Chapter

Citrus Biotechnology: Current Innovations and Future Prospects

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Abstract

Citrus is a valuable fruit crop worldwide. It not only provides essential minerals and vitamins but is also of great commercial importance. Conventional research has contributed a lot to the improvement of this fruit plant. Numerous improved varieties have been developed through conventional breeding, mutational breeding, polyploidization and tissue culture yet pathogens continue to emerge at a consistent pace over a wide range of citrus species. Citriculture is vulnerable to various biotic and abiotic stresses which are quite difficult to be controlled through conventional research. Biotechnological intervention including transgenesis, genome editing, and OMICS offers several innovative options to resolve existing issues in this fruit crop. Genetic transformation has been established in many citrus species and transgenic plants have been developed having the ability to tolerate bacterial, viral, and fungal pathogens. Genome editing has also been worked out to develop disease-resistant plants. Likewise, advancement in OMICS has helped to improve citrus fruit through the knowledge of genomics, transcriptomics, proteomics, metabolomics, interactomics, and phenomics. This chapter highlights not only the milestones achieved through conventional research but also briefs about the achievements attained through advanced molecular biology research.

Keywords: citriculture, conventional research, transgenesis, genome editing, multi-OMICS

1. Introduction

Citrus is one of the most diverse members of the family *Rutaceae* and is the leading tree fruit crop in the world. Citrus comprises different species of edible fruits like mandarins (*Citrus reticulata* Blanco), sweet oranges (*C. sinensis* Osbeck), grapefruit (*C. paradisi* Macf.), acid limes (*C. aurantifolia* Swingle), and sweet limes (*C. limettioides*), lemons (*C. limon* Burmf.) and their hybrids including tangerines, tangelos, tangors, etc. [1].

Citrus is being widely cultivated in the sub-tropical, tropical, and temperate regions of the world. Global citrus production is 157 million tons per annum from an area of 15 million hectares. About 50% of the area and production of citrus is being contributed by the northern hemisphere of the world. China (28%) and the Mediterranean regions (25%) are the major contributors to global citrus production followed by Brazil (13%). China is leading in grapefruit and mandarin production. Among Mediterranean countries, Spain is leading in global citrus

production (6 million tons) including mandarins, oranges, limes, lemons and exports. Brazil is leading in global fresh sweet orange and its juice production. Mexico and India are major lime producers [2]. Pakistan's share in global citrus production is quite low (1.6%) which includes mandarin and sweet oranges as major species whereas limes, grapefruit, and lemons have less production and are dealt as minor species. The global citrus industry is facing many biotic (Citrus greening, Citrus tristeza virus, sudden death, citrus canker, and Phytophthora) and abiotic stress (salinity, drought, and temperature fluctuations) which have a direct impact on fruit crop production and yield [3].

2. Origin and diversification

Citrus and other genera including *Poncirus*, *Clymenia*, *Fortunella*, *Eremocitrus*, and *Microcitrus* belong to tribe Citreae and sub-tribe Citrineae and are considered as true citrus [4]. Classification in citrus has been controversial since ancient times due to vast morphological diversity, interspecific and intergeneric sexual compatibility. However, molecular biology tools have revealed four species including mandarins, citron (*C. medica*), pummelos (*C. grandis* Linn.), and wild cultivar of papeda (*C. micrantha* Wester) as the true parental species that have contributed to the development of other species during the process of evolution [5, 6]. Based on phylogenetic and genomic studies it is revealed that mandarin originated in China, Vietnam, and Japan whereas citron was originated in northeast India and China. Pummelo originated in Indonesia and Malay whereas *C. micrantha* was originated in the Philippines [6]. Other citrus species including sweet oranges, grapefruit, lime, lemon, sour oranges, and hybrids (tangelos and tangors) have developed from these ancestral species through random hybridization and natural mutation events [7].

Among citrus genetic resource centers, major collections are found in the USA, China, Spain, France, Japan, and Brazil where a large number of wild species, their relatives, old and new varieties, and breeding lines are conserved [8]. In Pakistan, citrus genetic resources are conserved mainly in the field as orchards or germplasm units in Sargodha, Faisalabad, and Sahiwal in different academic and research institutes.

3. Conventional approaches for crop improvement

Citrus breeders have been using different approaches for their improvement including conventional breeding, mutation breeding, polyploidization and *in vitro* culture tools particularly somatic hybridization which has played an essential role in developing new somatic hybrids. These techniques have contributed towards the selection and development of new potential cultivars and are still being used as important fundamental tools for the development of genetically diverse germplasm which could be further screened and characterized using modern breeding technologies.

3.1 Classical and mutation breeding

Though conventional breeding has limitations in citrus due to its complex reproductive behavior, nucellar embryony, long juvenility, sterility, sexual incompatibility, and endogametic depression [9, 10]. However, still, many hybrids have been developed by conventional breeding and recovered using *in vitro* tools.

Mutation breeding has played a pivotal role in fruit crop improvement including citrus and has developed several mutants with improved phenotypic and genotypic traits [11]. Spontaneous or induced mutants do not have intellectual property rights (IPR) related issues that have to be faced in the case of conventional breeding and transgenics [12]. Both spontaneous and induced mutations have enhanced genetic diversity in existing varieties and have provided the raw material for making selections for the novel horticultural traits [13]. About 3365 mutant varieties belonging to 170 plant species have been released including citrus and 20 other fruit species [14]. Among continents, Asia is leading with 2052 mutants released followed by Europe (960 mutants). Among countries, China (817), Japan (479), India (341) and the USA (139) are leading in mutant development whereas Pakistan has released 59 mutants in different crops [15]. In citrus, a total of 15 mutants have been released since 1970 including mandarins and clementine (6), sweet oranges (6), grapefruit (2), and lemon (1) [10]. Pakistan has registered a single mutant variety in citrus, a Kinnow mandarin induced mutant having less number of seeds and named it as "NIAB Kinnow" in 2017.

The rate of spontaneous mutations has been much higher in citrus compared with other fruit crops, however, due to random genetic alterations it has been difficult to identify and utilize such mutants [16, 17]. Induced mutations using different irradiation sources including gamma rays (physical mutagens) and various chemical compounds have enhanced the frequency of genetic variability. Physical mutagens or ionizing radiations have been more commonly used for inducing genetic diversity, chromosomal aberrations, and point mutations. About 70% of the mutant varieties have been developed using physical mutagens [18]. In fruit crops, physical mutagens have altered key horticultural traits like seedlessness, precocious bearing, and dwarfism [19–21]. Other traits include fruit ripening time, fruit skin and flesh color, fruit aroma, self-compatibility, pathogen resistance, and fertility restoration in sterile hybrids. Among physical mutagens, gamma rays have been most used for the development of mutants due to their shorter wavelength and greater penetration [22], however, the ion beam is getting more popular and is being widely used due to its greater efficiency and precision compared with gamma rays [23]. Among chemical mutagens, ethyl methanesulfonate (EMS), diethyl sulfate (DES), ethylenimine (EI), sodium azide (SA) has been most frequently used for reliable and gene-specific mutations. A comprehensive review of the role of mutation breeding in mandarins and lime crop improvement has been discussed [24, 25]. Irradiation and chemical mutagen treatment of seeds and budwood have been commonly used by breeders for inducing variation followed by selection and clonal propagation. Mutation breeding applications have been reported in different fruit crops including papaya, peach, pear, grapes, sweet and sour cherries, banana, plum, almond [26], apple [27], and rough lemon [28]. Natural bud mutants include Washington navel orange, most of the early grapefruit varieties including Marsh, Foster, Shamber, Salustiana sweet orange, and Shamouti orange have originated as bud sports. Now there are several commercial seedless varieties including Daisy SL, Kinnow SL, Fairchild SL, and Tango that have been developed from their seedy parents through mutation breeding and are being commercially cultivated [29]. Other commercial mutants in citrus include sweet orange varieties Jin Cheng [30], Kozan [21], and NIAB Kinnow mandarin [31]. In grapefruit, Rio Red and Star Ruby are two induced mutants that have obtained commercial significance due to their better fruit color and seedlessness, respectively [32]. These are leading grapefruit varieties in Texas, USA. Star Ruby is the leading variety in Turkey, South Africa, Australia, and Spain. Rio Red is the main cultivar in China, India, and Argentine [33]. In Pakistan, Shamber is the main grapefruit variety that needs to be replaced with other potential candidate varieties like Star Ruby, Rio Red, and Flame [10].

Conclusively mutation breeding has shown its enormous potential in citrus crop improvement particularly in economically important horticultural traits. However, it is a slow and long-term process and takes more time to detection of desirable phenotypic variability. Utilization of modern breeding tools including molecular markers, advanced methods for phenotypic screening like Targeting Induced Local Lesions IN Genomes (TILLING) [34], using targeted mutagenesis and genome editing technologies [35] like Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) could enhance the efficiency and cost-effectiveness of variety development having novel traits in citrus.

3.2 Ploidy manipulations

Polyploid organisms have a greater number of chromosomes compared with their diploid progenitors. Breeders have utilized polyploidization for the investigation of inheritance patterns in genes of interest. Polyploids have shown tremendous success in nature due to higher heterozygosity, less inbreeding depression, and more tolerance to biotic and abiotic stress conditions compared with their diploid progenitors [36–38]. The duplicated genes may evolve new functionalization during evolution [39]. Polyploids have been reported in many fruit crops including grapes, apples, strawberry, and citrus [40, 41], however, the frequency of spontaneous polyploid events is quite low and breeders prefer to induce hyperploidy using different chemicals.

Among chemical mutagens, colchicine is mostly used for the induction of polyploids due to its more reliability, higher efficacy, and cost-effectiveness. Colchicine is an alkaloid derived from *Colchicum autumnale* (meadow saffron). It is used for inducing chromosome doubling or developing tetraploids by restricting the chromosomal segregation at metaphase in meiosis [42, 43]. Other methods of polyploid induction include interploid hybridization [44], unreduced gamete formation [45, 46], and endosperm culture [47, 48].

Members of the subfamily *Aurantioidae* including *Citrus*, *Fortunella*, and *Poncirus* are mainly diploid having chromosome number 2n = 18 [49]. The occurrence of spontaneous polyploids in citrus is known since the 1940s [50]. Important spontaneous polyploids include triploid Tahiti lime [51], Triphasia desert lime [52], *Clausena excavata* [53], tetraploid mandarins [54], sweet oranges [41, 42] and grapefruit [43]. In spontaneous polyploids, triploids and tetraploids are believed to be formed by doubling of chromosomes in nucellar cells and fertilization of the unreduced gametophytes [55, 56]. Polyploids have been induced using colchicine in several citrus species and tetraploids produced have been used for interploid crossing to develop triploid progenies that are usually seedless due to irregular distribution of the chromosomes during cell division particularly gamete formation and formation of unreduced gametes. In interploid crossing, the formation of tetraploids in addition to triploids indicates the predominant formation of the unreduced (2n) gametes which may be formed by the first division restitution (FDR) or second division restitution (SDR) during meiosis. Production of 2n gametes was predominantly via SDR in lemon [44, 45] and monoembryonic Orah mandarin [57]. Higher tetraploid: triploid ratio in the progeny of the interploid hybridization indicates greater production of the 2n megagemtophytes in that cultivar which is promising to produce a greater number of polyploids.

3.3 *In vitro* culture: somatic hybridization

Plant tissue culture tools offer advantages related to efficient regeneration, propagation, and crop improvement in citrus and other horticultural crops.

Endosperm cultures have been used for the development of triploids in citrus [48]. In interploid and wide hybridizations, the progeny may be sterile or have underdeveloped or shriveled seeds with viable embryos. The embryo rescue technique has shortened the breeding cycle and many plants have been recovered from these embryos through *in vitro* culture in different citrus species [45]. Similarly, micrografting is another tool in which a miniature bud is grafted under aseptic conditions on *in vitro* raised rootstocks and micrografted plants have been reported in many citrus varieties [58]. Micrografting is also useful for the production of virus-free citrus plants.

Another highly promising and most widely used approach is somatic hybridization which is utilized to overcome sexual incompatibility and to enhance genetic variability by combining nuclear and organelle (chloroplast and mitochondria) genomes followed by their characterization for hybrid confirmation and variability assessment [59]. The organelle genomes are known to encode genes related to photosynthesis and male sterility and new hybrids could be developed having novel genetic recombinations. Somatic hybrids may be developed through electrofusion of plant embryogenic protoplasts predominantly with mesophyll protoplasts. The plant progeny having nuclear origin could be characterized and separated using flow cytometry and molecular markers [60].

Protoplast fusion of distantly related citrus species bypasses the biological barriers and develops allopolyploids that could not be obtained through classical breeding. Somatic hybridization is an important tool and has been widely used in citrus scion and rootstock breeding. The first intergeneric allotetraploid somatic hybrid of Trovita sweet orange and *Poncirus trifoliata* was reported by Ohgawara et al. [61] followed by several interspecific and intergeneric hybrids in citrus from the USA [62], Japan [63], and other citrus-producing countries. Triploids were also reported from interspecific and intergeneric somatic hybridization of Citrus species, kumquats (*Fortunella japonica*), and *Poncirus trifoliata* by protoplast fusion [64]. Fusion of protoplasts from the haploid lines and diploid cultivars may also yield triploids and hundreds of triploids and tetraploids were developed and planted for field evaluations [65]. Polyethylene glycol may also be used to induce regeneration in the fused protoplast cultures as reported in Willow leaf mandarin (embryogenic parent) and Duncan grapefruit and sweet orange (mesophyll parents). The regenerated plants were identified as alloplasmic cybrids [66].

Polyploids developed through somatic hybridization have also shown enhanced tolerance to abiotic and biotic stress conditions. Allotetraploids of cv. FlhorAG1 (FL-4x) developed by somatic hybridization of diploid *Poncirus* and *Citrus* showed greater tolerance to cold and higher light conditions compared with parents (diploid) and their tetraploids [36]. Kumquats (*Fortunella* species) chloroplast have demonstrated higher resistance to canker in diploid kumquats and their tetraploid somatic hybrids developed with other citrus species including grapefruit [37].

4. Innovative approaches/technologies

4.1 Transgenesis

Since the advent of recombinant DNA technology, transgenesis has proved its significance, and 190.4 million hectares of transgenic crops were grown in more than 29 countries in 2019. They have significantly contributed to food security, climate change mitigation, sustainability thus uplifted the lives of 17 million biotech farmers worldwide. The first transgenic plant was developed in the 1980s and was available as commercial food in the 1990s. More than 400 transformation events

have been approved so far wherein 356 events have been approved for crop plants, 23 for ornamentals, 22 for fruit plants, and 2 for trees. Hence a wide range of plant species (maize, cotton, canola, papaya, rice, tomato, sweet pepper, squash, popular, petunia, sugarcane, alfalfa, and citrus) have been engineered for various valuable traits i.e. insect resistance, herbicide tolerance [67], abiotic stress tolerance, improved nutritional value, and disease resistance. In addition to nuclear transformation plastid genome has also been targeted and has proved to be of more value as multiple genes can be introduced at a specific target site, the transgene is contained owing to maternal inheritance, and hyperexpression of the transgene, etc. [68, 69].

Citrus is an economically important fruit crop worldwide. It not only provides essential minerals and vitamins but is also of great commercial importance. Conventional research has contributed a lot to the improvement of this fruit yet serious problems are evolving which are difficult to tackle with these conventional approaches [70]. Juvenility, sexual incompatibility, high heterozygosity, apomixes, large plant size, and nucellar polyembryony, and certain other biological limitations hinder the improvement of these plant species through conventional breeding. Genetic manipulation through advanced innovative techniques is a potential approach to improve crop plants as well as fruit species. Though citrus species are recalcitrant to transformation and subsequent rooting, yet consistent efforts by the researchers have resolved these bottlenecks and proficient protocols have been established. Likewise, various transformation methods i.e. Agrobacterium-mediated transformation [71], biolistic transformation [72], and chemically assisted uptake of recombinant DNA by protoplasts [73] have been attempted to introduce genes of agronomic value as well as to strengthen it against bacterial, viral, and fungal pathogens (**Figure 1**).

Genetic manipulation of vegetatively propagated crops like citrus is very tricky as the expression of transgenes over a long period during numerous cycles of graft propagation should be stable.

The first attempt to produce transgenic citrus was made in the 1980s wherein protoplast transformation was attempted but it was not successful. The first authentic report was published by Kaneyoshi et al. [74] who reported transforming NPT II and GUS genes into trifoliate orange through Agrobacterium. Epicotyls of the aforementioned citrus species were used to transform with the selectable marker gene as well as reporter gene and more than 25% transformation efficiency was achieved. Likewise, Yao et al. [72] reported the first successful transformation through gene gun. They transformed tangelo (*C. reticulata* × *C. paradisi*) embryogenic cells.

Since genetic transformation has successfully been performed in different species and hybrids including Carrizo citrange, Washington naval orange, *Poncirus trifoliata*, Sour orange, Mexican lime, sweet orange, *Citrus reticulata* [75], and a valuable rootstock, swingle citrumelo. Similarly, protocols have been optimized for the genetic transformation of different citrus species by using different explant tissues including seeds, embryogenic cells, epicotyls, embryogenic cells, callus, nodal stem segments, and protoplasts. The most responsive explant tissue has been epicotyl from the *in vitro* germinated seedlings and is preferably used for genetic transformation research. Duncan grapefruit was successfully transformed through *Agrobacterium* for the first time using epicotyl and confirmation of the transgene (NPTII and GUS) integration was carried in the resultant 25 transgenic plants by histochemical staining, PCR, and Southern blot hybridization. Transgenic grapefruit, sweet orange, and citrange plants were developed using epicotyls as target explant whereas selection was carried out on kanamycin [76]. Epicotyl has also been used for Agrobacterium-mediated transformation of citrange and sweet orange [77]. In addition, callus, as well as suspension cultures derived from different parts of flower and seed, have also been attempted to transform. The transformation

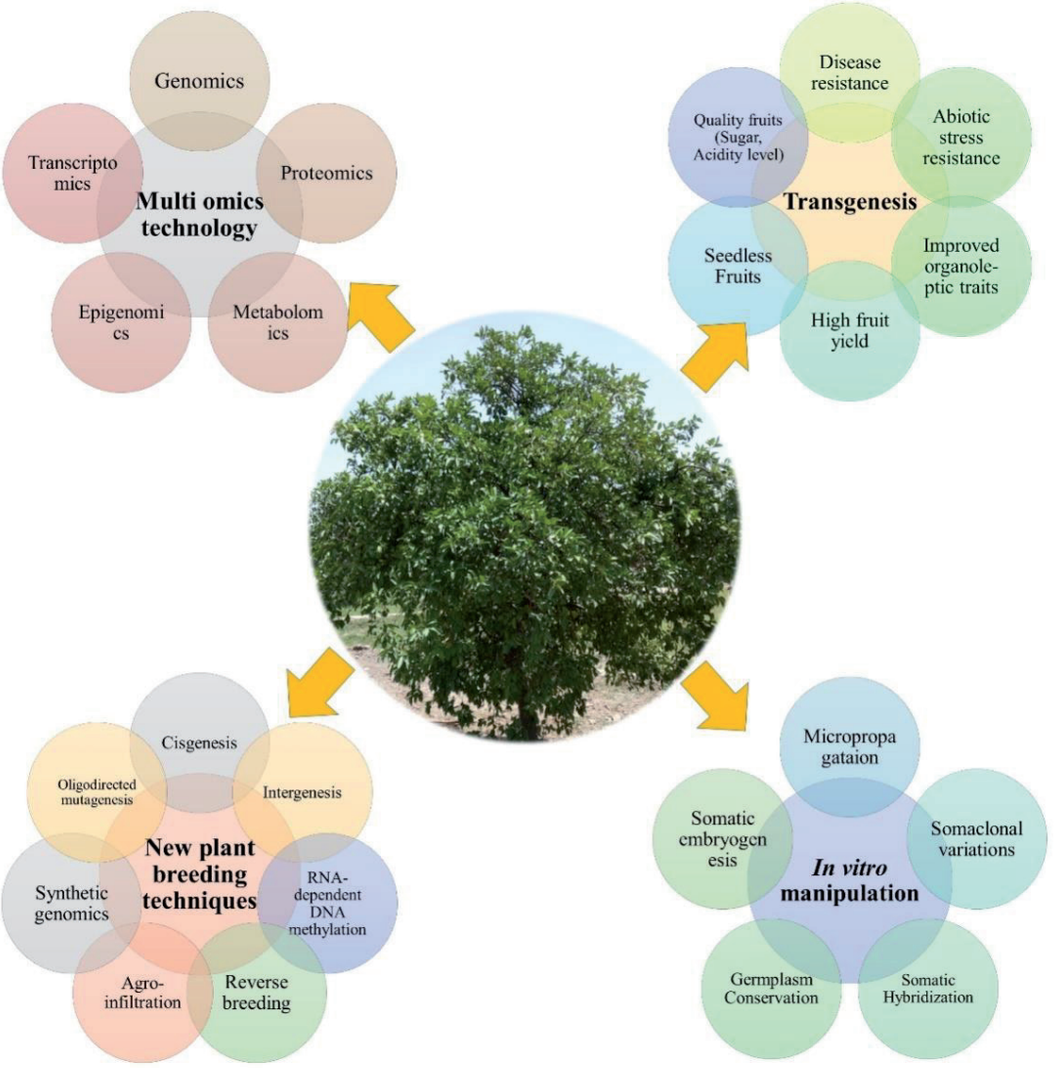

Figure 1.
Schematic sketch showing the importance of conventional and advanced innovative approaches for the improvement of different citrus species.

efficiency attained, in this case, was lower than 0.5%. Genetic transformation has also been optimized in pomelo (*Citrus grandis*) and sour orange wherein internodal stem segments were used as explants and a promising transformation efficiency was achieved (91%) [78].

The biolistic transformation has also been performed successfully in tangelo (*C. paradisi* Macf. x *C. reticulata* Blanco) using nucellar embryogenic cells raised from the suspension culture and more than 15 stable whereas 600 transient transgenic lines were attained per bombardment. The transformed calli cells showed proficient growth on kanamycin selection medium and positive GUS activity but were not able to regenerate into plants. Calli treatment with 0.3 M osmoticum sorbitol and 0.3 M mannitol appeared to have positive effects for enhanced transformation efficiency for stable and transient transformation. Thin epicotyl segments of germinated seedlings were also targeted through the biolistic gun and more than 93% transformation efficiency was attained for transient transgene integration in *C. citrange*. The incubation of explant on culture medium before bombardment appeared to have profound effects on transformation efficiency which was further improved [79].

Since transgenic technology is the most reliable intervention having the massive potential to improve the citrus crop. The introduction of alien genes is only possible through this technology so any of the desired traits can be engineered.

Recent research indicates that citrus growing farmers are facing severe problems due to biotic and abiotic stresses i.e. salinity, cold, drought, and diseases. Hence, the development of improved citrus varieties is direly needed to get a quality crop. Various citrus species have been engineered with alien genes to combat abiotic stresses including salinity and drought. Expression of HLA2 gene, isolated from yeast imparted salinity tolerance and resultant transgenic plants were able to tolerate a higher level of the salts as compared with non-transformants [80]. PsCBL and PsCIPK derived from *Pisum sativum* were transformed into *Citrus sinensis* and *Citrus reticulata* by targeting calli derived from mature seed. Bacterial strain LBA4404 was used to induce infection in the target calli cells. The putative transformants showed better performance as compared with control plants for salinity and drought tolerance when tested under *in vitro* conditions [81].

Citrus paradisi was transformed to improve carotenoid content by manipulating the genes involved in carotenoid biosynthesis i.e. phytoene synthase, lycopene-β-cyclase, and phytoene desaturase. The multigene transgenic citrus plants were aimed to supplement human nutrition with vitamin A along with antioxidants. Similarly, fruit juice quality has been attempted to improve in Valencia orange, a valuable variety that is majorly grown for its juice. Degradation of TSPME (thermostable pectin methylesterase) deteriorates the quality of the juice. This TSPME is encoded by the *CsPME4* gene. The protoplasts were isolated from embryogenic suspension cultures and transgene was introduced through the PEG mediated transformation method [82] aiming at down-regulation of the *CsPME4* resulting in the citrus with improved juice quality.

Citriculture is prone to be infected by a wide range of diseases that are controlled by chemicals, a drastic non-environmentally friendly strategy. Different types of viral, bacterial, and fungal pathogens infect these plants resulting in drastic losses to crop production and quality of the produce. A range of transgenic citrus lines have been developed varying from fully resistant to susceptible to the diseases. Coat protein (p25) from the CTV (Citrus Tristeza Virus) was expressed in Mexican lime and 33% of the transgenic plants were found to be resistant, neither symptoms appeared nor the viral load was detected. Accumulation of siRNA (small interfering RNA) in the transgenic lines resulted in resistant phenotype and plants were able to withstand viral infection [83]. Expression of viral coat protein (part of *p23* gene and the 3UTR), in the sense and antisense orientation also delayed viral infection in grapefruit [84].

Phytophthora is a noxious fungal pathogen that has been reported to infect a wide range of citrus species. Among these *Phytophthora parasitica* and *Phytophthora citrophthora* cause more severe damage in the citrus orchards and nurseries all over the world [85]. Expression of *bo* gene (bacterio-opsin) in Rangpur lime rootstock showed an elevated level of tolerance against *Phytophthora parasitica* infection. It was observed that expression of the aforementioned gene led to induce expression of defense-related proteins; chitinase, salicylic acid, and glucanase. Hence plants with an elevated level of transgene expression showed greater resistance against the oomycetic fungi including *Phytophthora parasitica*. Transgenic citrus plants expressing the tomato *PR5* gene showed an enhanced survival rate in the presence of pathogen (*P. citrophthora*). Transgenic grapefruit plants were able to better withstand citrus scab infection when transformed with the *attE* gene encoding for antimicrobial peptide [86].

Transgenic technology has also played a pivotal role to tackle another noxious disease in citrus i.e. Huanglongbing (HLB) which is supposed to be caused by phloem-restricted Gram-negative bacteria; *Candidatus Liberibacter* americanus, *Candidatus Liberibacter* asiaticus, and *Candidatus Liberibacter* africanus [87]. Various genetic strategies have been tested to develop HLB-resistant citrus lines

with decreased susceptibility to the pathogen. These include the expression of anti-microbial peptides from a bacterial, fungal, plant, or animal origin and engineering host-pathogen interaction pathways. The expression of antimicrobial proteins under phloem-specific promoters has been an effective strategy to control this phloem-resident pathogen. Overexpression of synthetic cecropin B gene under phloem specific promoter resulted in reduced bacterial population after one year of inoculation and no disease symptoms appeared even after two years of inoculation [88]. Overexpression of modified methionine under double 35S promoter also appeared to have an inhibitory effect on bacterial growth and lowered down the CLas (*Candidatus Liberibacter* asiaticus) titer in the roots of transgenic Carrizo citrange (rootstock). Further, newly emerging leaves from the rough lemon, grafted on this transgenic rootstock, also had a non-detectable bacterial titer. Expression of AtNPR1 and chimeric proteins (ThioninLBP and Thionin1-D4E1) demonstrated elimination of CLas providing tolerance against HLB infection [89].

Another economically important disease, the citrus canker has also been addressed through transgenesis resulting in enhanced tolerance against *Xanthomonas citri*. Engineering sweet orange genome with *Xa21* gene showed a significant reduction in disease severity upon inoculation in three lines Hamlin, Pera, and Natal. Expression of the *Xa21* gene under its promoter appeared to be more effective in disease resistance when expressed in highly susceptible Anliucheng sweet orange [90]. Transgenic Carrizo citrange and sweet orange plants were developed by introducing RpfF from *X. fastidiosa* which encodes for a quorum-sensing factor and can disrupt bacterial communication by reducing activation of virulence factors, thus enhancing the ability to tolerate pathogen infection. Similarly, the expression of AMP sarcotoxin from flesh fly also enhanced tolerance against *X. citri* [91].

4.2 Genome editing

Genome editing through CRISPR-Cas9 has emerged as a breakthrough technology for the precise modification and manipulation of targeted genomic DNA. It has extensively been exploited by several research groups [92] and certain successes have been achieved. Three major sequence-specific engineered nucleases that have so far been used for genome editing are CRISPR-Cas (clustered regularly interspaced short palindromic repeats), TALENs (transcription activators such as effector nucleases), and ZFNs (zinc finger nucleases). Among these, the CRISPR-Cas9 editing system has been established in many plant species through gene activation, repression, mutation, and epigenome editing in wheat, rice, maize, tomato, potato, carrot, apple, grape, and citrus. Even a few of the genome-edited crops have been approved for commercial cultivation. Through this technology, field crops as well fruit crops can not only be manipulated for improved agronomic traits but can also be manipulated for improved nutritional value [93].

For citrus, genome editing research is at infancy, yet few successes have been achieved by editing its genome for enhanced resistance against diseases. The CRISPR-Cas9 system was firstly used to target the *CsPDS* gene in Duncan grapefruit and sweet orange. The target gene was successfully modified through a transient expression method, Xcc-facilitated agroinfiltration [94]. The modified *CsPDS* sequence was not detectable in the leaves of sweet orange indicating that CRISPR/Cas9 has induced the desired mutation successfully.

Most of the research studies were carried out to target the *LOB1* (LATERAL ORGAN BOUNDARIES 1) gene which has been characterized as a citrus susceptibility gene for *Xanthomonas citri*. The said gene has been explored to be upregulated by TAL (transcription activator-like) effector PthA4 which binds to the EBE (effector

binding elements) in the promoter region of LOB1 thus activates expression of this canker-susceptibility gene [95]. Mutation in the single allele of effector binding elements of the *LOB1* gene resulted in minor alleviation of canker symptoms in Duncan grapefruit. Anyhow, a mutation in effector binding elements of both of the alleles of LOB1 promoters alleviated canker symptoms to great extent thus showing a high degree of resistance in Wanjincheng orange [96]. Another research group explored that editing the coding region of LOB1 in Duncan grapefruit, through the CRISPR-Cas9 system also provides resistance against *X. citri* infection.

A marker gene for pathogen triggered immunity (CsWRKY22) was knocked out in Wanjincheng orange and the resultant mutant plant showed a decreased level of susceptibility to the canker disease [97]. In addition to the CRISPR-Cas9 system, another improved genome editing system (CRISPR/Cas12a (Cpf1) has also been used to edit the *CsPDS* gene in the Duncan grapefruit gene. It appeared to be a more efficient editing system with lower off-target effects thus will prove a great milestone in citrus genome editing [98]. These studies indicate that CRISPR-mediated genome editing can be a promising pathway to generate disease-resistant citrus cultivars [99].

5. Multi-Omics technology: An integrated approach and useful strategy for the improvement of the citrus crop

MultiOMICS including genomics, transcriptomics, proteomics, metabolomics, interactomics, and phenomics approaches have massive potential for citrus improvement just like other crops and fruits. In all disciplines of OMICS, various techniques can be utilized for genome analyses, transcripts, proteins, metabolites, and interactions between different molecules to indicate the molecules which may result in crop improvement.

5.1 Genomics

The field of genomics is a highly applicable part of Omics technologies. It is based on sequencing technologies and the analysis of subsequent genome sequences. Many advanced techniques in genomics for example sequence determination DNA, marker-assisted selection, and transition from marker-assisted selection to genomic selection assist in quick varietal development. Genome sequencing technologies have brought about a revolution in the field of biology. It has also transformed the citrus breeding that helps to understand a relationship between the genetic makeup and response towards various abiotic and biotic stresses like Alternaria brown spot [100].

A specific pathotype of *Alternaria alternata* (Fr.) Keisel is a disease with heavy losses [101]. It causes necrosis and resultant lesions on the surface of fruits and young leaves. It leads towards defoliation and fruit drop [102]. Thus, exploitation of innate genetic resistance appears to be the most applicable and effective strategy of disease control. Currently, the control is primarily based on the application of 4–10 sprays of toxic and environmentally injurious fungicide per year [103]. Such limitations are compelling farmers to find alternative cultivars with resistant ones [104].

Usually, the female parent transmits the 2*n* gamete in 2x × 2x citrus crosses [105–108]. Cuenca et al. [109] recognized ABS resistance locus containing genomic region by using BSA-genome scan combined with HTA based. Trait segregation in crosses between two heterozygous ABS-susceptible or between heterozygous ABS-susceptible parents and resistance was used to confirm the recessive inheritance of the ABS resistance in triploid populations. ABSr locus was first located at 10 cM from a

centromere based on segregation of 368 SDR 2*n* gametes. A genomic region containing several markers with a high probability (> 99%) of association with phenotype variation was identified on chromosome III by performing BSA over 93 triploid hybrids from a Fortune × Willow leaf population. This identified region contains 25 significant SNP markers within an interval of 13.1 cM. The size of the genomic region among these two markers is 15 Mb. Linkage genetic mapping was performed on identified genomic regions by developing new SNP and SSR markers. A 268-diploid mapping population was performed by Cuenca et al. [110] from a heterozygous-susceptible × resistant hybridization. Fine mapping was performed to confirm the location of *ABSr* locus in a region of 1.1 cM between the markers SNP05/SNP06/SNP07/AT21 (at 0.7 cM) and SNP08 (at 0.4 cM). Another region containing eight genes with NBS-LRR repeats was identified by the SNP08 marker and considered ABS resistance genes.

In citrus plant, molecular markers are linked to some agronomic traits, e.g. SSR markers are linked to Citrus tristeza virus resistance from *Poncirus trifoliate*, PCR assay for the anthocyanin content of pulp [111], AFLP markers are associated with polyembryony [112] and RAPD markers are associated to dwarfism and fruit acidity [113]. Some other characteristics such as salinity tolerance and nematode resistance are linked to QTLs [114]. The selection of resistant genotype at the early growth stage was improved by the newly developed SNP08 marker. This marker was mapped at 0.4 cM from the ABD resistance gene and it has role in avoiding the selection of susceptible varieties. On the other side of the gene, some new markers were also identified at 0.7 cM from the *ABSr* locus. Combining these new markers with SNP08, the probability of selection of resistant genotype was increased by 0.0028%. This marker appeared to be very helpful in the selection of resistant and susceptible genotypes and for analyzing the resistant germplasm to configure the ABS genes. So, it is a very valuable tool for the selection of susceptible heterozygous cultivars which may be used as breeding parents allowing manipulation of genetic diversity in citrus and prevents susceptible homozygous genotypes.

About 40 mandarin genotypes (susceptible and resistant) were tested by the SNP08 marker and were used as breeding parents. An ultimate association was observed between response to *Alternaria* infections and SNP08 marker. Recently SNP08 is used in breeding programs of citrus performed at CIRAD and IVIA for the selection of ABS-resistant citrus genotypes. About 2187 resistant hybrids were selected from 4517 total hybrids rising from 10 different parental combinations by using the SNP08 marker since its development. This analysis was very helpful to prevent the growth of more than 2000 susceptible lines which were removed at the early growth stage after selection so, a lot of time, cost, personnel, and resources were saved.

5.2 Proteomics and metabolomics approaches

Proteomics is the comprehensive analysis of all the proteins found in a cell. It includes the identification of proteins, their location in the cell, their interactions with other proteins and other biological components in the cell, and most importantly post-translational modifications that a protein undergoes in the cell [115]. Metabolites are referred to as the last product of any biological activity in a cell and are found in very small quantities [116]. Metabolites are small molecules including intermediates of various metabolic reactions, signaling molecules, hormones, and other regulatory products found in a cell. Hence, metabolomics is defined as the study of metabolites of a cell [117, 118]. It is estimated that around five thousand metabolites are found in any cell depending upon the physical and chemical complexity of that cell [119].

Huanglongbing (HLB) is considered one of the most devastating citrus diseases that affect not only the production but also the quality of citrus fruit and its juice.

Using a combination of proteomics and metabolomics approaches it was found that in symptomatic fruit, the expression of proteins found in the cytoplasm for glycolysis, in mitochondria involved in the tricarboxylic acid (TCA) cycle, and in chloroplasts for the synthesis of amino-acids was downregulated. Similar downregulation was observed for genes involved in terpenoid metabolism for example valencene, limonene, 3-carene, linalool, myrcene, and aterpineol in fruit found on infected trees. Similar phenomena were observed for sucrose and glucose. Hence, the off-flavor found in symptomatic fruits was linked to the downregulation of the above genes and a decrease in the levels of the abovementioned secondary metabolites [120].

In another study, comparative iTRAQ proteomic profiling was carried out using the fruits of sweet orange which was grafted on sensitive and tolerant rootstocks infected by CaLas. The results showed that symptomatic fruit on sensitive rootstock exhibited a greater number of differentially expressed (DE) proteins as compared to the healthy fruit on a similar rootstock. It was also found that the expression level of various defense-related proteins was reduced in symptomatic fruit on sensitive rootstock, particularly the proteins related to the jasmonate biosynthesis, is signaling, protein hydrolysis, and vesicle trafficking. Hence, it was concluded that the down-regulation of these proteins is likely to be linked with the sensitivity of citrus to the CaLas pathogen [121].

5.3 Interactomics and metabolomics and phenomics

Interactomics bears a broad scope as it may cover a complete set of interactions in a cell [122]. It covers every type of interaction among interacting molecules including proteins and other molecules. It is a well-known fact that the Protein–protein interactions are major of all cellular processes [123].

To designate the complete phenotype of a plant, the term phenome is used. Similarly, a phenotype encloses a group of traits that are liable to be distinguished either by utilizing modern science analytical techniques or by a naked eye evaluation. These traits can also be attributed to being an interaction between external factors (environment) and Genotype. David Houle also termed phenomics as the collection of data from varying backgrounds and dimensions in a single entity [124]. Phenomics involves both "extreme phenotyping," referring to a comprehensive selection of a wide range of valid and correct phenotypes, and "phenome analysis" indicating towards an analysis of specimen and correlation between syndication of genotype and phenotype.

Plant phenomics utilizes screening of large populations to analyze genetic mutations found in the population for a specific trait (drought, salinity, or high-temperature stress tolerance). Various types of imaging techniques are employed in the phenotyping of plants for various growth and developmental processes. The techniques include visible-light imaging [125], Thermographic imaging [126], Hyperspectral imaging, Chlorophyll fluorescence, X-ray, MRI, PET [127].

Using the phenomics approach and tools, we can study the traits regarding plant growth, leaf growth, root growth, and architecture of soil/root interaction, etc. This extensive use of phenomics and its integration into OMICS is the need of the hour to combat food security issues and overcome adverse effects of climate change on crop production.

6. Conclusions

Conventional research has played a pivotal role in the improvement of citrus. Enhanced heterozygosity has helped in the development of genetically diverse

germplasm in most of the citrus species and numerous varieties have been released for commercial cultivation. However, with the advent of modern biotechnological tools, the period involved in crop improvement through indirect mutagenesis and polyploidization could be further reduced and enhancing cost-effectiveness. Transgenic technology and OMICS have great potential to improve this fruit crop. MultiOMICS, integrative-OMICS, or panOMICS technologies may result in better crops having better agronomic traits, enhanced yield potential, and less prone to insect pests. It will ultimately lead towards food security and poverty alleviation. Various OMICS technologies have been used for crop improvement, yet their integrated use will further strengthen the application of this robust technology. Still, there are many challenges associated with tolerant varieties which need to be fine-tuned. Moreover, three thousand reports of enhanced drought and salinity tolerance in wheat, sorghum, canola and rice are present but none of them is in use by farmers. A fundamental reason for this is that salinity and drought are complex multigenic traits. So, to induce tolerance in plants every gene needs to be fine-tuned precisely. However, their evaluation in the field is a long way, and distribution at the commercial level is also a hurdle in their production.

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