Brief communication

Engineering CRISPR/LbCas12a for highly efficient, temperature-tolerant plant gene editing

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The discovery of the CRISPR/Cas9 system was a milestone for plant biotechnology enabling targeted mutagenesis with an unprecedented simplicity and accuracy. An abundant G-rich protospacer-adjacent motif (PAM) constitutes the sole requirement for Cas9 cleavage. Nevertheless, targeting of T-rich sites in the genome such as non-coding sequences remains challenging. The characterization of the CRISPR/Cas12a system provided a tool to edit the genome such as non-coding sequences. However, the system shows an even higher temperature sensitivity. Only recently was a Cas12a variant identified which show solid genome editing activity in mammalian systems. For plants, Cas12a from Lachnospiraceae bacterium ND2006 (LbCas12a) is the most widely used orthologue for targeted mutagenesis. However, a certain divergence in editing efficiency was observed between plant species and targets, mainly due to the reduced activity of the enzyme at lower temperatures, which are mandatory for plant cultivation (Bernabé-Orts et al., 2019; Lee et al., 2019; Malzahn et al., 2019). The Cas12a from Acidaminococcus spec. BV3L6 (AsCas12a) shows an even higher temperature sensitivity. Only recently was a temperature-insensitive variant of AsCas12a established, enhanced AsCas12a (enAsCas12a), showing on average a twofold increase in activity at lower temperatures compared with wild-type AsCas12a in human cells (Kleinstiver et al., 2019).

We were interested in obtaining temperature-tolerant LbCas12a variants for application in plants. Since enAsCas12a was still outperformed by wild-type LbCas12a in in vitro cleavage reactions at 32 and 25 °C (Kleinstiver et al., 2019), application of enAsCas12a itself in plants did not seem promising. Therefore, we set out to use the knowledge obtained with AsCas12a to construct novel LbCas12a variants. Two variants were of special interest to us: The enAsCas12a variant variant performed best and harbours in total three amino acid substitutions E174R/S542R/K548R and the variant comprising the single substitution E174R that was identified as the main requirement for the enhanced efficiency at lower temperature (Kleinstiver et al., 2019). We performed a protein sequence alignment with different Cas12a orthologues showing an especially high conservation of E174 and K548. We identified the respective amino acids in LbCas12a: E174, S542 and K548 in AsCas12a correspond to D156, G532 and K538 in LbCas12a, respectively. Two LbCas12a variants were engineered, an enAsCas12a-analogue LbCas12a (enLbCas12a) harbouring three mutations, D156R/G532R/K538R and a temperature-tolerant LbCas12a (ttLbCas12a) that harbours the single mutation D156R. Using a codon-optimized Cas12a ORF (Wolter and Puchta, 2019), the variants were cloned under control of the constitutive Ubiquitin4-2 promoter from parsley (Fauzer et al., 2014) along with ribozyme-flanked crRNAs expressed by the Arabidopsis U6-26 promoter. We selected five genomic targets and transformed the respective crRNAs with all three Cas12a variants as T-DNAs into Arabidopsis thaliana using the floral dip method (Figure 1a). To exclude expression variations due to position effects, 10 different transgenic T1 plants were grown for two weeks at 22 °C and 28 °C, respectively, for each construct. After gDNA extraction, CAPS analysis was performed to evaluate the cleavage efficiencies. Gene editing was detected at all five targets for each of the variants. As an example, analysis of target two is shown in Figure 1b. The analysis revealed comparable low to medium activity levels for LbCas12a and enLbCas12a at 22 °C. Cas12a showed the highest mean efficiencies at 22 °C and 28 °C, respectively, for each construct. When the plants were grown at 28 °C, an increase in activity could be observed for LbCas12a, which is consistent with previous data (Malzahn et al., 2019), and also enLbCas12a. However, in most cases the cleavage efficiencies at 28 °C were still far lower than that of ttLbCas12a at 22 °C, demonstrating the superiority of the temperature-tolerance of this variant. Interestingly, incubation at 28 °C further enhanced ttLbCas12a activity. To validate the results obtained by CAPS analysis and enable quantitative comparison, TIDE analysis was conducted. The respective target sites were amplified for each individual transgenic T1 plant, subjected to Sanger sequencing, and the obtained data were subsequently processed for sequence decomposition. The calculated editing efficiencies are presented as the average values of the 10 plants in the table of Figure 1c, and the variation of the individual plants is documented as box plots in Figure 1d. ttLbCas12a showed the highest mean efficiencies at every single target (Figure 1c). On average, ttLbCas12a was between twofold to sevenfold more efficient at 22 °C compared to LbCas12a. The editing efficiencies of enLbCas12a were higher than that of the wild-type enzyme, but only for some sites and still below the level of ttLbCas12a. These results differ from the data obtained for the engineered AsCas12a variants in human cells, where the analogue enAsCas12a showed higher efficiencies, indicating that the additional mutations interfere with the activity of LbCas12a. Incubation at 28 °C resulted in increased efficiencies for each variant at most of the target sites. However, LbCas12a and enLbCas12a were unable to reach levels of ttLbCas12a incubated at 22 °C for any of the edited sites (Figure 1c). If the variability of the
different plants is taken into account (Figure 1d), a few more facts became apparent. There are sites such as target one that are almost completely resistant to editing by LbCas12a and enLbCas12a. Here, also increasing sample size does not raise the editing efficiency, and even ttLbCas12a shows low editing efficiencies across all samples. However, incubation at 28 °C
helps to further increase editing efficiency with the temperature-tolerant enzyme. For all the other targets even at 22 °C, tttLbCas12a achieves almost complete editing for single plants. tttLbCas12a dramatically outperforms LbCas12a especially at 22 °C, where almost all individual plants show much higher efficiencies for every single target. For some targets, enLbCas12a outperforms LbCas12a on the population level but not for others. At 28 °C, tttLbCas12a has uniform high efficiency across all plants for all targets tested. This is also demonstrated by the fact that a more detailed analysis of the sequencing chromatograms unveiled that for two plants edited by tttLbCas12a at 28 °C clonal biallelic mutations were already present at the T1 stage. Figure 1e shows a sequencing chromatogram from a biallelic mutant with a 13-bp and a 14-bp deletion, respectively. Analysis of the mutation profiles of plants edited by enLbCas12a or tttLbCas12a at 22 °C and 28 °C otherwise revealed the presence of chimeric mutations with patterns similar to that of the wild-type enzyme.

Together, this experiment demonstrates that the two newly designed variants enLbCas12a and tttLbCas12a provide moderate and strong temperature-tolerance, making especially the latter one a perfect tool for genome editing in plants that are cultivated at lower temperatures.

So far, several attempts have been conducted to establish a robust and highly efficient CRISPR/Cas12a system for plants. These include the incorporation of self-cleaving ribozymes to mature crRNAs, the engineering of variants with altered PAM sites and the expression of Cas12a and crRNA in a single transcriptional unit (Tang et al., 2017; Xu et al., 2019; Zhong et al., 2018). Depending on the target, it might be worth combining these approaches with tttLbCas12a to achieve even further improvements in DSB induction. While the repair of these DSBs by non-homologous end joining leads to knockout edits, the novel Cas12a variants might also be helpful for achieving higher rates of gene targeting by homologous recombination (Wolter and Puchta, 2019). The novel Cas12a enzyme might also be applied as a DNA-binding protein to improve Cas12a-based applications in plants as has been observed for enAsCas12a in transcriptional regulation or base editing (Kleinistiver et al., 2019).

Author contributions

H.P. conceived the research, P.S. designed and executed the experiments. Both authors wrote the article.

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Conflict of Interest

The authors declare no conflict of interest.

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