Addition of a New Nuclease Cpf1 in the Tool Box of CRISPR System

Anindya Bandyopadhyay

Summary

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has been setting the world of genome editing ablaze due to its ease of application and the precise nature of its targeting. Even though Cas9 has emerged as the most commonly used CRISPR effector for genome editing-based experiments, certain limitations in its targeting location (targets only GC rich area) and the creation of blunt double stranded DNA breaks makes its widespread and ubiquitous use difficult. Consistent research in the complex world of CRISPR effectors has given rise to a new effector called Cpf1 that can overcome the limitations that Cas9 faces. The following will elucidate what Cpf1 is and how it can be used, and show that Cpf1 can be used not only in mammalian models but also in model and crop plants.

Introduction

CRISPR was originally discovered as an immune response for bacteria against invading nucleic acid molecules of Phage-viruses. With time however, it has become one of the most elucidated, utilized, fast growing, and diverse genome editing tools in the scientific world. An array of previously discovered nucleases are being fine-tuned and employed as per the requirements of the genome editing experiments. Major components of CRISPR are the RNA guided effectors that act as nucleases to cleave the target nucleic acid. These effectors have now been classified in two broad classes. The first class includes the effectors comprised of multi-protein complexes, whereas the second class effectors have a single effector protein, of which Cas9[1] is a well characterized example. Cas9 has been established as an efficient and precise genome editing tool and has successfully been applied in diverse of organisms from bacteria to humans, as well as in unicellular algae to higher angiosperms.

Although the efficiency of Cas9 has been well characterized and established, it still has certain limitations. First, a trans-activating crRNA (tracrRNA) is required to target DNA recognition and to trigger the processing of CRISPR-RNA (Cr-RNA) in the presence of Cas9. Next, it uses NGG as a PAM (Protospacer Adjacent Motif) site, and the target sequences of Cas9 are usually GC rich, which might have advantages in some organisms but not all (**Fig. 1**). This limits the scope of Cas9 in genome editing. Moreover, enzymatic action by the nuclease Cas9 is a blunt-end cleavage; hence it yields blunt -end products, which restricts its usage for NHEJ-mediated editing processes, leading to error prone editing. These drawbacks in Cas9 mediated genome editing could be overcome by using another Class II CRISPR system: Cpf1 as the master nuclease. Since its discovery, Cpfl is considered an alternate or possibly a complementary approach to Cas9 mediated genome editing.

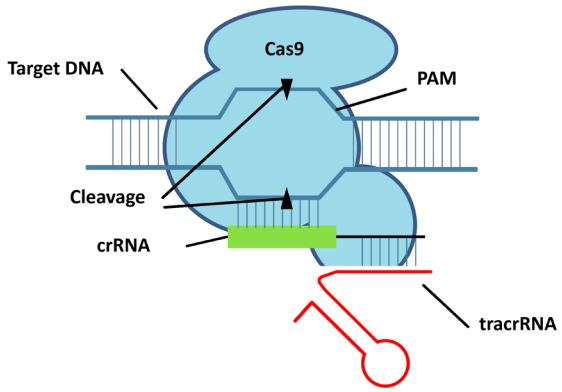


Figure 1. The structure and make up of Cas9. It relies on an NGG PAM for recognition and creates a double stranded break at the location of the target. The crRNA pairs with the target based on complementarily. Requires a tractrRNA for functioning.

Salient Features of Cpf1

Cpf1 (CRISPR from *Prevotella* and *Francisella* 1), like other CRISPR nucleases, functions as a defense molecule in the genome of a number of bacteria, with an ability to defend against plasmids or viral nucleic acid particles using CRISPR[2]. As stated earlier, Cpf1 has certain major advantages as a functional genome editing tool in molecular biology. The first major advantage of Cpf1 over Cas9 is it does not require a tracr-RNA to recognize the target DNA molecule. Secondly, it uses a T-rich PAM site (TTTN/TTN), and it mainly targets the AT rich sequences in the genome (**Fig 2**). Next, the Cpf1-mediated nuclease activity provides a staggered cut to the DNA, leaving a 4- to

Cpf1 Properties:

- **AT rich PAM, thus can target AT rich regions of the genome.**
- **Cleavage is away from the recognition/ seed sequence and PAM.**
- **Staggered cleavage resulting in over-hangs.**
- **Does not require an extra tracrRNA.**
- **Can process its crRNA by itself.**

5-nucleotide sticky overhang[2]. Another advantage of Cpf1 is that it is reported to cleave the DNA distal to the PAM region, compared to Cas9 which cleaves near the PAM and which may not support repeated cleavage by Cas9 in the already cleaved and mutated section of the genome. Whereas with Cpf1, the targets can be repeatedly cleaved, as the target recognition region is not disrupted as such; hence Cpfl promotes repeated cleaving of the target region, thus increasing the efficiency of editing, which can also be advantageous in HDR mediated genome editing.

Application and Validation of Cpf1

The first successful attempt to utilize Cpf1 as a genome editing tool was performed on the *DNMT1* gene where it successfully cleaved the PCR amplicon of the gene *in vitro*. This procedure was again tested on human embryonic kidney cells; two Cpf1 nucleases, AsCpf1 LbCpf1, efficiently produced nucleolytic cleavage resulting in insertions and/or deletions. In the same experiment, staggered cleavage by the Cpf1 molecules was also validated using Sanger sequencing. Further knockout mice were generated for *transformation related protein 53* (*Trp53*). The two Cpf1 molecules exhibited more precise genome editing[2], with high efficiency and minimum off target effects in the mice.

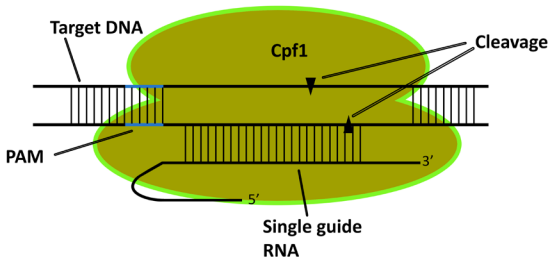


Figure 2. The structure and make up of Cpf1. It relies on a TTN PAM for recognition and creates a double stranded break at the location of the target. The crRNA pairs with the target based on complementarily. Does not require a tracrRNA.

Application of Cpf1 in Plants

After successful validation of the nuclease in the mammalian cell system, it was subsequently tested in the plant genome. To test whether Cpf1 has any nucleolytic effect on the plant genome, two marker genes were selected (*OsPDS* and *OsBEL*). Efficient Cpf1-mediated mutations were reported in transgenic rice, which established the activity and efficiency of Cpf1 in generating stable and inheritable targeted mutations in plants[3]. Furthermore, the Cpf1 molecule has been used as a transcriptional repressor molecule in *Arabidopsis* by deactivating its nuclease domain and fusing it to three copies of the SRDX transcriptional repressor. The transcriptional repression was carried into the T1 generation[4]. Further, FnCpf1 (from *Francisella novicida*), which uses an even shorter PAM sequence (TTN), was efficiently used to induce targeted mutagenesis in two genes of tobacco, *NtPDS* and *STENOFOLIA* ortholog (*NtSTF1*), and two genes in rice, *OsDL* (Drooping leaf) an *OsALS* (Acetolactone synthase).

The FnCpf1-mediated efficiency was as high as 28.2% and 47.2% for targeted mutagenesis in tobacco and rice plants[5], respectively. Very recently, a multiplex genome editing approach has also been reported using CRISPR-Cpf1. Four genes from the receptor-like kinase gene family (*OsRLKs*) and four members of the CYP81A gene family (*OsBEL*) were targeted by two Cpf1 nucleases (LbCpf1 and FnCpf1). There was not, however, any significant increase in the efficiency of cleavage by the multiplexing crRNA array. However no off-target effects were reported in this experiment, indicating higher fidelity in editing the Cpf1-mediated multiplex genome editing in plants[6].

Targeting Stomatal Developmental Rice Gene

At the International Rice Research Institute (IRRI), we have successfully managed to test the efficiency of the LbCpf1 system using a rice gene as a marker. The rice gene *OsEPFL9* (Epidermal Patterning factor) was used to test the cleavage efficiency of Cpf1. This gene is responsible for stomatal patterning and density in the development of a leaf. Knocking

down this gene would theoretically reduce the number of stomata in an adult plant. To validate this hypothesis, exon 1 of the *OsEPFL9* gene was targeted by both Cas9 and Cpf1, respectively, to evaluate cleavage efficiencies[7] (**Fig 3**).

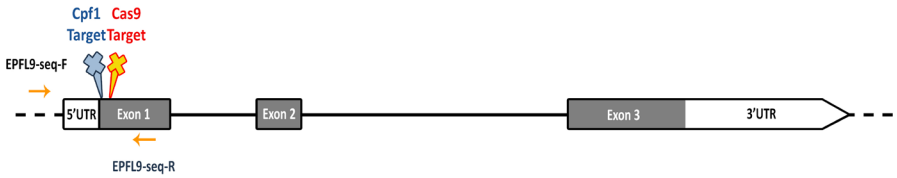


Figure 3. The location of the Cas9 and the Cpf1 targets selected in the Exon 1 of OsEPFL9 gene.

After the targets were cloned into the appropriate guide RNA scaffolds, constructs were transformed into rice immature embryos. The transformed plants were recovered and grown following transformation protocols. Initial screening of T0 plants for the nuclease transgene and the probable site of mutation were performed using the Surveyor assay (**Fig 4**). Subsequent sequencing analysis revealed bases were deleted in exon 1 of the gene. The deletions ranged from that of 5 bp to 63 bp in different events, resulting in truncated transcripts. To confirm the transmission of the mutation across generations, the plants were carried forward to the T1 generation. Southern blot analysis confirmed the absence of the transgene harboring the Cas9 insert, while subsequent analysis by Surveyor assay and sequencing confirmed the presence of the mutation; i.e., deletion of the bases were in the same pattern as that of the previous T0 generation. The editing efficiency of Cpf1 was higher compared to that of Cas9.

Phenotypically, the knockout plants obtained from both Cas9 and Cpf1 showed the predicted phenotype. There was a 6- to 8-fold reduction in the stomatal density of the leaves, indicating that editing was successful (**Fig. 5**). In addition, the analysis also revealed that the mutations were stable and heritable, and the plants were nuclease free and homozygous for the said mutation. With these reports of a more precise and error-free genome editing technique, the use of Cpf1 as a genome editing tool comprises a viable option for genome editing in plants and increases the horizon of CRISPR mediated gene manipulation.

Acknowledgement: The author would like to thank Dr. Abhishek Anand, Shamik Mujumdar, Dr. Xiaojia Yin, Dr. Paul Quick from C4 rice center of IRRI and Dr. Julie E. Gray from University of Sheffield, UK.

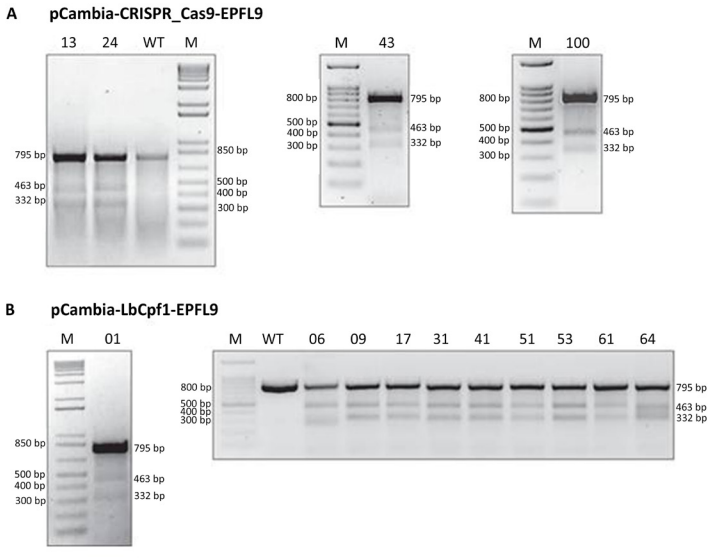
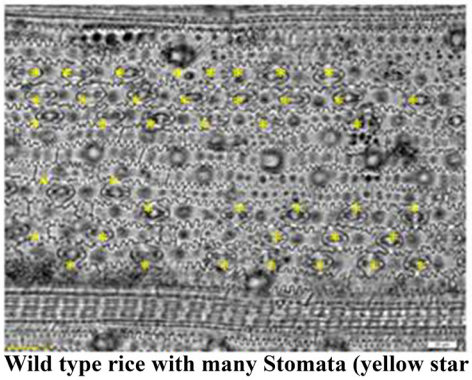
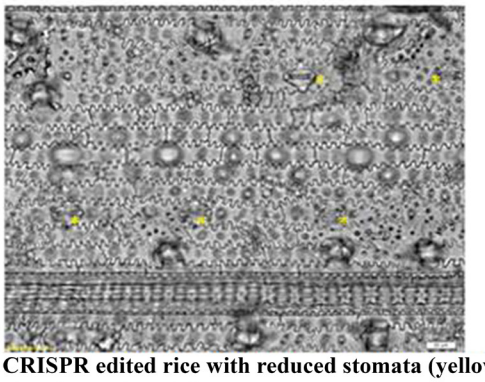


Figure 4. Surveyor Assay conducted on the TO samples of the CRISPR edited plants. The presence of multiple bands shows the presence of the mutation as the nature of the Surveyor enzyme is to cleave a double stranded DNA after encountering base pair mismatch.

Figure 5. The difference in the number of stomata between wild type and CRISPR edited rice plants. 8 fold reduction reported.



Wild type rice with many Stomata (yellow star shows stomata)



CRISPR edited rice with reduced stomata (yellow star shows stomata)

References

1. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier EA (2012) Programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 17;337(6096):816-21
2. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163(3):759-771
3. Xu R, Qin R, Li H, Li D, Li L, Wei P, Yang J (2016) Generation of targeted mutant rice using a CRISPR□Cpf1 system. *Plant Biotechnol J.* 2017 15(6):713-717
4. Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER (2017) A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nature Plants* 3:17018.
5. Endo A, Masafumi M, Kaya H, Toki S (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from Francisella novicida. *Sci. Rep* 6: 38169.
6. Wang M, Mao Y, Lu Y, Tao X, Zhu JK (2017) Multiplex gene editing in rice using the CRISPR-Cpf1 System. *Molecular Plant* DOI: http://dx.doi.org/10.1016/j.molp.2017.03.001
7. Yin X, Biswal AK, Dionora J, Perdigon KM, Balahadia CP, Mazumdar S, Chater C, Lin HC, Coe RA, Kretzschmar T, Gray JE, Quick WP, Bandyopadhyay A (2017) CRISPR-Cas9 and CRISPR-Cpf1 mediated targeting of a stomatal developmental gene EPFL9 in rice. *Plant Cell Reports* 36(5):745-57.

Dr. Anindya Bandyopadhyay
Scientist-II (Molecular Biology)
International Rice Research Institute,
DAPO 7777, Metro Manila, Philippines
a.bandyopadhyay@irri.org