Advances in Genetic Transformation of Litchi

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Abstract

Litchi (*Litchi chinensis* Sonn.) is one of the most delicious fruits fetching high values in the market, and the area under its cultivation has increased manyfolds. It is generally multiplied by vegetative propagation method, and breeding is being done by conventional and molecular marker-assisted methods to achieve the quality improvement. There are various hybrids and cultivars developed conventionally by plant breeders in litchi. But due to laborious process, linkage drag, low fertility, longer flowering and fruiting time and high levels of heterozygosity, these conventional methods haven't used to its potential in litchi. Plant genetic transformation can be a great tool in the modern molecular breeding of crops. It helps in transfer genes between unrelated plants resulting in genetically modified crop species with better agronomical traits, better nutritional values, disease resistance, insect tolerance and other desirable characteristics. Genetic transformation in plants is synergistic to conventional plant breeding technologies. By using this, the breeders can introduce novel genes irrespective of species barrier and can create phenotypes with desired characters. Over the last decade, some remarkable achievements have been made in the field of development of efficient transformation methods in field crops. Also in litchi genetic engineering technique can be used to introduce new traits in to popular genotypes, which can result into new cultivars with desirable traits. In this chapter we review the transformation methods which are being used or can be used for genetic improvement in litchi.

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18.1 Introduction

In the economy of developed and developing countries, fruit crops play a major role both commercially and nutritionally. Fruits are the major source of nutrients, fibre and anti-oxidants, which were essential for healthy diet. Amongst various fruits produced all over the world, litchi (*Litchi chinensis* Sonn.) is one of the most delicious fruits fetching high values in the market. Litchi is a tropical and subtropical fruit tree indigenous to parts of Southern China and belongs to Sapindaceae family. It is commercially grown in Thailand, China and Taiwan with major markets in Hong Kong, Singapore and Japan (Ghosh 2000; Menzel 2002). The other producers of litchi are Brazil, Malaysia, Myanmar, South Africa, Mauritius, New Zealand, Australia, Madagascar and Taiwan. China has the largest industry of litchi in the world, where it has been cultivated for more than two thousand years back (Li et al. 2013), but is relatively new to the rest of Asia and the Pacific region. The production of litchi per year is highest in China which is about 1.3 million tonnes and followed by India, i.e. 0.43 million tonnes.

In India, it was introduced in the eighteenth century and has well adapted to the climate of Eastern Indian states, viz. Jharkhand, Bihar, Tripura, West Bengal, Uttar Pradesh, Chhattisgarh, Punjab, Uttarakhand and Himachal Pradesh (Singh and Babita 2002). Because of the increasing demand, the area under its cultivation has increased manyfolds. There are numerous litchi cultivars grown in different climates producing different fruit qualities. India grows more than a dozen different cultivars, and during the season, the fruit remains in great demand and also fetches high premium in the market.

The fruit of litchi (white flesh called aril) consists of about 60% juice, 19% seed, 8% rag and 13% skin. The percentage of each constituent varies depending upon the variety of litchi and the climatic conditions under which it is grown. The principal constituents of litchi fruit are carbohydrates, proteins, fats, vitamins, minerals, pigments and organic acids. It is rich in sugar content, and the range of sugar in various Indian varieties varies from 6.74 to 18.0% with an average value of 11.85% (Abrol 2015). The dried form of litchi fruit is known as 'litchi nut' which tastes like raisin and can be also preserved in the form of canned fruit in syrup, squash and jelly (Sidhu 2012). The other parts of the plant like bark, leaves and roots can also be used for various medicinal purposes.

18.2 Litchi Breeding and Its Limitations

Litchi plant is generally multiplied by vegetative propagation method because through seed it grows slowly, has long juvenile period and also leads to genetically different progenies because of genetic segregation. The commonly used methods of vegetative propagation are cutting, air layering, grafting and budding (Menzel 1985). Litchi breeding is being done by conventional and molecular marker-assisted methods to achieve the quality improvement, and this aspect has been reviewed earlier (Sarin et al. 2009). There are various hybrids and cultivars developed conventionally by plant breeders in litchi. In China natural intergeneric hybrid – lungly – has been reported to be developed by crossing litchi (*Litchi chinensis* Sonn.) as female and longan (*Dimocarpus longan* Lour.) as male parent, and the hybrid produced was similar to the maternal parent except for the leaves which were comparatively smaller in size (McConchie et al. 1994).

Conventional breeding methods, like repeated backcrossing, multiline breeding and composite crossing used for crop improvement, are not commonly used in litchi breeding due to its several limitations. Due to more efforts and labour requirement, transfer of non-desirable genes, low fertility, long juvenile period (7–8 years), the length of time before horticultural traits can be evaluated, longer flowering and fruiting time and high levels of heterozygosity, the conventional breeding methods are not commonly used in fruit crops (Gómez-Lim and Litz 2004). Cross-breeding is not commonly used in litchi breeding because the genetic constituents of most litchi germplasm is unclear (Wu et al. 2007). This led to the advancement of many modern methods of breeding which uses the techniques of molecular biology like mutation breeding and genetic transformation. Fingerprinting and analysis of genetic diversity of litchi accessions has been studied by using microsatellite markers, AFLP markers and partial *rbcL* gene sequences (Lin et al. 2005; Madhou et al. 2013; Viruel and Hormaza 2004). In this chapter we review the transformation methods which are being used or can be used for genetic improvement in litchi.

18.3 The Need for Genetic Transformation of Litchi

The agricultural practices have improved a lot through the applications of modern technology, augmenting conventional breeding methods to improve quality and yield. However, there is demand for further improvements in fruit crops because of growing demand owing to rapid population growth, ecological considerations, environmental stress and renewable energy source. Plant genetic engineering offers new thoroughfare in this regard and has become the most important molecular tool in the modern molecular breeding of crops (Job 2002; Liu et al. 2013). Genetic engineering advancements in plants opened an avenue to transfer genes between unrelated plants resulting in genetically modified crop species with better agronomical traits, better nutritional values, disease resistance, insect tolerance and other desirable characteristics (Liu et al. 2013; Vain 2007). Genetic transformation in plants is considered as continuation of conventional plant breeding techniques (Visarada et al.

2009) by advanced technologies. It offers introduction of novel genes irrespective of species barrier and creates phenotypes with desired characters which are not available in the gene pool of original crop plant. Over the last decade, some remarkable achievements have been made in the field of development of efficient transformation methods in field crops (Mittler and Blumwald 2010).

Currently in case of fruit crops, the focus has also shifted to improve the quality of these crops using both conventional and genetic engineering (Kanchiswamy et al. 2015) techniques. Presently, several problems faced during the conventional and molecular breeding methods could not achieve the required potential genetic improvement in fruit crops including litchi. These methods made a relatively small contribution to global litchi supplies; therefore, reliable and speedy methods are needed to meet the future market requirement. To accelerate the process of genetic improvement in perennial fruit crops like litchi, the recently developed techniques can be used for integrating cross species DNA in plant genome resorting to different transformation tools. The main objective of transformation is creating phenotypes with desired traits that are not available in the germplasm pool of crop plants. During the last two decades, many genetically modified plants were generated with DNA that could not have been introgressed in its genome through any conventional breeding method. As a result, the number of genes for agronomically important traits such as herbicide tolerance, insect and pathogen resistance are being transformed in plants. Also in litchi genetic engineering technique can be used to introduce new traits into popular genotypes, which can result into new cultivars with desirable traits such as pest and disease resistance, herbicide resistance, drought and frost tolerance and improved fruit quality.

The recent developments in next-generation sequencing (NGS) technology have made it possible to produce transcriptomics and expressed sequence tag (EST) data from various tissues and cultivars of litchi (Li et al. 2013, 2014, 2015a; Lu et al. 2014, 2015; Wang et al. 2014, 2015; Zhang et al. 2014). This vast resource has opened up new possibilities in the area of litchi functional genomics. To achieve the potential, key requirement is the availability of efficient transformation system for executing the functional genomics strategies like gene overexpression and gene mutation. Despite significant advances over the last 20 years in genetic transformation of fruit plant, very limited studies have been conducted in litchi. The objective of this chapter is to provide an update on litchi genetic transformation and insight on unexplored venues of genetic improvement in it.

18.4 Transformation Systems for Fruit Trees and Their Use in Litchi

The gene transformation methods for delivering exogenous DNA into plant cells can be broadly divided into two main categories: indirect and direct DNA deliveries. In indirect gene transfer approach, the gene of interest is introduced into the target cell *via* soil bacteria, e.g. *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*

(Tzfira and Citovsky 2006) which naturally infects the plants. In contrast, direct gene transfer does not use bacterial cells, instead use direct DNA delivery methods like microinjection, electroporation and particle bombardment.

18.4.1 Direct DNA Delivery

18.4.1.1 Electroporation

Electroporation is a direct gene transfer technique used to introduce substances like drugs and piece of coding DNA into cell. In this process the permeability of plasma membrane is increased significantly by application of external electric field. Upon application of high voltage pulse for microseconds to milliseconds, aqueous pores are formed in the lipid bilayer through which different molecules like drugs, ions, tracers, dyes, antibodies, oligonucleotides, DNA and RNA pass inside cell (Faurie et al. 2005). First plant protoplast electroporation was reported in 1985 (Fromm et al. 1985), where the expression of DNA in protoplast of both dicots (carrots and tobacco) and monocots (maize) was achieved. The use of low voltage, typically 10–1000 V for 30–50 milliseconds, provides efficient transfection. Depending upon the cell type to be transformed, the optimal voltage strength, the pulse length and the number of pulse vary. Besides electric pulse parameters, other factors, such as electroporation medium composition and osmotic pressure, play significant roles in electroporation effectiveness (Fig. 18.1).

Electroporation technique needs viable protoplasts to transfect gene into it, and current advancement of protocols for efficient protoplast isolation and maintenance has made this simple and low-cost technique of interest to many researchers. In majority of the cases, only single copy of transgene is inserted in genome through protoplast transformation. This is one of the major advantages of electroporation over particle bombardment (Bates 1999), where it tends to insert multiple copies of transgene in the genome. High viability of cells after application of the electric pulse (up to 50% of the treated cells survive the treatment) is the another advantage of electroporation. It is also reported that it has higher DNA delivery rate (40–60% of the cell population received DNA under optimal electroporation conditions) (Sorokin et al. 2000). Single cells and cell clusters can be used efficiently in electroporation, which are susceptible to damage by other techniques. Also during the transfection, same culture condition is maintained which results in increasing the efficiency of selection. This is unlike the particle bombardment where the targeted cells require time to recover from particle damage.

Many studies have reported the successful transfer of DNA in plant tissues, cells or organelles with the help of electroporation. Studies with three different plant species – tobacco, soybean and alfalfa – and three different tissues, protoplasts, suspension cell culture and germinating pollen, have indicated that requirement of optimal field strength for each of these cells differs. Whereas protoplasts needed the lowest optimal pulse field strength, followed by suspension cells and finally germinating pollen requiring the strongest electroporation pulse (Saunders et al. 1995). In brief,

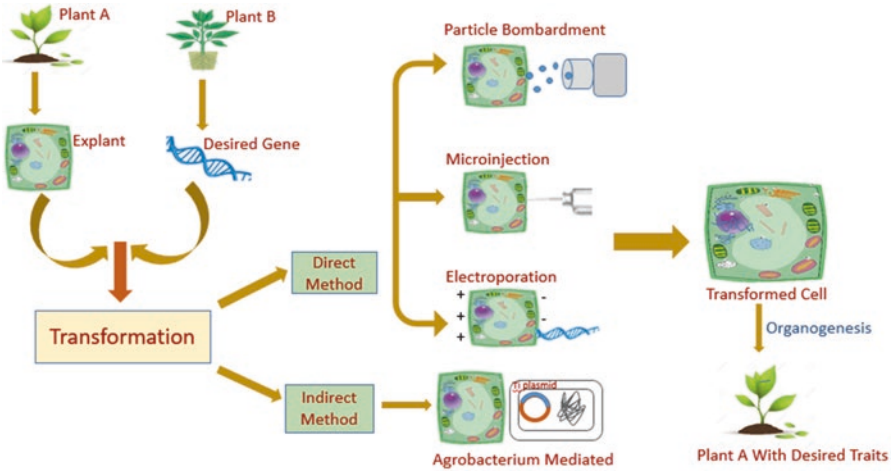

Fig. 18.1 Schematic representation of methods for transfer foreign genes to plant genome

electroporation is a useful technique that can be used to directly transfer desired gene into the litchi plant to produce cultivar with superior traits.

18.4.1.2 Particle Bombardment for Transformation

Particle bombardment with the help of gene gun is another gene transfer technique which utilizes high-velocity micro-projectiles to inject external DNA molecules into cells and tissues. In this method the DNA is precipitated on micron-sized gold or tungsten particles with spermidine and calcium chloride, and these DNA-coated particles are loaded into particle/gene gun and accelerated to high speed using pressurized helium gas to penetrate the plant cell walls and membranes. After the entry of micro-projectile, it releases the transgene from its surface, and it may transiently get expressed in cell or stably get incorporated into the plant's chromosomal DNA (Fig. 18.1).

The particle bombardment process was developed by Sanford and colleagues at Cornel University, and they termed it as "biolistics" gene gun (Sanford et al. 1987). This method is physical in nature and has simple methodology so can be used to transfer genetic material into a wide range of cells from a diverse group of organisms. An important feature of particle bombardment is the flexibility by which co-infection of various genomic components can be achieved. The ability of particle bombardment to transform a wide range of cell types without any biological limitation facilitates a wide range of applications which are difficult to achieve in other transformation methods.

Particle bombardment system is low cost and was successfully used in the transformation of many fruit plants like cherry (Li et al. 2015b) to slow down fruit ripening and drought tolerance and in avocado for transient expression (Chaparro-Pulido et al. 2014). This method was also used in American cranberry (*Vaccinium macrocarpon*) to transfer the *cry1* gene of *Bacillus thuringiensis* to increase the pest

resistance (Serres et al. 1992) and later on transformed by *bar* gene that confers tolerance to glufosinate herbicide (Zeldin et al. 2002). Until now, there are no reports of using this technique for transformation of litchi. However, this technique can be effectively used for litchi transformation and to deliver transgenes to a variety of cell types without any virus-based vectors or toxic chemicals. This is its distinctive advantage over other delivery methods.

18.4.1.3 Indirect DNA Delivery by *Agrobacterium tumefaciens*

A. tumefaciens is a pathogenic bacterium that lives in soil which causes crown gall disease characterized by a tumour in dicot plants. *A. tumefaciens*-mediated plant transformation (ATMT) is the most widely used method for introducing foreign genes into plant for making transgenic plants (Fig. 18.1). The first evidence that indicates this bacterium as causative agent of crown gall tumour goes back to 90 years or more (Smith and Townsend 1907). It is reported to induce tumours at wound sites on root, stem and crown of in about hundreds of dicot plants and some of the monocots and gymnosperms (De Cleene and De Ley 1976). This bacterium has the ability to naturally transfer DNA segment called T-DNA of Ti plasmid which after integration into host genome gets transcribed and produce crown gall disease (Binns and Thomashow 1988; Nester et al. 1984).

The T-DNA carries two types of genes: oncogenic genes and opine genes. Oncogenic genes encode enzymes which help in the synthesis of growth hormones-auxins and cytokinins that produce tumour; and opine genes help in the synthesis of opines which are consumed by *A. tumefaciens* as source of carbon and nitrogen. In Ti plasmid, outside the T-DNA opine catabolism genes, the genes help in T-DNA transfer from bacterium to plant cells, and genes involved in conjugative transfer of bacterial plasmids were present (Hooykaas and Schilperoort 1992; Zupan and Zambryski 1995).

The advanced molecular biology techniques have empowered the development of Ti binary vectors which are compatible with both *Agrobacterium* and *Escherichia coli*. The binary vectors are developed by placing virulence genes in one plasmid (large Ti plasmid) and gene to be transformed on a separate plasmid vector (small binary vector) (Hoekema et al. 1983). Binary vectors are designed in such a way that it can replicate both in *E. coli* and *Agrobacterium*. Recently the advancement in cloning techniques has led to the development of binary bacterial artificial chromosome (BIBAC) vectors that can transfer large-sized DNA into the host (Hamilton 1997; Rui-Feng et al. 2006).

The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The protein coded by the virulence region (*Vir* genes) of Ti plasmid and genes of bacterial chromosome cooperatively helps in the process of transfer of T-DNA. The virulence region is 30 kb in size and organized in six operons (*virA*, *virB*, *virC*, *virD*, *virE* and *virG*), all helps in the transfer of T-DNA (Hooykaas and Schilperoort 1992; Zupan and Zambryski 1995). The T-DNA of bacterium is genetically engineered to produce transgenic Ti plasmid

with desired characteristic of litchi and allowed to infect the plant and to express.*ATMT* has some advantages over direct transformation methods. It has reduced problems with transgene instability and cosupression because of less copy number of transgene (Hansen et al. 1997; Koncz et al. 1994). It is also a single-cell transformation process that does not form mosaic plants which are more frequent in direct gene transformation methods (Enríquez-Obregón et al. 1998; Enriquez-Obregon et al. 1997). *Agrobacterium*-mediated gene transfer method is widely used for transformation of a large number of fruit crops including almond, apple, banana, grapevine, orange, melon and litchi (Rao et al. 2009).

Very few reports on genetic transformation of litchi are available in literature, and all of them have used *Agrobacterium* as a tool for indirect gene transfer. In one study with the green fluorescent protein (GFP) (Puchooa 2004), the expression was achieved in the leaf tissues of litchi by ATMT. The GFP was observed in leaves and callus during fluorescent microscopy after 4 weeks of culture indicating successful transformation. This is one of the pioneer studies in litchi transformation, which opened the new venue of genetic transformation in this important fruit crop.

As we know, fungal diseases are one of the major causes of loss in the quality and yield of litchi worldwide (Crane et al. 1997). The most economically important fungal diseases of litchi in India and other countries are leaf spots and dieback caused by *Phomopsis* sp. and leaf blight caused by *Gloeosporium* sp. Transgenic plant resistant to *Phomopsis* sp. pathogen was developed by transferring bacterial chitinase (ChiB) gene into litchi cultivar *Bedana* by *Agrobacterium*-mediated transfer method (Das and Rahman 2010). Plants also produce pathogenesis-related (PR) proteins like chitinases which help in the plant defence system against pathogens (Legrand et al. 1987; Nishizawa and Hibi 1991). These pathogenesis-related proteins have been reported to provide resistance against many fungal diseases (Jayaraj et al. 2004; Punja 2006). Chitinase gene (Chi11) of rice has been reported to show resistance against many fungal pathogens (Das and Rahman 2012), so rice chitinase gene (RCC11) was transformed into *L. chinensis* cv. Bedana through ATMT. The resultant transgenic exhibited increased resistance to *Phomopsis* sp.

PISTILLATA (PI) gene of *Arabidopsis thaliana* helps in the floral organ development, and its mutation converts stamen to carpel and petal to sepal (Bowman et al. 1991; Goto and Meyerowitz 1994). Recently antisense strategy has been one of the important approaches for silencing gene for the study of its function and in assisting plant breeding. One cultivar of litchi, 'Brewster', was transformed with *Arabidopsis* antisense gene Pistillata cDNA by *Agrobacterium* method to induce parthenogenesis (Padilla et al. 2013). This interferes with the development of stamen and forces fruit production without pollination, and results have indicated that genetic transformation can be used to generate parthenocarpy in litchi.

Several factors affecting *Agrobacterium tumefaciens*-mediated gene transfer efficiency of litchi were studied by using beta-glucuronidase (GUS) gene. When three strains of *Agrobacterium* – LBA4404, AGL-1 and EHA105 – were compared for their virulence, it was found that EHA105 strain had the strongest virulence to litchi amongst them. Coculture for 2 days only gave higher GUS transient expression (Lihui and Liuxin 2003). The bacterial concentration of 0.5×10^8 cells/mL was found

to be enough for achieving best results. The embryogenic calli was found to be suitable starting material for transformation. Using these optimized inoculating conditions, callus with stable GUS expression was obtained (Lihui and Liuxin 2003).

We know that ATMT is generally dependent on host specificity and also the penetration of bacterium to proper cells in targeted tissues of plants. In order to increase transformation frequency in citrus, some modified methods like sonication-assisted *Agrobacterium*-mediated transformation (SAAT), vacuum infiltration and a combination of these two procedures were compared with conventional *Agrobacterium*-mediated inoculation method ('dipping' method) (de Oliveira et al. 2008). Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is a technique which uses a brief periods of ultrasound wave to the plant tissue in presence of *Agrobacterium*. The microscopic study has revealed that sonication produces small and uniform channel in cell wall allowing the bacterium easy access to internal plant tissues. Unlike other transformation methods, this system has the potential to transform meristematic tissue buried under several cell layers. This technique is being widely used in many crops and tissues like leaf, roots, shoot tips, immature cotyledons, embryogenic callus and also whole small seedlings (Trick and Finer 1997). The time of exposure to sonication along with vacuum may affect the transformation efficiency and regeneration capacity of tissues. Generally it is found that the combination of SAAT and vacuum infiltration treatments significantly increase the transformation efficiency.

18.4.1.4 Selection of Transformants

Now it is possible to introduce foreign DNA molecule into any plant cell or tissue using either direct gene transfer or *Agrobacterium*-mediated gene transfer method. In all the transformation methods, only a small fraction of cells become transgenic, while majority of cells remain untransformed. Therefore, it is necessary to select the transformed cells from the untransformed cells. So there was a need of selectable marker genes, which became an important tool in genetic engineering. The gene of interest to be transformed is introduced along with the selectable marker gene, and the transformed cells only will survive under the selection pressure imposed on them. The regenerated plants from surviving cells will contain the selectable marker gene along with the gene of interest. The genes for herbicide or anti-biotic resistance are being popularly used to make transgenic plant and selected on the basis of their growth on the media containing the corresponding toxic substances. The most commonly used selectable marker gene is neomycin phosphotransferase II gene (Fraley et al. 1986) that provides resistance to aminoglycoside anti-biotics neomycin, kanamycin, paromomycin and G-418 (Bevan et al. 1983; Guerche et al. 1987). There are some more selectable marker genes developed which show resistance to anti-biotics like bleomycin (Hille et al. 1986), bromoxynil (Stalker et al. 1988), glyphosate (Franz et al. 1997), hygromycin (Waldron et al. 1985), chloramphenicol (Fraley et al. 1983), 2,4- dichlorophenoxy acetic acid or phosphinothricin (De Block et al. 1987).

The reporter genes are like selection markers, which help in labelling and screening of transformed cells and also useful to investigate the transcriptional regulation

of gene expression. Expressions of reporter genes confirm the genetic modifications in transformed cells. They are generally fused with the coding sequence of gene of interest or under the control of regulating sequence like promoter of gene of interest. A wide range of reporter genes have been identified, and most of them are enzymes and can be a good resource for selection of a marker that is most suitable for the plant species or tissues to be transformed. Commonly used reporters include gene encoding neomycin phosphotransferase (NPT-II), chloramphenicol acetyl transferase (CAT), luciferase (LUC), ß-glucuronidase (GUS) and protein involved in the regulation of anthocyanin biosynthesis.

The enzyme glucuronidase (GUS)-encoding gene from *E. coli* is an important reporter system for screening the transformed plants (Jefferson et al. 1986; Vancanneyt et al. 1990). Being a hydrolytic enzyme, it catalytically cleaves a number of glucuronides which are commercially available as fluorometric, spectrophotometric and histochemical substrates. The luciferase enzyme-encoding gene is also a highly effective reporter gene because the enzyme assay is extremely rapid, easy to perform, sensitive and inexpensive (Ow et al. 1986). The enzyme produces light with highest quantum efficiency than the chemiluminescent reactions. Another benefit is that luciferase is a monomeric protein which does not require post-translational modification for enzymatic activity (De Wet et al. 1985).

The green fluorescent protein (GFP) isolated from coelenterate group like jellyfish *Aequorea victoria* can also be used as reporter molecule for monitoring the expression of gene in plants. The GFP emits green fluorescent light upon excitation with UV light. The molecular cloning of GFP encoding gene with the transgene of interest can be used to select the transformant. In this method there is direct fluorescent imaging of gene product which does not need other histochemical staining methods which may be lethal to the living cells. So this method can be used to select the genetic transformants in litchi.

18.5 Futuristic Methods for Transgenic Development in Litchi

The recently developed techniques like Zinc-finger endonucleases (ZFNs), transcription activator-like effector nucleases (TALENs), CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system can also be used in litchi breeding. The ZNFs are also called as molecular scissors which can be used to target specific genes in plant systems. These currently used engineered proteins have highly specific Zinc Finger domains (usually three) along with the sequence-independent nuclease domain of the Fok I restriction enzyme. After transformation, ZNFs introduce a targeted specific double-strand break in the genomic DNA of organism, which result in recombination at the genomic site of interest. ZFN-mediated gene targeting can be a powerful tool in inducing specific gene mutations (Townsend et al. 2009). But this promising tool can also be useful for homologous recombination of the transferred gene at site of double-strand DNA

break, help in transgene integration and successfully be demonstrated in tobacco. Based on this finding, the ZNFs can be useful tool in gene characterization and transgene integration in litchi in the future.

Transcription activator-like effector nucleases (TALENs) are recently developed techniques for gene editing and can be considered as improved alternative to ZNFs (Joung and Sander 2013). The concept of TALEN was developed after the identification and understanding of the working principles of the type III transcription activator-like (TAL) effectors secreted by *Xanthomonas* – a plant pathogenic bacteria (Boch and Bonas 2010). After entry into plant cells, the TAL effectors cross the nuclear membrane and bind to specific sequence in promoter of host gene and initiate its transcription (Moscou and Bogdanove 2009). The recognition of specific DNA sequence by TAL effectors is governed by tandem amino acid repeats (34 aa). Two repeat-variable di-residues (RVDs) that are located in 12th and 13th position in each of the 34 aa repeat are the key for binding specificity of these effectors. Similar to ZNF, the TALEN is generally engineered with two flanking domains – one with the specific DNA-binding domain at the N-terminal and a nonspecific *Fok I* nuclease domain at the C-terminal. Due to its simpler manipulation technique, TALEN has been successfully used for gene mutation, gene correction and transgene insertion in many crops (Xiong et al. 2015) and can also be applied in litchi breeding.

Most recently, a new revolutionary technique for genome editing is developed, and many laboratories are using it in many organism. The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system is based on the principle of the CRISPR/Cas system which was derived from a type II prokaryotic organism adaptive immune system (Jinek et al. 2012). The repeat-spacer-repeat (29-32-29 bp) pattern of CRISPRs functions through an RNAi-like mechanism, in which the flanking sequences used to recognize the specific sequences and induce cleave in DNA. Since 2013, this technique has been widely applied in gene modification in plants to modify the traits. All these three genome technologies can produce transgene expression in litchi, but the CRISPR/Cas method is the most promising because of its efficiency, low cost and user-friendly characteristics.

18.6 Conclusion

Litchi is an important tropical and subtropical fruit crop that is rich in nutrients, and the major constituents are carbohydrates, proteins, fats, vitamins, pigments and organic acids. The major breeding objective to increase yield and quality of fruit led to the development of various breeding methods and gene transfer technologies to produce high yielding and disease-resistance varieties of litchi. In India, various conventional breeding methods used for litchi breeding are backcross breeding, multiline breeding, hybridisation and composite breeding. But there are several limitations associated with conventional breeding methods, *viz*. it takes longer time to generate breeding lines, more resources and labour requirement, low fertility, transfer of non-desirable genes (genetic load), high levels of heterozygosity and

long juvenile period of about 7–8 years. This led to the development of new breeding approaches like mutation breeding and genetic transformation technologies which uses the principles of molecular biology. The gene transfer techniques for delivering genes into target cells are broadly divided into two categories: direct and indirect methods. The direct DNA delivery method includes electroporation, microinjection and particle bombardment in which the foreign genes are directly targeted into the cells. In indirect gene transfer method, the gene of interest is introduced into the target cell via bacterium *Agrobacterium tumefaciens* or *A. rhizogenes* and naturally infects the plants for inducing tumour. *Agrobacterium*-mediated gene transfer method has more advantage over direct gene transfer because of reduced transgene instability and cosupression; and also does not form mosaic plants which are frequent in direct transfer. So these genetic transformation approaches can be used to produce transgenic litchi varieties with better quality, yield and resistance to disease. After the genes are successfully transformed into the plant, cells need to be selected for transformation. The litchi transformants are selected by using a wide range of selectable marker and reporter genes. The genes of interest are transformed along with the selectable marker gene, and the transformed cells will only survive under the selective pressure. A wide range of reporter genes are also available which can be used for screening transformants. These gene transfer technologies and selection techniques can be future perspectives to produce litchi varieties with desired characteristics.

References

Abrol DP (2015) Subtropical fruits. In: Pollination biology, vol 1. Springer, pp 347–397

Bates GW (1999) Plant transformation via protoplast electroporation. Methods Mol Biol 111:359–366

Bevan MW, Flavell R, Chilton MD (1983) A chimeric anti-biotic resistance gene as a selectable marker for plant cell transformation

Binns AN, Thomashow MF (1988) Cell biology of Agrobacterium infection and transformation of plants. Annu Rev Microbiol 42:575–606

Boch J, Bonas U (2010) Xanthomonas AvrBs3 family-type III effectors: discovery and function. Phytopathology 48:419

Bowman JL, Smyth DR, Meyerowitz EM (1991) Genetic interactions among floral homeotic genes of Arabidopsis. Development 112:1–20

Chaparro-Pulido CA, Montiel MM, Palomo-Ríos E, Mercado JA, Pliego-Alfaro F (2014) Development of an efficient transient transformation protocol for avocado (*Persea americana* Mill.) embryogenic callus. In Vitro Cell Dev Biol Plant 50:292–298

Crane JH, Sanford RE, McMillan Jr RT (1997) Control of lychee anthracnose by foliar applications of tebuconazole, mancozeb, and copper hydroxide on 'Mauritius' lychee fruit under south Florida conditions. Paper presented at: Proceedings of the Florida State Horticultural Society

Das D, Rahman A (2010) Expression of a bacterial chitinase (ChiB) gene enhances antifungal potential in transgenic Litchi chinensis Sonn. (cv. Bedana). Curr Trends Biotechnol Pharm 4:820–833

Das D, Rahman A (2012) Expression of a rice chitinase gene enhances antifungal response in transgenic litchi (cv. Bedana). Plant Cell Tissue Organ Cult 109:315–325

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gossele V, Movva NR, Thompson C, Van Montagu M, Leemans J (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J 6:2513

De Cleene M, De Ley J (1976) The host range of crown gall. Bot Rev 42:389–466

de Oliveira MLP, Febres VJ, Costa MGC, Moore GA, Otoni WC (2008) High-efficiency Agrobacterium-mediated transformation of citrus via sonication and vacuum infiltration. Plant Cell Rep 28:387–395

De Wet JR, Wood KV, Helinski DR, DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. Proc Natl Acad Sci 82:7870–7873

Enríquez-Obregón GA, Vazquez-Padron RI, Prieto-Samsonov DL, Perez M, Selman-Housein G (1997) Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using anti-oxidant compounds. Biotecnol Apl 14:169–174

Enríquez-Obregón GA, Vázquez-Padrón RI, Prieto-Samsonov DL, Gustavo A, Selman-Housein G (1998) Herbicide-resistant sugarcane (*Saccharum officinarum* L.) plants by Agrobacterium-mediated transformation. Planta 206:20–27

Faurie C, Golzio M, Phez E, Teissie J, Rols MP (2005) Electric field-induced cell membrane permeabilization and gene transfer: theory and experiments. Eng Life Sci 5:179–186

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci 80:4803–4807

Fraley RT, Rogers SG, Horsch RB, Gelvin SB (1986) Genetic transformation in higher plants. Crit Rev Plant Sci 4:1–46

Franz JE, Mao MK, Sikorski JA (1997) Glyphosate: a unique global herbicide (American Chemical Society)

Fromm M, Taylor LP, Walbot V (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci 82:5824–5828

Ghosh S (2000) World trade in litchi: past, present and future. In: Paper presented at: I International Symposium on Litchi and Longan 558

Gómez-Lim MA, Litz RE (2004) Genetic transformation of perennial tropical fruits. In Vitro Cell Dev Biol Plant 40:442–449

Goto K, Meyerowitz EM (1994) Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Genes Dev 8:1548–1560

Guerche P, Bellini C, Le Moullec J-M, Caboche M (1987) Use of a transient expression assay for the optimization of direct gene transfer into tobacco mesophyll protoplasts by electroporation. Biochimie 69:621–628

Hamilton CM (1997) A binary-BAC system for plant transformation with high-molecular-weight DNA. Gene 200:107–116

Hansen G, Shillito RD, Chilton MD (1997) T-strand integration in maize protoplasts after codelivery of a T-DNA substrate and virulence genes. Proc Natl Acad Sci 94:11726–11730

Hille J, Verheggen F, Roelvink P, Franssen H, Van Kammen A, Zabel P (1986) Bleomycin resistance: a new dominant selectable marker for plant cell transformation. Plant Mol Biol 7:171–176

Hoekema A, Hirsch P, Hooykaas P, Schilperoort R (1983) A binary plant vector strategy based on separation of vir-and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature 303:179–180

Hooykaas PJ, Schilperoort RA (1992) Agrobacterium and plant genetic engineering. In: 10 years plant molecular biology. Springer, pp 15–38

Jayaraj J, Anand A, Muthukrishnan S, Punja Z (2004) Pathogenesis-related proteins and their roles in resistance to fungal pathogens. In: Fungal disease resistance in plants: biochemistry, molecular biology, and genetic engineering, pp 139–177

Jefferson RA, Burgess SM, Hirsh D (1986) beta-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc Natl Acad Sci 83:8447–8451

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821
Job D (2002) Plant biotechnology in agriculture. Biochimie 84:1105–1110
Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14:49–55
Kanchiswamy CN, Sargent DJ, Velasco R, Maffei ME, Malnoy M (2015) Looking forward to genetically edited fruit crops. Trends Biotechnol 33:62–64
Koncz C, Németh K, Rédei GP, Schell J (1994) Homology recognition during T-DNA integration into the plant genome. In: Homologous recombination and gene silencing in plants. Springer, pp 167–189
Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci 84:6750–6754
Li C, Wang Y, Huang X, Li J, Wang H, Li J (2013) De novo assembly and characterization of fruit transcriptome in *Litchi chinensis* Sonn and analysis of differentially regulated genes in fruit in response to shading. BMC Genomics 14(1): 552
Li WC, Wu JY, Zhang HN, Shi SY, Liu LQ, Shu B, Liang QZ, Xie JH, Wei YZ (2014) De novo assembly and characterization of pericarp transcriptome and identification of candidate genes mediating fruit cracking in *Litchi chinensis* Sonn. Int J Mol Sci 15:17667–17685
Li C, Wang Y, Huang X, Li J, Wang H, Li J (2015a) An improved fruit transcriptome and the identification of the candidate genes involved in fruit abscission induced by carbohydrate stress in litchi. Frontiers in Plant Science 6
Li Q, Chen P, Dai S, Sun Y, Yuan B, Kai W, Pei Y, He S, Liang B, Zhang Y (2015b) PacCYP707A2 negatively regulates cherry fruit ripening while PacCYP707A1 mediates drought tolerance. J Exp Bot 66:3765–3774
Lihui Z, Liuxin L (2003) A preliminary report on *Agrobacterium tumefaciens* mediated genetic transformation of Litchi. J Fruit Sci 4:011
Lin T, Lin Y, Ishiki K (2005) Genetic diversity of *Dimocarpus longan* in China revealed by AFLP markers and partial rbcL gene sequences. Sci Hortic 103:489–498
Liu W, Yuan JS, Stewart CN Jr (2013) Advanced genetic tools for plant biotechnology. Nat Rev Genet 14:781–793
Liu W, Xiao Z, Bao X, Yang X, Fang J, Xiang X (2015) Identifying litchi (*Litchi chinensis* Sonn.) cultivars and their genetic relationships using single nucleotide polymorphism (SNP) markers. PLoS One 10:e0135390
Lu X, Kim H, Zhong S, Chen H, Hu Z, Zhou B (2014) De novo transcriptome assembly for rudimentary leaves in *Litchi chinensis* Sonn. and identification of differentially expressed genes in response to reactive oxygen species. BMC Genom 15:1
Madhou M, Normand F, Bahorun T, Hormaza J (2013) Fingerprinting and analysis of genetic diversity of litchi (*Litchi chinensis* Sonn.) accessions from different germplasm collections using microsatellite markers. Tree Genet Genomes 9:387–396
McConchie C, Vithanage V, Batten D (1994) Intergeneric hybridisation between litchi (*Litchi chinensis* Sonn.) and longan (*Dimocarpus longan* Lour.). Ann Bot 74:111–118
Menzel C (1985) Propagation of lychee: a review. Sci Hortic 25:31–48
Menzel C (2002) The lychee crop in Asia and the Pacific. Food and Agriculture Organization of the United Nations, Regional Office for Asia and the Pacific, Bangkok, Thailand
Mittler R, Blumwald E (2010) Genetic engineering for modern agriculture: challenges and perspectives. Annu Rev Plant Biol 61:443–462
Moscou MJ, Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. Science 326:1501–1501
Nester EW, Gordon MP, Amasino RM, Yanofsky MF (1984) Crown gall: a molecular and physiological analysis. Annu Rev Plant Physiol 35:387–413
Nishizawa Y, Hibi T (1991) Rice chitinase gene: cDNA cloning and stress-induced expression. Plant Sci 76:211–218

Ow DW, De Wet JR, Helinski DR, Howel SH, Wood KV, Deluca M (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234:856–859

Padilla G, Pérez JA, Perea-Arango I, Moon PA, Gómez-Lim MA, Borges AA, Expósito-Rodríguez M, Litz RE (2013) *Agrobacterium tumefaciens*-mediated transformation of 'Brewster' ('Chen Tze') litchi (*Litchi chinensis* Sonn.) with the PISTILLATA cDNA in antisense. In Vitro Cell Dev Biol Plant 49:510–519

Puchooa D (2004) Expression of green fluorescent protein gene in litchi (*Litchi chinensis* Sonn.) tissues. J Appl Hortic 6:11–15

Punja ZK (2006) Recent developments toward achieving fungal disease resistance in transgenic plants. Can J Plant Pathol 28:S298–S308

Rao AQ, Bakhsh A, Kiani S, Shahzad K, Shahid AA, Husnain T, Riazuddin S (2009) The myth of plant transformation. Biotechnol Adv 27:753–763

Rui-Feng H, Yuan-Yuan W, Bo D, Ming T, Ai-Qing Y, Li-Li Z, Guang-Cun H (2006) Development of transformation system of rice based on binary bacterial artificial chromosome (BIBAC) vector. Acta Genet Sin 33:269–276

Sanford JC, Klein TM, Wolf ED, Allen N (1987) Delivery of substances into cells and tissues using a particle bombardment process. Part Sci Technol 5:27–37

Sarin N, Prasad U, Kumar M, Jain SM (2009) Litchi breeding for genetic improvement. In: Breeding plantation tree crops: tropical species. Springer, pp 217–245

Saunders JA, Lin CH, Hou BH, Cheng J, Tsengwa N, Lin JJ, Smith CR, McIntosh MS, Van Wert S (1995) Rapid optimization of electroporation conditions for plant cells, protoplasts, and pollen. Mol Biotechnol 3:181–190

Serres R, Stang E, McCabe D, Russell D, Mahr D, McCown B (1992) Gene transfer using electric discharge particle bombardment and recovery of transformed cranberry plants. J Am Soc Hortic Sci 117:174–180

Sidhu JS (2012) Tropical fruit II: production, processing and quality of guava, lychee, and papaya. In: Handbook of fruits and fruit processing, 2nd edn, pp 591–628

Singh H, Babita S (2002) Lychee production in India. Lychee Production in the Asia-Pacific Region Food and Agricultural Organization the United Nations, Bangkok, Thailand, pp 55–67

Smith EF, Townsend CO (1907) A plant-tumor of bacterial origin. Science:671–673

Sorokin AP, Ke X-Y, Chen D-F, Elliott MC (2000) Production of fertile transgenic wheat plants via tissue electroporation. Plant Sci 156:227–233

Stalker DM, McBride KE, Malyj LD (1988) Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science 242:419–423

Townsend JA, Wright DA, Winfrey RJ, Fu F, Maeder ML, Joung JK, Voytas DF (2009) High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459:442–445

Trick HN, Finer JJ (1997) SAAT: sonication-assisted Agrobacterium-mediated transformation. Transgenic Res 6:329–336

Tzfira T, Citovsky V (2006) Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. Curr Opin Biotechnol 17:147–154

Vain P (2007) Thirty years of plant transformation technology development. Plant Biotechnol J 5:221–229

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in Agrobacterium-mediated plant transformation. Mol Gen Genet 220:245–250

Viruel M, Hormaza J (2004) Development, characterization and variability analysis of microsatellites in lychee (*Litchi chinensis* Sonn., Sapindaceae). Theor Appl Genet 108:896–902

Visarada K, Meena K, Aruna C, Srujana S, Saikishore N, Seetharama N (2009) Transgenic breeding: perspectives and prospects. Crop Sci 49:1555–1563

Waldron C, Murphy E, Roberts J, Gustafson G, Armour S, Malcolm S (1985) Resistance to hygromycin B. Plant Mol Biol 5:103–108

Wang J, Liu B, Xiao Q, Li H, Sun J (2014) Cloning and expression analysis of litchi (*Litchi chinensis* Sonn.) polyphenol oxidase gene and relationship with postharvest pericarp browning. PLoS One 9:e93982

Wang B, Tan H-W, Fang W, Meinhardt LW, Mischke S, Matsumoto T, Zhang D (2015) Developing single nucleotide polymorphism (SNP) markers from transcriptome sequences for identification of longan (*Dimocarpus longan*) germplasm. Hortic Res 2:14065

Wu Y, Yi G, Zhou B, Zeng J, Huang Y (2007) The advancement of research on litchi and longan germplasm resources in China. Sci Hortic 114:143–150

Xiong J-S, Ding J, Li Y (2015) Genome-editing technologies and their potential application in horticultural crop breeding. Hortic Res 2:15019

Zeldin EL, Jury TP, Serres RA, McCown BH (2002) Tolerance to the herbicide glufosinate in transgenic cranberry (*Vaccinium macrocarpon* Ait.) and enhancement of tolerance in progeny. J Am Soc Hortic Sci 127:502–507

Zhang H-N, Wei Y-Z, Shen J-Y, Lai B, Huang X-M, Ding F, Su Z-X, Chen H-B (2014) Transcriptomic analysis of floral initiation in litchi (*Litchi chinensis* Sonn.) based on de novo RNA sequencing. Plant Cell Rep 33:1723–1735

Zupan JR, Zambryski P (1995) Transfer of T-DNA from Agrobacterium to the plant cell. Plant Physiol 107:1041