Double-strand break induced genome engineering in plants: Past, Present, Future

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Sequence-specific nucleases can be used to induce double-strand breaks (DSBs) in plant genomes. In the past we could show that thus gene targeting (GT) by homologous recombination (HR) can be enhanced and targeted mutagenesis can be achieved by error-prone non-homologous end joining (NHEJ). Moreover, by inducing several DSBs, sequences can be deleted out of the genome or chromosome arms exchanged. In the last years CRISPR/Cas became the major tool for targeted mutagenesis. We were able to demonstrate *Streptococcus pyogenes* (Spy)Cas9 nuclease induced, NHEJ mediated, heritable targeted mutagenesis in *Arabidopsis thaliana as well as homology dependent in planta GT*. Off-target effects might be avoided using two sgRNAs and a Cas9 protein that was transformed from a nuclease to a nickase, to induce adjacent single strand breaks (SSBs) in opposite strands. This "paired nickase" strategy has a mutagenic potential at the target site comparable to the nuclease. Interestingly; sequence duplications are a prominent outcome of this approach, hinting to the possibility that the repair of adjacent SSBs is a major cause of sequence duplications during genome evolution of plants. Recently we applied the Cas9 orthologues from *Streptococcus thermophilus* (Sth1Cas9) and *Staphylococcus aureus* (SauCas9) for NHEJ-mediated targeted mutagenesis in *A. thaliana* with efficiencies at least comparable to those of SpyCas9. We were also able to show that the SauCas9 and SpyCas9 proteins only work in the presence of their species-specific single guide (sg) RNAs and show no inter-species interference. Thus, the Cas9 proteins of *S. pyogenes* and *S. aureus* should be appropriate for simultaneously addressing different sequence motifs with different enzyme activities in the same plant cell. The simultaneous use of different Cas9 orthologues will offer the opportunity to control genetic information of plant cells on more complex levels than before and will lay the basis for future synthetic approaches in plant biology.

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