranded DNA break (DSB). (b) TAL effector nucleases (TALENs) work in a similar fashion as ZFNs, but use for DNA recognition a tandem array of 16 (or more) nearly identical protein domains of 34 amino acids each. At the 12th and 13th position in each repeat can be any of a set of two amino acids that can recognize one type of nucleotide (e.g. a histidine and aspartic acid pair recognizes a cytosine DNA base). Thus, arrays of repeats can be synthesized that can accurately recognize essentially any DNA sequence. By employing two TALENs recognizing adjacent DNA sequences on opposite strands, the two FokI domains attached to each TAL effector can dimerize and cause a DSB. (c) The CRISPR/Cas9/sgRNA system for gene editing relies on RNA-directed binding of the Cas9 nuclease to its target site on DNA. A 20 nucleotide sequence of the sgRNA hybridizes to the target DNA sequence, and this interaction triggers conformational changes in the sgRNA/Cas9 complex that result in activation of two independent nuclease domains in Cas9 and subsequent catalysis of a DSB. Once a DSB has been caused by ZFNs, TALENs or Cas9/sgRNA complexes, there are two alternative DNA repair processes. Most DSBs are repaired by a process called nonhomologous end joining, an error-prone process, in which the two ends of the DNA break are brought together—often with the insertion or deletions of one or a few nucleotides. If such errors occur in the reading frame of a target gene, the reading frame is disrupted and the gene is inactivated (i.e. ‘knocked out’). Alternatively, if a segment of DNA is present that is highly homologous to the broken DNA strand, the new segment can be incorporated in place of the original DNA by a process called homologous recombination (HR). This can result in a gene that produces a protein with a new or modified function as a result of the ‘gene replacement’.

2008). This was followed in 2009 with a pivotal paper demonstrating in tobacco that an inactive green fluorescence gene could be mutagenized and restored to activity using a set of ZFNs to target the inactive gene sequence (Cai *et al.*, 2009). More importantly, these researchers demonstrated that targeting a specific gene with ZFNs and simultaneously supplying a DNA sequence homologous to the target gene could lead to highly efficient gene replacement by homologous recombination. To accomplish this, they delivered ZFNs designed to bind and cleave an inactive phosphinothricin acetyltransferase (PAT) gene and, at the same time, they also delivered a DNA fragment homologous

to the PAT gene that contained a sequence to restore the herbicide resistance gene to full activity. Approximately 10% of resulting tobacco plants showed tolerance to treatment with glufosinate-type herbicides (of which phosphinothricin is one). The ability of ZFNs to stimulate homologous recombination (HR) and gene replacement has also been shown in *Arabidopsis* with experiments in which the protoporphyrinogen oxidase gene was mutated to allow resistance to butafenacil, a potent PPO inhibitor (de Pater *et al.*, 2013). An important step forward in making gene replacement through HR more facile was the recent design of plasmids containing DNA replication origins from gemi-

niviruses. These modified viruses allowed delivery of ZFNs (or Cas9/sgRNA genes) and, simultaneously, delivery of fragments of DNA that were homologous to target gene sequences and that contained a desired gene mutation. Activity of the viral replicase resulted in an abundance of copies of the replacement gene and a pleiotropic effect on homologous recombination (Baltes *et al.*, 2014).

Zinc finger nucleases have also been utilized to demonstrate that several other gene editing and chromatin modification techniques are possible with designer nuclease technologies. For example, cell- and tissue-specific gene expression is possible, as shown by the ability to localize gene expression to the egg cell in *Arabidopsis* (Even-Faitelson *et al.*, 2011). Likewise, carefully designed pairs of ZFNs have been effective in allowing creation of both small (Petolino *et al.*, 2010) and large (Qi *et al.*, 2013a) deletions of chromosomal segments. A preliminary *in vitro* study also has suggested a means of using ZFNs for inhibiting replication of begomoviruses (Chen *et al.*, 2014). Finally, ZFNs may also find their way into projects using other model photosynthetic eukaryotes, such as algae, for both fundamental and practical applications (e.g. Sizova *et al.*, 2013).

TAL effector nucleases

TAL effector nucleases, like ZFNs, are chimeric proteins composed of two major units, a domain capable of recognizing a specific DNA sequence and a *FokI* nuclease domain that can pair with the *FokI* domain of another TALEN bound to an adjacent DNA sequence on the other DNA strand (Figure 1b). This results in a DSB in the DNA sequence between the two TALENs. Ensuing repair is achieved by the error-prone NHEJ DNA repair mechanism or through homologous recombination with an exogenously supplied DNA fragment that generally differs from the target gene sequence by only one or a few nucleotides. The DNA recognition domain of TALENs is derived from TALEs (transcription activator-like effectors) produced by plant pathogenic bacteria (most generally, *Xanthomonas* species). Each TALE has the ability to recognize and bind to a specific DNA sequence in a target gene in the host plant. In nature, this recognition domain consists of 12–27 nearly identical repeat units, most typically 34 amino acids in length in majority of TALEs (Boch and Bonas, 2010). However, within each repeat, the amino acids at the 12th and 13th positions are more variable. These two amino acids, called the repeat-variable diresidues (RVDs), come in directly in contact with one nucleotide base of the DNA sequence targeted by the TALE. A set of codes exist that dictate which RVDs will pair with a particular nucleotide base (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). In one of the most widely used codes, an asparagine–isoleucine (NI) RVD recognizes adenosine (A) base, NG recognizes a T, HD recognizes a C, and NN recognizes a G or an A. Using this RVD code, scientists have been able to systematically assemble individual TALE repeat units into a string of TALE repeats that can recognize virtually any DNA sequence in any gene. Adding nuclear localization sequences (NLSs) and the *FokI* nuclease domain to the N-terminus produced one of a pair of TALENs needed to successfully target a particular gene sequence for cleavage. While the first successful uses of TALENs for gene editing were in yeast (Christian *et al.*, 2010; Li *et al.*, 2011a), the development of facile TALEN assembly methods (e.g. Cermak *et al.*, 2015; Li *et al.*, 2011b; Reyon *et al.*, 2012, 2013; Schmid-Burgk *et al.*, 2015) soon lead to applications in plants (Cermak *et al.*, 2011; Christian *et al.*, 2013; Li *et al.*, 2012; Mahfouz *et al.*, 2011; Zhang *et al.*, 2015) including demonstration of

TALEN-stimulated gene replacement by homologous recombination (Zhang *et al.*, 2013).

TALEs, as DNA recognition domains, can be coupled to gene activation or gene repressor proteins to control gene expression. For example, Mahfouz *et al.* (2012) coupled the EAR repression domain (SRDX) to a TALE recognizing the *Arabidopsis* RD29A promoter region and demonstrated strong repression of transcription of both the endogenous RD29A gene and an RD29A::LUC transgene. Conversely, TALEs associated with gene activation domains have been shown to activate both the arylsulfatase gene (Gao *et al.*, 2014) and HLA3 bicarbonate transporter gene (Gao *et al.*, 2015a) in *Chlamydomonas reinhardtii*. Likewise, TALEN-mediated high efficiency gene knockout and gene replacement in the diatom, *Phaeodactylum tricoratum*, allowed for engineering of cells that resulted in a 45-fold increase in triacylglycerol accumulation (Daboussi *et al.*, 2014).

CRISPR/Cas9

The newest and most facile technique for efficient gene editing, CRISPR/Cas9, is derived from a defence system in bacteria and archaea that wards off attacks by viruses and genetic transformation by plasmids and other DNA fragments present in the surrounding environment. In a laboratory optimized version of this system derived from *Streptococcus pyogenes* (Jinek *et al.*, 2012), a short RNA molecule known as a single guide RNA (sgRNA) molecule is bound by a conditional DNA nuclease called Cas9. The 20 nucleotides at the 5' end of the sgRNA guides the resulting Cas9/sgRNA (protein/RNA) complex along chromosomal DNA strands until an exact (or nearly exact) match is made between the 'guide' RNA and the 'target' DNA sequence. Once a match is made—and if a NGG trinucleotide (PAM) sequence is present just downstream of the DNA target site—the Cas9 molecule undergoes a conformational change (Jiang and Doudna, 2015; Jinek *et al.*, 2014) that activates two separate nuclease domains in the formerly inactive Cas9 molecule. This results in cleavage of both strands of the target DNA three nucleotides upstream of the PAM site (Jinek *et al.*, 2012) (Figure 1c). In early 2013, two laboratories (Cong *et al.*, 2013; Mali *et al.*, 2013) simultaneously published research showing specific targeting of genes in mammalian cells using the CRISPR/Cas9 system and the ability to target more than one DNA sequence in the same cell using multiple guide RNAs. Between August and the end of 2013, at least nine reports had appeared demonstrating that the Cas9/sgRNA system was functional in a variety of plant species (Feng *et al.*, 2013; Jiang *et al.*, 2013; Li *et al.*, 2013; Mao *et al.*, 2013; Miao *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013b; Upadhyay *et al.*, 2013; Xie and Yang, 2013). Numerous follow-up studies have provided ample evidence for normal Mendelian inheritance of Cas9/sgRNA gene modifications to T₁ and subsequent plant generations (e.g. Feng *et al.*, 2014; Jiang *et al.*, 2014b; Schiml *et al.*, 2014; Xing *et al.*, 2014; Zhou *et al.*, 2014).

Targeting interesting genes for elimination with Cas9/sgRNA

Perhaps, the most powerful initial use of Cas9/sgRNA technology in plants will be to interrogate gene function by targeting specific genes in metabolic or developmental pathways for knockout. For example, two key proteins in photosynthesis, highly homologous magnesium chelatase subunit I (CHLI) genes, CHLI1 (At4g18480) and CHLI2 (At5g45930), have been simultaneously targeted for elimination and resulted in the production of albino plants—a useful tool for understanding the role of these enzymes in

chlorophyll biosynthesis (Mao *et al.*, 2013). An elegant example of the use of Cas9/sgRNA to allow rapid progress in understanding the role of a particular gene in plant development was the recent demonstration that the auxin binding protein (ABP1) is not required for either auxin signalling or Arabidopsis development (Gao *et al.*, 2015c). These two examples point to the power of Cas9/sgRNA technology to greatly facilitate precise elimination of one or more specific genes to study the effects of such ablations on plant growth and development—from both an academic and practical perspectives.

Multiple simultaneous targets

Because sgRNA genes are relatively small and because several can be easily placed in a single plasmid construct, simultaneous targeting of multiple genes is possible (e.g. Cong *et al.*, 2013; Xing *et al.*, 2014). Of particular note are two recent publications that describe particularly facile assembly of sets of adjacent sgRNA genes. Ma *et al.* (2015) used both Golden Gate assembly and Gibson assembly techniques to build sgRNA gene sets that could simultaneously target multiple (4–8) genes. They used such constructs in both dicots (Arabidopsis) and monocots (rice) to successfully inactivate multiple genes. Xie *et al.* (2015) cleverly used the precise processing of tRNA precursors to create an elegant system in which multiple sgRNAs (up to 8) with exact start sites could be processed *in vivo* from a single transcript by the cell's own tRNA processing machinery.

Biallelic modifications

When diploid plants contain designer nuclease-stimulated modifications in only one of the two copies (alleles) of a target gene, simple genetic crosses can be used to obtain homozygous progeny plants with modifications in both alleles. However, the high efficiency with which biallelic mutations are obtained in rice and tobacco provides encouragement that relatively few transformation events may be needed to obtain adequate numbers of plants carrying desired mutations in both alleles of a specific gene in the primary generation (e.g. Gao *et al.*, 2015b; Zhang *et al.*, 2014; Zhou *et al.*, 2014).

Cas9/sgRNA design

In addition to the methods for assembling constructs containing multiple sgRNA genes, several other approaches to Cas9/sgRNA gene assembly have been published within the last 2 years (Jacobs *et al.*, 2015; Johnson *et al.*, 2015; Lei *et al.*, 2014; Xie *et al.*, 2014; Xing *et al.*, 2014; Yan *et al.*, 2014). Of particular note is a useful comparison of the relative efficiencies of different types of CRISPR/Cas nucleases produced with various codon optimizations (e.g. for human and Arabidopsis) and 12 different *de novo* synthesized sgRNA genes (Johnson *et al.*, 2015). The Cas9 system from *Staphylococcus aureus* is an interesting example of a Cas9 that is markedly smaller than the Cas9 from *Staphylococcus pyrogenes*, but still retains excellent gene targeting capabilities (Ran *et al.*, 2015). However, this new SaCas9 is yet to be tested in land plants. Perhaps unexpectedly, it has recently been reported that two portions of the Cas9 can be independently expressed from two separate genes and undergo self-assembly *in vivo* to form an active Cas9 nuclease if a sgRNA is present—potentially allowing for the development of an inducible dimerization system (Nihongaki *et al.*, 2015; Wright *et al.*, 2015). Although it is still to be verified in plant systems, a recent publication (Farboud and Meyer, 2015) indicates that, in addition to the NGG PAM site that is required in the DNA sequence

recognized by Cas9, the presence of another immediately adjacent GG in the guide sequence greatly increases the efficiency of gene recognition and cleavage by the Cas9/sgRNA system operating in the nematode, *Caenorhabditis elegans*.

Gene replacement by homologous recombination

Rates of gene replacement by HR are greatly increased if a DSB is created in the DNA sequence that is targeted for replacement. As with ZFNs and TALENs noted above, the creation of such cleavages by Cas9/sgRNA constructs in the presence of a homologous DNA replacement fragment has been shown to stimulate desired changes in various plant systems (e.g. Feng *et al.*, 2013; Li *et al.*, 2013; Zhang *et al.*, 2014). Also, as with TALEN-stimulated HR, the geminivirus-based system (Baltes *et al.*, 2014) that supplies a ready source of homologous DNA for gene replacement can be employed with even greater ease with the Cas9/sgRNA system because of the inherently simpler methods for designing and producing the targeting molecules (i.e. a single, small sgRNA gene compared with two complex TALEN gene constructs). Significant enhancements of gene replacement rates by homologous recombination have been achieved in mammalian cells and mice by targeting genes with Cas9/sgRNA complexes, while simultaneously suppressing genes required for NHEJ (i.e. genes encoding KU70 and KU80) and/or inhibiting DNA ligase VI with the compound, Scr7 (Chu *et al.*, 2015; Maruyama *et al.*, 2015). Such treatments greatly suppress NHEJ DNA repair and stimulate rates of homologous recombination at the site of Cas9/sgRNA-induced DNA breaks. Indeed, one report using ZFN-mediated gene targeting showed increased gene editing and HR in Arabidopsis containing mutations in *ku70* or *lig4* (NHEJ repair) and SMC6B (sister chromatid-based HR) (Qi *et al.*, 2013b).

Quite recently, efficient gene editing and gene replacement by HR has been demonstrated in maize and soya bean (Mark Cigan, Pioneer/DuPont, pers. commun.). Five different target genes in immature maize embryos were successfully targeted for biallelic editing using biolistic delivery of Cas9 genes and sgRNA genes. Surprisingly, delivery of sgRNAs as naked RNA molecules into embryos containing pre-integrated Cas9 genes worked as well as delivery of sgRNA genes. Success was also achieved in replacing large gene segments in two acetolactate synthase genes (to produce chlorsulfuron-resistant plants) and the *liguleless-1* gene using either double-stranded or single-stranded DNA molecules as replacement strands. These mutant genes and other edited genes were shown to be inherited in a normal Mendelian fashion.

Chromosome segment deletions

When two closely spaced DSBs are created by the actions of ZFNs, TALENs or Cas9/sgRNAs, it is common to observe deletion of the DNA fragment between the two sites during DNA repair by the NHEJ mechanism. In animal cells, large chromosome fragment deletions have been generated using two sets of designer nucleases to cause chromosomal breaks several kilobases apart. Reports now exist that similar small deletions can be made in tobacco and rice (Gao *et al.*, 2015b; Xie *et al.*, 2015) and deletions as large as 170–245 kb in rice (Zhou *et al.*, 2014). In the latter case, the deletion involved ten clustered genes on rice chromosome 2 involved in the diterpenoid synthesis pathway.

Gene regulation/plant development

The availability of mutations in Arabidopsis, tobacco and other plants that affect plant growth and development has been essential to our present understanding of how plants function

and how they can be manipulated to produce desired results. The ease with which gene knockouts and gene replacements can be generated in both model and crop plant species using Cas9/sgRNA promises an explosion of new findings within a few short years. One initial example of how our knowledge of basic plant development can be significantly expanded using Cas9/sgRNA technology is the recent observation that Cas9-/sgRNA-mediated double knockout of two novel NAC transcription factors alters gene expression and flowering time by eliminating the association of these transcription factors with the histone demethylase, JM14 (Ning *et al.*, 2015).

Gene activation and repression

Another means for modifying plant growth and development for academic and commercial benefit is to manipulate the timing and level of expression of specific genes. Cas9 molecules lacking nuclease activity (dead Cas9s; dCas9s) and coupled to either gene activator or gene repressor domains can be precisely directed to promoter regions of specific genes using the RNA-directed targeting afforded by bound sgRNAs. For example, Piatek *et al.* (2015) were able to fuse the dCas9 C-terminus with the activation domains of EDLL and TAL effectors to create transcriptional activators or, conversely, to fuse the dCas9 C-terminus with the SRDX repression domain to produce transcription repressors. These constructs were used to selectively activate or repress both artificial reporter transgenes and endogenous plant genes.

In addition to controlling gene expression with activators and repressors associated with dCas9/sgRNA constructs, more recent experimentation with human cells (Hilton *et al.*, 2015) has demonstrated that epigenetic control of gene expression can be achieved by attaching the human acetyltransferase, p300, to dCas9 and direct it to specific genome regions using sgRNA targeting.

Off-target DNA cleavage

In applying Cas9/sgRNA technology to human medicine, there can be no room for error in making gene edits. However, as pointed out by Wu and colleagues in their recent review (Wu *et al.*, 2014), several reports have surfaced of *bona fide* off-target gene modifications using any of the presently available designer nuclease systems. Because of the importance of this issue, numerous modifications of the earlier Cas9/sgRNA gene editing systems have been or are being devised (e.g. Fu *et al.*, 2014; Gulinger *et al.*, 2014; Tsai *et al.*, 2014)—as well as methods for detecting such erroneous events (Frock *et al.*, 2015; Kim *et al.*, 2015; Li *et al.*, 2015; Wang *et al.*, 2015a; Yang *et al.*, 2014). However, initial analyses in plants suggest that concerns regarding off-target gene modifications by the Cas9/sgRNA system may be less common (e.g. Gao *et al.*, 2015b; Zhang *et al.*, 2014). There is also the realization that such concerns are buffered by the likelihood that one or a very few erroneous DSB cleavages may have no notable effects on phenotypes—and, even if so, plants with defective phenotypes can be easily and inexpensively culled. Nonetheless, there are efforts to develop Cas9/sgRNA systems for plants that are inherently less mutagenic in regard to nontarget genes (e.g. by utilizing pairs of Cas9 nickases that must recognize two separate 20-bp DNA sequences to promote gene editing instead of a single Cas9 DSB nuclease that recognizes only a single 20-bp sequence (Schiml *et al.*, 2014). There is no doubt that it is important to continue efforts to develop methods to decrease off-target cutting by ZFNs, TALENs and Cas9/sgRNAs. However, the availability of inexpensive and accurate methods for

genome DNA sequencing and the ability to easily produce multiple plants carrying a particular desired gene editing offers plant biologists and breeders an opportunity to cull plants with off-target modifications and select only those bearing the desired mutations for study or for commercial release.

Other model systems

The value of the Cas9/sgRNA system to fundamental and applied research in other plant-like model systems is well recognized and attempts to utilize this technology are well under way (e.g. Jiang *et al.*, 2014a; Sugano *et al.*, 2014). Nonetheless, it should be noted that while the Cas9/sgRNA system has been implemented in most plant and animal systems, the model alga system, *Chlamydomonas reinhardtii*, is apparently recalcitrant to efficient gene editing—likely due to the apparent toxicity of Cas9 to this organism (Jiang *et al.*, 2014a).

Applications of designer nucleases to crop improvement

Rice

Proof-of-concept experiments

Because of the ease with which embryogenic rice cultures can be prepared, transformed (using either particle bombardment or *Agrobacterium tumefaciens*) and rapidly regenerated into fertile plants, rice has served as both a highly useful model monocot and an attractive target for agricultural improvements. Indeed, rice served as one of the first plant species to undergo gene editing using TALENs (Li *et al.*, 2012; Shan *et al.*, 2013a) and Cas9/sgRNA (Feng *et al.*, 2013; Jiang *et al.*, 2013; Shan *et al.*, 2013b). The ability to obtain biallelic gene modifications in a single generation (Feng *et al.*, 2013; Xu *et al.*, 2014; Zhou *et al.*, 2014), the opportunity to delete large segments of chromosomes (Zhou *et al.*, 2014) and the availability of gene replacement through homologous recombination (Feng *et al.*, 2013) coupled with positive/negative selection schemes (Shimatani *et al.*, 2015) bode well for the continued use of rice as a rapid means of generating genetically altered plants for basic understanding of monocot plant growth. In addition, the ability to simultaneously target several genes for knockout or modification (Endo *et al.*, 2015; Xie *et al.*, 2015) should greatly speed rice research and the development of improved rice varieties.

Practical applications

The ability to use a designer nuclease to simultaneously knockout both alleles of a single rice gene or to use sets of nucleases to ablate all genes involved in an entire pathway through chromosomal segment deletion can lead to significant changes in important traits of rice (Zhou *et al.*, 2014). For example, using TALENs to knock out the OsBADH2 gene encoding betaine aldehyde dehydrogenase (BADH2), Shan *et al.* (2015) were able to create a fragrant variety of rice from a nonfragrant variety. Likewise, Miao *et al.* (2013) were able to significantly change the branching pattern of rice by inactivating the CAO1 gene.

One of the most striking demonstrations of the utility of gene editing in creating crops with important new agronomic traits was the demonstration by Li *et al.* (2012) that TALEN-directed disruption of a natural TAL effector binding element (EBE) sequence in the promoter of the disease susceptibility gene, *Os11N3* (also called *OsSWEET14*), results in the production of rice

plants with resistance to the major rice disease, bacterial blight. Simple genetic crosses and progeny testing resulted in the selection of several plants that retained the disease resistance trait but were missing the TALEN genes that had been inserted into the T₀ generation progenitor plants. Stable inheritance of the disease resistance phenotype was observed in T₁ and T₂ generation plants. A second bacterial blight disease susceptibility gene, *OsSWEET13*, has been documented and its coding sequence modified using CRISPR/Cas9 technology to convert *OsSWEET13* from a disease susceptibility gene to a null mutant for disease resistance (Zhou *et al.*, 2015).

Maize

Proof-of-concept experiments

Both TALEN and CRISPR/Cas9 systems have been used successfully for precise simultaneous editing of multiple genes in *Zea mays* (Liang *et al.*, 2014). In subsequent studies, Char *et al.* (2015) demonstrated that the *GLOSSY2* (*GL2*) gene of maize could be efficiently modified using TALEN technology and provided evidence for normal Mendelian inheritance of the mutated genes.

In addition to ZFNs, TALENs and Cas9/sgRNAs, meganucleases also have been used effectively for gene editing in maize and other plants (Gao *et al.*, 2010; Marton *et al.*, 2013; Roth *et al.*, 2012). Of special note is the development of a novel gene injecting system for use in delivering meganucleases into maize cells using a novel biolistic bombardment particle (Martin-Ortigosa *et al.*, 2014).

It has now been shown that the Cas9/sgRNA system can be used effectively in maize to support efficient gene knockout and gene replacement (M. Cigan, Pioneer/DuPont, pers. commun.). Delivery of sgRNA genes or naked sgRNAs into immature embryos expressing the Cas9 gene leads to efficient gene knockout or to gene replacement if either a double-stranded or single-stranded DNA gene replacement fragment is supplied during particle bombardment.

Practical applications

Scientists at Dow AgroSciences and Sangamo, Inc. have teamed to demonstrate the power of ZFN technology to allow the introduction of important traits into maize. Among the plants developed were those with reduced phytate (inositol phosphate) created by insertion of an herbicide (Basta) resistance gene into the endogenous *IPK1* gene (Shukla *et al.*, 2009). Importantly, this feat was accomplished using homologous recombination to simultaneously deliver the herbicide resistance gene and knock out the phytate synthase gene. Of particular note has been the development by this group of facile methods for using ZFN-stimulated gene editing/homologous recombination to allow the rapid stacking of multiple transgenes into a single genetic locus (Ainley *et al.*, 2013)—a strategy that is of growing importance for efficient plant breeding as more and more transgenes become available for crop improvement.

Equally important (and as discussed in greater detail below), the ZFN transgenes that were located on one chromosome but caused a targeted mutation in a gene on a different chromosome could be eliminated from progeny plants by simple genetic crosses of the transgenic plant with nontransgenic plants (i.e. progeny plants could be selected that lacked the foreign ZFN genes but retained the desirable edited gene) (Ainley *et al.*, 2013). For this reason, the USDA has issued a statement that such

plants with precisely targeted ZFN-stimulated gene mutations (but lacking the ZFN genes themselves) are not regulated by USDA-APHIS under 7 CFR 340 and can be offered for commercial distribution (Ledford, 2013).

Male sterility is a trait in maize that can be of great benefit in plant breeding. Djukanovic *et al.* (2013) used a modified meganuclease derived from the *Chlamydomonas reinhardtii* I-Crel homing nuclease to successfully target both alleles of the MS26 gene of maize and create plants that were male sterile when homozygous for the gene knockout.

Soya bean

Proof-of-concept experiments

Zinc finger nucleases have been successfully employed to edit several genes in soya bean, including the paralogous *DCL4a* and *DCL4b* gene set (Curtin *et al.*, 2011, 2013). Specific targeting of a single gene (*DCL4b*) and normal Mendelian inheritance of this mutated gene in subsequent generations pointed to the accuracy of the ZFN system for selecting between highly homologous and redundant genes in a particular organism.

Cas9/sgRNA technology also has been successfully applied to soya bean (Jacobs *et al.*, 2015). Both the GFP and nine endogenous loci were targeted using either *Agrobacterium rhizogenes*-derived hairy root soya bean cultures or soya bean somatic embryo cultures. In the latter case, the number of gene editing events was seen to increase with increasing time in culture.

Practical applications

As a practical follow-up to the earlier use of ZFNs for targeting soya bean genes of academic interest, researchers have been successful in using TALENs to ablate the activity of the two fatty acid desaturase 2 (*FAD2*) genes of soya bean and creating plants that produce seeds high in monosaturated oleic acid (~80% vs. the normal ~20%) and low in the polyunsaturated fatty acid, linoleic acid (~4% vs. the usual ~50%) (Haun *et al.*, 2014). Importantly, progeny plants (as expected) were found that not only inherited the high oleic acid trait but also lacked the TALEN genes that had been inserted into a chromosomal site distant from the *FAD2* genes and, therefore, could be easily segregated away using normal genetic crossing techniques.

Cotton

Practical applications

Targeted gene stacking in cotton has been shown to be possible using specially designed meganucleases (D'Halluin *et al.*, 2013). In these studies, a site adjacent to an insect resistance gene in embryogenic cotton callus cells was targeted for DSB in the presence of exogenous DNA containing two different herbicide resistance genes flanked by DNA sequences with homology to the target site. Approximately 2% of independently transformed callus lines were shown to contain the correct insertion and to transfer the stacked traits to the following generations.

Canola

Practical applications

The oil content of canola seeds has been altered to decrease palmitic acid and increase total C18 fatty acids by increasing transcription of the two canola β -ketoacyl-ACP synthase II (KASII) genes using zinc finger proteins to which the VP16 transcriptional

activator domain had been attached (Gupta *et al.*, 2012). These experiments demonstrated that designed zinc finger protein transcription factors (ZFP-TFs) can be utilized to alter gene regulation in crop plants to gain desired changes in an economically important trait.

Wheat

Proof-of-concept experiments

Wheat was one of the first plants modified using CRISPR/Cas9 technology (Shan *et al.*, 2013b). A subsequent study (Upadhyay *et al.*, 2013) demonstrated knockouts of both the inositol oxygenase (*inox*) and phytoene desaturase genes in wheat. This study also showed that simultaneous expression of two multiplexed sgRNAs genes targeting two closely spaced target sequences in the wheat genome caused the DNA segment between the two sites to be efficiently deleted.

Practical applications

One of the challenges for successful gene editing in bread wheat is that its genome is hexaploid. Nonetheless, Wang *et al.* (2014) used TALEN technology to introduce targeted mutations in the three homoeoalleles that encode MILDEW-RESISTANCE LOCUS (MLO) proteins and, thereby, successfully confer heritable broad-spectrum resistance to powdery mildew. This was a truly pioneering venture, because no naturally occurring broad-spectrum resistance genes for powdery mildew are currently available to plant breeders. In the same studies, successful targeted DNA insertion by homologous recombination was demonstrated—along with the use of Cas9/sgRNA technology to create wheat plants that carry mutations in the TaMLO-A1 allele.

Sorghum

Proof-of-concept experiments

An initial report has indicated that CRISPR/Cas9 technology can be used in sorghum to target a nonfunctional out-of-frame red fluorescence protein gene for reactivation as a result of presumed insertions and/or deletions created after DSB repair by the NHEJ mechanism (Jiang *et al.*, 2013).

Barley

Proof-of-concept experiments

Barley is another monocot crop in which precise gene editing has been demonstrated. Wendt *et al.* (2013) used TALENs to demonstrate that several different types of mutations could be induced within a targeted gene sequence.

Practical applications

Use of genetically altered haploid cells to create desirable doubled haploids (instant inbreds) is a powerful tool with growing popularity in plant breeding. Thus, barley breeders should welcome the recent demonstration that genes in haploid barley cells can be specifically modified using TALENs and that such modified genes are inherited in a normal fashion in progeny of doubled haploids (Gurushidze *et al.*, 2014).

Vegetable, fruit and other crops

Proof-of-concept experiments

Genes in tomato plants have been targeted with TALENs to show the involvement of PROCERA gene in responses to gibberellic acid (Lor *et al.*, 2014). Transformation with Cas9/sgRNAs has been

used to demonstrate that disruption of the *ARGONAUTE7* (*SIAGO7*) gene has a distinct influence on leaf shape (Brooks *et al.*, 2014) and to show that *SHORT-ROOT* and *SCARECROW* gene function is conserved between *Arabidopsis* and tomato (Ron *et al.*, 2014).

Methods for transforming potato with TALEN constructs (Clasen *et al.*, 2015; Nicolai *et al.*, 2015) and Cas9/sgRNA construct (Wang *et al.*, 2015b) have resulted in efficient targeting of genes for editing and alteration of gene expression in regenerated plants.

Fruit trees such as apple and fig appear amenable to gene editing by ZFNs as evidenced by the targeted repair of a nonfunctional *uidA* (Gus) gene (Peer *et al.*, 2015). Likewise, genes in sweet orange can be targeted for disruption using Cas9/sgRNAs, in this case, to knock out the phytoene desaturase (*PDS*) gene (Jia and Wang 2014).

Practical applications

When, as discussed below, crop plants are mutagenized using designer nucleases, but are genetically crossed to eliminate the nuclease genes that caused the mutation and marker gene that facilitated selection of transformants, there is the possibility that such plants can be brought to market with fewer regulatory hurdles to overcome than those that exist for standard GMOs. The implications of such a situation for the fruit and vegetable industries are discussed in a recent article by Nagamangala Kanchiswamy *et al.* (2015). Indeed, recent results using TALENs to target all four copies of the vacuolar invertase (*Vinv*) gene in commercial tetraploid potatoes for knockout resulted in production of potato tubers with few, if any, reducing sugars following cold storage of the tubers. When such tubers were chipped and fried, the resulting French fries contained much lower levels of the nerve poison, acrylamide, and were much lighter in colour than potato chips made from conventional potatoes stored under identical conditions (Clasen *et al.*, 2015). As described earlier, simple genetic crosses can be used to rid potato plants of the TALEN genes. Thus, while similar strategies to decrease invertase levels using RNAi were successful (Ye *et al.*, 2010), the level of reducing sugar suppression was less—and that there is obligate need to permanently retain the gene expressing of the RNAi construct in production plants.

Regulation of non-GMO crop plants produced with designer nuclease technologies

One potentially significant advantage of improved crop plants developed using designer nucleases compared to plants enhanced through traditional genetic engineering techniques is that following genetic backcrossing with nontransgenic plants, the former can be selected to contain no foreign DNA sequences. As noted above, maize plants containing desirable genetic mutations created through use of ZFNs and lacking the original ZFN genes have been judged by US Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) as not requiring regulation by the agency (Ledford, 2013). Thus, it is possible in the future that many crop plants produced with ZFNs, TALENs, Cas9/sgRNAs, meganucleases and other designer nucleases that contain little or no foreign DNA may be brought to market in much shorter time frames and with significantly less expense than conventional GMO crops. Whether other US federal regulatory agencies such as FDA

(Food and Drug Administration) and EPA (Environmental Protection Agency) will rule in a similar fashion to APHIS is unknown, as are the responses of foreign countries. Initial thoughts and opinions in regard to these weighty matters have already surfaced (Nagamangala Kanchiswamy *et al.*, 2015; Riccio and Henard-Damave, 2015) and public dialogues (Bartholomaeus *et al.*, 2015; <http://nabc.cals.cornell.edu/Publications/NewsArchive/NABC-26-Overview.pdf>) are underway. If, in the future, the federal regulatory agencies (and perhaps similar agencies of foreign countries) decide to evaluate genetically enhanced plants based not on the process by which they are produced, but rather on whether or not they vary significantly in safety and nutrition from their conventional progenitors, it is possible that the distinction between standard GMOs and crops enhanced with designer nucleases will be of less consequence. Needless to say, how this plays out may have profound consequences for agriculture and how it is able, or not, to feed nine to ten billion people by mid-century.

A view to the future

Explosion in the adoption of ZFN, TALEN and, especially, Cas9/sgRNA systems for gene editing and the rapid development of new and improved techniques for their laboratory and field uses are opening significant opportunities for studying plant growth, development and productivity. These opportunities include, for example, modification of metabolic pathways (e.g. by changes in flux rates and end products through creation of gene knockouts or knockins and increases or decreases in gene expression levels). There are also possibilities for changing resistance/susceptibility to biotic and abiotic stresses, better understanding of cell cycle regulation and more in-depth investigations of hormone/growth regulator responses (and their effects on plant development). The availability of new gene editing tools offers the opportunity to help unravel the mysteries of diurnal/photoperiod/light quality responses and brings much needed new light to matters of water and nutrient uptake and utilization. There may even be renewed hope for unleashing the long-held promise of successfully manipulating photosynthetic pathways and efficiencies for increased food production.

Several potential applications of gene editing and gene replacement technologies that are not yet practical today can be envisioned. For example, there has been much discussion (Ledford, 2015) about the potential use of Cas9/sgRNA technologies to allow 'gene drive'. This is a process by which Cas9 and sgRNA gene cassettes are introduced into a specific target gene in an organism's genome which, in turn, can direct the insertion of this cassette into the target gene's sister allele on the homologous chromosome. When sperm or pollen from such an organism fertilizes the egg in a wild-type version of the organism, the target gene in the haploid egg is soon converted into a copy of the incoming modified gene from the sperm or pollen—a 'gene drive' process that ultimately allows rapid spread of a particular gene throughout the local, if not regional or global, environment. This technology has been successfully demonstrated under strict containment conditions to function in *Drosophila* (Gantz and Bier, 2015). Gene drive is perhaps the most often suggested as a means to potentially destroy the ability of another insect, mosquitoes, to transmit malaria. Gene drive technology could have many uses in plant biology and agriculture. For example, one approach to combating the significant problem with herbicide-resistant weeds

would be to introduce into these resistant weeds a Cas9/sgRNA construct that would disable the gene in the weed that confers herbicide resistance. Rapid spread of the gene carrying such a knockout system throughout the affected region could greatly suppress proliferation and further dissemination of the herbicide-resistant weed species. Along with introduction of crops containing one or more genes conferring resistance to herbicides to which the weed is vulnerable (Behrens *et al.*, 2007; Duke, 2015), use of the gene drive technology could have a dramatic impact in helping to solve a significant present-day challenge to agricultural production. Nonetheless, there is strong insistence among scientists (e.g. Ledford, 2015; Oye *et al.*, 2014) that careful thought and planning must be undertaken well before the release of gene drive technologies in the environment.

Given that the Cas9/sgRNA system functions effectively in providing protection to bacteria and archaea from invasion by DNA viruses, one wonders whether an appropriately engineered version of this system might be adapted for DNA virus protection in plants. Indeed, with the discovery that several Cas/gRNA systems are effective against RNA as well as DNA molecules (Plagens *et al.*, 2015), it may be possible that the large numbers of RNA viruses that plague agricultural crops might be controlled using modifications of these newly discovered systems.

From several points of view, it has been remarkable to see the rapidity with which ZFN, TALEN and, especially, Cas9/sgRNA technologies have evolved from newly emerging understanding of, respectively, zinc finger transcription factor binding to DNA, plant pathogen infection of plants and bacterial defences against virus attack. None of these technologies can be considered mature and yet they are already being used to make significant new discoveries in plant and animal biology and show every promise to have important practical applications. TALENs were hailed as 'the discovery of the year' only to be largely supplanted in 2 years by the Cas9/sgRNA system. In the light of human ingenuity and the speed with which that ingenuity can drive scientific progress, it is difficult to project what the future will bring. The only certainty is that such progress will come.

In the past, new resources such as the Arabidopsis T-DNA gene knockout libraries have had a powerful effect in rapidly advancing basic plant biology research. CRISPR/Cas9 and other gene editing technologies now have the same kind of potential to allow, at modest cost, the same kind of accelerated progress not only with model plants, but also with crop plants. There is every reason for the plant science community to organize itself to make sure the power of designer nuclease technology becomes widely available to advance both fundamental knowledge of plants and the application of that knowledge to better feed the world.

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