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Panchaal Bhattacharjee
University of Horticultural Sciences, Bagalkot, Karnataka, India

Utpal Das
University of Agriculture Science, Raichur, Karnataka, India

Venugopala Reddy M
University of Agriculture Science, Raichur, Karnataka, India

Sukanya Gogoi
Assam Agricultural University, Jorhat, Assam, India

Gangaraja MM
University of Agriculture Science, Raichur, Karnataka, India

Correspondence
Panchaal Bhattacharjee
University of Horticultural Sciences, Bagalkot, Karnataka, India

Role and recent advances in genetic engineering & biotechnology for improvement of fruit crops

Panchaal Bhattacharjee, Utpal Das, Venugopala Reddy M, Sukanya Gogoi and Gangaraja MM

Abstract
The main problem of genetic improvement of most fruit crops is their long juvenile period. This problem further aggravates by seedlessness, inter and intra specific incompatibility, high heterozygosity, sterility etc. Conventional breeding method of genetic improvement would not be a better option owing to its difficult, expensive and time consuming breeding techniques. Alternatively, non-conventional breeding method like recombinant DNA, a genetic engineering approach, is more precise in correcting the deficiencies in commercial cultivars or root stocks without disturbing their desirable genetic makeup. Transgenic plants develop through a number of gene delivery methods, the most common of which is Agrobacterium and particle bombardment mediated gene transfer. Generally, two tissue culture methods have been used for regeneration of transgenic plants: organogenesis and somatic embryogenesis. Genetic engineering technique has been used for resistance of a number of important diseases and pests. One successful example is transgenic papaya expressing coat protein of papaya ring spot virus (PRSV) against PRSV developing two cultivars 'SunUp' and 'Rainbow'. Despite the usefulness of genetic engineering in many aspects, public of many countries are reluctant to adopt such technique on the ground of their risk to health and environment linked to the introduction of transgene into crop species of genetic material derived from alien organisms. To overcome the notorious aversion against transgenic, new genetic engineering approach, namely 'cisgenesis' and 'intragenesis' has been proposed. The acceptance of science-based approaches like cisgenesis or intragenesis or use of selection marker free transgenic will encourage confidence, and bring the benefits of GM-products to consumers.

Keywords: Juvenile, transgene, gene, intragenesis, cisgenesis

Introduction
Fruit crops are mainlyvegetatively propagated perennials. Conventional genetic improvement of most species of fruit crops faces a realm of problems. This include the long juvenility period of some species, seedlessness, frequent inter and intra specific incompatibility, high heterozygosity, sterility and the presence of some specific traits only in wild species. These characteristics make conventional breeding techniques difficult, expensive and time consuming) (Mehlenbacher, 1995) [36]. Alternatively, recombinant DNA, a genetic engineering approach is more precise in correcting the deficiencies in commercial cultivars or root stocks without disturbing their desirable genetic makeup (Schuerman and Dandekar, 1993) [55].

Importance
Conventional plant breeding has had little success in improving fruit plants and is constrained –(1) Due to long juvenile period, breeding programs for such plants can involve the professional lifetimes of several generations of scientists.(2) Erosion of naturally occurring genetic variability (3) Transfer of undesirable genes along with desirable traits (4) Reproductive obstacles that limit the transfer of favorable alleles from diverse genetic resources (Gomez-Lim and Litz 2004; Varshney *et al*. 2011) [16, 72].
In the last 20 years, genetic transformation of fruitcrops has focused mainly on enhancing disease resistance like viruses, fungi, and bacteria; increasing tolerance against abiotic stresses like drought, frost, and salt; modifying plant growth habit and fruit quality. Although, there are only a few cases of field evaluation and commercial application of these transgenic plants (Litz and Padilla 2012) [32].

Genetic transformation of fruit plants
Mode of regeneration and transformation
Transgenic plants have been developed through a number of gene delivery methods. Agrobacterium and particle bombardment mediated gene transfer are the most popular methods for the development of transgenic plants.

In most fruit plants, both plant regeneration under *in vitro* condition and genetic transformation were slow to be developed, therefore, they are usually considered as 'recalcitrant' for *in vitro* culture and genetic transformation (Gomez-Limand Litz 2004) [16]. Usually, success of genetic transformation highly depends on the regeneration pathway adopted by individual species, which is influenced by several factors, namely, genotype or cultivar, the source of the ex-plant etc (Litz and Padilla 2012) [32].

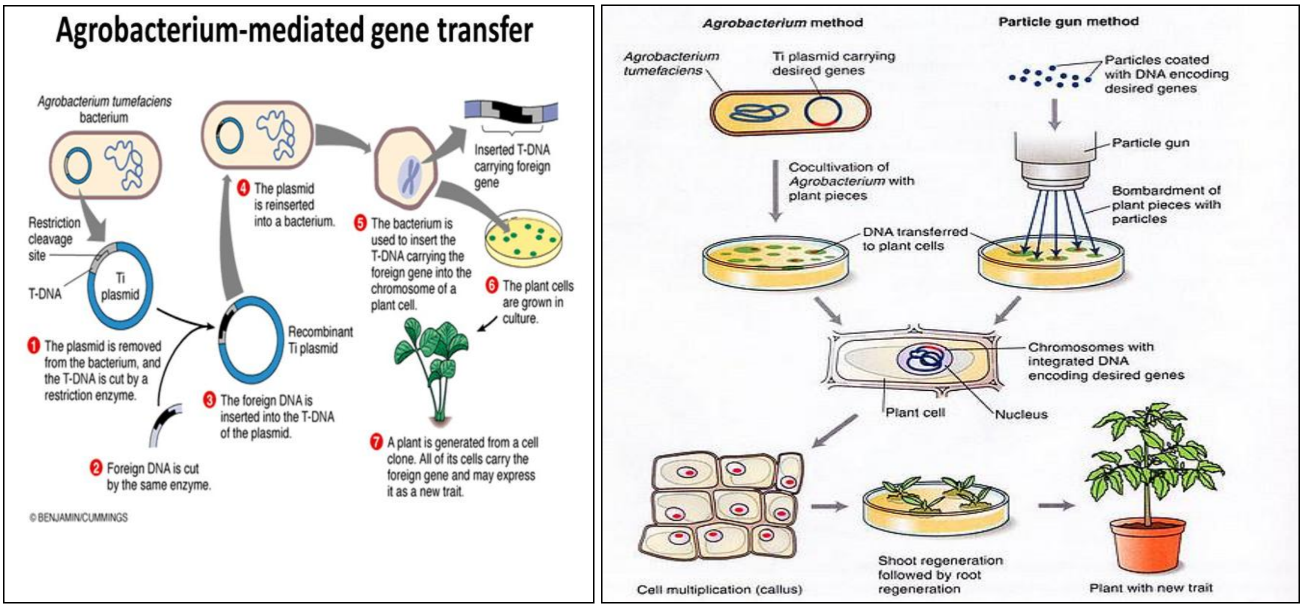


Fig 1: Agrobacterium mediated gene transfer

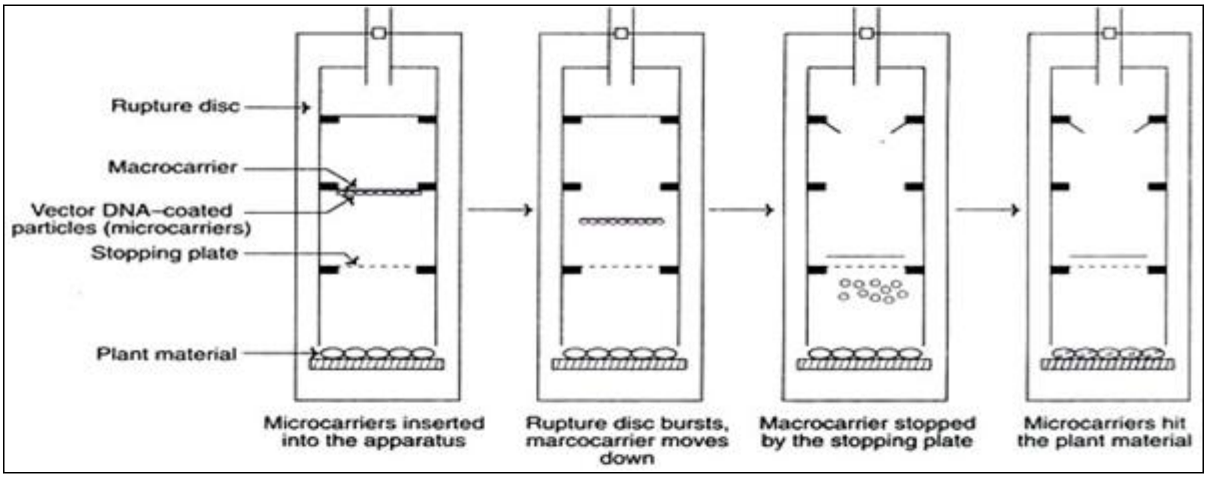
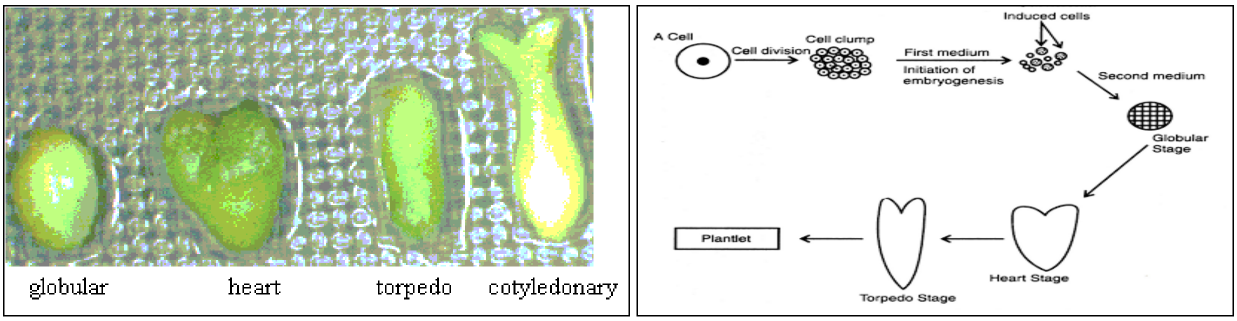


Fig. 2 A diagrammatic representation of particle bombardment (biolistics) system for gene transfer in plants.

Conventionally two tissue culture methods have been used for regeneration of transgenic plants: organogenesis and somatic embryogenesis. Organogenesis is the process in which plant regeneration occursby organ (shoot and root) formation on explants. Whereas, somatic embryogenesis is the formation of bipolar embryos from cell other than gametes or the products of gametic fusion (Pena and Seguin 2001; Rai *et al*. 2010) [45, 46]. Somatic embryogenesis appears to have many advantages over organogenesis, including its potentially high multiplication rates, potential for scale-up via bioreactor and delivery through synthetic seeds. Therefore, somatic embryogenesis has been emphasized as a suitable target for gene transfer (Merkle and Dean 2000) [38].



(A) (B)

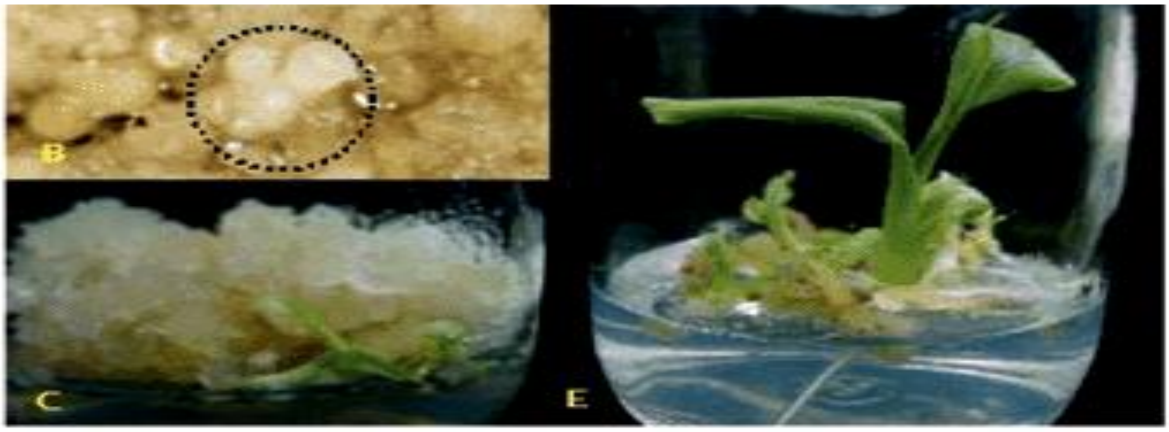


Figure 2. Somatic embryogenesis of banana cv. Maçã. A: embryogenic callus from cell suspension; B: somatic embryos; C and D: embryo plant conversion; E: plants from embryogenic callus.

Genetic engineering for pest and disease resistance

Virus resistance: The plant viruses reduce both the quality and quantity of crop yield by direct damage to plant. In several fruit crops, virus disease represents a particular problem, for example in grapes, the grape fan leaf virus (GFLV), in papaya the papaya ring spot virus (PRSV) and the grape vine chrome mosaic virus (GCMV) (Gonsalves, 1998) [18]. Transgenic resistance in fruitcrops can be obtained by pathogen derived resistance (PDR). PDR is operated in plants when genes from the virus is cloned and transferred to host genome. PDR is developed when the modulated viral gene products or virus related sequences in the plant genome interfere with the pathogenic virus infection cycle. Though many PDR strategies include coat protein (CP), antisense nucleic acids, satellite sequences, defective interfering molecules and non-structural gene (replicase, protease and movement protein), antibiotics and interferon related proteins are available (Kaniewski and Lawson, 1998) [28] for virus disease resistance, only CP mediated is successfully utilized in fruit crops. One such a successful example is transgenic papaya expressing CP of PRSV against PRSV. From a field trial (1992), two cultivars were developed and designated as 'SunUp' and 'Rainbow'. 'SunUp' is homozygous for the coat protein gene while 'Rainbow' is an F1 hybrid of 'SunUp' and the non-transgenic 'Kapoho'.Licenses to commercialize the transgenic papaya were obtained by the Papaya Administrative Committee in Hawaii by 1998. The transgenic fruit is currently sold in international market such as Canada and throughout the United States (Gonsalves, 2004) [19]. 'Honey Sweet' plum, a plum pox virus (PPV) resistant transgenic plum, has now been validated for cultivation in the USA (Scorza *et al*. 2013) [56].

There are other such reports of virus resistant transgenic plants expressing CP in many fruit crops (Table 1), but these are yet to be fully commercialized.

Table 1: Genetic modification of fruit crops for virus resistance.

Fruit crops	Gene(s)	System	Explants	Resistance in plants	Reference
Apricot (*P.armeniaca*)	CP-PPV	A.t	Cotyledons of immature embryo	PPV	Machado *et al*., 1992 [34]
Papaya(*Carica papaya* L.)	CP-PRV-4	PB	Zygotic embryos, hypocotyls	Some PRV strains	Fitch *et al*., 1992 [14]
Papaya	CP-PRSV	PB	Embryo geniccalli	PRV	Fitch *et al*., 1998 [15]
Plum (*P.domestica*)	CP-PPV	A.t	Hypocotyls	PPV	Scorza *et al*., 1994 [57] Ravelonandro *et al*., 1997 [47]
Plum (*P.domestica*) *L cv Bluefree*	CP-PPV	A.t	Leaf discs	PPV	Machado *et al*., 1994

PPV – plum pox virus (poty virus group): PRV/PRSV –papaya ring spot virus: A.t- *Agrobacterium tumefaciens*: PB-particle bombardment

Fungal resistance

Among diseases, fungi are the main cause of yield loss in fruit crops. They are controlled by several traditional techniques includingquarantine, sanitation, breeding and clonal selection of resistant varieties and application of fungicides. At present, research is focused on identifying the genes involved in resistance. Several proteins have been reported with antifungal activities which are otherwise called as pathogenesis-related proteins (PRs). Plant *b-1,3glucanases (PR-2) and chitinases (PR*-3) represent potential anti-fungal activity *in vitro* (Mauch *et al.,* 1988) [35]. In addition *b-1,3glucanases (PR-2)* release glucosidic fragments (secondary metabolites) from both the pathogen and host cell walls which could act as signals in the elicitation of host defences (Takeuchi *et al.*, 1990) [65]. The identified anti-fungal proteins are isolated from plants as well as from fungus such as *Trichodermaharzianum* (Melchers *et al.*, 1993) [37].

The transgenic fruit crops expressing the anti-fungal proteins are summarized in Table 2.

Table 2: Genetic modification of fruit crops for fungal disease resistance.

Fruit Crops	Gene(s)	System	Explants	Resistance in plants	Reference
Apple (*Malusdomestica*)	Endo-chitinase	A.t	Leaf	Against apple scab *Venturiainequalis*	Norelli *et al.*, 2000 [43]
Apple (*M. domestica*) Borkh McIntosh	ThEn-42	A.t	Maternal	Apple scab	Bolar *et al.*, 2000
Banana (*Musa spp*) Dwarf Parfitt (AAA-Cavendish)	-	Irradiation	shoots	Fusarium wilt-race 4	Smith *et al*., 1995
Kiwi fruit (*Actinidiadeliciosa*) cv. Hayward	osmotin	A.t	Maternal	Botrytis sp.	Rugini *et al*., 1999

ThEn-42- endochitinase from T. harzianum

Bacterial resistance: Important bacterial diseases of fruit trees are fire blight caused by *Erwiniaamylovora*in apple and pear, bacterial blight and canker (*Pseudomonads syringae)* and citrus canker (*Xanthomonascitri).* Research on resistance to bacterial disease has focused on genes producing the anti-microbial proteins like lytic peptides (cercopins, attacins and synthetic analogues shiva-1, SB-37), and lysozymes (egg white, T4 bacteriophage and human lysozyme).

Transgenic plants expressing these anti-microbial proteins against bacterial diseases are summarized as follows (Table 3).

Table 3: Genetic modification of fruit crops for bacteria disease resistance

Fruit crops	Gene(s)	System	Explants	Resistance in plants	Reference
Apple *(M. domestica)* M26	AttE	A.t	Leaf segment	E. amylovora	Norelli *et al*., 1994 [41]
Apple *(M. domestica)* cv. Galaxy	AttE: T4 lysozyme	A.t	Leaf segment	E. amylovora	Ko *et al*,. 1999 [29]
Apple *(M.domestica)* Bork. cv. Royal Gala.M7	AttE : SB-37: Shiva-1	A.t	Leaf segment	E. amylovora	Norelli *et al.*, 1999 [42]
Apple *(M. domestica)* M26	AttE	A.t	Maternal	E. amylovora	Norelli *et al.*, 1994 [41]
Apple *(M. domestica)* Bork	cercopin MB39	A.t	Maternal	E. amylovora	------
Pear *(Pyruscommunis)*cv. PasseCrassane	AttE	A.t	Leaf segment	E. amylovora	Reynoird *et al.*, 1999 [49]
Grape *(Vitisvinifera)*	pgip	A.t	Callus	*Xylellafastidiosa* causes Pierce’s disease	Aguero *et al.*, 2003 [1]

AttE- Lytic protein attacin E: pgip- Polygalacturonase- inhibiting proteins A.t: *Agrobacterium tumefaciens*

Nematode resistance: Many fruit crops are attacked by nematodes of the species of *Meloidogyne spp., Xiphinema spp., and Longidorus spp.* Nematodes are very difficult to eradicate from the infected soil and also their control by nematicides is not appropriate due to huge expenses and natural hazards. Potential anti-nematode genes have been reported and seem to be effective when they are constitutively expressed in plants, like gene over expressing collagenases which damage the nematode cuticle (Havstad *et al.* 1991) [21], exotoxin of *Bacillus thuringiensis* (Devidas and Rehberger, 1992) [13], anti-nematode monoclonal antibodies (Schots *et al.*1992) [53], etc. Molecular information on nematode resistance is limited and transgenic approaches by exploiting above nematode resistance genes need to be developed infruit crops.

Insect Resistance: Genetic engineering offers new approaches to more rapid implementation of anti-insect strategies in fruit crops. Several plants have been engineered with the aim of killing phytophagous insects by following strategies like genes encoding insecticidal crystal protein from *B.thuringiensis*, proteinase inhibitors, lectins, *a-amylase inhibitors*, chitinases, polyphenol oxidases and peroxidases, lipoxygenases, ribosome inactivating proteins (RIPs).Among the above techniques, crystal proteins and lectins were successfully employed in fruit crops. Some strains of *B. thuringiensis* have been known for insecticidal activity. This activity is due to their insecticidal crystal proteins which are toxic to several important insect pests of fruit crops. Genes encoding these crystal proteins have been cloned from *B. thuringiensis and are referred to as cry genes* (*cry1Aa, cry1Ab and cry1Ac).* They have been transferred into several fruit crops including apple, grapes and walnut (Table 4).

Table 4: Genetic modification of fruit crops for insect resistance.

Fruit crops	Gene(s)	System	Explants	Resistance in plants	Reference
Apple (*M.domestica*) cv Green leaves	Cry1Aa	A.t	Leaf segment	Lepidoptera	Dandekar, 1992 [11]
Apple (M. domestica) CV Green leaves	CpT1: Cry1Aa	A.t	Leaf segment	Lepidoptera Coleoptera	James *et al.*, 1992; 1993 [24, 25]
Grape (Vitisvinifera)	Cry1Aa	A.t	Stem segment	Lepidoptera	Singh and Sansavini,1998
Walnut *(Juglansregia)*	Cry1Ac	A.t	Somatic embryo	Lepidoptera	Dandekar *et al.*, 1998 [12]

Lectins are proteins with specific carbohydrate binding activity and seem to have multiple role in plant physiology. Over 300 purified lectins from seeds are toxic for animals. Some of them are toxic for coleopteran, lepidopteran, dipteran and homopteraninsects (Van Damme *et al*. 1998) [70]. The exact mode of action of lectins against insect pest is not yet clear. However, an insect diet containing mannose specific snowdrop lectin (GNA) is found effective in reducing larval growth of coleopteran and in reducing fecundity of adults of peach aphids (Sauvion *et al*. 1996) [52] reported that transgenic grapes expressing GNA had protection against lepidopteran pests.

Abiotic stress tolerance: Stresses like salinity, drought, heat, flood, frost, mineral toxicities, are often interconnected with oxidative stress that trigger a series of biochemical, physiological and molecular changes in plants, and may induce similar cellular damage (Ahmad *et al.*, 2010; Rai *et al.*, 2011) [2]. Plant responds to drought and/or salinity in mainly two phases, primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell and oxidative stress which may cause denaturation of functional and structural proteins (Jewell *et al*., 2010) [26]. Transgenic approach is now a widely used procedure for introducing genes from distant genepools, ranging from prokaryotic organisms such as *E. coli* to halophytes or glycophytes, into many plant species for the development of stress tolerant plants (Borsani *et al*. 2003) [9]. In recent years, a number of genes with diverse function and mechanism were employed for the development of transgenic fruit. However, in most cases this resistance/tolerance to abiotic stresses was linked to increased antioxidant capacity of the tissues or accumulation of compatible solutes through control of the genes involved in these mechanisms. Usually, abiotic stress tolerance through detoxification mechanism or accumulation of metabolites is likely to involve many genes at a time. Therefore, the attempts to develop transgenics for abiotic stress tolerance involved 'single action genes' i.e. genes involved in biosynthesis of a single metabolite that would confer increased tolerance, are unlikely to be sustainable (Bhatnagar-Mathur *et al*., 2008) [7].

Many studies have shown that stress-specific genes employed in transgenic experiments can be grouped into three major categories:(1) Genes involved in signal transduction pathways (STPs) and transcriptional control such as mitogen activated protein kinase (MAPK), CBL-interacting protein kinase (CIPK), SOS kinase and transcription factors like AP2/ERF, bZIP, MYB, MYC, NAC, Cys2His2 zinc-finger and WRKY; (2) Genes involved in membrane and protein protection functions such as heat shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins; synthesis of osmoprotectants like proline, betaine, sugars and sugar alcohol, and polyamines, and detoxification or elimination of reactive oxygen species (ROS) such as various enzymes and non-enzymatic antioxidants and (3) Genes involved in water and ion uptake and transport like aquaporins and ion transporters (Wang *et al*., 2003; Bhatnagar-Mathur *et al*., 2008; Ashraf and Akram 2009; Jewell *et al*., 2010) [72, 7, 5, 26].

Some genetically engineered crops for abiotic stress management traits are:

Table 1: Genes, Mechanisms and genetically modified fruit plant species implicated in plant responses to many stresses: some recent reports

Plant	Gene	Remarks	Perform of transgenic plant to abiotic stresses	References
Apple	Osmyb4	Encoding a transcription factor belonging to the Myb family, accumulation of several compatible solutes	Drought and cold	Pasquali *et al.*, (2008) [44]
	MdNHXI	Tonoplast Na^+/H^+antiporters	Salt	---
	PpCBFI	C-repeat binding factor (CBF/DREB). Transcriptional activator genes	Cold	Wisniewski *et al.*, (2011) [77]
	MdCIPK6L	Encode a CBL-interacting protein kinase (CIPK)	Salt, drought and chilling	Wang *et al.*, (2012) [73]
Banana	MusaDHN-1	Overexpression of dehydrin gene, belonging to a broader class of LEA proteins	Drought and salt	Shekhawat *et al.*, (2011a) [58]
	MusaWRKY71	Encode a WRKY transcription factor protein	Multiple abiotic stress	Shekhawat *et al*., (2011b) [59]
	MusaSAP1	Encode a zinc finger protein i.e. stress associated proteins (SAP)	Multiple abiotic stress	Sreodharan *et al*., (2012b)
Grapevine	DREB1b	Dehydration response element binding gene, a cold inducible transcription factor	Cold	Jin *et al.*, (2009) [27]
	VvCBF4	C-repeat binding factor gene, reduced freezing-induced electrolyte leakage	Cold	Tillet *et al.*,(2012) [67]
Kiwifruit	AtNHX1	Maintaining a relatively high K^+/Na^+ ratio	Salt	Tian *et al.*, (2011) [66]
Mulberry	*hva 1*	Encodes a group 3 LEA protein	Salinity and drought	Lal *et al.*, (2008) [31]
	Osmotin	Encode a group 3 LEA protein	Salt, drought and variety of fungal (biotic) pathogen	---
Papaya	C-repeat binding factor(CBF)	Encoding osmotin and osmotin-like proteins belonging to the plan PR-5 group of proteins	Cold	---
Pear	SAMDC2	Encode sadenosylmethionine decarboxylase, transgenic plants expressing polyamines	Salt	---
	SPDSI, SPDS	Encodes spermidine synthase, transgenic plants expressing polyamines	Salt, multiple abiotic stress	Wen *et al.*, (2008, 2009) [76, 75]
strawberry	Osmotin	Enhanced levels of proline, total soluble protein	Salt	Husaini and Abdin (2008) [22]

Genetically engineered crops for agro-horti traits

Reduction of the generation time

The long juvenile phase of trees is a limiting factor to their genetic improvement and preventing full domestication of most of the trees species (Pena and Seguin 2001) [45]. Meristem identity gene LEAFY (LFY), which is involved in flowering, has been characterized in *Arabidopsis thaliana* (Weigel *et al.,* 1992) [74]. LFY is a transcription factor which shortens the juvenile phase and promotes the transition from vegetative to the flower meristem. The juvenile period in apple has also been reduced by silencing the MdTFL1 gene (Kotoda *et al.,* 2006) [30] or overexpression of flower promoter FLOWERING LOCUS T(FT) or FT homologous gene (Trankner *et al*., 2010) [68]. In another study, European plum transformed with PtFT1 gene from *Populustrichocarpa* under the control of the 35S promoter produced fruits in the greenhouse within 1–10 months (Srinivasan *et al*. 2012) [64]. Transgenic lines showed the potential for accelerating breeding cycles, reducing juvenility time and adaptation to climate regimes.

Improvement in plant structure

During the last decade, many fruit plants have been transformed with rol A, B or C genes to improve rooting ability or dwarfism. The levels of plant hormones in plants were modified by over-expression of some specific genes i.e. rol A, B or C genes leading to morphological changes in transgenic plants (Pena and Seguin 2001) [45].

Improvement in nutritional status

Kim and his coworkers (2010) reported the development of transgenic kiwifruit lines with increased carotenoid content by over-expression of genes involved in its biosynthetic pathway i.e. geranylgeranyldiphosphate synthase (GGPS) and phytoene synthase (PSY). Ascorbic acid, one of important human dietary constituents, functions as an antioxidant to scavenge ROS and acts as cofactor for many enzymes (Conklin and Barth, 2004) [10].

Barriers and future prospects of genetically engineered crops

The public hesitance in European, Indian sub-continental and other countries to accept transgenic products and their risk to health and environment is also linked to the introduction of transgene into crop species of genetic material derived from alien organisms. To overcome the notorious aversion against transgenic, a new genetic engineering approach, namely ‘cisgenesis’ or ‘cisgenic’ has been proposed (Schouten *et al*., 2006; Molesini *et al.,* 2012; Vanblaere *et al.,* 2011) [54, 40, 69]. Cisgenic plants are those plants that have been genetically modified by one or more genes isolated from same speciesor from a sexually compatible one.By definition, cisgenic plants do not contain any foreign genes i.e. selection marker gene and they appear to be much closer to conventionally bred plants. Cisgenic approach has also been used todevelop disease-resistant apple (Vanblaere *et al.,* 2011) [69] andgrapevine (Dhekney *et al*., 2011). More recently, (Mlalazi *et al.* 2012) [39] isolated and characterized banana phytoene synthase (PSY) genes and suggested that this gene could be used as a cisgene for the development of cisgenic banana.

Another approach called ‘intragenesis’ differs from cisgenesis in the composition of the genetic construct, as intragene is not a perfect copy of a natural gene andcomposed of regulatory and coding sequences(gene editing and sequencing) derived from the same species itself or from sexually compatible species (Rommens *et al.,* 2007; Jacobsen and Schouten 2009; Molesini *et al.,* 2012) [50, 23, 40].

Application of cisgenesis and intragenesis

Recently, Joshi and his coworkera (2011) reported the role of the two genes HcrVf1 and HcrVf2 from wild malus spp. together with their native promoters and terminator (cisgenic approach) and combined with regulatory sequences of the apple rubiscogene (intragenic approach) in conferring resistance toapple scab. Genetic modification of fruit plants via cisgenesis or intragenesis may be other potential useful strategies. The acceptance of science-based approaches like cisgenesis or intragenesis or use of selection marker free transgenic will encourage confidence, and bring the benefits of GM-products to consumers. In addition, there is a great need for global coordination of regulations to remove artificial trade barriers, promoting technology transfer, and protecting developing countries from exploitation (Harfouche *et al.,* 2011; Gambino and Gribaudo 2012) [20, 17].

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