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Transgenic research in fruit crops for disease resistance

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Abstract

Fruits constitute an important part of the human diet. To meet the nutritional requirements of the increasing world population, a proportional increase in fruit production requires development of better varieties with higher yields under abiotic and biotic stress conditions. Many of the constraints faced by conventional methods of fruit breeding are overcome by use of the transgenic approach, which involves transfer of a target gene without altering the genetic makeup of a plant. Gene transfer through various methods can be done and it will be more useful where traditional breeding approach is not feasible. Tremendous progress has been made in different aspects of transgenic technology in the last few decades, that results in the development of commercial transgenic varieties in a few fruit crops. Now a days, major emphasis has been done on developing varieties resistant to diseases caused by fungi, bacteria and viruses. So that, the majority of target genes have been taken from a heterologous source. Breakthroughs in genome editing and high-throughput sequencing techniques hold the key for further progress in transgenic research in fruit crops.

Keywords: Fruit crops, transgenic, diseases, fungi, bacteria, viruses and resistant

Introduction

Horticulture contributes about 30% of GDP in agriculture, using only 17% land area. Horticulture production increased from 167 million tones in 2004-2005 to 283 million tones in 2013-2014 (Saxena and Gandhi, 2014) [57]. Fruit consumption is rising in India, reflecting the consumer's increased income, desire of diversity, and awareness of nutritional benefits. Some photochemicals found in fruits are strong antioxidants and are thought to reduce the risk of chronic disease by protecting against free radical damage, by modifying metabolic activation and detoxification of carcinogens, or even influencing processes that alter the course of tumor cells (Wargovich, 2000; Herrera *et al*., 2009) [75, 22]. Crop losses from diseases are of major concern due to devastating financial loss of farmers and starvation in developing countries. Applications of chemical pesticides are increasing day by day. These cause potential health hazards and even run-off of agricultural wastes from excessive use of pesticides and fertilizers can poison the water supply and cause harm to the environment. Biotechnology is a advanced powerful tool that has been added in most of the horticultural crops breeding programs. Genetically modified (GM) crops enable plant breeders to bring favorable genes, often previously inaccessible, into elite cultivars, improving their value considerably and offer unique opportunities for controlling insects, viruses and other pathogens, as well as nutritional quality and health benefits. Therefore, growing disease resistance GM fruit crops such as papaya, grapes, apple and plum etc. not only reduces the economic losses but also ensure to provide chemical free fruits.

Genetically Modified Horticultural Crops

Virus resistance

The plant viruses reduce both the quality and quantity of crop yield by direct damage to plant. In important fruit crops, virus disease represents a major problem, such as, the papaya ring spot virus (PRSV) in papaya, the grape fan leaf virus (GFLV) in grapes, plum pox virus (PPV) in plum and other stone fruits and citrus tristeza virus (CTV) in citrus (Gonsalves, 1998) [22]. Transgenic resistance in fruit crops can be obtained by pathogen derived resistance (PDR). PDR is operated in plants when genes from the virus is cloned and then transferred to host genome. PDR is developed when the modulated viral gene products /virus related sequences in the plant genome interfere with the infection cycle of pathogenic virus. PDR strategies include coat protein (CP), antisense nucleic acids, defective interfering molecules, satellite sequences and non-structural gene (replicase, protease and movement protein), antibiotics and interferon related proteins are available (Kaniewski and Lawson, 1998) [33] for virus disease resistance.

to achieve virus resistance in Generally CP mediated T technology is successfully utilized in horticultural crops. Gene silencing is another important biotechnological tool used horticultural crops. This method has provide transgenic plants which are able to switch off their endogenous genes and invading nucleic acids (Hannon, 2002; Scorza *et al.,* 2001; Voinnet *et al.,* 1999) [28, 62, 74].

Papaya: transgenic papaya expressing CP of papaya ring spot virus (PRSV) against PRSV is one of successful example of transgenic fruit crop. From a field trial (1992), two cultivars were developed and designated as SunUp and Rainbow. Sun Up is homozygous for the coat protein gene while Rainbow is an F1 hybrid of Sun Up and the non-transgenic cultivar Kapoho. Licenses to commercialize the transgenic papaya were obtained by the Papaya Administrative Committee and Rainbow and Sun Up have been successfully commercialized in Hawaii si
-+*09/8nce 1998, representing the first practical application of transgenic fruit crop (Gonsalves, 2002; Tripathi *et al*., 2007) [23, 69]. The transgenic fruit is currently sold in international market such as Canada and United States (Gonsalves, 2004) [24]. But CP-hemizygous line Rainbow is susceptible to non-Hawaiian PRSV isolates and the CP-homozygous line SunUp is resistant to a wider range of isolates from Jamaica and Brazil, but susceptible to isolates from Thailand and Taiwan (Gonsalves, 2002; Gonsalves, 1998; Tennant *et al*., 2001) [22, 23, 65]. In Taiwan transgenic papaya lines carrying the CP gene of a Taiwan severe strain- PRSV YK have also been successfully generated (Cheng *et al*., 1996) [11]. During the field trial, some transgenic papaya lines showed susceptibility to the new emerging papaya leaf distortion mosaic virus (PLDMV) that indicates, the virus is a threat for PRSV resistant transgenic papaya in Taiwan and elsewhere (Bau *et al*., 2008). Recently, transgenic papaya lines with double resistance against PRSV and PLDMV were developed using a chimeric construct strategy containing a fused cDNA fragment composed of truncated PLDMV P-TW-WF CP and PRSV YK CP coding regions. Three out of nine resistant transgenic papaya lines carrying the chimeric construct showed high levels of resistance to heterologous PRSV strains from Hawaii, Mexico and Thailand. Transgenic papaya lines found resistance to PRSV strains and PLDMV in Taiwan and elsewhere (Kung *et al*., 2009) [38].

Banana: *Xanthomonas* wilt caused by the bacterium *Xanthomonas campestris* pv. *musacearum* is one of the most devasting diseases of banana. The International Institute of Tropical Agriculture (IITA), in a partnership with Uganda's National Agricultural Research Organization (NARO) and other institutions have developed transgenic banana constitutively expressing the hypersensitivity response-assisting protein (*Hrap*) and the plant ferredoxin-like protein (*Pflp*) genes taken from sweet pepper (*Capsicum annuum*). These transgenic bananas have exhibited increased resistance to *X. campestris* pv. *Musacearum* (Tripathi *et al*., 2010) [67]. The best 65 transgenic plants expressing *Hrap* and *Pflp* and not showing any infection symptoms after artificial inoculation of potted plants with *Xanthomonas* wilt in the screenhouse were evaluated in a confined field trial in Uganda, against this wilt for two successive crop cycles (Tripathi *et al*., 2014) [68]. Most transgenic bananas had significantly higher host plant resistance than the non-transgenic control. After testing them as mother plants and first ratoon plants, 11 transgenic bananas were rated as having absolute resistance; means similar to the reference, resistant wild not-edible banana species *Musa balbisiana*. Transgenic bananas did not show significant changes in plant morphology as compare to the non-transgenic banana cultivar. Their plant phenotype and weight and size of bunch were similar to the non-transgenic counterparts. Transgenic bananas which showing wilt resistance will undergo multi-environment testing in Uganda. They will be further evaluated for environmental and food safety according to the country's biosafety regulations, risk assessment and management, plus procedures for seed registration and release (Tripathi, 2012) [66].

Stone fruits: *Plum pox virus* (PPV), the causing agent of Sharka, one of the worldwide most destructive diseases of plum and other stone fruits. In plum pathogen-derived resistance to PPV has been generated by the introduction of either wild type or mutated structural and non-structural genes into susceptible host plants. A gene silencing approach was used to generate transgenic European plums carrying a CP gene of PPV (Scorza *et al*., 2001) [62]. The diversity and dynamics of PPV and aphid population in transgenic European plums has been evaluated in the field for seven years under Mediterranean conditions (Capote *et al.*, 2008) [9]. After seven years of experimental trials, the transgenic line C5- cultivar Honey Sweet was observed to be free from PPV (Capote *et al*., 2008; Malinowski *et al*., 2006; Scorza, 2007) [9,43, 63]. The resistance mechanism of line C5 was confirmed to be based on PTGS (Hily *et al.*, 2005; Scorza *et al.*, 2001) [32, 61]. Now, Honey Sweet, a transgenic plum cultivar resistant to PPV has been validated for cultivation in the USA (Scorza *et al*. 2013) [61]. The transgene resistance technology and post-transcriptional gene silencing tested in Honey Sweet can be used to develop other resistant stone fruits, such as peach, Japanese plum, apricot and cherry, which are susceptible to PPV. A single chimeric transgene PTRAP6 was generated by the fusion of 400–500bp long fragments from six major Prunus fruit viruses - American plum line pattern virus (APLPV), Peach mosaic virus (PMV), Plum pox virus (PPV), Prune dwarf virus (PDV), Prunus necrotic ringspot virus (PNRSV), and Tomato ringspot virus (ToRSV). *Nicotiana benthamiana* plants transformed with PTRAP6 found resist to PDV, PPV, and ToRSV (Lopez-Gomez *et al*., 2008) [40]

Citrus: In citrus targeting citrus tristeza virus (CTV) using RNA interference (RNAi) strategies is the major strategy in plant transformation for CTV resistance. Citrus lines expressing p23 CTV- a silencing suppressor were found as resistant to CTV and transgenic viral protein accumulation was negatively correlated to resistance. In this study, post-transcriptional gene silencing (PTGS) was proposed as the mechanism for CTV resistance, since resistant citrus plants had multiple copies of the transgene, methylation of the silenced transgene, low levels of the corresponding mRNA and accumulation of the p23-specific small interfering RNAs (siRNAs) (Fagoaga *et al*., 2006) [16]. RNA-mediated resistance for CTV has also been generated in transgenic grapefruit (Rai, 2006; Febres *et al*., 2008) [55, 19]. CTV-resistant citrus plants that express the Coat Protein of the virus have been generated, in these plants virus resistance was linked to the accumulation of the transgenic viral protein in plant tissue, with Pathogen-derived resistance as the proposed mechanism of resistance (Valat *et al.*, 2006; Zanek *et al.*, 2008) [70, 80].

Baterial diseases resistance

Research on resistance to bacterial disease has focused on genes producing the anti-microbial proteins like lytic peptides (attacins, cercopins and synthetic analogues shiva-1, SB-37), and lysozymes (T4 bacteriophage, egg white and human lysozyme) (Panchaal *et al.*, 2017) [53].

Pome fruits: Expression of the lytic peptide attacin E in transgenic apples and pears provided resistance to the pathogenic bacterium (Ko *et al.*, 1999; Ko *et al.*, 2000; Hanke *et al.*, 2000) [2, 34, 35, 26] Another genes used to enhance fire blight resistance are epo, a gene encoding EPS-depolymerase (Hanke *et al.*, 2002; Flachowsky *et al.*, 2008) [27, 20], T4 lysozyme (Ko *et al.*, 2002) [26], SB-37 (Aldwinckle *et al.*, 1999) [2], hrpN (harpin), cecropin MB 39 (modified SB-37) (Liu *et al.*, 2001) [39] and Shiva-1 (Norelli *et al.*, 1999). GM apples resistant to fire blight have also been generated by partially silencing of certain genes encoding pathogen-protein receptors (Borejsza-Wysocka *et al.*, 2007) [7].

Walnut: To engineer resistance to *Agrobacterium tumefaciens*, a binary vector plasmid containing inverted repeats of portions of the iaaM and ipt genes was constructed and then transformed into walnut cultivar (Escobar *et al.*, 2002) [14]. Constitutive expression of this construct target RNAi-mediated degradation of the iaaM and ipt transcripts, demonstrating the use of RNAi to obtain resistance to a major bacterial disease (Escobar *et al.*, 2001) [13]. Because the construct and the oncogenes no need to have perfect homology for silencing to be effective, the resulting transgenic plants are resistant to a very large range of A*grobacterium tumefaciens* strains, displaying a broad spectrum durable resistance (Escobar *et al.*, 2002) [14]. Transgenic walnut plants containing the iaaM- and ipt-silencing constructs are currently under field trials.

Grapevine: Transgenic grapevines containing xylem-targeted effector proteins like polygalacturonase inhibiting protein (PGIP) and/or chimeric antimicrobial proteins have been obtained (Aguero *et al.*, 2005; Dandekar *et al.*, 2009) [12]. The use of PGIP to transform grapevines was based on the knowledge that *Xylella fastidiosa* causing Pierce's disease has genes that encode plant cell wall–degrading enzymes, including polygalacturonases (Aguero *et al.*, 2005) [1]. Five PGIP expression constructs were designed, each containing different signal peptide sequences in order to identify which most efficiently localizes PGIP to xylem tissues, as well as which provides the best distribution of PGIP through the graft union into untransformed scion tissues (Dandekar *et al.*, 2009) [12]. Chimeric antimicrobial protein strategy provides a protein-based therapeutic that targets the causative agent specifically resulting in its clearance, which results in resistance to the causative agent. Chimeric proteins have two domains, a surface binding domain and a clearance domain linked by a flexible linker such that both components can act independently(Dandekar *et al.*, 2009; Kunkel *et al.*, 2007) [12]. A chimeric antimicrobial protein was designed to obtain resistance to Pierce's disease that contained a surface recognition domain (SRD) and a clearance domain (CD). The SRD targeted the *Xylella fastidiosa* surface protein mopB, highly conserved among all *Xylella fastidiosa* strains and believed to be unique for *Xylella fastidiosa* linked to the CD, a lytic peptide with antimicrobial activity against Gram-negative bacteria. Transgenic grapevines expressing PGIP or the chimeric antimicrobial protein have been greenhouse propagated and mechanically inoculated with *Xylella fastidiosa* to validate their efficiency against Pierce's disease, the results obtained look promising (Dandekar *et al.*, 2009) [12]. Transgenic grapevines field trials in multiple locations started in 2010.

Citrus: Citrus cultivars has been transformed to improve Resistance against citrus canker disease by the expression of the *Sarcophaga peregrine* sarcotoxin, the attacin E antimicrobial peptide from *Tricloplusia ni* (Dutt *et al.*, 2009) [56], the Xa21 gene from *Oryza sativa* (Omar *et al.*, 2007) [51], and the harpin N gene (hrpN) from *Erwinia amylovora* (Barbosa-Mendes *et al.*, 2009) [3]. Such as, transgenic *Citrus sinensis* plants expressing hrpN under transcriptional control of a pathogen inducible promoter (gst1) showed up to 79% reduction in susceptibility to citrus canker compared with non-transgenic plants (Barbosa-Mendes *et al.*, 2009) [3].

Fungi disease resistance

Among diseases, fungi are the main cause of production loss in fruit crops. Fungal diseases are controlled by several traditional techniques including quarantine, sanitation, breeding and clonal selection of resistant cultivars and use of fungicides. At present, research is focused on identifying the genes imparting resistance. Several proteins have been reported with antifungal activities which are otherwise known as pathogenesis-related proteins (PRs). Plant *b-1,3glucanases (PR-2) and chitinases (PR*-3) represent potential anti-fungal activity under *in vitro* condition (Mauch *et al.*, 1988) [45]. In addition *b-1,3glucanases (PR-2)* release glucosidic fragments (a secondary metabolite) from both the pathogen and host cell walls which could act as signals in the elicitation of host defences (Takeuchi *et al.*, 1990) [64]. The identified anti-fungal proteins are isolated from plants as well as from fungus such as *Trichoderma harzianum* (Melchers *et al.*, 1993) [47].

Apple: Apple scab resistance locus- Vf, has been identified in the crabapple species *Malus floribunda* (Maliepaard *et al.*, 1998) [42]. The Vf locus confers resistance to five races of *Venturia inaequalis* 135 but not to races 6 and 7 (Guerin and Le Cam, 2004) [25]. Other scab resistance genes have been mapped onto the apple genome, but only the Vf locus has been analyzed in detail. Vf is a complex locus containing four paralogs, Vfa1, Vfa2, Vfa3, and Vfa4 (Xu and Korban, 2002) [76]. HcrVf2, a homolog to the *Cladosporium fulvum* resistance gene of tomato, was transformed into cultivar of apple 'Gala, imparting scab resistance (Belfanti *et al.*, 2004) [4]. In addition, the Vfa1, Vfa2, and Vfa4 genes were introduced into a plant cloning vector, pCAMBIA2301, and used for Agrobacterium-mediated transformation of 'Galaxy' and 'McIntosh' apple cultivars. Transformed lines expressing Vfa1 and Vfa2 exhibited partial resistance to scab, while transformed lines expressing Vfa4 were reported to be susceptible to scab (Malnoy *et al.*, 2008) [44]. The stilbene synthase (Vst) gene from *Vitis vinifera* L. is used to transform apple with apple scab resistance (Szankowski *et al.*, 2003) [63]. Alternative approaches to engineer resistance include expression of an antimicrobial peptide (Faize *et al.*, 2004) [18], chitinases (Mehlenbacher, 1995) [46], exochitinases and endochitinases. Transgenic apple plants expressing high levels of endochitinase are resistant to *Venturia inaequalis* (Bolar *et al.*, 2000) [6].

Grapevine: PGIPs have been reported to be effective in controlling *Botrytis cinerea*. Transgenic expression of pear PGIP in grape (Aguero *et al.*, 2005) [1] discourage fungal colonization by inhibiting fungal polygalacturonases and reducing susceptibility to *Botrytis cinerea*. Strategies based upon altering the expression of native plant cell wall–modifying enzymes have proved effective in controlling mold. Powdery mildew caused by *Uncinula necator*, is one of the most threatening fungal diseases in grapevine. Transgenic plants of grapevine harboring a constitutively expressing rice class I chitinase gene showed increased disease resistance to powdery mildew and anthracnose (Vidal *et al*., 2006) [72]. Another, transgenic 'Chardonnay' grapevines containing the magainin gene (mag2) - a peptide with broad spectrum antimicrobial activity showed symptom reduction in response to *Uncinula necator* under greenhouse conditions (Yamamoto *et al*., 2000) [78].

Strawberry: Strawberry has a lack of natural genetic resistance to gray mold, but transgenic expression of genes like ch5B encoding a chitinase (Vellicce *et al*., 2006) [71] and thau II encoding thaumatin II (Schestibratov and Dolgov, 2005) [59] from other plant species have been used to produce transgenic strawberry lines with resistance to *Botrytis cinerea*. Transgenic wild strawberry (*Fragaria vesca*) fruits over-expressing pectin methyl esterase gene (FaPE1) of *Fragaria* × *ananassa* showed high resistance to *Botrytis cinerea*. This resistance is related to the increase in pectin-derived oligogalacturonides which is essential components for elicitation of defense responses to *Botrytis cinerea* (Osorio *et al.,* 2008) [52].

Citrus: Orange plants have been engineered using the coding region of the tomato pathogenesis– related protein PR-5, a chitinase with antifungal activity. Transgenic plants as well as their detached bark were challenged with oomycete cultures and *Phytophthora citrophthora*. A significant reduction in lesion development was reported in one of the transgenic lines (Fagoaga *et al*., 2001) [15]

Table 1: Details of transgenic research in fruit crops for disease resistance:

Fruit crop	Disease	Research work	Reference
Apple *(Malus X domestica)*	Resistance to fire blight	Attacin E gene, cecropin MB-39 (modified SB-37 gene), Shiva-1	Liu *et al*., 2001 [39].
	Scab resistance	Ech42, Nag70, npt, AMP (antimicrobial peptide) gene, ThEN-42	Bolar *et al.,* 2000; Faize *et al.,* 2003 [17, 6].
Plum	Plum pox virus (PPV)	PPV coat protein	Hily *et al.,* 2004; Malinowski *et al.,* 2006; Polak *et al.,* 2008 [43. 31, 54].
Papaya (*Carica papaya*)	Papaya ringspot virus (PRSV)	Coat protein (cp) gene, PRSV replicase (rp) gene	Magdalita *et al*., 2004 [41].
	PRSV papaya leaf-distortion mosaic virus (PLDMV)	cp gene	Kung *et al*., 2010 [37].
Grapevine (*Vitis vinifera*)	Grapevine fan leaf virus (GFLV)	Coat protein gene, virE2	Xue *et al*., 1999 [77].
	Grapevine leaf roll virus (GLRV)	GLRa V-2, GLRa V-3 gene, npt11 gene	Gill *et al.,* 2004 [21].
	Xylella fastidiosa, Powdery	endogenous antifungal gene, albgene, defence gene,	Nirala *et al.,* 2010; Yamamoto *et al*, 2000; [49, 78].
	mildew and anthracnose	EGFP/NPT11, Lima-A, Lima-b, PR1 gene, RCC 2 (rice	Bornhoff *et al*., 2005
	Pierce's disease	chitinase gene) pPGIP (pear polygalacturonase inhibiting protein	Aguero *et al*., 2005 [1].
Banana (*Musa* spp.)	Bunchy top	Replicase associated protein, replicase inverted repeat, npt11	Tripathi, 2012; Vishnevetsky *et al.,* 2011; Yip *et al.,* 2011 [66].
	Xanthomonas wilt	Hrap, Pflp	
	Sigotoka leaf spot	pYc39	Fagoaga *et al*., 2001 [15].
	Virus	pAB6, pAHC17, pH1	Zanek *et al*., 2008 [80].
	Fusarium wilt	Pflp, npt11	Barbosa-Mendes *et al*., 2009 [3].
Orange (*Citrus sinensis*)	Fungi	Npt11, PR-5	
	Citrus psorosis virus	Coat protein	
	Citrus canker	hrpN gene from *Erwinina amylovora*	
Lime (*Citrus aurantifolia*)	Virus	Npt11, sgfp, p23	Fagoaga *et al*., 2006 [16]
Grapefruit (*Citrus paradisi*)	Citrus tristeza virus and aphid	Agglutinin, coat protein, GUS, npt11	Febres *et al*., 2008 [19]
	Citrus psorosis virus	RdRp (antisense and untranslatable version of replicase derived RNA polymerase	Cevik *et al*., 2006 2000 [10];
Guava (*Psidium guajava*)	Guava wilt	Endochitinase gene, npt11	Mishra *et al*., 2014; Biswas *et al*., 2005 [48, 5].
Strawberry	Verticillium wilt	cht 28 (plant origin chitinase gene)	Gill *et al*., 2004 [21].
	Grey mold	PGIP (from *Fragaria ananasa*)	Schaart *et al*., 2004 [58].

Challenges for Gm of Fruit Crops

Complex and costly regulatory processes, limited acreage of most horticultural crops makes it difficult to recover the costs of research and development and costly segregation of GM and non-GM commodities. Current practices in patenting and intellectual property protection is barriers to the use of biotechnology for the creation and commercialization of new horticultural crop varieties. Other challenges to the development and commercialization of GM horticultural crops include technical difficulties in the transformation of certain horticultural crops, barriers to regulatory approval in many countries, and the uncertainties of post-commercialization stewardship.

Conclusion

The consumption and production of horticultural products continue to increase with the rising demand for fruits by health conscious consumers. Biotechnological tool such as genetic transformation is ideal mean for speedy fruit production and have potential to resolve many of the world's hunger and malnutrition problems. And also help to protect and preserve environment by reducing reliance upon chemical pesticides.

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