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DNA-free gene editing in plants: a brief

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Abstract :

- ❖·**The conversion of bacterial CRISPR/Cas defense system into a simple and efficient tool for genome manipulations brought experimental biology into new dimensions.**
- ❖·**In plant biology and biotechnology, CRISPR/Cas gene editing became the second most important technology after plant transformation**
- ❖·**The main obstacle is that they include DNA delivery and, frequently, its subsequent integration into cellular genome. For this reason novel methods to achieve gene editing without the need of stable transformation and even without DNA delivery were developed. These new approaches include *in vitro* ribonucleoprotein complexes formulations use of virus-like particles and employment of bacterial secretory systems for Cas/gRNA delivery.**

Introduction:

- ❖·**DNA-free gene editing became a new and fast-developing trend in biological research due to its obvious advantages.**

- ❖·**Historically, genome editing became available in the 1990s upon the development of efficient plant transformation techniques. The first experiments used heterologous DNA (either relatively long fragments or oligonucleotides) for allele replacement *via* homologous recombination.**

- ❖·**The discovery of the bacterial immunity system based on RNA-guided endoDNAses opened a new possibility for precise genomic modifications.**

- **Gene editing became capable of reaching almost all research groups including those with minimal equipment and facilities.**

- **Since then CRISPR/Cas has been successfully applied on a number of species and for different purposes. In addition, almost all main plant transformation methods have been employed. Soon after a new problem emerged that needed to be addressed. The main obstacle was that the original gene editing includes DNA delivery (encoding either Cas or gRNA or both) and, frequently, its subsequent integration into the cellular genome.**

Related Works:

- ***Another Study focused its attention on vegetatively propagated plants. They used a cytidine based editor (CBE) to edit the acetolactate synthase (ALS) gene in potatoes and tomatoes via Agrobacterium infection. Point mutations in the ALS gene can lead to different types of resistance in plants. They successfully produced transgene-free (12.9%) chlorsulfuron-resistant tomatoes with a very high base editing efficacy (up to 71%). In potatoes, the transgene frequency was a little bit lower (10%). The main drawback of this method was the off-target effects, therefore there is a need for further protocol optimization.***

Methods:

1-Targeted plant species and delivery techniques used:

- **To achieve DNA-free editing, three approaches for Cas9/gRNA delivery have received main attention. The most popular one is by delivering *in vitro* assembled ribonucleoprotein. The second approach is to employ virus-mediated delivery of encoding RNA templates. The third approach is most intriguing. It is implementation of *Agrobacterium tumefaciens* Type IV secretory system for Cas9 delivery as protein into plant cells.**

- **It should be noted that in all reported cases DNA-free editing has resulted in inheritable modifications, regardless of the delivery systems used.**

2-CRISPR/Cas delivered as ribonucleoprotein complexes/nanoparticles:

- **The first and still most popular approach for achieving DNA-free editing is by delivering *in vitro* assembled Cas9-gRNA ribonucleoprotein complexes. It relies on the inherent ability of single-molecule Cas protein members (i.e. Cas9 or Cpf1) to interact with gRNA without the need of auxiliary factors.**

- **Cas9 is expressed and purified in *Escherichia coli*, whereas gRNA is either chemically synthetized or produced by *in vitro* transcription. Next, a ribonucleoprotein complex is obtained according to the transformation protocol used.**

- **A variation of this approach is the formation of more complex nanostructures rather that 'simple' ribonucleoproteins. Such complexes resemble but are not identical to virus-like particles. Nanoparticles allow not only delivery of premade protein-RNA complexes but also incorporation of mRNA and gRNA for successful expression of Cas9, followed by assembly of Cas9/gRNA in the plant cell and subsequent DNA-free editing.**

- **Ribonucleoprotein complexes and nanoparticles are delivered in plant cells mostly by particle bombardment or protoplast transformation.**

3- *CRISPR/Cas delivered as virus-like particles:*

- **Virus vectors have been successfully employed for editing the animal cell genome by delivering the CRISPR/Cas9 system both in vivo and in vitro. Viruses have also been utilized in plants for virus-induced gene silencing (VIGS) by introducing**

small interfering RNA and have become a promising tool for DNA-free plant genome editing.

- ❖·Engineering viruses with the CRISPR/Cas system for transgene-free plant genome editing proves to be a significant hurdle because of certain restrictions related to the viruses. First, mainly DNA and positive-strand RNA viruses have been used for editing the plant genome and their application is limited due to their cargo capacities.
- ❖·To achieve transgene-free plants. inserted the *Streptococcus pyogenes* Cas9 (SpCas9) and gRNAs into the SYNV genome. In order to confirm that the gRNA would be cut and expressed correctly, a (tRNA-gRNA-tRNA)-Cas9 cassette was constructed, relying on tRNA processing enzymes to cleave precisely the transcript and release an authentic gRNA.
- ❖·First, the system was tested by targeting two sites within the GFP gene of transgenic *Nicotiana benthamiana* plants. PCR-restriction digestion (PCR_RE) assays showed 77 and 91% mutagenesis frequencies respectively. The next target for mutagenesis were 3 endogenous *N. benthamiana* genes: phytoene desaturase (PDS), RNA-dependent RNA polymerase 6 (RDR6) and Suppressor of Gene Silencing 3 (SGS3). The mutagenesis frequencies remained high (40–91%).

4- *CRISPR/Cas delivery by Agrobacterium:*

- ❖·*Agrobacterium*-mediated plant transformation is the most widely used method for the delivery of the CRISPR/Cas9 cassette. The exogenous DNA carrying Cas9 and the gRNA is called T-DNA and is located on a Ti plasmid. This method has been employed successfully in more than 20 plant species. Furthermore, it is possible to use various explants as targets for transformation such as callus, leaf and floral organs of plants.
- ❖·It is also possible to eliminate transgenic plants by genetic segregation. In T_0 (the first plant generation) the transgene locus is usually heterozygous. The plants

carrying the T-DNA would segregate according to Mendelian genetics in the next generation (T_1). The transgene-free plants can therefore be selected by PCR-based methods.

- ❖·This method works only for sexually propagated plants and is rarely applicable for vegetatively propagated perennial plants because of the long time needed to reach sexual maturity. Furthermore, sexual reproduction would hinder the expression of genes responsible for many important traits in the plants because they are highly heterozygous for them.
- ❖·The first generation of plants (T_0) would include at least 3 populations: transgenic plants containing T-DNA, untransformed plants and the transiently transformed plants of interest.

Result & Conclusions:

- ❖·The main approach will rely on delivering premade ribonucleoprotein complexes or nanoparticles. Viral vectors are gaining popularity and some new opportunities might bring them into focus. Agrobacterium-mediated macromolecule delivery just emerges and, to our opinion, is the most promising and viable technology for DNA-free genome editing in plants. Of course, there is always a chance that continuous development in biological sciences might reveal a currently unknown delivery mechanism which will put all current tools in this Science.