SCIENCE CHINA
Life Sciences

•RESEARCH HIGHLIGHT•

https://doi.org/10.1007/s11427-020-1655-9

Application of CRISPR/Cas-mediated base editing for directed protein evolution in plants

Niklas Capdeville, Patrick Schindele & Holger Puchta*

Botanical Institute, Molecular Biology and Biochemistry, Karlsruhe Institute of Technology, Karlsruhe 76131, Germany

Received February 6, 2020; accepted February 21, 2020; published online March 5, 2020

Citation: Capdeville, N., Schindele, P., and Puchta, H. (2020). Application of CRISPR/Cas-mediated base editing for directed protein evolution in plants. Sci China Life Sci 63, https://doi.org/10.1007/s11427-020-1655-9

Breeding for improved cultivars of existing crops depends mainly on the available amount of genetic variability in yield or quality related genes. For thousands of years, humans have relied on naturally occurring mutations to recombine and select improved crops. Using chemical or physical mutagens, the amount and range of mutations could be increased, accelerating the development of new traits. Nevertheless, naturally occurring or randomly induced mutations lead to unpredictable breeding outcomes and therefore necessitate tedious rounds of backcrossing to eventually produce useful phenotypes (Pacher and Puchta, 2017).

Consequently, many methods for the directed evolution of genes and their products through locus-specific DNA diversification have been developed. Many of them, such as error-prone PCR, site saturation mutagenesis or chimera genesis are based on the generation of a sequence library and subsequent screening for improved protein variants either *in vitro* or *in vivo*. However, low transformation rates are the main limiting factor for these methods (Engqvist and Rabe, 2019). The application of gene editing methods that use programmable nucleases enables site-specific *in vivo* mutagenesis and therefore harbors the potential to be used for directed evolution. At present, targeted mutagenesis on a large scale was only enabled by the characterization of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated)-system due to its simplicity, versatility and high precision compared to previously used site-specific nucleases such as zinc-finger nucleases or transcription activator-like effector nucleases (Schindele et al., 2019; Zhang et al., 2018). However, the characterization of alternative CRISPR/Cas-systems with different target requirements has now further increased the amount of targetable sequences, covering almost any site within the genome (Shmakov et al., 2015). Recently, a CRISPR/Cas-based system for directed evolution (CRISPR/Cas-directed evolution, CDE) in plants was developed. CDE employs a Cas9 nuclease together with a sgRNA library to induce double-strand breaks (DSB) at all possible sites within the coding sequence of a specific target gene. Subsequent regeneration of the plants under selective pressure accelerates evolution and therefore this approach can be used to engineer crops that are resistant to biotic and abiotic stress (Butt et al., 2019).

CRISPR/Cas-induced DSBs are mainly repaired via the error-prone non-homologous end-joining (NHEJ) in somatic plant cells (Puchta, 2005). Consequently, the repair predominantly results in insertions or deletions (indel), which often results in frame shifts leading to non-functional proteins or premature translation-termination signals. Since many agronomical traits only vary in a few base changes within a gene, tools are required that can induce nucleotide changes without indel-formation. By providing a donor sequence composed of the desired changes flanked by sequences homologous to the target site, gene targeting can be used to induce single nucleotide substitutions, replace a longer sequence or to insert a new sequence at a specific site

*Corresponding author (email: holger.puchta@kit.edu)

via homology directed repair (HDR) of induced DSBs. However, although several approaches have been conducted to enhance gene targeting efficiency, it remains low in higher plants (Huang and Puchta, 2019). Furthermore, for each gene variant to be produced, a new donor sequence needs to be designed making gene targeting rather unsuitable for directed evolution purposes.

In 2016, a new technology was developed that enables DSB and donor template free base editing from C to T at a specific target site. These base substitutions are mediated by a cytidine deaminase that converts cytidine into uridine by removing an amino group. Subsequent DNA repair of the U-G mismatch results in a U-A base pair that is further resolved to a stable T-A base pair. The site-specificity of cytosine base editors (CBEs) is ensured by either a catalytically inactive Cas9 (dead Cas9, dCas9) or a Cas9 nickase that is fused to the deaminase (Komor et al., 2016). To overcome low editing efficiencies due to uracil DNA glycosylase (UDG) mediated uracil removal, a UDG inhibitor (UGI) was added to the protein complex. Several CBE variants with either different deaminase domains, engineered Cas9-proteins that recognize variable PAMs or other Cas nucleases such as Cas12a, which enables targeting of T-rich sequences, were developed and further increased base editing efficiency and the range of applications (Mishra et al., 2020). By fusing the human deaminase APOBEC3A to a Cas9 nickase, Zong et al. obtained high C-to-T conversion efficiency within an enlarged 17 nt window, thus making base editing suitable for crop improvement through targeted mutagenesis (Zong et al., 2018). The versatility of base editing was further increased with the development of adenine base editors (ABEs). Since no naturally occurring adenine DNA deaminases are known, Gaudelli et al. established the *Escherichia coli* tRNA adenine deaminase TadA to operate on DNA (*ec*TadA7.10) (Gaudelli et al., 2017). Deamination of adenosine converts it to inosine, which is recognized as guanosine by polymerases and therefore leads to an A-T to G-C conversion. Like CBEs, several ABE variants were developed. Based on ABE7.10, the most efficient ABE in human cells, the group of Caixia Gao designed an adenine base-editing system optimized for use in plants. Thereby, the enlarged editing window ranges from positions 4 to 8 of the protospacer (Li et al., 2018a).

Recently, the same group enabled simultaneous C to T and A to G conversions at a specific target site in plants by combining a cytidine deaminase with an adenine deaminase, therefore creating saturated targeted endogenous mutagenesis editors (STEMEs). These STEMEs consist of the cytidine deaminase domain of A3A-PBE (APOBEC3A) and the adenine deaminase domain of PABE-7 (*ec*TadA-*ec*TadA7.10) that are fused to a Cas9 (D10A) nickase and UGI. As with other base editing systems, Cas9 (D10A) mediated nicking of the non-edited strand stimulates cellular mismatch repair whereby the deaminated strand is used as template; the UGI ensures high C to T conversion efficiency (Figure 1). Several versions of the fusion construct were tested in rice protoplasts, of which STEME-1 (APOBEC3A-*ec*TadA-*ec*TadA7.10-nCas9-UGI) achieved the highest efficiency with up to 15.10% of the products showing simultaneous C to T and A to G conversions. On average, the efficiency of the dual base editor was about fourfold higher compared to individually codelivered base editors. Like for A3A-PBE and PABE-7, the editing windows for C to T and A to G conversion ranged from C_1 to C_{17} and from A_4 to A_8 of the protospacer, respectively (counting the PAM-distal end as position 1) (Li et al., 2020). With STEME-NG, a STEME-1 variant with an engineered Cas9-NG nickase that recognizes NGD (D=A, T or G) as PAM was established that increases the range of targetable sequences. By targeting the coding and non-coding strand of the acetyl-coenzyme A carboxylase in rice protoplasts with combinations of sgRNAs covering the majority of the gene, this system was successfully used to achieve a mutagenesis saturation of 73.21% of all targeted amino acids. In rice plants, a lower mutagenesis saturation, as well as unexpected transversion substitutions and indel-formation, could be observed, suggesting the need for further optimization of this system (Li et al., 2020).

CRISPR/Cas-based directed evolution is a growing field in which base editing plays a key role and in the last years, several groups have developed methods to improve BEs (Kuang et al., 2020; Liu et al., 2020). By using a catalytically inactive version of Cas12a, which requires a TTTV PAM across all orthologues, the set of target-sequences for CBEs was extended by T-rich sequences (Li et al., 2018b). The small editing window of ABEs could be overcome with a *Staphylococcus aureus* Cas9 (*Sa*Cas9)-based ABE, which showed high editing efficiency within an enlarged editing window ranging from position A_6 to A_{14} of the protospacer with no detectable off-target effects in rice (Hua et al., 2018). Editing efficiency of both CBEs and ABEs could be enhanced using codon-optimized bipartite nuclear localization signals (bpNLS) in mammalian cells (Koblan et al., 2018).

However, other CRISPR/Cas-based tools for directed DNA diversification must not be forgotten. EvolvR, for example, relies on an error-prone polymerase I, PolI3M, which is linked to a Cas9 nickase. Following site-specific nick-induction, PolI3M can bind to the nicked region and use it as a template. Thereby the old strand is displaced, cleaved and ultimately replaced with the newly synthesized sequence containing errors. The nick is then repaired by the cells' own machinery (Halperin et al., 2018). This system shows high efficiencies in prokaryotes and was successfully used to identify novel antibiotic resistance conferring mutations in *E. coli*. However, its use in eukaryotic cells still remains to be demonstrated.

Taken together, CRISPR/Cas-based directed evolution in

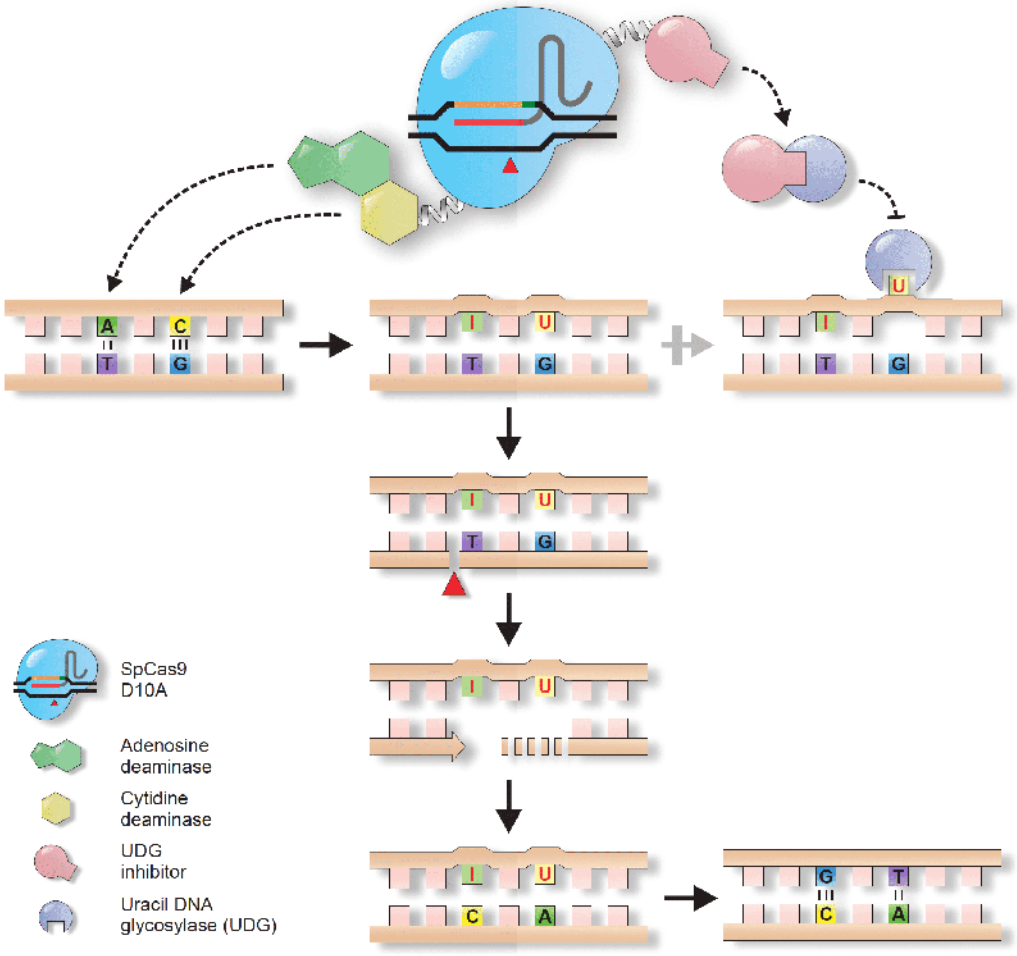

Figure 1 Schematic illustration of STEME-mediated dual base editing. STEME binding to the target DNA activates adenosine and cytidine deamination by the respective deaminases, resulting in their conversion to inosine and uracil, respectively. An UDG inhibitor prevents base excision repair while CRISPR/Cas9(D10A)-induced nicking of the non-targeted strand promotes mismatch repair. Mismatch repair using the deaminated strand as template results in the stable substitution of the edited base pairs.

plants is a steadily growing field and versatile and efficient tools are already available (Butt et al., 2020; Chen et al., 2019). Nevertheless, further research is needed to enable advances in basic research, as well as to address important agricultural issues. In particular, improved transformation methods are required to extend the scope of molecular breeding to other important crops.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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