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Transgenesis of tomato: Relevance in molecular research today

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Abstract

Tomato is not only one of the most important of vegetable crops but also is an important dicotyledonous model plant for scientific research. Genetic transformation of tomato is often the first step of various scientific researches to follow. Along with the view to generating tomato lines with diverse desirable traits transformed tomato serve as the background of doing functional studies. There are many transformation protocols for tomato and these have their advantages and dis-advantages. The selection of the desirable traits, the appropriate promoter/terminator sequences, suitable selectable markers and reporter genes are to be critically considered. A number of different transgenic lines of tomato are available today that have improved nutritional content, better shelf life, resistance to biotic/abiotic stresses and even for development of vaccines and as tools in immunotherapy. The importance of transgenesis of tomato in molecular researches today is discussed.

Keywords: Tomato, transformation, *Agrobacterium*, biotechnology, binary vector, tissue culture

Introduction

Tomato (*Lycopersicon esculentum* Mill), a member of the Solanaceae family, is not only one of the most important vegetable crops in the world but it is also a model plant for basic research on dicotyledonous plants. The entire tomato genome consists of 12 chromosomes. Genetic transformation of tomato is often the first step of various scientific researches along with the view to generating tomato lines with diverse desirable traits (Hansen *et al*, 1994). However, for any genetic engineering strategy to become successful, several protocol factors need to be optimized for the particular system. Other factors that need to be taken into consideration are the receptiveness of tissue to foreign DNA integration, availability of vectors and screenable markers and reproducible regeneration of intact plantlets from transformed tissues.

Major methods of transformation of tomato

Transformation methods followed in tomato are mainly of two types namely direct transformation and *Agrobacterium*-mediated transformation.

Direct or non-*Agrobacterium*-based methods

These methods again can be discussed under the following heads:

Protoplast based transformation, in which the desirable gene is transferred into the protoplast in the presence of calf thymus carrier DNA and polyethylene glycol. The prerequisite of this method is the preparation of receptive protoplasts from tomato tissues mainly leaf tissues. These have successfully been used by many workers initially and transgenic plants have been produced (Jones *et al*, 2005).

Non-protoplast based transformation, in which, unlike the use of *Agrobacterium*, is plant genotype-independent and relies upon the bombardment of accelerated noble metal particles coated with DNA. Most commonly used instruments for accelerating DNA coated particles are those powered by burst of helium generated by a rupture membrane mechanism or by a shock wave generated by high voltage discharge through a watered droplet (Jones *et al*, 2005).

The current protocols used for tomato transformation are based on shoot regeneration from leaf disk/cotyledon tissue co-cultivated with disarmed *Agrobacterium tumefaciens* harboring binary vector. The efficiency of such procedures is generally low because most of the transformed leaf cotyledon cells could not develop into shoots. *Agrobacterium* mediated transformation in tomato has been studied on different media and conditions have been optimized. The current protocols used for tomato transformation are based on shoot regeneration from leaf disk/cotyledon tissue co-cultivated with disarmed *Agrobacterium tumefaciens* harboring binary vector. The efficiency of such procedures is generally low because most of the transformed leaf cotyledon cells do not develop into shoots (Jones *et al*, 2005).

***Agrobacterium* mediated transformation**

This method includes infection of suitable explants using *Agrobacteium tumefaciens* carrying suitable recombinant binary vectors.

Agrobacterium strains and binary vectors

Agrobacterium mediated gene transformation is an effective and widely used approach to introduce foreign DNA into a dicotyledons plants like tomato. The ability of particular *Agrobacterium* strains to transform plant cells is defined by their chromosomal and plasmid genomes which between them must encode all the machinery necessary for attachment and DNA transfer (Vander *et al*, 2010).

There are several significant advantages to transferring DNA via *Agrobacterium*, including a low transgene copy number, the stable integration with fewer rearrangements of long molecules of DNA with defined ends and the ability to generate lines which are without selectable marker genes (Khan *et al*, 2012).

***Agrobacterium* infection process is divided into two steps**

The first step includes, a short period, normally a few minutes to a few hours, of inoculation by immersion of suitable explants in an *Agrobacterium* suspension. Then, after the majority of *Agrobacterium* cells are removed by pouring or pipetting, the explants are co-cultivated for a further 1–3 days. One or both these steps are carried out in darkness at approximately 25°C, although a two temperature co-cultivation step has also been tried with one day at 27°C then two days to 25°C (Velcheva *et al*, 2011).

Over view of *Agrobacterium* –mediated tomato transformation over view

In general, the *Agrobacterium* method is considered preferable to the gene gun, because of the greater frequency of single-

site insertions of the foreign DNA, making it easier to monitor (Chaudhury *et al*, 2009).

Agrobacterium tumefaciens is a soil bacteria that has the ability to infect plant cells and transfer a fragment of its DNA into the host cells. When the bacterial DNA is integrated into a plant chromosome, it effectively hijacks the plant's cellular machinery and uses it to ensure the proteins required for the proliferation of the bacteria in the host cells.

The infecting DNA in an *A. tumefaciens* cell is contained in the bacterial chromosome and also in Ti (tumor-inducing) plasmid. The Ti plasmid contains a stretch of DNA termed T-DNA (~20 kb long) that is integrated in to the plant genome during the infection process. *A. tumefaciens* can only infect a plant through wounds. Under natural conditions, when a plant root or stem is wounded it gives off certain chemical signals. In response to those signals, the *vir* genes of *A. tumefaciens* get activated and direct a series of events which results in the transfer of the T-DNA from the Ti plasmid to the plant genome.

Different *vir* genes help in copying the T-DNA to which a leader sequence is attached. For protection of the T-DNA it is coated with protein. A channel is opened in the bacterial cell membrane, through which the T-DNA moves out.

The T-DNA then enters the plant cell through the wound. It is not clear how the bacterial DNA moves from the cytoplasm to the nucleus of the plant cell, nor how the T-DNA becomes integrated into the plant chromosome.

To exploit the T-DNA as a transgene vector, scientists have removed the tumor-inducing section of T-DNA, while retaining the left and right border regions and the vir genes. The transgene is inserted between the T-DNA border regions, where it is transferred to the plant cell and becomes integrated into the plant's chromosomes. Gamma-amino-butyric acid (GABA) is a negative regulator of *Agrobacterium* infection and transgenesis. However a new strain of *Agrobacterium* expressing gamma-amino-butyric acid transaminase activity showed higher rate of tomato transgenesis in presence of low-GABA (Nonaka et al, 2017)

Difficulty in identifying and Locating Genes for Plant Traits to be used in tomato transformation

Identifying and locating genes for agriculturally desirable traits is presently the most limiting step in the transgenic process. We still know relatively little about the specific genes required to enhance plant growth, improve stress tolerance, modify specific properties of the harvested product, or otherwise affect plant characters. Usually identifying a single gene involved with a desirable trait is not adequate. Scientists must have an insight as to how the gene is regulated, what other effects it might have on the transformed plant, and how it interacts with other genes active in the same biochemical pathway. Public and private research programs are investing heavily into new technologies to rapidly sequence and determine functions of genes of the most important crop species. These efforts should result in identification of a large number of genes potentially useful for producing transgenic varieties for scientists to have a wider range of choices (Sederoff *et al*, 1999).

Designing Genes for Insertion during transgenesis

Once a gene has been isolated it must be cloned into a small cloning vector for maintenance. Typically *E coli* is the choice organism for this purpose of maintaining the vector with the cloned gene. Thereafter it must undergo several modifications before it can be effectively inserted into a plant.

Modification of cloned genes

Sometimes, the cloned gene is modified to achieve greater expression in a plant. For example, the Bt gene for insect resistance is of bacterial origin and has a higher percentage of A-T nucleotide pairs compared to plants. Plant translational machinery prefers G-C nucleotide pairs. During modification A-T nucleotides were substituted with G-C nucleotides in the Bt gene without significantly changing the amino acid sequence. This ensured in enhanced production of the Bt gene protein in plant cells (Deist *et al*, 2014).

Importance of promoter sequence

A promoter sequence must be added up stream of the cloned gene to be correctly expressed or in other words translated into a protein product (Agarwal *et al*, 2016). The promoter is the switch that controls when and where in the plant the gene will be expressed. To date, most promoters in transgenic crop varieties have been constitutive, causing genes to be expressed in all parts of the plant and throughout the life cycle of the plant across different developmental stages. The most commonly used constitutive promoter is CaMV35S, from the cauliflower mosaic virus, which generally results in a high degree of expression in plants. Other promoters are more specific and respond to cues in the plant's internal or external environment. An example of a light-inducible promoter is the promoter from the cab gene, encoding the major chlorophyll a/b binding protein or wound inducible promoters (Feng *et al*, 2014). Tissue specific promoters are important tools for site-specific expression of genes and improvement of crops. Fruit ripening is an important part of crop yield and is genetically regulated by irreversible changes in the fruit. Such a promoter of *Ripening induced protein 1* (*RIP 1*) in tomato is specifically expressed in fruits, more so in red ripe fruits of tomato (Sharma *et al*, 2016)

Importance of terminator sequence

The termination sequence signals to the cellular machinery that the end of the gene sequence has been reached. Current terminator sequences used are nopaline synthase terminator or rubisco terminator (Basu *et al*, 2015).

Current use of selectable marker genes

Selectable marker genes are added to the gene construct in order to "mark" plant cells or tissues that have successfully integrated the transgene for easy identification and selection. This is necessary because achieving incorporation and expression of transgenes in plant cells is a rare event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide resistance to chemicals that are normally inhibitory to plant cells, such as antibiotics or herbicides. Only those plant cells that expresses the selectable marker gene will survive when grown on a medium containing the antibiotic or herbicide within limited ranges. Just like other genes, the marker genes also require promoter and termination sequences for proper expression. Currently the most commonly used selectable marker gene for tomato is neomycin phosphor transferease (*nptII)* gene that imparts Kanamycin resistance.

Selection and Regeneration of transgenic tomato

The selection of transgenic tissues from expalnts is followed by regeneration of whole plants.

Selection of successfully transformed tomato tissues

Following the gene insertion process, plant tissues are transferred to a selective medium containing an antibiotic or herbicide,

depending on which selectable marker was used. Only
plants expressing the selectable marker gene will survive, as shown in the figure, and it is assumed that these plants will also possess the transgene of interest. Thus, subsequent steps in the process will only use these surviving.

Regeneration of whole tomato plants

To obtain whole plants from transgenic tissues such as immature embryos, they are grown under controlled environmental conditions in a series of media containing nutrients and hormones. For tomato the regeneration media usually contain a cytokinin and auxin. The regenerating plants are transferred onto fresh rooting media with antibiotic. Once whole plants are generated and produce seed, the progeny is evaluated. This regeneration step has been a stumbling block in producing transgenic plants in many species, but for tomato this step is nowadays fairly standardized (Khan *et al*, 2012).

Genetically modified tomato available today

The tomato originated from South America and was brought to Europe by the Spanish in the 16th century. Wild tomatoes are small, green and largely unappetizing but after centuries of breeding there are now thousands of varieties grown worldwide. *Agrobacterium*-mediated genetic engineering techniques were developed in the late 1980s that could successfully transfer genetic material into the nuclear genome of tomatoes. Genetic material can also be inserted into a tomato cell's chloroplast and chloroplast plastosomes using biolistics. This is called organelle transformation. Tomatoes were the first food crop with an edible fruit where this was possible.

Delayed ripening imparted by transgenesis

Tomatoes have been used as a model organism to study the fruit ripening of climacteric fruit. To understand the mechanisms involved in the process of ripening, scientists have genetically engineered tomatoes.

In 1994, the Flavr Savr became the first commercially grown genetically engineered food to be granted a license for human consumption (Francis *et al*, 2017). A second copy of the tomato gene *polygalacturonase* was inserted into the tomato genome in the antisense direction. During ripening the polygalacturonase enzyme degrades the pectin of the tomato cell wall, causing the fruit to soften. When the antisense gene is expressed it interferes with the production of the polygalacturonase enzyme, thereby delaying the ripening process. The Flavr Savr failed to achieve commercial success and was withdrawn from the market in 1997. Similar technology, but using a truncated version of the polygalacturonase gene, was used to make a tomato paste. DNA Plant Technology (DNAP), Agritope and Monsanto developed tomatoes that delayed ripening by preventing the production of ethylene, a hormone that triggers ripening of fruit. All three tomatoes inhibited ethylene production by reducing the amount of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor to ethylene. DNAP's tomato, called Endless Summer, inserted a truncated version of the *ACC synthase* gene into the tomato that interfered with the endogenous *ACC synthase*. Monsanto's tomato was engineered with the *ACC deaminase* gene from the soil bacterium *Pseudomonas chlororaphis* that lowered ethylene levels by breaking down ACC. Agritope introduced an S-adenosylmethionine hydrolase (SAMase) encoding gene derived from the *E. coli* bacteriophage T3, which reduced the levels of S-adenosylmethionine, a precursor to

ACC. Endless Summer was briefly tested in the marketplace, but patent arguments forced its withdrawal (Bawa and Anilakumar, 2013).

Scientists in India have delayed the ripening of tomatoes by silencing two genes encoding N-glycoprotein modifying enzymes, α-mannosidase and β-D-N-acetylhexosaminidase (Meng *et al*, 2016). The fruits produced were not visibly damaged after being stored at room temperature for 45 days, whereas unmodified tomatoes had gone rotten. In India, where 30% of fruit is wasted before it reaches the consumers due to a lack of refrigeration and delayed conveyance, genetic engineering of the tomato may decrease wastage.

Environmental stress tolerance

Abiotic stresses like frost, drought and increased salinity are a limiting factor to the growth of tomatoes. While no genetically modified stress tolerant plants are currently commercialised, transgenic approaches have been researched. An early tomato was developed that contained an antifreeze gene (*afa3*) from the winter flounder with the aim of increasing the tomato's tolerance to frost. The antifreeze protein was found to inhibit ice recrystallization in the flounders blood, but had no effect when expressed in transgenic tobacco. The resulting tomato was never commercialized, but raised ethical questions over adding genes from one kingdom to another.

Other genes from various species have been inserted into the tomato with the hope of increasing their resistance to various environmental factors. A gene from rice (*Osmyb4*), which codes for a transcription factor, that was shown to increase cold and drought tolerance in transgenic *Arabidopsis thaliana* plants was inserted into the tomato. This resulted in increased drought tolerance, but did not appear to have any effect on cold tolerance. Overexpressing a vacuolar Na^+/H^+ antiport (*AtNHX1*) from *A. thaliana* lead to salt accumulating in the leaves of the plants, but not in the fruit and allowed them to grow more in salt solutions than wildtype plants. They where the first salt-tolerant, edible plants ever created. Tobacco osmotic genes overexpressed in tomatoes produced plants that held a higher water content than wildtype plants increasing tolerance to drought and salt stress.

In another report, transgenic tomato plants with choline oxidase gene (*codA*) conferred more resistance under salt stressed conditions. They accumulated more glycine betaine than wild type plants. The photosynthetic rates were also higher in transgenic plants. Under salt-stress the Na^+/K^+ ratio is lower in transgenic plants than wild plants as the Na^+ efflux is greater than K^+ influx in the roots of transgenic lines. Accumulation of glycine betaine induced the expression of K^+-transporter, Na^+/H^+ antiporter and H^+-ATPase genes. Hence in transgenic tomato plants *codA* plays critical role in regulation of ion channels and transporters (Wei *et al*, 2017).

Sucrose is the most common form of sugar transported within plants. Sucrose transporters (SUTs) play a key role in sucrose partitioning. More over sucrose molecules can act as a signal to different pathways to manipulate gene expression and physiological adaptation under abiotic stress conditions. Over-expression of apple-SUT, *MdSUT1* in tomato conferred stress tolerance under low temperature. More anthocyanin pigmentation was observed under low temperature in *MdSUT1*-transgenic tomato plants than wild type. In addition to abiotic stress tolerance the transgenic tomato lines also showed better sucrose uptake, early flowering and fruit-ripening (Ma *et al*, 2017).

Transgenesis of tomato for resistant to biotic stress

The Brassica juncea 2S seed storage protein precursor gene has been used to produce insect resistant tomato lines in India (Mandal *et al*, 2002). In this work the novelty was that while the precursor of the protein had trypsin inhibitor properties, the processed protein did not have this property, thereby rendering the protein harmless during consumption. The insecticidal toxin from the bacterium *Bacillus thuringiensis* has been inserted into a tomato plant (Koul *et al*, 2014). When field tested they showed resistance to the tobacco hornworm (*Manduca sexta*), tomato fruitworm (*Heliothis zea*), the tomato pinworm (*Keiferia lycopersicella*) and the tomato fruit borer (*Helicoverpa armigera*). A 91 day feeding trail in rats showed no adverse effects, but the Bt tomato has never been commercialised. Tomatoes resistant to a root knot nematode have been created by inserting a cysteine proteinase inhibitor gene from taro A chemically synthesised *ceropin B* gene, usually found in the giant silk moth (*Hyalophora cecropia*), has been introduced into tomato plants and in vivo studies show significant resistance to bacterial wilt and bacterial spot. When the cell wall proteins, polygalacturonase and expansin are prevented from being produced in fruits, they are less susceptible to the fungus *Botrytis cinerea* than normal tomatoes.

Cucumber mosaic virus (CMV-Fny) in combination with its satellite RNA (77-satRNA FN) causes severe necrotic disease in tomato. Systemin signal peptide hormone is related to stress response in Solanaceae family and it induces proteinase inhibitors in plants just like jasmonate (JA) responsive pathways. In transgenic tomato lines over expressing prosystemin necrotic lesions were reduced in 50% of plants. In over expression lines JA-biosynthetic genes like *LoxD, AOS* and JA-induced genes like *Pin I* and *Pin II* were upregulated and salicylic acid (SA) responsive genes ere down regulated considerably (Bubici *et al*, 2017).

Improved nutritional content of tomato through transgenesis

Tomatoes have been altered in attempts to improve their flavour or nutritional content. In 2000, the concentration of pro-vitamin A was increased by adding a bacterial gene encoding phytoene desaturase, although the total amount of carotenoids remained equal The researchers admitted at the time that it had no prospect of being grown commercially due to the anti-GM climate. More recently, scientists have increased the production of anthocyanin, an antioxidant in tomatoes in several ways. One group added a transcription factor for the production of anthocyanin from *Arabidopsis thaliana* (Zuluaga *et al*, 2008). Whereas another used transcription factors from snapdragon (*Antirrhinum*) (Tohge *et al*, 2015). When the snapdragon genes where used, the fruits had similar anthocyanin concentrations to blackberries and blueberries, and when fed to cancer susceptible mice, extended their life span. Another group has tried to increase the levels of isoflavone, known for its potential cancer preventative properties, by introducing the soybean *isoflavone synthase* into tomato.

Transformation of tomato for improved taste

When geraniol synthase from lemon basil (*Ocimum basilicum*) was expressed in tomato fruits under a fruit-specific promoter, 60% of untrained taste testers preferred the taste and smell of the transgenic tomatoes **(**Bartoszewski, 2003**).** The fruits contained around half the amount of lycopene, reducing the health benefits of eating.

Transgenic tomatoes for production of vaccines and vehicle of vaccine delivery

Tomatoes (along with potatoes, bananas and other plants) are being investigated as vehicles for delivering edible vaccines. Clinical trials have been conducted on mice

using tomatoes expressing antibodies or proteins that stimulate antibody production targeted to norovirus, hepatitis B, rabies, HIV, anthrax, respiratory syncytial virus and IgA antibody against rotavirus (Merlin *et al*, 2017). Korean scientists are looking at using the tomato to expressing a vaccine against Alzheimer's disease. Hilary Koprowski, who was involved in the development of the polio vaccine, is leading a group of researchers in developing a tomato expressing a recombinant vaccine to SARS.

The Hepatitis B surface antigen (HBsAG) has been reported to accumulate to 0.01% of soluble protein level in transgenic tomato (MacKenzie, 1994). The antigens, delivered in a macromolecular form, are known to tolerate the digestive tract environment. Interestingly, the recombinant HBsAG was able to form virus-like particles of 22 nm diameter (comparable to yeast-derived HBsAG-based vaccine). Plant extract was used for parenteral immunization in mice. The immune response included all IgG subclasses as well as IgM against hepatitis B.

Tomato the popular model plants for transgenic research

Tomato is an important vegetable crop for India. According to FAOSTAT (2008) total world production was 129,649,883 tones out of which 10,260,600 tones were from India which ranked fourth. Yet heavy losses upto 50% is seen every year due to different abiotic and biotic stress conditions. Adverse abiotic conditions include salinity, drought, heavy metal toxicity. The biotic stresses including diseases like tomato leaf curl (caused by tomato leaf curl virus), early blight (caused by *Alternaria solani* f.sp. *lycopersici*) and insects are among the most destructive (McGarvey *et al* 1995). On the same note soil borne diseases are least understood and cause great damage to tomato production (Chowdhury *et al* 2013). Thus the improvement of this important vegetable crop in one of the two aspects for consideration. The other being, with the genome sequence now being available, tomato has become the model dicotyledonous plant of choice next to *Arabidopsis* in transgenic research.

On the whole it can be said that transgenesis of tomato is a part of the research work for many laboratories all over the world. Transgenic tomato with desirable traits is not only the end product of research, it is often the background which is the starting point of other research to follow.

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Conflict of interest

There is no conflict of interest.

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