**A gene-within-a-gene Cas9/sgRNA hybrid construct** **enables gene editing and gene replacement strategies in *Chlamydomonas reinhardtii***

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**Abstract**

Previous studies demonstrated highly inefficient gene editing in *C. reinhardtii* using conventional Cas9 and sgRNA genes (only 1 editing event using >1.5x109 initial cells). Design and testing of a hybrid gene-within-a-gene construct (composed of a Cas9 gene containing an artificial intron with an inserted sgRNA gene) demonstrated that such constructs were functional both in tobacco cells and *C. reinhardtii* cells. In tests with *C. reinhardtii*, approximately one in every ~3x107 initial cells contained an edited version of the targeted *FKB12* gene (i.e, an average of ~3 colonies with an edited *FKB12* gene per electroporation using 108 initial cells). Lack of an intact Cas9/intron-sgRNA gene in cells carrying either of two different edited genes strongly suggested that editing was due to transient expression of the Cas9/intron-sgRNA gene and the likely toxicity of long-term expression of Cas9 in *C. reinhardtii* cells. Co-transformation of the arginine-requiring mutant, *arg7-8*, with Cas9/intron-sgRNA constructs and appropriately designed synthetic, 80 nucleotide ssDNAs complementary to the argininosuccinate lyase (*ARG*) gene led to successful homologous recombination or nucleotide replacement andproduction of arginine prototrophs*.* As a practical application, a similar ssDNA oligonucleotide targeting the acetolactate synthase (*ALS*) gene and an appropriate Cas9/intron-sgRNA construct was used to create cells resistant to the herbicide, sulfometuron methyl.

**Introduction**

Adoption of the CRISPR (clustered regularly interspaced short palindromic repeats) system involving the use of only two genes, Cas9 and sgRNA, is revolutionizing gene editing in eukaryotic cells due to its simplicity, accuracy and speed (1, 2). The RNA-directed selection of a specific 20-22 bp nucleotide sequence within a target gene (or a set of closely related genes) by the Cas9/sgRNA complex allows the two nuclease domains of Cas9 to create a double-stranded DNA break (DSB) at a predetermined site within the gene of interest. Repair of the DSB by the error-prone nonhomologous end joining (NHEJ) DNA repair system can result in gene inactivation (i.e., gene knockout). Alternatively, replacement of the cleaved DNA segment with a closely related DNA fragment via homologous recombination (HR) can result in gene replacement (i.e., gene knockin). If the nuclease domains of Cas9 are inactivated, attachment of gene activation domains or gene repressor domains to the CAS/ sgRNA complex can be used to elicit marked increases or decreases in gene transcription and expression. Because of the high efficiency with which the Cas9/sgRNA system can be employed for gene editing in most animals and plants (2, 3), it holds great promise for driving significant improvements in medicine, industry and agriculture in the not too distant future. Indeed, several examples already exist of the practical applications of CRISPR technology and other “designer nucleases” [e.g., zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs)] to trait improvements in agriculturally important plants (3).

Unfortunately, the application of designer nuclease technologies to research with the widely used model alga, *Chlamydomonas reinhardtii* (hereafter, Chlamydomonas), has met with limited success. A single report has been published describing the use of ZFNs to target specific genes for modification and/or knockout via NHEJ DNA repair mechanisms and gene replacement by homologous recombination (4). Use of the Transcription Activator-like Effector (TALE), avrXa7, with its native transcription activation domain to construct artificial designer transcription activator-like effectors (dTALEs) targeting two Chlamydomonas arylsulfatase genes (*ARS1* and *ARS2*) and a dTALE targeting the HLA3 gene resulted in successful activation of transcription and expression of all three of these genes (5,6). However, numerous attempts to edit various Chlamydomonas genes using designer transcription activator-like effector nucleases (dTALENs) have failed to produce transformants containing edited target genes (unpublished observations).

When reports of successful use of the CRISPR (Cas9/sgRNA) system for targeted gene editing were published (7, 8), efforts were made to utilize this new tool for gene editing in Chlamydomonas. Unfortunately, as with the TALEN system, no gene editing was observed in colonies of Chlamydomonas cells that had been transformed with a Cas9 gene and sgRNA genes targeting endogenous genes (9). Moreover, greatly reduced rates of recovery of antibiotic resistant colonies were observed when cells were co-transformed with a vector containing Cas9 and sgRNA genes along with an antibiotic resistance gene. Subsequent experiments testing for potential Cas9/sgRNA activity in cells within 24 hours after transformation with Cas9/sgRNA gene constructs targeting either exogenously supplied target genes or the endogenous *FKB12* gene demonstrated that transient expression of the Cas9/sgRNA system lead to successful editing of the target genes (9). However, in experiments aimed at recovery of permanently transformed cells that were rapamycin resistant due to editing of the endogenous *FKB12* gene, only one transformant out of a starting population of >1.5X109 cells contained a *FKB12* target site with an editing event characteristic of Cas9/sgRNA cleavage coupled with NHEJ DNA repair (i.e., a 2 bp deletion at the predicted Cas9/sgRNA DNA cleavage site) (9).

In an attempt to alter the Cas9/sgRNA system for greater gene editing activity in higher plants and/or Chlamydomonas, we have made numerous modifications to Cas9 and sgRNA genes and to their expression levels. As part of these efforts, and to reduce the number of genes needed from two (a Cas9 gene and a sgRNA gene) to one, we designed and tested a construct in which the sgRNA gene was inserted into an artificial intron and this sgRNA-containing intron was placed in a potential intron splice site within the coding region of the Cas9 gene. This strategy envisioned that following transcription and excision of the intron, the sgRNA would be recognized and productively bound by Cas9 produced from the same “hybrid” Cas9/intron sgRNA gene. Here we report that such hybrid, gene-within-a-gene, Cas9/intron-sgRNA constructs function in transformed Chlamydomonas cells to cause gene sequence modifications in both exogenously supplied target genes as well as in the endogenous *FKB12, ALS* and *ARG* genes. Using such constructs, the efficiency of successful gene editing, while still not high, is sufficient for many, if not most, gene editing projects in Chlamydomonas. A simple, facile, two-step PCR method to rapidly produce Cas9/intron-sgRNA genes with new DNA cleavage target sites is provided. Importantly, we report that inclusion of a short ssDNA fragment with homology to the region targeted for cleavage by a Cas9/intron-sgRNA complex can result in modification of the target gene due to homologous recombination or nucleotide replacement. Potential uses of the hybrid Cas9/intron-sgRNA gene system along with short ssDNAs for gene replacement are discussed.

**Results and Discussion**

***Design of a gene-within-a-gene, hybrid Cas9/intron-sgRNA gene and initial testing in land plants***

The decreased rates of recovery of Chlamydomonas cells co-transformed with an antibiotic resistance gene and a Cas9 gene compared with cells transformed with only the antibiotic resistance gene (9) suggested that Cas9 expression in Chlamydomonas may be toxic. Attempts to limit Cas9 production through the use of various conditional promoters have thus far failed to increase the recovery of cells containing edited genes (unpublished results). As an approach to potentially increasing the efficiency of gene editing that we obtained in earlier studies with higher plants (10, 11, 12) and to potentially reduce the number of genes needed for successful gene editing from two (i.e., the Cas9 gene and a sgRNA gene) to only one, we designed and tested a hybrid, gene-within-a-gene construct. In this construct, the sgRNA gene is placed within the central portion of an artificial intron [derived from the second intron (IV2) of the potato (*Solanum tuberosum*) *ST-LS1* gene (13, 14) (Fig. 1). The initial construct was designed to target a sequence in a mutant, nonfunctional *GFP* gene (*mGFP*) containing an extra nucleotide in the 5’ coding region of the gene (Supplemental Data Fig. S1A). (A facile method for inserting a new sgRNA targeting region into the gene-within-a-gene construct is described in Fig. S2.) Successful cleavage of the mutant *GFP* gene by the Cas9/sgRNA complex produced from the hybrid Cas9/intron-sgRNA gene would trigger error-prone NHEJ DNA repair and, in approximately 30% of the cases in which there was nucleotide insertion or deletion, there would be restoration of GFP expression – as observed in our previous studies using constructs containing separate Cas9 and sgRNA genes (10, 11).

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**Figure 1.** Construct for testing the potential activity of an sgRNA gene located within an artificial intron of a Cas9 gene (i.e., a gene-within-a-gene). The Cas9 gene contains an inserted potato *IV2* intron in its coding region and the intron is embedded with an sgRNA gene targeting a specific site in a separate nonfunctional *mGFP* gene co-located on the same plasmid. The DNA sequence of the sgRNA gene embedded in the intron is displayed above the Cas9 gene image. Successful expression of the Cas9/intro-sgRNA in *Nicotiana benthamiana* (leading to Cas9/sgRNA-directed target site DNA cleavage in the out-of-reading-frame *GFP* gene and nucleotide insertion or deletion by NHEJ to correct the reading frame) is detected by the appearance of green fluorescence in leaf cells*.* The DNA sequence targeted by the Cas9/sgRNA complex is displayed above the image of the nonfunctional *GFP* gene. 35S: CaMV *35S* gene promoter region; Tnos: termination region of the *Agrobacterium tumefaciens* T-DNA nopoline synthase gene; PClSV, peanut chlorotic streak virus Flt36 gene promoter; rbcS3’, termination region of the pea Rubisco small subunit gene; nucleotides in bold blue: 20nt target sequence with restriction site *ApaLI* in italic; Nucleotides in bold red: PAM site.

To test the potential activity of the hybrid Cas9/intron-sgRNA gene, tobacco (*Nicotiana benthamiana*) leaves were infiltrated with an *Agrobacterium tumefaciens* strain carrying both the hybrid Cas9/intron-sgRNA gene construct and the *mGFP* gene in the T-DNA region of a binary Ti-plasmid. As documented by the images in Fig. S3C and S3E, such infiltration resulted in transient expression of the Cas9/intron-sgRNA gene and the appearance of GFP signals from leaf cells. Such signals can only occur if the proper reading frame has been restored in the mutant *GFP* gene. Infiltration of leaves with constructs carrying only the standard Cas9 gene (i.e., without an intron-sgRNA insert) and the mutant *GFP* gene (i.e., electroporation in the absence of a sgRNA gene) produced no cells exhibiting GFP fluorescence (Fig. S3A).

To verify that gene editing had occurred in the tobacco leaf cells, two assays were conducted. First, DNA was extracted from the section of a leaf expressing GFP and subjected to PCR amplification of a 268 bp DNA fragment containing the site targeted for cleavage by the Cas9/sgRNA complex. DNA was also extracted and PCR amplified from similar sections of leaves infiltrated with *Agrobacterium tumefaciens* carrying a construct containing only a Cas9 gene and no sgRNA gene. The point within the target sequence that was predicted to be the cleavage site for the Cas9/sgRNA complex was selected as a sequence containing an *ApaLI* restriction enzyme (RE) cut site. In this case, the *ApaLI* cleavage site was located at the very center of the amplified DNA. Thus, in those cases in which Cas9/sgRNA cleavage occurred and NHEJ DNA repair resulted in insertions or deletions of nucleotides within the RE site, the 268 bp PCR-amplified DNA fragment would no longer be cleaved by *ApaLI*. This provides a rapid, qualitative assay to determine if Cas9/sgRNA-modified genes are present (or absent) in treated cells. The data of Supplemental Data, Fig. S4, lane 4), show that a portion of the DNA from leaf sections infiltrated with the Cas9/intron-sgRNA construct contained 268 bp fragments resistant to *ApaLI* cleavage as well as, as expected, nonmodified DNA fragments that were severed into two 134 bp RE products. In the control experiment in which DNA extracts were made from similar leaf sections infiltrated with only the *mGFP* gene and the Cas9 gene, PCR/restriction enzyme (PCR/RE) showed that all DNA fragments were fully susceptible to *ApaLI* cleavage (Supplemental Data, Fig. S4, lane 3).

As a second verification of successful gene editing, samples of the 268 bp fragments PCR amplified from DNA of leaves infiltrated with Agrobacteria containing the gene-within-a-gene Cas9/intron-sgRNA construct were cloned and individually sequenced. The goal was to determine if the target sequence had been cut and subjected to NHEJ DNA repair at or near the site of predicted Cas9/sgRNA-mediated gene cleavage (i.e., 3 bp upstream of the NGG PAM sequence). The DNA sequencing data of Supplemental Data, Fig. S5 show that both short nucleotide deletions and insertions had occurred at or near the predicted Cas9/sgRNA cleavage site in seven different DNA samples. These data provide strong evidence for the proper function of Cas9/sgRNA complexes encoded by the hybrid Cas9/intron-sgRNA gene.

In an additional test of the function of the hybrid Cas9/intron-sgRNA gene in plants, two sites within the endogenous phytoene desaturase 3 (*PDS3*) gene of *Nicotiana benthamiana* were targeted for editing (Supplemental Data, Fig. S6). To facilitate PCR/RE analyses of potentially edited target sites, each target site contained a *MlyI* restriction enzyme cut site just upstream of the NGG PAM sequence and overlapping the predicted cleavage site for the Cas9/sgRNA complex. In two separate experiments, the two Cas9/intron-sgRNA hybrid gene constructs targeting the two independent sites within the *PDS3* gene were delivered to tobacco leaves via infiltration with Agrobacteria as described above. Analyses of the sensitivity of PCR-amplified DNA fragments to digestion with *MlyI* (Supplemental Data, Fig. S7) in this qualitative assay for Cas9/sgRNA activity showed that both target sites within the *PDS3* gene were resistant to cleavage, suggesting Cas9/sgRNA- and NHEJ-mediated nucleotide modifications at the two target sites. Such modifications were confirmed by DNA sequencing of cloned PCR-amplified fragments of the two targeted DNA regions (Supplemental Data, Fig. S8). Again, the modified target sites contained short nucleotide insertions or deletions at or near the Cas9/sgRNA cleavage site as expected from NHEJ-mediated DNA repair.

These experiments demonstrated that the gene-within-a-gene Cas9/intron-sgRNA construct was indeed functional in plant cells and allowed us the opportunity to test if such constructs might also be useful in producing successful gene editing in Chlamydomonas.

***Editing of exogenously supplied genes in Chlamydomonas expressing the hybrid Cas9/sgRNA gene***

The Cas9 gene used in previous experiments with both land plants and Chlamydomonas (9, 10, 11) (and in the experiments described above) was codon optimized for expression in Chlamydomonas. To allow tests of this gene incorporated into the gene-within-a-gene Cas9/intron-sgRNA construct in Chlamydomonas, an initial experiment was conducted in which the Cas9/intron-sgRNA gene (this time containing the sgRNA gene inserted into a DNA sequence copied from the Chlamydomonas RbcS2 first intron) was coupled in a single construct with a nonfunctional, mutant bleomycin (*Ble*) resistance gene (Supplemental Data, Fig. S9). The *Ble* gene was inactive because its 5' coding region was interrupted by insertion of an additional nucleotide. Cleavage of the sgRNA target site by the Cas9/sgRNA complex and repair of the cut by NHEJ DNA repair should result in indels near the DNA cleavage site and, in some cases, production of *Ble* genes in which the proper reading frame has been restored. Without such restoration, no zeocin-resistant Chlamydomonas colonies should be obtained. Following transformation in initial experiments, 7 zeocin-resistant colonies were recovered. DNA was extracted from cells of each colony and the sgRNA target region in each sample was PCR amplified prior to DNA sequencing (Supplemental Data*,* Fig. S10). In 7 of the genes, there was insertion of nucleotides in the vicinity of the presumed Cas9/sgRNA cut site and restoration of a proper reading frame.

Simultaneously with the experiment described above, we also sought to determine if we could use a specially designed exogenously-supplied mutant *Ble* gene as a means to enrich for cells containing an endogenous gene that had been modified by the same Cas9/intron-sgRNA construct that targeted the exogenous-supplied *Ble* gene. The strategy was to insert into the 5' region of the mutant *Ble* gene a 23 bp DNA sequence identical to an sgRNA target sequence in an endogenous gene (in this case, the putative *Ku70* gene). Thus, if the Cas9/sgRNA system successfully targeted the exogenous mutant *Ble* gene for modification to produce an active *Ble* gene, some of the resulting zeocin-resistant transformants might also contain a modification of the sgRNA target site within the *Ku70* gene. In an experiment performed to test this approach (Supplemental Data, Fig. S11), three zeocin resistant colonies were obtained, all containing a restored in-frame *Ble* gene coding region. DNA from one colony (colony #1) contained a 7 bp deletion at the site of predicted Cas9/sgRNA cleavage in the *Ku70* gene. These results indicate that such a strategy for creating modifications in genes lacking an easily selectable or scorable phenotype may prove useful. However, uncontrollable modifications (i.e., cleavage, deletions, rearrangements) of exogenously-supplied selectable marker genes by Chlamydomonas (15, 16) point to the desirability of using a mutant marker gene previously integrated into the genome such as the nonfunctional, mutant paromomycin gene employed in a similar strategy by Sizova et al.(4) in their work with zinc-finger nucleases. Selecting zeocin-sensitive Chlamydomonas cells following transformation with a construct that couples the present nonfunctional mutant *Ble* gene through a FMDV 2A bridge to a second selectable marker gene (17) may offer a facile means for accomplishing this task.

***Editing of the endogenous FKB12 gene and production of rapamycin resistant transformants***

Previous studies showed that the standard Cas9/sgRNA system could be used to cause typical Cas9/sgRNA-mediated disruptions in the endogenous *FKB12* gene of Chlamydomonas when DNA was extracted and analyzed 24 hours after electroporation (i.e., in a transient assay for Cas9/sgRNA activity) (9). However, in searching for viable mutants in which FKB12 gene disruption led to recovery of cells resistant to rapamycin, only one such mutant was recovered in experiments employing over 1.5x109 initial cells. Thus, it was important to determine if use of the new Cas9/intron-sgRNA construct might improve success rates. Using the Cas9/intron-sgRNA construct depicted Fig. S12 (and detailed in Supplemental Data, Fig. S1B), four independent experiments employing a total of 4x108 initial cells were conducted and yielded 13 independent, rapamycin-resistant transformants displaying deletions and insertions in the near vicinity of the predicted Cas9/sgRNA cleavage site (Fig. 2). (An unusual exception was one rapamycin resistant mutant containing an exact DNA sequence duplication of a 23 bp segment 9 bp downstream of the PAM site.) The recovery of 13 rapamycin-resistant Chlamydomonas colonies all containing target site modifications using only 4x108 initial cells calculates to a rate of 1 cell in ~3x107 initial cells being modified using the new Cas9/intron-sgRNA system. This is a rate approximately 50 times higher than the previous rate noted above. Although not exceptionally high, the rates of gene editing achieved with expression of the Cas9/intron-sgRNA system observed in the current studies suggest that this system may be more than adequate for use in most gene editing experiments with Chlamydomonas.



**Fig. 2.** DNA sequence confirmation of Cas9/intron-sgRNA-directed mutagenesis of the sgRNA target site within the Chlamydomonas *FKB12* gene of cells transformed with a Cas9/intron-sgRNA construct. Target regions of the *FKB12* gene in DNA from cells in single rapamycin-resistant Chlamydomonas colonies were PCR amplified, cloned and sequenced. Top line shows the DNA region containing the sgRNA target site while DNA sequences of the same region from individual mutant clones are shown below. The PAM site is in red, *ApaLI* recognition site is underlined and the 20 nucleotide target sequence is shown in blue. Deleted nucleotides are depicted as red dashes and inserted nucleotides are shown in green. Values indicating the net length of insertions and/or deletions (In/Del) and the number of each mutant clone are listed to the right in the first and second columns, respectively. Green nucleotide sequence is a 23 bp insert/duplication creating an exact tandem duplication within the *FKB12* gene. The 13 mutated *FKB12* genes were obtained in 4 experiments employing a total of 4x108 cells (i.e., a rate of one site-specific mutation in ~3x107 initial cells).

***Evidence for transient expression of the hybrid Cas9/sgRNA gene and toxicity of Cas9***

The two experiments above in which either the exogenous *Ble* gene or the endogenous *FKB12* gene were successfully targeted for modification provided an opportunity to determine if Cas9 is toxic when expressed in Chlamydomonas. If so, it would be predicted that randomly selected zeocin-resistant and rapamycin-resistant cells recovered from these experiments would lack a functional Cas9 gene. To determine if these transformants contained an intact Cas9/intron-sgRNA gene, two PCR primer sets were employed, one for amplification of a 5' segment of the gene and the other for amplifying a 3' segment of the gene. In most cases, neither 5’ nor 3' segments of the gene could be detected (Table 1). As testimony to the ability of Chlamydomonas cells to cut and scramble exogenously supplied DNA prior to integration (15, 16) some cells contained only 5' or only 3' segments of the Cas9/intron-sgRNA gene. In no case was there evidence for the presence of an intact gene. These results strongly suggest that the modifications of the *Ble* and *FKB12* gene observed in these experiments was due to transient expression of the Cas9/intron-sgRNA gene - a conclusion that is consistent with our previous suggestion (9) that constitutive expression of a Cas9 gene in Chlamydomonas may be lethal to the cell.

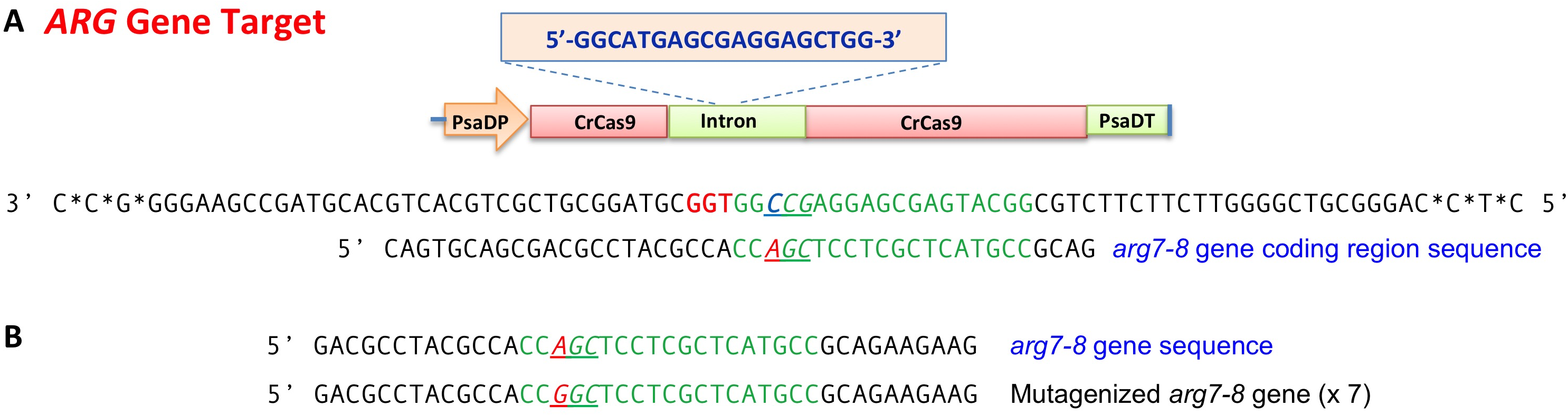
**Table 1.** Detection by PCR of the presence or absence of 5’ terminus (525bp) or 3’ terminus (137bp) of the Cas9/intron-sgRNA gene in individual rapamycin resistant (F*KB12* gene knock outs) or bleomycin (zeocin) resistant Chlamydomonas clones. Col., colony number; +, sequence present; -, sequence absent.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Rapamycin Resistant Cells** | | | **Zeocin Resistant Cells** | | | | |
| **Col.** | **5' end** | **3' end** | | **Col.** | | **5' end** | **3' end** | |
| 1 | - | - | | 1 | + | | - | |
| 2 | + | - | | 2 | - | | - | |
| 3 | - | - | | 3 | - | | + | |
| 4 | - | - | | 4 | - | | - | |
| 5 | - | + | | 5 | - | | + | |
| 6 | - | + | | 6 | - | | - | |
| 7 | - | - | |  |  | |  | |
| 8 | - | - | |  |  | |  | |

***Cas9/intron-sgRNA supports gene modification by homologous recombination or nucleotide substitution in the presence of short, synthetic, homologous ssDNAs***

Creation of DSBs by zinc-finger nucleases, TALENs or Cas9/sgRNAs and simultaneous provision of DNA with homology to the broken strands is known to allow for gene replacement by homologous DNA recombination in numerous eukaryotic cell types (1, 2, 18, 19). Indeed, creation of DSBs in Chlamydomonas DNA using zinc-finger nucleases (ZFN) and simultaneous delivery of long strands of homologous DNA has allowed conversion of a mutant paromomycin-resistance gene into an active gene (4), albeit at very low rates. As a potential step forward, we sought to determine if the Cas9/intron-sgRNA system might perform better. We also wished to determine if in place of long doubled-stranded or single-stranded DNAs we could substitute short, synthetic, single-stranded DNA whose 5' and 3' ends were protected from exonuclease attack by incorporation of phosphothioate bonds. Two separate endogenous Chlamydomonas genes were targeted for editing using a combination of the new Cas9/intron-sgRNA system and short (80 nucleotide), ssDNA fragments homologous to specific sites in the genes - genes whose modifications could create mutants with easily selectable phenotypes.

The first target gene was the mutant argininosuccinate lyase (*ARG*) gene (Cre01.g021251.t1.1 - (M=1) 4.3.2.1) of Chlamydomonas*,* cw 15 *arg7-8* mt+ (cc4350). This gene contains a GGC to AGC codon change that results in a glycine to serine substitution and loss of enzyme activity (21). Spontaneous reversion of this mutation is very rare (21). Transformation of 2x108 of these arginine-requiring cells with the Cas9/intron-sgRNA gene and the synthetic ssDNA shown in Fig. 3A resulted in the recovery of 7 colonies on growth medium lacking arginine. DNA sequencing revealed that in all cases, treatment with the Cas9/intron-sgRNA and synthetic DNA fragment resulted in restoration of the correct nucleotide in the *ARG* gene of these arginine prototrophs (Fig. 3B). Control transformation of 2x108 cells with the Cas9/intron-sgRNA gene in the absence of the synthetic ssDNA replacement strand produced no arginine prototrophs. Likewise, transformation with the 80 nt ssDNA molecule alone produced no arginine prototrophs.



**Figure 3.** Gene editing by homologous recombination or nucleotide replacement in the non-functional argininosuccinate lyase (*ARG*) gene (Cre01.g021251.t1.1 - (M=1) 4.3.2.1) of Chlamydomonas*,* cw 15 *arg7-8* mt+ (cc4350). **A)** Diagram: Design of a Cas9/intron-sgRNA gene targeting the *arg7-8* gene to produce cells able to grow without exogenous arginine following electroporation with the Cas9/intron-sgRNA gene and a short, synthetic, ssDNA fragment with homology to the targeted gene region. First line of sequence: a synthetic single strand DNA oligonucleotide mimicking the non-coding (NC) strand of the *arg7-8* gene with 5' and 3' terminal nuclease-resistant phosphothioate bonds (asterisks) and designed to create serine to glycine amino acid change (AGC -> GGC) if integrated into the *arg7-8* gene by homologous recombination or nucleotide replacement. Second line of sequence: DNA sequence of a segment of *arg7-8* gene coding strand. Red TGG sequence, PAM site. **B)** PCR-amplified DNA sequence of 7 independent colonies of arginine prototrophs generated by transformation of *arg7-8* cells with a combination of the Cas9/intron-sgRNA construct plus the NC single stranded gene replacement oligonucleotide. Red TGG, PAM site; green, sgRNA target site.

The second target gene was the Chlamydomonas acetolactate synthase (*ALS*) gene (Cre09.g386758.t1.1) for which it is known that a lysine to threonine (AAG to ACG) mutation at position 257 confers resistance to the herbicide, sulfometuron methyl (SMM) (20). Fig. 4A shows the sequence and composition of the ssDNA used for modifying the *ALS* gene and the DNA sequence in the non-coding strand targeted by the Cas9/intron-sgRNA complex. In two transformation experiments using a total of 2x108 initial cells, we recovered 5 colonies on plates containing SMM in the growth medium. In each case we observed the appropriate A to C transversion at the target site in the *ALS* gene when the Cas9/intron-sgRNA gene and the short, synthetic ssDNA was provided during electroporation. Transformation of 2x108 cells with the Cas9/intron-sgRNA construct, but without the synthetic ssDNA fragment, produced no SMM-resistant colonies.



**Fig. 4.** Gene editing by homologous recombination or nucleotide replacement in the Chlamydomonasacetolactate synthase (*ALS*) gene (Cre09.g386758.t1.1 ). **A)** Diagram: Design of a Cas9/intron-sgRNA gene targeting the *ALS* gene of Chlamydomonas to produce mutants resistant to sulfometuron methyl (SMM) following electroporation with the Cas9/intron-sgRNA gene and a short, synthetic, ssDNA fragment with homology to the targeted gene region. First line of sequence: a single strand DNA oligonucleotide with terminal nuclease-resistant phosphothioate bonds (asterisks) designed create a K257T change (AAG -> ACG) if integrated into the *ALS* gene by homologous recombination or nucleotide replacement. Second and third lines of sequence: DNA sequence of a segment of wild-type (WT) *ALS* gene coding strand and non-coding (NC) strand, respectively – the latter being complementary to the sgRNA targeting sequence. Fourth line: predicted DNA sequence in NC strand of an accurately mutagenized *ALS* gene. Red GGG sequence, PAM site. **B)** PCR-amplified DNA sequence of 5 independent SMM resistant colonies generated by transformation of WT cells with a combination of the Cas9/intron-sgRNA construct plus the single stranded gene replacement oligonucleotide. Red GGG, PAM site; green and blue, sgRNA target site.

Several reports exist of using short synthetic RNA/DNA chimeric oligonucleotides to obtain precise nucleotide replacement (22-30). Thus, whether the editing that is taking place in the present experiments is due to classical homologous recombination or to nucleotide replacement mechanisms will need to be determined in future studies. Regardless, the high rates of recovery of cells bearing desired nucleotide replacements (i.e., ~1 in every 3x107 cell) suggest that the present Cas9/intron-sgRNA system coupled with short, synthetic ssDNAs will likely be useful for numerous gene editing/gene replacement experiments in Chlamydomonas.

Development of the unique Cas9/intron-sgRNA (gene-within-a-gene) construct for gene editing and the discovery that a small ssDNA oligonucleotide with nuclease-protected ends strongly promotes gene replacement by HR or nucleotide replacement represent significant steps forward for researchers using Chlamydomonas and potentially other organisms. Most important in the short term is that the novel, Cas9/intron-sgRNA construct allows practical rates of gene editing and gene replacement (i.e., ~2-4 edited genes/electroporation) in Chlamydomonas - a critical tool for this widely used, haploid organism. The use of a single hybrid gene instead of separate Cas9 and sgRNA genes is convenient and may facilitate gene editing in those organisms for which the conventional CRISPR/Cas9 system currently fails to work and/or in those organisms for which the U6 promoter (for driving sgRNA gene transcription) has not yet been identified. An analogous, but distinctly different, “single transcript” CRISPR/Cas9 gene editing system utilizing a single polymerase II promoter to drive a single transcript containing a Cas9 gene and one or more sgRNAs bounded on either side by a ribozyme structure (to release mature sgRNAs following transcription) has been developed and successfully tested in land plants (31), but has not been tested in algal cells. Importantly, two recent reports (32, 33) suggest that Cas9/sgRNA ribonucleoprotein complexes formed *in vitro* can be delivered to Chlamydomonas cells and result in targeted gene editing. In these cases, the rates of successful gene editing were similar to the rates reported in the present study (i.e., approximately one successful gene editing event per 107 initial cells).

The small 80 nt ssDNAs with protected ends created in the course of this study coupled with Cas9/intron-sgRNA genes or conventional Cas9 and sgRNA genes (or other designer nucleases) to stimulate homologous gene or nucleotide replacement is likely applicable to most eukaryotic cells and has potential to improve success rates. The mechanism by which transient expression of the Cas9/intron-sgRNA construct succeeds in Chlamydomonas (e.g., more stoichiometric or better balanced production of Cas9 and sgRNA from the single hybrid gene) will be challenging to establish given the apparently low percentage of the cell population that is successfully modified. Nonetheless, the present Cas9/intron-sgRNA-based gene editing and replacement technology should be adequate to allow rapid advances in all of the fields of study in which Chlamydomonas is a leading model system such as flagellar/cilia structure and (dys)function, photosynthesis, photoreceptors/channelrhodopsins/optogenetics, algal cell cycles, circadian rhythms, hydrogen production, micronutrient homeostasis, and algal mating mechanisms. In addition, gene editing has strong potential for greatly improving Chlamydomonas (and other algae) for commercial applications in areas such as biomanufacturing of pharmaceuticals, nutriceuticals, specialty chemicals and certain types of biofuels.

**Methods and materials**

**Chemicals and reagents**

High purity rapamycin was purchased from LC Laboratories (Woburn, MA 01801 USA); zeocin was provided by RPI Research Products International Corp (Mt Prospect, IL 60056 USA); sulfometuron methyl (SMM) was purchased from CHEM SERVICE (West Chester, PA 19381 USA). L-Arginine, and other chemicals and reagents used in this study were purchased from Sigma–Aldrich (St. Louis, MO 63178 USA).

**Construction of a Cas9 gene containing an artificial intron with an inserted sgRNA gene**

The Cas9 genes codon optimized for expression in Chlamydomonas used in earlier studies(9, 10) was used as the starting point for creation of the gene-within-a-gene Cas9/intron-sgRNA constructs. To produce the original Cas9/intron-sgRNA constructs, a portion of this Cas9 gene flanked by *ApaI* and *Bsp1407I* restriction enzyme sites and containing an intron sequence with an inserted sgRNA gene (Supplemental Data, Fig. S1) was synthesized (Genscript, Piscataway, NJ 08854 USA) and inserted into the *ApaI* and *Bsp1407I* restriction enzyme sites within the Cas9 gene. Substitution of a different sgRNA gene within this construct was achieved in a two-step, overlap PCR reaction using appropriately designed PCR primers (Supplemental Data, Table S2). Steps in this process are described in Supplemental Data*,* Fig. S2 and its legend.

For testing transient expression of the gene-within-a-gene, Cas9/intron-sgRNA, construct in tobacco (*Nicotiana benthamiana*), a sgRNA gene targeting a nonfunctional mutant *GFP* (*mGFP*) gene (see below) was placed within the central portion of the artificial Cas9 intron derived from the second intron (IV2) of the potato (*Solanum tuberosum*) *ST-LS1* gene (13, 14)(Supplemental Data*,* Fig. S1A). [Choice of the intron insertion site within the Cas9 gene was based on the observations of Li et al. (18)]. Similarly, for vectors for testing Cas9/intron-sgRNA constructs in Chlamydomonas, the first intron of the Chlamydomonas nuclear gene, *RBCS2*, encoding the ribulose bisphosphate carboxylase/oxygenase small subunit (EC.1.1.39) [Sequence ID: emb (X04472.1)], was used for insertion of an appropriate sgRNA gene (Supplemental Data*,* Fig. S1B).

**Construction of Cas9/intron-sgRNA genes for targeting endogenous and exogenous genes in tobacco**

For expression in tobacco cells, the Cas9/intron-sgRNA gene was driven by the CaMV 35S promoter and terminated with an *Agrobacterium tumefaciens* T-DNA nopoline synthase gene (Tnos) terminator. The exogenous target *mGFP* gene was driven by the strong, constitutively-expressed peanut chlorotic streak virus promoter Flt36 gene promoter (34, 35) and terminated with a region from the pea Rubisco small subunit gene (rbcS3′) (35). The targeted exogenous *mGFP* gene was described in detail previously (10). The complete DNA sequence of the Cas9/intron-sgRNA gene targeting the exogenous mutant *GFP* gene is provided in Supplemental Data*,* Fig. S13.

For targeting the native phytoene desaturase 3 gene (*PDS3*) of tobacco, two sites (*NtPDS3-1* and *NtPDS3-2*) near the 5' end of the *PDS3* gene were chosen (Supplemental Data*,* Fig. S6). Target sequence in both genomic DNA and in the sgRNA gene are displayed in Supplemental Data*,* Table S1 and the complete DNA sequences of the Cas9/intron-sgRNA genes targeting the *PDS3-1* and *PDS3-2* genes are provided in Supplemental Data*,* Fig. S14 and Fig. S15.

**Construction of Cas9/intron-sgRNA genes for targeting endogenous and exogenous genes in *Chlamydomonas reinhardtii***

The exogenous *Ble* gene (17) was used as a selectable antibiotic resistance gene in initial experiments with Chlamydomonas. In a strategy similar to that employed with the *mGFP* gene, a 20nt target sequence (Supplemental Data*,* Table S1) was inserted downstream of the start codon of the *Ble* gene to create an out-of-reading-frame, mutant *Ble* gene. The 20nt target sequence was also embedded within the sgRNA of the Chlamydomonas-customized Cas9/intro-sgRNA gene as detailed in Supplemental Data*,* Fig. S1B. Both the Cas9/intron-sgRNA gene and the targeted exogenous mutant *Ble* gene were driven by the Chlamydomonas *PsaD* gene promoter (PsaDP) and terminated with the PsaD gene termination region (PsaDT) (Supplemental Data*,* Fig. S9). The targeted exogenous mutant *Ble* gene was placed in the same plasmid that contained the Cas9/intron-sgRNA gene. The complete DNA sequence for this expression vector is shown in Supplemental Data*,* Fig. S16.

For targeting the putative endogenous *Ku70* gene of Chlamydomonas, a site in exon 7 of this gene was chosen (Supplemental Data*,* Fig. S11). The 20nt target sequence is shown in Supplemental Data*,* Table S1. This same 20nt target was inserted downstream of the start codon of the exogenous *Ble* gene to create an out-of-reading-frame nonfunctional *Ble* gene. This strategy allowed simultaneous dual targeting of the mutant *Ble* gene and the endogenous *Ku70* gene with Cas9 and a sgRNA encoded by a single Cas9/intron-sgRNA gene. The complete DNA sequences of the mutant *Ble* gene and the Cas9/intron-sgRNA gene are shown in Supplemental Data*,* Fig. S17 and Fig. S18.

For targeting the endogenous *C. reinhardtii* peptidyl-prolyl cis-trans isomerase gene (i.e., the *FKB12* gene; Phytozome Cre13.g586300.t1.2), a site in exon 2 of the gene was selected. (Supplemental Data*,* Table S1). Accordingly, the corresponding 20nt target was inserted at the proper location in the intron-sgRNA of the Cas9 gene (following methods described in Supplemental Data*,* Fig. S2) to construct the Cas9/intron-sgRNA genes for targeting the *FKB12* gene. The DNA sequence of the Cas9/intron-sgRNA gene targeting *FKB12* gene is provided in Supplemental Data*,* Fig. S19.

For targeting the acetolactate synthase gene (*ALS* gene, Cre09.g386758.t1.1) in wall-less Chlamydomonas (cc503) and a mutant argininosuccinatelyase gene [*ARG* gene, Cre01.g021251.t1.1 - (M=1) 4.3.2.1] present in Chlamydomonas, cw 15 *arg7-8* mt+ (cc4350, referred to as *arg7-8*), a specific sgRNA target site was chosen that was in close proximity to the nucleotide targeted for change by homologous recombination or for nucleotide exchange. Exogenously supplied single stranded oligonucleotide templates were chosen for the *ARG* gene (Fig. 3) and for the *ALS* gene (Fig. 4). The corresponding 19-20nt targets were inserted into Cas9/intron-sgRNA genes as described in Supplemental Data*,* Fig. S2. Complete DNA sequences for the Cas9/intron-sgRNA genes targeting the *ARG* gene is shown in Supplemental Data*,* Fig. S20. The 80 nt ssDNA used for homologous recombination or nucleotide replacement (Fig. 4) has three 5' terminal and three 3' terminal phosphorthionate bonds and was synthesized by Eurofins (Huntsville, AL 35805 USA).

**Chlamydomonas growth and transformation**

The mutant strain of Chlamydomonas lacking an intact cell wall (CC-503) and the arginine-requiring mutant of Chlamydomonas, cw 15 *arg7-8* mt+ (CC-4350), were grown in Tris-Acetate-Phosphate (TAP) medium with or without 50 μM arginine, as appropriate. Growth conditions, transformation by electroporation, transformant recovery and DNA extraction procedures were as described previously (9). For selection of transformants resistant to sulfometuron methyl (SMM) selection, TAP medium containing 5µM final concentration of SMM was used. For selection of *FKB12* mutants, rapamycin at 20 µg mL-1 was used and for selection of zeocin-resistant cells, 10 µg mL-1 of zeocin was employed.

**Analyses of potentially mutagenized DNA**

Restriction enzyme analyses of PCR amplified regions of target genes in tobacco *(*Supplemental Data, Fig. S4 and Supplemental Data, Fig. S7) were performed as described previously (9, 10). Paired upstream and downstream primers for PCR amplification of the sgRNA target regions in the *mGFP*, *PDS3-1*, *PDS3-2*, m*Ble*, *FKB12*, *Ku70*, *ALS* and *ARG* genes are provided in Supplemental Data*,* Table S3. For DNA sequence analyses, PCR amplified fragments were cloned into pBlueScript for subsequent DNA sequencing (Eurofins, Huntsville, AL 35805 USA). For checking the presence or absence of Cas9 gene components in the Chlamydomonas genome, primer pairs for amplifying sequences in the 5' region and sequences in the 3' region of the gene were used and are shown in Supplemental Data*,* Table S4.

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The authors declare no conflict of interest.

References

1. Doudna, J.A. and Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346: 1258096.

2. Jiang, W. and Marraffini, L.A. (2015). CRISPR-Cas: New Tools for Genetic Manipulations from Bacterial Immunity Systems. Annu. Rev. Microbiol. [Epub ahead of print] PMID:26209264.

3. Weeks, D.P., Spalding, M.H. and Yang, B. (2015). Use of designer nucleases for targeted gene and genome editing in plants. *Plant Biotechnol. J.* doi: 10.1111/pbi.12448. [Epub ahead of print] PMID:26261084.

4. Sizova, I., Greiner, A., Awasthi, M., Kateriya, S. and Hegemann, P. (2013). Nuclear gene targeting in Chlamydomonas using engineered zinc-finger nucleases. Plant J. 73: 873-882.

5. Gao, H., Wang, Y., Fei, X., Wright, D.A. and Spalding, M.H. (2015) Expression activation and functional analysis of HLA3, a putative inorganic carbon transporter in Chlamydomonas reinhardtii. Plant J. 82: 1-11.

6. Gao, H., Wright, D.A., Ling, T., Wang, Y., Horken, K., Weeks, D.P. and Spalding, M.H. (2014). TALE activation of endogenous genes in Chlamydomonas reinhardtii. Algal Research 5: 52-60.

7. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A. and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems.Science 339: 819-823.

8. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. and Church, G.M. (2013). RNA-Guided Human Genome Engineering via Cas9.Science339: 823-826.

9. Jiang, W., Brueggeman, A.J., Horken, K.M., Plucinak, T.M. and Weeks, D.P. (2014). Successful transient expression of Cas9 and single guide RNA genes in Chlamydomonas reinhardtii. Eukaryot. Cell 13: 1465-1479.

10. Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B. and Weeks, D.P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice.Nucleic Acids Res. 41: e188.

11. Jiang, W., Yang, B. and Weeks, D.P. (2014). Efficient CRISPR/Cas9-mediated gene editing in Arabidopsis thaliana and inheritance of modified genes in the T2 and T3 generations.PLoS One 9: e99225.

12. Zhou, H., Liu, B., Weeks, D.P., Spalding, M.H. and Yang, B. (2014). Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res. 42: 10903-10914.

13. Vancanneyt, G., Schmidt, R., O'Connor-Sanchez, A., Willmitzer, L. and Rocha-Sosa, M. (1990). Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in Agrobacterium-mediated plant transformation. Mol. Gen. Genet*.* 220: 245-250.

14. Pang, S.Z., DeBoer, D.L., Wan, Y., Ye, G., Layton, J.G., Neher, M.K., Armstrong, C.L., Fry, J.E., Hinchee, M.A. and Fromm, M.E. (1996). An improved green fluorescent protein gene as a vital marker in plants. Plant Physiol. 112: 893-900.

15. Tam, L.W. and Lefebvre, P.A. (1993). Cloning of flagellar genes in Chlamydomonas reinhardtii by DNA insertional mutagenesis. Genetics 135: 375-384.

16. Zhang, R., Patena, W., Armbruster, U., Gang, S.S., Blum, S.R. and Jonikas, M.C. (2014). High-Throughput Genotyping of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and Endonucleolytic Cleavage of Transforming DNA. Plant Cell 26: 1398-1409.

17. Plucinak, T.M., Horken, K.M., Jiang, W., Fostvedt, J., Nguyen, S.T. and Weeks, D.P. (2015). Improved and versatile viral 2A platforms for dependable and inducible high-level expression of dicistronic nuclear genes in Chlamydomonas reinhardtii. Plant J. 82: 717-729.

18. Li, J.F., Norville, E.J., Aach, J., McCormack, M., Zhang, D., Bush, J., Church, G.M. and Sheen, J. (2013). Multiplex and homologous recombination–mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. Nature Biotechnology 31: 688-691.

19. Svitashev, S., Young, J., Schwartz, C., Gao, H., Falco, S.C. and Cigan, A.M. (2015). Targeted Mutagenesis, Precise Gene Editing and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA. Plant Physiol. pii: pp.00793. [Epub ahead of print] PMID: 26269544.

20. Kovar, J.L., Zhang, J., Funke, R.P. and Weeks, D.P. (2002). Molecular analysis of the acetolactate synthase gene of Chlamydomonas reinhardtii and development of a genetically engineered gene as a dominant selectable marker for genetic transformation. Plant J. 29: 109-117.

21. Mages, W., Heinrich, O., Treuner, G., Vlcek, D., Daubnerova, I. and Slaninova, M. (2007). Complementation of the Chlamydomonas reinhardtii arg7-8 (arg2) point mutation by recombination with a truncated nonfunctional ARG7 gene. Protist 158: 435-446.

22. Yoon, K., Cole-Strauss, A. and Kmiec, E.B. (1996). Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA.DNA oligonucleotide. Proc. Natl. Acad. Sci. USA 93: 2071–2076.

23. Cole-Strauss, A., Yoon, K., Xiang, Y., Byrne, B.C., Rice, M.C., Gryn, J. and *et al.* (1996). Correction of the mutation responsible for sickle cell anemia by an RNA-DNA oligonucleotide. Science 273: 1386–1389.

24. Kren, B.T., Bandyopadhyay, P. and Steer, C.J. (1998). In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. Nat. Med. 4: 285–290.

25. Alexeev, V. and Yoon, K. (1998). Stable and inheritable changes in genotype and phenotype of albino melanocytes induced by an RNA-DNA oligonucleotide. Nat. Biotechnol. 16: 1343–1346.

26. Zhu, T., Peterson, D.J., Tagliani, L., St, C.G., Baszczynski, C.L. and Bowen, B. (1999). Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. Proc. Natl. Acad. Sci. USA 96: 8768–8773.

27. Beetham, P.R., Kipp, P.B., Sawycky, X.L., Arntzen, C.J. and May, G.D. (1999). A tool for functional plant genomics: Chimeric RNA/DNA oligonucleotides cause in vivo gene-specific mutations. Proc. Natl. Acad. Sci. USA 96: 8774–8778.

28. Zhu, T., Mettenburg, K., Peterson, D.J., Tagliani, L. and Baszczynski, C.L. (2000). Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. Nat. Biotechnol. 18: 555–558.

29. Okuzaki, A. and Toriyama, K. (2004). Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. Plant Cell Rep. 22: 509–550.

30. Dong, C., Beetham, P., Vincent, K. and Sharp, P. (2006). Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system. Plant Cell Rep. 25: 457–465.

31. Tang X., Zheng X., Qi Y., Zhang D., Cheng Y., Tang A., Voytas D.F., Zhang Y. (2016) [A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants.](https://www.ncbi.nlm.nih.gov/pubmed/27212389) Mol Plant. 9:1088-1091

32. Shin S.E., Lim J.M., Koh H.G., Kim E.K., Kang N.K., Jeon S., Kwon S., Shin W.S., Lee B., Hwangbo K., Kim J., Ye H., Yun J.Y., Seo H., Oh H.M., Kim K.J., Kim J.S., Jeong W.J., Chang Y.K., Jeong B.R. (2016) [CRISPR/Cas9-induced knockout and knock-in mutations in Chlamydomonas reinhardtii.](https://www.ncbi.nlm.nih.gov/pubmed/27291619) Sci Rep. 6:27810. doi: 10.1038/srep27810

33. Baek K., Kim D.H., Jeong J., Sim S.J., Melis A., Kim J.S., Jin E., Bae S.. (2016) [DNA-free two-gene knockout in Chlamydomonas reinhardtii via CRISPR-Cas9 ribonucleoproteins.](https://www.ncbi.nlm.nih.gov/pubmed/27466170) Sci Rep. 6:30620. doi: 10.1038/srep30620.

34. Maiti, I.B. and Shepherd, R.J. (1998). Isolation and expression analysis of peanut chlorotic streak caulimovirus (PClSV) full-length transcript (FLt) promoter in transgenic plants. Biochem Biophys. Res. Commun. 244: 440-444.

35. Behrens, M.R., Mutlu, N., Chakraborty, S., Dumitru, R., Jiang, W.Z., Lavallee, B.J., Herman, P.L., Clemente, T.E. and Weeks, D.P. (2007). [Dicamba resistance: enlarging and preserving biotechnology-based weed management strategies.](http://www.ncbi.nlm.nih.gov/pubmed/17525337) Science 316: 1185-1188.