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Research News

Genome editing without the hassles of tissue culture: Hope for editing plants recalcitrant to *in vitro* manipulations

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ABSTRACT

Genome editing has become a breakthrough technology this decade to precisely modify the genome in short time. This technology has paved way for manipulating the traits of agronomic importance with ease in many crop plants. Even though plant transformation is necessary in the first step, the final product is non-transgenic; thus, attractive for social acceptance. Lack of robust transformation procedures is a limitation to use the genome editing in some crop plants. It becomes even harder when the frequency of precise editing effected by the reagents is low. Recent reports show that genome editing can be performed bypassing the tissue culture procedures; therefore, offer hope for its use in crops that are unresponsive. We briefly discuss these breakthrough techniques here.

Keywords: Biotic/abiotic stress, CRISPR/Cas9, Genome editing, Mobile sgRNA, Oilseed crops, Plant tissue culture

Conventional breeding methods that have been successfully employed so far in generating elite oilseed crop varieties are not only tedious and time taking but also are dependent on the unique combination of genes resulting from random recombination and segregation events of meiosis. In case there are agronomically superior genotypes lacking one or two traits, improving them precisely for just the target traits through recombination breeding approaches (the backcrossing method) is quite daunting as the changes brought about are not precise, often needs a number of cycles of backcrossing, and suffers from linkage drag. Hence, there is a hunt for simple and precise breeding methods that can specifically alter a genotype for only one or two traits. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated system (CRISPR/Cas) genome editing technology has come as a boon in this regard (Zhang *et al.*, 2020). CRISPR/Cas, a microbial adaptive immune system, has been established as a powerful tool to make precise genome modifications in plants. Explaining this technology briefly, Cas is an RNA-guided endonuclease that specifically targets and cleaves target DNA after recognizing protospacer adjacent motif (PAM) sequence present in close sequence context of the region of homology between guide RNA and the target sequence. The resultant cleaved DNA is repaired by two known *in-vivo* mechanisms i.e., non-homologous end-joining (NHEJ) which is error-prone and homology-directed repair (HDR) which is expected to precise but rare and less efficient. The NHEJ lead to the random nucleotide base insertions or deletions (InDels) at cleavage site which usually result in the formation of nonfunctional protein. CRISPR/Cas has revolutionized the pace of plant biology research (Manghwar et al., 2019) and made plant genome editing a reality (Zhu *et al.*, 2020). Thus, it has become as attractive and competitive field within a span of 8 years (first editing reported in 2012, Jinek *et al.*, 2012). It is noteworthy to mention here that the pioneer work by Emanuelle Carpentier and Jennifer Doudna, who sitting across the Atlantic did their basic work in establishing that CRISPR/Cas9 had evolved as a defense mechanism in bacterial system to fight the viral invasion, could be used for precise genome editing, earned them the Nobel prize in chemistry in 2020.

CRISPR/Cas9 (a Cas gene taken usually from *Streptococcus pyo-genes* has been adopted successfully in many crops for improving the traits of agronomic importance including disease resistance, quality improvement, plant architecture modification, reducing the duration, etc (Zhu *et al*., 2020). Even though this technology has made inroads into crop improvement programmes including that of oilseed crops, the limitation in adopting this technology routinely across crop plants is its dependence on protocols for obtaining the transgenic plants carrying required components (Cas9 protein and the single guide RNA) that bring about genome editing in the plant.

Delivering the machinery needed for genome editing, such as the guide RNA and Cas9, in the form of expressing cassettes into the plant cell is a critical step in realizing genome edited plants. Thus, plant transformation is a crucial and necessary step in adopting genome editing technology in any crop. Currently, two methods are widely used for DNA transfer in plants i.e., Agrobacterium-mediated and particle bombardment mediated. The former mode is often chosen owing to its simplicity and reliable transgene expression. However, in the first place, this approach demands the availability of an in vitro regeneration and transformation protocol. Besides, Agrobacterium-mediated plant genome alterations are labor-intensive, time consuming and costlier since this method is associated with aseptic *in-vitro* plant tissue culture (PTC). *In-vitro* genetic transformation and regeneration of plantlets are the major bottlenecks in PTC in

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many crop species (Altpeter *et al*., 2016). In fact, in most of the oilseed crops, high frequency transformation protocols are not available. Bypassing the PTC will certainly enhance the applications of plant genome editing technologies. Hence, innovating novel and alternative approaches that skip PTC are the need of the hour if genome editing has to become a reality on many crops including oilseed crops.

Overcoming tissue culture bottleneck: Several attempts have been made to overcome the tissue culture step for realizing transgenic plants - *in planta* transformation was a method that could successfully be employed in many crops. Floral-dip is the major *in-planta* transformation method known to date that skip PTC (Clough *et al*., 1998). This method has been followed in almost all the basic molecular biology labs worldwide. Nevertheless, this method is highly specific for Arabidopsis and related crucifers but not very successful in other major food and oilseed crops (Altpeter *et al*., 2016). Recently, two breakthrough techniques (Mahar *et al*., 2020; Ellison *et al*., 2020) have been developed that show the possible ways to generate plants without PTC procedure. Considering the new avenue these two techniques have opened in the area of plant genome editing, here we provide an overview of these techniques and also narrate the prospects of adopting them to alter oilseed crops for specific target traits. The first method uses the genes that are known to be involved in induction of meristems to induce de novo shoot production on *in situ* raised plants while the second one uses virus induced gene silencing (VIGS) vector system to transiently express the single guide RNA (sgRNA) in the transformed cells to effect genome editing.

Technique 1: De-novo induction of meristems: Molecular dissection of the development of a whole plant from the cells that are meristematic, led to understanding of the genes involved in the process. Plant developmental regulators (DRs) such as BABY BOOM (BBM), WUSCHEL (WUS), SHOOT MERISTEMLESS (STM), MONOPTEROS (MP), and ISOPENTENYL TRANSFERASE (IPT) play vital role in determination of apical meristem (Barton, 2010). This understanding led to manipulating the expression of DRs ectopically to realize increased transformation efficiency. Overexpression of the maize BABY BOOM (BBM) and WUSCHEL2 (WUS2) genes caused for high transformation frequencies in several recalcitrant maize inbred lines, sorghum immature embryos, sugarcane callus, and indica rice callus (Lowe *et al*., 2016). Ectopic expression of various combinations of these DRs induces differentiation of somatic cells into meristematic cells (Nelson-Vasilchik *et al*., 2018).

Based on this understanding, Maher *et al*. (2020) reported a first-of-its-kind plant transformation method called Fast-TrACC (Fast-Treated *Agrobacterium* CoCulture) to deliver desired plasmid DNA constructs to Nicotiana benthamiana through transient expression. They tested twelve combinations of different DRs (BBM /ipt /MPΔ /STM /Wus2 /All /BBM&ipt /BBM&Wus2 /IPT& MPΔ /STM&MPΔ /Wus2&ipt /Wus2&STM) driven with various promoters (*Zm*Ub1/*Cm*YLCV /35S /nos promoter) for successful meristem formation on in situ raised plants at the site of injury and transformation. Two combinations i.e., Wus2/STM and Wus2/ipt produced the transgenic meristems at high frequency. Encouraged with this result, they combined this observation along with genome editing process to obtain genome editing in the de-novo forming shoot meristems. To achieve this, Maher *et al*. (2020) used a technique called Agroinfection wherein the viral replicons could be introduced into the cells through Agrobacterium mediated procedure. *Agrobacterium* harboring desired viral replicons carrying expression cassettes of DRs, luciferase marker and guide RNA which targets two phytoene desaturase (PDS) homologs, was infiltrated at cut shoot apices of Cas9-expressing soil grown *N. benthamiana* plants. The premise was that DRs would increase the frequency of meristematic cells at the site of infiltration increasing the chance of transformation, and the gRNA would edit the PDS gene by using the Cas9 protein in the plant (encoded by the *cas9* transgene in the plant) leading to cells devoid of chlorophyll and thus enabling easy identification of transformed (genome edited) cells. This logic and rationale worked leading to a whopping 30% success rate of getting genome edited white shoots at the site of Agroinfection. This procedure is illustrated in Figure 1A. Thus, Maher *et al*. (2020) successfully bypassed the time-consuming PTC method and realized the genome edited tobacco plants in a short time. They extended the same protocol to other agronomically important crops like tomato, potato and grape and obtained similar results. Thus, this approach involving injecting of DRs and gene-editing reagents could be effectively used to create genome edited transgenic crops.

Technique 2. Editing with mobile single guide RNAs (sgRNA): Plant viruses have offered several tools (as transformation vectors) in plant biotechnology. The advantage is that they do not integrate into plant genome and do not pass through germline to the next generation. Ribonucleic acid (RNA) viruses are not much exploited as vectors in plant biotechnology except being used for viral-induced gene silencing (VIGS), a method used effectively in functional genomics studies to establish the role of selected genes. Thus, these vectors could be used for introducing transgene(s) for temporary expression and the effects caused by the introduced genetic elements are temporary. But the only limitation has been the 'carrying capacity of the cargo' (the size of the expression cassettes) of these RNA virus-based vectors. Hence, several researchers who wanted to use RNA vector based expression system for genome editing, successfully used RNA viruses such as tobacco mosaic virus-derived vector (TRBO), tobacco rattle

virus (TRV), pea early browning virus (PEBV) and barley yellow striate mosaic virus (BYSMV) to deliver sgRNAs against the target genes in plants that were already expressing Cas9 but that resulted in low frequencies of gene editing in somatic cells which was not inherited (Cody *et al*., 2017; Ali *et al*., 2018; Gao *et al*., 2019). Heritable gene editing could be possible if the sgRNA could get an access to the plant germline cells. To accomplish this, researchers took the advantage of plant endogenous mobile signals. Plant produce many mobile endogenous small RNAs i.e., FLOWERING LOCUS T (FT) RNA, methionine transfer RNA ($tRNA^{Met}$), isoleucine tRNA ($tRNA^{Ile}$), micro RNAs (miRNAs) and many other non-coding RNAs (ncRNAs). These small RNAs can systemically move throughout the plant including apical meristems and germlines (Notaguchi *et al*., 2015).

Based on this cumulative knowledge, in their ingenuous experiments Ellison *et al.* (2020) generated heritable mutations in *N. benthamiana* with remarkable efficacy. First, they generated SpCas9 expressing *N. benthamiana* plants and then infected them with *A. tumefaciens* carrying mobile sgRNA in tobacco rattle virus (TRV) vector. They effectively tested and confirmed heritable mutants with three mobile elements i.e., *FT* mRNA, $tRNA^{Met}$, and $tRNA^{Ile}$. They observed 90-100 % editing efficiency in somatic tissue that resulted in 65-100% heritable mutations. They succeeded in editing two essential genes i.e., phytoene desaturase (PDS) and AGAMOUS (AG) by multiplexing the sgRNAs against both the genes. The procedure followed has been illustrated in Figure 1B.

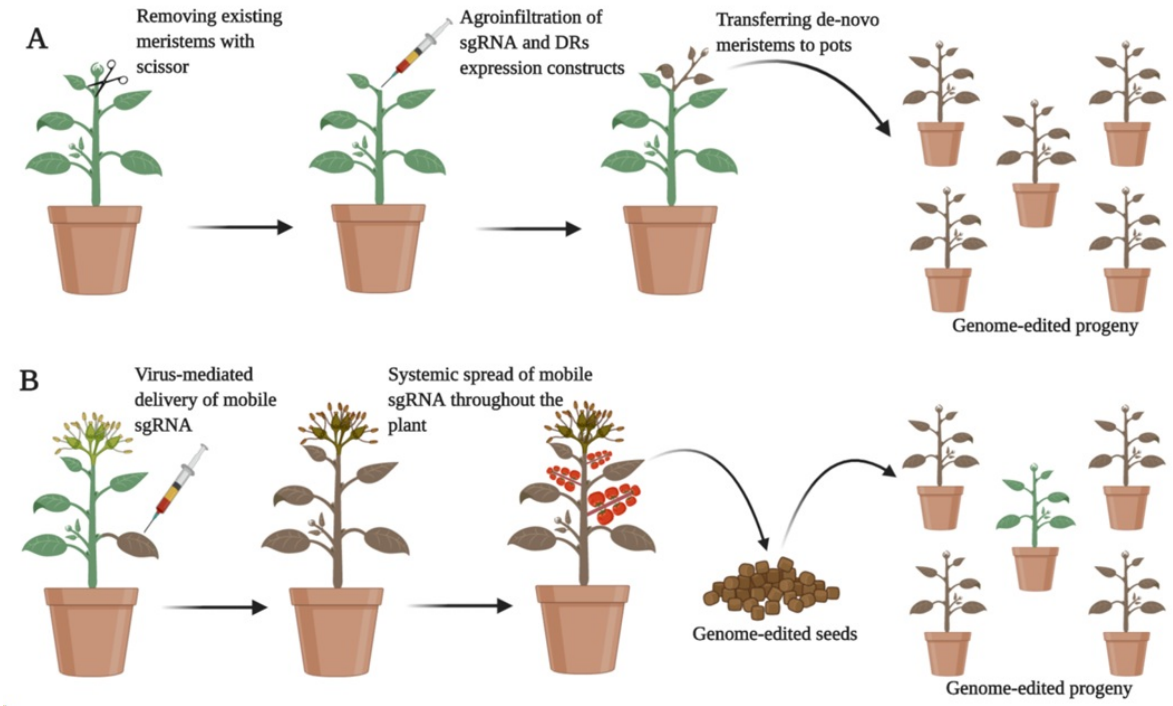

Fig. 1. Illustration of the methods for obtaining tissue culture-free genome editing in Cas9-overexpressing soil-grown plants. Genome-edited progeny shown in brown color while non-edited in green color. A) De-novo induction of meristems (method as described in Maher *et al*., 2020). The meristem is removed with scissor. The cut site is then perfused with *A. tumefaciens* cultures carrying developmental regulators (DRs), genome editing reagents (sgRNA against phytoene desaturase) and luciferase (for visual confirmation of construct delivery to plant cells). Over time, genome-edited plantlets that were chlorotic, produced from the site of delivery were transferred to the pots for next generation. B) Editing with mobile single guide RNAs (method as described in Ellison *et al.,* 2020). Viral vector comprising mobile sgRNA are infiltrated to bottom leaf by Agrobacterium infiltration. Mobile elements are known to move from leaf to shoot throughout the plant including germline cells. Seeds obtained from the infected plant screened for desired gene editing.

Caveats still to be addressed: As discussed above the two recent developments have opened up new vistas for exploiting the genome editing in crops that are difficult to regenerate through tissue culture. However, these two methods still depend on generation of constitutive *Sp*Cas9 over expressing transgenic plants which serve as the starting material and thus totally do not obviate the dependence on tissue culture. Also, in the first method (Maher *et al.*, 2020), it needs to be explored whether the developmental regulators would lead to formation of de-novo meristems in all crops and the resultant plantlets could be established in the soil to give the next generation plants. Wider application of the second technique (Ellison *et al.*, 2020) would depend on the availability of appropriate viral vectors that are effective in

the chosen crop. Between the two techniques, the second one could perhaps become more tissue culture independent if Cas9 variants that are smaller in size are developed/ identified so that and they could also be cloned within the RNA virus-based vector along with sgRNA cassette and thus the tissue culture step could be totally avoided.

There are other caveats also for application of CRISPR/Cas9 technique for precise editing of the genes of interest. These include, the requirement of PAM specificity around the region targeted for editing, random off-target mutations, selection of sgRNA, balanced in-vivo expression of sgRNA and Cas cassettes in the plant for high editing efficiency, availability of whole genome sequence of the crop for the selection of effective sgRNA through online web-tools so that homologs, if any, are not targeted for editing, and identification and optimization of suitable crop specific promoters for effective expression of Cas and sgRNA. Lower frequency of genome editing is yet another important criterion that necessitates production of a larger number of transgenic plants to achieve the manifestation of the traits as required.

Prospects of genome editing in oilseed crops: Breeding objectives in oilseed improvement programmes, besides increasing the seed and oil yield, include imparting biotic and abiotic stresses, reducing the seed losses due to shattering, improving the quality of oil produced, biofortification, reducing the duration, etc. Genome editing technology has already proven useful in tackling similar issues in other crops and thus could be an important technique in improving oil yielding crops as well. In plants, synthesis and accumulation of oils are controlled by complex gene network. Role of these networks is mostly unknown and need to be elucidated. Finding and tweaking these genes involved in desired networking pathways for oil accumulation can be exploited to increase oil production. There are several examples of successfully employing genome editing technique in oilseed crops and readers are referred to recent reviews for more information (Subedi *et al*., 2020).

In conclusion, the two techniques discussed here would be a major step towards achieving genome editing in crops that are recalcitrant to plant tissue culture methods. Further, if the other limitations of large-scale application of CRISPR/Cas9 mediated genome editing are overcome, oilseed crops could be altered precisely to meet at least a few breeding goals more rapidly and accurately.

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