



REVIEW ARTICLE

Genetic engineering and genome editing techniques in peanut plants

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ABSTRACT

Research has long been associated with human life. In the effort to make a living, many experts who have contributed to the modernization of traditional research methods by conducting various research activities. In this process, professionals, from farmers to senior researchers, have done their part by developing plants that can tolerate or resist to disease. The growing population, climate change and plant disease are having a devastating effect on food security. In particular, it is essential to increase food production by producing high yielding crops of good quality, that may ensure food security. Recently, different gene- editing technologies have been developed. These techniques have been applied in many research fields and their development has provided economic benefits to farmers. *Agrobacterium*-mediated and biolistic methods are very important techniques for transforming genetic materials in plants. Genome- editing technologies are recent and highly applied in plant research to improve genes associated with yield, disease resistance and drought resistance. For example, Zinc-finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats system (CRISPR/ Cas9) methods are now widely applied by researchers and are playing a positive role in increasing production and productivity. Of the gene- editing technology, CRISPR/ Cas9 is widely applied in plant breeding programme as it is easy to use and cost-effective. In this review, we mainly focus on peanut plant, which is an important oil-bearing allotetraploid crop. Therefore, peanut gene editing-technology could increase the oleic acid content in edible peanut oil. Thus, genome editing and gene transformation technologies are extensively explored in this review.

Introduction

Due to the development of different gene transformation and gene removal or addition methods, researchers are applying to solve plant disease problems, to increase the yield and quality of the product. Recently, gene- editing technology has proved to be a promising method. CRISPR/ Cas9 in particular is being used and is growing. Cas9 endonuclease and guide RNA can be delivered into plant cells as DNA, RNA or ribonucleoprotein (RNP) to cleave target DNA sequence (s) in the genome. However, in addition to the intended target (on-target) site, Cas9 can potentially cause off-target double strand breaks (DSBs) at genomic locations with significant sequence similarity to that of the intended target sequence (1, 2). Cause of off-target (creating unexpected mutation) may be a drawback of the technology which need improvement mechanism and further investigation to minimize related problem in the future breeding programme in plants.

Through germ plasm screening, plant breeders have identified crops having economically important traits. For example, legumes especially peanut with high oleic acid content was identified. This high oleate spontaneous mutant line (F435) contain 80% oleic acid (3). In this mutant line, two types of mutation were reported at 448base pair (bp) in the ahFAD2A gene and insertion between (441_442insA), in the ahFAD2B gene (4). Using mutant line as one of the parents helps to improve important trait in conventional breeding in the peanut genome. Since then, many high oleic acid to linoleic acid ratio (O/L) cultivars have been developed (5). Increasing oleic acid content in the peanut genome has a great effect to enhance the shelf life and has health benefits too. Reports are there on the new G451T mutation induced by CRISPR/Cas9 based gene-editing in the coding region of *Arachis hypogaea* FAD2B gene in the peanut (6). Using recently developed gene-editing technologies, peanut breeders will improve the oleic acid to linoleic acid ratio (O/L). China has produced several high oleate (HO) peanut cultivars and countries

like Argentina, Australia, Brazil, Israel, Japan and South Africa are also producing high oleate peanut products for consumption (7). Allotetraploid *Arachis hypogaea* (AABB, $2n=4x=40$) has two common diploid ($2n=2x=20$) ancestors (*Arachis dura-nensis* and *Arachis ipaensis* having (AA) and (BB) genome respectively (8-12).

The desaturase enzyme encodes ahFAD2A and ahFAD2B genes (4). This enzyme plays a great role in the conversion of oleic acid to linoleic acid. To reduce the linoleic acid and to increase oleic acid in the peanut genome, gene-editing method has a great effect on the future breeding programme. Therefore, the main focus of the current review is to assess genetic engineering and genome editing techniques in peanut.

Peanut oil contains about 12 fatty acids, of which nearly 80% is composed of oleic acid a mono unsaturated fatty acid (36-67%) and linoleic acid a poly unsaturated fatty acid (15-43%). Further, palmitic acid a saturated fatty acid contributes nearly 10%, whereas remaining 10% are constituted of up to nine other fatty acids (7, 13). The nutritional quality, flavor and shelf-life of peanut seeds and its products dependent on the presence of relative proportion of various fatty acids like saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids in its oil (14).

Peanut transformation via particle bombardment

Plant breeders are widely using different gene transformation techniques to transfer biologically important traits for various reasons. Producing high yielding crops with good quality, fungus resistance, bacterial resistance, pest resistance etc. are the main goal of plant breeding. Two genetic transformation methods (particle bombardment and *Agrobacterium* mediated transformation) have been widely applied to produce economically important trait. Genetic transformation by particle bombardment consists of the introduction of DNA in to intact cells and tissues by accelerated microparticles driven at high speed (15). The use of micro-projectile bombardment as a means of developing transformed peanut plant was first reported (16). Particle bombardment method was developed using immature peanut seeds as the source of explants. In the nucleus, exogenous DNA fragments are liberated and may be integrated in to chromosomal DNA through the process of illegitimate or homologous recombination, which depend exclusively on cellular components (17, 18). Some reports on micro-projectile bombardment of peanut tissue include: bombarding leaflets from mature embryos (19, 20), somatic embryogenesis regeneration system (21). Transformation through this technique is considered a more suitable to study gene function and transient gene expression (22). The main advantage of this technology is ability to transfer gene to a cell or tissue type and easier and quicker to use (23).

Peanut transformation via *Agrobacterium* mediated transformation

The *agrobacterium* mediated transformation has the ability to transform gene in plants (24). It can transfer DNA located on the tumour-inducing plasmid into the nucleus of the plant. Since this type of transformation is most effective and easy to use

nowadays, it is widely used in the molecular biology laboratories. Compared to particle bombardment, this technique is powerful and have a significant role in the production of transgenic plants (24).

Peanut is considered to be recalcitrant for tissue culture and genetic transformation. Many species are either resistant to this gene transformation system or show low transformation efficiency (25). However, using different explant sources such as de-embryonated cotyledons (26-28), embryo axes (9) and cotyledons (30) successful genetic transformation via *Agrobacterium*-mediated method has been reported in peanut research. Cotyledonary nodes (CNs) have relatively better regeneration ability than another explant source (31).

Several scientific reports on *Agrobacterium*-mediated transformation in peanut by using various explant sources have been reported, including a cotyledonary node (32-34), de-embryonated cotyledon (34-36), Leaflet (34), immature leaves (35, 29), decapitated half embryo (37), mesocoty (38), embryonic axes (39, 40), leaf and cotyledon (41), embryo axes (42), leaf (43), de-embryonated cotyledon (44). In addition to peanut, *Agrobacterium* mediated transformation has been widely applied in many crops. Some of these include soybean (45), barley (46), wheat (47), sorghum (48-50), maize (51, 52), rice (53).

Genetic transformation is a powerful tool for the investigation of gene function in crops, but in some systems its impact is reduced by limited transformation capability (54). With legumes *Agrobacterium tumefaciens* mediated transformation has been used for the study of gene function (55). Moreover, wider availability and cost effectiveness are also two advantages that can be achieved through this mechanism of gene transfer (56).

Zinc-finger Nucleases

Engineered zinc fingers were combined with the DNA cleavage domain of FokI, a type IIs restriction endonuclease, to form ZFNs. It targets specific DNA sequences and alter the genome by creating double strand break (DSB) at specific location. Double stranded breaks facilitates DNA-repair process in the cell by non-homologous end joining creating a loss of function mutations. In contrast, homology directed repair can create a precise mutation. One of the problems is to cleave off-target sites leading to cellular toxicity (57). Using this method large number of plants have been edited successfully. For example, rice (58), *Arabidopsis thaliana* (59-61), soybean (62), wheat (63), perennial fruit trees (64), tomato (65), tobacco (66), rapeseed (67).

Transcription Activator-like Effector Nucleases

This is restriction enzymes that can cut desired DNA sequence in the genome. TALEs protein contain DNA-binding domain which is composed of many tandems of amino acid repeats (68). It is highly specific and targets a nucleotide at the specific target site in the genome. Like ZFNs, TALENs targets the genome through protein-DNA interactions. Its off-target site recognition in the genome was reported challenge in TALEN and other gene editing technologies (ZFNs)

that need further studies to overcome unexpected gene mutation in the genome of the organism (69, 57). In many plant species including peanut, this gene editing- technology has been used (70, 71). Gene-editing by using TALEN has been reported for various crops include: peanut (72), rice (73-77), soybean (78), maize (79), Wheat (80), barley (81-83) *Arabidopsis thaliana* (84).

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR/Cas)

The CRISPR/ Cas9 system was derived from the prokaryotic type II CRISPR system which is developed from a gene editing system in bacteria. It is a recently developed technique giving the opportunity for researchers to alter an organism's genome for the intended purpose of study. This technology helps breeders to add, remove or change genetic materials at a specific location in the genome. Compared with ZFNs and TALENs the CRISPR/Cas9 system is characterized by its simplicity, efficiency and low cost and by its ability to target multiple gene (85). Gene-editing technology has a broad application prospects in peanut oleic acid improvement. In 2013, using CRISPR/ Cas9 the first gene editing in the model plants *Arabidopsis thaliana* (86) and *Nicotiana benthamina* (87) were reported. Since then, it is widely applied in many plant species for gene function analysis and its current wide usage in the breeding of crop species has promising application for the future breeding programmes.

In CRISPR/Cas9, the low specificity of sgRNA may cause off-target DNA sequence. Due to this effect an unexpected or unwanted mutation will occur in the genome of the organism. Even though cas9 nickase developed to reduce the off-target effect, still it needs

further investigation to avoid unnecessary mutation (88). Recently many crops that have been improved by CRISPR/Cas9 include: *Arabidopsis* and soybean (89), barley (90), *Brassica napus* (91), maize (92), peanut (6), rice (93), sorghum (94), soybean (95-97), wheat (98, 99). These reports provide evidence that the CRISPR/Cas9 could be used for targeted genome editing in crops.

Genome-editing approaches utilizing site-directed endonucleases capable of making chromosomal double-strand breaks (71, 119, 120) can help overcome the limitations of conventional breeding and accelerate development of improved crops. By harnessing natural cellular DNA repair process, double stranded breaks (DSBs) can be used to introduce targeted disease resistance, genome edits to improve agronomic traits such as yield, nutritional content (121, 122) (Table 1).

Conclusion

Among gene-editing technologies, ZFNs and TALENs target the genome through protein-DNA interactions, whereas genomic DNA editing by the CRISPR-Cas system is based on short RNA-DNA base pairing (69). Targeting one gene at two positions increase the overall mutation frequency and allows the recovery of homozygous mutants in one generation (123, 124). The ease of multiplexing with the CRISPR/Cas9 system is therefore an advantage for the generation of knockouts using this dual-gRNA approach. In contrast to ZFNs and TALENs, Cas9 generates blunt double strand breaks that are typically repaired by the formation of small (usually 1-bp) indels, leading to the frequency recovery of frame shift mutants when the target site is within an exon (125).

Table 1. Genome editing technology in different plant species.

Plant	Method of editing	Purpose of editing	References
Peanut	CRISPR/Cas9	Mutagenesis of <i>FAD₂</i> genes	(6)
Tomato	CRISPR/Cas9	Generate long-shelf life tomato	(100)
Potato	TALEN	Targeting sterol side chain reductase 2 (<i>SSR2</i>) gene	(101)
Arabidopsis	CRISPR/Cas9	Generate inheritable mutants of Arabidopsis	(89)
Soybean	CRISPR/Cas9	Generate inheritable mutants of soybean	(89)
Peanut	TALEN	Mutagenesis of fatty acid desaturase 2	(72)
Soybean	CRISPR/Cas9	Detect <i>Glyma06g14180</i> , <i>Glyma08g02290</i> and <i>Glyma12g37050</i> gene	(102)
Maize	CRISPR/Case 9	Evaluate specificity of CRISPR-cas9 editing	(103)
Maize	CRISPR/ Case9	Streamlines trait gene identification	(104)
Maize	TALEN	Induced targeted mutations in <i>ZmPDSZmIPK1A</i> <i>ZmIPK&ZmMRP4</i> genes	(79)
Barley	CRISPR/Cas9	Generating homozygous knockout mutants	(105)
Melon	CRISPR/Cas9	knockout phytoene desaturase gene	(106)
Barley	CRISPR/cas9	Elucidate genetic control of vitamin E composition	(107)
Rice	ZFNs	Mutagenesis of <i>SSIVa</i> gene	(58)
Wheat	TALEN	Evaluate mutation screening	(108)
Barley	CRISPR/Cas9	Creating targeted gene knockout	(109)
Wheat	CRISPR/Cas9	Examine the role of temperature to editing efficiency	(110)
Tomato	ZFNs	Targeted <i>LEC1-LIKE4</i> gene	(65)
Apple	CRISPR/Cas9	Mutagenesis of <i>MdCNGC2</i>	(111)
Apple	CRISPR/Cas9	Induction of <i>phytoene desaturase (PDS)</i> gene	(112)
Rice	CRISPR/Cas9	Improvement of rice blast resistance targeting the <i>OsERF922</i> gene	(113)
Arabidopsis	ZFNs	Targeted mutations	(59)
Apple	CRISPR/ Cas9	Reduced fire blight susceptibility	(114)
Grape vine	CRISPR/Cas9	Mutagenesis of <i>TAS4</i> and <i>MYBA7</i> loci	(115)
Sorghum	CRISPR/Cas9	Target <i>cinnamyl alcohol dehydrogenase (CAD)</i> and <i>phytoene desaturase (PDS)</i> genes	(116)
Cabbage	CRISPR/Case9	Cy3-Cas9 protein delivery in to the nucleus	(117)
Wheat	TALEN	gene editing	(80)
Rice	CRISPR/Cas9	knock out <i>OsSWEET14</i> gene	(107)
Tomato	CRISPR/Cas9	test novel sequence-specific mutations at <i>eIF4E1</i>	(118)

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Authors' contributions

AL, BS, YS, XH, HM, JZ, XL & DY wrote and revised the manuscript. AL and JZ revised and edited the manuscript. All authors read and approved the final manuscript.

Conflict of interests

The authors declare no conflict of interest.

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