OpenPlant Fund – Final report

Title of Project

Site-directed integration of transgenes into the nuclear genome of algae and plants using CRISPR/Cpf1/ssDNA

Team

Gonzalo I Mendoza-Ochoa*#, Aleix Gorchs-Rovira*†, Payam Mehrshahi*, Nandor D Hegyi*, Darius Zarrabian*, Tobias Stadelmann*, Pawel Mordaka*, Stefan Grossfurthner*, Oleg Raitskin§ and Quentin Dudley§

*Department of Plant Sciences, Cambridge University, UK
§ Earlham Institute, Norwich Research Park, UK
# Primary contact (gim23@cam.ac.uk)
† Deceased on September 2019

Abstract

The general goal of this project was to develop new methods for targeted gene insertion in the microalga *Chlamydomonas reinhardtii*, a model organism and potential biotechnological host. Heterologous genes can be easily inserted into the nuclear genome of Chlamydomonas, but this happens randomly and so copy number and chromosomal location is highly variable across transformed cell lines. Having the ability to insert genes in a targeted manner, would expand the number of synthetic biology approaches that can be applied to this charismatic organism. Therefore, our first aim was to achieve this, through homologous recombination, using the CRISPR/Cpf1/ssDNA technique (2). Our following aim was to try this method in a plant (e.g. *Nicotiana benthamiana).* Producing a long and protein-encoding ssDNA was not difficult but the yield was low, and we believe low yield is one possible reason why we were not able to achieve targeted insertion of an antibiotic selection marker. We therefore explored the use other types of repair templates, including dsDNA and chemically modified dsDNA. Our preliminary results suggest that, with this CRISPR/Cpf1 method, it is possible to edit genes using dsDNA as repair template, although we were not able to determine if editing efficiency is different than when using ssDNA. We believe that both the problems and results presented here will be useful for future follow-up studies.

Introduction

CRISPR/Cas9 is a method of choice for precision genome engineering in most model organisms (3, 4). It works by delivery a two-component system inside the cell: Cas9 nuclease and either crRNA and tracrRNA or sgRNA (5, 6, 7). The sgRNA or cr/tracrRNAs guide the Cas9 nuclease and activate it on the selected genomic loci to generate double stranded DNA breaks (8, 9). Customized DNA fragments can be inserted through these breaks and integrated into the genome either through the "error-prone" NHEJ pathway or homologous repair mechanisms (10). DNA fragments can be delivered as circular plasmids, as double stranded fragments (synthetic or PCR products) or as single stranded DNA, also called single stranded deoxyoligonucleotides (ssODN) (11,12,13,14). The advantage of the ssDNA is less nonspecific integration events and more preferable homologous repair-based integration (15).

In *Chlamydomonas reinhardtii* however, very low editing efficiency had been observed using CRISPR/Cas9 (2). In the last several years new CRISPR/Cas variants were discovered and characterized, largely increasing the existing repertoire of the genomic engineering tools and the pool of available genomic targets (16, 17, 18, 19, 20, 21). Therefore, the idea was born in the Molnar lab to test the use of ssDNA (ssDNA) as template for homologous repair, together with CRISPR/Cpf1 other than CRISPR/Cas9, for gene-editing in Chlamydomonas (2).

Ferenczi and others (2) have recently developed a highly efficient (~10% of colonies) method for knock-in of short sequences (~10-20 bp) into the genome of Chlamydomonas. The method works by delivering a ribonucleoprotein (RNP) complex of the Cpf1 (also called Cas12a) nuclease and guide RNA plus a single stranded DNA (ssDNA), as template for homologous recombination. We have successfully recapitulated this technique at the Smith lab, and obtained editing efficiencies comparable to those previously reported. However, the Cpf1-RNP/ssDNA editing strategy has not been used for inserting long (e.g. 500+ bp) sequences. The aim of this project is to enhance the technique to be able to insert long sequences, encoding heterologous proteins, into specific places of the genome.

To test the feasibility of this approach, our first objective was to insert a transcriptional unit (Ble) conferring resistance to an antibiotic (Zeocin), into FKB12. In principle, such recombination event is easily detectable, as it would result in resistance to both Rapamycin (FKB12 KO) and Zeocin (Ble gene). Our second objective was to insert different antibiotic resistance genes into different landing sites and, afterwards, to try this technique for other plant systems (*e.g.* protoplasts of *Nicotiana benthamiana* and *Arabidopsis thaliana*) and compare its efficacy with alternative CRISPR techniques.

Results and discussions

Ble insertion into FKB12

We constructed plasmid pNH101 containing a Ble gene, interrupted by the RBCS2 intron 1, under expression of a strong constitutive promoter and flanked by 600-bp homology arms (L_hom-Ble-R_hom). These homology arms were designed to target the same FKB12 PAM site used in Ferenczi et al. (2017) (Figure 1). Plasmid pNH101 was then used as template to produce around 2 ug of ssDNA using a kit from Takara. Cell wall deficient UVM4 was transformed with this long ssDNA Ble cassette, or with a short ssDNA for FKB12 KO as a control (2). This trial only produced a handful of rapamycin-resistant colonies – at least 10 times less than we previously experienced – indicating that there was a problem with the procedure. Nonetheless, we analysed a few rapamycin-resistant colonies by Sanger sequencing, 2 derived from the Ble cassette transformation and 3 from the FKB12 KO. This analysis showed that 3/4 colonies were bona fide FKB12 KOs but, the Ble cassette was not detected in any colony. A later attempt by undergraduate intern, Darius Zarrabian, to transform Chlamydomonas with dsDNA of the Ble cassette, but in much larger quantities (around 100 ug), did not produce the desired clone either. Although this time, we did observe a larger number of rapamycin-resistant colonies (>100), these numbers were still at least an order of magnitude lower than obtained by Ferenczi et al (2017). Therefore, we proceeded to troubleshoot this problem, as the low number of colonies obtained could explain our inability to obtain targeted insertions of Ble.

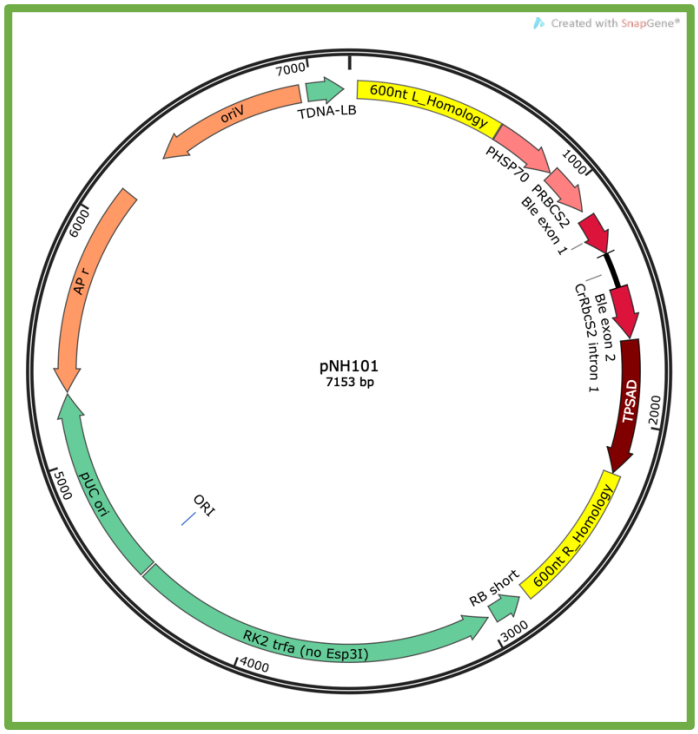

Figure 1. map of pNH101 plasmid containing Ble transcriptional unit flanked by homology arms for FKB12 targeted insertion (L_hom-Ble-R_hom).

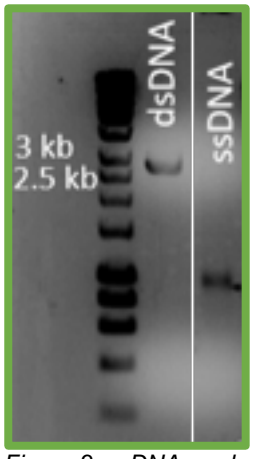

Figure 2. ssDNA product of L_hom-Ble-R_hom cassette using pNH101, as template, and the Takara ssDNA production system.

Improving and validating our Cpf1/ssDNA procedure

First, we test in vitro (2) whether our batch of Cpf1 is active. For this we compared our batch of Cpf1 with Cpf1 protein kindly provided by Aron Ferenczi (Molnar lab) and also with commercial Cpf1 from NEB (M0653T), in their capacity to cut a linear dsDNA fragment of FKB12 (Figure 3). For all three batches of Cpf1, the uncleaved DNA target was barely visible already at 15 minutes of incubation, and not observed at all at 30 minutes, confirming that our batch of Cpf1 is indeed active, at least in vitro. We also observed significant fainting of the cleaved bands with time. We speculate that this is caused by residual DNAse activity in the gRNA sample. As a precaution, from then onwards we eliminated the DNAse treatment step during the gRNA preparation, and this change in our procedure did not seem to impact the editing efficiency (data not shown).

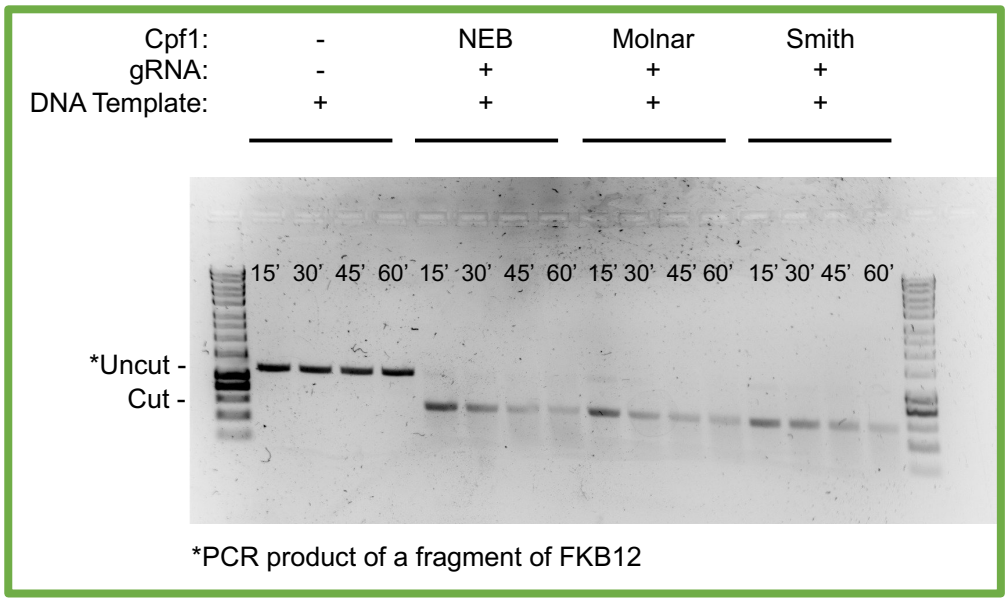

Figure 3. Cpf1 activity in vitro. In vitro analysis of different batches of Cpf1 protein samples (NEB, Molnar lab and our lab – the Smith lab). A PCR-amplified fragment of FKB12 was incubated with Cpf1 + gRNA for FKB12 KO, for 15, 30, 45 or 60 minutes, following the procedure described in ref.2. The larger band (Uncut) corresponds to the PCR amplicon of FKB12 and the smaller band (cut) corresponds to the cleaved product of this amplicon. The reaction is expected to produce only a single 'cut' band, as the two cleavage products are of very similar sizes.

To improve the transformation output, in number of rapamycin-resistant colonies, and at the same time re validate the efficiency of our procedure, we made changes to our spreading technique. Instead of spreading transformed cells into agar plates with a rod spreader – our current method – we tried spreading cells with the starch embedded method, which is the method used in Ferenczi et al. (2017), and is the recommended method for spreading cell wall deficient strains of Chlamydomonas (23). We also changed the strain of Chlamydomonas. Instead of UVM4, we transformed with the same strain used in the published protocol (2), CW15. Under these conditions we performed another FKB12 KO transformation using the standard ssDNA template. The number of rapamycin-resistant colonies we obtained were in the order of $1x10^3$ – about a 10-fold improvement from our previous trials. In this trial, we also compared the in vivo efficiency of the different batches of Cpf1 protein. We estimate that the editing efficiency of FKB12 was 12%, 11% and 7%, when using Cpf1 from the Molnar lab, NEB and our lab, respectively. These number are smaller but in the same order as the 22% efficiency previously reported (2). We then sequenced some of these colonies and the results confirmed that the rapamycin-resistant colonies we obtained with this assay were true FKB12 KOs.

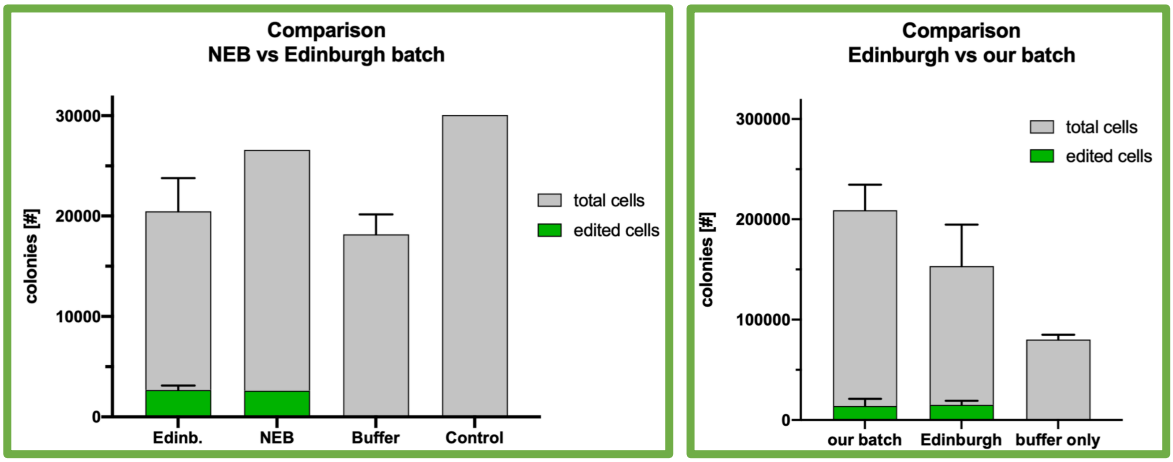

Figure 4. In vivo comparison of FKB12 KO efficiency using different batches of Cpf1 protein. Rapamycin-resistant cells are assumed to be edited. Editing efficiency is calculated as a ratio of rapamycin-resistant colonies/total colonies. Total colonies are estimated from the number of colonies that grow on plates without rapamycin. In this figure, Cpf1 protein generated by the Molnar lab is referred to as the Edinburgh batch. Buffer=cells electroporated without Cpf1, gRNA or DNA repair template, but only with Cpf1 storage buffer. Control=cells electroporated without any other additional component. Error bars represent standard technical error derived from three repeats of plate spreading.

Having improved and validated our procedure, we attempted to insert Ble into FKB12, with the approach described in the previous section of this report, but failed once again. However, we must note that our ssDNA product did not pass quality control. Although the ssDNA yield was as expected (around 2 ug) according to the nanodrop quantification, we were not able to observe a band corresponding to ssDNA in an agarose gel. So, we cannot conclusively conclude whether the approach works or not.

Testing dsDNA and PTO-modified repair templates

One of the technical challenges of this project was to produce ssDNA in large enough quantities. The 2.3 pmoles (2 ug) of ssDNA (total length, 2,770 nt; length of homology arms, 600 nt) that we generated using the Takara kit, is about 3 orders of magnitude less than the number of pmoles of short ssDNA (total length, 118 nt; length of homology arms, 47 nt average) that is added to the electroporation mix for knocking-out genes according to the published protocol (2). Although the relationship of ssDNA amount or length of homology arms with editing efficiency has not been established for this protocol, it is anticipated that more template translates into higher efficiency. As it is easier to produce high quantities of long dsDNA, than ssDNA, we investigated whether it is possible to KO FKB12 using different kinds of repair templates. The different templates that we tested are summarized in Table 1. Phosphorothioate (PTO) bonds (1 to 5 copies) are sometimes included at both ends of synthetic DNA to protect the DNA from exonuclease degradation. In this set, we included variants made of single (ss) or double strands (ds), PTO bonds in the last 4 nucleotides either at the 5' ends or in both 5' and 3' ends, and also a variant that here we refer to as ss/ds. This ss/ds variant was formed by two complementary strands that, when annealed, form a molecule with 5' ss overhangs of 24 nt on both ends.

Table 1. Summary of the different DNA repair templates tested for FKB12 KO. They all have the same sequence but only vary in the number of strands and if the ends are chemically modified (PTO) or not. ss=single stranded, ds=double stranded, NA=not applicable.

Transformation number	Template	Cpf1+gRNA	PTO in the 5' ends	PTO in the 3' ends
1	ss	+	-	-
2	ss	+	+	+
3	ds	+	-	-
4	ds	+	+	+
5	ds	+	+	-
6	ss/ds	+	-	-
7	-	+	NA	NA
8	-	-	NA	NA

Interestingly, cells transformed with either of the six different repair templates produced rapamycin-resistant colonies. In contrast, no colonies were observed in rapamycin plates when a repair template was not added to the transformations (number 7 and 8). Although from this experiment it was not possible to compare efficiencies quantitatively, due to lack repeats, the results do suggest that repair templates other than ssDNA can be used in this technique – including chemically unmodified dsDNA. With these results in mind, in the future we will try again to insert the Ble cassette in FKB12 using large quantities of dsDNA template.

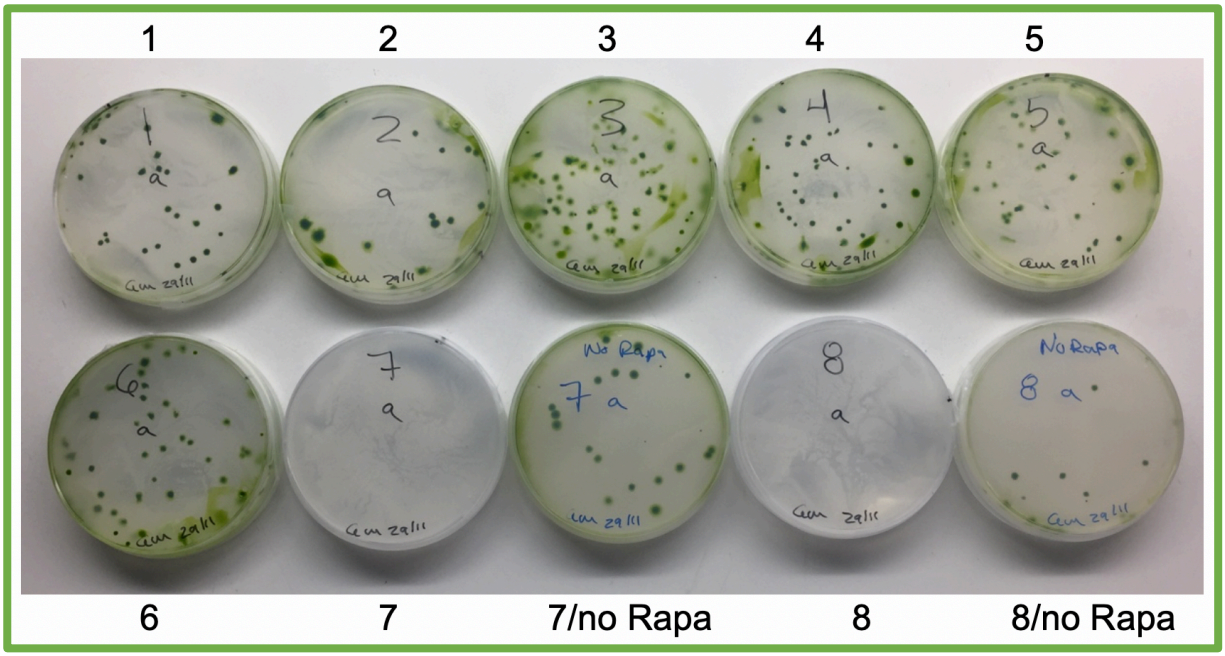

Figure 5. FKB12 KO using the different types of repair templates summarized in Table 1. All plates contain 10 uM of rapamycin except plates labelled "No Rapa 7 a" and "No Rapa 8 a". Transformations were serial diluted 4-fold 3 times then spread on plates. In this figure we show the set of plates containing 1.2% of the transformation, except plates "No Rapa 7 a" and "No Rapa 8 a" which contain 0.12% of the transformation.

Methods

Plasmid assembly

pNH101 plasmid was constructed through Golden Gate assembly using the Chlamydomonas MoClo kit (22). The plasmid map was generated in Snapgene.

ssDNA production

ssDNA was produced using the "Guide-it™ Long ssDNA Production System" kit from Takara, following the instructions in the manual.

CRISPR/Cpf1/ssDNA

Gene editing was carried out following the method of Ferenzci et al. (2017). Cultures of Chlamydomonas were grown at 24°C in high light (60-90 uE), except during the 24 h recovery step after electroporation – in this step cells were incubated at 30°C in low light (10-30 uE). gRNA was produced using the MEGAShortscript T7 Transcription kit from Thermo Fisher Scientific; and purified through a lithium chloride precipitation. All oligonucleotides were synthesized and purified by desalting by Sigma Aldrich.

Starch spreading

CW15 Chlamydomonas cells were spread on agar plates following the method described by Takemura et al. (2019).

Testing the in vitro activity of Cpf1 protein

The in vitro activity of Cpf1 protein samples was tested following the method described by Ferenczi et al. (2017). The cleavage template was generated by a PCR amplification of DNA from Chlamydomonas using primers with sequences ATGCACATGAAGAGACGTCGT and GCCAGTACTGCCGATTACCATA. Time course reactions were stopped by incubating aliquots of the samples at 65°C for 10 minutes. The DNA products were column-purified, using the Monarch® PCR & DNA Cleanup Kit (5 µg), and ran on an agarose gel for analysis.

Author contributions

GIMO conceived of the project. GIMO, OR and QD wrote the application for funding and part of that text was including in this report. GIMO and AGR recapitulated the CRISPR/Cpf1/ssDNA technique in the Smith lab. GIMO and AGR supervised summer intern NH. NH constructed plasmid pNH101 and generated the ssDNA shown in figure 2. GIMO supervised DZ. DZ attempted to insert Ble in FKB12 using ssDNA and also carried out the initial experiment of comparing different types of repair templates. GIMO, PMor and SG performed the experiment show in Figure 3. PMor recapitulated the starch spreading method in the Smith lab. PMer and GIMO supervised summer intern TS. TS validated our in-lab procedure for CRISPR/Cpf1/ssDNA and produced figure 4. GIMO performed the experiment shown in Table 1 and Figure 5.

Acknowledgements

We thank Aron Ferenczi and Attila Molnar for kindly providing Cpf1 protein used in Figures 3 and 4, and for their useful technical advice. We thank our lab technician Katie Sutherland for assisting in performing the experiment shown in Table 1 and Figure 5. We thank head of group Alison D Smith for her support and for kindly allowing us to perform the experiments in her laboratory. We thank OpenPlant for providing funding and for facilitating the interactions with our collaborators in the Earlham Institute.

References

1. Brodie J, Chan CX, De Clerck, O, Cock JM, Coelho SM, Gachon C, Grossma AR, Mock T, Raven JA, Smith AG, et al. (2017). The Algal Revolution. Trends Plant Sci. 1–13.

2. Ferenczi A, Pyott DE, Xipnitou A, and Molnar A. (2017). Efficient targeted DNA editing and replacement in Chlamydomonas reinhardtii using Cpf1 ribonucleoproteins and single-stranded DNA. Proc. Natl. Acad. Sci. 12, 201710597.

3. Knott GJ and Doudna JA. (2018). CRISPR-Cas guides the future of genetic engineering. Science. 2018 Aug 31;361(6405):866-869. doi: 10.1126/science.aat5011. Review. PubMed PMID: 30166482.

4. Jaganathan D, Ramasamy K, Sellamuthu G, Jayabalan S, Venkataraman G. (2018). CRISPR for Crop Improvement: An Update Review. Front Plant Sci. 2018 Jul 17;9:985. doi: 10.3389/fpls.2018.00985. eCollection 2018. Review. PubMed PMID: 30065734; PubMed Central PMCID: PMC6056666.

5. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE and Church GM. (2013). RNA-guided human genome engineering via Cas9. Science. 2013 Feb 15;339(6121):823-6. doi: 10.1126/science.1232033. Epub 2013 Jan 3. PubMed PMID:23287722; PubMed Central PMCID: PMC3712628.

6. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W,Marraffini LA and Zhang F. (2013). Multiplex genome engineering using CRISPR/Cas systems.Science. 2013 Feb 15;339(6121):819- 23. doi: 10.1126/science.1231143. Epub 2013

7. Jinek M, East A, Cheng A, Lin S, Ma E and Doudna J. (2013). RNA-programmed genome editing in human cells. Elife. 2013 Jan 29;2:e00471. doi: 10.7554/eLife.00471. PubMed

8. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A. (2012). Programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012 Aug 17;337(6096):816-21. doi: 10.1126/science.1225829. Epub 2012

9. Gasiunas G, Barrangou R, Horvath P and Siksnys V. Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. (2012). Proc Natl Acad Sci U S A. 2012 Sep 25; 109(39): E2579–E2586. Published online 2012 Sep 4. doi: 10.1073/pnas.1208507109

10. Yamamoto Y and Gerbi SA. (2018). Making ends meet: targeted integration of DNA fragments by genome editing. Chromosoma. 2018 Jul 12. doi: 10.1007/s00412-018-0677-6. [Epub ahead of print] Review. PubMed PMID: 30003320.

11. Bonawitz ND, Ainley WM, Itaya A, Chennareddy SR, Cicak T, Effinger K, Jiang K, Mall TK, Marri PR, Samuel JP, Sardesai N, Simpson M, Folkerts O, Sarria R, Webb SR, Gonzalez DO, Simmonds DH, Pareddy DR. (2018). Zinc finger nuclease-mediated targeting of multiple transgenes to an endogenous soybean genomic locus via non-homologous end joining. Plant Biotechnol J. 2018 Sep 16. doi: 10.1111/pbi.13012. [Epub ahead of print]

12. Ran Y, Patron N, Kay P, Wong D, Buchanan M, Cao YY, Sawbridge T, Davies JP, Mason J, Webb SR, Spangenberg G, Ainley WM, Walsh TA and Hayden MJ. (2018). Zinc finger nuclease-mediated precision genome editing of an endogenous gene in hexaploid bread wheat (Triticum aestivum) using a DNA repair template. Plant Biotechnol J. 2018 May 7. doi: 10.1111/pbi.12941. [Epub ahead of print] PubMed PMID: 29734518.

13. Richardson CD, Ray GJ, DeWitt MA, Curie GL and Corn JE. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol. 2016 Mar;34(3):339-44. doi: 10.1038/nbt.3481. Epub 2016 Jan 20. PubMed PMID: 26789497.

14. Quadros RM, Miura H, Harms DW, Akatsuka H, Sato T, Aida T, Redder R, Richardson GP, Inagaki Y, Sakai D, Buckley SM, Seshacharyulu P, Batra SK, Behlke MA, Zeiner SA, Jacobi AM, Izu Y, Thoreson WB, Urness LD, Mansour SL, Ohtsuka M, Gurumurthy CB. (2017). Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol. 2017 May 17;18(1):92. doi: 10.1186/s13059-017-1220-4. PubMed PMID: 28511701; PubMed Central PMCID: PMC5434640.

15. Zorin, B, Hegemann, P, and Sizova I. (2005). Nuclear-Gene Targeting by Using Single-Stranded DNA Avoids Illegitimate DNA Integration in Chlamydomonas reinhardtii. Eukaryot Cell 4, 1264–1272.

16. Bernd Zetsche, Jonathan S. Gootenberg, Omar O. Abudayyeh, Ian M. Slaymaker, Kira S. Makarova, Patrick Essletzbichler, Sara Volz, Julia Joung, John van der Oost, Aviv Regev, Eugene V. Koonin and Feng Zhang. (2015). Cpf1 is a single RNA-guided endonuclease of a Class 2 CRISPR-Cas system Cell. doi: 10.1016/j.cell.2015.09.038

17. F. Ann Ran, Le Cong, Winston X. Yan, David A. Scott, Jonathan S. Gootenberg, Andrea J. Kriz, Bernd Zetsche, Ophir Shalem, Xuebing Wu, Kira S. Makarova, Eugene Koonin, Phillip A. Sharp, Feng Zhang. In vivo genome editing using Staphylococcus aureus Cas9. Nature. 2015 Apr 9; 520(7546): 186–191. Published online 2015 Apr 1. doi: 10.1038/nature14299

18. Steinert J, Schiml S, Fauser F, Puchta H. Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus. Plant J. 2015 Dec;84(6):1295-305. doi: 10.1111/tpj.13078. PubMed PMID: 26576927

19. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, Aryee MJ, Joung JK. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. 2015 Jul 23;523(7561):481-5. doi: 10.1038/nature14592. Epub 2015 Jun 22. PubMed PMID: 26098369; PubMed Central PMCID: PMC4540238.

20. Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, Liu DR. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature. 2018 Apr 5;556(7699):57-63. doi: 10.1038/nature26155.

21. Matthew B Begemann, Benjamin N Gray, Emma January, Anna Singer, Dylan C Kelser, Yonghua He, Haijun Liu, Hongjie Guo, Alex Jordan, Thomas P Brutnell, Todd C Mockler, Mohammed Oufattole. Characterization and Validation of a Novel Group of Type V, Class 2 Nucleases for in vivo Genome Editing. doi: https://doi.org/10.1101/192799 https://www.biorxiv.org/content/early/2017/09/23/192799

22. Crozet, P., Navarro, F.J., Willmund, F., Mehrshahi, P., Bakowski, K., Lauersen, K.J., Pérez-Pérez, M.E., Auroy, P., Gorchs Rovira, A., Sauret-Gueto, S., et al. (2018). Birth of a Photosynthetic Chassis: A MoClo Toolkit Enabling Synthetic Biology in the Microalga Chlamydomonas reinhardtii. ACS Synth. Biol. 7, 2074–2086.

23. Shimogawara, K., Fujiwara, S., Grossman, A., and Usuda, H. (1998). High-Efficiency Transformation of Chlamydomonas reinhardtii by Electroporation -microalgae electroporation protocol. *125*, 1–8.

24. Takemura, T., Kobayashi, Y., Imamura, S. and Tanaka, K. (2019). Top Starch Plating Method for the Efficient Cultivation of Unicellular Red Alga Cyanidioschyzon merolae. Bio-protocol 9(4): e3172.

25. Greiner, A., Kelterborn, S., Evers, H., Kreimer, G., Sizova, I., and Hegemann, P. (2017). Targeting of Photoreceptor Genes in Chlamydomonas reinhardtii via Zinc-Finger Nucleases and CRISPR/Cas9 . Plant Cell 29, 2498–2518.