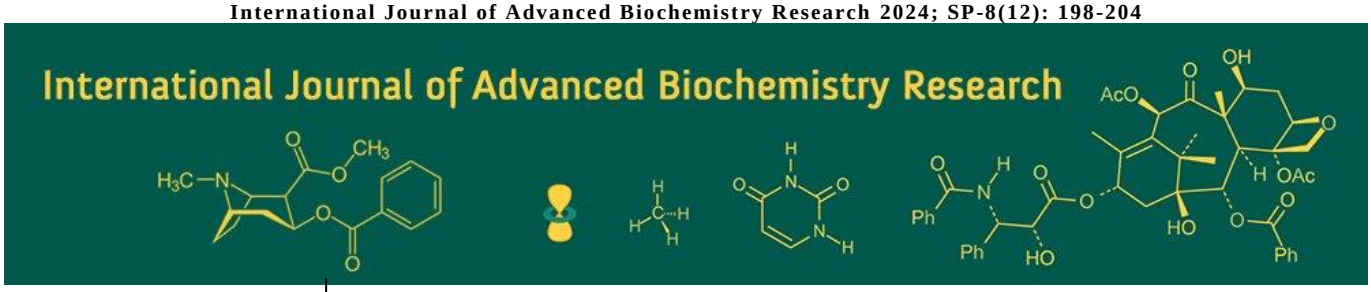

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Advance breeding technology in citrus for genetic improvement: A review

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Abstract
Conventional field selection methods struggle to identify desirable recombinants due to environmental and genetic factors affecting traits with low heritability. Fortunately, advances in molecular genetics, genomics, and biotechnology enable breeders to tackle challenging and costly traits. To breed for disease resistance, they must: screen elite germplasm (including commercial scions and rootstocks) against diseases, assess genetic variation, explore wild germplasm and related species if necessary, introgress genes between and within species, select suitable segregants with transgressive traits, and release scions and rootstocks for commercial cultivation.

Keywords: Citrus breeding, genetic improvement, molecular genetics, genomics

Introduction
Genetic improvement in citrus aims to create new recombinants with high yield potential, quality, and sustainable yield through resistance to biotic and abiotic stresses. Breeders use various tools like crossing, mutation breeding, and biotechnology to produce new genetic variations. After creating new recombinants, they screen and select for desired traits like yield, quality, and resistance to stresses. However, conventional breeding methods have limitations in citrus due to complex reproductive biology, long juvenile periods, and taxonomic relationships. Genetic transformation offers a solution by introducing specific traits into known genotypes without altering their elite background (Peña and Navarro, 2000) [42]. Biotechnological tools have assisted in fast germplasm improvement and new variety development. Due to environmental and genetic influences, selection of desirable recombinants is challenging, and advances in molecular genetics, genomics, and biotechnology are necessary to select difficult and expensive traits. Breeding for disease resistance requires screening elite germplasm, evaluating genetic variation, introgressing genes, and selecting suitable segregants for release to commercial cultivation.

Applications of New Plant Breeding Techniques in Citrus
Citrus species originated from a complex mix of four primary species: citron, pummelo, mandarin, and Citrus micrantha (Xu *et al*., 2013; Velasco and Licciardello, 2014; Wu *et al*., 2014; Curk *et al*., 2016; Wu *et al*., 2018) [105, 94, 103, 27, 104]. Most cultivated citrus varieties come from somatic mutations of a single ancestor, accumulated over time in different growing areas (Wu *et al*., 2014; Caruso *et al*., 2016; Curk *et al*., 2016) [103, 17, 27]. Traditional breeding methods are time-consuming and limited by difficulties such as plant size, long juvenile phase, and reproductive barriers like sterility, polyembryony, heterozygosity, and parthenocarpy.
Recently, the partial availability and knowledge of genes controlling traits of interest have slowed, making NPBTs in citrus a feasible option. The genes and markers mentioned herein refer to well-known traits, characterized after the availability of Citrus genomes (Xu *et al*., 2013; Wu *et al*., 2014; Curk *et al*., 2016; Wang *et al*., 2017; Wu *et al*., 2018) [105, 103, 27, 100, 104]. Key genes include: Ruby (MYB-like TF) and its promoter 3′LTR, controlling purple pigmentation due to anthocyanins in citrus fruits (Butelli *et al*., 2012) [13]; CsLOB1 and CsWRKY22, associated with susceptibility to citrus bacterial canker (CBC) in sweet orange

(Hu *et al*., 2014; Wang *et al*., 2019) [54, 100]; CitRWP, controlling polyembryony in sweet orange, grapefruit, lemon, and mandarin (Wang *et al*., 2017) [100]; and Noemi (Myc-like), controlling anthocyanins pigmentation and acidless trait in citrus fruits (Butelli *et al*., 2019) [15].

NPBTs in Citrus have focused on generating plants resistant to biotic stresses, particularly citrus bacterial canker (CBC). Genome editing has been successful in citrus using CsLOB1, the gene responsible for susceptibility to CBC (Hu *et al*., 2014; Duan *et al*., 2018) [54, 32]. CBC is a severe quarantine disease caused by Xanthomonas citri pathovar citri (Xcc) and aurantifolii (Xca). Genetic engineering is the best approach to induce resistance to CBC (Zhang *et al*., 2010) [109]. Recent methods involve intervening in the host-pathogen interaction mechanism using NPBTs. Genome editing of CsLOB1 has generated transgenic lines resistant to mutated Xcc strains (Jia *et al*., 2016) [56] and reduced symptoms in transformed plants caused by wild-type Xcc infection (Jia *et al*., 2016; Jia *et al*., 2017b; Peng *et al*., 2017) [56, 58, 112]. CRISPR/Cas9 approach on CsLOB1 promoter has generated homozygous mutants directly from citrus explants, decreasing susceptibility to CBC (Zhou *et al*., 2017) [112]. Cas12a has been used in Citrus, editing CsPDS in 'Duncan' grapefruit via Xcc-facilitated agro infiltration (Jia *et al*., 2019) [59]. Recently, Zhu *et al*. (2019) [113] used Fortunella hindsii to observe the effects of a successful CRISPR/Cas9 experiment. These results suggest that editing or cisgenesis could speed up gene characterization in functional genomic studies, especially for reproductive biology and fruit-related characters. However, the application of NPBTs in citrus relies on the availability of a transformation process using selectable marker genes, limiting the possibility of applying NPBTs for these species.

Promoter Sequences Used For Citrus Transgene Expression

The choice of promoter in a chimeric gene construct is crucial for achieving proper regulation of the desired trait in plant transformation. Despite the limited number of promoter sequences, selecting the right one can be challenging (Smirnova and Kochetov, 2020). Various promoters from viruses, bacteria, and plants have been used for citrus genetic transformation, highlighting the importance of promoter selection for effective trait expression.

Emerging Technologies: Cis and Intragenesis, Trans-Grafting and Gene Editing in Citrus

In citrus species, breeding programs using Agrobacterium or biolistic strategies have successfully inserted foreign DNA to improve desired traits without altering the genetic background. However, public concerns arose due to the use of genes from other species, selectable markers, and regulatory sequences from viruses or bacteria. To address this, new breeding technologies (NBTs) were developed to modify existing DNA sequences or modulate endogenous gene expression. These include cisgenesis, intragenesis, trans-grafting, and gene editing techniques. Although their application in citrus is limited, some examples include cisgenic and intragenic plants, which are genetically modified with DNA sequences from the species itself or closely related species. Recently, several citrus genes controlling traits of interest have been cloned and characterized for cis and intragenesis approaches. These genes include CsSAMT1, CsMADS5, CsMYB96, CiMADS43, and CiNPR4, which confer tolerance to HLB, regulate carotenoid biosynthesis, enhance fruit resistance, and control flowering and leaf development. To transform citrus plants with citrus-derived DNA sequences, a suitable transformation vector system is necessary. An intragenic vector system was developed by adding a T-DNA-like sequence from C. clementina, allowing for the recovery of positive events under non-selective conditions.

In citrus, researchers have made progress in using reporter genes based on anthocyanin production (Dutt *et al*., 2016, 2018b; Huang *et al*., 2019) [35, 113] and removing selectable markers (Zou *et al*., 2013) [114]. Additionally, Merritt developed a glyphosate-resistant selection system for citrus by transforming Duncan grapefruit with a mutated citrus EPSPS enzyme. This led to a 40% increase in transformation efficiency without inhibiting bud formation. Furthermore, researchers are working on characterizing citrus-derived promoters to link with the native target gene, meeting the third requirement for efficient transformation.

Genetic diversity

Genetic diversity within citrus germplasm is crucial for sustainable yield and disease resistance. Breeder selection for specific traits has narrowed genetic diversity, making it essential to evaluate and characterize germplasm to determine genetic diversity within and between citrus species. Molecular markers have proven useful in analyzing germplasm, showing high polymorphism and wide genomic distribution. Simple sequence repeats (SSR) have shown the highest level of polymorphism in citrus. Studies have utilized molecular markers to determine genetic distance between citrus species, revealing sister species, hybridization between closely related species, and distinct types within species. These findings highlight the importance of genetic diversity in citrus improvement programs and the potential for hybridization to manipulate heterosis or transgressive segregation.

Genetic variation within citrus germplasm for disease resistance is the first step in any breeding program for introgression of disease resistance. The initial step involves screening elite cultivars to determine the source of resistance and magnitude of genetic variation within the germplasm for disease resistance. If the desired resistance is absent in elite cultivars, then obsolete cultivars or related species are explored for the source of resistance. However, introgression from related and wild species is challenging due to ploidy, genetic, and phenological divergence among species. Moreover, breeders must combine high yield and quality with disease resistance to directly utilize the genetic recombinant as a cultivar, which further complicates introgression. This is because alien introgressions often exhibit linkage drag, incorporating undesirable genes that reduce yield and quality along with the gene of interest.

Evaluating germplasm for disease resistance involves screening by artificially or naturally inoculating it with the pathogen, followed by evaluating and characterizing host plants based on disease symptom severity. Phenotypic characterization can be challenging due to delayed symptom appearance in different genotypes, but molecular techniques like PCR and q-PCR can detect pathogens at low concentrations or multiplication within host species after a specific infection period. *In vitro* screening methods can

also be used for mass selection against particular diseases, where plant organs or seedlings are cultured on tissue culture media under aseptic conditions after inoculation with the pathogen. Various traits are evaluated, such as necrotic area, callus growth rate, and regeneration of embryos into plantlets. Successful experiments have been conducted using live pathogens, like Phytophthora parasitica, to identify resistant and susceptible genotypes. Additionally, deleterious phyto-chemicals or patho-toxins can be used for *in vitro* screening, including non-host toxins that interfere with plant defense responses.

Mutation breeding

Citrus genetic improvement is challenging due to factors like long juvenility, poly-embryony, self-cross incompatibility, and apomixes, making mutation breeding a promising approach to expand genetic variability and introduce new alleles. Mutation can be spontaneous or induced, and breeders can select spontaneous bud mutations for desired traits. Spontaneous mutations or tissue chimaeras occur at a frequency of 0.009-0.271% in citrus species and can lead to disease-resistant mutations and polyploidy mutants. Physical and chemical mutagens like gamma rays have been used to induce mutations in citrus, resulting in point mutations, structural aberrations, translocations, and polyploidy. Gamma rays have been extensively used in citrus improvement programs, with LD50 values ranging from 40-100 Gy depending on the species and plant organ. Scions, seeds, floral stage embryos, immature seeds, and *in vitro* materials can be treated with gamma rays. While significant efforts have been made to develop seedless citrus through mutation breeding, little has been done to induce and screen disease-resistant mutants. However, studies like Gulsen have used gamma radiation to induce mutations in 'Kutdiken' lemon and identified Mal secco-tolerant plants with desirable traits like high yield and early maturity.

Hybridisation/introgression citrus for disease resistance

If the desired level of resistance is absent in elite or obsolete cultivars, wild germplasm is a potential source of resistance for particular diseases, as it is less influenced by human selection and may still hold resistant alleles. However, introgression is challenging due to significant genetic divergence between cultivated and wild germplasm. Conventional crossing is also difficult in citrus due to polyembryonic seedlings, apomixes, and long juvenility periods. Biotechnological tools like protoplast fusion can facilitate introgression, while genetic engineering has been used to produce transgenic disease-resistant stocks. Various transgenes have been exploited to induce resistance in citrus species, and Agrobacterium strains have been used to transform explants using binary vectors. Transformation frequency varies depending on the citrus species, and transgenic plants are confirmed using marker genes and tests like Southern analysis. The level of transgene expression in the host species is evaluated using reverse transcriptase and northern analysis.

Need for genetic resources

The global citrus production is facing an existential threat due to the rapid spread of Huanglongbing (HLB) disease in the past two decades, which poses a significant risk to the limited native, wild citrus germplasm in Asia, Oceania, and Australia. The interaction between CLas (the bacterium that causes HLB) and citrus germplasm is a recent phenomenon, and the genetic diversity within the global germplasm pool is at risk of being lost forever. It is essential to identify gaps in collections and across taxa to collect and preserve the remaining germplasm for future generations. This urgent task requires a global strategy that all nations can collaborate on to protect the world's citrus production and genetic diversity. The window of opportunity to act is limited, and it is crucial that we work together to prevent the loss of this vital genetic resource.

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