Re-engineering plant gene targeting

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The genome sequence of *Arabidopsis* is complete and the genomes of plants representing legumes (*Medicago truncatula*) and grasses (rice) will soon follow. The rate at which new genes have been discovered has far outstripped the pace at which their function is determined. The greatest hurdle that plant biologists face in assigning gene function and in crop improvement is the lack of efficient and robust technologies to generate gene replacements or targeted gene knockouts. Many of the factors underlying these events remain to be elucidated. This review addresses the current status of plant gene targeting and what is known about the associated plant DNA repair mechanisms.

Since the development of plant transformation technologies in the early 1980s, functional characterization of newly discovered genes has occurred in a broad range of plant species. In addition to its application to basic research, the ability to express foreign genes or to attenuate expression levels of endogenous genes has made a huge impact on crop improvement.

Given the tremendous resources being invested in plant genomics, data are being generated at an unprecedented rate. Unfortunately, our understanding of gene function lags far behind. The ability to delete, or specifically alter, chromosomal sequences of interest would greatly enhance our ability to test gene function. To date, targeted gene replacement occurs in higher plants at such low frequency as to render it an unfeasible approach for everyday use by biologists. The ability to generate targeted gene changes in plants would have as great an impact on fundamental research and crop improvement as did the original plant transformation experiments that took place more than two decades ago.

In this review, we discuss methods to disrupt plant gene function and the mechanisms by which they operate, in an attempt to better address questions of gene targeting in plants.

Reverse genetic approaches to plant functional genomics

The ability to knockout genes or suppress their expression has been a powerful tool for plant biologists. Common approaches include insertional mutagenesis, antisense and co-suppression in transgenics, and virus-induced gene silencing. Collections of random T-DNA or transposable element insertion mutants are currently available to the *Arabidopsis* research community through a variety of

sources. These collections total >200 000 independently derived T-DNA mutants. The flanking DNAs of many of these insertions have been sequenced, and mutants in specific genes can be identified by BLASTing public databases; other mutants can be identified via PCR [1] (<http://signal.salk.edu/> and www.tmri.org). These collections are tremendously useful, but they do not always produce null alleles, and smaller gene targets might not be represented at all.

Homology-directed gene silencing (HDGS) [2] provides another avenue for downregulation of gene function. RNA-directed gene silencing was discovered in transgenic plants in which interactions between similar host genes and transgenes resulted in the reduction of mRNA levels for both loci. This phenomenon occurs throughout most plant species, is similar to gene silencing events in other organisms [3,4] and can be induced at the transcriptional (TGS) or post-transcriptional (PTGS) levels [5]. TGS is associated with hyper-methylation of regulatory regions resulting in a substantial decrease in transcription of the targeted gene while PTGS is associated with cleavage of a double-stranded RNA into small interfering RNAs (siRNA) of 21 to 25 nucleotides [6]. siRNAs, which spread throughout the plant, are thought to direct a nuclease complex that degrades the target mRNA and a methylation complex responsible for the methylation of silenced genes [7]. PTGS can be efficiently induced, at least in somatic tissues, by the expression of dsRNAs and therefore provides a useful method for dominant inhibition of gene function [8,9]. The downside of HDGS is its somewhat unpredictable nature because of its lack of controllable tissue specificity.

Plant gene expression can also be suppressed through the infection of virus vectors harboring exon-containing regions of the host gene. This approach, referred to as virus-induced gene silencing (VIGS), has been used to silence a variety of plant genes in multiple plant species. Early examples of VIGS used tobacco mosaic virus-based vectors [10] and potato virus X-based vectors [11] that contained a portion of a phytoene desaturase (PDS) gene. The mechanisms by which VIGS occurs are still being elucidated. However, it is known that certain elements are conserved between the various HDGS phenomena.

The various approaches to RNAi each have their own limitations, including incomplete or partial silencing and a lack of member-specificity within a multigene family. VIGS is highly dependent on finding a suitable viral vector for the recipient host species and, like other forms of PTGS, lacks tissue specificity because of the systemic nature of the silencing signal. Nevertheless, these

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approaches have provided plant biologists with powerful tools to alter gene function.

Transgene insertion via double-strand-break repair: two competing pathways

In *Saccharomyces cerevisiae*, transgenes (including T-DNAs [12,13]) with homology to the yeast chromosome integrate via homologous recombination (HR), producing gene replacement events. In most vertebrate tissues and in all higher plants, transforming plasmids and T-DNAs integrate via nonhomologous end joining (NHEJ), resulting in random gene addition events [14]. What accounts for this difference? It's conceivable that the plant genome, perhaps owing to its larger size, is an impossibly difficult substrate for gene targeting. Most vertebrate cells, like plant cells, have large genomes and integrate transgenes nonhomologously. But the exceptions to this vertebrate rule prove that genome size does not have to be an impassable barrier to gene replacement. Adult mouse cells ($C = 3$ gigabases) integrate transgenes nonhomologously, but mouse embryonic stem cells can integrate transgenes via homologous recombination frequently enough to make directed knock-outs feasible in this relatively expensive model system [15]. Chicken ($C = 1.2$ gigabases) DT40 cells integrate transgenes primarily via homologous recombination [16]. In addition, the *Physcomitrella* genome is three times as large as that of *Arabidopsis*, and yet gene replacement occurs efficiently in the chloronemal cells of this moss [17–19] (D.G. Schaefer, PhD thesis, University of Lausanne, 1994). Thus, a large genome might or might not be a good target for gene replacement, apparently depending on the pattern of expression of proteins involved in the process, presumably double-strand-break-repair proteins. Yeasts, plants and vertebrates all possess the ability to repair double-strand breaks (DSBs), or integrate transgenes, via both HR and NHEJ [20–23], but choose to employ these pathways to varying extents. Thus, by altering the pattern of expression of these genes it might be possible to alter the mode of transgene integration.

The relative rarity of transgene replacement events versus nonhomologous events (~ 1 replacement event to 3000 nonhomologous events [24–26]) suggests that substantial reengineering of the DSB repair process will have to be performed. This might be an impossibly complex task, requiring just the right level of expression of a variety of collaborating gene products. More optimistically, one might hope that the enhanced expression of a protein that's currently a bottleneck in the homologous repair pathway, or perhaps the elimination of the competing NHEJ pathway, might enhance the ratio of homologous to nonhomologous events. Many, although not all, of the genes required for both the NHEJ and HR DSB repair pathways have been identified in yeast and also in vertebrates, although the biochemical functions of many of these proteins remains to be determined. The completion of the *Arabidopsis* genome project has shown that plants carry what seems to be the vertebrate suite of genes required for double-strand-break repair [27]. Screens for ionizing radiation- or bleomycin-sensitive mutants [28–34], which can be defective in DSB repair, as well as

assays for the direct identification of plants defective in T-DNA transformation [35–37], have been developed and will continue to be employed to identify potentially novel genes involved in this process. Thus, *Arabidopsis* appears to have an established infrastructure for the study and manipulation of transgene integration pathways.

Transgene integration via HR: possible targets for upregulation

There are many models, and perhaps just as many pathways, for the repair of DSBs via homologous recombination. For further guidance, see Ref. [38] for a discussion of models for gene replacement. In Fig. 1, we present two different mechanisms for homologous recombination repair of DSBs and list the proteins involved in these processes. The essential difference between the two mechanisms is the establishment of a Holliday junction in the double crossover (Szostak) model; this presents an

Fig. 1. Double-strand break repair via homologous recombination. *Arabidopsis* homologs thought to be involved, based on mammalian models [51] are written in red. Proteins written in black are required for homologous recombination (HR) in some other organisms but obvious homologs do not exist in plants. These include NBS1 (in *S. cerevisiae*, the distantly related protein XRS2), EME1 (in *Schizosaccharomyces pombe*, with no obvious sequence homolog in mammals), and RAD52 (crucial for most HR events in yeasts, but less so in mammals [63,64]). The eukaryotic genes required for branch migration have not yet been identified, although a mammalian branch migration activity (with an associated resolvase unrelated to MUS81) has been biochemically characterized [75]. *Arabidopsis* mutants defective in RAD50 are viable and have been characterized, and evidence suggests a role in the maintenance of genomic integrity [76,77] and meiotic recombination [78]. Arrowheads represent 3' ends; the invading (damaged, or transgene) strands are depicted in purple; the template strands are in green.

opportunity for chromosome arm exchange. Although these exchanges are required for correct segregation of homologs during meiosis, they might be hazardous during repair of DSBs on nonaligned chromosomes or chromatids; exchange between dispersed repetitive sequences might generate deletions, duplications or translocations. However, the synthesis-dependent strand-annealing (SDSA, Fig. 1) mechanism eliminates the need for a Holliday junction and the hazards associated with its resolution. Evidence points to SDSA as a major pathway in plants for the repair of chromosomal double-strand breaks induced by restriction enzymes [39] or transposable elements [40].

How might these DSB repair pathways be employed for the integration of transgenes via HR? Presumably the free ends are found on the transgene (rather than at the recipient locus) and these must be either spliced into (Szostak model) or somehow copied onto (SDSA) the target. It is possible to develop SDSA models for the integration of transgenes; these models require either the break-induced replication (BIR) of the entire chromosome (a phenomenon known to occur in yeast [38]) or the encounter of a nick on the displaced strand, with subsequent trimming of flaps. An SDSA model can also explain the integration of 'ends-in' constructs such as those employed in the *Drosophila* gene-targeting system [41]. The Szostak model might explain the integration of 'ends out' constructs (Fig. 2).

Homologous transgene integration via the Szostak model requires the induction of nicks in the target site. These might be generated by Holliday junction resolvases, by a flap endonuclease, or through replication to a pre-existing nick. Enzymes specifically required for the generation of target-site nicks during gene replacement have recently been identified in mice. Using *mErcc1*^{-/-} embryonic stem cell knockouts, it has been shown [42] that the structure-specific 3' flap endonuclease [43] encoded by *mErcc1* and *mXPF* is required for gene replacement. The complex is involved in the integration of gene replacement constructs that involve the insertion of a significant piece of heterologous sequence (such as a selectable marker) within the target gene (Fig. 2). It might also be involved in the trimming of nonhomologous flanking ends [44]. Homologs of both *ERCC1* and *XPF* have been identified in the *Arabidopsis* database (The *Arabidopsis* Genome Initiative, 2000), and mutants in both genes identified [33,45,46]; the mutants are gamma-sensitive, UV sensitive, and sensitive to the crosslinking agent mitomycin C. The role of *UVH1* (the *Arabidopsis* *XPF* homolog) in nontargeted T-DNA integration has been investigated, with conflicting results [47–49]. The requirement for this endonuclease in gene replacement in plants has yet to be investigated.

Attempts have been made to stimulate the HR pathway in plants, with the goal of improving the efficiency of gene replacement. These include the overexpression of *E. coli* RecA [24,50] and the expression of RuvC Holliday junction resolvase proteins [51]. Both efforts have resulted in an increase in spontaneous homologous recombination, which might reflect either an increase in the efficiency of HR or an increase in the rate of induction of recombinogenic DNA damage. The

Fig. 2. A model for gene replacement in mammals [43]. (a) Resection of transgene ends. (b) RAD51-driven homology search. (c) Branch migration ends at the heterology-homology border. (d) ERCC1/XPF-dependent incision of the target site just 5' of the displaced 3' strand. (e) Nuclease or helicase activity eliminates the targeted DNA segment. (f) DNA synthesis, a 5' flap endonuclease (FEN1) and DNA ligase complete the gene-replacement process. Arrows indicate canonical ERCC1/XPF recognition sites [43]. The question marks indicate more questionable sites; sites that might or might not be cleaved by ERCC1/XPF. Arrowheads represent 3' ends; the invading (damaged, or transgene) strands are depicted in purple; the (homologous) template strands are in green. The pink insert represents an insertion (such as a selectable marker) with no homology to the target.

fidelity of repair of a targeted (I-SceI-induced) break in tobacco lines overexpressing a nuclear-targeted RecA was characterized [51], and the frequency at which breaks were repaired entirely through HR was found to have increased. Interestingly, however, the frequency of gene replacement events did not increase.

Overexpression of RAD51 and/or RAD52 has also been investigated in mammalian cell lines, and has produced conflicting results, including an increase in gene conversion at a targeted break [52], a decrease in HR repair of a similar site [53], and an increase in spontaneous HR. It is possible that an excessive level of overexpression of RAD51 inhibits repair of DSBs by HR.

Nonhomologous end joining: a target for elimination

Our understanding of the nonhomologous end-joining pathway is largely derived from research in vertebrate

cell lines, confirmed by experiments in *S. cerevisiae* employing a HR-defective (*rad52*) background. Mammalian mutations affecting NHEJ and ionizing radiation (IR) sensitivity include cell lines defective in Ku80 and Ku70 (DSB binding proteins), DNA ligase IV, and its cofactor, XRCC4 [54]. Genetic evidence in *S. cerevisiae*, as well as recent *in vitro* evidence, indicate that the MRE11/RAD50/XRS2 complex also plays an essential role in NHEJ, perhaps in maintaining the alignment of ends [55–57]. The *Arabidopsis* genome encodes obvious homologs of Ku70, Ku80, Lig4, XRCC4, MRE11 and RAD50. In vertebrates, DNA-PK_{cs} interacts with Ku to assist in bringing DSBs together. Neither *Arabidopsis* nor yeast encodes a DNA-PK_{cs} homolog, but they do encode homologs of the closely related proteins ATM [58] and ATR, which are involved in response to DNA-damaging agents. *Arabidopsis* mutants defective in Ku70 and ATM are sensitive to gamma radiation; other mutants have not yet been tested for IR sensitivity [59,60]. The *Arabidopsis* Ku80, Ku70, DNA ligase IV and RAD51 genes are transcriptionally induced in response to IR or bleomycin, suggesting that they play a role in DSB repair [60–62].

It is possible that the HR and NHEJ repair pathways not only coexist but also compete for DSBs. An end captured by Ku complex might become inaccessible to either RAD51 or the nucleases and accessory proteins required to prime the substrate. For this reason, elimination of the NHEJ pathway might not only enhance the ratio of homologous to nonhomologous transformation events, but might raise the absolute frequency of gene replacement events per introduced transgene. It will be interesting to determine whether lines with deficiencies in the early stages of NHEJ (Ku mutants) have enhanced frequencies of gene targeting.

Oligonucleotide-directed gene targeting

Self-complementary chimeric DNA/2'-O-methyl RNA (chimeras) and modified DNA oligonucleotides direct site-specific base changes in chromosomal and episomal targets in mammalian cells [63–66]. Designed to pair with a homologous sequence within genomic DNA, these molecules introduce single base changes at specified targets (Fig. 3). Initial studies using mammalian culture cells were successful in correcting a targeted mutation in β -globin [64] and in an alkaline phosphatase gene [63]. Subsequent experiments in animals were directed toward restoring expression of mouse dystrophin [65] and canine muscle [66]. Gene correction efficiencies reported in these findings were in the 1 to 20% range.

A proposed mechanism by which these molecules act involves the DNA strand of the chimera functioning as a template for gene repair acted upon by the host DNA repair machinery while the RNA strand enhances targeting efficiency by stabilizing complex formation with the target DNA sequence [67].

The utility of chimeras to introduce site-specific base changes has also been demonstrated in plants. Chimeras were introduced into tobacco [68] and maize [69,70] to target endogenous and transgenes resulting in selectable

Fig. 3. Integration of a targeting oligonucleotide into double-stranded DNA [68,72]. The chimeric oligonucleotide (RNA: red, DNA: purple) interacts through homologous base pairing with the target DNA (green) and results in the formation of a double D-loop structure. The mismatched base pairs are resolved through a series of DNA repair processes.

phenotypes. Unlike the high level of efficiencies observed in mammalian systems, oligonucleotide-directed gene conversion occurs at a low frequency (10^{-4}) in plants [68,69].

Data from chimera-directed gene-targeting experiments in plants yielded results never before observed in other systems. Nonspecific conversions *in planta* were detected at the targeted base and at the position immediately 5'. Whether this *in vivo* lack of precision, or slippage, is limited to certain plant species is unknown. However, these 'ectopic' conversions are not observed in plasmid targeting experiments involving maize or *Arabidopsis* cell free extracts (CFE) and have only been observed *in vitro* when using tobacco CFEs [71].

To help elucidate the factors involved in the conversion mechanism and as a system to study plant DNA repair mechanisms, a cell-free assay utilizing protein extracts from either plastids or whole cells has been established [71,72]. This work has also provided a mechanistic explanation of the *in vivo* plant results. Chimeras and modified, single stranded DNA oligonucleotides containing phosphorothioate linkages can both convert or insert a targeted base within a plasmid DNA substrate [72].

Current examples of oligonucleotide-directed plant gene targeting were directed toward base changes

resulting in a selectable phenotype. The current low level of targeting efficiency precludes the use of addressing genes yielding a non-selectable phenotype. Improvements in vector design and an increase in our knowledge of plant DNA repair mechanisms will expedite the applicability of this technology. Oligonucleotide-directed gene targeting provides us with one possible approach to target precise regions of the plant genome. It is unlikely that this technology will be applicable in efforts directed at the attenuation of plant gene expression. However, it should be useful for generating systematic amino acid substitutions or for targeting select members of complex gene families.

Perspectives

A milestone in the progression of plant biology and crop varietal improvement will be the ability to target readily and to modify specifically genes of interest in a wide range of plant species. The ability to make precise gene modifications in crop species might help to alleviate many of the concerns associated with the current biotechnological approaches that are integral to their improvement.

With the exception of the lower land plant *Physcomitrella*, homologous recombination remains an elusive tool for use in plant genetic engineering. Given limited successes in higher plant species, *Physcomitrella* might provide an excellent model system for determining the mechanisms by which homologous recombination and gene targeting occurs in plants [73]. The manipulation of the expression of genes involved in recombination might alter transgene integration pathways and make higher plants more *Physcomitrella*-like in this respect. In addition, the judicious use of counterselectable flanking markers (which kill plants that have integrated the transgene nonhomologously) has already been shown to bring transgene replacement to the verge of feasibility in rice [74] (see the Research Focus article by Barbara Hohn and Holger Puchta in this issue of *Trends in Plant Science*).

In efforts to use the vast amounts of genomic information, we will continue to rely upon insertional mutagenesis and RNAi approaches to assist us in assigning function to newly discovered plant genes. The processes of homology-directed transgene integration and *in vivo* site-specific gene changes through the use of chimeric and modified-DNA oligonucleotides are currently too inefficient to compete with the less precise, but more accessible techniques of RNAi and random insertional mutagenesis.

It is much too early to predict whether more precise genetic engineering techniques will ever become a readily accessible technology in all higher plants. However, the growing interest and participation in this field and the rate at which plant DNA repair and gene targeting discoveries are being made is an encouraging and essential step in the right direction.

Acknowledgements

Research performed by A.B.B.'s laboratory in the area of DNA double-strand-break repair was funded by the National Science Foundation, the

USDA and BARD. Oligonucleotide-directed gene-targeting efforts in G.D.M.'s laboratory was supported by the Samuel Roberts Noble Foundation.

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