Editorial Prerogative and the Plant Genome

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17 In the coming decades, plant agriculture must produce the food needed to sustain the
18 world's burgeoning population. This challenge is made more daunting in light of a
19 changing climate, scarcity of inputs such as water and fertilizer, and the need to grow
20 more crops on increasingly marginal land. To achieve food security, we will need to fully
21 exploit plant genetics to develop new crop varieties that produce higher yields of
22 healthier food. Crop improvement through breeding has been the foundation of modern
23 agriculture, resulting in steady increases in productivity of the major commodity crops
24 (Sleper and Poehlman, 2006). Additional genetic variation of value has been created
25 through mutagenesis strategies (Micke et al., 1990). More recently, gene addition
26 through transgenesis has provided valuable traits that impact yield, including herbicide
27 tolerance and resistance to insect pests (Gepts, 2002). Now, with the advent of genome
28 editing, we can directly alter the genetic blueprints of crop species – a powerful
29 approach to create genetic variation needed to produce abundant, healthy food.

30 Current approaches for editing plant genomes rely on introducing double strand
31 breaks in target loci; the manner in which the break is repaired achieves the desired
32 DNA sequence modification (Voytas, 2013). For many years, genome editing was
33 difficult to perform due to the lack of effective methods to produce nucleases that target
34 and cleave specific loci in complex genomes. The first programmable nucleases
35 required considerable protein engineering; for example, to alter the DNA sequence
36 specificity of rare-cutting endonucleases or zinc finger proteins (Cathomen and Joung,
37 2008; Arnould et al., 2011). The characterization of the DNA binding motif from TAL
38 effectors was an important advance for the field (Boch et al., 2009; Moscou and
39 Bogdanove, 2009). Variant TAL effector motifs in nature recognize each of the four
40 bases, and the motif is highly modular; that is, when arrays of motifs are assembled to
41 target novel DNA sequences, they recognize their intended targets >90% of the time
42 (Cermak et al., 2011). More recently, the development of CRISPR/Cas reagents has

irrevocably altered the field (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013). Protein engineering is now largely obsolete, since targeting with CRISPR/Cas is achieved simply through base-pairing between a guide RNA and the DNA target of choice. Simplicity in reagent design and high activity of CRISPR/Cas nucleases make it possible for the average molecular biologist to target DNA sequences with ease.

At present, editing one or a few genes can be performed with any of the available nuclease platforms, as long as the reagent effectively binds and cleaves its intended target site *in vivo*. For most scientists, particularly those in academia, the CRISPR/Cas system is preferred, due to its effectiveness and simplicity. Companies with intellectual property on the other nuclease platforms, however, will continue to use different reagents to develop products such as new crop varieties. However, with academic scientists largely unified around a single platform, improvements will be rapid. Already available are Cas9 variants with improved target specificity, one of the initial concerns about the system's efficacy (Kleinstiver et al., 2016; Slaymaker et al., 2016). Further, CRISPR/Cas reagents have been developed that perform functions other than gene editing, such as modulating transcription or altering the epigenetic status of a locus (Thakore et al., 2016). Finally, since a single Cas nuclease can be directed to multiple target sites by simply expressing multiple guide RNAs, it is straightforward to edit several genes simultaneously (Cong et al., 2013).

Will other programmable nuclease platforms emerge? Most likely; however, it is doubtful they will supplant CRISPR/Cas. For one, it is hard to imagine a simpler targeting mechanism than base-pairing. Further, the rapid adoption of CRISPR/Cas reagents for genetic analysis across diverse organisms, including plants, indicates they serve the needs of most researchers; hence, there is little motivation to develop new platforms. The field has moved from an era of reagent design to an era of reagent deployment.

Editing genomes to increase biological understanding

Before we can fully capitalize upon genome engineering for crop improvement, we must have a sophisticated understanding of plant genomes and how they work. Already available are high quality DNA sequences for genomes of dozens of important crop plants; however, we really only understand the functional role of a fraction of plant genes. The tools of genome engineering can help elucidate plant gene function. Targeted mutagenesis makes it possible to connect the dots between genotype and phenotype.

When double strand breaks are introduced in somatic cells, the broken chromosomes are most typically rejoined through a mechanism called non-homologous end-joining (NHEJ) (Puchta, 2005). NHEJ repair is sometimes imprecise, and, consequently, the rejoined chromosome suffers a loss of nucleotides at the break site (or less frequently, sustains a small insertion). If the insertion/deletion (indel) occurs within a coding sequence, it can create a frameshift mutation, leading to gene inactivation. Many reagents, including TALENs and CRISPR/Cas, create NHEJ-induced mutations at remarkably high frequencies. For example, when protoplasts (plant cells lacking a cell wall) are transformed with constructs expressing CRISPR/Cas reagents, it is not uncommon for >25% of surveyed chromosomes to have mutations created by imprecise NHEJ (Shan et al., 2013). For a handful of plant species, mutagenized protoplasts can be regenerated into plants, making it possible to assess the phenotypic consequence of NHEJ-induced mutations at the whole plant level (Clasen et al., 2016).

Only a handful of plant species, however, can be regenerated from protoplasts, and so mutations in most plants are created by first generating a transgenic plant that expresses the sequence-specific nuclease and a selectable marker gene (Voytas,

2013). As the transgenic plant grows, cells are mutagenized, including those in the meristem that ultimately produce flowers and seed. Mutagenesis, therefore, occurs *in planta*, and heritable mutations can be reliably obtained in many species by this strategy (Weeks et al., 2016). For some plants, such as *Arabidopsis*, mutagenesis frequencies have been increased by using promoters that express the nuclease preferentially in the germline (Wang et al., 2015; Mao et al., 2016). An advantage of *in planta* strategies is that they use standard transformation methods, which are available for diverse plants. Since it is unlikely that the targeted mutation and the nuclease-encoding transgene are genetically linked, the latter can be segregated away through crossing, leaving a plant with only the desired mutation.

The efficiency of sequence-specific nucleases for *in planta* mutagenesis is striking. For example, six homeoalleles in wheat were simultaneously knocked out, resulting in a fungus-resistant cultivar (Wang et al., 2014). Similarly, eight genes were simultaneously mutated in *Arabidopsis* (Ma et al., 2015). Although resource intensive, it is not unrealistic to contemplate creating a mutant plant library for species such as rice, in which each member of the library is a plant with a different gene inactivated. Further, the extensive collections of T-DNA insertion mutants in plants like *Arabidopsis* could be augmented through targeted mutagenesis to create a complete knockout library for this model species. For scientists working in baker's yeast, the completion of a knockout collection proved invaluable for dissecting gene function (Giaever et al., 2002). One of the most important outcomes of our newfound editorial control over plant genomes will be the wealth of basic biological information we glean regarding plant gene function.

Clearly targeted mutagenesis can help advance basic biology. However, there are also some traits of value that can be created through gene inactivation, such as removal of anti-nutritionals or toxins (e.g. trypsin inhibitors in soybean, ricin in castor oil). Further, metabolic pathways can be blocked at specific steps to accumulate desirable

precursors. This has been done in soybean by knocking out a seed-expressed fatty acid desaturase, thereby lowering the production of downstream products, namely polyunsaturated fats, and enriching for healthier precursors, namely monounsaturated fats (Haun et al., 2014). Plants with traits enabled by targeted mutagenesis are likely to soon enter the food supply. In the US, the USDA has ruled that in several cases, transgene free, NHEJ-induced mutants are not regulated (Jones, 2015). This sets an important precedent, indicating that mutations created with sequence-specific nucleases will be treated in the same way as mutations created by chemical mutagens or ionizing radiation. Plants with targeted mutations can therefore quickly be tested in the field; field trials provide the most rigorous assessment of a trait's value.

Editing plant genomes with finesse

Whereas targeted mutagenesis with sequence-specific nucleases has worked well in many species and is beginning to facilitate a greater understanding of gene function, sequence modifications other than NHEJ-induced indels are often desired. For example, specific nucleotide substitutions, which alter key amino acid residues in a protein or change promoter function, might be of value. Such precise modifications allow more sophisticated tests of gene function, and by extension, agronomically relevant genetic variation can be moved into the genomes of crop species to introduce traits of value.

Precision genome editing is best enabled by harnessing the other mechanism by which broken chromosomes are repaired, namely homologous recombination (HR). In HR, a template is provided with the desired sequence alterations to be incorporated into the genome (Puchta, 2005). Sequence alterations on the template are copied into the break site, thereby altering the locus. The types of sequence modifications that can be

introduced through HR are expansive, ranging from single nucleotide substitutions to the insertion of one or more transgenes. A challenge in achieving HR, however, is the need to simultaneously deliver the template and nuclease. Further, HR is inefficient in somatic cells, and the competing pathway – NHEJ – predominates (Puchta, 2005). Because of these challenges, remarkably few examples of HR-mediated genome editing have been reported in plants.

For the few plants species that can be regenerated from protoplasts, direct delivery of the nuclease and donor can achieve HR at reasonable efficiencies – in the range of 1% of transformed cells (Townsend et al., 2009). Populations of edited cells can be regenerated into plants and screened to identify those with the desired DNA sequence modification. For other plant species, in which differentiated tissues are transformed by *Agrobacterium* or biolistics, recovering edited plants is a bit of a numbers game. Only subsets of cells in the tissues are transformed, and of those, only a fraction undergoes HR. Donor templates that incorporate selectable markers at the target locus can help identify cells having sustained HR (Sun et al., 2007). The downside is that the edited plant contains the selectable marker gene, which may be undesirable for product development. Further, if the goal is precise genome editing, then incorporating a marker gene certainly lacks the sought-after precision.

Similar to the strategies described above for NHEJ-induced mutagenesis, *in planta* strategies have also been used for HR-based targeted modification (Fauser et al., 2012). In this approach, a nuclease cassette and the repair template are integrated into the genome at a random chromosomal site. The template is flanked by nuclease cut sites, such that when the intended genomic target site is cut, the template is released by the nuclease, enabling it to move throughout the nucleus and recombine with the target. Other strategies to improve gene targeting have involved developing vectors to improve delivery of the nuclease and donor template. For example, geminivirus-based DNA

replicons amplify to high copy number in plant cells (Baltes et al., 2014; Cermak et al., 2015). Thus, the replicons produce an abundance of nuclease and repair template, often increasing HR frequencies an order of magnitude or more. Finally, it is also possible to knockout function of key enzymes in the NHEJ pathway, thereby increasing the frequency with which broken chromosomes are repaired through HR (Qi et al., 2013).

Despite these promising approaches, editing plant genomes with precision remains difficult. With so much interest in genome engineering among plant biologists, and with the availability of powerful easy-to-use reagents to make targeted chromosome breaks, it is likely that the field will witness considerable technical improvements in the coming years. Such improvements should soon make it easy and routine to perform plant genome editing with finesse.

Conclusions

The advent first of TALENs and then CRISPR/Cas reagents made it possible for many plant scientists to begin modifying plant genomes in sophisticated ways. NHEJ-induced targeted mutagenesis is now efficient in many plant species and promises to be a powerful approach for elucidating plant gene function. Efficient methods to precisely edit plant genomes through HR are the next challenge; however, improvements are likely to be rapid. Genetic variation created through genome editing will shortly enter the food supply, and it is the hope that traits created through genome editing will help meet the world's increasing need for more and healthier food.

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