

## Review

# Citrus breeding, genetics and genomics in Japan

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Citrus is one of the most cultivated fruits in the world, and satsuma mandarin (*Citrus unshiu* Marc.) is a major cultivated citrus in Japan. Many excellent cultivars derived from satsuma mandarin have been released through the improvement of mandarins using a conventional breeding method. The citrus breeding program is a lengthy process owing to the long juvenility, and it is predicted that marker-assisted selection (MAS) will overcome the obstacle and improve the efficiency of conventional breeding methods. To promote citrus molecular breeding in Japan, a genetic mapping was initiated in 1987, and the experimental tools and resources necessary for citrus functional genomics have been developed in relation to the physiological analysis of satsuma mandarin. In this paper, we review the progress of citrus breeding and genome researches in Japan and report the studies on genetic mapping, expression sequence tag cataloguing, and molecular characterization of breeding characteristics, mainly in terms of the metabolism of bio-functional substances as well as factors relating to, for example, fruit quality, disease resistance, polyembryony, and flowering.

**Key Words:** Citrus, genome, breeding, EST, functional genomics.

## Introduction

Citrus is one of the most cultivated fruits in the world, and the total citrus fruit production for 2012 has been estimated at 131.3 million tons (8.8 kha) (FAO 2014: <http://faostat3.fao.org/>; accessed 08/05/2015), which was 20.0% of total fruit production. Among citrus production, sweet oranges account for 52.6%, followed by mandarins (21.1%), lemons and limes (11.2%), and pummelos and grapefruits (6.2%). There are wide variations in citrus in terms of, for example, fruit shape, quality, embryo types, inflorescence, tree growth and habit, and adaptability. Mainly owing to sexual compatibility between *Citrus* species, a wide diversity has been generated during a long history of cultivation and wide dispersion (Nicolosi *et al.* 2000); this has led to a complicated system of citrus taxonomy and nomenclature over a long period. Taxonomy and phylogeny have been based mainly on morphological data and geographical data, and two different classification systems are commonly accepted for the citrus taxonomy: the system of Swingle (1943, 1967) and that of Tanaka (1954, 1961) (Nicolosi 2007). The major difference between the two systems is the number of recognized species: Swingle recognized 16 species in the genus *Citrus*, whereas Tanaka recognized 162 species. The latter

system has been essentially accepted in most Japanese citrus researches.

Japan is located to the east of the natural distribution location of citrus, and there are two endemic species: tachibana (*Citrus tachibana* Tanaka) and shiikuwasha (*C. depressa* Hayata). During prehistoric ages and historical ages, many types of citrus have been introduced, or naturally migrated to Japan, or occurred as natural hybrids. These have been cultivated widely in the coastal region of southwest Japan, including satsuma mandarin, kinokuni (*C. kinokuni* hort. ex Tanaka), kunenbo (*C. nobilis* Lour.), iyo (*C. iyo* hort. ex Tanaka), natsudaidai (*C. natsudaidai* Hayata), hassaku (*C. hassaku* hort. ex Tanaka), hyuga-natsu (*C. tamurana* hort. ex Tanaka) and yuzu (*C. junos* Sieb. ex Tanaka). Using these genetic resources, citrus industry has developed through breeding by cross hybridization and selection, as well as improvement of culture management. At present, predominantly satsuma mandarin is cultivated, and accounted for 62.5% (45.5 kha) of all citrus acreage (72.6 kha) in 2014 in Japan ([http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou\\_kazyu/index.html](http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou_kazyu/index.html)), followed by ‘Shiranuhi’ (3,139 ha), iyo (2,854 ha), yuzu (2,216ha), ponkan (2,056 ha), natsudaidai (2,023 ha), hassaku (1,747ha) and ‘Kiyomi’ (1,019 ha), among others. The first artificial hybrid ‘Tanigawabuntan’ was released in the Taisho period. A large scale breeding program was began in 1937 in Japan, and thereafter numerous promising varieties have been developed by the citrus breeding program of the national institute, so the

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citrus industry has benefited from conventional breeding methods (Nesumi and Matsumoto 2003). ‘Shiranuhi’ and ‘Kiyomi’ have also been developed through the breeding program and their cultivated acreages are ranked within the top ten varieties. Conventional citrus breeding is a long-term process, requiring approximately 20 years until a new variety is developed and cultivated for commercial shipment. The efficiency of breeding is affected by long-juvenility, heterozygosis, gametophytic cross-incompatibility, male sterility, apomixis, seedlessness and instability of characteristics under different environmental conditions. To produce new attractive and promising cultivars in the future, it is expected that the use of marker-assisted selection (MAS) will overcome these obstacles and improve the efficiency of conventional breeding programs. To promote citrus molecular breeding, a genome mapping program was initiated in Japan in 1987 and numerous efforts have been conducted to develop the experimental tools and resources necessary for citrus functional genomics. On the one hand, the International Citrus Genome Consortium (ICGC, composed of researchers from Australia, Brazil, China, France, Israel, Italy, Japan, Spain, and the USA) was inaugurated in 2003 to sequence the genome of sweet orange and Clementine mandarin. The genomes of sweet orange (diploid) and mandarin (haploid) were sequenced (Wu *et al.* 2014), and their draft sequences are available to view from public databases (<http://phytozome.jgi.doe.gov/pz/portal.html>). This progress in genome research has extended to molecular breeding and the isolation of agronomically important genes, resulting in many worldwide reviews and publications on *Citrus* genomics, genetics and breeding (Gmitter *et al.* 2007, 2012, Khan 2007, Talon and Gmitter 2008). In this review, we introduce the current research activities relating to citrus breeding in Japan, along with the progress of genome researches in genetic mapping and EST analysis, which have focused on the molecular characterization of breeding characteristics in Japan, mainly bio-functional substance metabolism in citrus. Such information may contribute not only to the advance of citrus molecular breeding and functional genomics but also to botanical science because *Citrus* species have various unique characteristics such as polyembryony, oil gland and juice sac development in fruit, and terpenoid metabolites.

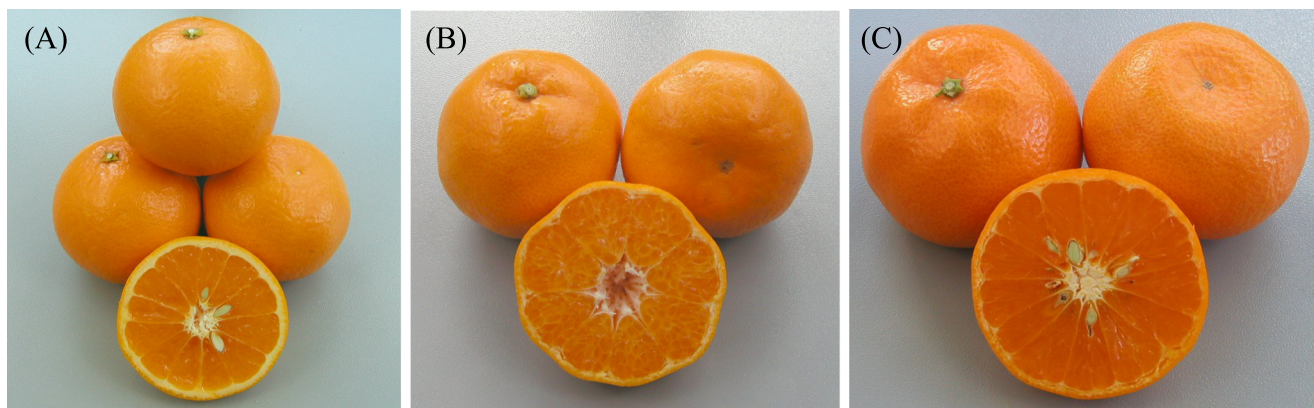
### Citrus breeding in Japan

Before modern ages, the introduced or migrated citrus and their hybrids have been selected and cultivated. Since the national institute of Horticultural Research Station (present organization: the NARO Institute of Fruit Tree Science (NIFTS)) was founded in 1902, citrus germplasm has been collected from a number of countries as well as from Japan, and the breeding program was begun by the cross breeding and screening of mutations (Nishiura 1964). The satsuma mandarin is suited to the Japanese climate and has been predominantly cultivated for more than 100 years in Japan

because this citrus fruit offers many favorable characteristics that facilitate its cultivation and consumption, such as seedlessness, easy peelability, early maturing, disease resistance, and high productivity. Within the citrus breeding program, the primary objectives have been improving the satsuma mandarin to add more excellent fruit quality, fragrance, stable production and stress resistance as the most important breeding objects. To generate a new cultivar with desirable traits and harvesting season from August to early spring, satsuma mandarin and the other mandarins were pollinated with sweet orange, pummelo, other citrus species and their hybrids (Nesumi and Matsumoto 2003). However, the many mandarins and the derived hybrids produce polyembryonic seeds which arise when the nucellar tissue surrounding the embryo sac generates somatic embryos so that that seed contains more than one embryo. Because most embryos in polyembryonic seeds originate from maternal tissue; this is a serious barrier to conventional breeding in addition to the prolonged juvenile periods until flowering from seedling. Several efforts have been conducted to mitigate serious limitations imposed by polyembryony and juvenility. Iwamasa *et al.* (1967) researched the heredity of polyembryony among 220 hybrids, and found that polyembryony was likely controlled by single dominant gene. Okudai *et al.* (1980) established the top-grafting method to promote and flowering and fruiting in the seedlings, and succeeded to shorten the breeding cycle. By using a culture of separate embryos obtained from ‘Miyagawa wase’ (the most popular satsuma mandarin cultivar) pollinated by ‘Trovita’ orange (sweet orange), a hybrid was developed and launched as ‘Kiyomi’ (tangor) in 1979. ‘Kiyomi’ is also a superior seed parent cultivar owing to monoembryonic (strictly zygotic) seed and male sterility, so it has contributed to the production of many promising cultivars with excellent quality such as ‘Shiranuhi’, ‘Harumi’, ‘Setoka’ and ‘Harehime’. Over the past 25 years, 49 cultivars have been released as progeny cultivars of ‘Kiyomi’ through the breeding program (Fig. 1). The cultivation of ‘Harumi’, ‘Shiranuhi’ and their progeny cultivars has been replacing the cultivation of traditional mid and late ripening citrus varieties.

In addition to the breeding program in national institute, more than 200 cultivars including kumquat (*Fortunella spp.*) and trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) have been discovered/selected and registered for cultivar license since 1981 (<http://www.hinsyu.maff.go.jp/vips/CMM/apCMM110.aspx?MOSS=1>). Among them, bud sports and nucellar seedlings of satsuma mandarin accounted for 90 cultivars, which varied in fruit maturity season, fruit shape, rind color and texture, reduced degree of rind puffing, and sugar content. Somatic hybridization, ploidy breeding and graft chimera techniques also contributed to the development of 6, 9 and 17 cultivars, respectively.

From breeding activities aimed at further improvement, many excellent cultivars have been developed and launched, for example ‘Tamami’, which is rich in  $\beta$ -cryptoxanthin,



**Fig. 1.** ‘Kiyomi’ and its progeny cultivars released from the NARO Institute of Fruit Tree Science (NIFTS). (A): ‘Kiyomi’ (‘Miyagawa wase’ (*C. unshiu* Marc.) × ‘Trovita’ orange (*C. sinensis* (L.) Osbeck.)) (B): ‘Harumi’ (‘Kiyomi’ × ponkan (*C. reticulata* Blanco)) (C): ‘Tamami’ (‘Kiyomi’ × ‘Wilking’ (*C. deliciosa* Ten. × ‘King’ (*C. nobilis* Lour.))

‘Chuukanbohon Nou-5’ (Nou-5) and ‘Chuukanbohon Nou-6’ (Nou-6) exhibiting seedlessness, and ‘Chuukanbohon Nou-8’ (Nou-8) incorporating the citrus tristeza virus (CTV) resistant gene from *Poncirus*. The present breeding program has 2 major processes for seedling selection. In the primary selection, 30–40 combinations of parental cultivars are cross-hybridized, and approximately 3,000 seeds are disseminated. Seedlings are subjected to grafting onto trifoliate orange rootstock, and are selected through the evaluation for tree habits, fruit quality and disease tolerance. The primary selection of seedlings generally takes 9–10 years and the population size varies depending on feasibility of cross-hybridization among parent varieties, and target of breeding traits. In the secondary selection, the evaluation of selected individuals is carried out at multiple research stations in different environments, and is mainly focused on fruit quality, cultivation, and stable fruit production. Secondary selection of individual generally takes 7–8 years, and the promising seedling with high evaluation progress to the process of the cultivar registration. The following characteristics remain important breeding targets: attractive fruit color, excellent flavor and aroma, rich health-promoting substances, easy-peelability, seedlessness, parthenocarpy, resistance to biotic and non-biotic stress, and a stable crop yield and tree habit. Since the cross-hybridization program was started, the current fifth- and sixth-generation cultivars by intercross between the improved cultivars and lines have piled on the favorable genetic traits. Present breeding cultivars and populations have been developed from the inter-crossing among the limited number of progenitor cultivars, and the accumulation of favorable genes has progressed in breeding populations in each successive generation. Recent demands for new cultivars enriched with various health-promoting substances in addition to seedless fruit with high quality and stable flower generation requires the need to identify suitable germplasm as well as the application of new strategies to single out individuals with favorable genes using genomic and marker information.

### Cytological information and genome analysis

The basic chromosome number of *Citrus* has been recognized as  $x = 9$  since the studies conducted in the 1920s (Frost 1925, Nakamura 1929), and this largely applies to species from the *Rutaceae* family. The genome size was estimated as 0.76 or 0.82  $\mu\text{g DNA}/2C$ , equivalent to 367 or 396 Mb/1C, in *C. sinensis* L. (Arumuganathan and Earle 1991), but varied among species, ranging from the largest citron (*C. medica* L., average value 398 Mb/haploid genome) to the smallest mandarins (*C. reticulata* Blanco, 360 Mb). The pummelo (*C. grandis* L.) had an intermediate genome size of 383 Mb (Ollitrault *et al.* 1994).

The fluorescent staining of chromosomes (Guerra 1993) showed karyotype variation in the size and distribution of chromomycin A+ (CMA+) bands (Befu *et al.* 2000, Miranda *et al.* 1997). Proximal heterochromatin with CMA+ was highly variable among various *Citrus* species (Befu *et al.* 2000, 2001). Variations were also observed between possible homologous chromosomes. Fluorescence *in situ* hybridization (FISH) detection of ribosomal DNA (rDNA) sites also showed variations in signals between homologous chromosome pairs in sweet orange (Matsuyama *et al.* 1996, Roose *et al.* 1998), suggesting that commercial citrus cultivars such as *C. sinensis* originated from hybridization from basic species and are characterized by high heterozygosity (Herrero *et al.* 1996).

The establishment of reference whole-genome sequences involved the identification and development of the haploid or the doubled haploid from anther culture (Hidaka *et al.* 1979, 1982, Xu *et al.* 2013), interploid hybridization (Germanà and Chiancone 2001, Oiyama and Kobayashi 1993), or pollination with irradiated pollen (Aleza *et al.* 2009). A reference genetic map of Clementine mandarin was developed (Ollitrault *et al.* 2012) and sequence of haploid sweet orange and haploid Clementine were released (Wu *et al.* 2014, Xu *et al.* 2013). Recent comparative genome sequencing has revealed that species of possible interspecific

hybrid origin, such as sweet orange (*C. sinensis* L.), sour orange (*C. aurantium* L.), lemon (*C. limon* L.) and grapefruit (*C. paradisi* Macf.), have mosaic genomes with large DNA fragments inherited from the basic taxa of citron (*C. medica* L.), pummelo (*C. grandis* L.) and mandarin (*C. reticulata* Blanco) and the others.

### cDNA cataloging and expression profiling

The availability of EST and genome sequences of *Citrus* species from public databases was quite limited in 1990, and the random sequencing of EST clones from various fruit tissues of the satsuma mandarin such as pulp, albedo, ovary and young seed has been promoted since 1994 (Fujii *et al.* 2003, Hisada *et al.* 1996, 1997, 1999, Kita *et al.* 2000a, 2000b, Moriguchi *et al.* 1998a, Shimada *et al.* 2003) (Table 1). To date, 21,976 independent sequences have been registered in the DDBJ database (the DNA Data Bank of Japan). In addition to the utilization of the ESTs for genetic mapping, they were used for the physiological and molecular analysis of flower- and fruit-related traits, including the metabolism of bio-functional substances.

A microarray analysis is one of the most powerful tools in functional genomics for addressing transcriptional profiling for a huge number of genes in one experiment. In terms of the first generation of microarray systems, where cDNA inserts were spotted onto glass slides, Shimada *et al.* (2005a) reported a cDNA microarray containing 2,213 cDNA probes which were independent genes from 8,333-cDNA collection. At the same time, the Spanish citrus genomic consortium generated 25 cDNA libraries covering different tissues, developmental stages and stress conditions and developed a cDNA microarray containing 12,672 cDNA probes, corresponding to 6,875 putative unigenes of a 22,635-EST collection (Forment *et al.* 2005). In terms of the second generation of microarray systems, where short oligonucleotide sequences were printed onto glass slides, Affymetrix (CA, USA) developed and released a GeneChip citrus array in 2006. The sequence information for this array was selected from Citrus HarvEST <http://harvest-web.org/hweb/hmain.wc?versid=19> and cDNA clustering database. This array contains 30,171 probe sets representing up to 33,879 citrus transcripts. Moreover, a citrus 22K oligoarray comprising 21,495 independent EST probes derived from citrus species was developed on an Agilent oligoarray system (Fujii *et al.* 2007).

Using these microarray platforms, various researches have been reported. Cercós *et al.* (2006) identified more than 2,200 putative unique genes that exhibited significant changes of expression level during fruit development and these included genes in the metabolism of carbohydrates, acids, secondary metabolites, the genes in cell expansion and transcription regulatory genes. Fujii *et al.* (2007, 2008a) characterized ethylene and GA-responsive genes in satsuma mandarin mature fruit using a citrus 22K oligoarray and found that carotenoid biosynthetic genes show reverse re-

sponses to ethylene and GA treatments compared with chlorophyll biosynthetic genes. In addition, a type-II ethylene receptor (ETR2) plays a major role perception sensing the ethylene signal in citrus mature fruit.

### DNA marker development and application

Up to the present, numerous DNA markers, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) and cleaved amplified polymorphic sequences (CAPS) were developed and applied to cultivar identification (Matsuyama *et al.* 1992, Omura *et al.* 2003, Sugawara *et al.* 2002, Ueda *et al.* 2003). Numerous EST-SSR and genomic SSR markers have been developed from citrus genome sequences and assigned to the genetic map of the Clementine mandarin (Luro *et al.* 2008, Ollitrault *et al.* 2012). Among these markers, CAPS markers are generally less polymorphic than the other molecular markers but represent a convenient method that does not require special analysis instruments for genetic diagnosis. Therefore, CAPS markers were utilized as the principal DNA marker for cultivar identification and for phylogenetic and linkage analyses in Japan. To the present, 3,562 primer sets have been tested for DNA marker construction, and 708 CAPS markers were found to be eligible for genetic analysis (Shimada *et al.* 2014a). Using CAPS markers, cultivar identification was carried out using 33 representative Japanese cultivars (Ninomiya *et al.* 2015) and a database was created for the genotypes of the examined CAPS markers. Based on “MinimalMarker”, which is a computer program for the identification of minimal sets of discriminating DNA markers for efficient cultivar identification (Fujii *et al.* 2013a), nine CAPS markers were sufficient to discriminate examined 33 cultivars. Fujii *et al.* (2008b, 2010) also developed the computer programs “MarkerToolkit” and “MARCO” which conduct various calculations automatically and estimate parentage based on the genotypes of DNA markers. Application of these DNA markers and computer programs enables us to evaluate the parentage of cultivars and resolve several areas of confusion regarding certain cultivars, and to secure rights for the citrus breeders. CAPS markers were also applied to evaluate the diversity of 103 various mandarin and related species using chloroplast DNA polymorphisms (Yamamoto *et al.* 2013), dividing them among seven groups.

Fujii *et al.* (2013b) developed a single nucleotide polymorphism (SNP) genotyping array using Illumina’s GoldenGate assay system. Among 1,497 SNPs candidates, 384 SNPs were selected for a high-throughput genotyping array based on the physical parameters of Illumina’s BeadArray criteria and applied to genotyping for a hybrid population of 88 progenies and 103 citrus accessions, for breeding in Japan. Genome-wide genotyping of citrus cultivars indicated that the chromosome transmission to progeny tended to be inherited in the large linkage blocks from both of the parent cultivars (Omura *et al.* 2003) and

**Table 1.** Citrus EST catalogs and its application in Japan

Library	Name	Source cultivar	Latin name	Tissue (organ)	Stage	NO of EST analyzed	ESTs-gene homologs used for functional analysis (example)	Reference	No. of frame loci (AGI map) <sup>a</sup>
	VSS	Valencia orange	<i>C. sinensis</i> (L.) Osbeck	Young seed	1 month after anthesis	577	<i>CuMFT1</i> promoter, etc.	Hisada <i>et al.</i> 1996	17
	FRI	Miyagawa wase	<i>C. unshiu</i> Marc.	Fruit pulp	Fruit developing	1,051		Hisada <i>et al.</i> 1997	11
	FRM	Miyagawa wase	<i>C. unshiu</i> Marc.	Fruit pulp	Maturation	385	<i>CiFT</i> , <i>CiMT45 promoter</i> , <i>CitPAP</i> , <i>CitCHS</i> , etc.	Moriguchi <i>et al.</i> 1998a, 1998b	21
	ALM	Miyagawa wase	<i>C. unshiu</i> Marc.	Albedo	Maturation	623	<i>CitLGT</i> , etc.	Kita <i>et al.</i> 2000	57
	OVA	Miyagawa wase	<i>C. unshiu</i> Marc.	Ovary	Flowering	827	<i>CitSERK</i> , <i>CiMADS</i> , <i>CitSEP</i> , <i>CitSEP</i> , <i>CitFUL</i>	Shimada <i>et al.</i> 2003	34
	ALP	Miyagawa wase	<i>C. unshiu</i> Marc.	Albedo	Initiation stage of rind peeling	941		Fujii <i>et al.</i> 2003a	26
	WFY	Miyagawa wase	<i>C. unshiu</i> Marc.	Whole fruit	Young (1 month after flowering)	1,689		Direct submission on DDBJ	40
	BFC	Miyagawa wase	<i>C. unshiu</i> Marc.	Fruit rind	Rind coloring	1,650	<i>CuMTSE2</i> promoter, monoterpene synthases, etc.	Nishikawa <i>et al.</i> 2009b, Shimada <i>et al.</i> 2004, etc.	54
	FBI	Miyagawa wase	<i>C. unshiu</i> Marc.	Flower bud	1 month prior to anthesis	2,367			57
	GSA	Miyagawa wase	<i>C. unshiu</i> Marc.	Seed	4 days after imbibition	1,920			1
	RGP	Miyagawa wase	<i>C. unshiu</i> Marc.	Root	2 week seedling	960			
	SLG	Miyagawa wase	<i>C. unshiu</i> Marc.	Shoot	2 week seedling	1,920			
	YJS	Miyagawa wase	<i>C. unshiu</i> Marc.	Juice sac	60 days after flowering	1,926			2
	PCC	Miyagawa wase	<i>C. unshiu</i> Marc.	Callus	Culture for proliferation	960			1
	EIC	Miyagawa wase	<i>C. unshiu</i> Marc.	Callus	Culture for embryogenesis	1,152			
	STG	Miyagawa wase	<i>C. unshiu</i> Marc.	Stigma	Flowering	3,552			21
	ANT	Miyagawa wase	<i>C. unshiu</i> Marc.	Anther	Flowering	2,600			6
	LLL	Lisbon lemon	<i>C. limon</i> (L.) Burm. f.	Leaf	Young (before full expansion)	2,016			10
	EGJ	Kimokuni	<i>C. kinokuni</i> hort. ex Tanaka	Ovule	60–70 days after flowering	2,112			14
	Total					29,228			372

<sup>a</sup> Refer to Shimada *et al.* (2014a).

suggested that the frequency of recombination in a chromosome was low. To accumulate and combine the favorable genes distributed through-over the genome in cross-breeding procedure, detailed genotyping of breeding resources using recent SNP analysis (Fujii *et al.* 2013b) might provide the genetic information needed to select suitable parental candidate lines and to perform genomic selection in the near future for breeding purposes.

### Linkage mapping and marker-assisted breeding

Genetic maps are useful tools for breeding program to select hybrids with economically important traits, and high density genetic maps provide information on quantitative trait loci (QTLs) associated with pathogen resistance and fruit quality. Numerous molecular markers have been developed and used to construct citrus genetic maps over the past decade. The initial citrus genetic maps were constructed from RFLPs and isozymes (Durham *et al.* 1992, Jarrell *et al.* 1992). Subsequently, SSR markers were adopted for mapping (Kijas *et al.* 1997). Thereafter, genetic maps have been extended to localize important traits, such as cold-acclimation (Cai *et al.* 1994), citrus tristeza virus (CTV) resistance (Cristofani *et al.* 1999, Fang *et al.* 1998, Gmitter *et al.* 1996), fruit acidity (Fang *et al.* 1997), apomixis (Garcia *et al.* 1999, Kepiro and Roose 2010), nematode resistance (Ling *et al.* 2000) and *Phytophthora gummosis* resistance (Siviero *et al.* 2006). These individual linkage maps provide us with useful information, but they are somewhat arbitrary and are difficult to compare. Recently, reference genetic maps have been developed. Ollitrault *et al.* (2012) constructed a genetic map of the Clementine mandarin using a SNP and EST-SSR marker, and Shimada *et al.* (2014a) constructed a framework genetic map of mandarin progenies using CAPS and SNP markers (Table 2). These maps were constructed using co-dominant genetic markers associated with a rather conserved and functional genome-

coding region, and include functional annotation and positional information about the Clementine genome sequence. Therefore, framework genetic maps represent a useful resource for structural, functional and evolutionary studies in *Citrus* species. Using these framework genetic maps, Ohta *et al.* (2015) conducted graphical genotyping of pseudo-backcrossed F<sub>2</sub> (BC<sub>2</sub>) progeny to introduce a CTV resistance from trifoliate orange into *Citrus* via introgression breeding, and developed CTV linkage markers (Ohta *et al.* 2011).

As an approach to breeding a seedless cultivar in Japan, the male sterility derived from ‘Kiyomi’ was genetically analyzed (Nakano *et al.* 2001) and mapped on linkage group 8 (Nakano *et al.* 2003). The seedlessness derived from ‘Mukaku kishu’ (Yamasaki *et al.* 2007a) was mapped on linkage group 9 (Shimada *et al.* 2014a). Nakano *et al.* (2008) also mapped a polyembryony locus and constructed bacterial artificial chromosome (BAC) contigs covering this region. Nakano *et al.* (2012) characterized genomic sequences for 380 kbps of BAC contigs and elucidated that the polyembryony locus consisted of 70 predicted open reading frames (ORFs), developing DNA markers that discriminated polyembryony from monoembryony. Recently, QTL mapping and expression QTL (eQTL) mapping were carried out to identify DNA markers that could be used to increase the amount of β-cryptoxanthin within a cultivar (Sugiyama *et al.* 2011, 2014).

### Flowering

Citrus has a long juvenile period, for example, sweet orange needs more than 10 years until growing up to adult and flowering from seed. This juvenile period has hampered conventional breeding and genetic studies and flowering control is required to reduce juvenile period. In plants, flowering genes such as *APETALA1* (*API*), *LEAFY* (*LFY*) and *FLOWERING TIME* (*FT*) promote flower development, and

**Table 2.** Mapping of breeding objective traits in Japan

Parents of population	Population size	Trait	Linkage group	Reference
Kiyomi × Miyagawa wase	125	Seed number (QTL)	6	Omura <i>et al.</i> 2003
Kiyomi × Miyagawa wase	125	Polyembryony	1	Nakano <i>et al.</i> 2012
Kiyomi × Okitsu-41	92	Male sterility	8	Nakano <i>et al.</i> 2003
Nou-8 × Siamese acidless	92	CTV resistance	2	Ohta <i>et al.</i> 2011
Okitsu-46 × Nou-5	87	Seedlessness	9	Shimada <i>et al.</i> 2014a
Okitsu-46 × Nou-5	87	Carotenoids (QTL)	6	Sugiyama <i>et al.</i> 2011

‘Kiyomi’ is derived from the cross between ‘Miyagawa wase’ (*C. unshiu* Marc.) and ‘Trovita’ orange (*C. sinensis* (L.) Osbeck).

Okitsu-41 is selected breeding line derived from the cross between ‘Kiyomi’ and × ‘Wilking’ (‘King’ tangor (*C. nobilis* Lour.) × ‘Willowleaf’ mandarin (*C. deliciosa* Ten.).

Okitsu-46 is selected breeding line derived from the cross between ‘Sweet spring’ (‘Ueda unshiu’ (*C. unshiu* Marc.) × Hassaku (*C. hassaku* hort. ex Tanaka)) and ‘Trovita’ orange.

Nou-8 is a CTV resistant line derived from the cross between ‘Kiyomi’ and ‘H-FD-1’ (Hassaku × ‘Hiryu’ (*P. trifoliata* var. *monstrosa*)).

‘Siamese acidless’ is acidless variety of pummelo (*Citrus grandis* [L.] Osb.).

Nou-5 is a seedless line derived from the cross between ‘Lee’ (Clementine (*C. clementina* hort. ex Tanaka) and ‘Orland’ (‘Dancy tangerine’ × ‘Duncan’ grapefruit)) and ‘Mukaku kishu’ (*C. kinokuni* hort. ex Tanaka).

Only the QTL showing the highest LOD score.

MADS-box genes are involved in floral organ development and/or flower meristem identification (Kobayashi *et al.* 1999, Wigge *et al.* 2005). Several flowering genes have been functionally characterized using transgenic plants to understand the processes regulating the phase transition from vegetative to reproductive growth in citrus. Peña *et al.* (2001) reported that constitutive expression of *AtLFY* or *AtAPI* genes derived from *Arabidopsis* dramatically reduced their generation time in transgenic citranges (hybrids between sweet orange and trifoliolate orange). Endo *et al.* (2005) reported that the *35S::CiFT1* trifoliolate orange exhibited extremely early flowering. These results suggest that *FT*, *LFY* and *API*, which are key genes promoting flowering in *Arabidopsis*, may regulate the phase transition from the juvenile to the adult phase in citrus and its relatives. Pillitteri *et al.* (2004) reported that juvenility in sweet orange was positively correlated with the transcription accumulation of *TERMINAL FLOWER LOCUS (CtTFL)*, a key negative regulator of floral timing. Endo *et al.* (2006) characterized five MADS-box cDNA clones (*CitMADS1*, *CitMADS3*, *CitMADS5*, *CitMADS6* and *CitMADS8*) from the fruit tissues of satsuma mandarin. Most of them, except *CitMADS1* and *CitMADS3*, were expressed in the seedlings before the phase transition from vegetative to reproductive growth. In *35S::CiFT1* trifoliolate orange, the transcripts for some of these genes were found to be more significantly accumulated than in normal plants (Nishikawa *et al.* 2010). These results indicate that *CiFT* plays an important role in flowering induction and is more closely involved in seasonal periodicity than *LFY* and *API*.

*CiFT* shows a seasonal increase during the floral induction period in satsuma mandarin, and low temperature promotes floral induction via the activation of *CiFT* transcription in adult satsuma mandarin trees (Nishikawa *et al.* 2007). The endogenous expression of flowering-related genes has been investigated in satsuma mandarin and trifoliolate orange, which are evergreen and deciduous, respectively (Nishikawa *et al.* 2009a). In satsuma mandarin, in which floral induction is triggered by low temperatures, mRNA levels of *CiFT* increased during fall and winter, corresponding to the floral induction period, and mRNA levels of *CsLFY* and *SEPALLATA* homologs (*CuSEP*) increased during early spring just before blooming. *CsAPI* and *FRUITFULL* homologs (*CuFUL*) do not show a significant association with seasonal flowering. In trifoliolate orange, in which floral induction and flower bud development occur during early summer, as in many deciduous trees, expression of *CiFT*, *CsLFY*, *CsAPI*, *CuSEPs* and *CuFUL* increases during early summer, corresponding to the period of floral induction and flower bud development. The *CuSEPs* expression peaks again during early spring just before blooming. In both species, *CsTFL* shows low transcript levels during the period of floral induction and flower bud development. Thus, despite the difference in flowering season, in both species transcriptional changes in *CiFT*, *CsLFY*, *CsTFL* and *CuSEPs* are correlated with seasonal flowering. In contrast, the corre-

spondence between *CsAPI* and *CuFUL* expression and seasonal flowering differs between species. Similar results have been observed in kumquat (genus *Fortunella*), a close relative of species in the genus *Citrus* and *Poncirus* (Nishikawa *et al.* 2011). Elucidation of the flowering mechanism is important to obtain a new insight into resolving the problem of stable fruit production due to the alternate bearing of flower and fruit.

### Sugar and acid metabolism

High sugar content and low organic acid content in fruits is one of the most important traits of fruit quality in citrus breeding. A high ratio of sugars to organic acids in fruit is a kind index of taste. Generally, the sugar (sucrose, glucose and fructose) content of the fruit increases during fruit maturation while the content of organic acids (citric and malic) decreases. These storage components in fruit originate from sucrose as a main translocation form of photoassimilate in leaves (Goldschmidt and Koch 1996) and mainly accumulate in the vacuoles of juice sac cells. Sucrose synthase activity in juice sac tissues plays an important role during the early stages of fruit development, when cell division, cell wall synthesis and respiration rate are occurring at a maximum rate (Lowell *et al.* 1989). Sucrose synthase activity is high in the early stages and decreases until mid-developmental stage, then rapidly increases during the maturation stage (Komatsu *et al.* 2002). The increase in sucrose synthase activity directly promotes sucrose accumulation during fruit maturation. The three sucrose synthase genes (*CitSUS1*, *CitSUS2* and *CitSUSA*) have been isolated, and their gene expression patterns correspond with changes in sucrose content during fruit development and maturation. Komatsu *et al.* (1996) also characterized sucrose-phosphate synthase (SPS), which is another key enzyme in the synthetic pathway of sucrose. SPS is involved in many processes relating to important agronomic traits such as the growth and yield of plants, and their transcripts accumulated in flower, leaves and mature fruits in citrus. Katz *et al.* (2011) reported the results of a metabolite analysis of citrus fruit during fruit development using label-free shotgun proteomics. In all of these studies, the protein change responsible for sugar metabolism shows a similar pattern. The invertases, an important family of proteins responsible for sucrose degradation to glucose and fructose, did not change in the proteomics analysis. The transcription activity of two acidic and one neutral invertase genes showed peaks at the early stage of fruit development and then decreased in the later stages (Kubo *et al.* 2001). Interestingly, an invertase inhibitor protein, responsible for the reduction in invertase activity, was up-regulated in the later stages of fruit development.

Citrus fruit accumulate large amounts of organic acids, mainly citric acid, in juice sac cells. Citric acid is produced through the TCA cycles and is an intermediate metabolite in energy production, accumulating in the vacuole during early-mid fruit development. Toward fruit maturation, citric

acid is released from vacuole to cytosol to assist with a vacuolar citrate/H<sup>+</sup> symporter (Shimada *et al.* 2006). In the cytosol, the released citric acid is catabolized by cytosolic aconitase, to be converted to amino acids (Cercós *et al.* 2006, Sadka *et al.* 2000). In terms of the consumption of citrus fruits, a high citric acid content reduces fruit quality, but the mechanisms regulating citric acid accumulation and degradation are still unknown. Degu *et al.* (2011) reported that inhibition of aconitase activity by citramalate and oxalomalate were effective at reducing citric acid metabolism. Citramalate, as an endogenous inhibitor, inhibited mitochondrial aconitase activity and resulted in an increase in citric acid accumulation, although it did not inhibit cytosolic aconitase activity. In addition, oxalomalate inhibited cytosolic aconitase activity and resulted in a metabolic shift towards amino acid biosynthesis by inducing the gamma-aminobutyric acid (GABA) shunt. The usage of citramalate and oxalomalate is one of the manipulations to control fruit acidity in citrus.

### Aroma production and diversity

The molecular identification and characterization of genes related to chemical constituents of citrus is very informative on genetic and physiological improvement of the target traits involved in fruit quality. Most aerial citrus tissues and organs have oil glands that contain and emit a wide diversity of volatile terpenoids, such as hemiterpenes, monoterpenes and sesquiterpenes, including their alcohol, ester and acetate derivatives as fragrance components (Sawamura 2000, Vekari *et al.* 2002). Monoterpenes and sesquiterpenes have roles in communication between plants and insects, and plants and pathogens, and in attracting pollinators (Pichersky and Gershenzon 2002). Plant terpene synthases comprise a large gene family, of which 49 putative members have been estimated in *Citrus* germplasm through the use of *in silico* characterization of ESTs (Dornelas and Mazzafera 2007). Several citrus cDNAs encoding monoterpene and sesquiterpene synthase genes have been functionally characterized (Lücker *et al.* 2002, Murayama *et al.* 2001, Sharon-Asa *et al.* 2003, Shimada *et al.* 2004, 2005a, 2005b, 2012, 2014b). Most of these genes encode cyclic monoterpene and sesquiterpene synthases, and they share the common structural features of a tandem arginine (RR) motif and a DDXXD motif. In addition, most produce a specific main product accompanied by significant amounts of by-products. This enzymatic feature may contribute the complex factor in aroma breeding programs as a quantitative trait. In rough lemon (*C. jambhiri* Lush), *in situ* hybridization revealed that the RlemTPS4 (delta-elemene synthase gene) was specifically expressed in specialized epithelial cells surrounding the oil secretory cavities of leaf tissues (Yuya *et al.* 2015).

Certain citrus volatiles, including the most predominant monoterpenes of fruit peels (*d*-limonene,  $\alpha$ -pinene,  $\beta$ -pinene and myrcene), have stimulatory effects on host recognition,

germination and growth of *Penicillium digitatum* and *P. italicum* (Droby *et al.* 2008). The essential oils of ponkan have been proposed as a promising source of fungicidal substances because they show antifungal activity *in vitro* against several diseases caused by, for example, *P. italicum* and *P. digitatum* (Chutia *et al.* 2009, Tao *et al.* 2014). In rough lemon leaves, monoterpene volatiles likely play an important role in defense against microbes and herbivores (Yamasaki *et al.* 2007b). In studies of a transgenic orange with downregulated *d*-limonene biosynthesis, the aroma components of the transgenic fruits were significantly altered (i.e., levels of certain oxygenated monoterpenes strongly increased) to generate broad-spectrum resistance against pathogens (Rodríguez *et al.* 2011, 2014). Thus, citrus aroma components from leaves and fruit peels have clear biological roles in plant resistance and susceptibility responses to pests and pathogens, but the activity of each aroma component and particularly that of specific oxygenated monoterpenes in such biological interactions has not yet been clarified in detail. Shimada *et al.* (2014b) reported that linalool, which is a major oxygenated monoterpene in citrus, showed anti-bacterial and antifungal activities against the citrus major diseases of canker (*Xanthomonas citri* subsp. *citri*) and molds (*P. digitatum* and *P. italicum*). The biosynthetic genes of linalool were transcriptionally induced by wounding and elicitor treatments [bacteria, fungi and jasmonic acid (JA)]. Ponkan, one of the breeding materials with field resistance against cankers and molds, contains a high level of linalool in its leaves and mature fruit. In rice, JA signaling plays an important role in resistance to rice bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), and is accompanied with linalool accumulation (Taniguchi *et al.* 2014). In transgenic orange fruits harboring bacterial and mold resistances, *cis*-(+)-12-oxophytodienoic acid (OPDA), as a precursor of JA biosynthesis, has been found to be highly accumulated and to produce extraordinary amounts of JA after elicitor treatments compared with normal orange (Rodríguez *et al.* 2014). From these findings, we will gain a new insight into elucidating the molecular mechanisms involved in the field resistance of ponkan.

### Limonoid metabolism

Limonoids are highly oxygenated triterpenes present in *Rutaceae* spp. and a few other plants such as *Meliaceae* spp.; 36 limonoids have been identified in *Citrus* and its related species (Berhow *et al.* 2000). Limonoids have several biological functions linked to chemically induced carcinogenesis in mice, hamsters and human breast cancer cell growth (Lam and Hasegawa 1989), which may offer commercial benefits. However, limonoids in fruits cause a gradual bitterness called delayed bitterness in navel orange after juice processing, and reduce the quality of the juice. In contrast, mature fruits of satsuma mandarin cause much less delayed limonoid bitterness than sweet orange; probably because the concentration of limonoid aglycones in the juice from



satsuma mandarin is low. Limonoid metabolism is characterized by radioactive tracers (Hasegawa *et al.* 1984, Ou *et al.* 1988) but many of the corresponding enzymes and biosynthetic genes have been not isolated. Kita *et al.* (2000c) isolated cDNA clones encoding limonoid UDP-glucosyltransferase (LGT) that regulated the conversion of limonoid aglycones such as limonin bitter compound to their nonbitter glucosides. Satsuma mandarin has 2 LGT allelic genes (*CitLGT1* and *CitLGT2*), and their expression patterns were found to be different among fruit development (Kita *et al.* 2003). *CitLGT2* is the gene responsible for converting limonoid aglycones to their nonbitter glucosides throughout fruit development. The delayed bitterness in sweet orange was presumed to be caused by a lack of *CitLGT2*. Endo *et al.* (2002) performed a study involving genetic engineering to maximize the formation of limonoid glucosides in a suspension cell culture system through *Agrobacterium* transformation of the functional coding region of *CitLGT1*. The suspension cell culture system study attempted to evaluate limonoid metabolism; genetic modification resulted in an accumulation of limonoid glucoside in transgenic cells but the quantity was very low due to other unexpected factors. Thus, limonoid metabolism is still not fully understood, and further researches are required to isolate a series of biosynthetic genes and elucidate the regulators of limonoid metabolism.

### Flavonoid metabolism

Flavonoids are a group of pigments contained in plants and are responsible for flower and fruit coloration, and for bitterness of citrus fruit. Approximately 60 flavonoids have been identified in citrus and categorized into six classes: flavones, flavanones, flavonols, isoflavones, anthocyanidins and flavanols (or catechins), according to their molecular structures (Peterson *et al.* 1998). The yellow or orange color of general citrus fruits originates from an accumulation of carotenoid pigments, while the red color of blood orange predominantly originates from an accumulation of anthocyanin pigments. *Citrus* species accumulate large amounts of flavonols, especially flavanone glycosides (Horowitz 1964). Flavanone, which is synthesized from naringenin chalcone by the catalysis of chalcone isomerase, is modified stepwise to various derivatives by hydroxylation, methylation and glycosylation, then rhamnosylation (Lewinsohn *et al.* 1989). The most common glycosidic group attached to the flavonoids in citrus is rhamnoglucose diglycoside. Flavanone neohesperidosides mainly accumulate in citrus species related to the pummelo, such as grapefruit, sour orange and natsudaidai, and lend a bitter taste to citrus fruit (Horowitz 1964, Rouseff 1980). Other species related to citron and mandarin mainly accumulate the tasteless flavanone rutosides naringin and hesperidin (Nishiura *et al.* 1971). In addition to the bitter property of citrus flavonoids, pharmacological investigations have shown that several flavonoids such as naringenin and hesperetin function to prevent athero-

sclerosis and cancer (Tripoli *et al.* 2007). Moreover, citrus peels contain several polymethoxylated flavonoids (PMFs) tangeretin and nobiletin that are enriched in orange and tangerine peel (Kurowska and Manthey 2004). They are O-methylated flavones and are reported to have several anticancer, anti-inflammatory, anti-atherogenic and anti-diabetic properties (Miyata *et al.* 2011).

Moriguchi *et al.* (2001) isolated and characterized major flavonoid biosynthetic genes (*CitCHS1*, *CitCHS2*, *CitCHI* and *CitF3H*) and reported that flavonoid accumulation and transcription of the related genes were correlated and abundant in the young tissue of leaves and fruits. Frydman *et al.* (2004) isolated and characterized the gene encoding 1,2 rhamnosyltransferase, a key enzyme in the biosynthesis of the bitter flavonoids of citrus. However, the other metabolic genes responsible for hydroxylation, methylation and glycosylation of flavonoids are still not fully known.

Recently, the mechanism involved in the accumulation of anthocyanin pigments in blood orange has been elucidated. The transcription level of the gene that specifies dihydroflavonol 4-reductase (DRF), which is a key gene in anthocyanin biosynthesis and catalyzes the conversion of dihydroflavonols to leucoanthocyanins, is lower in non-red orange cultivars than in blood orange cultivars (Lo piero *et al.* 2006). Anthocyanin biosynthesis is regulated mainly at a transcriptional level in plants controlled by a regulatory complex that is composed of proteins of the Myb, bHLH and WD-repeat family. Butelli *et al.* (2012) isolated R2R3-type Myb gene (*Ruby*) which is responsible for anthocyanin biosynthesis, and the insertion of a *Copia*-like retrotransposon adjacent to *Ruby* activated the production of anthocyanin in blood orange. The cultivation of blood orange is becoming more widespread in Japan, but the poor blood coloration of both rind and flesh is a principal issue owing to physiological and climatic factors. Further research would be valuable in the creation of a new citrus cultivar with a different color.

### Transgenic research on gene function relating to citrus breeding traits

Transgenic research is considerably important to progress the functional genomics of *Citrus*. Transformation efficiency is generally low in mandarins (Hidaka *et al.* 1990) and the varieties suitable for transformation are limited to fruits such as trifoliate orange and sweet orange (Kaneyoshi *et al.* 1994, Peña *et al.* 2008). The reduction in generation time by genetic engineering could accelerate the research in terms of understanding the complex regulatory mechanisms involved in biological and agronomical traits as well as in manipulating the nutritional value and quality of fruits. Several efforts have been made to achieve a reduction in long juvenile periods in citrus using flowering genes. Peña *et al.* (2001) succeeded in reducing the generation time of citrange by the introduction of either *APETALA1* (*API*) or *LEAFY* (*LFY*). Endo *et al.* (2005) introduced citrus

*FLOWERING LOCUS T* (*CiFT*) to trifoliolate orange and succeeded in developing a precocious flowering phenotype. The precocious flowering phenotypes in 35S::*CiFT* trifoliolate orange may result primarily from the conversion of a vegetative shoot apical meristem into an inflorescence meristem rather than by the transition from the juvenile to adult phases. Early flowering phenotype caused by *CiFT* could be inherited in F<sub>1</sub> progenies obtained by crossing ‘Kiyomi’ tangor (*C. unshiu* × *C. sinensis*) with pollen from a transgenic line. Mendel’s segregating ratio (1:1) was fitted to the segregation of the transgene, resulting in the complete co-segregation of the transgene and the early flowering phenotype. Interestingly, the null segregant, which lost the transgene in the progress to the next generation, showed no early flowering phenotype. In maize, a seed production technology (SPT) process was developed to improve the efficiency of hybrid seed production by using the segregation-out of transgene. In Europe and the United States, the SPT seeds are outside of the regulation governing genetically modified organisms. Because the introduced *CiFT* in a transformed trifoliolate orange could be also segregated out, *CiFT* would be useful to accelerate the cycles of breeding generations of backcrossing with citrus and produce non-GMO citrus hybrids. In addition, Endo *et al.* (2009) developed a *CiFT* co-expression binary vector, which drives the target gene under El<sub>2</sub>-Ω-cauliflower mosaic virus P35S (El<sub>2</sub>-35S) promoter with *CiFT*. Using this vector, they succeeded in modifying aroma components in transgenic trifoliolate orange to repress the biosynthesis of *d*-limonene using antisense transgenes of *d*-limonene synthase genes. Transgenic trifoliolate orange formed normal flowers and fruits within 18 months of infection by *Agrobacterium*. Thus, the functional analysis of transgenic citrus facilitated evaluate the mature traits of flowers and fruits, unlike in past research. The utilization of flowering genes contributes to overcome problems relating to long juvenile periods in transgenic research.

Meanwhile, additional efforts should be made to develop collections of tissue-specific and inducible promoters for the control of incorporated gene expression. To the present, several promoters controlling tissue-specific expression

have been reported in citrus. Endo *et al.* (2007) developed a *CitMT45* promoter that was derived from genomic clones of type 3 metallothionein gene, which was exclusively expressed in the fruit tissue of citrus (Moriguchi *et al.* 1998a). Nishikawa *et al.* (2008) developed a *CuMFT1* promoter that was derived from a *FT/TFL1* homolog (*CuMFT1*), which may be useful in regulating seed-specific expression in transgenic plants in *Citrus*. Nishikawa *et al.* (2009b) developed a *CuMTSE2* promoter that was derived from *d*-limonene synthase gene 2 with peel-specific expression in mature fruit.

The establishment of transgenic technology combined with the technical improvement in gene function assays could accelerate the identification of genes related to breeding factors such as fruit quality and bio-functional substance production.

### Conclusion and future prospects

The development of experimental tools and resources for citrus genome analysis has contributed to elucidating the genetic composition and molecular mechanism of agronomically important traits and to advancing the isolation of such genes. In breeding programs, DNA marker-based selection methods can be further developed and applied to the selection of seedlings with new favorable traits such as CTV resistance and seedlessness. In addition to these traditionally important breeding traits, the breeding of new cultivars enriched with health-promoting substances has progressed. Research into the mechanisms involved in the metabolism of monoterpenes, limonoids, flavonoids and carotenoids has advanced, and the development of DNA markers is under way as part of the practice of selection in target of β-cryptoxanthin, auraptin and nobiletin (Table 3).

The use of molecular technologies will help to overcome the obstacles of juvenility and polyembryony in citrus breeding. The utilization of *CiFT* will succeed in reducing the time needed to evaluate fruit traits in transgenic plants and will shorten the cycles of breeding generation. Furthermore, researches on the genes involved in flower bud formation will contribute to improving breeding technology as

**Table 3.** Target of functional metabolites aimed to enrichment in fruit

Compounds	Breeding resource (Latin name)	Target gene or metabolite
Limonoids	Satsuma mandarin ( <i>C. unshiu</i> Marc.)	UDP-glucose: limonoid glucosyltransferase
Polymethoxyflavonoids	Nou-6 (‘King’ ( <i>C. nobilis</i> Lour.) × ‘Mukaku kishu’ ( <i>C. kinokuni</i> hort. ex Tanaka)), King ( <i>C. nobilis</i> Lour.)	Flavonoid O methyltransferases (nobiletin and tangerine from sinensetine)
Hesperisin	Satsuma mandarin ( <i>C. unshiu</i> Marc.), Hassaku ( <i>C. hassaku</i> hort. ex Tanaka)	Conversion of rhamoside
Auraptene	‘Aura star’ (‘H-FD-1’ (Hassaku ( <i>C. hassaku</i> hort. ex Tanaka) × ‘Hiryu’ ( <i>P. trifoliata</i> var. <i>monstrosa</i> )), ‘Banpeiyu’ ( <i>C. grandis</i> Osbeck))	Conversion of geranyloxy group
β-cryptoxanthin	‘Tamami’ (‘Kiyomi’ (‘Miyagawa wase’ ( <i>C. unshiu</i> Marc.) × ‘Trovita’ orange ( <i>C. sinensis</i> (L.) Osbeck)) × ‘Wilking’ (‘King’ tangor ( <i>C. nobilis</i> Lour.) × ‘Willowleaf’ mandarin ( <i>C. deliciosa</i> Ten.))	Carotenoid biosynthetic genes
Monoterpenes	Ponkan ( <i>C. reticulata</i> Blanco), sweet orange ( <i>C. sinensis</i> (L.) Osbeck)	Monoterpene biosynthetic genes

well as regulating the development of flower buds so as to avoid alternate fruit bearing in cultures. Seedlessness is perhaps one of the most important traits relating to citrus fruit breeding in the future, so the isolation and identification of genes involved in seed and embryo development, as well as male sterility, are vitally important. The genes regulating polyembryony have been investigated through a positional cloning procedure combined with expression analysis (Nakano *et al.* 2012). The isolation of a gene corresponding to the locus is likely to be achieved in the near future. This will provide a new strategy for the production of seeds from heterozygous plants and a practical application for MAS.

In citrus, most of the important breeding objects such as fruit quality and fruit yield are quantitatively inherited, and the development of the QTL marker is urgently needed (Table 2). The established experimental tools and resources used in genome analysis are expected to accelerate the discovery of the loci associated with various quantitative traits and contribute to the design of breeding programs supported by MAS, including genomic selection in citrus breeding.

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