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SCENARIO OF CITRUS BIOTECHNOLOGY- NATIONAL/ INTERNATIONAL

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ABSTRACT

Citrus is one of the world's prime fruit crops having great economic and health value. Citrus genetic improvement is impeded by barriers to sexual hybridization like nucellar embryony, long juvenility, self incompatibility and hybrid nature of important cultivated species. Further there is risk of destruction by diseases threatening to the citrus production and future availability for human consumption. Recently rapid technological advances made in citrus cell and tissue culture techniques, ploidy manipulation, genetic transformations, genome mapping and quickly adapted to mitigate the challenges to citrus biology. The classical areas of tissue culture regeneration pathways are micrografting for disease free planting material, nucellar embryogenesis for uniform rootstock seedlings (micropropagation), production of haploids and seedless citrus triploids through somatic embryogenesis for will open vistas for citrus genetics breeding and cultivar improvement. In this status report/ review the current state of various aspects of citrus biotechnology was reviewed and future strategies / application are speculated.

INTRODUCTION

Citrus is one of the most important and widely grown fruit crops, with total global production reported to be 117.7×10^6 t in 2010 (http://www.fao.org). Citrus crop is grown globally in all the tropical and subtropical regions, where temperatures are warm enough for tree survival but cool enough for adequate chilling and where adequate water and suitable soils are to support tree growth and subsequent fruit production. The most prominent production areas are found in the Brazil, the United States, Mexico, Argentina and the Mediterranean basin especially Spain, Italy, Egypt etc., and the South and East Asian regions predominantly China, India, and Japan. Despite the economic significance of the genus, genetic improvement of Citrus through conventional breeding is limited by their genetic and reproductive characteristics. Citrus species has a complex reproductive system, with many cases of cross and self compatibility, apomixes, heterozygocity and juvenility and mode of inheritance of important traits is unknown. Traditional varietal improvement programmes in Citrus have relied on limited sources of genetic variation, including: spontaneous mutants (e.g. limb sports); conventional breeding using local and exotic germplasm; and irradiation of seed and budwood. Advances in biotechnological approaches such as plant cell and tissue culture, genetic engineering, genomics made during past 5 decades have led to the employment of several new strategies for Citrus improvement. Some of these strategies combine biotechnology with traditional methods to maximize overall efficiency of improvement efforts. This paper is a consolidation of the current status of biotechnology relevant to Citrus breeding and improvement.

Regeneration, Micropropagation and Micrografting

The direction of genetic improvement in citrus is greatly impacted by the advances in plant cell and tissue culture. The amenability of citrus to be regenerated via organogenesis and somatic embryogenesis is the fundamental basis that makes possible much of the potential for these genetic advances. Plant regeneration systems are potentially useful for obtaining genetic change through cell transformation or mutagenesis. Organogenesis has been induced *in vitro* from various explants such as shoot meristems of seedling and mature trees, stem internodes, leaf sections and root tissues. *In vitro* culture of excised, fully developed embryos , early heart-shaped embryos, globular embryos within undeveloped ovules of mature fruits, and immature embryos (Cavalcante-Alves *et al*. 2003) had been used to recover plants. *In vitro* seedling explants were used for multiple shoot formation and/or regeneration (Yang *et al*. 2006). Regeneration has also been achieved by culturing

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thin sections of mature stem segments (Soneji *et al*. 2007; Sharma *et al* 2009; Montoliu *et al* 2010; Vijayakumari 2014). Many genotypes of citrus have the capacity to regenerate through nucellar embryony, hence citrus somatic embryogenesis has become more researched topic. Somatic embryogenesis has been induced directly in cultured nucelli and undeveloped ovules (Gmitter and Moore 1986) or indirectly via callus formation (Tomaz *et al*. 2001; Kayim and Koc 2006). Embryogenesis has also been induced from endosperm-derived callus (Gmitter *et al*. 1986), juice vesicles , anthers (Chiancone *et al*. 2006), styles (Calovic *et al*. 2003), and pistil thin cell layers.

Investigations to achieve embryo to embryo proliferations, their maturity and convertibility, yielding multiple plantlets are required, particularly in case of rootstocks. Production of nucellar in scion species is undesirable due to the prolonged juvenile phase, like zygotic seedlings, and slightly poor fruit quality (Chaturvedi and Agnihotri 2002).

Micrografting for rejuvenation of old orchards/ establishment of healthy orchards/ mass production of disease free planting material.

The micro grafting technique was implemented and successfully demonstrated to revolutionize citrus production making Spain the top citrus fruit exporter in the world (Navarro *et al* 2004). Virus sick citrus industries of Spain and other countries were rejuvenated by successfully employing this technique. *In vitro* micro-grafting is well established as a method for eliminating viruses and other pathogens from Citrus. These techniques are now used in programs to supply 'clean' bud wood to Citrus nurseries and growers in many countries including California, Florida, Spain, Argentina, Australia, Pakistan and South Africa,. (Gumpf *et al* 1996,). Such programs are fundamental to the continued success of Citriculture as increased travel and more rapid dissemination of new cultivars have increased the risk of introduction of new diseases. At Central Citrus Research Institute, a successful STG based Citrus sanitation programme was implemented and tristeza, greening and major graft transmissible pathogens were eliminated through micro grafting from *C. reticulata and* 21 *Citrus* introductions (Vijayakumari and Karihaloo 2012; Vijayakuimari 2019). Later protected and field foundation blocks of elite, certified, STG derived healthy nucleus stock were established . About 3.50 lakh STG derived disease free planting stock was released during 2002 to 2015 to the Citrus growers/nurserymen facilitating raising of 1000 ha of orchards predominantly in Central India. The results of trials on vegetative growth parameters including yield indicated shoot tip grafted plants are either significantly superior or at par in growth performance in terms of plant height, stem height, stock girth, scion girth and canopy volume and number of roots compared with commercial budded plants throughout central India. (Vijayakumari, 2013, 2014,2017,2019).

Somaclonal Variations

Somaclonal Variation is defined as genetic variation present among *in vitro* regenerated plants that is either uncovered or induced by a tissue culture process. Somaclonal Variation can be exploited to alter the maturity dates, sugar/acid ratio, color, seed content and other characteristics of existing varieties. This phenomenon is mostly studied in sweet orange cultivars and preliminary data suggests that populations of plants regenerated via organogenesis and from protoplasts via somatic embryogenesis are more viable than those derived from embryogenic callus or directly from nucellar seed.

Somaclonal variation can certainly be exploited as a tool to expand the maturity dates and reduce the seed content of commercially important Citrus cultivars. Long-term cultures may accumulate minor genetic changes (i.e. mutations/movement of transposons (Kubis *et al*. 2003) or cytological aberrations such as inversions or translocations) that do not significantly affect the mitotic index or interfere with the plant regeneration process, thereby resulting in regenerated plants with the observed minor but significant variation.

Citrus plants rejuvenated from nucellar callus of monoembryonic 'Clementine' mandarin have been detected with somaclonal variation. It is being exploited to identify sweet orange clones with improved traits such as fruit quality improvements across an extended season of maturity. Somaclones of 'Hamlin' and 'Valencia' have been obtained via regeneration of adventitious shoot buds, regeneration of secondary embryogenic callus via somatic embryogenesis, and/or regeneration from protoplast via somatic embryogenesis. Of these, early- and late-maturing somaclones, somaclones with fresh market potential, as well as somaclones with elevated soluble solids of 'Valencia' and 'Hamlin' with improved color are under field trial. 'Femminello' lemon somaclones have also been evaluated for tolerance to mal secco by artificial inoculation (Gentile *et al*. 2000).

In vitro mutagenesis and somaclonal variation techniques were used for fruit crop improvement to induce salt tolerance in a commercial citrus rootstock, 'Rough Lemon' (Citrus jambhiri Lush.). For induction of in *vitro* mutations, gamma rays (physical mutagen), ethyl methane sulphonate and methyl methane sulphonate (chemical mutagens) were used. Calli of 40 and 60 days old (DOC) were treated with gamma rays (0, 10, 20, 30, 40, 50 and 60 Gy) under physical mutagenesis. Calli of 45 and 60 DOC was subjected to chemical mutagens (EMS and MMS) at different concentrations (0.1, 0.2, 0.3 and 0.4 %) by culturing them directly (control I) or prior to culturing, shaked in liquid medium without (control II) or with mutagens (EMS and MMS) treatment After observing the survival and the regeneration potential, 10–20 and 20– 30 Gy were regarded the optimum mutagenic doses of gamma rays for 40 and 60 DOC, respectively. For chemical (EMS and MMS) mutagenesis for 45 DOC, 0.1 % each was found most suitable dose, whereas 60 DOC failed to regenerate after mutagens treatment. For in vitro salt screening of the somaclones and mutants salt concentration of > 25 mM (NaCl) was found to be optimum. Calli of 170 days old when exposed to different concentrations of NaCl (0, 25, 50, 75 and 100 mM), four somaclones were obtained at 25 mM and one each at 50 and 100 mM (Krishnan Kumar *et al* 2010).

Ploidy Manipulation

Polyploid plants may offer considerable prospective for cultivar improvement through exploitation of their horticulturally useful characteristics and as parents in breeding programs, particularly the triploid and tetraploid lines. Production of triploid hybrids is the most promising approach

to obtain cultivars that do not produce seeds even with substantial cross-pollination (Navarro *et al*. 2004). Recovery of citrus sexual triploid hybrids (3x = 27) has been reported from 2n x 4n, 4n x 2n crosses. 'Shasta Gold1'or 'TDE2' (a late maturing), Tahoe Gold1'or 'TDE3' (mid-season maturing), and Yosemite Gold1'or 'TDE4' (mid-late season maturing) are triploid mandarin hybrids of tetraploid female parent ('Temple' tangor, 'Dancy' mandarin) and diploid male parent 'Encore' mandarin that combine large fruit size, attractive deep orange rind color, rich fruit flavor, and the virtual absence of seeds (Roose and Williams 2006 ; Russo *et al*. 2003). 'Tacle' and 'Clara' seedless triploid mandarins were obtained by crossing diploid female parent 'Monreal' Clementine and tetraploid male parent 'Tarocco' orange, while 'Camel' mandarin was a triploid hybrid of diploid female parent 'Nules' Clementine and tetraploid male parent 'Willowleaf' mandarin . Embryo rescue and culture *in vitro* are necessary because embryos that arise when diploid seed parents are crossed with tetraploid pollen sources do not undergo normal development. Triploids have also been regenerated by *in vitro* culturing of hybrid endosperm. However, this method has not been adapted as a breeding strategy because it is species and cultivar dependent, and is far less efficient than creating triploid offspring by interploid hybridization. Incorporation of colchicine in standard tissue culture media has made it possible to recover tetraploid plants of elite diploid selections or cultivars.

Anther culture is another useful tool to the breeders/ researchers to recover plants of reduced ploidy level for Citrus cultivar improvement and genetic studies (Gmitter *et al*. 1992). Citrus and Poncirus (Benelli *et al* 2010) anthers have been cultured in attempts to produce haploid plants (Chen *et al*.2011; Germana *et al*. 2011). Haploid plants may have many applications for citrus breeding: easier mutations detection; double-haploids formation by diploidization of their chromosomes, allowing recovery in just one generation of homozygous plants, which is very difficult in tree crops.

Haploids are plants with a single set (haploid set) of chromosomes and doubled haploids are formed when *haploid* cells undergo chromosome *doubling*. The production of haploids and doubled haploids (DHs) permits a single-step development of complete homozygous lines from heterozygous parents, reducing the time required to produce homozygous plants compared to conventional breeding. The production of haploids and DHs provides a particularly attractive biotechnological tool and had a significant impact on agricultural systems. currently, these biotechnologies represent an integral part of the breeding programmes of many agronomically important crops. Among the available methods to obtain haploids and double haploidss, *in vitro* anther or microspore culture are the most effectively used.(Germana 2011).

Haploid and doubled-haploid plants obtained in some fruit species through anther culture (Chiancone *et al*. 2006; Germana` and Chiancone 2003), microspore culture (Ho¨ffer 2004), *in vitro* pistil culture pollinated with pollen of a triploid plant, in situ parthenogenesis obtained using irradiated pollen grains (Froelicher *et al*. 2007) and ovule culture (Chen *et al*. 2011). However, Citrus, like most other genera, have shown to be most genotype-dependent for gametic embryogenesis, and gametic embryos and plant recovery successfully obtained only in few cultivars of clementine and mandarin (Germana and Chiancone, 2003). In sweet orange genotypes, only two reports are available on the induction of haploid and double-haploid callus, due to intense recalcitrance for gametic embryogenesis using anther or isolated microspore culture, which makes the task of obtaining homozygous plants very difficult. Among the various factors affecting anther culture, genotype, anther pre-treatment (e.g. high or low temperatures) and osmolarity of the culture medium demonstrated to be very effective to induce microspore-derived embryos, also with different levels of ploidy. In Citrus, only a few cultivars of *Citrus clementina* showed a good capacity of induction ofgametic embryogenesis. (Germana` 2011)

Divya & Grosser (2010) reported induction of auto tetraploids in pummelo through colchicine treatment of meristematically active germinating seeds in University of Florida. Near the home Vijayakumari (2017) Standardized a novel methodology of producing tetraploids based on *in vivo* colchicine treatment of microbudded plants of two commercial citrus rootstocks viz Rough lemon & Rangpur lime. Few surviving Rough lemon & Rangpur lime microbudded plants after ploidy analysis by flowcytometry were confirmed as tetraploids and mixoploids. Further Vijayakumari (2017)Devised a new method for production of Stable tetraploids in commercial citrus rootstocks viz., Rough lemon and Rangpur lime through colchicines treatment of meristematically active seeds in vitro. The ploidy of seedlings at 1-2 expanded leaf stage of seedling was confirmed using flow cytometry and chromosomal counts . Tetraploid in scions are essential for interploid hybridization to create triploid seedless citrus.Tetraploid citrus rootstocks will facilitate advanced production systems & sustainable citruculture

Somatic Hybridization

Plant somatic hybridization via protoplast fusion has become an important tool for ploidy manipulation in plant improvement schemes, allowing researchers to combine somatic cells from different cultivars, species, or genera, resulting in novel allotetraploid and autotetraploid genetic combinations.

Somatic hybridization allows production of somatic hybrids that incorporate genomes of the two parents without recombination, thus avoiding the problem of the high heterozygosity in citrus (Navarro *et al*. 2004). In citrus, somatic hybridization has been extensively utilized and has many important inferences. The first successful protoplast isolations were reported as early as 1982 and the first citrus somatic hybrid was obtained between *C. sinensis* and *P. trifoliata*.

The greatest level of success for fruit breeding has occurred in Citrus, primarily due to the highly successful model of fusing embryogenic suspension derived protoplasts with leaf-derived protoplasts, resulting in the regeneration of somatic hybrid plants from nearly 500 different parental combinations (Grosser and Gmitter 2011).

In Citrus, somatic hybridization is being used to generate key allo-tetraploid breeding parents for use in interploid crosses to generate seedless triploids . Seedlessness has become a primary breeding objective of all citrus fresh fruit improvement programs, as seedless fruits are much preferred in the marketplace. Somatic hybridization via protoplast fusion technology provides the opportunity to combine

complementary elite diploid scions into allotetraploid somatic hybrids. Flowering somatic hybrids are being used as breeding parents in interploid crosses with selected complementary diploid parents to generate triploid progeny. Autotetraploids, often a by-product of somatic hybridization experiments and also produced by other *in vitro* techniques, are also used as parents in interploid crosses. However, more variation in triploid progeny is generally observed when using allotetraploid parents.University of Florida/ IFAS, CREC generated more than 12,000 triploid citrus hybrids from interploid crosses, with a few thousand of these being fathered by somatic hybrids (Grosser and Gmitter 2011).

Somatic hybridization has provided a means of producing heterozygous tetraploid hybrids, which have incorporated complementary traits from donor parents. It has made production of hybrids from sexually incompatible or difficult to hybridize citrus relatives that possess valuable attributes possible, thus broadening the germplasm base available for rootstock improvement. Somatic hybridization in rootstock breeding program is facilitating combining of desirable characteristics such as the biotic and abiotic resistances with wider soil adaptation, productivity, and the ability to control tree size. The strategy for rootstock improvement was to combine complementary diploid rootstocks via protoplast fusion to generate tetraploid somatic hybrid rootstocks. More than 100 such somatic hybrid rootstock combinations have been produced, propagated by rooted cuttings, and many have entered commercial field trials. Tetraploid citrus rootstocks have continuously shown the ability to reduce tree size, including somatic hybrid rootstocks. Somatic hybrids such as sour orange + Rangpur lime or sour orange + Palestine sweet lime, can yield over 22 tons fruit per acre on trees approximately 3–4 m in height.

Somatic hybridization technology facilitated scion improvement by generating superior allotetraploid breeding parents for use in interploid crosses to generate seedless triploids and enabled rootstock breeding, incorporating disease and insect resistance, wide adaption, high yields of quality fruit and tree size control (Gmitter *et al* 1992)

Transformations

Genetic transformation, a biotechnological tool, allows the release of improved cultivars with advantatious characteristics in a shorter period of time and therefore may be useful in citrus breeding programs. A number of laboratories has now been achieved Citrus transformation by various methods. The Citrus transformation researches which are currently being implemented in the countries are USA, Brazil, Spain, China, Pakistan, India etc in the following species C. *sinensis* L. Osb, C. *paradisi* Macf, C. *unshiu* Marc, *Poncirus* trifoliata L. Raf, C*arrizo* citrange, C. *aurantium*, C. *macrophylla,* C. *limon Troyer citrange*, C. *limonia* Osb, C. *jambhiri* Lush, C. *reticulata* Blanco and *Tangelo* by following mainly A. tumefaciens transformation method and exceptionally in some cases by A. rhizogenes, particle bombardment, Electroporation, PEG, RNA interference for the traits viz., resistance to pests, fungi(phytophthora), bacteria (citrus greening disease), virus (CTV), and improvement of agronomical traits (fruit quality and parthenocarpy) (Soler *et al*. 2012).

Genetically modified crops were first launched in the U.S in the mid-1990s. The GM Citrus trees which are currently in field trials are citranges (Spain), oranges & Lemons (Italy) for resistance to fungi & characteristics of flowering and fruiting and grapefruit, lime, citrange (USA), orange (Argentina), lemon (Mexico) and orange (Brazil).

Transformed sweet orange and citrange lines carrying the selectable marker genes that are most commonly used in citrus transformation are substantially equivalent to the non-transformed controls during long-term agricultural evaluation trials at IVIA, Spain. This information is essential to be able to focus mainly on the pleiotropic effects that may be induced by the insertion of gene(s) of interest in future experiments with GM citrus. Citrus tristeza virus (CTV), the causative organism of the most fastadious viral disease of citrus, has evolved three silencing suppressor proteins acting at intra- (p23 and p20) and/or intercellular level (p20 and p25) to overcome host antiviral defense. Using an intron-hairpin vector carrying, untranslatable versions of the genes p25, p20, and p23 from CTV strain T36 Mexican lime was genetically transformed to silence the critical gene expression in infected CTV cells. Complete resistance was displayed by three transgenic lines to viral infection, with all their subsequent propagations remained virus free and showing no disease symptoms after attempting graft inoculation with CTV-T36, either in the non-transgenic rootstock or in the transgenic scion. For CTV resistance accumulation of transgene-derived siRNAs was one of the major requirements. Partial breaking the resistance was recorded after Inoculation with a divergent CTV strain, indicating the role of sequence identity in the underlying mechanism. Employing RNA interference (RNAi) the three viral silencing suppressors appears crucial, for developing transgenic resistance to CTV.

Genetic transformation is an fascinating field of biotechnology for genetic improvement of citrus. In general transformation efficiencies are low and cultivar dependent. Many of the commercially important *citrus* species are recalcitrant to the efficient regeneration process which resulted in the unsuccessful transformations (Pena *et al* 2007). Further, difficulty in rooting the transgenic shoots of few citrus cultivars has been reported. Hence genotype specific *in vitro* regeneration protocols need to be developed for evolving successful genetic transformants. Standardization of direct genetic manipulation techniques has provided novel opportunities for plant improvement. Plant transformation protocols facilitated modification of one or two traits without loosing unique characteristics of original cultivar. For genetic transformation of Citrus the traits that could potentially be manipulated, include pest and disease resistance, tree growth habit, and fruit quality parameters. Development of efficient regeneration pathways and genetic transformation systems are essential for successful application in citrus improvement.

Table 1 Important Transformations researches in Citrus

Species	Transferred genes	Transformation method	Refer-ences
C. jambhiri Lush	*GUS* and *npt*II	*A. tumefaciens*	[1]
C. sinensis L. Osb.	*gfp* and *pme*	PEG	[29]
Swingle citrumelo	*uid*A, *npt*II, and *GUS*	*A. tumefaciens*	[41]
Carrizo citrange	*GUS* and *npt*II	*A. tumefaciens*	[50]
Carrizo citrange	*GUS, GFP*, and	*A. tumefaciens*	[17]

and *C. aurantifolia*	*npt*II		
Carrizo citrange	Citrus blight-associated	*A. tumefaciens*	[35]
C. sinensis and *C. limonia*	*GUS*	*A. tumefaciens*	[2]
Carrizo citrange	*uid*A and *npt*II	Particle bombardment	[5]
C. sinensis	*pTA29-barnase*	*A. tumefaciens*	[39]
Citrus sinensis	*PMI*	*A. tumefaciens*	[7]
Citrus sinensis	*GUS* and *npt*II	*A. tumefaciens*	[3]
Citrus aurantium L.	*GUS* and *npt*II	*A. rhizogenes*	[13]
Citrus paradisi Macf.	*cp* and GUS	*A. tumefaciens*	[19]
C. sinensis L. Osb.	*GUS*	Electroporation	[46]
Carrizo citrange and *C. sinensis* L. Osb.	*GUS*	*A. tumefaciens*	[66]
Citrus sinensis L. Osbeck	*GUS* and *npt*II	*A. tumefaciens*	[36]
C. reticulata Blanco	*pTA29-barnase*	*A. tumefaciens*	[40]
C. paradisi Macf.	Carotenoid biosynthetic genes	*A. tumefaciens*	[16]
Carrizo citrange	*LFY* and *AP1*	*A. tumefaciens*	[49]
C. aurantium L.	*Cp*	*A. tumefaciens*	[25]
C. paradisi Macf.	*CP* and *T36*	*A. tumefaciens*	[44]
Troyer citrange	*Bar* and *uid*A	*A. tumefaciens*	[52]
C. aurantifolia Swing.	*Cp*	*A. tumefaciens*	[18]
C. sinensis (L.) Osb.	*Gfp*	PEG	[20]
C. aurantifolia Swing.	*GUS*	*A. tumefaciens*	[37]
C. paradisi Macf.	*GUS, uncp, gna*	*A. tumefaciens*	[63]
Carrizo citrange	*uid*A and *npt*II	*A. tumefaciens*	[11]
C. sinensis L. Osb.	*GUS*	*A. tumefaciens*	[06]
C. aurantifolia (Christm.) Swing.	*GUS* and *npt*II	*A. rhizogenes*	[19]
C. aurantium L.	*Cp*	*A. tumefaciens*	[31]
Tangelo	*GUS* and *npt*II	Particle bombardment	[65]
Carrizo citrange	*GUS* and *npt*II	*A. tumefaciens*	[47]
C. sinensis L. Osb.	*GUS* and *npt*II	*A. tumefaciens*	[48]
C. reticulata Blanco	*GUS*	Electroporation	[30]
Citrus spp.	*GUS* and *npt*II	*A. tumefaciens*	[42]
Citrus spp.	*cat* and *npt*II	PEG	[59]

Dicle Donmez *et al*. 2013 (http://dx.doi.org/10.1155/2013/491207).

Genomics/ Genome Characterization

There is a lack of knowledge and understanding of the genetic mechanisms that control important traits such as disease resistance, cold tolerance, juvenility/maturity, and aspects of fruit ripening process (Gmitter *et al*. 1992). The entire field of citrus biology and genetics can be revolutionized by expanding the potential capabilities of genomics and bioinformatics to cultivar improvement through precise and targeted manipulations of the genome.

A number of molecular marker techniques based on identification of specific DNA fragments are now available to the plant biotechnologists, pathologists, microbiologists and breeders. The molecular markers are primarily of three major categories: (i) southern hybridization markers (Restriction Fragment Length Polymorphism-RFLP, Variable number of tandem repeats- VNTR), (ii) PCR based markers (Random Amplified Polymorphic DNA- RAPD, Simple Sequence Repeats-SSR, Inter Simple Sequence Repeats- ISSR, and (iii) Combination of RFLP and PCR based markers (Amplified Fragment Length Polymorphism-AFLP. Among the diverse range of molecular markers techniques, SSRs or microsatellites are well known for their potentially high information content and versatility. In addition to the above, single nucleotide polymorphism (SNPs) functional diversity analysis, functional genomics involving allele mining, association genetics and comparative genomics analysis. With respect to use of molecular markers for identification, the following applications have been made in citrus: Cultivars, genotypes, pest and pathogens, true to type plants at juvenile stage, pure seeds and hybrids, Mutants and chimeras, Nucellar and zygotic embryos, Somatic hybrids in fusion experiments, Somaclonal variations.

Phylogenetic and taxonomic studies in citrus have also been widely carried out by molecular markers. Some efforts have also been made in the areas of resistance gene candidates, satellites, microsatellites (Chen *et al* 2011), variations from fragment restriction, methylation, and individual gene expressions. The rapid development of molecular marker technologies has made it possible to investigate gene expression and has helped in construction and integration of genetic and physical maps of the economically beneficial traits such as CTV resistance (Gmitter *et al*. 1992), nematode resistance, fruit acidity, and dwarfing .These genetic maps may provide the basis for early screening procedures, thus permitting breeders to make initial selection among very young progeny based on the phenotype predicted by their genotype at molecular loci known to co-segregate with a particular phenotype. These are very essential for map-based cloning (MBC) approach and marker-assisted selection (MAS) breeding programs.

DNA-based molecular markers may be used to select rootstocks that may contain many of the desired resistances to CTV (Gmitter *et al*. 1992), nematode ,Phytophthora, etc. This will prove to be highly cost-efficient as compared to traditional greenhouse or field screening facilitating the selection in a shorter period.

MBC is also called as positional cloning. It is different approach to isolate gene(s), without prior knowledge of gene product, using tools of comprehensive genetics, genomics, and bioinformatics. MBC of genes for CTV resistance from *P. trifoliata* has provided target gene sequences (Gmitter *et al*. 1992).

To support genetic improvement efforts for *Citrus* crop, the international Citrus Genetics Community has collaborated with International Sequencing Centers in the development of freely available genomic resources. Prominently, two full-length annotated genome assemblies have been produced and made available to the global research community. The first genome, based substantially on Sanger sequencing, is from a haploid plant derived from 'Clementine' mandarin, to serve as the reference genome for citrus. Production of the sweet orange clone 'Ridge Pineapple', was a second genome assembly. Extensive EST datasets and a number of microarray platforms for investigating the transcriptomic responses of *Citrus* species and hybrids to a wide range of conditions have been shared to support exploitation and utilization of genome sequence information. As many researchers in the citrus genomics community (Chen *et al;* 2006; Tao *et al*; 2007) are actively engaged in genetic improvement programs, there has been a natural integration of improvement efforts with the rapidly developing genomic tools.

Work is in progress (In Dr. F. G Gmitter's laboratory in University of Florida, Citrus Research and Education Centre, Lake Alfred, Riverside, USA and by different scientists in Brazil, France and Japan) to expand and improve the citrus genome sequence resources and tools to enable application of sequence-derived knowledge in improving citrus plants and to better managing their interactions with biotic and abiotic factors.

CONCLUSION

Biotechnology holds considerable promise as a means of developing new cultivars with improved quality, diseases and pests resistance, stress tolerance and productivity. Advances in plant cell and tissue culture have major impact on genetic improvement of *Citrus*. Somatic hybridization can create new combinations that were previously impossible because of sexual incompatibility. Recovery of monoploids, triploids and tetraploids have expanded the range of germplasm valuable to Citrus breeders. Interploid hybridization for developing seedless triploid clones can be facilitated by embryo rescue *in vitro* and applying flow cytometry to detect hybrid ploidy levels. Improved endosperm culture methods may be useful for producing seedless hybrids. Molecular markers are already enabling breeders to better understand the origin and diversity of *Citrus* germplasm and to improve the efficiency of breeding through markers aided selection. The fundamental reference genome from the triploid Clementine will serve well for accessing and utilizing the newly produced genome sequences. Deep sequencing of Citrus transcriptomes is already underway in gene expression studies of host response to pathogens; with a major emphasis currently on Huanglongbing (*Citrus* greening or HLB) most severe disease ravaging Citrus production on nearly global basis. Genetic transformation has great potential use in Citrus cultivar development. Efficient protocols for transformation of several genotypes have been developed & introduction of genes of potential agronomical interest has been accomplished but transformation will require evaluation before release.

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