

Genomic insights into citrus domestication and its important agronomic traits

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<https://doi.org/10.1016/j.xplc.2020.100138>

ABSTRACT

Citrus originated in Southeast Asia, and it has become one of the most important fruit crops worldwide. Citrus has a long and obscure domestication history due to its clonal propagation, long life cycle, wide sexual compatibility, and complex genetic background. As the genomic information of both wild and cultivated citrus becomes available, their domestication history and underlying traits or genes are becoming clear. This review outlines the genomic features of wild and cultivated species. We propose that the reduction of citric acid is a critical trait for citrus domestication. The genetic model representing the change during domestication may be associated with a regulatory complex known as WD-repeat-MYB-bHLH-WRKY (WMBW), which is involved in acidification and anthocyanin accumulation. The reduction in or loss of anthocyanins may be due to a hitchhiking effect of fruit acidity selection, in which mutation occurs in the common regulator of these two pathways in some domesticated types. Moreover, we have summarized the domestication traits and candidate genes for breeding purposes. This review represents a comprehensive summary of the genes controlling key traits of interest, such as acidity, metabolism, and disease resistance. It also sheds light on recent advances in early flowering from transgenic studies and provides a new perspective for fast breeding of citrus. Our review lays a foundation for future research on fruit acidity, flavor, and disease resistance in citrus.

Keywords: citrus, domestication, fruit acidity, genome, wild germplasm

Rao M.J., Zuo H., and Xu Q. (2021). Genomic insights into citrus domestication and its important agronomic traits. *Plant Comm.* 2, 100138.

INTRODUCTION

Citrus is widely grown in subtropical and tropical areas in more than 140 countries and regions, among which China, the United States, Mexico, Brazil, India, Spain, and Argentina are the most significant producers (according to FAO statistics, 2016, <https://www.fao.org/faostat/en/>). Citrus has a high economic value, it has an annual yield of 150 million tons and covers a cultivation area of more than 14.4 million Ha worldwide (FAO, 2018; <http://www.fao.org/faostat/en/#data>). Citrus fruit is a good source of nutrition and provides sugars, volatiles, organic acids (citric acid), dietary metabolites, amino acids, fibers, vitamin B6, vitamin C, and macro- and micronutrients (Liu et al., 2012). Citrus fruits are also rich secondary in metabolites (such as limonoids, alkaloids, flavonoids, coumarins, anthocyanins, essential oils, phenol acids, and carotenoids) and therefore have beneficial effects on human health (Rouseff and Nagy, 1994; Economos and Clay, 1999). In addition, citrus fruits are widely used for cosmetics, food, beverage, and pharmaceutical industries for the production of spices, medicines, additives, chemoprophylactic drugs, and others (Krishnaiah et al., 2011; Vun et al., 2015). In addition, some primitive and wild citrus

species, such as *Citrus medica* L., *Citrus wilsonii* Tanaka, and *Atalantia buxifolia*, are used for traditional medicine, and others are used as herbal medicine (Gmitter and Hu, 1990; Dave, 2009; Vun et al., 2015).

Because it is a long-lived perennial tree species, it is considered to have been domesticated at least 2,000 years ago. An ancient Chinese work titled *Records of the Grand Historian* recorded the commercial production of citrus and the management of citrus industrial affairs by specific government officials during the Han dynasty. Long juvenile phases, extensive hybridization, widespread outcrossing combined with clonal propagation, cultivar-wild gene flow, and multiple origins have contributed to the domestication of perennials (Mckey et al., 2010; Miller and Gross, 2011). Citrus is one of the most important fruit crops commercially cultivated worldwide. However, how citrus trees were domesticated remains largely unknown (Figure 1). This

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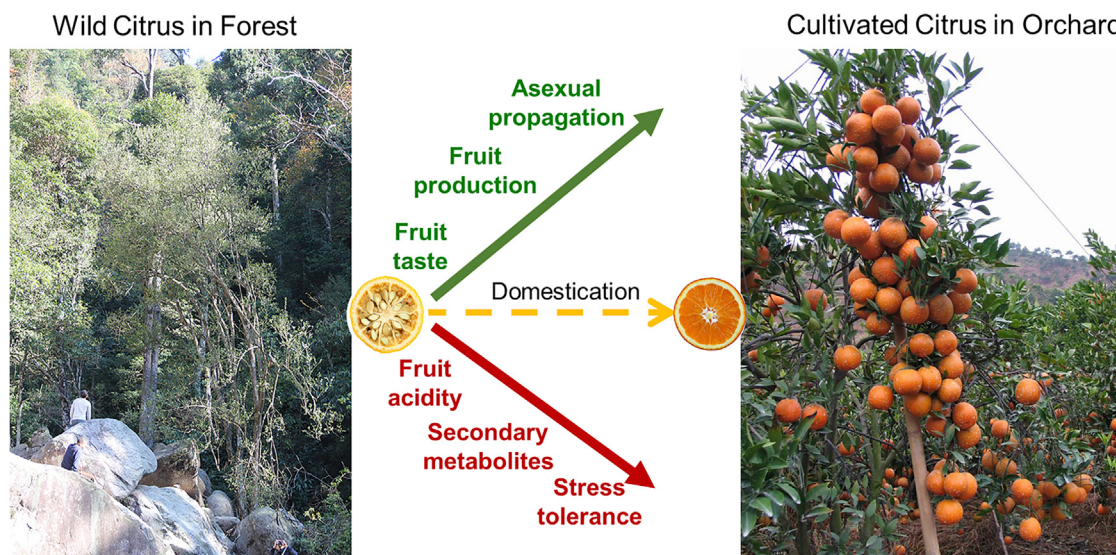


Figure 1. Morphological changes in cultivated citrus species.

Compared with wild citrus, the cultivars exhibit decreased fruit acidity, secondary metabolite levels (such as bitterness compounds), and tolerance to biotic or abiotic stresses but increased fruit production and taste, which mainly depends on the sugar/acid ratio. Asexual propagation, such as apomixis, is also popular among cultivated citrus species.

review focuses on the genomes of wild and cultivated citrus and outlines the progress in the identification of genes associated with key agronomic traits in citrus.

CITRUS GENOMES: FROM CULTIVARS TO WILD SPECIES

The *de novo* assembly of various citrus genomes is summarized in Table 1. Sweet orange is highly heterozygous, making its assembly by the short-read sequencing technology extremely difficult. Sweet orange is of global interest because it accounts for ~60% of the total citrus production, and both the fresh fruit and juice are consumed. Using a homozygous dihaploid line derived from anther culture, the genome of Valencia sweet orange was assembled (Xu et al., 2013). The genome of Clementine mandarin was first assembled using a haploid line and the Sanger sequencing technology by an international expert group with members from the United States, France, Italy, Spain, and Brazil (Wu et al., 2014). The above-mentioned studies showed that mandarins have a complex genetic background with intergenic introgressions from “pummelo” (*Citrus maxima*).

With the rapid development of sequencing technology, the pummelo and satsuma mandarin reference genomes were assembled using the long-read sequencing technology (Wang et al., 2017a; Shimizu et al., 2017). For the first time, the pummelo (*C. maxima*) haploid clone was used to produce a *de novo* sequence assembly using the PacBio RS II single-molecule sequencing system, and 307.3× coverage of Illumina short reads and 56.8× coverage of long reads were used to assemble the pummelo genome.

The genomes of several wild citrus species, such as *Citrus ichangensis* (Ichang papeda), Mangshan wild mandarin, citron, and *A.*

buxifolia (known as Chinese box orange), have been sequenced and assembled (Wang et al., 2017a, 2018). Comparison of the genomes between wild and cultivated citrus revealed that unique genes in the wild type and diversified genes in the cultivars are enriched in pathways related to defense response, proteolysis, pectate lyase activity, and reproduction (Wang et al., 2017a).

ORIGIN AND DOMESTICATION OF CITRUS

The *Citrus* genus and its associated genera (*Fortunella*, *Microcitrus*, *Clymenia*, *Oxanthera*, *Eremocitrus*, and *Poncirus*) belong to the Rutaceae family, and they are widely distributed in Southeast Asia, covering the Bismarck Archipelago, the south East Indian Archipelago, New Guinea, New Caledonia, north-eastern Australia, the western Polynesian islands, and Malaysia (Reuther, 1967). Despite major morphological and physiological differences, *Poncirus*, *Citrus*, *Fortunella*, *Eremocitrus*, and *Microcitrus* are sexually compatible (Krueger and Navarro, 2007; Garcia-Lor et al., 2013, 2015; Wu et al., 2018). The origin, domestication, and taxonomy of *Citrus* are complex and controversial. *Citrus* is thought to have originated in Southeast Asia (Figure 2), a biodiversity hotspot region influenced by both south Asian and east Asian monsoons (Jacques et al., 2011, 2014). Some regions in southwestern China, such as the Yunnan province; northeastern India in the Himalayan foothills; and Myanmar are also considered to be places of citrus origin (Reuther, 1967; Gmitter and Hu, 1990). A recent study indicates that the Nanling mountain region in central-south China may also be place of origin for mandarin (Wang et al., 2018).

There are two major taxonomic systems, namely, Swingle’s 10 species (Swingle, 1967) and Tanaka’s 162 species (Tanaka, 1977). In most studies, citrus is categorized as one of the three

Serial No.	Species	Common name	Domestication status	Ploidy level	Genome status	Sequencing technology	Total number of scaffolds	Size of assembled scaffolds (Mb)	Longest contig (Mb)	Contig N50 (*L50) (kb)	Reference
1	<i>Fortunella hindsii</i>	Hong Kong kumquat	Wild	Diploid	High quality	PacBio/Illumina	900	373.6	12.00	2209	Zhu et al. (2019)
2	<i>Citrus reticulata</i>	Mangshan wild mandarin	Wild	Diploid	Draft	Illumina	42 714	334	0.30	24.76	Wang et al. (2018)
3	<i>Citrus unshiu</i>	Satsuma mandarin	Cultivar	Diploid	Draft	Illumina/PacBio	20 876	359.7	0.54	24.26	Shimizu et al. (2017)
4	<i>Atalantia buxifolia</i>	Chinese box orange	Wild	Diploid	Draft	Illumina	25 600	316	0.20	23.89	Wang et al. (2017a)
5	<i>Citrus ichangensis</i>	Papeda	Wild	Diploid	Draft	Illumina	14 915	357	0.77	76.56	Wang et al. (2017a)
6	<i>Citrus grandis</i>	Pummelo	Cultivar	Haploid	High quality	PacBio RS II/Illumina	1612	346	10.62	2183	Wang et al. (2017a)
7	<i>Citrus medica</i>	Citron	Wild	Diploid	Draft	Illumina	32 731	405.0	0.41	46.50	Wang et al. (2017a)
8	<i>Citrus × clementina</i>	Clementine	Cultivar	Haploid	High quality	Sanger	1398	301.4	1.23	118.9*	Wu et al. (2014)
9	<i>Citrus × sinensis</i>	Ridge pineapple	Cultivar	Diploid	Draft	Sanger/454	12 574	319.2	0.119	6.6*	Wu et al. (2014)
10	<i>Citrus sinensis</i>	Valencia sweet orange	Cultivar	Dihaploid	Draft	Illumina	4811	327.8	0.323	49.89	Xu et al. (2013)

Table 1. Summary of *de novo* citrus genome assemblies and their features.

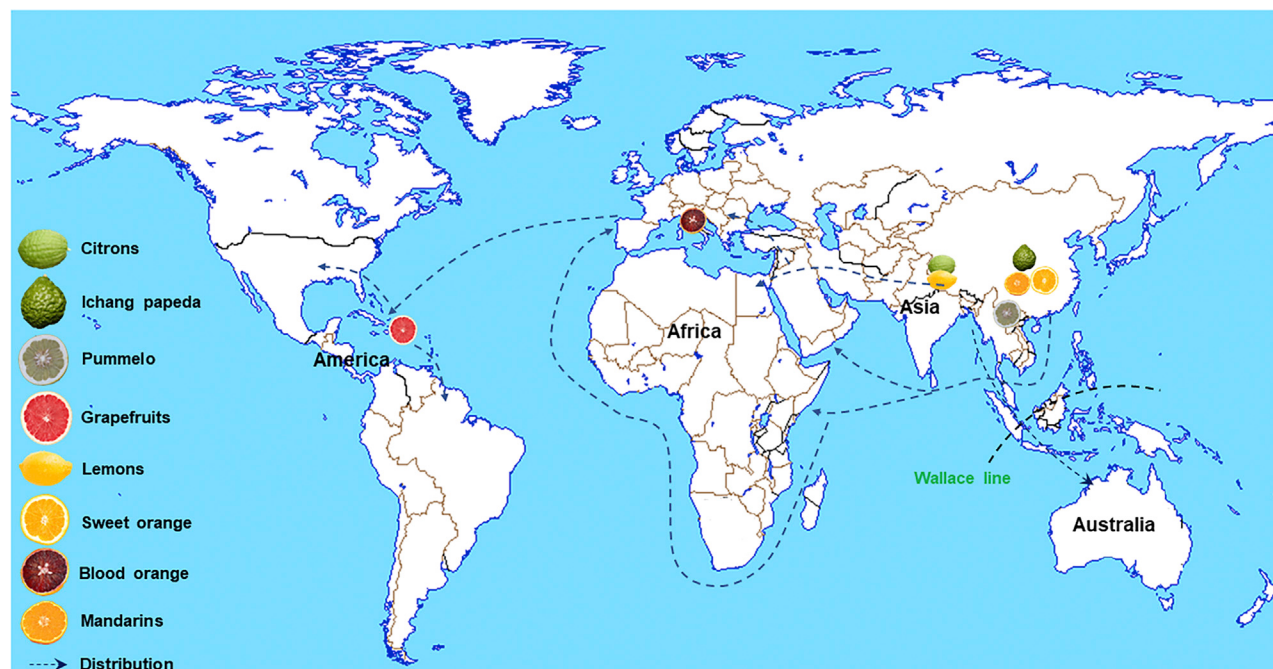


Figure 2. The origin and distribution of major citrus species.

The dashed lines indicate possible distribution directions rather than actual dispersal routes.

basic species, *Citrus reticulata* (mandarin), *C. maxima* (pummelo), and *C. medica* (citron). Most commercial cultivars are either from these three basic species or their hybrids. Nucleotide diversity reflects the degree of intraspecific variation and interspecific divergence, which can reveal the genomic origin of each species (Curk et al., 2016; Wu et al., 2018). Hybrid cultivars (lemon, sour orange, non-Australian limes, and calamondin) are identified from two or more ancestral citrus species with high segmental heterozygosity (~1.5%–2.4%) and intraspecific diversity (0.1%–0.6%). Bimodal distribution of heterozygosity has been observed in some highly heterozygous citrus accessions, such as grapefruits, sweet orange, and some mandarins, revealing high interspecific heterozygosity and complicated backcross mechanisms. Some pure genotypes without interspecific admixture, such as citrons (a monoembryonic species), display significantly reduced (~0.1%) intraspecific diversity compared with other species (0.3%–0.6%) (Curk et al., 2016).

The three basic species (mandarin, pummelo, and citron) probably originated from different places as indicated by updated whole-genome sequencing information on citrus evolution and origins (Xu et al., 2013; Wu et al., 2014, 2018; Wang et al., 2017a). Citron and pummelo are most likely to have originated in a triangle region of northwestern Yunnan (China), northeastern India, and northern Myanmar (Wu et al., 2018), whereas mandarin is most likely to have originated in Mangshan, a branch of the Nanling mountains (central-south China) (Wang et al., 2018). The Mangshan wild mandarin has many primitive characteristics. For example, its fruit is small and the fresh fruit is highly acidic (as acidic as lemon). The Mangshan wild mandarin has a long existence in old-growth forest and a pure genetic background. It has genetic introgressions

from a wild citrus variety, whereas Ichang papeda and the cultivated mandarin have wide introgressions from pummelo. Two independent domestication events occurred in south China, resulting in two geographically distinct groups of cultivated mandarins MD1 and MD2 in the north and south of the Nanling mountains, respectively (Figure 3).

The origin of hybrid citrus species is also getting clearer despite their complex genetic backgrounds that cause wide sexual compatibility between species and even between genera. For example, the origin of sweet orange, as one of the mysteries, is not yet fully understood. Sweet orange is known to be a hybrid between pummelo and mandarin. There are two proposed models for the possible origin of sweet orange. One is a simple model constructed based on the genomes of dihaploid and diploid sweet oranges: sweet orange = (pummelo × mandarin1) × mandarin2, and a comparison between the pummelo and mandarin genomes indicates that sweet orange probably arose from two rounds of hybridization between pummelo and mandarin (Xu et al., 2013). Subsequent genome sequencing data indicate that mandarins yielded from the two rounds of hybridization are different. The other model is complex: sweet orange = (pummelo × mandarin) × pummelo × mandarin2 (Wu et al., 2014), and it remains to be further verified due to the lack of genotypic data that support (pummelo × mandarin) × pummelo as the female parent of sweet orange. Moreover, whole-genome sequence data have confirmed that lemon is a hybrid between sour orange and citron, grapefruit is a hybrid between pummelo and sweet orange, and sour orange (*Citrus aurantium*) is a hybrid between pummelo and mandarin (Wu et al., 2018).

The Asian radiation of citrus species is assumed to have originated in the late Miocene (about 6–8 mya), during which the

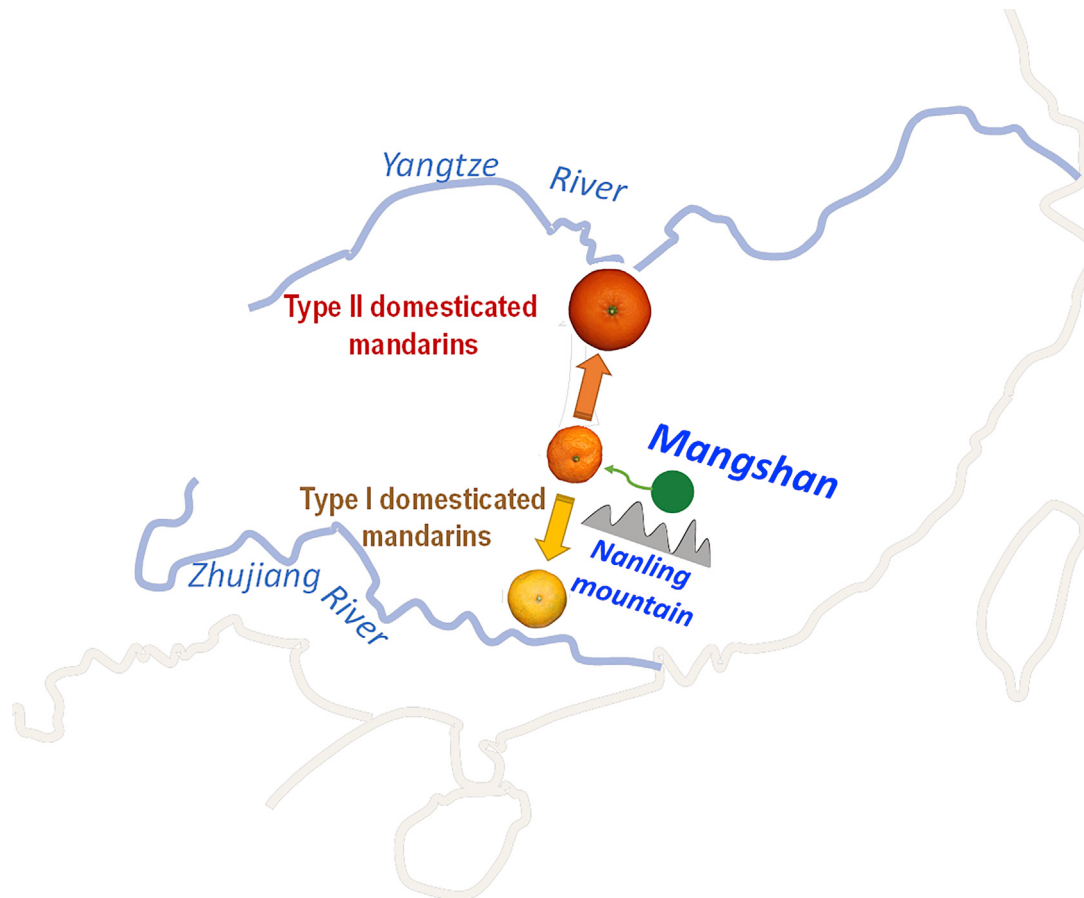


Figure 3. Origin and domestication of mandarin (*C. reticulata*) in Mangshan of the Nanling mountains in south China.

The model is based on the genomic analysis of Mangshan wild mandarins, Daoxian wild mandarins, semidomesticated mandarins, and domesticated mandarins in the surrounding regions (Wang et al., 2018).

climate changed from wet to dry as the monsoon weakened (Clift et al., 2014), resulting in major variations in biota, including the rapid radiation of several plant lineages (Wen et al., 2014; Favre et al., 2015). A fossil specimen from the late Miocene epoch, *Citrus linczangensis*, was discovered in the Lincang county of Yunnan province. It has been identified to have the characteristics of the current major citrus family (Xie et al., 2013). A distinct clade of Australian citrus species was formed, which is assumed to relate to citrons (Curk et al., 2016), and Swingle assigned different genus names to these species (*Microcitrus* and *Eremocitrus*) in his botanical classification (Batchelor, 1948; Reuther, 1967). However, a whole-genome phylogenetic analysis (Wu et al., 2018) and a molecular dating analysis (Pfeil and Crisp, 2008) suggest that citrus is not of Australian origin (Beattie et al., 2008). Citrus somehow migrated from Southeast Asia to Australasia via intercontinental dispersals, which might be attributed to the elevation advantage of Wallacea and Malesia in the Miocene and Pliocene epochs (Van welzen and Alahuhta, 2005; Hall, 2009). A genomic analysis suggests that the Australian radiation emerged in the Pliocene era (about 4 mya). Phylogenies of the chloroplast and nuclear genomes show clear signs of admixture between two Australian finger limes and round limes (Wu et al., 2018). The diversity among citrus species in northeast Australia is observed in both rainforests and dry

environments (Brophy et al., 2001). *Citrus tachibana* (tachibana mandarin) is naturally found in Japan, Taiwan, and the Ryukyu archipelago (Tanaka, 1931), it separated from Asian mainland mandarins during the early Pleistocene era (about 2 mya). Genomic data suggest that tachibana mandarin does not have a separate taxonomic position but shows close affinity with *C. reticulata* (Hirai et al., 1990; Shimizu et al., 2016).

KEY GENES ASSOCIATED WITH CITRUS DOMESTICATION AND THEIR IMPORTANT TRAITS

Yield, fruit quality, and taste, including juiciness, texture, acidity, reduction in seed number, and peel color (Goldenberg et al., 2014; Zheng et al., 2019), and some reproductive traits such as apomixis (Wang et al., 2017a) are important traits related to citrus domestication. In addition, disease (especially the citrus canker and Huanglongbing disease) and insect/pest resistance of cultivated citrus generally decreased (Bernet et al., 2005; Bastianel et al., 2009; Asins et al., 2012; Cuenca et al., 2013). Comparative analysis of the wild and cultivated species revealed remarkable changes in reproduction mode from sexual reproduction to apomixis during citrus evolution (Wang et al., 2017a). One recent comparative study of wild species

and cultivated mandarins indicated that the fruit acidity of cultivars has reduced significantly (Wang et al., 2018). Fruit acidity is one of the key factors that determine fruit taste, it can be evaluated by the sugar/acid ratio. Based on this, we speculate that fruit taste might be the first selected trait during domestication.

Asexual reproduction through apomixis

Apomixis is a natural phenomenon of asexual reproduction that produces progenies that are genetically identical to the mother plant (Conner et al., 2015). Apomixis is uncommon for most important agricultural crops except for citrus and apple. Apomixis was first reported in citrus in 1719 the same seed was found to produce two plantlets (Batygina and Vinogradova, 2007). Since then, it has been confirmed that some important commercial citrus species, such as sweet orange and grapefruit, develop more than one embryo from somatic nucellar cells and thus share the same genotype (Kepiro and Roose, 2010). Generally, one seed produces 2–10 embryos, but in some cases, it can produce 30 or more embryos (Koltunow, 1993). This phenomenon is known as “polyembryony” (a phenomenon referring to the development of two or more embryos from one fertilized egg). Polyembryony has been used extensively for propagation programs in citrus nurseries to produce uniform rootstocks from seeds. Moreover, the polyembryony trait, as a form of apomixis, has been fixed in citrus cultivars because it allows growers to maintain and disperse an elite line with desirable variations and traits without segregation.

Most commercially grown citrus cultivars are polyembryonic in nature. Relatively fewer citrus cultivars are monoembryonic, these include all citron, some mandarin hybrids, clementine cultivars, and pummelo (Wang et al., 2017a; Zhang et al., 2018). Recently, the citrus *CitRWP* gene has been shown to associate with polyembryony by both genetic mapping and association analysis (Table 2). Sequence analysis of this gene revealed a 203-bp miniature inverted-repeat transposable element (MITE) insertion in the promoter region of the *CitRWP* gene in polyembryonic genotypes in the *Citrus* genus (Wang et al., 2017a). Notably, *CitRWP* expression is higher in the ovules of polyembryonic cultivars than in those of monoembryonic cultivars, suggesting that it is a key candidate gene that governs polyembryony. A recent gene function study indicates that antisense interference of *CitRKD1* (the same gene as *CitRWP*) expression in sweet orange abolishes nucellar embryogenesis in T_1 regenerants (Shimada et al., 2018). Therefore, knockout of the *CitRWP* gene from polyembryonic citrus cultivars will probably reduce polyembryony in cultivated citrus and provide promising insights into apomixis research in citrus.

Fruit acidity

Some citrus species, such as lemon, lime, wild mandarin, and sour orange, show an extremely low pH value of 2–3 due to a high level of acidification of vacuoles in juice vesicles. Based on the evidence from a recent population analysis of wild and cultivated mandarins, we speculate that reduced citric acid level is a remarkable trait for citrus domestication (Wang et al., 2018). This is different from the traditional view that increased sugar content

is likely the most significant event of the domestication of fruit crops and reduced acidity is a hitchhiking effect.

The two citrus vacuolar P-ATPase homologs, *CitPH1* and *CitPH5*, are strongly induced in highly acidic citrus species such as lime and lemon fruits, by contrast, their expression levels are significantly reduced in acidless mutants (Strazzer et al., 2019). Vacuolar ATPases can regulate the pH gradient across the tonoplast (Müller and Taiz, 2002; Nishi and Forgac, 2002; Shimada et al., 2006; Pittman, 2012; Riemmüller et al., 2012). A steep proton gradient across the vacuolar membrane due to differences in pH can drive the transport of citrate into the vacuole, however, the underlying mechanism remains unclear. The expression levels of *CitPH1*, *CitPH5*, and the PH3 (WRKY), PH4 (MYB) (Strazzer et al., 2019), and basic helix-loop-helix (bHLH) (citrus Noemi or *CitAN1*) transcription factors were reduced in the mutants (Butelli et al., 2019). In petunia, the homologs of these transcription factors are involved in the activation of *PH1* and *PH5* expression. Homologs of petunia PH4, which is a MYB transcription factor, has been reported to activate the promoter of the proton pumps (PH1 and PH5) in petunia as well as in citrus (Quattrocchio et al., 2006; Faraco et al., 2014; Butelli et al., 2019; Strazzer et al., 2019). These findings provide a guidance for regulating citric acid levels in fruits.

A previous genetic analysis revealed that the insertion of large retrotransposon fragments or deletion in the Noemi (*CitAN1*) gene results in acidless phenotypes and loss of proanthocyanidin and anthocyanin in sweet lime, citron, sweet orange, lemon, and lime-ta accessions (Butelli et al., 2019; Figure 4). Moreover, a variation in the core promoter region of the Noemi gene in two lime-ta accessions reduces its expression and increases the pH of the juice, indicating that Noemi is a key gene that contributes to fruit acidity. Previous studies revealed that a bHLH transcription factor (AN1) is involved in both vacuolar acidification and anthocyanin accumulation in petunia (Spelt et al., 2002; Quattrocchio et al., 2006; Faraco et al., 2014). Recent studies showed that a specific mutation in *CitAN1* is responsible for the reduced expression of *CitPH1* and *CitPH5* in acidless Faris lemon and other acidless citrus fruits (Strazzer et al., 2019). These data suggest that the reduction or loss of anthocyanins may be due to the hitchhiking effect of fruit acidity selection in some domestication types, when mutations occur on the bHLH transcription factor, a common regulator for both citric acid and anthocyanin metabolism.

Fruit color: anthocyanins

Anthocyanins are pigment compounds that are abundant in several wild citrus accessions but absent from most cultivated citrus (Butelli et al., 2017; Huang et al., 2018). Anthocyanins are a subclass of flavonoids, which endow young leaves, flowers, petals, and fruits with different colors. Transcription factor complexes that regulate anthocyanin levels and acidification have long been studied in many species such as *Arabidopsis*, maize, and petunia (Spelt et al., 2002; Quattrocchio et al., 2006; Verweij et al., 2016). Complexes composed of the WD40-repeat proteins, as well as MYB, bHLH, and WRKY transcription factors are named WD-repeat-MYB-bHLH-WRKY (WMBW for short). Specifically, the regulatory complex is composed of three anthocyanin pathway-related transcriptional regulators, AN1

Serial No.	Gene details	Identified/cloned	Function study	Target trait	Reference
1	Papain-like cysteine protease	Sweet orange	–	Citrus Huanglongbing	Clark et al. (2018)
2	Accelerated cell death 2	Sweet orange	Duncan grapefruit		Pang et al. (2020)
3	2-oxoglutarate and Fe(II)-dependent oxygenase gene	US-897 (<i>Citrus reticulata</i> × <i>Poncirus trifoliata</i>)	–		Albrecht and Bowman (2011)
5	<i>PtCDR2</i> and <i>PtCDR8</i>	<i>P. trifoliata</i>	–		Rawat et al. (2017)
6	<i>AtNPR1</i>	<i>Arabidopsis</i>	Duncan grapefruit and Hamlin sweet orange		Robertson et al. (2018)
7	<i>CsLOB1</i>	Sweet orange Duncan grapefruit	Editing of gene	Citrus canker	Hu et al. (2014) Jia et al. (2017)
8	<i>CitRWP</i>	Citrus	–	Polyembryony	Wang et al. (2017a)
9	<i>CgMYB58</i>	Pummelo	Expressed in citrus callus	Lignin biosynthesis	Shi et al. (2020)
10	S-RNase gene	Lemon	–	Self-incompatibility	Zhang et al. (2015)
11	Sm-RNase	Mandarin	–		Liang et al. (2020)
12	FT gene	Citrus	Grapefruit	Early flowering	Sinn et al. (2020)
13		<i>P. trifoliata</i>	Trifoliolate orange and satsuma mandarin		Endo et al. (2005) Nishikawa et al. (2007)
14	<i>CcISBP7</i>	Clementine mandarin	<i>Arabidopsis thaliana</i>		Zeng et al. (2019)
15	miR3954	Citrus	Hong Kong kumquat (<i>Fortunella hindsii</i>)		Liu et al. (2017a)
16	<i>CitdGlcTs</i>	Citrus	Tobacco	Flavor	Chen et al. (2019)
17	<i>Cit1,2RhaT</i>	Citrus	Tobacco		Chen et al. (2019)
18	<i>Cm1,2RhaT</i>	Pummelo	Hong Kong kumquat (<i>F. hindsii</i>)		Chen et al. (2019)
19	<i>CsVPP-1</i> and <i>CsVPP-2</i>	Sweet orange	–		Hussain et al. (2020)
20	<i>CitAco3–CitIDH1–CitGS2</i>	Sweet orange	–	Acidity	Chen et al. (2013)
21	<i>CsAPD2</i>	Sweet orange	–		Bai et al. (2020)
22	<i>CsPH8</i>	Sweet orange	Pummelo, tomato, and strawberry		Guo et al. (2016); Shi et al. (2019)
23	<i>CitPH1</i> and <i>CitPH5</i>	Sour lemon, orange, pummelo and rangpur lime fruits	–		Strazzer et al. (2019)
24	<i>CWINVs</i> , <i>VINV</i> , <i>SPS2</i> , <i>SUT2</i> , <i>VPP-1</i> , and <i>VPP-2</i>	Sweet orange	–	Sugar	Hussain et al. (2020)
25	<i>V-PPase1, 2</i>	Sweet orange	–		Hussain et al. (2020)
26	<i>CitPH1</i> and <i>CitPH5</i>	Sour lemon, orange, pummelo, and rangpur lime fruits	–	Anthocyanin and flavor	Strazzer et al. (2019)
27	<i>CsCYP75B1</i>	Sweet orange	<i>A. thaliana</i>	Flavonoid biosynthesis	Rao et al. (2020)

Table 2. Genes controlling important traits in citrus.

(Continued on next page)

Serial No.	Gene details	Identified/cloned	Function study	Target trait	Reference
28	<i>CsUDP78D3</i>	Sweet orange	<i>A. thaliana</i>	Anthocyanin	Rao et al. (2019b)
29	<i>CgRuby1</i> , <i>CgRuby2</i> , <i>AbRuby2</i>	Atalantia and pummelo	<i>A. thaliana</i>		Huang et al. (2018)
30	Ruby and Noemi (bHLH)	Citrus	–		Catalano et al. (2020)
31	MYB3	Sweet orange	<i>Arabidopsis</i>		Huang et al. (2020)
32	CDG1	Pummelo	<i>Arabidopsis</i> and tobacco	Delayed leaf greening	Yu et al. (2020)
33	<i>CCD4</i>	Mandarins and its hybrids	–	Carotenoids	Zheng et al. (2019)
34	<i>CsDxs</i> and <i>CsPsy</i>	Sweet orange	–		Fanciullino et al. (2008)
35	<i>CitDXS1</i> and three <i>CitPSY1</i>	Citrus	–		Peng et al. (2013)
36	<i>CsMADS5</i> and <i>CsMADS6</i>	Sweet orange	–		Lu et al. (2018)
37	<i>PSY2</i> , <i>LYCB2</i> , <i>LYCE</i> , and <i>CCD4</i>	Pummelo	–		Jiang et al. (2019)

Table 2. Continued

(bHLH), AN2 (MYB), and AN11 (WD-repeat), as well as two pH-specific transcription factors, PH3 (WRKY) and PH4 (MYB). Ruby1, which is homologous to the AN2 gene, is the first identified MYB transcription factor that plays a critical role in the regulation of anthocyanin biosynthesis in blood orange (Butelli et al., 2012). *Ruby2* and *Ruby1*, two neighboring genes, form a cluster to regulate anthocyanin levels in citrus (Huang et al., 2018). In the distant wild citrus (*A. buxifolia*), *Ruby2* and *Ruby1* both function as anthocyanin activators but *Ruby2* functions in tender leaves, whereas *Ruby1* functions in fruits. *Ruby2* mutations are found in both recent wild citrus and domesticated citrus (Huang et al., 2018). Recently, MYB3 has been identified as a balance factor in anthocyanin metabolism because it is subjected to *Ruby1* regulation and shows an expression pattern similar to *Ruby1*, however, MYB3 acts as a repressor of anthocyanin biosynthesis (Huang et al., 2020).

The citrus HLB and canker disease

Huanglongbing (HLB), also known as the greening disease, is the most devastating citrus disease in the world (Wang and Trivedi, 2013; Wang 2019). HLB is caused by three phloem-limited, gram-negative, and fastidious bacteria known as “*Candidatus Liberibacter asiaticus*” (CLAs) (Bové, 2006; Gottwald et al., 2007; Ma et al., 2014), “*Candidatus Liberibacter americanus*,” and “*Candidatus Liberibacter africanus*” (Teixeira et al., 2005). HLB can reduce citrus yield by 30%–100% (Bové, 2006) and result in small, greenish, and poor quality fruits, causing huge losses to growers (Bassanezi and Stuchi, 2009; Wang et al., 2017b; Dagulo et al., 2010; Bassanezi et al., 2011). The infected fruits are highly acidic, with reduced sugar contents (Brodersen et al., 2014; Massenti et al., 2016), a metallic, bitter taste, and less juice (Kiefl et al., 2017; Dala Paula et al., 2018).

Recently, some wild citrus germplasms or distantly related citrus species, such as *Eremocitrus glauca*, *Microcitrus australasica* (Ramadugu et al., 2016), orange jasmine (*Murraya paniculata*)

(Miles et al., 2017), *Poncirus trifoliata* (Killiny and Hijaz, 2016), and papeda (*C. ichangensis*) (Wu et al., 2020) have been identified as highly tolerant to HLB, whereas cultivated citrus such as *C. maxima* (Ramadugu et al., 2016), *Citrus sinensis*, and mandarins (*C. reticulata*) are considered highly susceptible to HLB (Folimonova et al., 2009). The tolerance was speculated to be related to the defense response and/or antimicrobial secondary metabolites (Hammond-Kosack and Jones, 1996; Christeller and Laing, 2005; Liu et al., 2013; Rao et al., 2019a). Interestingly, several transcriptomic studies of HLB-infected citrus revealed that several genes related to the WRKY family, the PR family, and secondary metabolites were stimulated in response to CLAs invasion (Rawat et al., 2015; Hu et al., 2017; Yu et al., 2017). Generally, HLB-tolerant citrus species show a fast and resilient response to CLAs invasion (before Las stability), whereas susceptible species show no or a delayed response (Hu et al., 2017; Wu et al., 2020). Furthermore, the CLAs pathogen releases some virulence proteins and enzymes to degrade salicylic acid and its derivatives, thus damaging the host defense system (Li et al., 2017a) and resulting in severe symptoms (Tolba and Soliman, 2015). Recent studies have advanced the understanding of interactions between CLAs effectors and citrus genes, such as the interaction between defense-inducible papain-like cysteine proteases and Sec-delivered effector 1 (SDE1) (Clark et al., 2018) and that between accelerated cell death 2 (ACD2) in susceptible citrus and SDE15 (Pang et al., 2020).

Citrus canker is another devastating bacterial disease caused by *Xanthomonas* strains, such as *Xanthomonas citri* subsp. *citri* and *Xanthomonas axonopodis* pv. *aurantifolii* (Schubert et al., 2001; Sun et al., 2004). Citrus canker causes severe necrosis symptoms on leaves and fruits (Stover et al., 2014), resulting in considerable yield losses (Gottwald et al., 2002). Almost all cultivated citrus species are canker susceptible (Gottwald et al., 1993), whereas some wild citrus species show tolerance or resistance to canker. Previous studies have reported that canker bacteria inject transcription activator-like effectors to

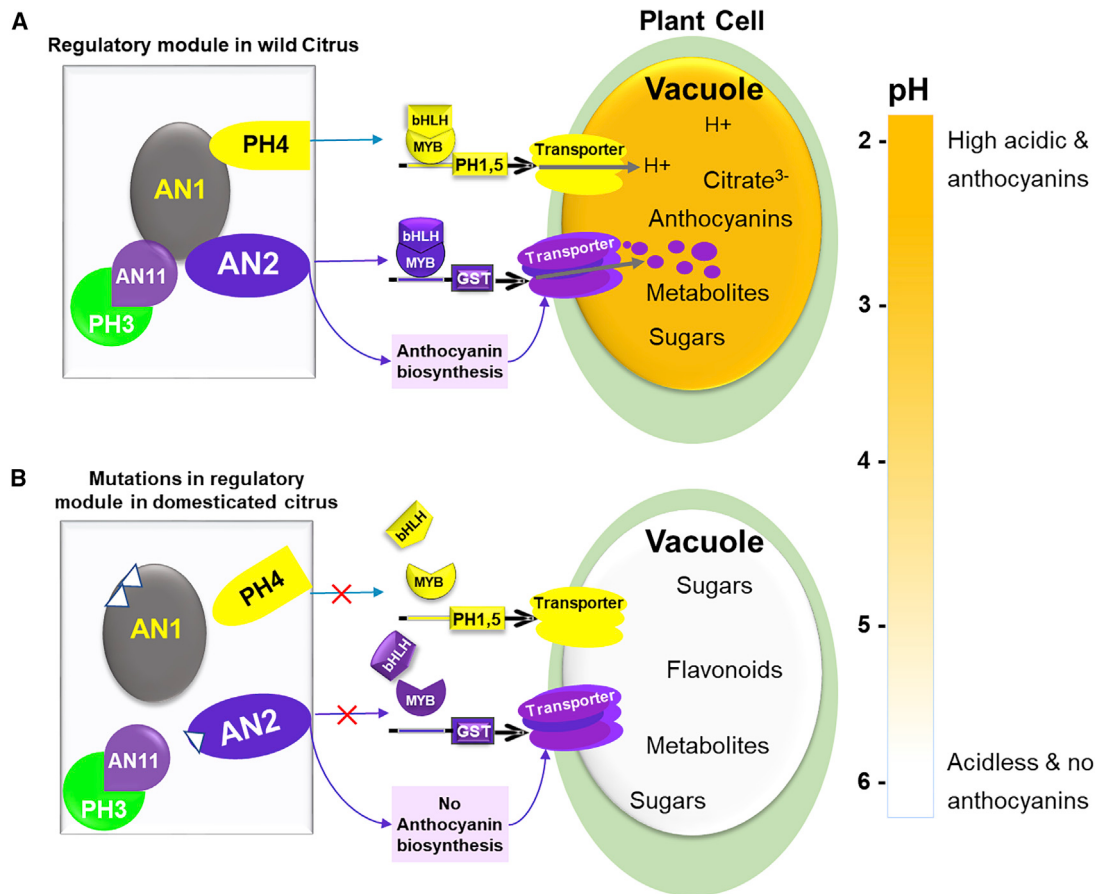


Figure 4. Domestication modules of fruit acidity and anthocyanin accumulation in citrus.

The regulatory module is designated WD-repeat-MYB-bHLH-WRKY, it is composed of three anthocyanin pathway-related transcription regulators AN11 (WD-repeat), AN2 (MYB), and AN1 (bHLH) and two pH-specific transcription factors PH3 (WRKY) and PH4 (MYB). These regulators are named after their petunia homologs (Spelt et al., 2002; Quattrocchio et al., 2006; Verweij et al., 2016). **(A)** Most wild citrus can accumulate high levels of citric acid in fruits. The regulatory module can activate the *CitPH1* and *CitPH5* genes, which encode the proton-pumping complex and are responsible for generating a discrepancy in proton gradient to drive citrate transport. Anthocyanin accumulation in tender leaves, early flowers, and fruits is mediated by anthocyanin transporter genes. The AN1 transcription factor, also known as Noemi, which encodes a bHLH transcription factor, is involved in the regulation of both pH and anthocyanin accumulation (Butelli et al., 2019). AN1 can interact with both PH4 (a key regulator of fruit pH) and AN2 (also known as Ruby1, a key regulator of anthocyanins), both encode MYB transcription factors (Butelli et al., 2017; Huang et al., 2018). Another bHLH transcription factor named bHLH1 can interact with only AN2 to regulate anthocyanin accumulation in fruits (Huang et al., 2018). **(B)** Published studies indicating that in domesticated citrus, mutations mostly occurred in the regulatory module containing the AN2 or AN1 transcription factor. The AN2 mutation disrupts anthocyanin biosynthesis, whereas the AN1 mutation affects the accumulation of anthocyanins, proanthocyanins, and citric acid (Butelli et al., 2019).

bind to the promoter elements of susceptible host genes such as the citrus *LATERAL ORGAN BOUNDARIES 1* (*CsLOB1*) gene to stimulate *LOB1* expression through type III secretion pathways (Hu et al., 2014). The high expression level of *CsLOB1* (a transcription factor) can promote pustule formation and bacterial growth (Xu et al., 2016; Zhang et al., 2017b). *A. buxifolia*, belongs to primitive citrus, It is tolerant to some abiotic and biotic stresses and is considered canker tolerant (Shi et al., 2014; Yang, et al., 2013). A recent study has suggested that natural variations in *LOB1* and *TFIIA γ* , which encodes a transcription factor that stabilizes the interaction between the effector and *LOB1* to confer resistance to the citrus canker disease in *A. buxifolia* (Tang, et al., 2021).

Recent studies have reported that citrus relatives (*M. paniculata*) and primitive (*A. buxifolia*) and wild (*E. glauca*, *M. australasica*) cit-

rus show strong tolerance against the devastating HLB and canker diseases. The availability of the bacterial (CLas) and various citrus genomes has facilitated the understanding of pathogenicity, as well as the citrus tolerance and susceptibility mechanisms. We propose that identifying resistant genes from citrus wild relatives and comparing these genes with those in cultivated citrus are important to understand the loss of resistance in cultivated species, and that the the knock down/out of susceptible genes offers an alternative way to improve disease resistance in citrus cultivars.

Juvenility

Normally, citrus seedlings have a very long juvenile phase (about 3–20 years), which hinders the breeding and improvement of citrus. In the past 2 decades, efforts have been made to minimize the juvenile

phase (Cervera et al., 2009; Endo et al., 2009; Flachowsky et al., 2009). To promote early flowering in citrus, the Arabidopsis *APETALA1* (*AP1*) and *LEAFY* (*LFY*) genes were introduced into citrange (*P. trifoliata* L. Raf. × hybrid of *C. sinensis* L. Osbeck) (Peña et al., 2001), and transgenic citrange plants overexpressing the *AP1* and *LFY* genes flowered early and produced fertile and normal flowers. Fruits were obtained from first-year transgenic plants that were only 2–20 months old and bore their first flowers, suggesting that the juvenile phase was significantly reduced to less than 5 years in transgenic citrange compared with control plants (Peña et al., 2001). Zygotic seedlings obtained by crossing *AP1* and *LFY*-transgenic citrange also showed early flowering with normal fruit setting in the first spring. These results confirmed that early flowering can be induced in cultivated citrus species by introducing the *AP1* and *LFY* genes.

In citrus, the use of endogenous genes has also been reported to minimize the juvenile period (Endo et al., 2005; Sinn et al., 2020). The citrus (ortholog) *FLOWERING LOCUS T* (*FT*) gene, which is involved in the photoperiodic induction of flowering (Koornneef et al., 1991), was introduced into *P. trifoliata* (trifoliate orange) under the 35S CaMV promoter. The transgenic trifoliate orange expressing the citrus *FT* gene showed early flowering just 6–11 months after transformation and produced fertile pollen and normal flowers. Zygotic seedlings obtained by crossing these transgenic plants with the monoembryonic “Kiyomi” tangor (*C. sinensis* × *C. unshiu*) also showed a greatly shortened juvenile phase, these seedlings flowered right after germination (Endo et al., 2005). Flowering-related orthologous genes in citrus, such as *FT*, *CsTFL1* (*TERMINAL FLOWER 1*), *CsLFY* (*LEAFY*), and *CsAP1* (*APETALA1*) (Nishikawa et al., 2007, 2010), were highly expressed during putative induced flowering. These outcomes suggest that *FT* overexpression might have reduced the juvenile period in cultivated citrus species. Moreover, the overexpression of miR3954 facilitated early flowering in transgenic kumquat (Liu et al., 2017a). One recent study reported that chimeric *FT* protein successfully reduced flowering time in grapefruit (*Citrus paradisi*) without negative effects. Therefore, transgenic expression of chimeric *FT* proteins provides a promising alternative tool for minimizing juvenility in cultivated citrus species (Sinn et al., 2020).

Recently, SQUAMOSA-promoter binding protein (SBP/SPL)-box genes have been shown to play critical roles in plant growth, flowering, and fruit development (Chen et al., 2010; Shalom et al., 2015; Liu et al., 2017b; Long et al., 2018). A total of 16 and 15 SBP-box genes have been identified in *Arabidopsis* and citrus, respectively. To understand the function of these genes, clementine mandarin SBP-box genes were cloned and overexpressed in *Arabidopsis*, and transgenic *Arabidopsis* plants expressing the *CcISBP7* gene showed flowered earlier than control plants (Zeng et al., 2019). These results indicate that *CcISBP7* from is involved in early flowering in clementine mandarin and can induce early flowering in other citrus species to shorten the juvenile phase.

Other important traits

The appearance of red fruit of some mandarin landraces is the outcome of long-term selection in recent breeding history. Using an integrated genetic approach, we revealed that a change in a *cis*-regulatory element in *CCD4b*, which encodes CAROTENOID

CLEAVAGE DIOXYGENASE 4b, is a major genetic determinant of natural variation in C30 apocarotenoids responsible for the red color of citrus peel (Zheng et al., 2019). The *CsMADS6* transcription factor modulates carotenoid metabolisms by directly binding to a set of carotenogenic genes, including those encoding the rate-limiting enzyme phytoene synthase (PSY), the branch-limiting Lycopene b-cyclases, phytoene desaturase (PDS), and carotenoid cleavage dioxygenase1 (CCD1). The overexpression of *CsMADS6* increases carotenoid content (Lu et al., 2018). Recently, Citrus Delayed Greening gene 1 (CDG1) has been reported to contribute to delayed leaf greening by inhibiting the synthesis of chlorophyll (Yu et al., 2020).

Some citrus species show self-incompatibility. A molecular study in lemon indicates that the *S-RNase* gene is related to self-incompatibility (Zhang et al., 2015). One recent study has reported that a predominant single-nucleotide mutation in *Sm-RNase* causes the loss of its natural function, in turn producing self-compatible citrus. A large-scale genetic analysis indicates that most wild citrus are self-incompatible, and that the transition from self-incompatibility to self-compatibility is because *Sm-RNase* initially arose in mandarin, and then passed to its hybrids and became fixed in cultivated cultivars (Liang et al., 2020).

MINI-CITRUS (HONG KONG KUMQUAT): A WILD SPECIES AS A CANDIDATE MODEL FOR GENETIC AND GENE FUNCTION STUDIES

Fortunella hindsii, commonly known as Hong Kong kumquat, is a wild citrus species (Figure 5). Its fruit is spicy but has an attractive color, it is thus frequently used for gardening and ornamental purposes. Its tree canopy is considered the smallest of all citrus species (Swingle, 1967). In addition, Hong Kong kumquat is also famous among citrus species due to its distinctive features, such as continuous and very early flowering with a very short juvenile period of ~8 months (Ye, 1985; Zhu et al., 2019). Among all citrus, *F. hindsii* has the highest callus induction rate and callus can be induced from its roots and seedlings, making it an ideal model species for citrus research (Deng and Zhang, 1988). The *F. hindsii* transgenic system has been established to unravel the genetic basis of carotenoid biosynthesis (Zhang et al., 2009; Cao et al., 2015). A recent study has reported that *F. hindsii* exhibits both asexual and sexual reproduction modes (Zhu et al., 2019), and the latter is ideal for both genetic studies and transgenic segregation because most citrus species undergo asexual reproduction.

The successful applications of CRISPR-Cas9 in citrus have been reported recently (Zhang et al., 2017a; Jia et al., 2017; Peng et al., 2017), and this method has also been applied to the mini-citrus (Zhu et al., 2019). The application of CRISPR-Cas9 strategy in the functional genomics studies of citrus species has been restricted due to their heterogeneous genetic backgrounds and their nature as complicated and unstable chimeras (Zhang et al., 2017a; Jia et al., 2017), with different citrus species exhibiting asynchronous growth patterns and distinctive organs (Guo et al., 2012; Zeng et al., 2013; Liu et al., 2016; Lu et al., 2016). Furthermore, a recently published report reveals that CRISPR-Cas9-based targeted



Figure 5. Mini-citrus (*F. hindsii*): a candidate model species for citrus genetics studies and gene functional studies.

Left panel: Fruits on a young *F. hindsii* seedling; right panel: a mini-citrus plant transformed with the GFP marker.

mutations successfully reproduced transgenic T₁ (heterozygous, biallelic, chimeric, and homozygous) uniformly mutated plants (Ma et al., 2015). Conversely, in citrus, attaining a suitable T₁ plant is restricted by polyembryony (asexual reproduction) features and the long juvenility. Hence, it is anticipated that the CRISPR method is applicable in *F. hindsii*, which has a short juvenile phase, and the application of this method will facilitate the functional study of desired traits and unravel the genomic features of citrus.

CONCLUDING REMARKS AND PERSPECTIVES

This review summarizes the genomic resources of various citrus species. It reveals current progress in the understanding of citrus origin and domestication with a focus on key agronomic traits and the corresponding genes. We have highlighted that fruit acidity has been targeted during citrus domestication, whereas anthocyanin was very likely selected due to the hitchhiking effect during domestication, considering that the *AN1* gene was found to regulate both anthocyanin and acidity metabolism. In addition, we have addressed the latest progress in the identification of genes controlling apomixis (polyembryony), early flowering, and resistance against HLB and citrus canker.

Future research based on more citrus genomes is suggested to comprehensively investigate the genomic changes from the wild to cultivated citrus. The assembly of genomes of wild species and landraces is particularly necessary for fulfilling such purpose. One type of the largely neglected changes during domestication is probably associated with transposable elements. Several studies have revealed that the insertion of MITE into key genes is responsible for apomixis (Wang et al., 2017a), the red color of fruit peel (Zheng et al., 2019), and anthocyanin accumulation (Butelli et al., 2017; Huang et al., 2018). It will be interesting to investigate whether MITE or other types of transposable elements have participated in citrus domestication.

The change in disease resistance/tolerance during citrus domestication is another interesting topic. Citrus HLB and canker are the most devastating diseases when a uniform cultivar is planted in a

large farm. Because wild citrus has grown in natural environments for tens or hundreds of years without any artificial protection or management, future studies should focus on revealing the mechanisms of HLB susceptibility, tolerance, and host-pathogen interactions in wild citrus relatives.

The genes related to important agronomic traits provide targets for precise breeding. One way to develop molecular markers is

to select ideal genotypes at the young seedling stage. Monoembryonic (sexual reproduction) and polyembryonic (apomixis) species can be selected for different breeding purposes using markers developed from MITE insertions in the *CitRWP* gene (Wang et al., 2017a). Moreover, using the latest genome editing technology, we can separate linked traits selected during domestication. For example, anthocyanin- and acidity-related genes or their promoters can be manipulated independently to increase anthocyanin content, which is beneficial to human health, while reducing fruit acidity in new cultivars. By tracing the domestication history of thousands of years, we provide a theoretical basis for developing new citrus varieties to meet the consumers' demands in the coming decades.

FUNDING

This project was supported by the National Natural Science Foundation of China (31925034), the National Key Research and Development Program of China (2018YFD1000101), and Fundamental Research Funds for the Central Universities.

AUTHOR CONTRIBUTIONS

M.J.R., Q.X., and H.Z. wrote the original draft. M.J.R. prepared the figures and tables. Q.X. provided resources and funding and revised/edited the original manuscript.

ACKNOWLEDGMENTS

Great gratitude goes to linguistics professor Ping Liu from the Foreign Language College, Huazhong Agriculture University, Wuhan, China, for her work of English editing and language polishing. No conflict of interest declared.

Received: October 9, 2020

Revised: December 4, 2020

Accepted: December 25, 2020

Published: December 30, 2020

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