Studies on Development and Application of High-throughput Genomic and Bioinformatics Tools for Citrus Fruit Physiology and Breeding

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（カンキツ果実の生理学と育種学のためのハイスループットなゲノム及び
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Chapter 1: INTRODUCTION

Citrus is one of the most economically important fruit species in the world. During the long history of the natural evolution, the fruits had diversified in the colors, shapes, fragrances and tastes as well as abundant secondary metabolic elements possessing great health values. These diversities have been used as the resources for citrus breeding to obtain attractive fruit. The efforts on breeding have generated the cultivars with seedless fruit. Along with the development of seedless cultivars, citrus breeding program has become complicated and difficult to improve through traditional breeding approaches (Talon and Gmitter, 2008) because the obtaining of hybrids was interfered by polyembryony, male sterility and self-incompatibility.

In this decade, genomic technology has rapidly advanced. The biological challenges can now be addressed also in citrus plant to understand genetic and physiological events on fruit traits (Talon and Gmitter, 2008). For the purposes, many genome analysis projects have been performed. They included expressed sequence tag (EST) analysis (Bausher et al., 2003; Shimada et al., 2003; Fujii et al., 2003a; Forment et al., 2005; Terol et al., 2007), EST database analysis (HarvEST http://harvest.ucr.edu; Fujii et al., 2003) and development and application of DNA marker. They were developed by cleaved amplified polymorphic sequences (CAPS) maker analysis (Omura, 2005), simple sequence repeat (SSR) marker analysis (Chen et al., 2006), single nucleotide polymorphism (SNP) marker analysis (Ollitrault et al., 2012; Distefano et al., 2013), and applied to the linkage mapping (Omura, 2005), quantitative trait loci (QTL) analysis (Sugiyama et al, 2011), and cultivar typing (Omura, 2005). EST analysis made much progress in recent years to microarray technology for gene expression profiling (Shimada et al, 2005; Terol et al., 2007).

The cataloguing of ESTs has emerged in 1990s as a powerful tool capable of

obtaining a large set of expressed genes from genome. The citrus genome analysis team (CGAT) of the National Institute of Fruit Tree Science (NIFTS), National Agriculture and Bio-oriented Research Organization of Japan (NARO), started the EST analysis program in the 1990s (Hisada et al., 1996; Hisada et al., 1997; Moriguchi et al., 1998; Kita et al., 2000; Shimada et al., 2003; Fujii et al., 2003a). The EST program stimulates and supports molecular and physiological research on citrus fruit. Through the program, CGAT/NIFTS has collected 29,228 ESTs on 19 cDNA libraries covering different tissues and developmental stages (Fujii et al., 2006). Among the 19 libraries, 16 were derived from *C. unshiu* and the six remaining libraries were derived from *C. sinensis*, *C. limon*, and *C. kinokuni* hort. ex Tanaka. The 20,525 ESTs of adequate quality were submitted to the DNA Data Bank of Japan (DDBJ) and released (Table 1-1). Fujii et al. (2003b) also constructed an in house EST database to manage EST sequences, accession numbers, and functional annotations as user-friendly database.

The large collection of ESTs has been applied to reveal the gene expression patterns, gene regulation, and sequence diversity (Brandle et al., 2002), and development of EST databases have contributed to discover the genes associated with fragrance (Shimada et al., 2005a; Shimada et al., 2005b; Shimada et al., 2005c) and to induce the precocious flowering while assaying the gene functions in fruit (Endo et al., 2005, Endo et al., 2006). The gene repertory analysis indicated that the easy peeling of citrus fruit rind, which is an important trait for commercial value in citrus, is related with the gene expression involved in relaxation of the cell wall (Brummell and Harpster, 2001). After a prototype cDNA microarray with 2,213 spots has been produced to promote the molecular analysis of fruit development and quality using the EST database (Shimada et al., 2005d), the custom citrus 22K oligoarray had been developed as the tools for functional genomics (Fujii et al., 2006). In the procedure of EST microarray

design, the EST sequences were subjected to clustering. The collection of 29,228 ESTs grouped into 13,896 putative unigenes. Each unigene was translated into its amino acid sequence and subjected to a similarity search against amino acid and motif databases using Fasty, Blastx, and motif search algorithms. Among the 13,986 unigenes, 6,759 (48.6%) showed similarity to genes with known functions and 759 (5.5%) showed similarity to only functional domains.

In addition to the use of EST information on fruit physiology and molecular biology, the ESTs have been used to generate DNA makers for genome mapping. The CAPS markers were used to construct linkage maps of several mapping populations of citrus and they have been applied to obtain the selection markers for breeding (Omura, 2005). The traits related to fruit quality, such as sugar and acid contents, peel thickness, rind and pulp color and carotenoid content, and seed characteristics, such as polyembryony, embryo color, seed number, and seedlessness, were analyzed and mapped on the CAPS linkage maps as QTLs (Omura, 2005; Sugiyama et al., 2011). The CAPS markers also provided the molecular tools to identify cultivars (Omura, 2005).

Recently, the international consortium on citrus genome analysis publicly released the haploid Clementine (*Citrus clementina*) and the diploid sweet orange (*C. sinensis*) genomes (Gmitter et al., 2012; Citrus Genome Database http://www.citrusgenomedb.org/). Furthermore, the draft genome sequence of the dihaploid sweet orange has been produced (Xu et al., 2013) and made available to the global research community (*Citrus sinensis* annotation project. http://citrus.hzau.edu.cn/orange/). Despite the challenges of working with citrus, understanding important characteristics from the gene expression level is insufficient. It is believed that the important characteristics of citrus fruits are under complex genetic regulation. In addition, the heterozygosity of the citrus genome makes more difficult to

understand genotype-phenotype relation and to identify the key regulatory gene. It is necessary to make excellent use of the high-throughput genomic tools available to understand the regulations. In this thesis, high-throughput genomic technology, such as the oligo-microarray, SNP genotyping array, and analytical software, were developed and applied to citrus to provide the basis for comprehensive use of citrus genome information, which has been accumulated quickly. Chapter 2 details a gene expression analysis using the 22K citrus oligo-microarray that was performed to profile gene expression in mature mandarin fruit undergoing plant hormone treatment. In Chapter 3, the development of an algorithm and computer program for efficient cultivar identification using DNA makers is described. Chapter 4 discusses the development of a 384 SNP genotyping array for high-throughput genotyping and how the array was applied to 98 citrus accessions and a population. The results obtained in this study, the expression analysis of many genes related to important characters, the analysis of genome-wide genotyping among many varieties and the software for efficient cultivar identification, or the combination of these three analyses will be necessary to understand important characters of citrus.

Table 1-1. The EST catalogs analyzed in CGAT/NIFTS

Library	Originating cultivar	Species	Tissue and stage	No. of clones	No. of DDBJ registered	Accession number	Reference
VSS	'Valencia' orange	*Citrus sinensis*	Young seed	577	577	C21828-C21914 DC899990-DC900479	Hisada et al. 1996
FRI	'Miyagawa wase'	*C. unshiu*	Fruit pulp, developing	1,051	1,051	C21915-C24319 DC893414-DC893590	Hisada et al. 1997
FRM	'Miyagawa wase'	*C. unshiu*	Fruit pulp, maturation	385	385	C81631-C81927 DC893591-DC893680	Moriguch et al. 1998
ALM	'Miyagawa wase'	*C. unshiu*	Albedo, maturation	623	623	C95196-C95572 DC892843-DC893089	Kita et al. 2000
OVA	'Miyagawa wase'	*C. unshiu*	Ovary, flowering	827	827	AU186170-AU186562 DC893681-DC894116	Shimada et al. 2003
ALP	'Miyagawa wase'	*C. unshiu*	Albedo, initiation stage of rind peeling	941	941	AU300309-AU300928 DC893090-DC893413	Fujii et al. 2003a
WFY	'Miyagawa wase'	*C. unshiu*	Whole fruit, young	1,689	1,689	DC894117-DC895805	
BFC	'Miyagawa wase'	*C. unshiu*	Rind, coloring	1,650	1,650	DC884963-DC886612	
FBI	'Miyagawa wase'	*C. unshiu*	Flower bud, 30 days before flowering	2,367	2,367	DC888010-DC890376	
GSA	'Miyagawa wase'	*C. unshiu*	Seed, imbibition 4 days	1,920	1,042	DC890377-DC891418	
RGP	'Miyagawa wase'	*C. unshiu*	Root, seedling 2 weeks	960	553	DC896389-DC896941	
SLG	'Miyagawa wase'	*C. unshiu*	Shoot, seedling 2 weeks	1,920	991	DC897089-DC898079	
YJS	'Miyagawa wase'	*C. unshiu*	Juice sac, 60 days after flowering	1,926	1,035	DC900480-DC901514	
PCC	'Miyagawa wase'	*C. unshiu*	Callus, proliferating	960	583	DC895806-DC896388	
EIC	'Miyagawa wase'	*C. unshiu*	Callus, embryogenesis	1,152	752	DC887258-DC888009	
STG	'Miyagawa wase'	*C. unshiu*	Stigma, flowering	3,552	1,910	DC898080-DC899989	
ANT	'Miyagawa wase'	*C. unshiu*	Anther, flowering	2,600	1,480	DC883483-DC884962	
LLL	'Lisbon' lemon	*C. limon*	Leaf, young	2,016	1,424	DC891419-DC892842	
EGJ	Kisyu-mikan	*C. kinokuni*	Ovule, 60-70DAF	2,112	645	DC886613-DC887257	
			Total	29,228	20,525		

Chapter 2: Oligoarray analysis of gene expression in mature mandarin fruit

During fruit development and ripening, complex physiological and biochemical changes are regulated by hormonal, nutritional, and environmental controls (Giovannoni, 2004). Citrus fruit is generally classified as non-climacteric fruit (Kader, 1992) but can respond to exogenous ethylene, which stimulates fruit ripening along with chlorophyll degradation and carotenoid accumulation in peel (Goldschmidt et al., 1993). Many ripening-related genes have been isolated and characterized in *Citrus* species, and it is well documented that ethylene regulates chlorophyll degradation and regulates carotenoid accumulation at the transcriptional level (Jacob-Wilk et al., 1999; Kato et al., 2004; Kato et al., 2006; Rodrigo et al., 2004; Rodrigo et al., 2006).

Gibberellin (GA_3) delays ethylene-, or sucrose- induced peel color change by the repression of chlorophyll degradation and by the repression of carotenoid accumulation (Cooper and Henry, 1968; Trebitsh et al., 1993; Iglesias et al., 2001; Rodrigo and Zacarias, 2007). Iglesias et al. (2001) consider that GA appears to control the timing of chlorophyll disappearance by inhibiting or reducing chlorophyll biosynthesis. After the natural reduction of endogenous GA levels in mature fruit, color change may be stimulated by the basal level of endogenous ethylene, along with the de novo synthesis of chlorophyllase. Thus, ethylene and GA are assumed to play important roles in the endogenous regulation of maturation and senescence in mature citrus fruit, but little is known about the effects of GA on transcriptional regulation during fruit ripening.

In tomato and *Arabidopsis*, ethylene-regulated genes were investigated using microarray analysis, and it was demonstrated that a large number of transcription factors and some putative signaling components, which were transcriptionally associated with fruit maturation and ripening, were highly regulated by ethylene, providing a new insight into the molecular basis of ethylene-mediated ripening (Zhong and Burns, 2003;

Alba et al., 2005).

Recently, 2.2K and 12K cDNA microarrays (Shimada et al., 2005) and (Forment et al., 2005) were developed in *Citrus* species and applied to the global analysis of transcriptome dynamics during the development and ripening of citrus fruit. Using 12K cDNA microarrays, Cercós et al. (2006) identified more than 2,200 putative unigenes with significant expression changes during fruit development, which were involved in the metabolism of carbohydrates, acid, secondary, cell expansion, and transcription regulators.

In this Chapter, the citru*s* custom 22K oligoarrays were used to understand complicated transcriptional regulation during fruit development and ripening. It will provide a new insight of the ethylene or gibberellin regulatory mechanism in citrus.

Section 1. Profiling ethylene-responsive genes in mature mandarin fruit using a citru*s* 22K oligoarray

Mature citrus fruit exhibit a relatively low respiration rate and level of ethylene production and are generally classified as non-climacteric fruit (Kader, 1992). This low level of exogenous ethylene is assumed to play a role in the endogenous regulation of maturation and senescence (Goldschmidt, 1998). Ethylene has significant effects on plant development to regulate germination, senescence, abscission, fruit ripening, drought, wounding, chilling, and pathogen infection (Abeles et al., 1992). In climacteric fruit, such as tomato, numerous studies of ethylene biosynthesis and response have been reported, and ethylene has been shown to control the ripening process through the regulation of gene transcription (Giovannoni, 2004). However, the ripening mechanism in non-climacteric fruit remains unclear, and it would appear that a unique program regulates the development and ripening of citrus fruit.

In general, ethylene treatment is ineffective with regard to the ripening of non-climacteric fruit, such as grape (Brady and Speirs, 1991), strawberry (Atta-Aly et al., 2000), and cherry (Given et al., 1988), however, citrus fruit responds to exogenous ethylene, which stimulates fruit ripening by enhancing respiration and changes in peel color (chlorophyll degradation and carotenoid accumulation) (Goldschmidt et al., 1993). In addition, some reports have indicated that there have been marked increases in the endogenous levels of ethylene production following various events, such as wounding (Hyodo and Nishino, 1981), pathogen attack (Achilea et al., 1985), chilling temperature (McCollum and McDonald, 1991), and detached young fruit (Katz et al., 2005), although mature citrus fruit produces only small amounts of ethylene and lacks an autocatalytic rise in its production. Thus, complex regulations of ethylene production and perception might exist during fruit development. Recently, ripening-related genes have been isolated and characterized in *Citrus* species, which are involved in chlorophyll degradation (Jacob-Wilk et al., 1999), carotenoid biosynthesis (Kato et al., 2004; Kato et al., 2006; Rodrigo et al., 2004; Rodrigo et al., 2006), and ethylene biosynthesis and perception (Katz et al., 2004; Katz et al., 2005). Most of these genes respond to exogenous ethylene, and their transcriptions are up-regulated in mature fruit. In contrast, significant transcriptional changes of ethylene biosynthesis and receptor genes were not detectable against ethylene and propylene treatments in mature fruit (Katz et al., 2004). Therefore, a full understanding of the ethylene regulatory mechanism in citrus fruit will be of value.

In this experiment, the ethylene-responsive genes in citrus mature fruit were investigated using a citrus 22K oligoarray containing 21,495 independent ESTs from *Citrus* species. Seventy-two hours after ethylene treatment, 1,493 genes were identified as ethylene-responsive genes with more than 3-fold expression change; an interesting

aspect of gene regulation by ethylene was observed, namely, that more than half of the ethylene-responsive genes were repressed, and it was assumed that these transcriptional changes might enhance the ripening process. In addition, transcriptional regulations related to chlorophyll degradation, carotenoid biosynthesis, and ethylene perception in the mature fruit were also discussed.

Materials and methods

Plant material and ethylene treatment

Satsuma mandarin (*C. unshiu* Marcovitch, cv. Miyagawa wase) cultivated at the Citrus Research Division Okitsu (Shimizu, Shizuoka, Japan) of NIFTS were used as materials. Samples of fruit at 150 days after anthesis (DAF) were collected. For the ethylene treatment of fruit, higher concentration of ethylene ($100\mu l \cdot L^{-1}$) was applied in each container in order to complete degreening within 72 h and monitor ethylene responsive genes during short time period. Both ethylene treatment and air treatment were conducted at 25°C. The flesh flavedo tissue was excised and immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction and the quantification of carotenoids and chlorophylls.

Carotenoid and chlorophyll quantification in flavedo

Quantification of 6 representative carotenoids, all *trans*-violaxanthin (*trans*-Vio), 9-*cis*-violaxanthin (*cis*-Vio), lutein (Lut), β -cryptoxanthin (B-Cry), α -carotene (A-Car), and phytoene (Phy), was carried out by the method of Kato et al. (2004). Samples were homogenized in 40% (v/v) methanol containing 10% (w/v) magnesium carbonate basic. Pigments were extracted from the residues using an acetone : methanol (7:3 [v/v]) solution containing 0.1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and

partitioned into diethyl ether. The extracts containing carotenoids esterified to fatty acids were saponified with 20% (w/v) methanolic KOH. After the saponification, water-soluble extracts were removed from the extract by adding NaCl-saturated water. The pigments repartitioned into the diethylether phase were recovered and evaporated to dryness. Subsequently, the residue was redissolved in 5 mL of an MTBE: methanol (1:1 [v/v]) solution. An aliquot (20 μL) was separated by a reverse-phase HPLC system (Jasco, Easton, USA) fitted with a YMC Carotenoid S-5 column of 250- x 4.6-mm-i.d. (Waters, Milford, USA) at a flow rate of 1 mL min^{-1}. The eluent was monitored using a photodiode array detector (MD-910, Jasco). The peaks were identified by comparing their specific retention times and absorption spectra with the authentic standards. The standard curves for the carotenoid quantification were prepared with those of the authentic standards at 286 nm for Phy and 452 nm for *trans*-Vio, *cis*-Vio, Lut, B-Cry, and A-Car. The carotenoid concentration was estimated by the standard curves and expressed as milligrams per gram fresh weight. According to the method of Shimada and Shimokawa et al. (1978), the chlorophyll (*a* + *b*) content was determined by measuring the absorbance at 642 and 662nm. Carotenoid and chlorophyll quantification was performed in three replications.

RNA isolation and fluorescent labeling of probes

Total RNA was extracted by the methods of Ikoma et al. (1996) from flavedo tissues of non-treatment at 0 h and at 24 h, 48 h, and 72 h after ethylene treatment or air treatment. At least three independent RNA extractions were used in probe labeling for experimental reproducibility. The total RNA (400 ng) of all samples was labeled with the fluorescence Cy5, while non-treatment at 0 h was labeled with Cy3 according to the instructions for the Low RNA input linear amplification and labeling kit (Agilent

technologies, Santa Clara, USA). Labeled cRNA was purified using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Hybridization and washing were performed according to the manufacturer's instructions. Glass slides were hybridized overnight at 60°C in a hybridization buffer containing a fragment of Cy3- or Cy5-labeled cRNA. After hybridization, slides were washed in 6×SSC, 0.005% Triton X-100 for 10 min at room temperature and 0.1×SSC, 0.005% Triton X-100 for 5 min at 4℃. After drying the slides with gaseous nitrogen, hybridized slides were scanned with the use of a microarray scanner (Agilent technologies). The intensities of the Cy5 and Cy3 fluorescent signals from each spot were automatically normalized, and the ratio value (Cy5/Cy3) was calculated using Feature Extraction version 7.1 software (Linear & LOWESS analysis, Agilent technologies). Data analysis was carried out using GENESPRING 7.00 (Silicon Genetics, Redwood City, USA). Genes with more than a 3-fold expression change between ethylene treatment and air treatment at each experimental time (24 h, 48 h, and 72 h) were accepted as ethylene-responsive genes in this experiment.

Northern gel blot analyses

For Northern blot analysis, total RNA was extracted by the methods of Ikoma et al. (1996) from flavedo tissues at 0 h, 24 h, 48 h, and 72 h after ethylene treatment. Ten microgram from each RNA sample was subjected to electrophoresis on a 1.2% agarose gel containing 8% (v/v) formaldehyde and transferred to a nylon membrane (Hybond-NX, Amersham Pharmacia Biotech, Little Chalfont, UK). The cDNA probes of 7 representative ethylene-regulated genes identified by microarray analysis were prepared with the use of a PCR DIG labeling kit (Roche Molecular Biochemicals, Tokyo, Japan). Hybridization and detection were conducted according to the

manufacturer's directions (Roche Molecular Biochemicals).

Results and discussion

Identification and functional classification of 1,493 ethylene-responsive genes

A citrus 22K oligoarray including 21,495 independent EST probes derived from *Citrus* species and 1,080 control spike probes was used in this study to identify ethylene-responsive genes in mature fruit. The fold change of each gene expression was calculated based on the mRNA expression ratio between ethylene treatment samples and air treatment samples at every 24h. In the 72 h after the ethylene treatment, 1,493 genes showed more than a 3-fold change in the mRNA expression ratio. Table 2-1 showed representative ethylene resopnsive genes with 3-fold expression change between Ethylene and air treatments. Of 1,493 genes, the expression of 554 genes was up-regulated, while 939 genes were down-regulated, indicating that ethylene tended to repress transcription in this fruit stage. Ethylene-induced esterase, pathogenesis-related (PR) protein, and 9-*cis*-epoxycarotenoid dioxygenase had high ethylene sensitivity, and they were radically induced by exogenous ethylene within 24h with more than a 30-fold change. In contrast, the chlorophyll a/b-binding protein (CAB), ribulose-1,5-bisphosphate carboxylase (RBC), and extensin-like protein were down-regulated by more than 30-fold. To confirm the results from the microarray analysis, 7 representative genes, each with a different responding pattern against ethylene, were selected and subjected to Northern blot analysis (Fig. 2-1). As shown in Fig. 2-1, aminocyclopropanecarboxylate (ACC) oxidase 1 (ACO1), ethylene-induced esterase, and PR protein were significantly induced, and xyloglucan endotransglycosylase (*XET*), *RBC*, and flowering time (*FT*) genes were suppressed after exogenous ethylene treatment. The regulation patterns were different among these genes,

but the genes were either induced or suppressed by exogenous ethylene or by constitutive activation of the ethylene-signaling pathway. The signal intensities of each Northern band visually reflected changes detected in the microarray, demonstrating the fidelity of the experiments.

A total of 1,493 ethylene-responsive genes were compared by TBLAST X similarity search (e-value <1e-5) with all cDNAs of *Arabidopsis* (downloaded from the TAIR. Since each cDNA of *Arabidopsis* provided functions according to gene ontology annotations for *Arabidopsis* (GOSLIM in TAIR), the genes were assigned the functions according to GOSLIM on the basis of their similarity with the cDNA of *Arabidopsis*. As a result, 939 genes were assigned to three aspects of GOSLIM (Table 2-2). Certain genes were often assigned to more than one category in each aspect of GOSLIM; thus, the total did not equal 100%. Among the molecular functions, the category of "other enzyme activity" was the most affected by ethylene, and 176 genes (11.8% of 1,493 genes) responded to ethylene treatment. Among the biological processes, the categories of "other metabolic processes" (22.4% of 1,493), "other physiological processes" (19.9%), and "other cellular processes" (19.9%) were significantly affected by ethylene. Among the cellular components, the categories of "other membranes" (18.0%), "chloroplast" (8.0%), and "other cellular components" (7.5%) were affected by ethylene treatment. Thus, more than one half of the ethylene-responsive genes were repressed in these Go Term categories. This aspect might suggest that ethylene demotes numerous biological processes and plays an important role in fruit ripening and senescence.

Hierarchical clustering of 1,493 ethylene-responsive genes

To visualize ethylene-responsive expression patterns in 72 h, the 1,493 genes were subjected to cluster analysis and divided into 2 major clusters (Fig. 2-2). As shown in

Fig. 2-1, ethylene treatment caused drastic transcriptional changes of these genes in comparisons with air treatment, and most of the genes quickly responded to exogenous ethylene within 24 h of the treatment. Cluster 1 consisted of 939 genes that were down-regulated after the ethylene treatment. Many genes related to photosynthesis, chloroplast biogenesis, sugar metabolism, transcription, and cell wall metabolism were quite evident. Interestingly, ethylene repressed the transcription of most genes involved in photosynthesis and chloroplast biogenesis, such as the CAB, the photosystem I subunit, and RBC. This result indicated that repression of photosynthesis-associated genes was controlled at the transcriptional level by ethylene. Similar repression of photosynthesis by ethylene was observed in *Arabidopsis* (Zhong and Burns, 2003). In the sugar metabolism, starch synthase, gulcose-6-phosphogluconate dehyrogenase and hexokinase 2 were down-regulated, while hexose carrier, a sucrose transporter, and acidic invertase were up-regulated. The expression of genes related to the sugar metabolism is generally reduced during ripening, although not all of them are similar (Hennig et al., 2004). In ripening fruit of 'Fortune' mandarin, sucrose translocation rather than sucrose synthesis was considered to play a major role in the maintenance of the sucrose levels in flavedo due to the low activity of sucrose phosphate synthase (Holland et al., 1999), and sucrose broken down to hexoses was mediated by sucrose synthase, acid invertase, and alkaline invertase. Cell wall modification genes were also regulated by ethylene. Most genes were down-regulated by exogenous ethylene, such as cellulose synthase, pectate lyase, polygalacturonase, pectinacetylesterase, xyloglucan and endotransglycosylase. In contrast, expansin, ethylene-induced esterase and beta-galactosidase, UDP-galactose-4-epimerase, and germin-like protein were up-regulated. There is less information for the transcriptional regulation of cell wall genes against ethylene in citrus mature fruit. In grapefruit, arabinosyl and galactosyl

residues were most abundant in flavedo tissue, and fruit ripening accelerated softening through hydrolysis for these galactosidase galactosyl and arabinosyl residues of cell wall by β-galactosidase and UDP-galactose-4-epimerase (Mitcham and McDonald, 1993). However, it was reported that ethylene had no effect on the loss of mature fruit weight and firmness in 'Shamouti' orange (Porat et al., 1999). This result suggested that drastic cell wall modification was not occurred by ethylene treatment during mature fruit, unlike climacteric fruits, and unique regulation system of cell wall genes should exist in citrus mature fruit. Interestingly, divergent effects of ethylene have reported in peach, so that regulatory activity by ethylene can either be positively and negatively according to the different genes (Trainotti et al., 2003). In strawberry, exogenous ethylene decreased pectin esterase in ripe and senescing fruits (Castillejo et al., 2004). Therefore, it is possible that cell wall genes such as pectate lyase and polygalacturonase were down-regulated by ethylene in mature fruit. Ethylene activates pathogen defense and several cell-wall-related genes were also induced by pathogen attack (Maleck et al., 2000). In orange, expansin was induced by glassy-winged sharpshooter (GWSS) -derived elicitors (Mozoruk et al., 2006).

Cluster 2 contained 554 genes that were radically up-regulated after ethylene treatment. There were the genes involved in resistance, defense, stress, amino acid synthesis, protein degradation, secondary metabolism, protein kinase, and other signaling components. Cysteine proteases, polyubiquitin, and proteasome were up-regulated, and these proteins were implicated in the ubiquitin-mediated protein degradation pathway, which might be associated with the initiation of the fruit senescent process, as reported by Cercós et al., (2006). Ethylene is known to play a key role in various aspects of plant defense against abiotic stress, such as wounding and ozone exposure as well as insect and microbial attack (Kunkel and Brooks, 2002). Genes such

as osmotin, beta-glucanase, chitinase, and the PR protein were induced, as well as oxidative-burst proteins of peroxidase and glutathione S-transferase. Reactive oxygen molecules were generated in the initial steps of response to pathogen attack (Bolwell and Wojtaszek, 1997). Recently, the GWSS - derived elicitors induced genes that were characterized in orange using a nylon filter cDNA microarray, and significant transcriptional changes occurred for the genes involved in direct defense, defense signaling, cell wall modification, photosynthesis, and abiotic stress (Mozoruk et al., 2006). Several ethylene-responsive genes characterized in our experiment were overlapped in these elicitor-induced genes. Plant defense responses are regulated through a complex signaling network with a cross talk among salicylic acid (SA), jasmonic acid (JA), and ethylene-signaling pathways. Some of them might be activated positively or negatively through this cross talk among plant hormone-signaling pathways.

Ethylene regulates chlorophyll degradation at the transcriptional level

It is well known that ethylene results in the enhancement of color change by increasing chlorophyll degradation and the promotion of carotenoid biosynthesis (Goldschmidt et al., 1993). In this experiment, the application of exogenous ethylene accelerated chlorophyll breakdown, and degreening was completed within 72 h (data not shown). The chlorophyll contents and ratio of chlorophylls a to b were investigated in flavedo tissues at 0 h and 72 h after treatments (Table 2-3). In a comparison of air treatment, ethylene accelerated the loss of chlorophyll, and the content of chlorophyll became one-half. The chlorophyll a content in the ethylene-treated fruit decreased along with chlorophyll degradation, indicating that chlorophyll a was more predominantly degraded than chlorophyll b. In citrus 22K oligoarrays, 4 chlorophyll-related gene

homologues were included: magnesium chelatase (accession no. CK665296), chlorophyllase (accession no. CF838747), chlorophyll synthase (accession no. CD575834), and NADPH-protochlorophyllide oxidoreductase (accession no. DC885363). The gene expression of chlorophyllase was extremely up-regulated by exogenous ethylene, while magnesium chelatase was down-regulated (Fig. 2-3A). Other genes showed similar expression patterns between ethylene and air treatments. This ethylene-enhanced chlorophyllase gene expression is in good agreement with the result of Jacob-Wilk et al. (1999). In addition, ethylene treatment significantly suppressed the transcription of magnesium chelatase, which mediates the insertion of Mg^{2+} into protoporphyrin IX and is the first unique enzyme of the chlorophyll biosynthetic pathway. Thus, ethylene was found to play binary roles in enhancing the decomposition of chlorophyll and suppressing chlorophyll biosynthesis at the transcriptional level.

Ethylene regulates the transcriptional changes of carotenoid biosynthesis genes and affects carotenoid composition

The contents of 6 representative carotenoids (*trans*-Vio, *cis*-Vio, Lut, B-Cry, A-Car, and Phy) in the flavedo tissue were characterized in ethylene-treated and air-treated fruit at 0 h and 72 h (Table 2-3). Within 72 h of the ethylene and air treatments, the total carotenoid contents increased from 58.0 μg·g-1 up to 220.4 μg·g-1 (air treatment) and 234.8 μg·g-1 (ethylene treatment). It was reported that optimum ethylene and temperature treatments improved fruit color development (Wheaton and Stewart, 1973). In Satsuma mandarin, more than 20°C temperature treatment enhances carotenoid accumulation in peel of detached fruit (Hasegawa and Iba, 1983). Interestingly, the total carotenoid contents of ethylene- and air-treated fruit for 72 h were almost identical, but their carotenoid composition differed. For example, B-Cry in ethylene-treated fruit was

almost twice that of air-treated fruit. On the other hand, the *trans*-Vio and *cis*-Vio ratio (29.39%) of total carotenoids was lower in ethylene-treated fruit than air-treated fruit (46.42%). Thus, ethylene treatment affected the ratio of B-Cry and violaxanthin (Vio) content during the 72 h treatment.

A citrus 22K oligoarray allows the profiling of 10 genes related to carotenoid biosynthesis in flavedo tissue (Fig. 2-3B), including phytoene synthase (*CitPSY*), phytoene desaturase (*CitPDS*), ζ -carotene desaturase (*CitZDS*), lycopene ε -cyclase (*CitLCYe*), lycopene β -ring hydroxylase (*CitLCYb*), β -ring hydroxylase (*CitHYb*), zeaxanthin epoxidase (*CitZEP*), carotenoid isomerase (*CitCRTISO*), and carotenoid cleavage dioxygenases (*CitCCD1* and *CitNCED2*). Comparing these gene expression patterns in ethylene- and air-treated fruits, it is noteworthy that ethylene treatment exclusively enhanced the transcription of *CitCCD1* and *CitNCED2*, and their fold change in expression was, at maximum, 39 times higher than that in air-treated fruit. They radically responded to exogenous ethylene within 24h and maintained a higher transcriptional level up to 72 h in spite of the lack of response in air-treated fruits. These enzymes mediate the cleave reaction of epoxycarotenoids into xanthoxin, which is the main regulatory step in abscisic acid (ABA) biosynthesis in citrus (Kato et al., 2006; Rodrigo et al., 2006). A similar result was reported, namely, that *CsNCED1* was up-regulated in orange flavedo by exposure to ethylene (Rodrigo et al., 2006). The expressions of *CitPSY*, *CitHYb* and *CitZDS* were also up-regulated in ethylene treatment within 24h, while *CitZEP* expression was not affected. This high response of carotenoid cleavage dioxygenases to ethylene could explain the lower Vio content in ethylene-treated fruit than air-treated fruit for 72h. The higher amount of *trans*-Vio and *cis*-Vio in air-treated fruits than ethylene-treated one could be explained by highly ethylene-induced *CitCCD1*and *CitNCED2,* which mediated these epoxycarotenoids into

xanthoxin. In addition to this, most upstream carotenoid biosynthesis genes were up-regulated by ethylene while *CitZEP* gene expression was not so induced. These balance change of these transcription led to the increase of B-Cry.

Thus, ethylene up-regulated the transcription of most carotenoid biosynthesis genes. The responsive pattern and sensitivity to ethylene were different among these genes. Their different responding patterns to ethylene would cause a change in the transcriptional balance of carotenoid biosynthesis genes, directly affecting the carotenoid composition in the fruit. Similar result was obtained in orange that the change of carotenoid composition was consistent with the change of related gene expression caused by ethylene treatment (Rodrigo and Zacarias, 2007).

Ethylene perception signal transduction

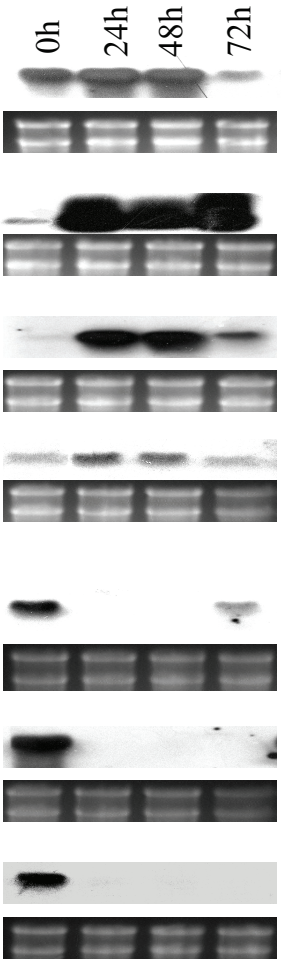
Ethylene regulates its own biosynthesis and receptor genes (Wang and Ecker, 2002). Many components of the ethylene signal transduction pathway have been isolated and characterized in recent years in *Arabidopsis* (Bleecker and Schaller, 1996) but little is known about the transcriptome dynamics of ethylene signal transduction in citrus fruit. A citrus 22K oligoarray allows the profiling of the following ethylene biosynthesis and ethylene signal transduction components functionally characterized in plants: ACC synthase (ACS), ACC oxidase (ACO), the ethylene receptor (ETR), basic leucine zippers, the carbon catabolite repressor-associated factor (CTR1), mitogen-activated protein kinases, 14-3-3 proteins, ethylene-responsive factors, and ethylene-responsive element-binding proteins. Most biosynthesis genes and signal transduction components did not show any significant expression change (< 2 fold) after exogenous ethylene treatment (data not shown). Only 2 genes, ACO1 (accession no. DC894173) and ethylene receptor homologue 2 (ETR2) (accession no. CF931498), showed more than

2-fold expression changes by exogenous ethylene treatment (Fig. 2-3C). Katz et al., (2004) reported that the gene expressions of *CsACS1*, *CsACS2*, *CsACO1*, *CsETR1*, and ethylene response sensor 1 (*CsERS1*) were independent from ethylene and propylene treatments in mature citrus. Similar results were obtained in this experiment, except for *CsACO1*. *ETR2* has different structures from *CsETR1* and *CsERS1* and was newly identified as an ethylene-responsive gene in mature citrus fruit. Genetic and biochemical studies have revealed that ethylene receptors work as a negative regulator in the ethylene perception-signaling pathway and that the binding of ethylene with the receptor inactivates them (Chang and Stadler, 2001). Recently, a new interesting finding was reported, namely, that the amino-terminal domain of CTR1 could interact with the His kinase domains of the ethylene receptor (Clark et al., 1998) and that the binding affinity of CTR1 has a higher type I (ETR1 and ERS1) than ETR2 (Cancel and Larsen, 2002), suggesting the possible hypothesis that the structural variation of these receptors might affect ethylene sensitivity. Therefore, our results would provide a new insight for ethylene perception in citrus fruit, namely, that type II ethylene receptors might be related to low sensitivity to ethylene in mature fruit. Interestingly, *FaETR2* showed highly induced by exogenous ethylene in strawberry (Trainotti et al., 2005). They considered that CTR1 might be released by type II ethylene receptor by lower amounts of ethylene and small amount of endogenous ethylene might be sufficient to trigger some physiological response. The biochemical function of these ethylene receptors (*CsETR1*, *CsERS1*, and ETR2) should be elucidated to understand the different ethylene sensitivities between young and mature fruit.

Ethylene-responsive transcription factors

The citrus 22K oligoarray contained 350 probes with DNA-binding domains

corresponding to the orthologues of *Arabidopsis* transcription factors. In the experiment, 24 transcriptional factors were identified as ethylene-responsive transcription factors with 3-fold expression changes. The functional classification of 24 responsive genes was conducted in reference to the functional classification of *Arabidopsis* transcriptional factors. There are 5 MYB family cDNAs, 2 WRKY family cDNAs, and 2 bHLH family cDNAs, among others. The 6 genes showed low homologies against *Arabidopsis* transcription factors. The expression of 13 genes showed down-regulation in response to exogenous ethylene treatment, and 11 genes showed up-regulation. These transcription factors are particularly interesting because their transcriptions were ethylene-regulated and their transcriptional accumulation might be associated with fruit ripening. Recently, MADS-box factors have been involved in many other aspects of plant development in addition to the regulation of flowering time. Vrebalov et al. (2002) revealed that the MADS-box transcriptional factor controlled the tomato never-ripening phenotype, a ripening inhibitor. In fact, the mRNAs of citrus MADS-box transcription factors accumulated during fruit development and were assumed to play some roles in fruit development and ripening (Endo et al., 2006). Causier et al., (2002) proposed that transcription factors, such as the MADS-box family, might regulate ripening in non-climacteric fruit, which do not require the ethylene pathway to ripen and act as global regulators of fruit development. Therefore, some of the identified transcription factors might play an important role to regulate gene expressions involved in fruit ripening, such as chlorophyll degradation and carotenoid accumulation. Toward a better understanding of these actual gene functions, a gene silencing or ectopic expression experiment will be required.



			Fold expression change		
	ID	Annotation	24h/0h	48h/0h	72h/0h
	MWYAR52A	ACO1	7.2	7.3	-3.3
	VS28993A	Ethylene-induced esterase	48.5	120.5	116.1
	CF653559	PR protein	30.4	41.2	10.8
	ANT0329	MYB	8.7	9.7	1.7
	MAM9A24A	XET	-11.5	-10.2	-2.73
	BFC5B47A	RBC	-64.4	-72.6	-102.7
	AB027456	CiFT	-50.2	-49.3	-48.3

Fig.2-1. Northern blot analysis of 7 representative ethylene responsive genes identified by microarray analysis. Ten μg of total RNA from ethylene treated peels was loaded in each lane (0 h, 24 h, 48 h and 72 h after ethylene treatment). To the right of each blot is the EST ID, EST annotation, the ratio of fold expression change between ethylene treatment (E24h, E48h, E72h) and non treatment(C0h).

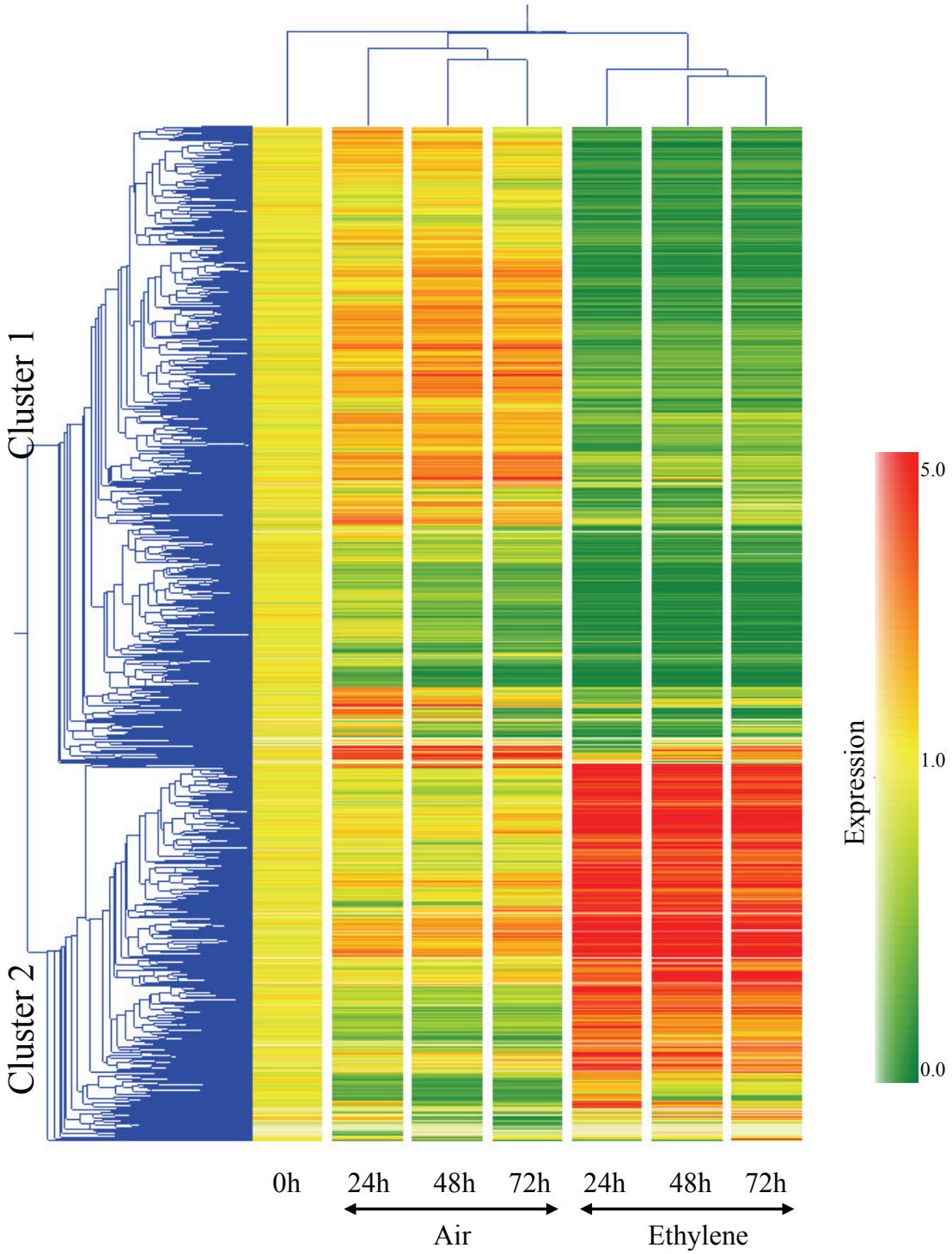


Fig. 2-2. Hierarchical cluster analysis of 1439 ethylene responsive genes with more than 3-fold expression changes between ethylene and air treatments (ethylene/air signal intensity ratio). The color scale indicates a signal intensity of each gene. Tree at the left side of the matrix represents gene relationship and upper tree indicates experiment relationship.

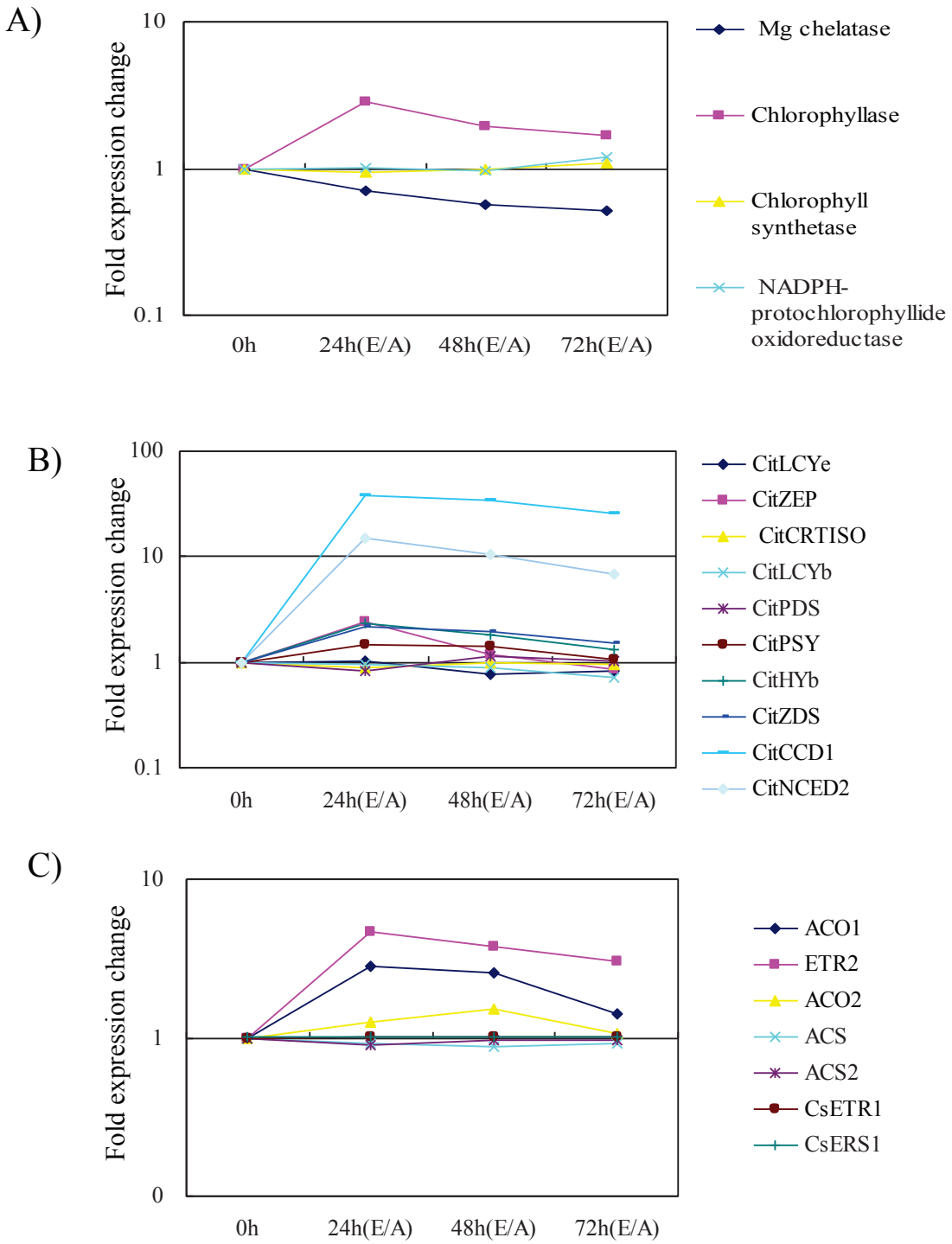


Fig. 2-3. Expression profiles of chlorophyll (A), carotenoid (B) and ethylene (C) related genes during 72 h after ethylene and air treatments. Fold expression change between ethylene treatment and air treatment (ethylene/air signal intensity ratio) was calculated for each gene. Log scale is applied to the X-axis.

Table 2-1 Representative ethylene resopnsive genes with 3-fold expression change between Ethylene and air treatments (Ethylene/Air ratio).

EST code	Annotation	E24h/A24h	E48h/A48h	E72h/A48h	Up/Down
Amino acid synthesis					
CK938622	Amino acid carrier protein	13.45	8.40	8.01	UP
VS28295A	2-oxoisovalerate dehydrogenase	2.64	3.80	1.93	UP
CO912599	Alanine-glyoxylate aminotransferase	3.41	3.45	4.32	UP
ANT2_0344	Branched-chain amino acid aminotransferase 2	4.70	2.70	3.18	UP
MWYAR88A	Cobalamine-independent methionine synthase.	0.49	0.31	0.39	Down
MOADE54R	Coffea arabica methionine synthase	0.43	0.27	0.33	Down
FBI1456C	Glutamate decarboxylase	0.32	0.42	0.41	Down
ANT2_1143	Glutamine synthetase	3.65	3.14	3.04	UP
CK701455	Glycine hydroxymethyltransferase	0.07	0.09	0.12	Down
FBI1086A	L-asparagine amidohydrolase	9.85	13.87	15.25	UP
ANT2_1463	Nitrate transporter (ntp gene)	0.14	0.06	0.09	Down
MFI7HD2D	Serine hydroxymethyltransferase	0.20	0.21	0.24	Down
CN188023	Tryptophan synthase	3.12	3.77	3.39	UP
Cell wall metabolism					
LLL0411	Alpha-glucan phosphorylase	0.28	0.27	0.32	Down
BFC4E30A	Beta-galactosidase	14.15	17.27	13.84	UP
CF509249	Cellulose synthase	0.27	0.24	0.24	Down
MAPF194R	Cellulose synthase catalytic subunit	0.29	0.24	0.26	Down
ANT0028	Endo-xyloglucan transferase	0.06	0.06	0.05	Down
VS28993A	Ethylene-induced esterase	31.46	25.86	26.75	UP
VS28642A	Expansin 1	3.07	1.32	2.05	UP
MAPFF03A	Extensin-like protein	0.03	0.04	0.04	Down
BFC4D19A	Germin-like protein	0.65	0.20	0.31	Down
MOA16892	Pectate lyase	0.04	0.04	0.04	Down
FBI0771A	Pectin methylesterase	0.29	0.21	0.21	Down
CK934694	Pectinacetylesterase	0.42	0.38	0.23	Down
MFI7J67D	Pectinesterase	0.47	0.24	0.20	Down
ANT2_0794	Polygalacturonase	0.29	0.22	0.73	Down
CK939533	UDP-galactose-4-epimerase	12.35	10.09	7.24	UP
MOA16779	Xyloglucan endotransglycosylase	0.17	0.20	0.62	Down
CK936995	Xyloglucosyl transferase	3.24	2.18	1.60	UP
Fatty acid biosynthesis and oxidation					
MFI87D1D	Omega-6 fatty acid desaturase	0.27	0.31	0.31	Down
STG1068	Acyl-CoA synthetase	3.33	1.54	1.52	UP
Lipid degradation					
ANT0310	13-lipoxygenase	0.46	0.35	0.25	Down
FBI1121R	Fatty acid hydroperoxide lyase	0.09	0.10	0.13	Down
CK665268	GDSL-motif lipase	0.25	0.24	0.25	Down
CF507211	Steryl ester lipase-like protein	0.24	0.12	0.18	Down
Photosynthesis and chloroplast biogenesis					
BQ624944	10kd polypeptide of photosystem II	0.33	0.42	0.54	Down
MOA16603	Early light-induced protein-like protein	0.28	0.27	0.41	Down
MOA16819	Geranylgeranyl hydrogenase (Ggh)	0.31	0.35	0.41	Down
FBI1909D	Glyceraldehyde-3-phosphate dehydrogenase	0.45	0.32	0.37	Down
CK934598	NADP-dependent glyceraldehydephosphate dehydrogenase subunit B	0.25	0.25	0.25	Down
CO913035	NADPH oxidase	0.29	0.44	0.39	Down
EGJ_1273	33kDa precursor protein of oxygen-evolving complex	0.32	0.34	0.30	Down
LLL0543	Chloroplast matK	0.33	0.66	0.63	Down
SHA01H03_F1	Chloroplast nucleoid DNA binding protein	0.88	0.30	0.30	Down
FBI2160A	Chloroplast oxygen-evolving enhancer protein	0.13	0.19	0.12	Down
MOA16447	Chloroplast phosphoglycerate kinase	0.34	0.75	0.79	Down
CD576128	Crystallinum phosphoribulokinase	0.06	0.12	0.09	Down
MWYF162R	Gamma subunit of ATP synthase.	0.24	0.23	0.22	Down
FBI1693R	Geranylgeranyl reductase	0.18	0.28	0.29	Down
MWYF573F	Glycolate oxidase	6.19	5.99	5.82	UP
LLL1100	Light inducible tissue-specific ST-LS1	0.32	0.43	0.43	Down

Continued

EST code	Annotation	E24h/A24h	E48h/A48h	E72h/A48h	Up/Down
ANT2_0766	Phosphate transporter	0.10	0.10	0.11	Down
EGJ_0860	Phosphate-responsive protein	0.18	0.20	0.22	Down
BFC2E01R	Phosphoenolpyruvate carboxykinase	0.30	0.34	0.30	Down
EGJ_0741	Phosphoglycolate phosphatase	0.30	0.34	0.34	Down
EGJ_1317	Photosystem I psaH protein.	0.15	0.18	0.17	Down
BFC3A60D	Photosystem I reaction center subunit PSI-N	0.16	0.23	0.26	Down
CK933507	Photosystem I subunit XI	0.08	0.13	0.11	Down
LLL0827	Photosystem II reaction center (PsbW)	0.23	0.19	0.21	Down
BFC3A44A	Phototropic-responsive NPH3 family protein	0.35	0.27	0.21	Down
ANT2_0849	Phytochelatin synthetase	0.14	0.12	0.11	Down
MWYF542A	Plastidic glucose 6-phoaphate	0.12	0.08	0.09	Down
MWYAR05A	Plastocyanin	0.08	0.08	0.10	Down
LLL0930	PSI-K subunit of photosystem I f	0.09	0.09	0.09	Down
LLL1995	Ribulose-1,5-bisphosphate carboxylas	0.08	0.08	0.07	Down
VSSJ011D	Rubisco activase beta form precursor (RCA2)	0.08	0.09	0.10	Down
MOAFA81R	Type I chlorophyll a/b binding protein	0.20	0.14	0.12	Down
CK934974	Type II chlorophyll a/b binding protein	0.08	0.10	0.10	Down
BFC4A24A	Thioredoxin F isoform.	0.38	0.76	0.76	Down
EGJ_1324	Triose phosphate translocator	0.08	0.09	0.08	Down
Plant horomone related					
FBI2162E	Allene oxide cyclase	0.23	0.32	0.37	Down
BQ625110	ABA-responsive protein	0.21	0.33	0.41	Down
ANT2_1369	Aux/IAA protein	0.17	0.23	0.25	Down
MAPDR18A	Auxin-associated protein	0.15	0.45	0.42	Down
FBI1682A	Auxin-regulated IAA8	0.20	0.23	0.34	Down
CF931498	Ethylene receptor (ETR2)	4.62	3.77	3.02	UP
FBI1182R	Ethylene-inducible protein	4.56	3.74	2.57	UP
CF509669	Ethylene-responsive family protein	0.24	0.32	0.47	Down
CF837667	GH3-like protein	5.74	5.57	7.63	UP
CK933029	Gibberellic acid-induced gene Gasa4	0.29	0.37	0.37	Down
ANT2_0636	Ripening-related protein	6.33	5.02	3.11	UP
Protein degradation					
YJS0628	Delta proteasome subunit	5.96	3.63	3.90	UP
ANT2_0868	Fasciclin-like AGP 12	0.37	0.32	0.23	Down
STG1185	Polyubiquitin	3.75	2.16	1.64	UP
BFC4D36S	Adenosylhomocysteinase (AHC2)	4.07	3.64	3.04	UP
CK938754	Aspartic proteinase 5	0.22	0.38	0.46	Down
CK934091	Formate dehydrogenase	6.78	2.88	2.06	UP
MWYB720A	Phytochelatin synthetase family protein	0.14	0.11	0.12	Down
MAPAT76A	Cystein proteinase	1.61	2.61	3.47	UP
MFI6MA5D	Small ubiquitin-like modifier 2	3.30	2.92	2.50	UP
MFI6MA0R_2	Urate oxidase	3.13	4.95	3.60	UP
Protein kinase and other signaling components					
MWYAV31D	Leucine-rich repeat transmembrane protein kinase	3.87	3.23	2.87	UP
CD576318	APS-kinase	3.00	2.14	1.41	UP
MAPF178F	CBL-interacting protein kinase 5 (CIPK5)	0.19	0.18	0.39	Down
FBI0632R	Cyclin-dependent kinases CDKB	0.27	0.22	0.32	Down
ANT2_0895	Cytokinin signal transduction regulator (RR2)	14.97	6.86	8.95	UP
FBI1751A	Leucine-rich repeat transmembrane protein kinase	0.32	0.33	0.31	Down
MOA16936	Protein kinase family protein	3.31	2.33	2.04	UP
MWYBU53F	SOS2-like protein kinase	4.35	3.91	3.38	UP
Resistance, defense, stress and PR					
MAP9C16R	Dehydrin	0.19	0.25	0.27	Down
ANT2_0655	Glutathione S-transferase	0.37	0.29	0.26	Down
CK936454	Peroxidase (POX2)	22.47	9.23	7.17	UP
CN187002	Peroxidase (POX3)	10.62	6.55	4.64	UP
ANT2_1324	Polygalacturonase-inhibitor protein	0.35	0.42	0.29	Down
SHA02H08_F1	Type I proteinase inhibitor-like protein	18.08	47.18	15.07	UP

Continued

EST code	Annotation	E24h/A24h	E48h/A48h	E72h/A48h	Up/Down
STG2_0541	Gamma-thionin protein	0.25	0.34	0.38	Down
BFC2B72A	NADPH-cytochrome P450 oxydoreductase	4.79	5.31	3.60	UP
STG1140	Chitinase III	9.29	13.87	9.06	UP
VSSK008D	Cold stress protein	0.04	0.04	0.03	Down
CD575783	Cytochrome P450	0.21	0.19	0.19	Down
ANT0147	Dehydration-responsive protein-related	0.13	0.15	0.13	Down
ANT0966	Elicitor-inducible cytochrome P450 (CYP92A5)	3.27	2.34	2.20	UP
CD573771	Fiddlehead-like protein (FDH)	0.26	0.34	0.72	Down
CO912812	Gamma-glutamylcysteine synthetase	6.38	3.91	3.47	UP
BQ624413	Heat shock protein 83	2.10	3.33	2.19	UP
BFC2E35A	Hydroxycinnamoyl transferase	6.38	8.26	10.33	UP
CN186287	Metallothionein-like protein (MT45)	17.71	13.87	17.19	UP
MAMBH57A	Miraculin-like protein 3	4.39	7.89	5.49	UP
MOA16155	Nodulin family protein	5.23	4.80	3.80	UP
PCC0717	Osmotin	4.34	5.55	4.76	UP
MAMB485R	Polygalacturonase-inhibiting protein	0.35	0.48	0.31	Down
CF653559	PR1b protein p	32.10	37.54	31.40	UP
LLL1689	PR4-type protein	3.41	2.94	1.23	UP
CO913068	Putative aconitate hydratase	2.90	2.61	2.67	UP
MAM8881A	Stearoyl-ACP desaturase	3.27	3.78	3.08	UP
CN182240	stearoyl-acyl carrier protein desaturase	2.89	2.77	2.28	UP
YJS1644	wound-induced protein.	2.27	8.53	2.32	UP
Secondary metabolism					
MAMBH04A	Tropinone reductases	4.53	5.12	6.05	UP
MWYF940R	Ascorbate oxidase-related protein.	0.17	0.17	0.38	Down
ANT0201	Geranylgeranyl pyrophosphate synthase	4.67	4.02	3.72	UP
LLL0814	Limonoid UDP-glucosyltransferase	2.19	3.47	2.37	UP
MOA15608	3-hydroxy-methylglutaryl coenzyme A reductase	0.28	0.25	0.26	Down
BFC3A26A	9-cis-epoxycarotenoid dioxygenase 1 (NCED1)	33.24	23.00	26.40	UP
CF836703	9-cis-epoxycarotenoid dioxygenase 2 (NCED2)	15.14	10.55	6.86	UP
STG2_1091	Caffeoyl-CoA 3-O-methyltransferase	0.13	0.18	0.22	Down
EGJ_1463	Chalcone isomerase	0.16	0.11	0.19	Down
MOA16374	Chalcone reductase	3.62	1.04	1.03	UP
FBI0692A	Chalcone synthase	0.10	0.09	0.09	Down
CN189470	Cinnamoyl-CoA reductase	2.61	3.33	3.45	UP
VSSH017D	Flavanone 3-hydroxylase	0.36	0.32	0.27	Down
BFC4G87C	Geranylgeranyl pyrophosphate synthase	4.57	3.39	3.43	UP
ANT2_0601	Isoflavone reductase homolog 2 (IFR2)	1.58	1.92	3.81	UP
LLL0283	Mg protoporphyrin IX chelatase (Chl H) mRNA	0.04	0.07	0.07	Down
MOA14689	Oxidoreductase （2OG-FeII)	3.88	3.99	4.11	UP
LLL1313	Phenylalanine ammonia-lyase	3.23	1.78	1.81	UP
MAMB463R	Terpene synthase	2.13	6.39	7.28	UP
CK933805	Transcription factor LIM, putative	0.38	0.37	0.32	Down
STG0952	UDP-glucose-flavonoid-3-O-glucosyl transferase	0.23	0.24	0.21	Down
Sugar metabolism					
EGJ_0068	Carbohydrate oxidase gene	0.21	0.15	0.31	Down
GSA1095	Chloroplast granule-bound starch synthase (GBSSI) gene,	0.35	0.58	0.58	Down
ANT2_1130	(1-4)-beta-mannan endohydrolase, putative	0.36	0.28	0.29	Down
CO912461	1-deoxy-D-xylulose-5-phosphate reductoisomerase	3.24	2.84	2.54	UP
MWYGA88A	Acid invertase	3.67	1.86	1.96	UP
CK939901	ADP-glucose pyrophosphorylase small subunit	0.21	0.26	0.85	Down
CN188922	Aldose 1-epimerase family protein	0.23	0.31	0.26	Down
CF508941	Carbonate dehydratase	0.30	0.31	0.42	Down
FBI1584R	Glucosyltransferase-5	0.12	0.17	0.14	Down
BFC4G65D	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) subunit A	0.04	0.05	0.04	Down
FBI1629A	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) subunit B	0.04	0.04	0.03	Down
BE208888	Glycosyl hydrolase family 9 protein	0.58	0.28	0.51	Down
CK932841	Glycosyl transferase family 8 protein	0.15	0.14	0.11	Down
ANT0194	Gulucose-6-phosphogluconate dehydrogenase（G6PDH）	3.39	2.84	2.29	UP
MOA15223	Hexokinase 2 (Hxk2)	0.30	0.25	0.21	Down

Continued

EST code	Annotation	E24h/A24h	E48h/A48h	E72h/A48h	Up/Down
ANT2_0396	Hexose carrier (Hex9)	26.58	17.62	13.70	UP
CN192432	Putative sugar transporter (st3 gene)	3.40	3.60	2.90	UP
BFC3C07A	Starch synthase	0.11	0.11	0.15	Down
MOA14956	UDP-glucose dehydrogenase	0.28	0.16	0.16	Down
STG2_1368	UDP-xylose synthase	0.27	0.25	0.23	Down
Transcription Factor					
CK934325	Aux22d	0.10	0.09	0.08	down
CF509669	Ethylene-responsive protein	0.25	0.32	0.48	down
STG0694	Homeobox leucine zipper protein	7.85	6.90	6.84	up
ANT0329	Myb family transcription factor	7.29	6.51	5.37	up
BQ623221	Myb family transcription factor	3.12	3.45	3.04	up
CF509156	Myb family transcription factor	0.16	0.16	0.21	down
CF838547	Myb family transcription factor	11.25	9.10	7.80	up
EGJ_0492	Myb family transcription factor	6.54	5.80	3.71	up
MWYB731F	NAC domain protein	3.66	3.84	3.17	up
BFC5E05D	Putative transcription factor	5.68	3.75	3.69	up
CB293768	Putative transcription factor	0.19	0.21	0.19	down
BFC4G38R	Putative transcription factor	0.30	0.27	0.23	down
MOA9P37A	Putative transcription factor	0.29	0.23	0.21	down
CK938765	Putative transcription factor	0.20	0.25	0.25	down
EGJ_0316	Putative transcription factor	0.30	0.39	0.46	down
CK933805	Putative transcription factor	0.39	0.37	0.33	down
ANT2_1578	Putative transcription factor	0.35	0.20	0.21	down
BFC2A96S	Putative transcription factor	0.30	0.35	0.39	down
CK938806	Putative transcription factor	10.12	8.64	6.18	up
CN189405	Putative transcription factor	5.62	5.27	4.96	up
MWYBO18A	Putative transcription factor	0.24	0.16	0.21	down
STG1783	Putative transcription factor	6.16	7.10	4.58	up
CN190833	WRKY family transcription factor	6.26	5.40	4.10	up
MAPEM69E	WRKY family transcription factor	0.27	0.37	0.34	down

Table 2-2.
Gene ontology annotations for *Arabidopsis* (GO SLIM) functional assignments for ethylene responsive 1493 genes with more than 3-fold expression changes.

Go Term	Total (%)	No. of down-regulated genes	No. of up-regulated genes
Molecular function			
DNA or RNA binding	27 (1.8%)	21	6
Hydrolase activity	97 (6.5%)	60	37
Kinase activity	25 (1.7%)	16	9
Nucleic acid binding	2 (0.1%)	1	1
Nucleotide binding	26 (1.7%)	9	17
Protein binding	40 (2.7%)	23	17
Receptor binding or activity	5 (0.3%)	3	2
Structural molecule activity	5 (0.3%)	4	1
Transcription factor activity	53 (3.5%)	37	16
Transferase activity	99 (6.6%)	60	39
Transporter activity	41 (2.7%)	24	17
Other binding	116 (7.8%)	80	36
Other enzyme activity	176 (11.8%)	91	85
Other molecular functions	49 (3.3%)	32	17
Molecular function unknown	157 (10.5%)	112	45
No similarity to *Arabidopsis* cDNA	554 (37.1%)		
Biological process			
Cell organization and biogenesis	25 (1.7%)	19	6
Developmental processes	37 (2.5%)	19	18
DNA or RNA metabolism	2 (0.1%)	2	0
Electron transport or energy pathways	78 (5.2%)	47	31
Protein metabolism	56 (3.8%)	43	13
Response to abiotic or biotic stimulus	89 (6.0%)	56	33
Response to stress	88 (5.9%)	50	38
Signal transduction	21 (1.4%)	14	7
Transcription	37 (2.5%)	24	13
Transport	130 (8.7%)	81	49
Other biological processes	160 (10.7%)	95	65
Other cellular processes	274 (18.4%)	160	114
Other metabolic processes	334 (22.4%)	191	143
Other physiological processes	297 (19.9%)	178	119
Biological process unknown	185 (12.4%)	116	69
No similality to *Arabidopsis* cDNA	554 (37.1%)		
Cellar component			
Cell wall	18 (1.2%)	12	6
Chloroplast	119 (8.0%)	83	36
Cytosol	19 (1.3%)	9	10
ER	9 (0.6%)	7	2
Extracellular	11 (0.7%)	7	4
Golgi apparatus	1 (0.1%)	1	0
Mitochondria	58 (3.9%)	31	27
Nucleus	56 (3.8%)	33	23
Plasma membrane	9 (0.6%)	7	2
Plastid	55 (3.7%)	49	6
Ribosome	3 (0.2%)	2	1
Other cellular components	112 (7.5%)	91	21
Other cytoplasmic components	101 (6.8%)	76	25
Other intracellular components	90 (6.0%)	76	14
Other membranes	269 (18.0%)	190	79
Cellular component unknown	231 (15.5%)	128	103
No similarity to *Arabidopsis* cDNA	554 (37.1%)		

Table 2-3. Chlorophyll and carotenoid contents in the examined fruit peels.

Pigment	Control 0h	Air 72h	Ethylene[a] 72h
Total carotenoids ($mg^{-1}FW$)	58.0 ±1.5	220.4 ± 9.8	234.8 ± 13.3
All trans-Violaxanthin	11.98 ± 3.6	52.3 ± 4.5	35.3 ± 5.8
9-cis-Violaxanthin	11.0 ± 2.3	49.7 ± 6.8	33.7 ± 8.5
Lutein	16.8 ± 2.4	78.62 ± 8.2	92.4 ± 5.6
β -cryptoxanthin	8.31 ± 2.4	27.1 ± 3.9	52.52 ± 3.6
α -carotene	1.4 ± 0.2	0.7 ± 0.1	1.9 ± 0.3
Phytoene	8.93 ± 1.4	11.6 ± 2.1	18.9 ± 2.3
Total chlorophylls ($mg^{-1}FW$)	12.5 ± 1.6	11.2 ± 2.1	4.2 ± 0.8
Chlorophyll *a*	9.5 ± 0.7	8.4 ± 0.6	1.7 ± 0.3
Chlorophyll *b*	3.0 ± 0.6	2.8 ± 0.4	2.6 ± 0.4
Chlorophyll *a*/*b* ratio	3.2	3.0	0.6

[a]Treatment: see text.

Section 2: Profiling gibberellin (GA_3)-responsive genes in mature fruit using a citrus 22K oligoarray

In Section 1,1493 ethylene-responsive genes were identified and found that ethylene repressed the transcription of most genes involved in photosynthesis and chloroplast biogenesis, while it induced the transcription of several genes related to resistance, defense, stress, amino acid synthesis, protein degradation, and secondary metabolism. Therefore, transcriptional profiling using microarray technology is expected to provide new insight into the GA regulatory mechanism of citrus fruit. In this experiment, GA_3-responsive genes in mature citrus fruit were investigated using a citrus 22K oligoarray. 231 genes were identified as GA_3-responsive genes; genes that showed an expression change of 3-fold or greater in the 72 h after GA_3 treatment, compared to expression after air treatment. It was found that GA_3 up-regulated the expression of genes related to photosynthesis and of pathogen-related genes and repressed the expression of some of the ethylene-inducable genes that are involved in fruit ripening.

Materials and methods

Plant material and gibberellin treatment

Satsuma mandarin (*C. unshiu* Marc.), cultivated at the Citrus Research Division Okitsu of NIFTS, was used. Samples of fruit at 150 DAF were collected. For the gibberellin treatment of fruit, 60 μM GA_3 was sprayed on fruits. Both GA_3 treatment and air treatment were conducted at 25°C. The flavedo tissue was excised and immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction.

Chlorophyll and carotenoid quantification in flavedo

Chlorophyll (*a* + *b*) content was determined by measuring the absorbance at 642 nm and 662 nm according to the method of Shimada and Shimokawa et al. (1978). Quantification of 6 representative carotenoids (*trans*-Vio, *cis*-Vio, Lut, B-Cry, A-Car, and Phy) was carried out by the method of Kato et al. (2004). An aliquot (20 μL) was separated by a reverse-phase HPLC system (Jasco) fitted with a YMC Carotenoid S-5 column of 250- x 4.6-mm-i.d. (Waters) at a flow rate of 1 mL min^{-1}. The eluent was monitored using a photodiode array detector (MD-910, Jasco). Chlorophyll and Carotenoid quantification was performed in three times.

RNA isolation and microarray analysis

Total RNA was extracted by the methods of Ikoma et al. (1996) from flavedo tissues of untreated fruit at 0 h and from either GA_3-treated or air-treated fruit at 24 h, 48 h, and 72 h after treatment. At least three independent RNA extractions were used in probe labeling for experimental reproducibility. The total RNA (400 ng) of all samples was labeled with Cy5, while non-treatment at 0 h was labeled with Cy3 according to the instructions for the Low RNA input linear amplification and labeling kit (Agilent technologies). Labeled cRNA was purified using the Qiagen RNeasy mini kit (Qiagen). Hybridization and washing were performed according to Section 1. The intensities of the Cy5 and Cy3 fluorescent signals from each spot were automatically normalized, and the ratio value (Cy5/Cy3) was calculated using Feature Extraction version 7.1 software (Linear & LOWESS analysis, Agilent technologies). Data analysis was carried out using GENESPRING 7.3.1 (Silicon Genetics). The fold change of each gene expression was calculated based on the mRNA ratio between GA_3 treatment samples and air treatment samples at equivalent time points. Genes with a 3-fold or greater expression

change between GA_3 treatment and air treatment at each experimental time (24 h, 48 h, and 72 h) were accepted as GA_3-responsive genes.

Northern blot analysis

Ten microgram from each RNA sample was subjected to electrophoresis on a 1.2% agarose gel containing 8% (v/v) formaldehyde and transferred to a nylon membrane (Hybond-NX, Amersham Pharmacia Biotech). The cDNA probes of 6 representative GA_3-responsive genes identified by microarray analysis were prepared with the use of a PCR DIG labeling kit (Roche Molecular Biochemicals). Hybridization and detection were conducted according to the manufacturer's directions (Roche Molecular Biochemicals).

Results and discussion

Identification and functional classification of 231 GA_3-responsive genes

A citrus 22K oligoarray was employed to identify GA_3-responsive genes in mature fruit. Out of 21,495 independent EST probes, 231 genes showed a 3-fold or greater change in the ratio of mRNA levels 72 h after GA_3 treatment compared to mRNA levels after 72 h of air treatment. To monitor the results of microarray analysis, the signal intensity of several representative genes was compared between Northern blot and microarray analysis. The fidelity of the experiments was confirmed (Fig. 2-4). The 231 GA_3-responsive genes were compared by TBLAST X similarity search (e-value <1e-5) against all cDNAs of *Arabidopsis* (downloaded from TAIR. Since each *Arabidopsis* cDNA entry in TAIR provided functional information (GOSLIM in TAIR), the Satsuma mandarin genes were assigned functions according to GOSLIM on the basis of their similarity to cDNAs of *Arabidopsis* (Table 2-4). GA_3 treatment affected genes that had

been assigned to the following functional categories: ‘other enzyme activity’ (15.2%), ‘hydorase activity’ (12.1 %) (in the molecular function categories) and ‘other metabolic processes’ (30.7 %), ‘other physiological processes’ (28.6 %), ‘other cellular processes’ (28.6 %) (in the biological processes categories). In the cellular components catagories, ‘other membranes’ (25.1 %) and ‘chloroplast’ (9.5 %) were affected by GA_3 treatment. Ethylene treatment had the effect of down-regulation on similar categories as shown in Section 1. GA_3 treatment, however, in this fruit stage, had the predominant effect of up-regulating genes within these categories. 79 genes showed this contrasting response between ethylene and GA_3 treatments. Only 27 genes were functionally annotated and, of these, most genes were related to ‘secondary metabolism’, ‘photosynthesis and chloroplast biogenesis’, and ‘resistance, defense, stress and PR’ (Table 2-4). GA_3 treatment increased the expression of genes related to ‘photosynthesis and chloroplast biogenesis’, including 6.1 kDa polypeptide of photosystem II, CAB type I, chloroplast sedoheptulose-1,7-bisphosphatase (Table 2-5), all of which are down-regulated by ethylene treatment. The effect of GA on photosynthesis is controversial because contradictory results have been obtained from different plants, such that GA increased or decreased photosynthetic capacity and photosynthetic rate (Dijkstra et al, 1990; Yuan and Xu, 2001; Ashraf et al., 2002). These results indicate that GA_3 has a positive effect on photosynthesis in mature citrus fruit peel.

Clustering analysis of 213 GA_3-responsive genes

To visualize GA_3-responsive expression patterns 72 h after GA_3 treatment, the 231 genes were subjected to cluster analysis and divided into 2 major clusters (Fig. 2-5). Drastic transcriptional changes of these genes were seen following GA_3 treatment compared to that seen following air treatment. Cluster 1 consisted of 95 genes that were

down-regulated after GA_3 treatment, listed in Table 2-4. GA_3 treatment repressed some of the genes that had been ethylene-induced in Section 1. For example, NCED1 is one of the cleave reaction enzymes converting epoxycarotenoids into xanthoxin, which is the main regulatory step in ABA biosynthesis in citrus (Rodrigo et al., 2006; Kato et al., 2006). *NCED1* was one of the highly inducible genes in mature fruit by ethylene treatment. GA_3 treatment down regulated the mRNA levels of this gene. This would result in the repression of the metabolic conversion of carotenoids to ABA. In *Arabidopsis* seed germination, GA reduced ABA levels by affecting ABA biosynthesis (Ogawa et al., 2003).

Cysteine proteases have been implicated in the ubiquitin-mediated protein degradation pathway and might be associated with the initiation of the fruit senescent process (Cercós et al., 2006). P450, (CF507320), which was down-regulated by GA_3, had high homology to brassinosteroids-6-oxidase of grape, which was a key gene in brassinosteroid (BR) biosynthesis and mediates the conversion of 6-deoxocastasterone to castasterone in grape (Symons et al., 2006). They considered that BR level was associated with ripening in grapes, which is a non-climacteric fruit, as is citrus. Citrus invertase 1 (*CitINV1*) is associated with the brake-down of sucrose to hexoses, regulates sucrose concentration during fruit ripening and regulates sucrose synthase and acid invertases (Holland et al., 1999; Kubo et al., 2001). In tomato fruit (Jeffery et al, 1984) and in citrus fruit, ethylene treatment enhanced enzyme activity and gene expression of invertase. GA_3 reduced the transcription of these ethylene-inducable genes, which are associated with ripening in mature citrus fruit.

Cluster 2 contained 136 genes up-regulated by GA_3 treatment, listed in Table 2-5. Several genes involved in resistance, defense and stress, or cell wall modification were either up- or down-regulated by GA_3 treatment. Some cell wall modification genes are

also induced by pathogen attack (Maleck et al., 2000; Mozoruk et al., 2006). Some genes showed similar patterns of response to ethylene treatment, however, the opposite response was also observed. Chitinase is a well-known antifungal protein and belongs to the pathogenesis-related (PR) group of proteins, and its gene expression was markedly induced by elicitor treatment in flavedo (Porat et al., 2001). GA_3 treatment induced chitinase expression whereas ethylene did not induce chitinase expression. A similar result was obtained in tomato; chitinase expression was induced by MeJA, GA and wounding signal, but not by ethylene and ABA (Wu and Bradford, 2003). GA up-regulated several citrus flavor related genes such as *(E)-*β*-*ocimene synthase, gamma-terpinene synthase and HMG-CoA synthase. Monoterpenes play ecological roles in pollinator attraction, allelopathy, and plant defense. Several monoterpenes and sesquiterpenes were reported to take part in direct plant defense (Langenheim, 1994). In addition, citrus miraculin-like protein was reported to have protease inhibitor activities and defensive function against pathogen (Tsukada et al., 2006). Various WRKY-DNA binding proteins, belonging to a large group of zinc-finger proteins, are implicated primarily in defense responses but are also implicated in plant development (Eulgem et al., 2000). Thus, it was considered that GA_3 treatment, directly or indirectly, might induce the transcription of these genes related to resistance, defense and stress. Generally, plant defense responses are regulated through a complex signaling network with cross talk between SA, JA, and ethylene-signaling pathways. Some pathways might be activated positively or negatively through this cross talk. Therefore, these results indicate that the GA response pathway takes part in cross talk with the pathogen-related pathways in mature citrus fruit.

Profiling GA_3 regulation of chlorophyll, carotenoids and ethylene biosynthesis

It is well known that ethylene promotes chlorophyll degradation and carotenoid biosynthesis and that GA represses these color changes (Goldschmidt et al., 1993). In this experiment, chlorophyll contents and 6 representative carotenoids were investigated in flavedo tissues at 0 h and 72 h after treatments (air or GA_3) (Table 2-6). No significant difference was seen in either chlorophyll content or in Chlorophyll a/b ratios between fruits at equivalent time points. Total carotenoid content increased from 105.9 μg·g-1 to 217.0 μg·g-1 (air treatment) and 209.1 μg·g-1 (GA_3 treatment), 72 h after treatment, possibly due to moderate temperature (Wheaton and Stewart, 1973). No significant difference was not observed between carotenoid composition of GA_3 and air treated fruits. Similar results were obtained in orange, where GA_3 did not have a significant effect on total carotenoid content and prevented most of the ethylene-induced carotenoid changes (Rodrigo and Zacarias, 2007).

Citrus 22K oligoarray enabled the profiling of 4 chlorophyll metabolic genes and 10 carotenoid metabolic genes. Concerning chlorophyll metabolism, GA_3 treatment only affected magnesium chelatase and it up-regulated its transcription (Fig. 2-6A). The expression levels of chlorophyll synthase, NADPH-protochlorophyllide oxidoreductase, and chlorophyllase did not significantly change between GA_3 and air treated fruits. Magnesium chelatase is the first unique enzyme of the chlorophyll biosynthetic pathway and mediates the insertion of Mg^{2+} into protoporphyrin IX. Ethylene treatment repressed gene expression of magnesium chelatase and enhanced chlorophyllase gene expression. Of the genes examined that relate to chlorophyll biosynthesis, GA_3 affected only magnesium chelatase but induced an opposite effect to ethylene. This result agreed with the hypothesis of Jacob-Wilk et al. (1999), that chlorophyll levels are determined by the balance between synthesis and breakdown. In carotenoid metabolism, GA_3 treatment down-regulates almost all biosynthesis genes (Fig. 2-6B). Particularly, it

highly repressed the gene expression of *CitCCD1*and *CitNCED2*, *CitPSY* and *CitHYb*. In orange, it was reported that GA reduced the ethylene-induced expression of early carotenoid biosynthesis genes and the accumulation of Phy, phytofluence and β -citraurin (Rodrigo and Zacarias, 2007). In Satsuma mandarin, the transcriptional accumulation of carotenoid cleavage dioxygenases was higher compared to orange (Kato et al., 2006) and their mRNA accumulation results in a natural carotenoid component (high content of B-Cry) during fruit ripening. Ethylene treatment enhanced mRNA accumulation of these carotenoid cleavage dioxygenases as well as accumulation of *CitPSY and CitHYb*. Thus, GA_3 treatment appeared to have a contrasting effect to ethylene, as it repressed the transcription of carotanoid biosynthesis genes. Our results are in accord with the hypothesis that GA levels are important in the formation of peel coloration (Iglesias et al., 2001). GA_3 treatment did not cause any significant expression changes in ethylene biosynthesis genes and signal transduction components, except for *ACO1*. There is possibly cross talk between plant hormone and pathogen-resposne pathways, with regard to the transcriptional regulation of *ACO1*, because *ACO1* expression also responded to ethylene treatment.

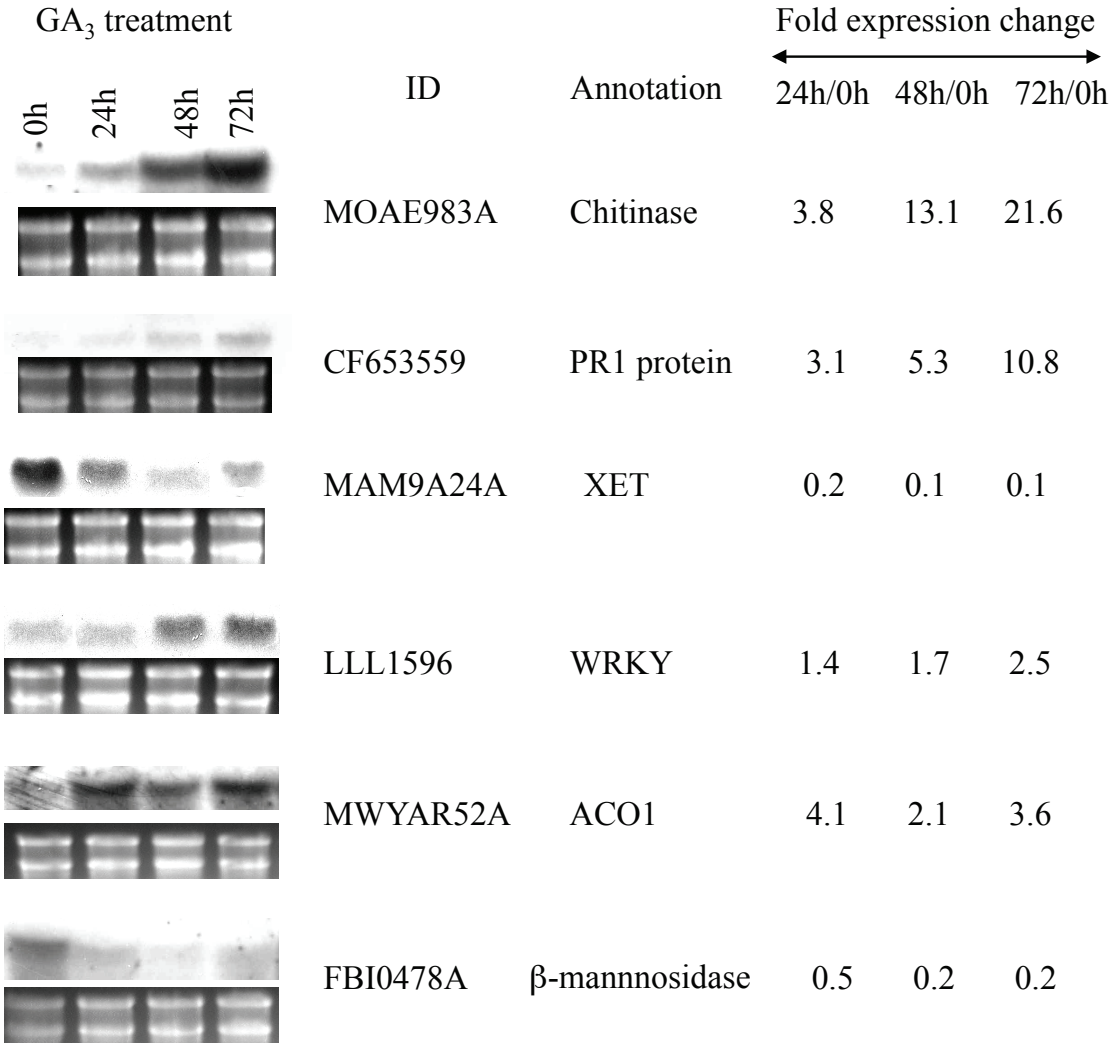


Fig. 2-4. Northern blot analysis of 6 representative GA_3-responsive genes identified by microarray analysis. Ten μg of total RNA from GA_3 treated flavedo was loaded in each lane (24 h, 48 h and 72 h after GA_3 treatment). To the right of each blot is the EST ID, EST annotation, the ratio of fold expression change between GA_3 treatment (24 h, 48 h and 72 h) and air treatments (0 h).

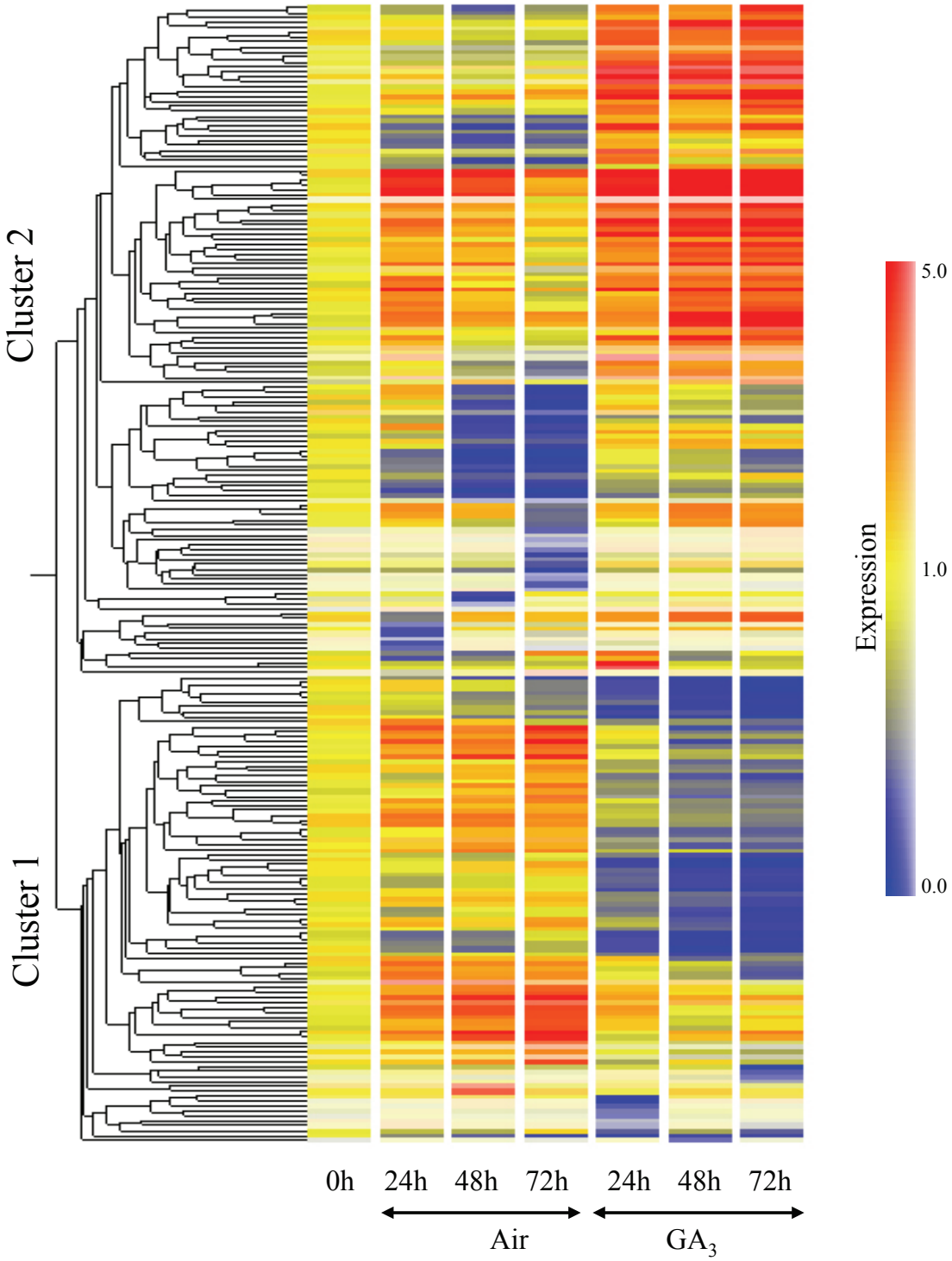
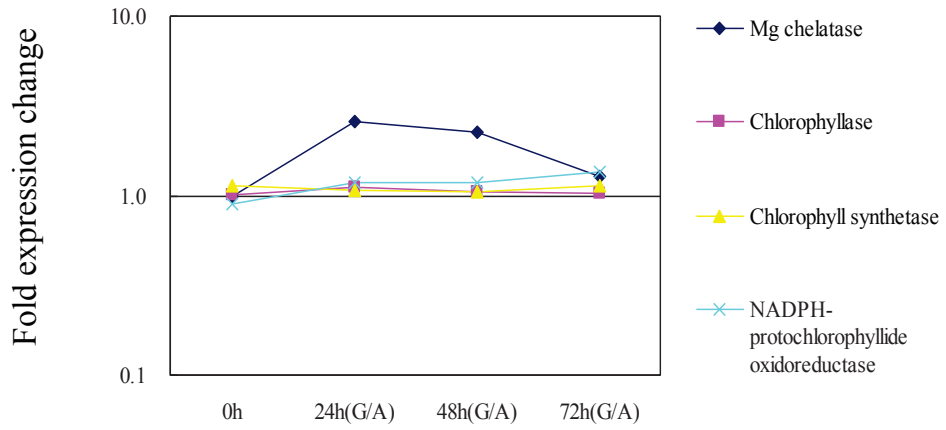


Fig. 2-5. Hierarchical clustering of 213, GA_3-responsive, genes that showed a 3-fold or greater expression change between GA_3 and air treatments (GA_3 / air signal intensity ratio). Two major clusters showed distinctive expression profiles either up- or down-regulated by GA_3 treatment. The color scale indicates a signal intensity of each gene. Tree at the left side of the matrix represents gene relationship.

A)



B)

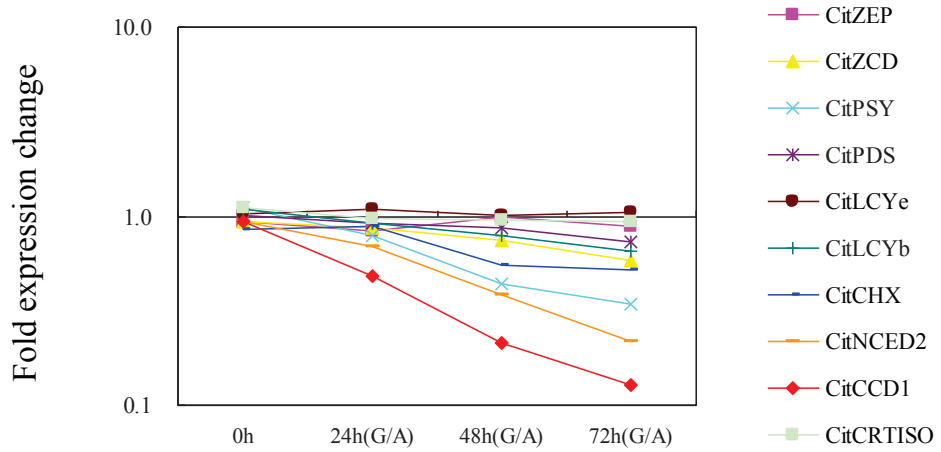


Fig. 2-6. Expression profiles of chlorophyll (A) and carotenoid (B) related genes 72 h after GA_3 or air treatments. Fold expression change between GA_3 and air treatments (GA_3 / air intensity ratio) was calculated for each gene. Log scale is applied to the X-axis.

Table 2-4.
Gene Ontology annotations for *Arabidopsis* (GO Slim) functional assignments for GA_3-responsive 231 genes with 3-fold expression changes.

Go Slim Term	Total (%)	No. of down-regulated genes	No. of up-regulated genes
Molecular function			
DNA or RNA binding	6 (2.6%)	3	3
Hydrolase activity	28 (12.1%)	15	13
Kinase activity	2 (0.9%)	1	1
Nucleic acid binding	0 (0.0%)	0	0
Nucleotide binding	5 (2.2%)	4	1
Protein binding	12 (5.2%)	8	4
Receptor binding or activity	0 (0.0%)	0	0
Structural molecule activity	2 (0.9%)	0	2
Transcription factor activity	7 (3.0%)	3	4
Transferase activity	22 (9.5%)	5	17
Transporter activity	7 (3.0%)	5	2
Other binding	24 (10.4%)	8	16
Other enzyme activity	35 (15.2%)	12	23
Other molecular functions	14 (6.1%)	2	12
Molecular function unknown	33 (14.3%)	13	20
No similarity to *Arabidopsis* cDNA	54 (23.4%)	26	28
Biological process			
Cell organization and biogenesis	7 (3.0%)	2	5
Developmental processes	4 (1.7%)	3	1
DNA or RNA metabolism	0 (0.0%)	0	0
Electron transport or energy pathways	1 (0.4%)	1	
Protein metabolism	9 (3.9%)	5	4
Response to abiotic or biotic stimulus	18 (7.8%)	8	10
Response to stress	23 (10.0%)	6	17
Signal transduction	10 (4.3%)	6	4
Transcription	8 (3.5%)	3	5
Transport	8 (3.5%)	4	4
Other biological processes	37 (16.0%)	16	21
Other cellular processes	66 (28.6%)	23	43
Other metabolic processes	71 (30.7%)	27	44
Other physiological processes	66 (28.6%)	24	42
Biological process unknown	43 (18.6%)	17	26
No similality to *Arabidopsis* cDNA	54 (23.4%)	26	28
Cellar component			
Cell wall	10 (4.3%)	3	7
Chloroplast	22 (9.5%)	10	12
Cytosol	8 (3.5%)	0	8
ER	0 (0.0%)	0	0
Extracellular	6 (2.6%)	2	4
Golgi apparatus	0 (0.0%)	0	0
Mitochondria	15 (6.5%)	3	12
Nucleus	14 (6.1%)	3	11
Plasma membrane	2 (0.9%)	0	2
Plastid	8 (3.5%)	4	4
Ribosome	0 (0.0%)	0	0
Other cellular components	15 (6.5%)	7	8
Other cytoplasmic components	20 (8.7%)	7	13
Other intracellular components	12 (5.2%)	7	5
Other membranes	58 (25.1%)	25	33
Cellular component unknown	39 (16.9%)	13	26
No similarity to *Arabidopsis* cDNA	54 (23.4%)	26	28

Table 2-5. Representative GA_3-resopnsive genes with 3-fold or greater expression change between Ethylene and air treatments (GA_3/Air ratio).

EST code	Annotation	$GA_3$24h/C24h	$GA_3$48h/C48h	$GA_3$72h/C72h	Up/Down
Amino acid synthesis					
MOAHE09R	Phenylalanine-ammonia lyase	1.5	2.5	3.1	Up
CD575911	Aspartyl aminopeptidase	0.6	0.3	0.3	Down
CF417508	Phenylalanine ammonialyase 1	0.6	0.4	0.3	Down
CN190923	Tyrosine aminotransferase	0.6	0.4	0.3	Down
FBI1086A	L-asparagine amidohydrolase	3.7	2.6	2.6	Up
MOACM40A	S-adenosylmethionine synthetase	1.0	3.0	4.1	Up
MOA15207	Tryptophan synthase, alpha subunit	1.0	1.3	3.3	Up
Cell wall metabolism and fatty acid biosynthesis					
CK665263	Cellulase	0.4	0.5	0.3	Down
MWYFM39D	Pectate lyase	0.4	0.4	0.3	Down
MAM9A24A	Xyloglucan endotransglycosylase XET2	0.3	0.2	0.1	Down
BFC3A85A	Lipoxygenase	0.9	3.4	4.3	Up
BQ623531	Germin-like protein 3	0.8	1.5	3.8	Up
CF509179	Germin-like protein 6	0.8	1.7	4.4	Up
FBI1121R	Fatty acid hydroperoxide lyase (HPL)	1.2	3.2	3.7	Up
Photosynthesis and chloroplast biogenesis					
SLG1643	6.1 kDa polypeptide of photosystem II	1.2	1.0	3.2	Up
MOAFA81R	Chlorophyll a/b binding protein type I	1.6	3.1	2.8	Up
CO913035	NADPH oxidase	1.2	1.5	3.0	Up
EGJ_0860	Phosphate-responsive protein	3.0	2.4	1.3	Up
FBI1693R	Geranylgeranyl reductase	1.3	2.2	3.0	Up
FBI1909D	Glyceraldehyde-3-phosphate dehydrogenase	1.0	1.8	3.1	Up
FBI2160A	Chloroplast oxygen-evolving enhancer protein	3.1	2.3	3.1	Up
LLL0930	PSI-K subunit of photosystem I	1.2	2.4	3.0	Up
MWYAR05A	Plastocyanin	1.3	1.9	3.1	Up
MWYF542A	Plastidic glucose 6-phoaphate	1.0	2.2	3.1	Up
Plant hormone related					
MWYAR52A	ACC oxidase	12.0	11.1	13.1	Up
VS28993A	Ethylene-induced esterase	4.3	4.0	3.1	Up
LLL0654	Salicylic acid carboxyl methyltransferase	5.8	6.1	5.0	Up
STG2_0165	Ethylene response factor 5 (ERF5)	0.6	0.4	0.3	Down
Protein kinase and degradation					
MAPAT76A	Cystein proteinase	0.6	0.3	0.3	Down
STG1600	Miraculin-like protein 3	1.1	2.3	8.7	Up
SHA02H08_F1	Type I proteinase inhibitor-like protein	8.6	3.3	15.0	Up
CK935793	Ser/Thr protein kinase	10.2	1.1	1.1	Up
Resistance, defense, stress and PR					
CF507320	Cytochrome P450 enzyme	0.7	0.5	0.3	Down
RGP0454	17.6 kD class I small heat shock protein	0.5	0.9	0.3	Down
MAM8881A	Stearoyl-ACP desaturase	0.5	0.4	0.2	Down
FBI2074F	Acidic chitinase 1	1.0	1.7	3.0	Up
MOAE983A	Acidic chitinase 2	1.2	4.0	16.4	Up
MAPAT10R	Cold stress protein	1.1	1.8	4.5	Up
CD575783	Cytochrome P450	3.2	3.2	3.3	Up
STG2_0974	Elicitor-inducible cytochrome P450	3.1	2.0	1.8	Up
FBI1167F	Peroxidase 1	4.4	5.0	5.0	Up
LLL1596	Peroxidase 2	4.4	5.4	4.7	Up
CF653559	PR1b protein.	2.9	5.9	10.5	Up
Secondary metabolism					
BFC3A26A	9-cis-epoxycarotenoid dioxygenase 1 (NCED1)	0.5	0.2	0.1	Down
MAMB463R	Monoerpene synthase	0.5	0.5	0.2	Down
BFC4F50A	(E)-b-ocimene synthase	1.9	6.4	4.4	Up
MWYB722A	Ascorbate oxidase	2.2	7.8	8.1	Up
CK934829	Caffeate O-methyltransferase	1.0	2.8	4.9	Up
EGJ_1059	g-terpinene synthase	0.8	2.3	3.2	Up
FBI0917R	HMG-CoA synthase 2	1.3	3.4	3.2	Up
Sugar metabolism					
STG2_0179	Acid invertase (CitINV1)	0.6	0.3	0.4	Down
EGJ_0068	Carbohydrate oxidase	1.7	3.2	4.0	Up
STG2_0661	Sorbitol transporter	3.3	2.1	2.0	Up
Transcription factor					
CK935601	Flowering time (FT)	0.7	0.3	0.4	Down
MOA16528	DNA-binding protein	1.1	1.7	3.6	Up
LLL0373	WRKY family transcription factor	1.6	2.6	3.3	Up

Table 2-6. Chlorophyll and carotenoid contents in the examined fruit peels.

Pigment	Control	Air	Gibberellin[a]
	0h	72h	72h
Total carotenoids (mg^{-1} FW)	105.92 ±10.3	217.0 ± 8.7	209.1 ± 11.3
All trans-Violaxanthin	22.87 ± 3.6	54.5 ±5.2	50.3 ± 7.8
9-cis-Violaxanthin	22.3 ± 3.5	46.3 ± 6.8	54.7 ± 6.8
Lutein	34.2± 3.2	77.5 ± 7.9	73.7 ± 9.3
β -cryptoxanthin	16.82 ± 4.5	26.5 ± 3.4	17.9 ± 2.3
α -carotene	1.8± 0.3	0.7 ± 0.1	1.1 ± 0.3
Phytoene	7.93 ± 1.5	11.5± 2.0	11.4 ± 2.1
Total chlorophylls (mg^{-1} FW)	12.5 ± 1.6	11.2 ± 2.1	13.5 ± 0.4
Chlorophyll *a*	9.5 ± 0.7	8.4 ± 0.6	10.0± 0.4
Chlorophyll *b*	3.0 ± 0.6	2.8 ± 0.4	3.5 ± 0.3
Chlorophyll *a*/*b* ratio	3.2	3.0	2.8

[a]Treatment: see text.

Section 3: Conclusion

We have identified 1,493 ethylene-responsive genes and 213 GA_3-responsive genes with more than 3-fold expression change in the ratio of mRNA levels after ethylene and GA_3, respectively using a citrus 22K oligoarray. Although the level of a specific gene transcript does not necessarily mean a corresponding alteration at the protein level, the obtained results provide a new insight into the role of ethylene in the chlorophyll and carotenoid metabolism and the ethylene signal transduction in citrus fruit. GA_3 oppositely regulated these gene transcriptions, which were either induced or repressed by ethylene. Considering that citrus fruit produce tiny amounts of ethylene, the endogenous level of GA_3 might be important for the endogenous regulation of maturation and senescence in mature citrus fruit. In addition, it was found that the GA response pathway was likely to take part in cross talk with the pathogen-related pathway in mature citrus fruit.

More than half of the identified genes are functionally unknown but may also play significant roles. The identification and determination of the biological function of these unknown genes will contribute to an understanding of the unique ethylene and GA_3 biology in citrus fruits. Further experiments will be required to understand their function. Meanwhile, it will be necessary to advance the bioinformatics study. Specifically, there is a possibility that the information of the gene expression is obtained from EST database and the database of high throughput gene expression data.

Chapter 3: An algorithm and computer program for the identification of minimal sets of discriminating DNA markers for efficient cultivar identification

Development of a new algorithm and software for data mining is one of aspect of bioinformatics study to obtain newly biological knowledge from data by the experiment.

Fruit tree varieties such as citrus, apple, sweet cherry, peach, Japanese pear andchestnut are frequently bred in Japan, and more than 1,100 fruit tree varieties are listed in the Japanese Ministry of Agriculture, Forestry and Fisheries' most recent catalog of fruit tree varieties (http://www.hinsyu.maff.go.jp/tokei/contents/9_2011kaju.pdf). In recent years, protection of breeders' rights for these varieties has become of central importance to the fruit tree cultivation industry; at least one infringement case related to a Japanese fruit tree-breeder's rights to a specific sweet cherry variety has already made its way through the courts (Tahira, 2008). Also of key interest to this industry are concerns about the origins of specific food products resulting from fruit-tree cultivation (e.g. orange juice). Inadequate identification of specific varieties can hinder governmental food-inspection and -labeling efforts.

Thus, we suggest that accurate identification of fruit tree varieties at a genetic level is necessary for both the protection of breeders' rights and the improved management of food-inspection mandates. Various types of DNA markers, including restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR), have previously been studied in fruit trees (Wunsch and Hormaza 2002). Recently, SSRs have become the markers of choice in mapping plant genomes because of this technique's co-dominant inheritance, large number of alleles and suitability for automation (Yamamoto et al., 2003). SSR markers have been used for variety

identification of peach (Aranzana et al., 2003), grapevine (This et al., 2004), apple (Galli et al., 2005), almond (Dangle et al., 2009), olive (Ercisli et al., 2011) and Japanese pear (Yamamoto et al., 2002) trees, among other fruit tree varieties; this includes several Japanese fruit tree varieties with documented breeders' rights (e.g. sweet cherry (Takashina et al., 2009), Japanese pear (Terakami et al., 2010), peach (Yamamoto et al., 2003), apple (Moriya et al., 2011) and Japanese chestnut (Yamamoto et al., 2008). Such genotypic analysis typically results in the creation of computer-generated variety/marker "summary" tables that list all possible markers for the studied fruit tree varieties.

In many cases where DNA markers are used, it is not necessary to use all of the markers listed in such summary tables to differentiate between specific fruit tree varieties. Thus, we determined that the development of a minimal marker set—a marker set that can differentiate between all fruit tree varieties shown in a particular summary table and that is as small as possible—could be expected to simplify and streamline the marker-identification process. Not only would this aid in determining the genetic background of specific varieties to help enforce breeders' rights, but it would also permit easier inspection of large-quantity fruit imports due to the minimal marker set's concomitant reduction in the number of markers required for accurate inspection. However, as the number of markers or varieties increases in a summary table, it becomes increasingly difficult to accurately identify a minimal marker set without a concrete methodology and an appropriate computer program capable of doing so.

Several previous studies have attempted to identify minimal marker sets. The Minimum SNPs computer program (Robertson et al., 2004) has identified highly informative sets of single-nucleotide polymorphisms (SNPs) in entire multi-locus sequence typing (MLST) databases for bacteria (Aanensen and Spratt, 2005). To choose

optimum marker sets for grapevines, RAPD primers were evaluated using the discrimination power parameter (*D*), as defined from polymorphic information, and attempts were made to identify the most efficient RAPD primer set for differentiating between 224 grape varieties (Tessier et al., 1999). In Gerber et al., (2003), 20 SSR markers were used for 4,370 accessions of grapevine germplasm, with these SSR markers evaluated by the probability of identity (PI) as computed using the Famoz software package (Gerber et al., 2003). As a result, a minimal set of nine SSR markers is now routinely used in the laboratory for identification purposes and for checking the homogeneity of the accessions (Laucou et al., 2011). However, the intent of these methods for identifying a minimal marker set was to evaluate the identification capability of each individual marker statistically by some index, and to select only those with the highest index value. That is, these methods did not consider the combination of markers in the first place and thus were unable to provide a solution for accurately obtaining minimal marker set(s) because the studied subjects were often a wide group of varieties and genetic resources used for the characterization of germplasms.

Another previous study reported an algorithm and its related GGDS software program, which was based on an integer-linear programming (ILP) formula (Gale et al., 2005). This particular program was capable of accurately identifying minimal marker sets for a given summary table for wheat, but the program is not available at present; in addition, it was designed exclusively to analyze binary data for dominant markers and thus was incapable of examining co-dominant markers.

In this study, we designed an algorithm and a related computer program that identifies with certainty all minimal marker sets for a given summary table using co-dominant markers. While our research focuses on the practical use of such technology for the genotypic identification of fruit tree varieties to protect breeders'

rights for the fruit tree cultivation industry, we expect that the algorithm and computer program described here would also prove useful to other fields.

Implementation

Program architecture

Our platform-independent, Perl-based MinimalMarker software is licensed by the National Agriculture and Food Research Organization, and can be downloaded from http://fruit.naro.affrc.go.jp/eng/MinimalMarker_en.html. The cost-free program can be easily run on standard laboratory-type computer systems and does not require access to high-capacity servers. Successful execution of the program requires a Perl5 (or higher) environment. The MinimalMarker program operates in the command line and requires input files in a comma-separated value (CSV) format; results are output to a text file. Optional features included with the software allow users to obtain optimal discriminating marker sets by considering the experimental features of particular DNA markers and by accelerating the computing process.

Algorithm

The goal of this study is to devise an algorithm and an accompanying program that uses co-dominant markers for the identification of all minimal marker sets for a summary table such as that shown in Supplemental Table 3-1 (Yamamoto et al., 2003).

As such, we first simulated a testable dataset by producing a “pretend” sample summary table (Table 3-1). We then used this sample table to test the algorithm against five DNA markers and five fruit tree varieties for the identification of appropriate minimal marker sets.

When developing a workable algorithm for accurate identification of a specific

marker, the most important principles are as follows:

1) The marker set(s) that can discriminate all varieties in a given summary table is the marker set(s) that can discriminate between any pair of varieties displayed in a given summary table.
2) Minimal marker set(s) is (are) the marker set(s) that can discriminate between any pair of varieties in a given summary table and that is (are) as small as possible. The algorithm we designed (described below) does just that. Fig. 3-1 shows the flow diagram for the algorithm. The algorithm shown in Fig. 3-1 involves an exhaustive method without adding considerable mathematical novelty, and the use of an improved algorithm (as described later) does not necessarily mean that the new method can identify minimal marker sets. However, in the past it has been thought that there was no example of this application to the discrimination of minimal marker sets for cultivar identification by DNA markers.

Producing the possibility-of-discrimination table

The first step in our algorithm is to calculate whether two or more varieties have exactly the same genotypes in all markers. If such pair of varieties is found, they are treated as the same variety following steps because at least one marker type must be different for each pair of varieties in a given summary table as a principle of this algorithm. This is not unusual for the fruit tree since there are many bud mutation varieties. The next step is to produce the possibility-of-discrimination table that serves as the foundation for all subsequent actions. In our example, Table 3-1 is used to produce the possibility-of-discrimination between all pairs of varieties for each marker, shown in Table 3-2.

That is, if the genotypes of a variety pair in Table 3-1 are the same for a particular

marker, a value of "0" is assigned to the corresponding position in Table 3-2; this indicates that the specific marker cannot discriminate between these two varieties. If the genotypes are different, a value of "1" is assigned, indicating that the marker is capable of that particular discrimination. For example, in Table 3-1, marker *M1* identifies genotypes “aa” and “ab” in varieties *V1* and *V2*, respectively. As these two genotypes differ, we conclude that *M1* can be used to discriminate between *V1* and *V2*, and thus a "1" is placed at the intersection of column *V1V2* and row *M1* in Table 3-2.

Discrimination of minimal marker set

Using the aforementioned possibility-of-discrimination table, we can search for the minimal marker set. For example, in Table 3-2 the sum of each column is greater than 1 in all combinations of two displayed varieties, which shows that there exists more than one markers capable of distinguishing between every pair of varieties. Consequently, a combination of the fewest markers that satisfies all the column sums being at least one is a minimal marker set. We must therefore examine whether this requirement is met while concomitantly incrementally increasing the number of markers. In the case of one marker, no single marker satisfies this condition, as there is a zero in every row in Table 3-2. However, in the subset of combinations of two markers, both *M1* with *M2* and *M2* with *M4* meet the condition, as shown in Tables 3-3A and 3-3B; these are each therefore minimal marker sets.

In general, in our algorithm, let V be the number of varieties and M be the number of markers in a given summary table, and let n be the number of markers in a subset. The dimension of the table of discrimination probability is $M \times {}_{v}C_{2}$. The number of marker subsets is ${}_{M}C_{n}$, where n is increased incrementally from 1. Next, we examined whether the column sum in all columns is at least one for all subsets that consist of n

markers. Thus, the first subset of n markers that meets the condition is defined as a minimal marker set. As more than one minimal marker set may meet the aforementioned condition, it is necessary to calculate the column sums for all subsets of the same size as the first minimal marker set found; this permits accurate identification of all of the summary-table minimal marker subsets. In the process of the marker development for variety discrimination, we needed to first develop numerous redundant markers until we were able to obtain markers that provided adequate discrimination power to protect breeder's right. By overviewing markers that compose all minimal marker sets, we could distinguish essentially important markers. In practice, when the DNA marker is applied to unknown varieties, the marker type might at times obtain more than one null result. This may be due to mispriming or non-priming of the primer. Therefore, it is important that the algorithm provide with two or more minimal marker sets.

Computational effort

An exhaustive search such as that suggested in the aforementioned algorithm is generally infeasible or at the very least time consuming (Martin, 2011).

In consideration of the computational cost of the algorithm:

Let *m* be the number of markers in a minimal marker set. The number of combinations of two varieties is shown in Eq. (1) below:

$$ {}_{v}C_{2} = \frac{V(V-1)}{2} = \frac{1}{2}V^{2} - \frac{1}{2}V \qquad (1) $$

This leads to polynomial time.

The number of subsets that must be checked until all minimal marker sets are found is

shown in Eq. (2) below:

$$\sum_{n=1}^{m} {}_{M}C_{n} = \sum_{n=1}^{m} \frac{M!}{n!(M-n)!} \qquad (2)$$

When $M=m$, this number is maximized as shown in Eq. (3) below:

$$\sum_{n=1}^{M} {}_{M}C_{n} = \sum_{n=1}^{M} \frac{M!}{n!(M-n)!} = 2^{M} - 1 \qquad (3)$$

The algorithm is thus likely to be exponential if m is not small enough. The time and memory required for an algorithm are usually measured in terms of asymptotic notation. For our present algorithm, the asymptotic notation can be denoted as $O(2^n)$. This shows a possibility that the computing time increases exponentially as the number of markers increases.

Method of using the largest discrimination power

To avoid the problems of exponential running time when m is too large, we incorporated into our approach a method using the largest discrimination power as an optional software feature to accelerate the computation. The possibility-of-discrimination table such as Table 3-2 can be simplified through transformation by row subtraction, row and column translocation. Eventually, one will find the minimal marker set. This procedure is shown below:

1) Search for all the two-variety combination(s) in the table where the sum of the column is one. If this requirement is met, add the corresponding contributing marker(s) in the candidate minimal markers set(s).
2) Find the row with the maximum discrimination and move it the first row. Through

column translocation, ensure the upper-left cell to be 1. Subtract all the other rows with the first row. If a cell has a number -1, it can be redefined as 0. *M2* is added as the candidate minimal markers set(s) in Table 3-4A and 3-4B. This adds up to $(M-1) \times {}_vC_2$ times of subtraction.

3) Find a row excepting the first one to have the maximal discrimination. Through the table transformation, move it to the second row and make sure the second cell from the left is 1. Subtract all other rows with this second row, which is *M1* in Table 3-4C and 4D. This should be no more than $(M-2) \times {}_vC_2$ times of subtraction.

4) Repeat the above procedure and stop when one cannot find a row with number 1 in it. All these calculations add up to $< (M-1)(M-2)/2 \times {}_vC_2$ number of calculations. The markers corresponding to the first row to the last row containing 1 then constitute the minimal marker set. In this case, *M1* with *M2* are obtained as a minimal marker set.

5) Even if the table transformations are considered, the asymptotic notation can be denoted as $O(M^2 \times V^2)$. We can assume the number of varieties is a constant, even if it is large. The asymptotic notation can then be denoted as $O(M^2)$, polynomial complexity.

A concrete example demonstrating the method of using the largest discrimination power to greatly decrease computational effort is shown in below “Results” section. In this case, the result corresponds to those discovered using the basic algorithm. However, the method of using the largest discrimination power does not guarantee whether the marker sets obtained are minimal or whether all minimal marker set(s) are identified for the given summary table (The acceleration achieved using the option is shown in the below “Results” section.).

In addition, the program was designed with nine additional options that allow users to obtain optimal discriminating marker sets by considering the experimental features of

particular DNA markers. For example, when the smallest number of markers of minimal marker set(s) is three, and the –s option defines four markers and the –e option defines four markers, MinimalMarker searches the marker set composed of four markers. In practice, this option proves significant in that the required labor might not change for assessing three or even four of the markers, depending on experimental conditions. In addition, there are markers that the experimenter may wish to use or to avoid. In this case, MinimalMarker does not select marker set(s) including redundant markers. That is, MinimalMarker never selects marker set(s), such as the marker set composed of three markers (=minimum number of markers), added as an arbitrary marker. Therefore we opted to use the term "minimal marker set". Moreover, the –m option accelerated the computation while providing shielding from potential faults associated with exponential running time (Table 3-5).

Results

Validation of the identification of minimal marker set(s) with both sample and published datasets

As described below, our algorithm and the accompanying MinimalMarker program proved effective in finding minimal marker sets in multiple fruit tree-specific datasets. When we applied MinimalMarker to the sample data (Table 3-1), two minimal marker sets that each contained two markers, *M1* with *M2* and *M2* with *M4*, were discovered. These results are consistent with those shown in the above description of the algorithm. For discrimination among grapevine varieties, researchers in a previous study identified six microsatellite markers with many (13–23) allelic forms (This et al., 2004), and proposed that these could be used as a standard optimal marker set. In the present study, we applied MinimalMarker to this grapevine dataset, and found that all varieties could

be discriminated using two markers, *VVMD27* and *VVMD5*, which was consistent with the results in the above-mentioned report (This et al., 2004).

The previous study reported that the GGDS software program (Gale at al. 2005) provided a variety/marker summary table with 22 dominant markers and 22 varieties, and showed only one minimal marker sets, *{A, C, G, I, S, T, U}*, as a concrete example. We applied MinimalMarker to the dataset, and found 24 minimal marker sets. One of them was *{A, C, G, I, S, T, U}*.

We also applied MinimalMarker to a summary table that contained 18 peach varieties analyzed by 17 SSR markers (Supplemental Table 3-1) (Yamamoto et al., 2003). However, all the SSR markers for the "Akatsuki" variety and its bud mutation derivative "Gyosei" were exactly the same in the table. MinimalMarker could consider "Akatsuki" and "Gyosei" to be the same variety and could thus keep running the algorithm after outputting the message that "Akatsuki" and "Gyosei" had the same genotypes in all markers. Accordingly, the following six minimal marker sets each containing three markers were obtained: *{M1a, M4c, MA023a}*, *{M4c, MA007a, MA023a}*, *{M4c, MA007a, MA035a}*, *{M4c, MA014a, MA023a}*, *{M4c, MA015a, MaA027a}* and *{M4c, MaA027a, MA035a}*. We also confirmed that these results were consistent with those calculated by hand.

The program was also applied to summary tables for sweet cherry (Takashina et al., 2009), Japanese pear (Terakami et al., 2010), apple (Moriya et al., 2011) and Japanese chestnut (Yamamoto et al., 2008); results were validated by the researchers who produced the tables.

Performance test

For the performance test, we applied MinimalMarker to an unpublished dataset of

citrus varieties compiled by us in which 98 varieties were analyzed using 256 SNP markers described in Chapter 4. The entire dataset and seven subsets subsequently generated from the original dataset by reducing the number of varieties and markers were tested. The number of varieties in the tested datasets was either 49 or 98, with each run using 64, 128, 192 and 256 markers (Table 3-6). All varieties in the eight datasets had different genotypes with the SNP markers. Tests were performed under default conditions (see Table 3-5) using the method of using the largest discrimination power option –m2 (Table 3-5) for acceleration on a Macintosh machine (OS: Mac OS X 10.6.8; CPU: 28GHz Intel Core 2 Duo; Memory: 4GB), and the elapsed time was measured.

Computation times are shown in Table 3-6. Using the default settings, the computation time increased exponentially with the number of markers, taking 1,322,428 seconds—approximately 15 days—to compute the entire 98-variety/256 SNP-marker dataset. This increased computation time might thus limit the size of datasets used when MinimalMarker is run using regular (non-laboratory-grade) personal computers. Similarly, as the computation time increased as the number of markers in a minimal marker set increased, the elapsed time might be longer for SNP markers—where the allele number is four in theory but two in practice—than for SSR markers, which typically have rich polymorphism.

With the method of using the largest discrimination power option, the elapsed time was substantially shorter than with the default option, and increased linearly as the number of markers increased (Table 3-6). As the discrimination power between the markers often have large overlaps, the algorithm may not take much time, making it closer to an equation of linear complexity. It took 129 seconds to compute the entire 98-variety/256SNP marker dataset; the ratio between the time required for the 49 and 98

variety-datasets was about four, even as the number of markers changed. Therefore, the elapsed time can be estimated by using the method of the largest discrimination power option with a subset of the dataset that has a reduced number of markers or varieties.

The reduced number of marker subsets found using the power option was ${}_{M}C_{m} = {}_{256}C_{7}$ = 13,161,885,792,000, representing 97.2% of the number that should be investigated normally in the entire 98-variety/256 SNP-marker dataset. The reduced number of combinations of two varieties by the option was 3,045 (= the largest discrimination power) and it was 64.1% of the total number of combinations of two varieties (=4,753) in the entire 98-variety/256 SNP-marker dataset.

The number of markers in a minimal marker set was the same for the default computation and for computation with the method of using the largest discrimination power option in seven of the eight datasets shown in Table 3-6; in these seven datasets, the minimal marker sets found using the power option were among those found by the default computation. Therefore, if identifying a strictly minimal marker set is not required for a specific task, the option might be useful for faster variety identification of sufficiently small marker sets.

Discussion

Several approaches using genetic statistics have been attempted for finding minimal marker sets for the identification of fruit tree varieties (Robertson et al., 2004; Tessier et al., 1999; Laucou et al., 2011; Martin, 2011). However, these techniques cannot be used to find marker sets containing the smallest number of markers for discriminating all varieties in a particular summary table.

The previously reported GGDS software program (Gale et al., 2005) can be used to identify all minimal marker sets for a given summary table. Yet because GGDS was

designed for use with only dominant markers because input data are restricted binary data, we developed MinimalMarker to use with summary tables containing co-dominant markers such as the SSR markers that are more commonly used for discriminating between varieties of fruit tree varieties (Takashina et al., 2009; Terakami et al., 2010; Moriya et al., 2011; Yamamoto et al., 2008). Incidentally, MinimalMarker also operates with dominant markers or with both co-dominant markers and dominant markers, regardless of the number of alleles, because the markers' genotypes are replaced by alphabetic characters in the software and subsequently treated via string manipulation. The method using GGDS (Gale et al., 2005) should combine ILP solvers with GGDS, therefore the knowledge of ILP would be requested from the researchers. The algorithm of MinimalMarker is simple does not need other software.

To increase the usability of MinimalMarker, we have added a method of using the largest discrimination power option (Table 3-5) to accelerate computation speeds, although this option does not ensure that strictly minimal marker sets will be found. It should be noted that missing data caused by an inability of the marker primer to hybridize with a specific variety's genome cannot be included in MinimalMarker calculations (i.e. missing data lead to incorrect summing of column figures).

In DNA-marker-based discrimination of fruit tree varieties and other species, a sample genotype can be evaluated as a different variety if the genotype differs from a known variety for at least one marker. In contrast, even if all the genotypes for a sample and a variety are identical, then the varieties are not necessarily the same. When an unknown sample differs from the known varieties, a minimal marker set can determine that the sample is different from the varieties listed in a given summary table using the fewest markers. If the sample is a variety that appears in the table, the minimal marker set will identify the variety because the minimal marker set discriminates all varieties in

the table; in this case, it becomes necessary to apply more markers to prove it to be the same variety, but MinimalMarker greatly simplifies such analysis. This makes finding a minimal marker set advantageous when a large number of samples are involved. Whenever a new variety or a new marker is added, calculation by MinimalMarker is required.

In Japan, the implementation of MinimalMarker has contributed to the selection of SSR markers used to establish the official protocol for identifying Japanese pear and sweet cherry varieties for the protection of breeders' rights (Takashina et al., 2007); similarly, the protocol for genotypic identification of apple and Japanese chestnut (Yamamoto et al., 2008) was prepared using MinimalMarker.

The genotyping process can be costly in terms of laboratory consumables, labor and time when a large number of samples and SSR markers are involved (Gale et al., 2005), but establishing a minimal marker set permits efficient discrimination of varieties. Such concerns led us to devise an algorithm and the accompanying MinimalMarker software to identify all appropriate minimal marker sets that contain the smallest number of markers while still discriminating all varieties in a particular summary table.

Although MinimalMarker is currently used primarily for the genotypic identification of fruit tree varieties, the program is also useful for analyzing other species' markers. We similarly expect that this program would prove useful for both genomics researchers and inspection agencies that perform large-scale analysis of DNA markers to authenticate food labeling.

Availability and requirements

As previously described, the MinimalMarker computational tool is made available cost-free to the scientific community and general public by Japan's b Website

(http://www.naro.affrc.go.jp/org/fruit/eng/MinimalMarker_en.html). Successful execution of the program requires a Perl5 (or higher) environment.

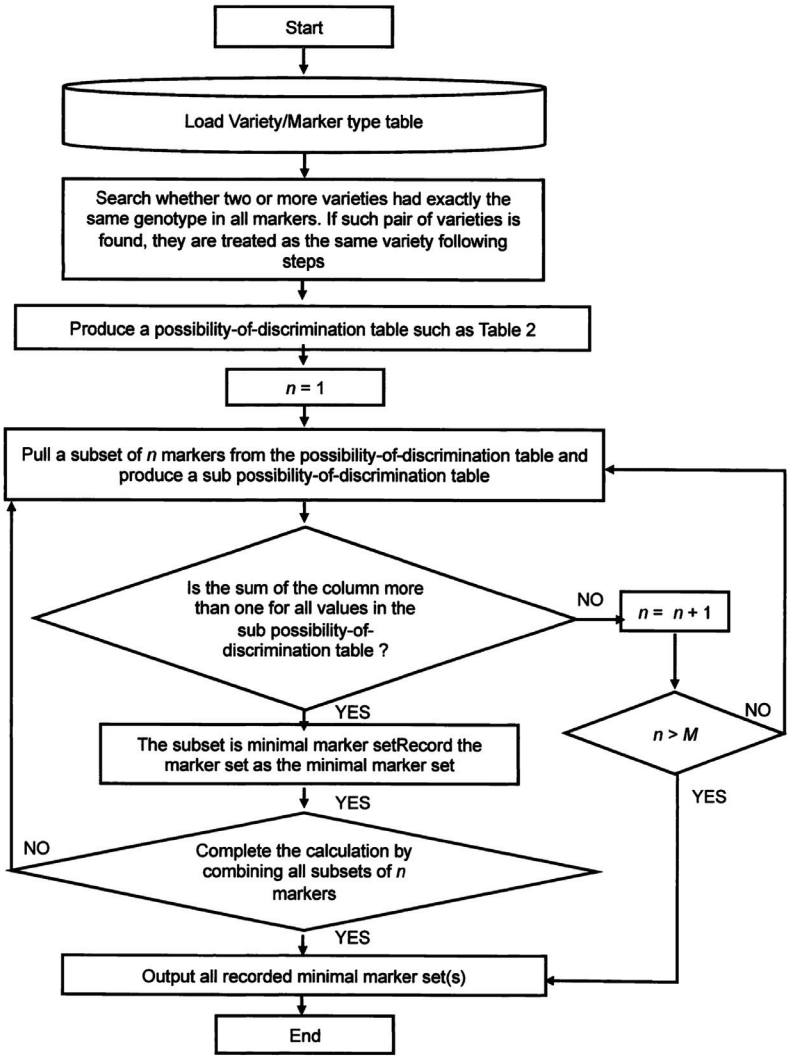


Fig 3-1. Flow diagram of the MinimalMarker algorithm. *M* is the number of markers in a given summary table, and *n* is a counter.

Table 3-1. A summary table with five co-dominant markers (M1−M5) and five diploid varieties (V1−V5) as imaginary test data

Marker	Variety				
	V1	*V2*	*V3*	*V4*	*V5*
M1	aa	ab	aa	ab	aa
M2	aa	ab	ab	aa	bb
M3	aa	aa	aa	aa	bb
M4	aa	aa	ab	ab	aa
M5	ab	ab	bb	ab	aa

Table 3-2. Possibility of discrimination between two varieties by each marker, as derived from Table 3-1

Marker	Possibility of discrimination between two varieties					
	V1V2	*V1V3*	*V1V4*	*V1V5*	*V2V3*	*V2V4*
M1	1	0	1	0	1	0
M2	1	1	0	1	0	1
M3	0	0	0	1	0	0
M4	0	1	1	0	1	1
M5	0	1	0	1	1	0
Sum of column	2	3	2	3	3	2

Note: "1" and "0" indicate discriminating and non-discriminating markers, respectively.

Table 3-3. Possibility of discrimination between two varieties by each marker in two minimal marker sets, as derived from Table 3-2

A

Marker	Possibility of discrimination between two varieties									
	V1V2	*V1V3*	*V1V4*	*V1V5*	*V2V3*	*V2V4*	*V2V5*	*V3V4*	*V3V5*	*V4V5*
M1	1	0	1	0	1	0	1	1	0	1
M2	1	1	0	1	0	1	0	1	1	1
Sum of column	2	1	1	1	1	1	1	2	1	2

B

Marker	Possibility of discrimination between two varieties									
	V1V2	*V1V3*	*V1V4*	*V1V5*	*V2V3*	*V2V4*	*V2V5*	*V3V4*	*V3V5*	*V4V5*
M2	1	1	0	1	0	1	1	1	1	1
M4	0	1	1	0	1	1	0	0	1	1
Sum of column	1	2	1	1	1	2	1	1	2	2

Note: "1" and "0" indicate discriminating and non-discriminating markers, respectively. (A) and (B) show that both the combination of *M1* and *M2*, and of *M2* and *M4*, are minimal marker sets because all column sums are at least one.

Table 3-4. Possibility-of-discrimination table reduced by the method of using the largest discrimination power option from Table 3-2

A

Marker	Possibility of discrimination between two varieties										Sum of row
	V1V2	*V1V3*	*V1V5*	*V2V4*	*V2V5*	*V3V4*	*V3V5*	*V4V5*	*V1V4*	*V2V3*	
M2	1	1	1	1	1	1	1	1	0	0	8
M1	1	0	0	0	1	1	0	1	1	1	4
M3	0	0	1	0	1	0	1	1	0	0	4
M4	0	1	0	1	0	0	1	1	1	1	4
M5	0	1	1	0	1	1	1	1	0	1	6
Sum of column	2	3	3	2	4	3	4	5	2	3	

B

Marker	Possibility of discrimination between two varieties										Sum of row
	V1V2	*V1V3*	*V1V5*	*V2V4*	*V2V5*	*V3V4*	*V3V5*	*V4V5*	*V1V4*	*V2V3*	
M2	1	1	1	1	1	1	1	1	0	0	8
M1	0	0	0	0	0	0	0	0	1	1	2
M3	0	0	0	0	0	0	0	0	0	0	0
M4	0	0	0	0	0	0	0	0	1	1	2
M5	0	0	0	0	0	0	0	0	0	1	1
Sum of column	1	1	1	1	1	1	1	1	2	3	

C

Marker	Possibility of discrimination between two varieties		Sum of row
	V1V4	*V2V3*	
M1	1	1	2
M3	0	0	0
M4	1	1	2
M5	0	1	1
Sum of column	2	3	

D

Marker	Possibility of discrimination between two varieties		Sum of row
	V1V4	*V2V3*	
M1	1	1	2
M3	0	0	0
M4	0	0	0
M5	0	0	0
Sum of column	1	1	

Table 3-5. Program options for MinimalMarker

Option	Arguments	Significance
-p[0/1/2]	0: Do not print the table（default） 1: Print the table 2: Print the table and the program quits	The table shows the possibility of discrimination between two varieties by each marker, as in Table 2.
-w		Under the condition that at least two markers are required for discriminating between every pair of varieties, minimal marker set(s) are searched.
-s[number]	Number of markers which starts search	Specifies the number of markers required to start the search. In case of dominant markers, at least 7 markers are required to identify 100 varieties. In this case, the search is completed quickly using the -s7 option switch.
-e[number]	Number of markers which stops the search -1: Search the total number of markers.	Specifies the number of markers required to end the search. Even if no minimal marker set is identified, the search can be stopped when it is expected that the computation time will be too large to be practical.
-n[number]	Row number of the marker One or more specification is possible (ex. -n1 -n2)	Specifies markers that must be included in the minimal marker sets.
-x[number]	Row number of the marker One or more specification is possible (ex. -x1 -x2)	Specifies markers that must be excluded from the minimal marker sets.
-v[number]	Column number of the variety(ies) One or more specification is possible (ex. -v1 -v2)	Specifies variety(ies) and the minimal marker set(s) that discriminate the specified variety(ies) from other varieties in a summary table.
-m[0/1/2]	0: Enumeration method 1: Branch-and-bound method (default) 2: Method of using the largest discrimination power	Computation is accelerated using the branch-and-bound method. The same result is obtained by the enumeration method and the branch-and-bound method. Computation is drastically accelerated using the method of using the largest discrimination power, but it does not guarantee whether the marker sets obtained are minimal nor whether all minimal marker set8s) are identified.
-b[0/1]	0: Arithmetic operation 1: Bit operation (default)	Computation is accelerated using the bit operation when the amount of computation time required to obtain the results becomes too large. The same result is obtained by the arithmetic operation and the bit operation.

Table 3-6. Performance test of MinimalMarker under default (A) and the largest discrimination power option (B)

A Default

Number of SNP markers	98 varieties			49 varieties			[a/b]
	Elapsed time (seconds) [a]	Number of markers in a minimal marker set	Number of minimal marker sets	Elapsed time (seconds) [b]	Number of markers in a minimal marker set	Number of minimal marker sets	
64	329	8	255	172	7	8560	1.9
128	5214	7	21	700	6	210	7.5
192	136965	7	288	14417	6	8569	9.5
256	1322428	7	4607	77961	6	176341	17

B With method of using the largest discrimination power option for acceleration (refer Table 5)

Number of SNP markers	98 varieties			49 varieties			[a/b]
	Elapsed time (seconds) [a]	Number of markers in a minimal marker set	Number of minimal marker sets	Elapsed time (seconds) [b]	Number of markers in a minimal marker set	Number of minimal marker sets	
64	25	8	6	6	7	89	4.1
128	34	8	234	8	6	19	4.3
192	74	7	26	18	6	164	4.1
256	129	7	80	30	6	478	4.3

Supplemental Table 3-1. The summary table obtained using 17 SSR markers for 18 peach tree varieties from Yamamoto et al. (2003).

SSR marker	Variety								
	Akatsuki	Yuuzora	Saotome	Chiyohime	Yoshihime	Masahime	Akizora	Natsutome	Hakuhou
M1a	80/84	80/84	84/84	80/84	80/84	80/80	80/80	80/84	80/84
M4c	78/94	78/94	78/88	88/94	80/94	80/94	74/94	80/94	74/78
M6a	193/197	193/197	195/197	195/201	193/197	193/197	193/197	193/197	193/197
M12a	177/195	195/195	177/195	177/177	177/195	177/195	177/195	177/195	177/195
M15a	136/136	136/136	132/136	136/136	136/136	136/136	116/136	136/136	116/136
MA006b	295/295	295/295	295/301	295/301	295/295	295/295	295/295	295/295	295/295
MA007a	111/133	111/133	111/111	111/121	133/133	133/135	133/133	111/133	111/133
MA013a	197/213	197/213	211/227	197/227	197/213	197/213	197/211	197/213	197/211
MA014a	167/167	167/167	150/167	167/167	163/167	167/167	163/167	163/167	163/167
MA015a	178/178	178/263	180/185	178/180	178/178	178/263	178/185	178/178	178/185
MA017a	165/165	165/165	177/177	177/177	165/165	165/165	165/177	165/165	165/177
MA023a	192/214	214/214	192/192	192/206	214/214	214/214	192/192	192/214	192/214
MA024a	245/245	245/245	243/245	243/245	245/245	245/245	245/245	245/245	245/245
MaA027a	147/160	147/160	145/147	147/160	147/191	147/191	147/191	147/160	147/191
MA030a	238/238	238/238	238/238	238/238	238/238	236/238	238/238	238/238	238/238
MA031a	123/131	123/123	123/131	123/123	123/131	123/123	123/131	123/131	123/131
MA035a	167/179	179/179	167/167	167/167	179/179	167/179	167/167	179/179	167/179

Note: The figures in each cell show the length of the SSR fragments.

SSR marker	Variety								
	Hakutou	21-18	Gyosei	Hikawa Hakutou	Abe Hakutou	Kawanaka jima Hakutou	Kouyou Hakutou	Shimizu Hakutou	Ookubo
M1a	80/80	80/80	80/84	80/84	80/80	80/80	80/80	80/84	80/84
M4c	78/94	78/80	78/94	80/94	78/80	74/94	80/94	80/94	78/80
M6a	193/197	193/197	193/197	193/201	197/197	197/201	197/201	193/201	197/201
M12a	195/195	195/195	177/195	177/195	177/195	177/195	177/195	177/195	177/195
M15a	136/136	136/136	136/136	116/136	136/136	136/147	136/136	136/136	136/136
MA006b	295/295	295/295	295/295	295/297	295/295	295/295	295/295	295/295	295/295
MA007a	121/133	133/135	111/133	133/135	133/133	121/133	121/121	111/133	111/133
MA013a	197/213	197/213	197/213	197/213	213/213	197/213	197/213	197/197	197/213
MA014a	163/167	163/167	167/167	163/167	163/167	150/167	160/167	163/167	163/167
MA015a	178/263	178/263	178/178	178/263	178/178	178/185	178/263	178/185	178/185
MA017a	165/165	165/165	165/165	173/177	165/165	165/177	165/177	165/177	165/177
MA023a	214/214	214/214	192/214	192/192	192/214	214/214	206/214	206/214	192/216
MA024a	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245	245/245
MaA027a	160/191	189/191	147/160	189/191	147/160	147/160	153/160	153/191	147/160
MA030a	238/238	236/238	238/238	236/238	236/238	238/238	236/238	236/238	236/238
MA031a	123/123	123/123	123/131	123/123	123/123	123/123	123/131	123/131	123/123
MA035a	179/179	179/179	167/179	167/167	179/179	167/179	167/179	167/179	167/179

Chapter 4: SNP genotyping by custom genotyping array in citrus accessions

Citrus cultivars contain a variety of secondary metabolites, such as flavonoids (Murakami et al., 2000), carotenoids (Tsushima et al., 1995), and limonoids (Lam and Hasegawa 1989; Lam et al., 1989), which have health promoting functions in humans. Recently, it has become important to breed new cultivars enriched with such substances. For example, increasing the content of B-Cry, which is a carotenoid component with cancer preventative activity (Tsushima et al., 1995), is an important breeding objective for citrus in Japan. However, there are many genetic factors involved in increasing B-Cry (Kato et al., 2004). Quantitative trait loci (QTL) analysis is a powerful approach to map the genetic factors for important traits, such as B-Cry content (Sugiyama et al., 2011). The accuracy in mapping a trait loci, including QTLs, depends on the DNA marker density and the number of individuals, provided the genetic background of the mapping population is the same. To improve mapping efficiency and quality, high-throughput technologies have been required to perform genotyping with a large number of markers on a large number of progeny. Until now, many genetic maps of citrus have been constructed by traditional DNA markers such as restriction fragment length polymorphism (RFLP), cleaved amplified polymorphic sequences (CAPS), or simple sequence repeats (SSR). However, it was difficult for researchers to map each target trait locus in the different segregating populations because generating new linkage maps using traditional markers was a time- and labor-consuming procedure.

Compared with other DNA markers used for genetic mapping, single nucleotide polymorphisms (SNPs) have two advantages as genotyping markers. First, SNPs are the most frequently detectable variation in the genome sequences of various organisms. For example, it has been shown that one SNP occurs per 21 bp in potato (Rickert et al., 2003), per 78 bp in barley (Russell et al., 2004), per 164 bp in citrus (Jiang et al., 2010),

and per 232 bp in rice (Feltus et al., 2004). In comparison one SSR occurs per 8000 bp in rice (Goff et al., 2002).

Second, several high-throughput technologies utilizing SNPs have been developed in human genome analysis (Kwok 2001; Steemers et al., 2006; Syvänen 2005) and also in some crop species such as rice (Masouleh et al., 2009; Tung et al., 2010).

Among the SNP genotyping technologies, the bead array, such as the GoldenGate® Assay (Illumina Inc.), has been applied successfully to various plant species, including rice (*Oryza sativa* L.) (Tung et al., 2010), barley (*Hordeum vulgare* L.) (Rostoks et al., 2006), soybean (*Glycine max* (L.) Merrill) (Hyten et al., 2008), white spruce (*Picea glauca* Moench) and black spruce (*Picea mariana* (Mill.) Britton) (Pavy et al., 2008), loblolly pine (*Pinus taeda* L.) (Eckert et al., 2009) and sugi (*Cryptomeria japonica* D. Don) (Uchiyama et al., 2011). Because the assay is capable of multiplexing from 96 to 1536 SNPs in a single reaction, applying the high-throughput genotyping system would quickly create high-density genetic maps for marker-assisted breeding of various important traits.

There have been attempted to develop arrays for SNP markers in citrus (Close et al., 2006; Ollitrault et al., 2011). However, a detailed flowchart of the high-throughput SNP genotyping system, from SNP screening to validation of genotyping results, has not been established so far in citrus. In this study, we surveyed SNPs by direct sequence comparison of the sequence tagged site (STS) fragment amplified from genomic DNA of cultivars representing the genetic diversity of citrus breeding in Japan, and developed a prototype multiplexed SNP genotyping GoldenGate platform to establish the high-throughput genotyping system in citrus. The assay using the SNP genotyping platform was applied to a hybrid population of 88 progeny and 103 citrus accessions. The reliability of the SNP genotyping call results was confirmed using parentage

analysis, since datasets derived from DNA markers often contain missing or questionable genotype calls (Close et al., 2009).

Development of high-throughput SNP genotyping array, which incorporates the reliable SNPs, is able to finish the genotyping for any citrus population within a few months. The technology will actively promote the genetic analysis of citrus, such as QTL analysis, linkage mapping.

Materials and Methods

PCR primer design for STS sequence comparison

To design PCR primers for SNP discovery in genomic sequences of citrus cultivars, we evaluated expressed sequence tags (ESTs) in the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/index-e.html), HarvEST (http://harvest.ucr.edu/), and our private citrus EST database (Fujii et al., 2003b). The EST sequences were clustered by Visual Bio Clustering software (NTT software, Tokyo, Japan) to select representative ESTs from redundant sequences. The representative ESTs were used as candidate sites of STS amplification and SNP discovery. The exon-intron-junction of ESTs were predicted using software MAEZATO (Fujii et al., 2010a) to design intron spanning primers as introns are more polymorphic regions. STS primers for amplification and sequencing were designed using OLIGO primer analysis software (Molecular Biology Insights Inc., Cascade, USA). In addition to the newly developed STSs, STSs that were previously developed to generate CAPS markers (Omura, 2005) were also used for sequence comparisons among cultivars to detect SNPs.

PCR amplification and SNP discovery

PCR was performed on citrus genomic DNA of Clementine (*C. clementina* hort. ex

Tanaka), ‘Miyagawa wase’ (*C. unshiu* Marc.), ‘Trovita’ orange (*C. sinensis* Osbeck), ‘Duncan’ grapefruit (*C. paradisi* Macf.), Kishu mikan (*C. kinokuni* hort. ex Tanaka), Ponkan (*C. reticulata* Blanco), Mediterranean mandarin (*C. delicious* Tenore), and a haploid clone derived from Clementine (Oiyama and Kobayashi 1993). The haploid was used to obtain reference sequences. These eight citrus cultivars cover the majority of alleles used in citrus breeding programs for table fruits in Japan. The PCR products were purified by Whatman DNA binding unifilters (Whatman Ltd.) to remove the excess primers, subjected to direct sequencing by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.), and sequenced by ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Inc.). The ABI sequence file obtained was directly imported to CodonCode Aligner (CodonCode Corporation) to detect mutation sites. SNPs were discovered using CodonCode Aligner’s “Find Mutation” tool. For array analysis of candidate SNPs, only those that displayed base substitutions occurring in more than 2 of the 8 cultivars were selected. To detect SNPs in a shortcut analysis, the genomic sequence comparison of a subset of STSs was performed on ‘Okitsu 46 Gou’ (‘Sweet Spring’ (‘Ueda unshiu’ (*C. unshiu*) × Hassaku (*C. hassaku* hort. ex Tanaka)) × ‘Trovita’ orange) and ‘Kankitsu Chukanbohon Nou 5 Gou’ (‘Lee’ (Clementine × ‘Orlando’ tangelo (‘Duncan’ grapefruit × ‘Dancy’ tangerine (*C. tangerina* hort. ex Tanaka)) × ‘Mukaku-kishu’ (*C. kinokuni* hort. ex Tanaka), the parents of the hybrid population for the SNP genotyping assay. In addition, a small subset of 31 genes was deduced from *in silico* identification of SNPs in contigs assembled from EST sequences of *C. unshiu* and *C. sinensis* that were downloaded from DDBJ.

SNP selection and bead array construction

The candidate SNPs were used to construct Illumina bead arrays of 384 SNPs for the GoldenGate Assay. To optimize the SNPs chosen, three conditions were selected: SNP without any other SNP within 60 bp or less in the sequence; SNP without any other SNP in the zone located 20 bps downstream; and SNP with an appropriate designability rank score. A designability rank score, which summarizes a number of parameters, was given to each SNP by Illumina's algorithm, with the score ranging from 0 to 1.0 based on specificity and likelihood of success of genotyping in the GoldenGate Assay. A rank score of <0.4 had a low success rate, 0.4 to <0.6 had a moderate success rate, and >0.6 had a high success rate. A total of 384 SNPs with a designability rank score of 0.8 or higher were selected. In some cases more than one SNP was chosen in the same gene to allow validation of haplotype mapping.

Annotation of STSs used for SNP analysis

EST sequences used as SNP sources were annotated by a TBLASTX similarity search against the coding sequence of *Arabidopsis* in The Arabidopsis Information Resource (http://www.arabidopsis.org/), and the annotation was assigned (Table 4-1).

Plant materials for genotyping assays

The hybrid population consisted of 88 progeny generated by crossing 'Okitsu 46 Gou' and 'Kankitsu Chukanbohon Nou 5 Gou'. The two parent clones were used to evaluate the parental genotypes, and the results for SNP genotyping can be compared with previously performed CAPS genotyping. These plants were cultivated in the research field of the Citrus Research Division Okitsu of NIFTS. Ninety-seven diverse genotypes were selected from cultivars bred in Japan with their parents and accessions

from NIFTS Citrus Germplasm Collection (Table 4-2). Among them, HF, 'Kankitsu Chukanbohon Nou 5 Gou' and 'Tamami' had the duplicated analysis with sample code of TY9 with TY10 and TY12, TY62 with TY104, and TY48 with TY57, respectively.

All genotypes were cultivated at the Citrus Research Division Okitsu or the Citrus Research Station Kuchinotsu (Minamishimabara, Nagasaki) of NIFTS. Genomic DNA of the 88 hybrid progeny and 98 germplasm accessions including the hybrid parents with replicates (Table 4-2) for breeding was isolated from young leaves. After freeze-drying, leaf material was ground using a MM 300 Mixer Mill (Retsch Inc.), and DNA extraction was performed using a QIAmp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. A total of 192 samples were adjusted to concentrations of 80 ng/μl using distilled water.

SNP genotyping assay and validation

Based on the description by Fan et al. (2003), Illumina's GoldenGate Assay utilizing a bead array platform was performed on the candidate SNPs. The assay was outsourced for SNP genotyping (Moritex Inc.), performed as per the manufacturer's protocol, and genotyping reports were generated.

According to the manufacturer's description (http://www.illumina.com/Documents/products/technotes/technote_gencall_data_analysis_software.pdf), the scores generated by the SNP signal calling system software were used to validate the SNP genotyping for each DNA sample and for each SNP.

We also examined the parentage analysis as another evaluation step for the reliability of SNP genotyping, since an SNP genotype should be the allele combination derived from the parental genotypes according to the co-dominant inheritance mode. As listed in Table 4-2, 74 germplasm accessions and 88 individuals of the hybrid

population genotyped in this assay could be used for parentage analysis. The computer program MARCO (Fujii et al., 2010b) was used for this lineage test.

Results

Construction of SNP genotyping array

A total of 1497 SNPs in 416 STSs and 18 EST contigs were detected from our three data sources (Table 4-3). The SNPs, along with the flanking sequences, were screened by the design rules of the GoldenGate Assay as described in the Materials and Methods, and 25.7% of all the SNPs detected were selected for the assay (Table 4-3). Using the 384 SNPs selected from 283 independent STSs (Tables 4-2, Table 4-3, Table 4-4), an Illumina custom GoldenGate Array for citrus was constructed and termed *CitSGA-1* hereafter. Among the 384 SNPs set on *CitSGA-1*, 286 SNPs were selected from the sequence comparisons among the eight citrus, 82 from comparing between two cultivars, and the remaining 16 from the *in silico* analysis (Table 4-3). From the 283 STSs, 199 STSs produced 1 SNP each. From the remaining 84 STSs, 71 STSs produced 2 SNPs each. In addition, 10 STSs, 2 STSs and 1 STS produced 3 SNPs, 4 SNPs, and 5 SNPs, respectively (Table 4-1, Table 4-5).

Screening of SNP markers for genotyping

1) Screening of SNPs and DNAs by signal call scores for genotyping on CitSGA-1

One hundred and ninety-two DNA samples (Table 4-2) were genotyped using *CitSGA-1* with 384 SNPs, which resulted in a total of 73,728 individual SNP calls. However, the results included 2169 (2.9%) "No Calls," based on the aforementioned criteria, obtained using the GenCall software. The GenCall scores generated are primarily designed for ranking and filtering out failed genotypes, sample DNAs, and/or

SNPs, and the scores have values from 0 to 1. Scores below 0.2 indicate poor quality of signal and were termed as No Calls for failed genotypes, while scores above 0.7 usually indicate well-behaving genotypes.

To evaluate the typing accuracy for each SNP on the array, the call frequency score, which is the ratio of the number of samples on which the genotyping succeeded among the total 192 samples, was applied. The call frequency scores over 0.9 indicate valid SNPs according to the manufacturer's criteria. Thus, 15 SNPs (SI066, SI087, SI090, SI121, SI147, SI225, SI249, SI256, SI303, SI310, SI319, SI354, SI364, SI370 and SI377) were invalid and omitted by the call frequency scores from the following analyses (Table 4-3).

For evaluation of DNA samples, we used "GC10" and "GC50" scores that are calculated by taking the 10th and 50th (median) percentile of the GenCall scores. DNA samples with GC10 ≥0.5 and GC50 ≥0.7 were identified as valid according to the manufacturer's criteria. As a result, eight DNA samples, QT18, QT73, QT86, QT87, QT88, TY50 ('Osceola'), TY62 ('Kankitsu Chukanbohon Nou 5 Gou'), and TY99 ('Kincy'), were excluded using the proceeding analyses. Therefore, a total of 184 DNA sample calls were selected as reliable DNA samples. The 184 DNA samples included replicates, three 'HF9' (TY009, TY010, and TY012), and two 'Tamami' (TY048, TY057); therefore, the number of independent DNA samples was 181.

The analysis on 184 DNA samples, including the replicates, with 369 SNPs resulted in a total of 67,896 (92.1%) individual genotyping calls, in which 142 No Calls remained.

2) Exclusion of Monomorphic SNPs

We searched for monomorphic SNPs among the remaining 369 SNPs. Consequently, 26 SNPs were called termed as the single homozygous genotype across

all the DNA samples, and 17 SNPs were also called as the single homozygous genotype but with No Calls in some DNA samples. Thus, these 43 SNPs were judged as monomorphic SNPs and were omitted from the following analyses (Table 4-3). A total of 326 SNPs, including 59,784 individual genotyping calls with 80 No Calls, were used for further analyses.

Monomorphic rates among the three sources of SNP discovery, the detection from comparing eight citrus cultivars, the detection by comparing two citrus cultivars and the detection *in silico*, were 8.3%, 14.6% and 43.8%, respectively.

3) SNP typing validation by parentage discrepancy

We used the parentage analysis as another evaluation step for evaluating the reliability of SNP genotyping, since the genotype of an SNP should be the combination of the parental genotypes according to the co-dominant inheritance mode. In a certain SNP, if a discrepancy of parentage exists between the parents and progeny, at least one of the parents or progeny will have been mis-genotyped. Therefore, the analysis provides important information on the evaluation of accuracy of SNP genotyping and the omission of the invalid SNPs.

Table 4-2 shows all the 77 combinations of parent-progeny relationships among cultivars used in SNP genotyping. Among them, TY50, TY62 and TY99 had already been omitted by the GenCall score-based criteria, and TY2 (No.971594), TY3 (No.971614), TY21 (‘Kuchinotsu 36 Gou’), TY28 (KyEn5-En-6), TY79 (‘Reiko’), TY47 (‘Encore’), TY56 (‘Setoka’), TY97 (‘Kinnow’), and TY91 (‘Southern Red’) were omitted from the analysis because they showed mismatches in a previous lineage test using MARCO (Fujii et al., 2010b). The remaining 65 combinations of parent-progeny, in which discrepancies had not been detected previously, were used in the genotype

assay. However, 382 discrepancies of parentage were detected in 29 SNPs including 35 No Calls. Fifty of 65 combinations of parent–progeny had discrepant SNPs. Therefore, these discrepancies would be derived from miscalling the genotyping of 29 SNPs but not from a misunderstanding of lineages. Thus, the 29 SNPs were omitted from the following analyses. At that point, a total of 297 SNPs, including 54,648 individual genotyping calls with 45 No Calls remained (Table 4-3).

Re-construction of a set of SNP markers screened by the validation cut-off procedure

1) Reduction of SNPs that have No Call

Previous validation procedures for SNP genotyping lead to the deletion of 2124 No Calls from a total of 73,728 individuals analyzed. Only 45 No Calls remained in 20 SNPs representing 3680 genotyping calls in 184 DNA samples. We decided to delete the No Call SNPs to keep the robustness of the SNP markers for many citrus cultivars and progenies. Consequently, the 20 SNPs were omitted from the following analyses. Therefore, the 277 SNPs that remained, which included 50,968 individual genotyping calls, were selected as reliable SNPs (Table 4-3).

2) Reproducible and non-redundant SNP set

Among the remaining 277 SNPs that were selected by previous steps as reliable, nine SNPs appeared in replicate to check the reproducibility of the genotyping. Those included pairs of SI15 and SI337, SI19 and SI30, SI28 and SI292, SI95 and SI181, SI124 and SI284, SI170 and SI302, SI201 and SI252, SI220 and SI315, and SI272 and SI327. Among the paired SNP markers, no different genotyping were observed. Therefore, we eliminated the replicates from the 277 SNPs and selected the remaining 268 as independent reliable SNPs in *CitSGA-1*.

Reproducibility was also investigated for the 268 reliable SNPs. The reproducibility of the assay was evaluated using three sample replicates of HF9 (TY9, TY10, and TY12) and two sample replicates of 'Tamami' (TY48 and TY57) (See Table 4-2). All genotypes were concordant among replicates in all reliable 268 SNPs. Moreover, the reproducibility of the assay was evaluated using two SNP replicates from the nine SNP pairs mentioned above. All genotypes were concordant among replicates in the 184 samples.

3) Source of SNP detection and detection rate of reliable SNPs

The 277 reliable SNPs, including replicates, were classified by the sources of SNP detection. As described previously, the first class of SNPs were derived from the comparisons of eight citrus cultivars, and these comparisons provided 212 (77.2%) of the 286 SNPs placed on *CitSGA-1*. In the second class, where SNPs were derived from the comparison of two citrus cultivars, 'Okitsu 46 Gou' and 'Kankitsu Chukanbohon Nou 5 Gou', 57 reliable SNPs (69.5%) were observed out of 82 SNPs tested. In the third class, using the *in silico* source, the number of reliable SNPs was 8 (50.0%) of 16 assayed SNPs. The success rate of the *in silico* source was obviously inferior to that of the wet sequence comparison sources, especially since 7 of 16 (43.7%) assayed SNPs were monomorphic (Table 4-3).

Evaluation of Applicability of SNP genotyping

1) Ratio of heterozygous loci

The percentage of heterozygous SNP loci in the 268 reliable SNPs varied among 98 cultivars from 8.9% (TY69, 'Hirado Buntan' pomelo, *C. grandis* Osbeck) to 70.7% (TY59, 'Trovita' orange) with an average of 41.2% and a median of 44.7% (Table 4-6).

The percentage of heterozygous SNP loci in the 268 reliable SNPs varied from 1.0% (SI021, SI021, SI074, SI115, SI333, and SI360) to 80.6% (SI202) with a median of 45.9% (Table 4-4).

2) Mapping to Citrus genome of STS that originates SNP

The whole genome sequences for Clementine (*C. clementina*), as constructed by JGI, are now available on the Citrus Genome Database (http://www.citrusgenomedb.org/). The Clementine genome (v. 0.9) is 296 Mb spread over 1128 scaffolds with 2.3% gaps at 6.5× coverage.

We have mapped STSs that originate SNPs to both scaffolds by BLASTN. Of 384 STSs that originated SNPs, the e-value of 3 in Clementine were larger than 1E-10 (Table 4-4) and scaffolds were not identified between them.

3) Minimal marker sets for cultivar identification

It is sometimes unnecessary to use all the DNA markers listed to discriminate all the cultivars of a particular crop. That is, if a minimal marker set, a marker set that can differentiate between all cultivars shown in a cultivar/marker type table and that is as small as possible, is found, it is possible to discriminate cultivars efficiently both in terms of labor and time. We attempted to identify SNPs in the present study using the program, MinimalMarker described in Chapter 3.

We used a genotyping subset consisting of 98 germplasm accessions and 246 SNPs that showed independent and reliable genotyping results for MinimalMarker. The program outputted 4607 minimal marker sets containing seven markers, *i.e.* SI001, SI006, SI009, SI131, SI191, SI247, and SI259. By using 7 markers, all 98 germplasm accessions could be discriminated from each others.

Discussion

Throughput of SNP genotyping in citrus

In this research, we constructed a prototype 384 SNP citrus array (*CitSGA-1*) for the GoldenGate Assay and in addition performed a survey to obtain reliable SNPs using this system. We successfully applied this array to SNP genotyping in citrus. In total, SNP genotype data were obtained for 351 (91%) of the 384 SNPs on *CitSGA-1* (Table 4-5). The 91% success rate is comparable to the 90% success rate previously reported in barley (Rostoks et al., 2006), 89% in soybean (Hyten et al., 2008), and 81.6% and 82.0% in white spruce and black spruce, respectively (Pavy et al., 2008).

Marker data sets derived from DNA markers often contain missing or questionable genotype calls (Close et al., 2009). Therefore, we have eliminated missing or questionable genotype calls in several steps, such as monomorphic SNPs, that have discrepancies of parentage or No Calls. Consequently, 268 independent reliable SNPs have been obtained. Through the analysis, it was recommended to validate the following procedures: (1) Adoption of SNPs by call frequency scores (over 0.9) according to the manufacturer's criteria, (2) Adoption of sample by GC10 and GC50 scores according to the manufacturer's criteria, (3) Removal of monomorphic SNPs, (4) Removal of SNPs with discrepancy of parentage, and (5) Removal of No Call SNPs.

Comparison of sources for SNP discovery on reliability of genotyping

To detect SNPs for *CitSGA-1* we used three sources, two re-sequencing sources and an *in silico* source. As shown in the results, 212 of the 286 (77.2%) reliable SNPs were from the eight citrus source and 57 of the 82 (69.5%) reliable SNPs were from the two cultivars source. SNPs from the genomic sequence comparison among the eight citrus

cultivars had a higher SNP genotyping success rate than those from the comparison of the two cultivars. However, SNP success rates were not significantly different. The reason may be that the cultivars used as the two cultivar sources, 'Okitsu 46 Gou' and 'Kankitsu Chukanbohon Nou 5 Gou' are hybrids derived from plural sources with diversified genome sequences. SNPs in genomic sequences from these two cultivars would widely cover those obtained from the eight cultivars.

Compared with the two re-sequencing sources, the number of reliable SNPs using the *in silico* source was 8 of the 16 (50.0%) assayed SNPs. The success rate of the *in silico* source was obviously inferior to that of the wet sequence comparison sources, especially since 7 of the 16 (43.7%) assayed SNPs were monomorphic (Table 4-3). A similar tendency was also observed in the monomorphic ratios. Among the SNPs detected by comparisons of two citrus cultivars, the incidence of monomorphism may be caused by plural targeted genes existing in the genome. The high monomorphic rate of SNPs detected by the *in silico* source may be due to the EST sequence errors and the assembly of ESTs that were derived from different transcript regions. However, the cause of monomorphisms in SNPs detected from comparing eight citrus cultivars remains unclear.

Applicability of cultivar genotyping of CitSGA-1

When the number of No Calls of each DNA sample was counted, it was the largest in TY69 ('Hirado Buntan' pomelo) with 20. Five of the 20 No Calls occurred in only 'Hirado Buntan'. This may be due to mis-priming or non-priming of the primer. Because pomelo is classified distantly from other mandarins used in this assay by DNA markers (Federici et al., 1998), it suggested that there were a lot of SNPs in pomelo germplasms, which were not revealed while comparing the limited cultivars mainly

consisting of mandarins and sweet orange. It was considered that the above mentioned primers would not function only in ‘Hirado Buntan’ for the SNP assay; however, pomelo is an important breeding parent in Japan so we eliminated SNPs that would be less available for pomelo. For further analysis, allele or haplotype diversity in pomelo and mandarins in relation to the lineages of grapefruit or sweet oranges should be discussed based on detailed data.

Possible application of CitSGA-1

The number of chromosomes in haploid citrus is nine generally, but the number of SNPs per chromosome is 43 in average when all the 384 SNPs on the genotyping array were available. Therefore, the prototype 384 SNP array could be useful to roughly map the breeding trait loci at the marker density depending on the number of heterozygous loci for each cultivar. However, the information obtained by the prototype analysis system in the experiment would be applicable to construct a higher multiplex custom assay such as an over 1500 SNPs system. In addition, since most of the STSs were mapped on scaffolds by whole genome analysis of clementine, as shown in Table 4-4, tight mapping could be performed.

Moreover, it was shown that 98 germplasm accessions could be identified by combining the reliable SNPs obtained in this study. Therefore, these SNPs are immediately applicable for citrus cultivar identification.

Table 4-1 Relationship of assigned *Arabidopsis* loci, and their annotation, to STSs and SNPs on the citrus genotyping array *CitSGA-1*.

STS name	SNP name[a]	DDBJ EST accession number for STS primer design	Species of EST derived[b]	Assigned *Arabidopsis* locus	Annotation of *Arabidopsis* locus
Al0014	SI058:SI153:SI312	C95210	Cu	AT5G25110.1	CIPK25; SnRK3.25
Al0218	*SI350*	C95269	Cu	AT4G11150.1	TUF (VACUOLAR ATP SYNTHASE SUBUNIT E1)
Al0302	*SI090*:SI311	C95329	Cu	AT3G57520.2	AtSIP2 (Arabidopsis thaliana seed imbibition 2)
Al0304	SI259:*SI293*	C95332	Cu		no homology
Al0307	*SI029*	C95335	Cu	AT1G69530.2	ATEXPA1 (ARABIDOPSIS THALIANA EXPANSIN A1)
Al0308	SI045	C95336	Cu	AT4G21490.1	NDB3; NADH dehydrogenase
Al0327	SI065:SI344	C95360	Cu	AT1G12440.2	zinc finger (AN1-like) family protein
Al0329	*SI185*	DC892963	Cu		no homology
Al0409	SI201:SI252	C95388	Cu	AT4G15560.1	CLA1 (CLOROPLASTOS ALTERADOS 1)
Al0413	SI275:SI299	C95392	Cu	AT3G23920.1	BAM1 (BETA-AMYLASE 1)
Al0415	*SI120*:*SI310*	C95396	Cu	AT5G19010.1	MPK16
Al0417	SI244:SI255	C95398	Cu		no homology
Al0515	SI178	DC893061	Cu	AT2G26330.1	ER (ERECTA)
Al0524	SI043:SI341	C95551	Cu	AT5G02290.2	NAK
Al0625	SI329	C95496	Cu	AT5G60870.2	regulator of chromosome condensation (RCC1) family protein
Al0633	SI123	C95520	Cu	AT1G28520.2	VOZ1 (VASCULAR PLANT ONE ZINC FINGER PROTEIN)
Al0636	SI313	C95540	Cu	AT3G13225.1	protein binding
Al0637	SI353	C95541	Cu		no homology
Bf0003	*SI354*	DC884981	Cu	AT3G51860.1	CAX3 (CATION EXCHANGER 3)
Bf0004	SI273	DC884983	Cu	AT3G49140.1	unknown protein
Bf0005	SI194	DC884984	Cu	AT2G33700.1	protein phosphatase 2C putative
Bf0008	SI309	DC885005	Cu	AT3G17980.1	C2 domain-containing protein
Bf0011	SI218	DC885033	Cu	AT5G20080.1	NADH-cytochrome b5 reductase putative
Bf0024	*SI093*	DC885129	Cu	AT1G43900.1	protein phosphatase 2C putative
Bf0027	SI372	DC885151	Cu	AT4G25100.4	FSD1 (FE SUPEROXIDE DISMUTASE 1)
Bf0028	SI001	DC885165	Cu	AT1G11720.1	ATSS3 (starch synthase 3); starch synthase
Bf0029	*SI193*:*SI283*	DC885170	Cu	AT3G25820.1	ATTPS-CIN (terpene synthase-like sequence-18-cineole)
Bf0033	SI356:SI379	DC885228	Cu	AT4G30210.2	ATR2 (ARABIDOPSIS P450 REDUCTASE 2)
Bf0103	SI155	DC885333	Cu	AT5G08100.1	L-asparaginase
Bf0109	*SI233*	DC885420	Cu	AT2G40490.1	HEME2; uroporphyrinogen decarboxylase
Bf0110	SI297	DC885426	Cu	AT3G63520.1	CCD1 (CAROTENOID CLEAVAGE DIOXYGENASE 1)
Bf0115	*SI054*:*SI295*	DC885474	Cu	AT4G34640.1	SQS1 (SQUALENE SYNTHASE 1)
Bf0116	SI260	DC885486	Cu	AT2G13360.2	AGT (ALANINE:GLYOXYLATE AMINOTRANSFERASE)
Bf0123	SI141	DC885557	Cu	AT5G01220.1	SQD2 (sulfoquinovosyldiacylglycerol 2
Bf0130	SI063:SI188	DC885666	Cu	AT5G51380.1	F-box family protein
Bf0145	SI272:SI327:*SI368*	DC885769	Cu	AT5G02810.1	PRR7 (PSEUDO-RESPONSE REGULATOR 7)
Bf0147	*SI165*	DC885795	Cu	AT4G12320.1	CYP706A6
Bf0150	SI316:*SI370*	DC885821	Cu	AT1G79440.1	ALDH5F1
Bf0158	SI376	DC885888	Cu	AT5G51190.1	ERF (ethylene response factor)
Bf0159	SI074	DC885890	Cu	AT3G27090.1	unknown protein
Bf0161	SI089:SI348	DC885944	Cu		no homology
Bf0164	SI263	DC885962	Cu	AT1G76550.1	pyrophosphate-fructose-6-phosphate 1-phosphotransferase alpha subunit putative
Bf0165	*SI280*	DC885967	Cu	AT5G47120.1	ATBI1 (BAX INHIBITOR 1)
Bf0171	SI381	DC886014	Cu		no homology
Bf0174	SI282	DC886036	Cu	AT5G63120.2	ethylene-responsive DEAD box RNA helicase putative (RH30)
Bf0177	SI060	DC886078	Cu	AT1G18640.2	PSP (3-PHOSPHOSERINE PHOSPHATASE)
Bf0183	*SI308*	DC886132	Cu	AT5G59530.1	2-oxoglutarate-dependent dioxygenase putative
Bf0193	SI100:SI109	DC886216	Cu	AT5G48150.2	PAT1 (phytochrome a signal transduction 1)
Bf0195	SI369	DC886240	Cu	AY607026.1z	*Citrus reticulata*: NHX1 gene
Bf0200	SI335	DC886299	Cu	AT1G25350.1	OVA9 (ovule abortion 9)
Bf0204	SI326	DC886340	Cu	AT5G67030.1	ABA1 (ABA DEFICIENT 1)
Bf0205	*SI236*	DC886360	Cu	AT2G23320.1	WRKY15; calmodulin binding
Bf0212	*SI332*	DC886446	Cu	AT3G01500.3	CA1 (CARBONIC ANHYDRASE 1)
Bf0213	*SI097*	DC886474	Cu	AT4G12770.1	heat shock protein binding
Bf0229	SI253	DC886577	Cu	AT4G14040.1	SBP2 (SELENIUM-BINDING PROTEIN 2)
Bf0233	SI359	DC886598	Cu	AT2G45550.1	CYP76C4
Bf1117	SI224	DC885323	Cu		no homology
Bf1186	*SI245*	DC885875	Cu		no homology

[a]Italics indicate 'Not Reliable' SNP. [b]Cu: *Citrus unshiu* Marc., Pt: *Poncirus trifoliata* (L.) Raf., Cn × Ck: *C. nobilis* Lour. × *C. kinokuni* hort. ex Tanaka, Cs: *C. sinensis* Osbeck, Cl: *C. limon* (L.) Burm. f.

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STS name	SNP name[a]	DDBJ EST accession number for STS primer design	Species of EST derived[b]	Assigned *Arabidopsis* locus	Annotation of *Arabidopsis* locus
Bf1204	*SI364*	DC885762	Cu	AT1G20120.1	family II extracellular lipase putative
Bf1208	*SI108*	DC885159	Cu	AT4G26600.1	nucleolar protein putative
Bf2018	SI232	DC886503	Cu	AT4G09020.1	ISA3 (ISOAMYLASE 3)
Cp0813	*SI047*	DC898430	Cu	AT2G36290.1	hydrolase alpha/beta fold family protein
Cp0849	*SI355*	CD574105	Pt	AT2G38550.1	unknown protein
Cp0870	SI338	DC892827	Cl	AT2G39730.3	RCA (RUBISCO ACTIVASE)
Cp0996	SI340	CK938441	Cu	AT1G56560.1	beta-fructofuranosidase putative
Cp1624	SI307	CF835489	Cu	AT4G27460.1	CBS domain-containing protein
Cp1738	SI237:SI296	CK933293	Cu	AT4G40080.1	epsin N-terminal homology (ENTH) domain-containing protein
Cp2154	*SI164*	DC887048	Cn × Ck	AT5G54600.1	50S ribosomal protein L24 chloroplast (CL24)
Edp002	SI092	DC885494	Cu	AT5G60910.1	AGL8 (agamous-like 8)
Fb0110	SI246	DC888023	Cu	AT1G22940.1	TH1 (THIAMINE REQUIRING 1)
Fb0124	SI167:*SI212*	DC888039	Cu	AT2G42600.1	ATPPC2 (PHOSPHOENOLPYRUVATE CARBOXYLASE 2)
Fb0143	SI361	DC888059	Cu	AT2G26330.1	ER (ERECTA); transmembrane receptor protein kinase
Fb0144	SI191	DC888061	Cu	AT1G02205.1	CER1 (ECERIFERUM 1
Fb0159	*SI227*	DC888077	Cu	AT5G24318.1	catalytic/ cation binding / hydrolase hydrolyzing O-glycosyl compounds
Fb0180	SI209	DC888100	Cu		no homology
Fb0223	*SI240*	DC888148	Cu		no homology
Fb0233	SI025:SI079:SI169	DC888160	Cu	AT4G29730.1	NFC5 (Nucleosome/chromatin assembly factor group C 5)
Fb0234	SI250:SI265	DC888161	Cu	AT2G42840.1	PDF1 (PROTODERMAL FACTOR 1)
Fb0293	SI198:SI374	DC888231	Cu	AT4G35220.1	cyclase family protein
Fb0301	SI053	DC888236	Cu	AT5G63380.1	4-coumarate--CoA ligase family protein
Fb0357	SI384	DC888303	Cu		no homology
Fb0364	SI373	DC888316	Cu	AT4G10480.1	nascent polypeptide associated complex alpha chain protein putative
Fb0365	*SI036*:*SI320*	DC888317	Cu		no homology
Fb0372	*SI052*:*SI343*	DC888326	Cu	AT1G27530.1	unknown protein
Fb0827	SI215:SI301	DC888835	Cu	AT4G15560.1	CLA1 (CLOROPLASTOS ALTERADOS 1)
Fb0976	*SI229*	DC888997	Cu	AT1G11430.1	plastid developmental protein DAG putative
Fb0995	SI026	DC889015	Cu	AT2G17930.1	binding / inositol or phosphatidylinositol kinase
Fb1611	SI071	DC889716	Cu	AT5G57360.2	ZTL (ZEITLUPE)
Fb1751	SI264	DC889876	Cu	AT1G53730.1	SRF6 (STRUBBELIG-RECEPTOR FAMILY 6)
Fb1916	*SI336*	DC890051	Cu	AT5G13960.1	SUVH4 (SU(VAR)3-9 HOMOLOG 4
Fb2130	SI069:SI117	DC890291	Cu	AT1G07270.1	cell division control protein CDC6b putative (CDC6b)
Fb2159	*SI205*:SI300	DC890329	Cu	AT2G43120.1	pirin putative
Gn0014	SI009	AF296158	Cu	AT4G25700.1	BETA-OHASE 1 (BETA-HYDROXYLASE 1)
Gn0040	SI015:SI337	DC893194	Cu	AT4G18960.1	AG (AGAMOUS)
Gn0043	SI323	DC893225	Cu	AT5G60690.1	REV (REVOLUTA)
Gn0048	SI289	DC895343	Cu	AT1G69780.1	ATHB13
Gn0051	SI306:SI366	DC895375	Cu	AT1G27340.1	F-box family protein
Gn0064	SI144:SI380	AB075547	Cu	AT5G67030.1	ABA1 (ABA DEFICIENT 1); zeaxanthin epoxidase
Gn0066	SI352	AB114651	Cu	AT1G06820.1	CRTISO (CAROTENOID ISOMERASE); carotenoid isomerase
Gn0067	SI204:SI349	AB114652	Cu	AT5G57030.1	LUT2 (LUTEIN DEFICIENT 2); lycopene epsilon cyclase
Gn0069	SI086:SI375	AB114655	Cu	AT5G57030.1	LUT2 (LUTEIN DEFICIENT 2); lycopene epsilon cyclase
Gn0071	SI363	CK935329	Cs	AT3G63520.1	CCD1 (CAROTENOID CLEAVAGE DIOXYGENASE 1)
Gn0074	SI176	AB046992	Cu	AT4G14210.1	PDS3 (PHYTOENE DESATURASE 3); phytoene dehydrogenase
If0003	SI130	C22334	Cu		no homology
If0205	*SI256*	C22047	Cu	AT3G52730.1	ubiquinol-cytochrome C reductase UQCRX/QCR9-like family protein
If0206	SI208	C22133	Cu	AT3G53990.2	universal stress protein (USP) family protein
If0208	SI083:SI317	C22162	Cu	AT1G48460.1	unknown protein
If0210	SI042	C22223	Cu		no homology
If0211	SI226	C22289	Cu		no homology
If0216	*SI174*	C23944	Cu	AT4G00380.1	XH/XS domain-containing protein / XS zinc finger domain-containing protein
Is0001	*SI177*	DC883613	Cu	AT1G67350.1	unknown protein
Is0002	*SI339*	DC884052	Cu	AT5G44340.1	TUB4
Is0003	SI318	DC895944	Cu	AT2G29420.1	ATGSTU7 (ARABIDOPSIS THALIANA GLUTATHIONE S-TRANSFERASE TAU 7)
Is0004	SI268	DC896307	Cu	AT4G25810.1	XTR6 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6)
Is0005	*SI279*	DC896365	Cu	AT4G27740.1	unknown protein
Is0006	*SI358*	DC896409	Cu		no homology

[a]Italics indicate 'Not Reliable' SNP. [b]Cu: *Citrus unshiu* Marc., Pt: *Poncirus trifoliata* (L.) Raf., Cn × Ck: *C. nobilis* Lour. × *C. kinokuni* hort. ex Tanaka, Cs: *C. sinensis* Osbeck, Cl: *C. limon* (L.) Burm. f.

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STS name	SNP name[a]	DDBJ EST accession number for STS primer design	Species of EST derived[b]	Assigned *Arabidopsis* locus	Annotation of *Arabidopsis* locus
Is0007	SI032	DC896765	Cu	AT2G18370.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
Is0008	SI314	DC896803	Cu	AT5G14570.1	ATNRT2.7 (Arabidopsis thaliana high affinity nitrate transporter 2.7)
Is0009	*SI162*	DC897578	Cu	AT3G24200.2	FAD binding / monooxygenase/ oxidoreductase
Is0010	*SI206*	DC898118	Cu	AT5G54160.1	ATOMT1 (O-METHYLTRANSFERASE 1)
Is0011	SI334	DC898183	Cu	AT4G30080.1	ARF16 (AUXIN RESPONSE FACTOR 16)
Is0012	SI173	DC899883	Cu	AT5G05960.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
Is0013	SI012	DC901296	Cu	AT1G68220.1	unknown protein
Lp0024	SI010:SI302	AU300371	Cu	AT2G18050.1	HIS1-3 (HISTONE H1-3)
Lp0032	SI286	DC893131	Cu		no homology
Lp0102	SI080:*SI319*:SI360:*SI378*	AU300902	Cu	AT4G25310.1	oxidoreductase 2OG-Fe(II) oxygenase family protein
Lp0105	SI113:SI163:*SI254*	AU300400	Cu	AT2G17820.1	ATHK1 (histidine kinase 1)
Lp0112	SI170	AU300448	Cu	AT3G52300.1	ATPQ (ATP SYNTHASE D CHAIN MITOCHONDRIAL)
Lp0119	SI276	AU300466	Cu		no homology
Lp0226	SI210	AU300802	Cu	AT4G34110.1	PAB2 (POLY(A) BINDING 2)
Mf0003	SI228	C81792	Cu		no homology
Mf0010	SI048:SI149	C81689	Cu		no homology
Mf0012	SI131	C81631	Cu		no homology
Mf0039	*SI016*:*SI147*	C81786	Cu		no homology
Mf0070	*SI017*	C81837	Cu	AT5G04750.1	F1F0-ATPase inhibitor protein putative
Mf0079	SI067	C81861	Cu	AT5G58490.1	cinnamoyl-CoA reductase family
Mf0084	SI383	C81872	Cu	AT3G62290.1	ATARFA1E (ADP-ribosylation factor A1E)
Mf0086	SI220:SI315	C81880	Cu	AY261671.1	*Citrus x paradisi*:HSP19 class II
Mf0092	SI138	C81907	Cu	AT2G36530.1	LOS2; copper ion binding
Mf0095	*SI290*	C81912	Cu	AT3G57280.1	unknown protein
Mf0096	*SI249*:SI333	C81915	Cu	AT3G18040.2	MPK9 (MAP KINASE 9)
Mf0098	SI235:*SI365*	C81916	Cu		no homology
Ov0002	SI110:*SI179*	AU186184	Cu	AT1G14320.1	SAC52 (SUPPRESSOR OF ACAULIS 52)
Ov0005	*SI243*:*SI331*	AU186258	Cu	AT5G17920.1	ATMS1
Ov0015	SI304	AU186310	Cu	AT4G25150.1	acid phosphatase putative
Ov0020	SI055:SI143:SI213	AU186345	Cu	AT3G56940.1	CRD1 (COPPER RESPONSE DEFECT 1)
Ov0104	SI216	AU186385	Cu	AT3G19270.1	CYP707A4
Ov0105	*SI126*	AU186386	Cu	AT5G15080.1	protein kinase putative
Ov0106	*SI278*	DC901398	Cu	AT5G25150.1	acid phosphatase ctivity
Ov0109	SI172:SI202	AU186389	Cu	AT5G25230.1	elongation factor Tu family protein
Ov0117	*SI040*	AU186413	Cu		no homology
Ov0118	*SI357*	AU186414	Cu	AT2G45440.1	DHDPS2 (DIHYDRODIPICOLINATE SYNTHASE)
Ov0127	SI124:SI284	AU186445	Cu	AT5G14670.1	ATARFA1B (ADP-ribosylation factor A1B)
Ov0301	SI078	AU186450	Cu	AT5G47390.1	myb family transcription factor
Ov0305	SI028:SI292	AU186464	Cu	AT5G58420.1	40S ribosomal protein S4 (RPS4D)
Ov0306	*SI382*	AU186465	Cu	AT4G35550.1	WOX13 (WUSCHEL-RELATED HOMEOBOX 13)
Ov0314	*SI107*	AU186489	Cu	AT2G16600.2	ROC3; peptidyl-prolyl cis-trans isomerase
Ov0403	*SI136*:SI298	DC893934	Cu	AT2G05100.1	LHCB2.1
Ov0412	SI050:SI222:SI325	DC893973	Cu		no homology
Ov0426	*SI051*:*SI291*:SI351:SI371	DC894006	Cu	AT3G44110.1	ATJ3
Ov0429	SI342	AU186558	Cu	AT3G13510.1	unknown protein
Ov0508	SI322	DC894044	Cu	AT2G39730.3	RCA (RUBISCO ACTIVASE)
Tf0004	SI219:SI330	DC884099	Cu	AT1G61140.1	EDA16 (embryo sac development arrest 16
Tf0013	SI161:*SI362*	DC886674	Cn × Ck	AT1G44900.1	ATP binding / DNA binding / DNA-dependent ATPase
Tf0016	SI345	DC886929	Cn × Ck	AT3G55730.1	MYB109 (myb domain protein 109)
Tf0017	SI148:*SI171*:SI217:SI242:SI285	DC886985	Cn × Ck	AT5G47390.1	myb family transcription factor
Tf0019	SI056:SI347	DC887091	Cn × Ck	AT1G75710.1	zinc finger (C2H2 type) family protein
Tf0020	SI154	DC887181	Cn × Ck	AT4G36740.1	ATHB40 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 40)
Tf0026	SI111:SI288	DC898171	Cu	AT2G17190.1	ubiquitin family protein
Tf0027	*SI046*	DC898182	Cu	AT5G25190.1	ethylene-responsive element-binding protein putative
Tf0028	*SI377*	DC898260	Cu	AT4G09960.3	STK (SEEDSTICK); protein binding / transcription factor
Tf0045	*SI303*:*SI321*	DC899852	Cu	AT1G27050.1	ATHB54 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 54)
Tf0049	SI328	BQ623105	Cs	AT3G13350.1	high mobility group (HMG1/2) family protein
Tf0050	SI104:SI346	BQ623221	Cs	AT3G47600.1	ATMYB94 (MYB DOMAIN PROTEIN 94)

[a]Italics indicate 'Not Reliable' SNP. [b]Cu: *Citrus unshiu* Marc., Pt: *Poncirus trifoliata* (L.) Raf., Cn × Ck: *C. nobilis* Lour. × *C. kinokuni* hort. ex Tanaka, Cs: *C. sinensis* Osbeck, Cl: *C. limon* (L.) Burm. f.

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STS name	SNP name[a]	DDBJ EST accession number for STS primer design	Species of EST derived[b]	Assigned *Arabidopsis* locus	Annotation of *Arabidopsis* locus
Tf0051	SI027:SI214	BQ623496	Cs	AT3G48440.1	zinc finger (CCCH-type) family protein
Tf0053	SI305	BQ624296	Cs	AT1G53670.1	MSRB1 (methionine sulfoxide reductase B 1)
Tf0054	SI238	BQ624467	Cs	AT1G76580.1	transcription factor
Tf0056	*SI061*:SI137	BQ624834	Cs	AT1G66230.1	MYB20 (myb domain protein 20)
Tf0058	*SI257*	BQ624935	Cs	AT3G20740.1	FIE (FERTILIZATION-INDEPENDENT ENDOSPERM)
Tf0059	*SI101*	BQ624977	Cs	AT3G17850.1	protein kinase putative
Tf0061	SI150:SI186	BQ625130	Cs	AT5G65670.2	IAA9 (INDOLE-3-ACETIC ACID INDUCIBLE 9)
Tf0062	SI223	CB290239	Cs	AT3G09600.1	myb family transcription factor
Tf0065	SI142	CB290624	Cs	AT5G62000.3	ARF2 (AUXIN RESPONSE FACTOR 2)
Tf0066	SI221	CB290927	Cs	AT1G52890.1	ANAC019 (Arabidopsis NAC domain containing protein 19)
Tf0067	SI033:*SI187*:SI239	CB291001	Cs	AT3G16350.1	myb family transcription factor
Tf0068	*SI106*	CB291749	Cs	AT2G26150.1	ATHSFA2
Tf0069	SI199	CB292181	Cs	AT1G43700.1	VIP1 (VIRE2-INTERACTING PROTEIN 1)
Tf0070	SI277	CB292225	Cs	AT1G35460.1	basic helix-loop-helix (bHLH) family protein
Tf0071	SI084:SI146	CB292689	Cs	AT2G41350.2	unknown protein
Tf0075	SI059:SI248	CB293244	Cs	AT3G12020.1	kinesin motor protein-related
Tf0076	*SI266*	CB293271	Cs	AT5G04410.1	NAC2
Tf0077	SI135	CB293496	Cs	AT4G30935.1	WRKY32
Tf0079	SI270:SI294	CB293578	Cs	AT4G11660.1	AT-HSFB2B
Tf0081	SI038:SI324	CB293768	Cs	AT2G31380.1	STH
Tf0083	SI129	CB293916	Cs	AT4G22920.1	NYE1 (NON-YELLOWING 1)
Tf0085	SI094:SI211	CD573622	Pt	AT5G03415.1	DPB
Tf0087	SI166:SI271:SI287	CD573723	Pt	AT4G18020.2	APRR2
Tf0088	*SI066*	CD573726	Pt	AT1G27660.1	ethylene-responsive protein -related
Tf0092	SI099	CD574584	Pt	AT4G32880.1	ATHB-8 (HOMEOBOX GENE 8)
Tf0094	SI006	CD574865	Pt	AT1G69580.2	transcription factor
Tf0143	SI127	DC885186	Cu	AT5G24120.1	SIGE (SIGMA FACTOR E)
Tf0144	SI125:SI189:SI192	DC885340	Cu	AT3G19860.2	basic helix-loop-helix (bHLH) family protein
Tf0148	*SI119*	DC885880	Cu	AT5G13080.1	WRKY75
Tf0149	SI128:SI269	DC886255	Cu	AT3G04730.1	IAA16
Tf0150	*SI262*	DC886590	Cu	AT1G03840.1	MGP (Magpie)
Tf0151	*SI159*:SI281	DC888225	Cu	AT4G24440.2	transcription initiation factor IIA gamma chain
Tf0164	SI251	DC889433	Cu	AT2G19260.1	ELM2 domain-containing protein
Tf0166	SI156	DC890056	Cu	AT5G13960.1	SUVH4 (SU(VAR)3-9 HOMOLOG 4)
Tf0167	SI230:SI241:SI247	DC890115	Cu	AT1G08540.1	SIG2 (RNA POLYMERASE SIGMA SUBUNIT 2
Tf0168	SI175	DC890126	Cu	AT3G43240.1	ARID/BRIGHT DNA-binding domain-containing protein
Tf0170	SI116	DC891569	Cl	AT3G12680.1	HUA1 (ENHANCER OF AG-4 1)
Tf0177	SI133	DC900310	Cu	AT4G31420.1	zinc finger (C2H2 type) family protein
Tf0199	SI023:*SI157*	DC900398	Cu	AT1G54610.3	protein kinase family protein
Tf0201	SI195	BQ625052	Cu	AT1G16060.1	ovule development protein putative
Tf0203	*SI121*	CB291458	Cu	AT5G44180.1	homeobox transcription factor putative
Tf0205	SI274	CB292412	Cu	AT1G04850.1	ubiquitin-associated (UBA)/TS-N domain-containing protein
Tf0208	SI181	CX546428	Pt	AT3G02380.1	COL2 (constans-like 2)
Tf0210	SI183	CD574434	Pt	AT2G02080.1	AtIDD4 (Arabidopsis thaliana Indeterminate(ID)-Domain 4)
Tf0212	SI196	CD574499	Pt	AT1G13220.2	LINC2 (LITTLE NUCLEI2)
Tf0214	SI020	CD574660	Pt	AT4G00050.1	UNE10 (unfertilized embryo sac 10)
Tf0219	SI076:SI267	CD576023	Pt	AT3G57800.2	basic helix-loop-helix (bHLH) family protein
Tf0221	SI057:*SI225*	CF418134	Cs	AT5G66055.1	AKRP (ANKYRIN REPEAT PROTEIN)
Tf0230	SI140	CF509456	Cs	AT5G55760.1	SRT1 (sirtuin 1)
Tf0232	*SI258*:*SI261*	CF509665	Cs	AT2G17900.1	SDG37
Tf0238	SI114	CF653350	Cs	AT2G17730.1	zinc finger (C3HC4-type RING finger)
Tf0240	*SI184*	CF828177	Cs	AT1G04600.1	XIA (MYOSIN XI A)
Tf0243	SI231	CF829056	Cs	AT2G37060.3	NF-YB8 (NUCLEAR FACTOR Y SUBUNIT B8)
Tf0245	SI021	CF830462	Cs	AT3G15510.1	ATNAC2 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 2)
Tf0251	SI005	CF831040	Cs	AT5G23150.1	HUA2 (ENHANCER OF AG-4 2)
Tf0257	SI049	CF832981	Cs	AT1G63650.2	EGL3 (ENHANCER OF GLABRA 3)
Tf0258	*SI234*	CF833637	Cs	AT1G19700.2	BEL10 (BEL1-LIKE HOMEODOMAIN 10)
Tf0263	*SI013*	CF835628	Cs	AT3G24520.1	AT-HSFC1

[a]Italics indicate 'Not Reliable' SNP. [b]Cu: *Citrus unshiu* Marc., Pt: *Poncirus trifoliata* (L.) Raf., Cn × Ck: *C. nobilis* Lour. × *C. kinokuni* hort. ex Tanaka, Cs: *C. sinensis* Osbeck, Cl: *C. limon* (L.) Burm. f.

Continued

STS name	SNP name[a]	DDBJ EST accession number for STS primer design	Species of EST derived[b]	Assigned *Arabidopsis* locus	Annotation of *Arabidopsis* locus
Tf0265	SI197	CF835803	Cs	AT3G61150.1	HDG1 (HOMEODOMAIN GLABROUS 1)
Tf0279	SI073	CK665314	Cs	AT1G63650.1	EGL3 (ENHANCER OF GLABRA 3)
Tf0280	SI158:*SI200*	CK665679	Cs	AT4G36920.1	AP2 (APETALA 2)
Tf0289	SI207	CK933416	Cs	AT4G23860.1	PHD finger protein-related
Tf0301	SI182	CK934342	Cs	AT4G16780.1	ATHB-2 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 2)
Tf0302	SI105	CK934596	Cs	AT3G03750.2	SET domain-containing protein
Tf0303	SI082	CK934654	Cs	AT5G04240.1	ELF6 (EARLY FLOWERING 6)
Tf0309	SI190	CK935320	Cs	AT2G47900.1	AtTLP3 (TUBBY LIKE PROTEIN 3)
Tf0317	SI145	CK936562	Cs	AT3G47640.1	basic helix-loop-helix (bHLH) family protein
Tf0319	SI152	CK937012	Cs	AT5G20510.1	AL5 (ALFIN-LIKE 5)
Tf0320	SI203	CK937275	Cs	AT2G42830.1	SHP2 (SHATTERPROOF 2)
Tf0321	SI003	CK937318	Cs	AT1G69310.2	WRKY57
Tf0323	SI030	CK937389	Cs	AT1G69490.1	NAP (NAC-like activated by AP3/PI)
Tf0326	SI085	CK938083	Cs	AT2G23740.1	nucleic acid binding / transcription factor/ zinc ion binding
Tf0328	SI039:SI180	CK938765	Cs	AT1G59640.1	ZCW32
Tf0330	SI064:SI139	CK938961	Cs	AT5G48150.2	PAT1 (phytochrome a signal transduction 1)
Tf0332	*SI081*	CK939458	Cs	AT1G77450.1	anac032 (Arabidopsis NAC domain containing protein 32)
Tf0334	SI034	CK939708	Cs	AT5G63470.1	NF-YC4 (NUCLEAR FACTOR Y SUBUNIT C4)
Tf0335	SI014:*SI168*	CK939747	Cs	AT2G40815.1	unknown protein
Tf0339	SI019	CK940145	Cs	AT1G69490.1	NAP (NAC-like activated by AP3/PI)
Tf0342	SI151	CN182953	Cs	AT5G41370.1	XPB1
Tf0345	*SI011*	CN183639	Cs	AT5G60450.1	ARF4 (AUXIN RESPONSE FACTOR 4)
Tf0348	SI118	CN185079	Cs	AT3G53340.1	NF-YB10 (NUCLEAR FACTOR Y SUBUNIT B10)
Tf0350	SI132	CN185280	Cs	AT5G23050.1	AAE17 (ACYL-ACTIVATING ENZYME 17)
Tf0351	SI122	CN185598	Cs	AT3G23240.1	ERF1, ATERF1
Tf0353	*SI087*	CN186263	Cs	AT1G09770.1	ATCDC5 (ARABIDOPSIS THALIANA CELL DIVISION CYCLE 5)
Tf0354	SI070	CN186267	Cs	AT1G01520.1	myb family transcription factor
Tf0356	SI134:*SI160*	CN186308	Cs	AT3G51960.1	bZIP family transcription factor
Tf0357	SI018	CN186402	Cs	AT5G66730.1	zinc finger (C2H2 type) family protein
Tf0358	*SI098*	CN186577	Cs	AT5G48150.2	PAT1 (phytochrome a signal transduction 1)
Tf0360	SI112	CN188939	Cs	AT1G76880.1	trihelix DNA-binding protein putative
Tf0362	*SI062*	CN189405	Cs	AT1G20700.1	WOX14 (WUSCHEL RELATED HOMEOBOX 14)
Tf0363	SI002:SI096	CN189628	Cs	AT3G61150.1	HDG1 (HOMEODOMAIN GLABROUS 1)
Tf0368	SI102	CN191477	Cs	AT3G04070.1	anac047 (Arabidopsis NAC domain containing protein 47)
Tf0373	SI088	DC901064	Cu	AT3G10030.1	aspartate/glutamate/uridylate kinase family protein
Tf0376	*SI072*	DC898619	Cu	AT4G25470.1	ATCBF2
Tf0386	SI044:SI095	CD573987	Pt	AT3G02380.1	COL2 (constans-like 2);
Tf0396	SI035	CD575233	Pt	AT3G57800.2	basic helix-loop-helix (bHLH) family protein
Tf0397	*SI008*	CF417964	Cs	AT3G30530.1	ARABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 42 (ATBZIP42)
Vs0002	SI103:SI115	C21910	Cs	AT1G75310.1	AUL1
Vs0003	*SI077*	C21853	Cs		no homology
Vs0005	SI022:*SI075*	C21850	Cs		no homology
Vt0032	SI024	DC900011	Cs	AT3G24240.1	leucine-rich repeat transmembrane protein kinase putative
Wy0015	SI041	DC894276	Cu	AT2G36790.1	UGT73C6
Wy0016	*SI068*	DC895604	Cu	AT4G15270.1	glucosyltransferase-related
Wy0019	*SI031*	DC894422	Cu	AT2G26580.2	YAB5 (YABBY5)
Wy0020	SI007	DC894174	Cu	AT1G04410.1	malate dehydrogenase cytosolic putative
Wy0023	SI037	DC895741	Cu	AT2G41680.1	NTRC (NADPH-DEPENDENT THIOREDOXIN REDUCTASE C)
Wy0024	*SI091*	DC894238	Cu	AT3G27890.1	NQR (NADPH:QUINONE OXIDOREDUCTASE)
Wy0025	*SI004*	DC895710	Cu	AT5G13180.1	ANAC083 (ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 83)
Wy0034	*SI367*	DC894870	Cu	AT4G36750.1	quinone reductase family protein

[a]Italics indicate 'Not Reliable' SNP. [b]Cu: *Citrus unshiu* Marc., Pt: *Poncirus trifoliata* (L.) Raf., Cn × Ck: *C. nobilis* Lour. × *C. kinokuni* hort. ex Tanaka, Cs: *C. sinensis* Osbeck, Cl: *C. limon* (L.) Burm. f.

Table 4-2 *Citrus* plant materials for the GoldenGate Assay[a].

Sample ID	Plant material	Seed parent	Pollen parent	Sample ID	Plant material	Seed parent	Pollen parent
TY1	No.960203	TY15	TY77	TY54	Clementine mandarin		
TY2	No.971594	TY32	TY17	TY55	'Shiranui'	TY52	TY73
TY3	No.971614	TY32	TY17	TY56	'Setoka'	TY22	TY74
TY4	No.980389	TY8	TY37	TY57[c]	'Tamami'	TY52	
TY5	2700OIy-25		TY49	TY58	'Dancy' tangerin		
TY6	E-647	TY52	TY50	TY59	'Trovita' orange		
TY7	EnOw21	TY47	TY75	TY60	'Nankou'	TY75	TY54
TY8	HF15	TY75	TY59	TY61	'Nishinokaori'	TY52	TY59
TY9[b]	HF9	TY75	TY59	TY62[de]	'Kankitsu Chukanbohon Nou 5 Gou'	TY78	TY76
TY10[b]	HF9	TY75	TY59	TY63	'Kankitsu Chukanbohon Nou 6 Gou'	TY53	TY76
TY11	HF24	TY75	TY59	TY64	'Hayaka'	TY75	TY73
TY12[b]	HF9	TY75	TY59	TY65	'Harumi'	TY52	TY73
TY13	HF9En-29	TY12	TY47	TY66	'Harehime'	TY6	TY75
TY14	JHG	TY75	TY68	TY67	'Hareyaka'	TY47	TY73
TY15	'Kuchinotsu 18 Gou'	TY30	TY47	TY68	'Hyuganatsu'		
TY16	'Kuchinotsu 26 Gou'	TY7	TY77	TY69	'Hirado' buntan		
TY17	'Kuchinotsu 27 Gou'	TY7	TY77	TY70	'Page'		TY54
TY18	'Kuchinotsu 33 Gou'	TY29	TY47	TY71	'Benibae'	TY12	TY47
TY19	'Kuchinotsu 34 Gou'	TY29	TY47	TY72	'Benimadonna'	TY60	TY45
TY20	'Kuchinotsu 35 Gou'	TY30	TY47	TY73	Ponkan-F2428		
TY21	'Kuchinotsu 36 Gou'	TY27	TY74	TY74	'Murcott'		
TY22	'Kuchinotsu 37 Gou'	TY52	TY47	TY75	'Miyagawa wase' satsuma mandarin		
TY23	'Kuchinotsu 38 Gou'	TY30	TY80	TY76	'Mukaku Kisyu'		
TY24	'Kuchinotsu 40 Gou'	TY30		TY77	'Youkou'	TY52	TY73
TY25	'Kuchinotsu 49 Gou'		TY37	TY78	'Lee' mandarin	TY54	TY90
TY26	'Kuchinotsu 50 Gou'		TY37	TY79	'Reikou'	TY27	TY74
TY27	KyEn5	TY52	TY47	TY80	'Robinson'	TY54	TY90
TY28	KyEn5/En-6	TY27	TY47	TY81	No.990343	TY22	TY71
TY29	KyOw14	TY52	TY75	TY82	'Setomi'	TY52	TY73
TY30	KyOw21	TY52	TY75	TY83	'Sweet Spring'	TY75	TY101
TY31	KyOw21/Cc-33	TY30	TY54	TY84	'Seminole' tangelo		TY58
TY32	KyOw21/D-4	TY30	TY58	TY85	Shikaikan		
TY33	LeeAo35	TY78	TY75	TY86[e]	'Okitsu 46 Gou'	TY83	TY59
TY34	LeeAo9	TY78	TY75	TY87	'Kara' mandarin	TY75	TY53
TY35	No.1010	TY60	TY5	TY88	'Okitsu 60 Gou'	TY86	TY65
TY36	No.1011	TY60	TY5	TY89	'Temple'		
TY37	No.1408	TY7	TY38	TY90	'Orlando'		TY58
TY38	No.2681	TY52	TY49	TY91	'Southern Red'	TY87	TY73
TY39	'Okitsu 45 Gou'	TY52	TY48	TY92	'Okitsu 56 Gou'	TY39	QT89
TY40	'Okitsu 57 Gou'	TY86	TY65	TY93	'Okitsu 47 Gou'	TY52	TY48
TY41	'Okitsu 58 Gou'	TY86	TY65	TY94	'Akemi'	TY52	TY84
TY42	M5	TY52	TY80	TY95	'Okitsu 55 Gou'	TY86	
TY43	'Aki' tangor	TY75	TY59	TY96	Mediterranean mandarin		
TY44	'Amaka'	TY52	TY47	TY97	'Kinnow'	TY53	TY96
TY45	'Amakusa'	TY29	TY70	TY98	'Bakamikan'		
TY46	'Ariake'	TY59	TY54	TY99	'Kincy'	TY53	TY58
TY47	'Encore' mandarin	TY53	TY96	TY100	'Pixie'		
TY48[c]	'Tamami'	TY52		TY101	Hassaku		
TY49	'Otani-iyokan'			TY102	Tankan-T132		
TY50	'Osceola'	TY54	TY90	TY103	'Kuchinotsu 39 Gou'	TY47	TY75
TY51	'Kanpei'	TY61	TY73	TY104[de]	'Kankitsu Chukanbohon Nou 5 Gou'	TY78	TY76
TY52	'Kiyomi' tangor	TY75	TY59	QT1-88[f]	AG population	TY86	TY104
TY53	'King' mandarin						

[a]Illumina, Inc. [b]Repeat of plant material; HF9. [c]Repeat of plant material; 'Tamami'. [d]Repeat of plant material; 'Kankitsu Chukanbohon Nou 5 Gou'. [e]Parents of mapping population. [f]Hybrid population including 88 individuals.

Table 4-3 Comparing the success rate of the GoldenGate® Assaya using a 384 SNP genotyping array, CitSGA-1, among three sources of SNP discovery.

The method of SNP discovery	(a) Number of candidate SNP (STS)	(b) Number of assayed SNP (STS) Percentage of SNP selected for CitSGA-1 (b/a%)	(c) Number of invalid SNP that call frequency score was under 0.9[h] (c/b%)	(d) Number of monomorphic SNPs (d/b%)	(e) Number of SNP including discrepancy in parentage (e/b%)	(f) Number of SNP including "No Call	(g) Number of relaiable SNP (b - c - d - e - f) Percentage of relaible SNP (g / b %)
Detection form eight citrus[b]	1174 (332[e])	286 (219[g]) 24.4% (66.0%)	11 3.80%	24 8.30%	22 7.70%	17	212[i] (169) 74.1% (77.2%) 18.1% (50.9%)
Detection form two citrus[c]	277 (84[e])	82 (63[g]) 29.6% (75%)	4 4.90%	12 14.60%	6 7.30%	3	57[j] (44) 69.5% (69.8%) 20.6% (50.4%)
Detection by *in silico*[d]	46 (18)	16 (16) 34.8% (88.9%)	0 0.00%	7 43.80%	1 6.30%	0	8[k] (8) 50% (50%) 17.4% (44.4%)
Total	1497 (434[f])	384 (283[f]) 25.6% (65.2%)	15	43	29	20	277[l] (221) 72.1% (78.1%) 18.5% (50.9%)

[a] Illumina, Inc.

[b]Clementine (*Citrus clementina* hort. ex Tanaka), 'Miyagawa wase' (*C. unshiu*), 'Trovita' orange (*C. sinensis*), 'Duncan' grapefruit (*C. paradisi*), Kishu mikan (*C. kinokuni* hort. ex Tanaka), Ponkan (*C. reticulata*), Mediterranean mandarin (*C. deliciosa*) and a haploid derived from Clementine (Oiyama and Kobayashi. 1993).

[c] 'Okitsu 46 Gou' and 'Kankitsu Chukanbohon Nou 5 Gou'.

[d] *in silico* SNPs were detected in aligned ESTs derived from *C. sinensis* and *C. unshiu.*

[e] Including 15 repeats.

[f] Excluding repeats.

[g] Including 15 repeats.

[h] Invalid data criteria according to manufacturer's criteria.

[ijkl]each figure has SNP repeats: repeats whithin i is one, repeats between i and j is five, repeats between i and k is one, repeats within k is one. Totally l has nine repeats.

Table 4-4 STS sequences including SNPs and ratio of heterozygous loci in SNPs, and the result of BLASTN analyses of SNP sequences against *Citrus clementin*a scaffolds derived from Citrus Genome Database [http://www.citrusgenomedb.org/].

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]	
SI001	8	TATCAGCTCCATCAAAACTAAATCCATTTGGTTCAAGATCTAGG[C/G]CTTGTGCTCTCTCTTTATC ATGGTCAACATCAAATACAGTGTCATAAAGTCCTACAGGAG	59.2	19	**
SI002	8	CCAAAATCAGCAGGCAATGTTGTGGTCACGGTACTGCTTAAACCACCAAAACCATTAATG[T/G]T ACCAACACCAAGTTCTAAACTTGAGTTTGGCATTGGAGGAGGACCCATTGA	4.1	3	**
SI003	8	AAAACAGAGGATTACATCAGAAACCGTATTAAATCCGTCAAC[C/G]GTTTGTCTGACTCAATTCT TTCTGTTGTCGTTTCTGTAACGGGTGCAAATG	46.9	39	**
SI005	8	GCACGTGTTTGCTCTGCTGGTGATGCATGACCATCTTC[A/G]TTAAATGTAATGCTAGGAAGATCT TCTTCTTCATCATCTTCAAATACATGAGAAGACAAA	36.7	10	**
SI006	8	TTTGGTGCTCTTGGTCTTACGGGAAGAAAAAAGGAAAAGATTTTTTTTCTGAGTGTATAC[A/G]C ATTTGCTTTCTTGTTGCAGAAATACAGACTGGGGAAGAGCCAACATGTGGAAGCCTGCA	35.7	39	**
SI007	8	AATAACTCAACCAAAAAAAATCACCAAACTTTACTATTAATCAAGGGGAGAAAAATATTT[A/G]A GTTTAATACCATGCATCATCCTTGACAAGCTCACGGACAGGCTTTTCCCCAGCTGCAGT	55.1	27	**
SI009	8	CCTGTACGGATACATAACATGTAAGCATCATCAAAAGAGGGGCGAAATTTTCTGTTCATA[T/C]AA CCAACCAATAACATTAGGAC	51	6	**
SI010	2	GCGAAGTGCTTCAGCTGCACTGCCAATATTTTCCTGAAATTCGCTGGGAGCTCATC[T/C]TTGTG CTTCTCTTCCATGTACTTGGCTATTGCGTATGGGCTTGAACCGCTCTTATCTTGC	56.1	25	**
SI012	*in silico*	ACAAAAGACACCGAAACTTTGTGAATTAATCTACACAGACAATAATTAAGCTTTCTCAAA[T/G]T CATTGCCAGGTTGCTGCTGCTGCATAGCCATGCCCAGGCCTTCATTTC	48	67	**
SI014	8	AGACGCGGCGCTGCACGTTGAAGTCTATAGCAGAGAGCCTATTTTTCTTAGGGAAAGGCT[T/C]C TGGGAACCGCAACTATTGCCTTGAAAGAATTTTTGGCCAAGTATAGTAAAAATTCTGAG	32.7	16	***
SI015	2	CTGGTAGATGCAGCTATCCTGCAATTCAACTGTGCCAAAATATCAAATTCAAGCGACACA[A/C]A TACATATTCATACGTTGTTTTCATAATTTATAT	38.8	21	**
SI018	8	ATCAAAGACCTGGAAAATACAGCCCCACAAT[T/C]GCATTTGTACTCTTTAGTGCCACAAACTTT GGAGTGTGCTTTATAATCAGACTGCAC	25.5	25	**
SI019	8	TTTGCAAATTATGTATGAAAATGTAGAGAAAGCTGAATTCGG[A/G]GAGAAGGAATGGTACTTTT TCAGCCCACGAGACAGGAAGTATCCCAATGGGACGAGGCCT	41.8	39	**
SI020	8	AAAATGCTTAGCTCTTAGAACTTGAA[C/G]CTGGGGGCTGCTGCTGCATCTGCTGATACATTGCA GCCATTCTGTTATAAGCATCCATGC	3.1	3	**
SI021	8	TACCGGAACAGATAAGCCAGTGATGAGTTCAGGGAGTAATCAAAAAGTTGGTGTGAAAAA[A/G] GCACTTGTATTCTATGGAGGCAAGCCTCCTAAAGGTATCAAGACCAATTGGATCATGCAT	1	69	***
SI022	2	TTGAACAAAATGGCTCCCTTCAGGCAAATGTATGATCTCCAAATTGGGCACAAGGTCTTT[T/C]G CTTTGCCGCTTCTTATGTAGTCCTCAATCCCTGGAAATTTGAGGAAATAGTCTTTGTC	56.1	19	**
SI023	8	GAAGGTTAAATGTTACATGCATCAACTGCTATCTGGACTTGAGCACTGTCACAACAATGG[T/C]G TGCTTCACCGGGACATTAAAGGCTCTAATCTTCTAATTGATGATGATGGAGTGCTTAAA	15.3	1	***
SI024	8	CAGGCAAGCACTCGCTACATGCAAGTGTTGAAGAAGCTCAATCTCGATGTTTGGATCCTC[T/C]T TCATGAGTTCTGGATCAAAGACATCGCTTATTTTCAGCTTCGCATGCTGCTTTACCCAC	31.6	24	***
SI025	8	ACACTAACAATCGTCCATGGATCAGATGCATTCCAATGGAAGTCAACAACTTTGTCTCTG[A/C]A TCATATGCAAAAAAAATACTAAATAGTAAGTGACAATCTTATGAAATTCAGCAATAAAG	20.4	32	**
SI026	8	TCCTCCAAGACCATGATAAAAGCTCATCACGAAAAAACATGCCTAAATGATACCATAGAT[A/G]C TCACTTTGCTGCAACATTACAAAAAGAGAAAAAGCCAATCAAAACTTCTATAACACCAG	56.1	25	***
SI027	8	CTTTTTATCAGTTTTTGACTTTTAGCATAAGAATCTGAACTTTGTTTGACCACATCAAGC[T/C]AC TGAGTTTTCTATTTTGTGACCTCTAGTTCTTGGTTTTTGAAATACAGGGTCAAAACGT	45.9	12	***
SI028	8	AATCTTGATTTTAGATCTATTAGTTATATAGGAGTAGCAGTTTCTTTTCATTCTTCTGCC[T/C]GGCC TATTCTTTGCAGAATTGTTGGTTTGAGGTACTGAGATTGTTATGTCCTTTTTTTTC	56.1	2	**
SI030	8	TTGTTAGTAATGATTGTGTTTGCAAATTATGTATGAAAATGTAGAGAAAGCTGAATTCGG[A/G]GA GAAGGAATGGTACTTTTTCAGCCCACGAGACAGGAAGTATCCCAATGGGACGAGGCCT	22.5	39	***
SI032	*in silico*	AAGAGTGGATCTGGGAAACCACCAGCTGCATGTTGCAGTGGAGCCTCAGCTCTTGCATCT[A/G] CTGCAACAAGCACTGCTGATAAGAAAACCGCTTGTGGATGCATTAAGACAGCAGCCCAGA	41.8	151	***
SI033	8	GAAATGAATGGAATGGCAGTTTCGTAATTTCGGAGGTCCTGTTTGGTTGTTTTGGGTTTC[A/C]A ATTTGATTTTTTTGAGGTTTTTGTTTGTTAATGATGATGTTATGTCTTGGTAAATCTTC	3.1	33	*
SI034	8	CGCTATATCATTCTTTTGCAGTGTTCTCCTTTTATTCTCTTCTGTGTGGATCCAAGACCG[T/C]AGA GTTAGTTCCAAAATAAACATCTCACAAGCCTTGGCAAATATGACAGGGGCCTCTGCT	4.1	6	***
SI035	2	GTTTGCCTTTAAAACCTGTACAGGG[A/G]AGTGGGTCAGACTAGGACCACCCTATTTTACCTGCT CAAATTTACACTTCCGACAATCAA	61.2	6	**
SI037	8	AAAACAAGTTTCAGTTCCACTTGTTTTATTTTAGGTAACCACCCATGTATTCTACCATCT[A/C]AA AATCCTACATAAGTTCTGAGTCCCATAGTATACCGCCACCAAA	51	25	**
SI038	8	TAAATCTTTTTTGCTTTTATTTGTTTTCT[T/G]TCTTTTTATGATTTTCCTTGGTTTTGATATGTAATT TGTTGATTTTTTCCTGTTATTTTT	57.1		-
SI039	8	GAATGGGGAATATGGGTCATGGGTTCGGGGAAAGTTCGGCTCTCAGAGATGGGTCGATGG[A/G] GGAATCAACAGTGACTGAGCAGAGTGGTGGTGGGTGTGGCAGAAAGAGAAGGGATTTGAG	55.1	12	***
SI041	8	TGTAACGGAATTTTATTTAACCACGAATCGCCTAGACG[T/C]GGCGAAAATTTTGTTACTAGAAA AATTACGCGTGCGGTTGGGAGGATCAAGATAGGATTG	35.7	25	**
SI042	2	ATGTACACGGGTCAGTTCGTGTACTGCGGCAAGAAAGCCACTCTGATGGTTGGTAATGTG[T/C]T GCCCGTTAGATCTATCCCTGAAGGAGCTGT	45.9	25	**
SI043	8	TTGTATCATCCTAATCTTGTAAAATTGATCGGCTATTGCTTAGAGGATGACCACCGGCTT[T/C]TGG TGTATGAATTTATGCCCAAGGGCAGCTT	16.3	16	**
SI044	2	ATCAGTCATCAATGTTGCGGAGAACATTTGATCAACTTCAACTTCAGCATCGGTTCTCTT[A/C]GC GAACCTGCCCCTGATACGTGGTCTAGTTTCCGCATAGGCC	33.7	80	**
SI045	8	CCAACACTTGACGGAGCTCTAATCTT[T/G]ATCCCTGCACGTTTGATGTATTTGCTAAA	65.3	3	*
SI048	8	AAGCAACAACAACATCCACAGAGACCATCCCAACAACCCCAGTAGAAACTAAGGTCCAAA[T/C]CAAATCTCTCTCCCTCTCTACATATGTGTGTTTATGTGTACTGTTTC	27.6	10	*
SI049	8	TCTCTAAGATGGACAAAGCCTCTCTGCTTGGTGATGC[T/C]ATTACCTATATCACTGACCTCCAGA TGAAAATCAGGGTTTTGGAGACAGAGAAGGATATG	5.1	3	**
SI050	2	TACTCGTTAATCTTATTTCCCTTTGCTTCTTCTAATTAATCAAATTTGATGGGGGTCTTT[T/C]GTTT TGATTGATTGCAGATAATTATTAATGCTCCATTTTGGTACTATGCTTTGAATGCTC	59.2	15	***
SI053	8	CAGCAACCTCCGGATGGGACAGAAGCAAGTGTTCTAGTTCTGCAGGGGCAACCTACACAA[T/C] GTAACAACAACGGAAATGGAAATGGAAATATTAGCTGTATGATCTTAACAAACCAAATCA	5.1	5	***
SI055	2	GTTGGCTCACTTGGCTGGTACACAATGTGCTGTCAAGTTCTACATATTGACAATTGATT[T/C]GCA GCACAGAAATCTTCATAAACTTCTGTAAAAAGCACACTAACAAGCTCAGCCACAGAC	54.1	47	**
SI056	8	GCTGTAAACGGCTCATCAACTCGGTCTGCTTCCGTGTCGGCGAGATCCAACGGTTGTCGG[A/G] CGTATTCGTCATCTTCCAGAGGCATGCAATTCAGGAAACTTTCTGGGTGTTATGAATGTC	36.7	22	***
SI057	8	TTTTTTATTTATAACTCTTAAAATGATTCAATACCAA[T/G]AAGTGGTTTCTGCAGTTTAATCTTAG GTCAGATTGAGTAGTTAGTTAGAGCTGGAATGTT	32.7	25	**
SI058	8	TAGAATTTTGTCTTTTGAATCAGGACTATAATTGTATCCCTATGAATTATCAATTATGAT[A/G]ATAA TACTGCGATAAGGGGATCCAAGAA	19.4	63	**

Continued

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]	
SI059	8	TATGCCTATGGTAAGTTATTAAATAGTCTT[T/C]AGTGGAATTACATACTGTGGTTGGCCGCATAAC TTGTTGATGAAAAGCTTGAAACATGCT	42.9	2	*
SI060	8	GGTTGCACTGAAGCAGCAACGGAATTGAATGATTTAGAATGCTTCATCATCCCAACTCGA[C/G]C TCTACATAAATTTGTCCTCAATTTCAATGAAAATTTCGGAATAAAAACAGAGTGTTGTT	64.3	29	***
SI062	8	TATATACTGTGACCCATTAATGACATCTGGTGGTGGGCACAAGATCAGTGCCAGACAGAG[A/G]T GGACTCCAACTCCAGTTCAGCTTCAAATTCTGGAGAGCATTTTGATCAAGGGACTGGGA	52	25	**
SI063	8	TCTCATGACCAGTTGACCACGATCCCA[A/G]CCACTGTCCAAATGGTCTTTCTGTGCGAAATTCT CTTCATCAAATATAATCTGATTCCTT	58.2	6	**
SI064	8	TCCACCAGGCCGTTTTGCAAGAGCATGGAGGAGGAACATCCACTGGGTGCCCTGAGCAAT[T/C] TGGAAATCAATGATGTGAATTATATGTTCATTCTCCACTGCTTCCCCAATGACGACATTT	12.2	19	***
SI065	2	GTCTGCTGCGGCCTCTCAACAGTTGAGTGTTGA[T/G]CAGCCTGAGCCCCAAGCCAAGGGGCCT ACCAGGTGCTTGAGTTGCAACAAGAAGGTCGGA	55.1	2	**
SI067	2	TCAAGAGATAAATCCCTTGGCCTTCAAACTCTCGA[T/C]AGAGTCCTTGATGATTTGATCCATGG GAATGAATTGTAAACCCAAGTCCATTAGCTTTTT	57.1	8	**
SI069	8	CATGCCGGGATGAAGATACTCAG[A/C]TCCAAGGGAGAACAAATCAACAAAATGAAAAACCACA TAAAAGCAAAAAATTTAGAAGCT	58.2	8	-
SI070	8	TAAGCAATCTTATTGGCAGATACGAAGTCATGCACAAAAGTACTTTCTAAAGGTTCAGAA[A/G]A ATGGGACAAGTGAACATGTACCTCCCCCTCGACCAAAGAGAAAAGCAGCTCATCCATAC	2	3	***
SI071	8	AGATAAGAAGGAGAACGAGGAACATTGAATAACCTGCAATGGAGGTATGTTGTCTGCTGC[A/G] AATAACACCACTCGCGCAAGTTTCAAATCATGATGTGAGCTCAAAACACATTGCACAAAT	34.7	95	***
SI073	8	AACTTATATTTTCACCTTTCATTTGCAATTTTTTTCCTCTTATACATAGCATTCTTATCA[T/C]ACGG GGGTTTTAATTAACATGGTCACCTTCCACTTTGTCTTGTAGTTTG	37.8	3	**
SI074	8	AAAAAGTGAGAGCAATAACTGGAAAGGAAATGCATG[A/G]TTATGATACCTCAGGAACAGAGAG CTCAAGACGAAATTTAGGACCATCGTAGTGATGCAA	2	5	**
SI076	8	ACGAGTCTTTACAGACATAACTACAACA[A/G]TCAAGTGCATTTAGCTCAGGCCATGACAATCAT TTTGATTGTCGGAAGTGTAAATTTGAG	61.2	6	**
SI077	2	AAGCTCCACTTGCCTAGCACAACCTCCATAATGTCTAGCTTGAGGTTGTATGGA[T/C]AGCAGCT ACAGATGAGACCATGGAGTCGACCCCACAGACATTGCGACCCATGCAGATCTT	50	22	**
SI078	2	CTACTTTGAAATCTCTGTGTAATTTTAAGCTTAATTTCGGTGTTTTTCCAGTTGTCTTCA[T/C]TGT AATTTTGGTGTTAAAATATTCTAGTATAATAATAAAATTAGGTCATAGAGTTCGGGC	62.2	2	**
SI079	8	GAGTTTCCTATTTTCTGATCTTTACTGTTGACCAAATTGGAACCTCAATTCATAAC[A/G]AGGTGA TCCATTTGTATGTTGTGCATGTGTAAGACTAAAGTCAATTTTCCTATACCTGTA	43.9	32	**
SI082	8	CCTACTTTATCCGATAATTTGCC[A/G]TCACTCTCAGCTATTTCCTGCCTTTTCTTCT	23.5	114	*
SI083	2	AGTCAAGAAACAACCTCAAAGATGATAGGAATTAAGGGCCAACATGA[T/C]GACCCACGCTTGT CAAGATTGGTCTCAAATGCAAGTTGAGCCGCACAACCAACCAGAAAT	46.9	32	**
SI084	8	CGCAAAAGAGGTCCCCGCCTTCGAATACACGTCTCGGAGCGTTGCGCATTTGTACAACCT[C/G] GCAACTCTCTCGCAGGCCAAAACTAAAGCCGCCAACATCGTCGCCGCCGATTTTCGCCAA	55.1	13	-
SI085	8	CTAATAXACATTACTCAAGCTACCCTTTAGTAATTTTGTT[T/G]GAAGCTGTAAAATTGTACTTTTT TAGGCGGTGGATTATGGAGATTGTTATGTTCTCATGG	42.9	25	**
SI086	8	AGGCGCTACTCTTTACTTTGTTAT[A/G]AGTGGAATCCACCAAATTATTTAGTTGTTCGTTAAAAG AACATGAAAACTATCACGAGTA	46.9	22	**
SI088	8	AGCTATATGTGGAGACCAAGTTGGTACATTTATTGATCGGACAGGAAGAATGAGCTAGTA[T/G]G TGGTTCTTTACTTGGTATGACCTCTGACTGTGTCTGTTATGGTTCAGTGGACTCT	54.1	47	**
SI089	8	GCAATCTGCTGGCCGGAGTGTCTTTGAGATGGAAATGGCTAGGATTTTTCAAGACCCCTC[A/G]A AGTCAATGTGGGAGAGAAGGGAGTGGATTAAGAAGCAGAAGTTGGCACTTCAGAATCAA	13.3	20	***
SI092	8	GGCAGCAGAGCGTTAGCTCGATGTCCTGTCGGAGTATCCTC[A/G]TCCTCCTCTCCGCTGCCCCC TCCCATTTGGTAAGAGCTGCATGCATGCAGCGTATTAGAA	14.3	29	**
SI094	8	GAATTAGGATTTCAATAAATAAGAAGTAAGACGGATGCCAATTATTTGAGAAGCTGACAT[A/T]TT GAGTACCTCATGTGGAAATCAGTTCCTACATGCTTTATCTTCCCTCTGATTGGATATG	51	42	***
SI095	2	GCGAACCTGCCCTTGATACGTGGTCTAGTTTCCGCATAGGCCTTCCTTGAGGCATACC[T/G]GATT GTCTTCTCGAATTTTCTTGTCTTCTTTTTTTCCCTGTATCTCAAGACTCTGGCCTC	53.1	80	**
SI096	8	ATCCATGGCAGCCAAAGCAAGCTCCAAAAACATTGATCTTTCAATGGATCTATCGAGGCC[T/C]G TAACGCCGGGACCTGATCTGTTCGGAGGCATAACAACTGGCAAGGCATTAGAAATCCCA	18.4	3	***
SI099	8	TATGCAAGTACTAATAAATATGAACATCGTTTG[T/C]TGTTTGTCTCACCATGGGACTTTTT	6.1	9	*
SI100	8	TATTGTAGGGAAGAGACTGTCGGAGTTTGCTGCATCCTGCAATGTCCCATTTGAATTCCA[A/T]GA TACTGCCATGACTGCTTGTGAGGTTGAACGACAACATCTAAGGGTTCAACCTGGGGAA	59.2	19	***
SI102	8	CAAGGCTGCCTTCGGTGAGAAAGAGTGGTATTTTTTCAGTCCTAGAGACCGAAAGTACCC[T/G] AATGGAGCCAGGCCGAACAGGGCAGCTGCATCCGGGTATTGGAAGGCGACAGGCACGGAT	59.2	98	***
SI103	2	CTCTATTCATGTGAATTATCATAGATCAGCTGATCTTTATCTGCCACTACTGGTCCCCTC[A/C]TTC CCAGTCACAATTTCTTTATAAAGAGATGTTACCTTCCAATGAGATAAATTTTGCATA	46.9	22	***
SI104	8	TGCTTTTTCTTTAAGTGCATGCCTT[T/G]TTCTTTTATCAGAAGTGATTAGTTGGATGAGCACTGTT TGATGGTTGTTGCAGATGGGC	48	53	**
SI105	8	TCTGGAGGATGTGGGCATAGTGAGTGAGTGCGGACCGAGTTGCGGGTGCGGGTCTGAGTG[T/C] GGGAATCGGTTGACTCAGAGAGGAATATCGGTGA	10.2	3	**
SI109	8	AAATGTCGTCATTGGGGAAGCAGTGGAGAATGAACATATAATTCACATCATTGATTTCCA[A/G]AT TGCTCAGGGCACCCAGTGGATGTTCCTCCTCCATGCTCTTGCAAAACGGCCTGGTGGA	12.2	19	***
SI110	2	GGCAAGGATGCTTTCCATTTGCGAGTGAGGGTGCACCCCTTCCATGTTCTGCG[T/C]ATTAACAA GATGCTTTCGTGTGCTGGGGCTGAT	45.9	16	**
SI111	8	ATTTTGGGGATTTTAAACTTTTAACTTTCGTTGAATTGCTTTAACAACGATTAACCTTG[T/C]GG GAAAAATTTACAAATGGTCTTAGGTAACCCTAGACTCATGTTTATGGCCCCATTTAGT	57.1	25	**
SI112	8	TGAAGATACGTTCTGATATGGATCAAGTGTTTAGAGACTCCAGTCTCAAAGGTCC[A/G]TTATGG GAAGAGATTTCCAGGTGATGATTTTATTAACTTTTGTCAAAGCTAATTTCTAGC	39.8	34	**
SI113	2	CCTAAGCGTTTTGCTTGTTGAAGATCAAGCAGTACTACAAAGGATAGGAATCAGAATGCT[A/T]A AAAAGCTTGGAGCTGGTGTTACTCTAGTCAAAGATGGAGAGGCTGCCGTTGAAGCCATG	52	25	***
SI114	8	CAGATGAGTCTGGGATCGGGTGTCTCCTGTATTTGGTGAGCCATTTAGCCAGCGTCATTG[T/G]TG CTTATCTGCTTAGCCAACTTGATGATTCCACAAATTGATCTT	2	25	**
SI115	2	CCTTCCAATGAGATAAATTTTGCATAACTGCTCTCCCGCACTTGGTATTAACTGGTTTTT[A/T]AC ACTTGCAGATCCTTGGGCCTGATAGTGGTTGGCAGCCAATTCCACTAACAGATCTTAT	2	22	***
SI116	8	TGAAGTTTTTTAGTGGTCACATTCCTTATTGTTAATTAATCGAATTGTGATAATTTGTGT[C/G]TATC TATGCTGTTGTCAGGCCCTCATAATTTGAGCTTTTAGACTAAATAGTGTTTAAAAA	34.7	6	***
SI117	8	GCCTTAAAAATAATCAAAATATTCAGTGTCATCGACATTCCTTCTAGATA[T/C]GTGCTTCTGGTCT TGTGCATAGTATTCAAAATAGAGGAGATTATAAATCCAGATT	58.2	8	**
SI118	8	GAGGAAAAATAAAGTTACCTCCATCTGCACA[A/G]CATTGCAATCTCAGTATCAACA	56.1	101	*
SI122	8	ACAGCTACAGCTAGTTGATGGTGATCGCGATCTTGATTATTCATTTT[A/G]ATTTTCTTGTTACTCA CCGAAGATGATGATGAAGATTTGGACTTCTTTCTGATAGAGTGT	61.2	3	**

Continued

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]
SI123	8	CTTGAAGAGATCATACTACATGGATCCACAACCATTGAACCATTTCGAGTGGCACCTTTA[T/C]G AGTATGAGATCAATAAGTGTGATGCTTGCGCCTTGTATAGATTAGAACTGAAGCTTGTT	5.1	19 ***
SI124	8	ATGTACATATGAATGTGTCTTTTGCCTTGTAACAATG[T/C]GTCTCTTGAAGGTTGAGGGTGGTCG ACCATAGAGTGAAGCATATTGTTTGCCTTTTGTGG	63.3	3 **
SI125	8	TTTTGGATTTTCTTTGTCCTGATGGAAGATCCTATAGAACAATTTCA[A/T]TTCAGTAAAATGGCT CAATCAGATAATTA	7.1	28 **
SI127	8	GTTCATACCATGTGCTGGCGAAGATAATCAACTCGAGTTGGATGCTTGAGTTTCATCAGA[C/G]C CTTAACTTCATGTTTACGGACCATTTCCCTTGAAATATTTAAGTTCCCAGCAATTTCTC	45.9	6 ***
SI128	8	AACTTACTCCCATGGTACATCACCAACAAGCATCCAATCTCCATCTTTGTCTTCATAAGT[A/G]GG TACATAATCTGAGCCATTCAAAAGATCAATCAGTTTGCTCTCATTCATAAAATCCTTC	3.1	1 ***
SI129	8	TTCATGGAGATGGCAATTTGTTAAACAATCACCCGGAATTACAGGAGGCTTTGGTTTGGG[T/C]C TATTTTCATTCCAATATTCCTGAATTCAATAAAGTCGAATGCTGGGGTCCACTCAAAGA	39.8	2 ***
SI130	2	TATGTGTGGGCTGAGCCTCAAAGGGAAGGATTGCGTCCCTTTCCTTGAGAAGCTTGTGAT[T/C]G CTGATGTTGCTGGACTTGCCCCAGGAACTGGGACTCTTACAGTCTTTACAAATGAAAAT	59.2	19 ***
SI131	2	AATGAGTGAAAACTTATCAAAACGGAAGGACAAATGAAAATTGGAACAGAGGCAAAAAGA[A/ C]GGAAACTCCATTTCTCAACTACTAAGGAAAAGAAAAAAATTGATCATAAATTTTCTTAGG	57.1	13 **
SI132	8	GATGGGCTAGGTTTGAAGAGCTGAAATCAGTACCTTGAACAGAGGATTTAGTTTCTTTTG[T/C]A CTGCTGAATTGAAAGACATCCTCAGTTGATTCAAATCTGGTGGC	66.3	29 **
SI133	8	CACCTTGAGGCCAGCGCAGCTGTAATGGCCACATTATTTGCCGGTGACGGGCGGGGTTTC[T/C]G GCGATAATACCGCAGATATTCACGGGAGCCGAACGTTTTAGTCGATGTTCCTTTATCTG	7.1	63 ***
SI134	8	TCAATATCAAAATTCCAAATTTACCTCTTTTGTGGGTT[T/C]AGAGTTTAATTTGAAATGGATGATG GGGAGGTTGATTTCTCGAACCAAGAAATGCTTAGC	43.9	26 **
SI135	8	GGAGCGAGTCAACTCACTGAGCCACACCAACTGCAACTCACCGAGAATAATAAACAC[T/C]ATA AAGGTAGTTTCTTTCTTTCTTTCGCTTTTTTTATTTTTCATTTTTATTTTAGTTTTG	43.9	4 *
SI137	8	CAATGGCCAATGCTGCTGGAGAGCTGTTCCCAAGCTTGCAGGTCTCTGATTGATCATCAG[T/C]T CTTTCAGCATATATAGACTCATCTTTCGCAGCTTCATTTTGAGCTCTTTCTTTCATTTG	45.9	47 ***
SI138	2	GGTCAAATGATCTTTCAAGATGAAATAAGAAGATGCTGACCTGATTATACTTTGCAAG[A/G]CGC TCTGACCTGCATGGTGCTCCTGTCTTAATTTGACCCTGATCATGAACAAGAAAACAA	49	2 **
SI139	8	TTCCCCAGGTTGAACCCTTAGATGTTGTCGTTCAACCTCACAAGCAGTCATGGCAGTATC[A/T]T GGAATTCAAATGGGACATTGCAGGATGCAGCAAACTCCGACAGTCTCTTCCCTACAATA	59.2	19 ***
SI140	8	TGAAGAATGGTAATAGAGTGAATTATGCTTTTTATCGTTGACTGTTGAAGAAAGACATTC[A/G]TT GGTTAGCTTGAGATTGAATTTTACAACAATGTGGATCATGATTC	54.1	4 **
SI141	8	ATTGTGTGTTCCAATTGTGATGTCGTATCACACTCATGTTCCAGTGTGAGTTTAATTCTC[A/T]TAT GATTTTCCTGTCAAAAGTTTTTGCATGCACATGTTCTGTTTAGCTTAAATACAATTA	58.2	2 ***
SI142	8	GTAATTAATTGTTATATGAATTTGATTACAGGCTGAACCTGATACTGATGAGGTGTTTGC[A/T]CAA GTGACTTTACTTCCAGAGTCAAACGTAACTACTTCATCGATTTTCATCATGGCTTTG	18.4	84 ***
SI143	2	ACTGACACTGTTAGATGTCTTACTAAAACT[A/G]AAAATGCAAATGAAGAAAAGCAGGATATTGT TAAAGTGAGCTT	42.9	47 **
SI144	8	AATTGTCATTTTCCTTGGGTTGCTGCTTAGTTGTAAGAGGAGGAGAAGATGGAGGAGGGG[A/T] AGCTGCAGGTGATGGAGGGTATTTTGGGGACTGCTTGGGGAAATGAAGAATGGAAGGTGT	15.3	17 ***
SI145	8	CATTTGAATGATCTCTTTCTTGATCTTGCCAATGCTGTTGGTAATTTAGGACTCTTTAAT[A/T]GGC TTCTTTGCCATCCTTCTCCATACTGTGTCAACAAAATTGTCTGAAGTTCAAACCTGT	65.3	12 ***
SI146	8	CAGTTTCCTTGTAGCAATGGGCGACATTTCGCTGAGAAAAACTGCCGTGGAGGAGAAGAG[A/G] GCTAAAGTACAGAAGGAGTCCAAAATTCTT	54.1	13 **
SI148	8	TGCAATTGTTTATGCTTTTAAAATCATTCCCCTAATGTTTGTTAACTATTCTTGGTGTGT[A/G]TCA CTTATGTGCAGCCACTCGACACTCCAGCTGCATCACAGGACTTGTTTTCTGCCAACC	67.3	2 ***
SI149	2	GCAACCCCAGAATCACTCACAGCTGAAGAAGCAACAACAACATCCACAGAGACCATCCCA[A/G]CAACCCCAGTAGAAACTAAGGTCCAAA	29.6	10 **
SI150	8	TTGGCCACCAATTAGATCATTTAGGAAGAATTCATTGGCCACTTCGTCTAAGAATAATGA[T/C]GA AGTAGATGGAAAAGCAGGTTCCAGTGCTCTGTTTGTTAAGGTCAGCATGGATGGTGCT	55.1	1 ***
SI151	8	AAGGTGAGAAAGAAGAGAGAAAACGTGATTTTAGCAAACTAGAACTGAAACCTGATCATG[T/C] TAATCGGCCTCTGTGGGCTTGTGCTGATGGACGAATATTTCTTGAAACATTCTCTCCATT	19.4	12 ***
SI152	8	TGACTTGGATTTGTTGGTACTATGATTTGAGACTGATGACTTCTCCTTAACTTGTTTCTT[A/G]GCT GCACCAGTTACAACTTCAAATAACGATGGAAGTTCATTTATCATG	38.8	32 **
SI153	8	TTTGCCACCTTAAAGTTCATCCCCTTGGCAACCCC[T/C]TCAATCTTTTCCATTATATGCCTGGCCG AATACCTTGACGTGAACATGGACCCCGTTTTC	12.2	63 **
SI154	8	GGATACTACTTTTATACTTGCCACCTCACTTTTGACGGCAAAAGCCACCAAAATGATACA[T/G]GT TCATAACTTGGTATTGATTGATAATAATAATAACACGGTGAGCATTACGTTAGTCTTT	44.9	25 ***
SI155	8	GGATTTTGCTTATGAATAGCTATCCGCAGTTACTTAACAGTGGGTAACTAAAATGCAACT[T/C]AC TGATTTCAATTGCCACAGGCTTACTACATGGCCTTGCCTTTTCCAA	48	4 **
SI156	8	GGGAATGAAAGATGCTATTAACCAATCTTCAAAGGTCTACGTATATGATGGACTCTATAC[A/G]GT TCAAGAGTCGTGGACTGAAAAGGGGAAATCAG	22.4	76 **
SI158	8	GTAACCACCAGTGTAAAAGTAATATCATAACTACTCAACACAGGCATAGATGGTATACCC[A/C]AA TGTACCAACCACGAATGGTCTTCAAACAAATACTATCG	62.2	3 **
SI161	8	CCATAGCCGTGAGACACATAGAATCAATGATACGAATGTCTGAAGCTCATGCCAGAATGC[A/G]C CTCAGACAGCATGTAACACAAGAAGATGTGAACATGGCCATCCGTGTCCTACTTGATTC	62.2	52 ***
SI163	2	GGCAGCCAACAGGTGTTCCTGCGCTGGCTTGTTGAGAAAAGTATCCATGCCAGAATTGAA[C/G] CATTCTCGTTCATTATCTGCGGTTACCAGAGCTGTAAATGCAATTATGGGAATGCTTTGA	10.2	25 ***
SI166	8	CATGTTGCACACTTCTAATGCAGTACGTACTTTCTTCTATTGCAGTAACTTCAAATATTC[A/T]TTG CTTGAGCACCATGATGAAGTGCATAGCGGTGAGATTTATTCTAATCTCCATGCACTG	4.1	61 ***
SI167	8	TGGCTGTCATTGCTACAAAGGAATATCGTTCTATAGTATTCCAGGAACCTCGTTTTGT[T/C]GAATA TTTTCGCCTTGTAAGTATTTCACCCATTCATCATCATTTTGTCTTTTCAATTTTT	38.8	124 **
SI169	8	ACTATAAAGATAATCTGCTCATCAATGC[A/C]TGCTGATTCTTCTGCTTTACAGCAGAATGACCAT TTCTGGATTTATGATGGGCTCTATTG	40.8	32 **
SI170	2	GGACTTGATGGATTTGGGCTGCTTTGGTTTGGCAGCAGC[T/C]GATTTCTTTGCCTTCTTAGGCGG AGGAGGAGCGGCGGCGGC	51	25 **
SI172	2	GATGTCAAGACGAAATGTTTACAGCTTGTCCTCAAACTCAGATAGTGGTGTCATTTGCTC[T/C]TT CAAGCCCTTCCTCTTCCTGATATCAGCAACAAGCTGTGCAGCCTGTGTACCAGGCTCC	28.6	6 ***
SI173	*in silico*	TGGCCAGAATCCAAGCTGCCTCTGTGCTGTTATGCTGTCTAATGTTGCTAAGAGCTCTGG[T/C]AT CAAGCCTGATGTTGCAATAACCATCCCCAAGCGCTGCAACCTTGCTGATCGCCC	28.6	16 **
SI175	8	TACTTGTCTGCATGTCATTGTTAGTGTGCAACGAA[T/G]TAGTTTCTTAATGCTGGTAGTAACCAC TGCTAGGTTTAGTGGTTATAACCATACTTTGGT	48	4 **
SI176	8	GAATTAGATAGTGTCTACAAATACTGTGATGGTTTGCCTGAAAATTCCAGTGAATGGAAG[A/T]G AATAAGTTGGTCACGTGTGAAGTTTCTTGTAATATCTGAAATGGTATGAAAATGCAAGT	55.1	30 ***
SI178	8	TTAACTCAGCTGGAAGTTATCGAACTCAG[T/C]GAAAACTCGTTCACCGGGACACTTGAAGCGT GGTTCTTCCTGTTGCCAGCACTCCAGCAA	65.3	26 **

Continued

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]
SI180	8	TTTTTTTGACGCTCAATTGACGGG[A/G]GGATTTCCTTTCTTTCAAAGTTACCAGATATTTAGATTTTTAAGATTTGAGATTCTGGTT	56.1	12 **
SI181	8	GCGAACCTGCCCTTGATACGTGGTCTAGTTTCCGCATAGGCCTTCCTTGAGGCATACC[T/G]GATTGTCTTCTCGAATTTTCTTGTCTTCTTTTTTTCCCTGTATCTCAAGACTCTGGCCTC	53.1	80 **
SI182	8	TCAAGAAAGAAGCTGAGACTTTCTAAAGATCAGTCAGC[T/C]ATTCTTGAAGAGAGCTTCAAAGAGCACAACACTCTCAACCCAGTA	6.1	2 **
SI183	8	TTATTAAGATCTACAACAGCTAATTAATTGATCAATTAATTCTCTCTTCATAATTTTCTT[T/C]CCTTCAATTTTTTTTCTCTCCGGCTGCTAGTAGC	67.3	15 *
SI186	8	AATAACAGTAGCAGTGCACCTGCTGCCAAGTAAGTCCAAAGTTGGTTTTGCGTTAAATCT[T/C]GGATTCAGTAAGATAGAAAAATTACAATCTTCCTTATTCGGTGACAGGGCACAGGTTGTG	10.2	1 ***
SI188	8	CAAAGGCAGATTGTTGTTTAGTCTCATCCTTTCTGGTATGAGTGTGGAATACCAGCCGAG[A/G]GATGAAAGAGTATGATAAATTTGCAGATCTCCTTTCTTGATGCACATCAGGCCGAAGACG	59.2	6 ***
SI189	8	GCGGACTGTTGTTCCACCAGTGTATTAGGA[C/G]CCATATACGGGACAAAAGTTGAACAGGGGTTAGGAATGACCCCAGGATTCTGATTTCCAA	48	28 **
SI190	8	TGGTCTCCTTGATGACAGGTCCAACTTTTTGGGCACCAAGTTCACAATCTATGATGGGCA[T/G]CCTCCAAATGCTGAAGCAAGAGTTACTAAATGTCGCTCCACAAGACAGGTTAATATGAAA	58.2	3 ***
SI191	8	TACCACCCAGGTTTGCAATTTGAGTTTGTCCAAAGTGTTGCTCTCTGAAACAAAAGTCCG[A/G]CCATAGATCCAACTCACAAGGACAGAACAAGAAGCTGTGAAAGGCCACAACAGCTGTAGA	52	121 ***
SI192	8	GTTAGAGTCCTCATTCTTCTCAATCTTGCTCTCCCCTGATGACTTGTTTCCAGAATCTT[C/G]TTTAGCTGAACCTTGGGACCTACCACTTGGCTGTGCAACTGCAGATGCATATTG	69.4	28 **
SI194	8	GGCTCAGGCGGCACAATGATCCGCAACAATGTGCTCGAGAACTTGTCATGGAAGCATCAC[A/G]CCTGAATTCATCTGATAATCTCACTGTGATTGTTATCTGCTTCTCCTCTCTTGCAC	33.7	7 **
SI195	8	TTTTCAAAAGTGCAAGAAAGAAAGAGAGAAAAAAGTGTAACGT[A/G]AAACAAAATGCTTTGTGTGATGGCACTATTGTCTTC	56.1	9 **
SI196	8	CAGAAAACCCACTCTTTCCTTTACAACTAACTCTGCTAGTCGACTATTTATTTCATCCTC[A/C]CTTTCTTTCAACTTTGAGCTGGACAAATCAATTTTCTTTTCCAACTCTTCAAGGTCCC	6.1	10 **
SI197	8	TTTTAGTTTGTCCCGTAGAGATAAAAGGTTAAATTGAAAGGACAAAGTTTAGAATTTGGC[A/G]GTATCTGTTGGCAGGAGATAGATAGAGAAAGAGA	4.1	3 **
SI198	8	CCACCGTATTACGAGCGACATGCCGTCGTTTGGCTCGAAGGAGGGGCTGGGGCAGTACTT[A/G]TGGCTGCCCAAGAGTATTAAGAACGGGTCGCTTGCTAATAACTCGGAGATGAAGCTGCCG	29.6	26 ***
SI199	8	CCGTAGGGCCCACTCCGACACCTCCTTCCGCTTCGATGACTTCTTACTCTTCGACCCCTC[A/C]GACCTCGACCTCTCCGCCCTCGACCTCCCCTCGCCCAATCCGACGCCGCCACGTGGCGTC	51	34 ***
SI201	8	TTCAAGCTGATCGGCTGGTGATCCATGGTC[A/G]ATGTATCTATCAGGAAGCACCATTGATCTCAACTGCAGA	37.8	23 *
SI202	8	GGAGCCTGGTACACAGGCTGCACAGCTTGTTGCTGATATCAGGAAGAGGAAGGGCTTGAA[A/G]GAGCAAATGACACCACTATCTGAGTTTGAGGACAAGCTGTAAACATTTCGTCTTGACATC	28.6	6 ***
SI203	8	TTATTAGTTAAATGATACCTGAAGTCATATATTTTGGATTTCTTATTTTGTACCTAGACC[T/C]TCCTGCAATTGTATAAGAATTTGGTTGTTCCTTGTCTGTGAAGATCTATTTAATTAGTAC	42.9	21 ***
SI204	8	GCAAAAATGCATAACCAATGGTGTTAAGTT[T/C]CACCAAGCTAAAGTTATTAAGGTTATTCATGAAGAGTCCAAATCTTTGTTGATTTGCAAT	55.1	13 **
SI207	8	CAGAAAATGCAATTTAAAGCATCTACATA[A/G]CATCAACAAGTGAAAATGGCTGAACACCATGGTTTGATATTTTACAATAAAAATATGAAT	64.3	12 **
SI208	2	GCATCTCCCCAGTAAATTTTGGCAACAATATTCGCCTGCAAAATCACATCATTACAATTC[T/C]AAACATTCGATCCAACCATAACAAAGTAACGATTTAATTAATTAATGTCCAAGGCCAGTA	35.7	2 ***
SI209	8	CCCCTCGGTCGCCGGCTGGTGGAAGACCGTACAGCTGTACACGGACCA[A/G]ACTGGCGCGAACATCTCCCGCACGGTGCGGTTAGGCCAGGAGAAGAACGACCGTTTCTAC	54.1	6 **
SI210	8	AGCTAAGGTTACGGGAATGCTTTTGGAGATGGATCAGACTGAGGTTTTGCACTTGCTCGA[A/G]TCACCAGAAGCTCTGAAAGCGAAAGTGGCTGAGGCAATGGAGGTTCTGAGGAATGTTGCT	66.3	26 ***
SI211	8	TTGATCCATTCTAGTGTGTGGTGTCCTTGAGAATGCCTTGACTTCCTGAAGTGATTACAG[T/C]AGACCTGTGCGGATATTATCTCCATGCCTTTGTGCTGATCCATATTTTTGTT	25.5	42 **
SI213	2	ATCCAACCTCCTCTTGAACTCTGGGTTTTCAACATCCAGCACAGCAGGGAAAATTCTAGC[T/C]GTTGTGCGATTGGTCTGTAGAAAAAGAATTTCAGATACACAAGTTCAAGTATCATAAAAA	30.6	47 ***
SI214	8	ACGACCTGGCCAGCCTGAGTGCAGTTACTTC[C/G]TAAGAACGGGAGACTGTAAGTATAAGTCTAATTGCAAATATCATCATCCAAAAAATCG	68.4	12 **
SI215	8	CTCTTGGGCTATGGTACAGCCGTTCAGAGCTGTTTAGCTGCTTCTGCTTTATTGGAATCC[A/C]ATGGGTTACGGCTGACTGTAGCTGATGCTCGATTCTGTAAACCACTGGATCATGCCCTCA	36.7	7 ***
SI216	2	GACCCGTGAAGGTAAAGAATACGACATACCAAAGGGACACATAGTTGGAACATCACCAGC[A/T]TTTGCAAACCGGCTTCCTCATATTTACAAGAATCCAGACAGTTACGATCCTGATAGGTTT	48	65 ***
SI217	8	GTCAAAGCAGAAGCTGGTTTCTTCCCCTGGGCT[T/C]GACTGTTAATGCATGCTCATTTTTGCAATTGTTTATGCTTTTAAAATCATTCCCCTAATG	43.9	2 **
SI218	8	ATGTATCCTACGCCTCCTTTCCAATTCTTTGTTGGATTGTCTACAGTGTAGAACACCTGA[C/G]GTTTTTATCCCAAAGAATTAAATAAATAAGACTAATTCAAAGTTATCTATTTGTAACTAC	40.8	9 **
SI219	8	TGCTGCCCTGGAGATTCAAATGATCTTCATGGAGGAGATACACTTGATAATATCTCTGAT[A/G]AGAACGAAAAGATCGCTGCAAAATGTTCGATTGATTCAATTAAGTTAGGTGGGGAGAAAG	58.2	19 ***
SI220	8	AGAGAGGAAGCGGGAGGAGGAGAAAGATGGGGCCAAGTATGTGAGAATGGAGAGGAGGGT[T/C]GGCAAGTTTATGAGGAAATTTGTGCTGCCTGAGAATGCTAACGTTGAAGCCATTTCGGCT	51	4 **
SI221	8	TTACCCGTATGCCCTGCTCTTCATATTATTAACTAGCTTAATCACCCTCTTTAACAAGTT[A/G]ATTGGGTTTTGTTTATTTTTGCAGTTAGATGATTGGGTTCTGTGCAGA	49	69 **
SI222	2	GAGCATTCAAAGCATAGTACCAAAATGGAGCATTAATAATTATCTGCAATCAATCAAAAC[A/G]AAAGACCCCCATCAAATTTGATTAATTAGAAGAAGCAAAGGGAAATAAGATTAACGAGTA	59.2	15 ***
SI223	8	TCTTTTTTTTTTCAATTGTTTTAAAGTAACTTGGGTTGGTTTTTACGTTGCGTTTACTTG[T/C]ACTGTCAACAAAATTGAAGCTTTAGGCTTTAGGCTTCTTCAAAGTTTTGAATTGGTTGGC	32.7	7 **
SI224	8	GAAAACAAGTATGTAGACAAGGCTAAGGATTATCTTCACCAATACCACTCTTCTCATTCC[A/G]CTGCCCAGACCGACACCTCTGGCCACTCAGCCCAAGGTGGTC	22.4	39 **
SI226	2	GATAATTAAAACGCGGCAACTCGCTTTTCTTTTTCTCACGGTAAAGTCTCTTAATCAGCT[T/G]TCGCTTTCTTTATTTGCATGCGAATCGCCTCTCCTGCAACAACATGTTAATCAGACTATG	51	34 **
SI228	2	AGCGCACGCATCACATAATTAATTAAAAGAGATGGTGGTGCTTCTTTCCATGAGCTTCCT[C/G]GTCTTCTTCCTTGGCTTCTTTCTTCTCATGGTGCTCGTGGAACGCAAATCCTCCCGATCC	34.7	34 ***
SI230	8	AAAAAAATGAAAGAGATGTCATGATGC[A/G]TCTGGATCTGTATGTGTATGGCATATAAGAGCTACTAGTACCAGTTTTGTCATATTTATA	33.7	91 **
SI231	8	ATTTTATAACAGCGAATTGGTGTGTTTGGAAAGTGTGTAGCTGGCTC[A/G]ATTTTGATTTGAGAATGTGATGGTGTTTTGGAAAATAAAGGTCCTATAGTTT	24.5	16 **
SI232	8	TGCAATTTAGATGCTACATATTGTGTTTGTTCAAGCAATATGCATGCCAACCGTATATTC[A/G]AGTTTTCTGATTTGTCTTTGAAATTCA	40.8	1 **
SI235	2	CTTAACTCTTCCCTTTGTTTTATGGGTGTTTTCGCAAATTCTTGAAGTTGAAGGGAG[A/G]GGAGATGATGATGGCAAATTCAGAAACTGGAGGAAGAACTGAAGGCCAAGTACAAGTTTT	40.8	10 **

Continued

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]
SI237	8	AAACTTCAGAGACAACACGACGCCGCTTACTTGGGAACTCTCTTCGTGGGTCAGATGGTA[T/C]G CTTTATATCTCGAACACCTCTTGTCCACTTCTAGGGTTTTGGGTTTCTTCCTT	36.7	23 **
SI238	8	ATAACAAGCTGGTGGCTCGTAAACTTGCTGACAGAAGAAATGGTCAAGTTACAATACCAG[T/C]T GGAGTAGAGATAGAGCAATCTGGCTTGGCAAAGGAGCAGGTACATGGGTTAAGTTCCCA	51	22 ***
SI239	8	AGCTGTAGTTGACAAGTACCTCATGAATTTGGTTGATTGCTAATTTCATCAGAAATCGGT[T/C]GC TGATCTTGTGGTTTATGACAAAAAAGGGAGAATATGTTGCTTCATTTTTTTTCCCTTT	43.9	33 **
SI241	8	TACCAGTAGTTCCAGGCTTCT[C/G]ACTGCCAATGAAGAAATGCAGTTGTCAGCAGGAATACAGG TGTGACATACATACTAGTTA	41.8	91 **
SI242	8	CTACTTTGAAATCTCTGTGTAATTTTAAGCTTAATTTCGGTGTTTTTCCAGTTGTCTTCA[T/C]TGT AATTTTGGTGTTAAAATATTCTAGTAT	80.6	2 **
SI244	2	TTCCCAATTATCAGTACTCGGATCATAGACCTGACCTCTCGGAGAGACAAAAAATGGCCA[T/C]A ACCAGCCTTCCGTCACAAGAAGTTTGCCATCTAGAACTGCAGCATCATAAGATGCCATG	59.2	19 ***
SI246	8	GAAAGATGCTGACACAAGGGGCTTTCTCGAAGCAGCGAAAGCATGTCTTCAAATTTGTTG[T/C]G TCCATGGAGTACCTCTGCTGATTAATGACCGCATCGATATTGCCCT	43.9	36 **
SI247	8	GAAAGCCATTAGATATAACAAATGACTTTAT[A/G]AGCCTTTGAAAATGAAGCTGGTCCATCCTGA GATTGCACTGTTTGGTACACTCTAATCAT	54.1	91 **
SI248	8	CTCACTCATGCATGCTGCCTCCATTTATCCACAATGCTTTGATTCTCCTCCTCCTTTCT[A/C]CTCT TCTCTCTCTTTCTCTCCAGGTGAGCAACCTCCACAAATATATTT	42.9	2 *
SI250	8	GGGATTGATATGGGGTCTGCTGGGCTGGTGGGGTACATTGGGCGGCATAGGTGTGCCTAG[T/C]G TTCCTGGTTTTGGGTCAAGTACGAGCCTCCTGCAAGCTCTTTCCAACACTCACACTGAT	37.8	21 ***
SI251	8	ATTATGTTTGTCTATCACAAACAATTTCCTTCTAGTCA[C/G]CTGGTACAATAACAAAATGACGAG CAATAACTGTAAAAGGGCAAAGGACAATCAGACCAA	43.9	4 **
SI252	2	TTCAAGCTGATCGGCTGGTGATCCATGGTC[A/G]ATGTATCTATCAGGAAGCACCATTGATCTCAA CTGCAGA	37.8	23 *
SI253	8	CAAAACCCAAGATGGATCATGGAATCATGAGGTATGTAAATCTCTGTTTATGCCA[A/G]TGTGAGT GAATGGTTGGCCTTAAATTTTGTTCTGTTGTCTTGCACTATGATCTGCAGCAT	54.1	59 **
SI255	2	GCCACATATATCATCCCCCCAATCACCCCACTAGCAAAAAATGATCT[A/G]GCTGTAATCATCTTGT TCATGACTGTCCAACGGTTTTTTTGCATTTCATACTT	17.3	19 **
SI259	8	TACTTGTTAATTTCTCTCTTCATTCGAATTGATCAT[A/G]TTTTATTTTGATAAGTGGACAAAATCAT TANGGGGNAGGTTTCTCAA	51	47 *
SI260	8	ACAACATCTTATTTGGTAATTGGAGCCTGTAGTAAAACCATCCCGAAAGAAATTTATAAT[A/G]GA TGTTTTGAAACTCTTGATAATCCGTATATCTCGTTTTGCTCTTCTGTTGAAAGAATAT	39.8	20 ***
SI263	8	CACTCAAAGCTGCTTTCAGTACCTCTTGTGAACAACCTGGTTTCACTATGGTTCGAACCT[A/G]G GAAATAAATTGGAAAAAGAAAAAAAAAAAACA	44.9	22 *
SI264	8	ATCATTTCCAATCGTTCTTTTGCTCATGTTGGCTCGCTGAACTAACCGAACCAATGCTTG[A/G]AC GACTTCGGACATAGGTGGTCGAAACTCAGGTTCCGGCTGCACAAGCCAGTGAGTCAAT	44.9	33 ***
SI265	8	GCACACAGTTTTTATCACAACTTGCACATGTTAATAGTTTCATGCATTTATATGTTTATT[T/C]GCAG TTACTGGAGTTCTCACCCGGGATTGATATGGGGTCTGCTGGGCTGGTGGGGTACAT	7.1	21 ***
SI267	8	TTGATTGTCGGAAGTGTAAATTTGAGCAGGTAAAATAGGGTGGTCCTAGTCTGACCCACT[T/C]C CCTGTACAGGTTTTAAAGGCAAAC	61.2	6 **
SI268	*in silico*	GCAGTAATTGTAAATCATGTAATTCTTCTGCACCCATTTCAGCCTA[T/C]TTTGATTTGTTGAATCT AGCTCTTGTGAGAACCAAGCACCGTTGCTGGATGTAGAGGAAG	49	4 **
SI269	8	AGATATTCACTTGTACTTTTATTGAACTTACAA[A/G]CAACCAATCAACACATGACATCGTGAC	22.4	1 *
SI270	8	TTTATTCTTCGCTTGTTAGGTTTGGTGAGAAAATAATCAAAATTCCTCTTCATTACCCAA[C/G]GTA CAGGTACAATGATTCAGTCCTCGTTTATTTATATCTCATACTGTGCTAA	65.3	29 **
SI271	8	TTTCGTTAATTTTTTACATAGCAATGTCTATGTGCAAAGTAAGGGT[T/G]CTTTGTTCACAAACTCC AGACCTCCAAAATAGAAGGGAACAATCTTTGTTGTCTGAGGCT	32.7	61 **
SI272	8	CAGTGACAATGGGGCAGCAGGAAATAGTGGAGTTGGTGGCATAACTGAAAGAATCAGTGG[A/G] AATGTGGTAGATGAAGTTTGGGTTGCTCAGAGAGAAGCTGCCTTGACTAAATTTCG	56.1	8 **
SI273	8	ACATTGCAACATGTTAGCTTTAACATGCTTAAAGTATCACATATTTGCTTGGATCATGGT[T/G]GAT ATTCATTTATCAGCTACAAGAGTGG	23.5	6 **
SI274	8	AATGCCTGATTGACCAGAAATATCAAATGGATGATG[T/C]GCATGGATAAACATTTTAAGTAGAGT GATTGGTTCTGCAAAAGA	55.1	59 **
SI275	8	TGGATATTACAATACACGATTCCGTGATGGTTACCTCCCTATTGCCCAGATGCTAGCACG[A/G]CAT GGTGCCATATTCAACTTTACTTGCATAGAGATGCGTGATCATGAACAACCACAAGAT	55.1	51 ***
SI276	2	GGCTCTCTCATCCTCTTGGTCCTATCTCAAACGTATCTTCTCTAATTCAAAG[A/G]CCCTGAAACC CAGCTTGTCCCAGACTAGCTCTACCTCATCAACATC	12.2	8 **
SI277	8	AGTTACGATTATGTCGATGTTTCTCCTAATTCCAATAAGAGAGCTAGGGAAGACAACAAT[A/G]CA CAATTTCCATCTCCAACTGCAAAACTGAACTTTCACTCTCACTTGGTAAATAAGTATT	55.1	32 ***
SI281	8	ATGTCCCCTAACATTTGCACTCACAAAAATTGGCAAACACAAATGATTACAATATGAGTC[A/G]A ACCTGTGCATAAAAGTAAGCATCCAATCAGACTTAAA	54.1	1 **
SI282	8	ATTGTGCAAATGATGACTTAACCGTTCTTCCTCGCGAAGCCGAACTTGAGCTGAGCTCAA[T/C]T TGTCTGATCTGTTCTGCAACCTATGTCTTTAATAATTTTCTTATGTTTGATAAACCTTA	59.2	12 ***
SI284	2	ATGTACATATGAATGTGTCTTTTGCCTTGTAACAATG[T/C]GTCTCTTGAAGGTTGAGGGTGGTCG ACCATAGAGTGAAGCATATTGTTTGCCTTTTGTGG	63.3	3 **
SI285	8	GGTTGGCAGAAAACAAGTCCTGTGATGCAGCTGGAGTGTCGAGTGGCTGCACATAAGTGA[T/C] ACACACCAAGAATAGTTAACAAACATTAGGGGAATGATTTTAAAAGCATAAACAATTGCA	67.3	2 ***
SI286	2	CGCCTAGCCAACCCGTCATCCGCGTTGACCTTAAACATCTTGCCCGTATTGGATTGCACC[A/G]CG AGCAAATACCCCTTGCTCACGTAAGCTATCCCGTTTAATCCACACTCGCTAAACGGCG	52	56 ***
SI287	8	CCTCATTCATCTTTTGTTAATTATTTCCCAATGATGCCAGCAGCTAACAG[T/C]ATTGCAAGATTCA CTTTATTTAATTTGTTAGCTCAAAAATTATTTTGATAAATTTC	15.3	61 **
SI288	8	TATTAGAGGTGTCATTTTTGGGAGTATGGTTCTCATCTTGGCT[A/G]GCTTTCAAGCACCACTTTCT GTTATGCTTATTATTCTGCAAT	52	25 **
SI289	2	GAGAAAGACTATGATCTTCTCAAGAGACAATATGAAGCAGTCAAAGCCGA[T/C]CGCGAAGCAC TCCAAGCTCAAAATCAGAAGCTTCATGCAGAGGTTTGTCTTGAATTCCA	43.9	10 **
SI292	2	AATCTTGATTTTAGATCTATTAGTTATATAGGAGTAGCAGTTTCTTTTCATTCTTCTGCC[T/C]GGCC TATTCTTTGCAGAATTGTTGGTTTGAGGTACTGAGATTGTTATGTCCTTTTTTTT	56.1	2 **
SI294	8	CAGCGACGGATCGACCTTCATAGTCTGGAGACCAGCGGAATTCGCCAGAGATTTGTTGCC[T/G]A AATATTTTAAACACAACAATTTCTCTAGCTTCGTCCGCCAGCTCAA	67.3	29 **
SI296	8	GGAAGCTTCATTCTCCAAGATCAGCTCTCTGTTTATCCCTCCGC[T/C]GGTGGAAGAAACTATCTT AAATTGTCAAACTTCAGAGACAACACGACGCCGCTTACTTGG	17.3	23 **
SI297	8	AGGAGAGGTTAAAAAGTATTTTTACGGCGATAATAAATATGGCGGCGAGCCATTTTTCTT[T/G]CC AAGAGATGCTAATAATTCTGAAAATGAAGACGATGGTTATATTCTTGCATTTGTTCAT	61.2	19 ***
SI298	2	GATATGCCATTAGTTTTCAGCTTATGTAAAAATAAATAAAGAGATCAGACATAACTTCAA[T/G]TTA AAACAAAAAGCTCTAGGCGCAAGAAAA	65.3	117 **
SI299	2	ATCTTGTGGTTGTTCATGATCACGCATCTCTATGCAAGTAAAGTTGAATATGGCACCATG[T/C]CGT GCTAGCATCTGGGCAATAGGGAGGTAACCATCACGGAATCGTGTATTGTAATATCCA	55.1	51 ***

Continued

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]
SI300	8	TCTGGGATGCTCTGGTGAATTTGAGC[T/C]CTGGGTTTCAAAGTAAAATCAAGAAACATCGTCGG TGTTCTTGTGTAAACCGGAGACCGG	58.2	87 **
SI301	8	TTACTGTGGAGGAAGGATCAATTGGGGGTTTTGGATCTCATGTTGTTCAGTTCCTCGCTC[A/T]CG ATGGTCTTCTTGATGGCAC	26.5	7 **
SI302	2	CTTGGCTTTCTTAGCAGCAGAGGACTTGATGGATTTGGGCTGCTTTGGTTTGGCAGCAGC[T/C]G ATTTCTTTGCCTTCTTAGGCGGAGGAGGAGCGGCGGCGGC	29.6	25 **
SI304	8	ATTATTATTGCTTGGAATTAAGATTGTATTCCTCACGGGAAGACCAGAAGATCAGAGAAA[T/C]GT TACAGAAGCTAATTTAAAGCATGCTGGATTCNACACNTGGGAGAAGCTCATCCTCAAG	37	33 **
SI305	8	CAACAGGGTTGATTATGCTTCTATAAGTGATGAAGAGTGGAAA[A/G]GAAGGCTCACAGGGGAG CAATATTACATAACTCGACAAAAAGGGACTGAGAGGGCTTTTA	54.1	17 **
SI306	2	ATTATCCTGTCCAAAGCACTCAAATCTTTCAGCTGATAGCCTTAATAAAGCTCGAAAATA[T/C]TTT GGCGGCATCCTACTCATCTCCACCCACATAATCTTTGAATGATCCAATTCCCAAATT	52	53 ***
SI307	8	ACTCCGACGAGAGCTTCATAAGCGTGTGGGATTGCAATTGTTGTAGCAATCATCGCAAGC[T/C]G GGTGATGCGTGTGAGTGCCAGTGCGTTGGCAAAGTTTGTATGGTTGATGTGATTTGTTA	52	69 ***
SI309	8	CCCCTTTTTCCTTTTGTAATCATTTTTGTGTGTTTAGTTCCAC[T/C]TCTGGCTTCTAGGCTTGCCG AACTTTGAATTTTTTGATGATGTTTTATTTATCTGTTTAT	51	47 **
SI311	8	TTATGTTTGAGAGATGGGCTACATAGGAAAAGCAAATGAAATTGTGCTGATGC[A/G]ATGTATGAT TTGCAGAGTTTACACCCAGCTGCAGAGTATCATGGTGCGGCTCGTGCAGTA	55.1	6 **
SI312	8	ATTCCTTCTTCAATTGATCCTTGTTGATCACTTTGATCGCCACGCTCTCCCCTGTCATCA[C/G]CTG CTTCCCGTAATACACTTTCGCGAACGTGCCCTTGCCTAATAACCTCCCCATCTCGTA	45.9	63 ***
SI313	8	TGATTTATAACCTGTTATTGGTTTTGCGGC[T/C]TTTTGCTTTTCTCAATAGTTCGAAACAAAAAGC GGACAGTTGCTGTTGCTCCCTCCTTAA	55.1	4 **
SI314	*in silico*	CGTGAGGCCACCGGCAGCTTGAACGAACACAGAAAACACACACATGACAAGTACAGAACC[C/G]CAAAGCGAGTTAACTCGTCCAAGCAACACACACAGCAACCCGGCCGCCGTCTGCACGGCC	59.2	20 **
SI315	2	AGAGAGGAAGCGGGAGGAGGAGAAAGATGGGGCCAAGTATGTGAGAATGGAGAGGAGGGT[T/ C]GGCAAGTTTATGAGGAAATTTGTGCTGCCTGAGAATGCTAACGTTGAAGCCATTTCGGCT	51	4 **
SI316	8	AACGCTTGAGGCTCACACTATTCCAATCCGTCTTCAAAATACAGTTCAAAGGCAGTACTA[A/C]A GGCTCCTTCCTTCTGCATAAAATAAATCATTTAACTTTAAAAACAAATGTTGTCAAATA	27.6	6 ***
SI317	2	TTTCTTCACGACGGGCTTAAAGGTTGTTGCCCCTCTCTTTGCCGTCTATGTAACTTGGCC[A/T]GT GCTTCGACTGCCAGCCTTGGTTGCAGTGCTTCCATTTCTGGTTGGTTGTGCGGCTCAA	46.9	32 ***
SI318	*in silico*	ATAAACAATTGAACCAAACGCTAGGTCCACTAAGCCAATATTGTCTCCATTGAAAAATTT[T/C]TT CTCTCCCAAGCCATGCTCTTCAACGGTTTGTAGCATTTCCAAGGCTTCCTTCATTGTG	2	51 ***
SI322	2	NTTTTTTTGGGTTTGAGGATATGTTTGGTGCGTACTTACTCTGTGATGTTATGATAATGA[A/T]TTTT CAGCCCAATTATGATGAGTGCCGGAGAATTGGAAAGTGGCAATGCTGGAGAACCCG	28.6	16 **
SI323	2	AATCAATCCCCCCACCCCAATTTCATGATAAATATTCAATGAATTAGATGCATGTAGTTG[A/G]CTT CCATAGTCTGGACAAGGTCAATTTCAGTCGCAAAAGAGAGTT	59.2	8 **
SI324	8	GCTTACTGTGTGTCTTATATGATGTTCAGGAAAAGGCAGCTTTCATTTTCTGTGT[T/G]GAAGACA GAGCACTCTTCTGTAAGGACTGTGATGAACCTATCCATTCACCTGGTAGTCTT	58.2	46 **
SI325	2	CGTCGATCGGGAAGGTCACCCTGTTTGCTACAACATTTATGGGGTGTTTGAGAGTGATGA[A/G]C TTTATCAGAAGACTTTTGGTACTGAGGAGAAGCGTGGCCAGTTCTTGAGATGGAGGCTG	53.1	15 ***
SI326	8	TATGACATTTCTTGTGCTGTGCTC[T/G]CTACTCTGATCCTACATTTGTATACAAGGCAGGGCATCA GACTTGCATTAGTGCGCCAAT	46.9	25 **
SI327	*in silico*	CAGTGACAATGGGGCAGCAGGAAATAGTGGAGTTGGTGGCATAACTGAAAGAATCAGTGG[A/G] AATGTGGTAGATGAAGTTTGGGTTGCTCAGAGAGAAGCTGCCTTGACTAAATTTCG	56.1	8 **
SI328	8	TCTGATCTTTTCTGGGCTACTCTCGAAGCATTTCACAAGTCTTTTGGTGACAA[A/G]TTCAAGTTT GTGACTTTTGCTTTTCTTCATGTTTTCTTTTTCCCCCTCCGTAAATTTTGT	54.1	4 **
SI329	8	TATTGATAATTTTCATTTCCTTGATGTTATATGTTGATAATATGCTGATTAGGTCTAGCA[A/G]CATG CATGGCTAGATTCCAGATTAAATTTCAATACAAAGGTTCTAGGAACCGCATAAAAG	40.8	2 ***
SI330	8	TTTTTATCCACTTATATTGGTATTCCATCTGAATTTACTTCC[A/C]ATATTTGATCCTTTTGCGAGTAG GTCTCGGTTATGATTATGTCTTTGAAAGCTGCCAGTC	58.2	19 **
SI333	2	ACATTGTAAAAGAATTGCCTACGACTTTGGGTGATAATGACTCGTCTGGTACTCTTCTAT[T/C]TGT CCCTAAAACGCACTTTTCAGTCGCAATATTTTCCGTTGACACCCACACATTTTGAAG	2	56 ***
SI334	*in silico*	CATCAGATGGGCATTCAGATAAAGTTAAAGCATCTTCTGATGGTTCAGGATCTA[T/C]TCATGAGC AGAGAATTTCAATAGAAAAGTCCTCCAGTTCTGAATTTTTCTGGAATCGAAG	37.8	8 **
SI335	8	AAAATTTATCTAAGTCTGCATGATTTGGCTGCTGCATTTAAGAATTTGAGCTCAAATTTT[C/G]TTT TGGTGACATGTGAAAAGAAGTTTCTGGTTCATGCTAACC	53.1	67 **
SI337	8	CTGGTAGATGCAGCTATCCTGCAATTCAACTGTGCCAAAATATCAAATTCAAGCGACACA[A/C]AT ACATATTCATACGTTGTTTTCATAATTTATAT	38.8	21 **
SI338	8	TGTGTGCAAGGGTATCTTCAGGAATGACAATGTGGCTGATGACGACATTGTCAAGCTTGT[T/C]G ACACCTTCCCCGGCCAATCCATTGGTAAGTTTCCAGTTCCGTTGATGATGCAAACTCCC	29.6	16 ***
SI340	8	TAAGTGGGATGATCTTGTTGCTCATATGCCTCTTAAGATCTGTTACCCTGCTTTGGAGTA[T/C]GAG GAATGGCGAATAATCACTGGCGGTGACCCAAAGAACACGTGAGGCTCTTGTTTTGTG	35.7	7 ***
SI341	8	TTTTCCTTTTTTGAAGGACATCTAAC[T/C]GCGAAAAGCGATGTGTACAGTTTTGGGGTTGTTCTC CTCGAAATGTTAAG	11.2	16 **
SI342	8	ATGAACGTAGATCATGAAGCGTCGAGCTTCCTGAGCTCTAAGATAGTCTGAATCATCCTC[T/C]GG CCTTTCTGCAGGTTCATATTCACCACTCCTCTTCACCTCCCGATTCCCAAGGCAGAAG	54.1	88 ***
SI344	2	TGCCTCCGCAAAAGCCGCTATGGAGAAATCTCTCAATTTGAAGTCACCGAAGCAAAT[A/C]CATC AGACGCCAGAATTGGAGACTGCCAAGGTGGCTGCTGAGCCCTTTGTGGGTTCCTCG	29.6	2 **
SI345	8	GGCCGAGCAATAATATTTCCGGGTTGTGTTTGAACCCGGGTAGTCCATCTGGATCCGATT[T/C]GA GTGATTCAAGCTTACCCGGTGTGGCTCAGTA	46.9	32 **
SI346	8	GGCCACCTGTTGTGACAAAATTGGTATCAAGAAAGG[A/G]CCATGGACTCCAGAAGAAGATATCA TTTTAGTTTCTTATATTCAAGAGCATGGCCCTGGA	53.1	53 **
SI347	8	CCCGTTAATTTTGGATTTACCGCACTAACTTTACTTACTTTTAGCCGAATCGTGCAATGG[T/G]AAC ACCTCTTCTTTTGATTTTACCTTCAATGGGCCAGTTAGTACAATAATACTACAACTG	48	22 ***
SI348	8	TAAGAAGCAGAAGTTGGCACTTCAGAATCAAAGAGTCAGCATTCAGGCTCAAGCTTTTGA[A/G] CTTGAGAAACAACACTTAAAGTGGCTAAGATATTGCAGCAAGAAAGACCGGGAACTGGAG	30.6	20 ***
SI349	8	CCCTATAATCCAGGTTACCAAGTGGCATATGGAATACTAGCTGAGGTAGAA[C/G]AGCACCCGTTT GATTTAGACAAGATGGTTTTCATGGATTGGAGAGATTCGCATCTGAACA	2	13 **
SI351	2	TGTTTTTGGAGGCTTCCTTAGATAGCTTTATTGATGCAATTTAGCTAGTTTCTTAAGACT[C/G]TTG TCGAAGGCATCATACATGTTGAATTATGTGATGATATGATCTATTCTAGATTCAGAT	14.3	129 ***
SI352	8	TGCCCCTCTGGAGCCAAAGATGAATCAAGAAC[T/C]GTTGGAATGCTTAAAAATATACTTCCATAA GGCTCCTCCAGTCTGTTCCAGTCATCCTAA	61.2	2 **
SI353	8	AGGAGCCAAGCTCTGGGTTCTAATGAAGTCTCAGTTTATTGCAACTCAGTTTCGTGTTCT[T/C]AT CCGAGCAATCGCTACGGCTCTCGACGTTTTTCCATTGGATACAGTGGAXATTTGTGGT	61.2	12 **
SI356	8	ATTTTACTAGTGAGAAGACTCAGGATCAGAAGGATGGTCACCATACATCACG[T/C]AGATACCTG CCAGTCATTTGCAAATTCTTCACCATGCTCTCAGCTTTAGAGCTGTCAAGA	40.8	4 **

Continued

SNP name	Method of SNP discovery[a]	STS Sequence including the SNP[b]	Heterozygous loci (%)	*Citrus clementina* Scaffold number mapped STS sequence[c]
SI359	8	TCCTCAATTGGATTGCCTTTGCCAATCGTTTGTTCTAACTCTAATCG[T/G]ACTTTTGATAAAATAT CTGGGTTGCGAAGTAGTTCTGTCATTGCCCATTCCAATGTACTC	29.6	2 **
SI360	2	GCCAAGCTCGACTTCGCATGCAAAGAATGGGGTTTTTTCCAGGTATAATGCTTTAGCAAA[A/G]TT AGAAAATTGTTTCGCCAGAACATGCTTGTTTGTGAACAAGAATCGAGAAATATGCAAA	1	17 ***
SI361	8	TTAAATTGTTTTCTTTGAATACCGAAGTCTTATGCATTTTGGTATCTCAGGTCCAACAAA[A/G]AA GAAAAAGCTTGACTGGGACACTCGACTGAAAATAGCACTAGGAGCTGCACAAGGGCTA	46.9	4 ***
SI363	8	ACTTCATTCTCAACGACTAACCTCTGACACAAAGAAGGCATGAAATCCGTATGGAACCCT[A/T]T GTGGTAATTCGACAACTGCAACAGGATCAGCTGACATTGTTTTTGCATCAATCACGTTC	41.8	39 ***
SI366	2	GAGGAGGTTAAAAGTGAAGATTATACCCTCATAAACCTCTCAATAGAAAATAAAATGTTA[A/G]G CCCCGAAGCTAAAAATTTCCAGCTCAATAGATGGAAGCATCAAATCTTGGCTCATAAAA	49	53 ***
SI369	8	AAGCGTCCGTGGAGGACAGTGAGAGAAGGAAAACTGTACCATAATTGTAAGTAAACGTGC[A/G] GTGCATTGAAAACAAGCAAGTGCATGGCTGAAAACCTTTTTCATTACTTTTTGTTGAGGC	55.1	1 **
SI371	2	GGTAGTACGGTCTCCAGCATCTTGCACTGGTCTGGGCTCAGTGACTCAGGGAAGTCAACA[C/G]T GAAATGAATGTACAGTTTGCCCCTCATAAATGGCCTCTGATACATAGGCATTCCTTCAT	14.3	129 ***
SI372	8	GTATCCCAAAAACAGGTATATGTGACCATTTCTGAAAAAAAAGGGCGC[A/G]CATAACCATTTTAT CAAATCATAAGAGTGCTGCTGTCATTTAT	18.4	84 *
SI373	8	GCCCAACTTCAGCATTGCCTTGCGGCTCTTTTTCTCACTTCTGCTCTGCTTTGAACTCCC[A/T]CC TGCAACTCAGTAACCAATCAAGACTACTTAAATCACTGTAGAAACTACATCCATTGCA	58.2	18 ***
SI374	8	CCGGTACGGCGAGAGGTGTACGAAAACGGC[C/G]AAATTTACGACATCACCCACCGTATTACGAG CGACATGCCGTCGTTTGGCTCGAAGGAGG	65.3	26 **
SI375	8	TAGTTATTGGTTTATGATTTTCTGCATCACACTCTTACTGCATTGCGAATTTACATTTTG[T/C]CCAG ATTTGGATTTGCCTACAGTTGTGTATACCACAGAAGTTTTTTTCTTTCTTTTAATT	5.1	22 **
SI376	8	AGCGAAGAAGAGGAGAGAAAAGCCAAGGTGGTGAAAACGGAGTCTGCGGATAC[A/G]GTTAAT GAATTTCCTTTGACGCCGGCGAGTTGCGCGGCGTTTTGGGACTTG	64.3	2 **
SI379	8	TCAGGAAAGGTTTGCTCTGAAGGAAGCTGGAACAGAACTGGGACCCTCTCTATTATTTTT[T/C]G GATGCAGGAACCGTAAGATGGTAAGTGCT	39.8	4 **
SI380	8	AGTGATTTCGGTGGGCTTTTTTAAACTAAAAGCAATTCTTGCTCTTGAAGAAGAAAAAGA[A/C]G GAGAAGAACAAGAATCTTTACAAGAGCCCAAGTCTATGAGTGAAGAAGTAGTAGTTGCT	60.2	17 **
SI381	8	GAGTCTTGAAATGATAACAAGACCCTAT[T/C]CTTAATACTTGAAGCGCACGTCATGTTTCTTCCG TGGCTAATTATTGTGTATGAATTATG	53.1	27 **
SI383	2	CAATCAAATATCTAACACAAACAAATACATTCT[A/T]CTGAACGTAGGTAGTAAAGCCCAAAAAG AATCCCCGACAAAATAAACAAAAGCATAGTAA	55.1	72 **
SI384	8	ATTGATCATATGTTGGGATTTTCTAGTTGTGATC[T/C]GTTATGCAAGGGGCAATGCTGTACTTCAT GTGTCTTCAATTAGATTCTTTTAAGTGAATT	56.1	32 **

[a] '8', '2' and 'in silico' indicate 'The SNP is detected from comparison of eight citrus genotypes', 'The SNP is detected from comparison of two citrus genotypes' and 'The SNP is detected in silico', respectively.

[b]SNP is indicated by '[/]' in the sequence.

[c]Number of asterisk shows expect of blastn: ***=2.00E-58 (minimam value), ** <1.00E-30, *<1.00E-10, - >1.00E-10.

Table 4-5 Number of STS from which genotyping SNPs were derived for the array *CitSGA-1*.

Number of SNPs per STS	Number of STS from which SNPs derived on *CitSGA-1* (%)	Number of STS from which relaiable SNPs derived on *CitSGA-1* (%)
1	199 (70.3%)	156 (73.6%)
2	71 (25.1%)	48 (22.6%)
3	10 (3.5%)	7 (3.3%)
4	2 (0.7%)	1 (0.4%)
5	1 (0.4%)	0 (0.0%)
Total	283 (100%)	212 (100%)
Number of SNP	384	277

Table 4-6 Ratio of heterozygous loci in citrus germplasm samples.

Sample name	Heterozygous loci (%)	Sample name	Heterozygous loci (%)	Sample name	Heterozygous loci (%)	Sample name	Heterozygous loci (%)
TY001	44.7	TY028	29.3	TY054	56.5	TY081	35.4
TY002	41.5	TY029	49.6	TY055	42.7	TY082	51.2
TY003	35.4	TY030	50	TY056	35	TY083	50
TY004	50.8	TY031	50	TY058	27.6	TY084	58.9
TY005	54.5	TY032	45.1	TY059	70.7	TY085	30.1
TY006	45.9	TY033	50.8	TY060	44.7	TY086	58.1
TY007	43.9	TY034	45.5	TY061	57.7	TY087	43.9
TY008	55.7	TY035	44.7	TY063	42.7	TY088	57.7
TY009	43.9	TY036	63.4	TY064	39	TY089	66.7
TY011	52.4	TY037	41.9	TY065	42.7	TY090	49.6
TY013	39.4	TY038	41.9	TY066	61	TY091	37.4
TY014	49.2	TY039	45.1	TY067	30.9	TY092	29.7
TY015	45.1	TY040	35.8	TY068	54.5	TY093	43.1
TY016	38.6	TY041	44.7	TY069	8.9	TY094	50.8
TY017	41.9	TY042	54.9	TY070	49.6	TY095	47.2
TY018	50.8	TY043	56.5	TY071	42.7	TY096	21.1
TY019	44.7	TY044	39.4	TY072	55.3	TY097	28
TY020	39	TY045	54.1	TY073	37.8	TY098	33.3
TY021	36.2	TY046	57.3	TY074	38.2	TY100	30.1
TY022	40.7	TY047	26	TY075	37.4	TY101	59.8
TY023	53.7	TY048	39.8	TY076	37.4	TY102	51.2
TY024	50.4	TY049	55.7	TY077	42.7	TY103	32.5
TY025	34.1	TY051	48.4	TY078	48.4	TY104	58.5
TY026	32.1	TY052	66.3	TY079	43.5		
TY027	41.5	TY053	48	TY080	37.4		

Chapter 5. General Discussion

This thesis describes the development and application of high-throughput genomics tools, the oligoarray and the SNP genotyping array, based on recent technologies and the software for efficient use of genotyping result. It was shown that a large amount of gene expression analysis and the genome structure analysis have been available in citrus.

The results in Chapter 2 show that the citrus custom oligoarray is an important tool for profiling gene transcription. An example of its use was the gene transcript profiling of mature mandarin fruit subjected to plant hormone treatments. Consequently, a gene encoding a transcription factor of a carotenoid metabolic enzyme gene was screened. In the further study its function confirmed in recombinant tomato (Endo et al. 2013). Chapter 3 detailed that the software MinimalMarker was effective for producing a minimal set of DNA markers to identify cultivars when a large amount of genotyping data are available. Afterward the software has been applied to identify cultivars of various crops (Takashina et al., 2008; Yamane et al., 2012) and haplotype of allele among several cultivars. Chapter 4 describes the development of a 384 SNP genotyping array, and its use for high-throughput genotyping of 98 citrus accessions and one citrus population. Using this method, a linkage map can be constructed rapidly and SNP markers can be used to identify cultivars. Consequently, this study has provided genomic tools and higher-level genomic analyses for citrus. Their applications to citrus to establish a foundation for advanced genomic analyses. The high-throughput genome analysis tools will be indispensable for the essential resolution of important traits of citrus to the gene level.

However, it remains difficult to achieve important breakthroughs, even when powerful genomic tools are used. For instance, the microarray analyses in Chapter 3

identified 24 genes that showed 3-fold changes in transcript levels in response to ethylene as ethylene-responsive transcription factors. Based only on the results of microarray analyses, it is difficult to identify which transcription factors might play important roles in regulating expressions of genes involved in fruit ripening, such as those associated with chlorophyll degradation and carotenoid accumulation.

Two previous studies serve as references for the limitation of genomic tools. Sugiyama et al. (2010a) performed an eQTL analysis to quantify expression levels of genes involved in carotenoid metabolism in citrus fruit. Their aim was to identify possible *cis*- and *trans*-regulating regions to refine selection markers for carotenoid accumulation. In other eQTL analyses (Jansen and Nap, 2001; Doerge 2002), transcript levels were analyzed as quantitative traits and their variations were used to map eQTL. Once eQTL are identified in a population, they provide the necessary information to identify genes or loci that control quantitative traits. In plants, global eQTL analysis of gene expression has been used to detect *cis*-polymorphisms controlling individual genes, as well as to search for *trans*-eQTL that regulate individual genes from remote loci (DeCook et al., 2006; Keurentjes et al., 2007; Potokina et al., 2008; West et al., 2007; Sugiyama et al., 2014). The eQTL methodology combines two types of genomic data; the expression levels of genes, and a linkage map composed of DNA markers. Together, these data provide sufficient information to search for genes related to particular functions. Nagano et al. (2012) collected transcriptome data using a 461 microarray from the leaves of rice plants grown in a paddy field, and obtained the corresponding meteorological data, and used both data sets to develop statistical models to explain the endogenous and external effects on gene expression. Their models will help to translate the knowledge amassed in laboratories to solve agricultural problems. Thus, genomic analyses that combine various data, including genomic data, are required to further

research on citrus.

New opportunities have arisen in citrus genome analysis research in recent years. Three full-length annotated genome assemblies have been produced and made available to the global research community (Gmitter et al., 2012; Citrus Genome Database. http://www.citrusgenomedb.org; Xu et al., 2013; *Citrus sinensis* Annotation project. http://citrus.hzau.edu.cn/orange/). The first genome assembly, which serves as the reference genome for citrus, is from a haploid plant derived from 'Clementine' mandarin. The sequencing project was the work of the International Citrus Genome Consortium (ICGC http://www.citrusgenome.ucr.edu/). This version of the assembly (v1.0) is 301.4 Mb spread over 1,398 scaffolds with 2.1% gaps at 7.0x coverage. Over 96% of the assembly is accounted for by the 9 chromosome pseudo-molecules ~21-51 Mbp in length. The current gene set (clementine1.0) integrates 1.560 M ESTs with homology and *ab initio*-based gene predictions (by GenomeScan, Fgenesh, exonerate). 24,533 protein-coding loci have been predicted. Each encodes a primary transcript. There are an additional 9,396 alternative transcripts encoded on the genome generating a total of 33,929 transcripts. 16,963 primary transcripts have EST support over at least 50% of their length. A third of the primary transcripts (8,684) have EST support over 100% of their length (http://www.citrusgenomedb.org/species/clementina/genome1.0). A second genome assembly was produced from the sweet orange clone 'Ridge Pineapple' (Gmitter et al., 2012). This version (v.1) of the assembly is 319 Mb spread over 12,574 scaffolds. Half the genome is accounted for by 236 scaffolds 251 kb or longer. The current gene set (orange1.1) integrates 3.8 million ESTs with homology and *ab initio*-based gene predictions. 25,376 protein-coding loci have been predicted, each with a primary transcript. An additional 20,771 alternative transcripts have been predicted, generating a total of 46,147 transcripts. 16,318 primary transcripts have EST

support over at least 50% of their length. Two-fifths of the primary transcripts (10,813) have EST support over 100% of their length (Citrus Genome Database. http://www.citrusgenomedb.org/species/sinensis/genome1.0). The third was produced from a doubled-haploid callus line of sweet orange (Xu et al., 2013). The version was assembled using SOAP denovo, resulting in 4,811 scaffolds. The total contig length (320.5 Mb) covers about 87.3% of the sweet orange genome. The scaffolds were aligned and oriented to the *Citrus* linkage map and about 80% of the assembled genome was anchored and organized as nine pseudo-chromosomes (*Citrus sinensis* Annotation project. http://citrus.hzau.edu.cn/orange/).

The production of these genome assemblies enabled advanced genome analysis. For example, it became possible to identify repression after meiotic recombination by comparing the physical map with the genetic map, and to analyze expression of individual members of multi-gene families. Moreover, recent rapid developments in DNA sequencing technologies have dramatically cut both the cost and the time required for sequencing.

To make the most of these opportunities, it is necessary to advance genomic analyses of citrus, especially analysis of individual alleles. For example, the carotenoid metabolism enzyme ZEP is a key regulator of carotenoid accumulation in citrus fruit. The expression level of alleles of the gene encoding this enzyme, *ZEP*, accounts for differences among varieties in their ability to convert zeaxanthin to Vio during fruit development. The expression levels of *ZEP* alleles in the fruit of three heterozygous citrus cultivars were compared using allele-specific RT-PCR. Sugiyama et al. (2012b) showed that there was a stronger allele and a weaker allele for expression, and suggested that the difference between the expression levels of the two alleles was at least partly due to differences in *cis*-structures located in the 5′ - UTR of the *ZEP*

genes. Since eight genes are related to the carotenoid biosynthesis pathway in citrus, it was proposed that the combination of gene alleles expressed at each step was related to differences in accumulation of various carotenoid components among cultivars. Most citrus cultivars and strains cultivated in Japan are derived from only 14 ancestral cultivars (Imai, personal communication). Hence, each cultivar and strain has two of only 28 alleles derived from the 14 ancestral cultivars. Sequencing of all these gene regions in 14 ancestor cultivars and analysis of the data using a SNP genotyping array would confirm the haplotypes of this gene. This would clarify the allele type in most citrus cultivars and strains in Japan. Such analyses might be able to explain the differences in various traits among cultivars based on their combination of alleles.

SUMMARY

Citrus is one of the most economically important fruit species in the world. The fruit is rich in the second metabolites for beneficial for human health, with diverse colors, fragrances and tastes. In addition, citrus is among the most difficult plants to improve through cross breeding approaches and to analyze physiologically varietal characteristics because of its the polyembryony, male sterility or self-incompatibility. Genome science technologies have advanced rapidly over the last decade, and have been adapted to address the challenges of the citrus breeding and physiological analysis. Expressed sequence tag (EST) analysis, DNA markers, linkage mapping and quantitative trait locus (QTL) analysis have promoted the efficient selection systems and analysis of gene expression. Remarkably, three genome assemblies have been released to the public since 2011. Despite the challenges of working with citrus to understand the important characters of citrus, the result is insufficient. Expression analysis of many genes related to important characters and analysis of genome-wide genotyping among many varieties or the combination of these two analyses is necessary to understand important characters of citrus. This study was performed to provide the basis for comprehensive use of citrus genome information, which has been accumulated quickly.

1. 22K citrus oligoarray analysis of gene expression in mature mandarin fruit

1) Profiling ethylene-responsive genes in mature mandarin fruit using a citrus 22K oligoarray

A comprehensive transcriptome analysis using a citrus 22K oligo-microarray was performed to identify ethylene-responsive genes and gain an understanding of the

transcriptional regulation by ethylene in Satsuma mandarin fruit (*Citrus unshiu* Marc.). Seventy-two hours after ethylene treatment of mature fruit, 1,493 genes were identified as ethylene-responsive, with more than 3-fold expression change. Interestingly, more than half of the ethylene-responsive genes were repressed after ethylene treatment. This might suggest that ethylene inhibits various biological processes and plays an important role in fruit ripening and senescence. Ethylene repressed the transcription of many genes involved in photosynthesis, chloroplast biogenesis, and sugar metabolism, while it induced the transcription of several genes related to resistance, defense, stress, amino acid synthesis, protein degradation, and secondary metabolism.

2) Profiling gibberellin (GA_3)-responsive genes in mature fruit using a citrus 22K oligoarray

Gibberellin3 (GA_3)-responsive genes were investigated with a citrus 22K oligo-miciroarray to gain further the understanding of the transcriptional regulation by GA_3 treatment in Satsuma mandarin fruit. 213 GA_3-responsive genes were identified that showed a 3-fold or greater expression change after 72h of GA_3 treatment of mature fruit, compared with expression after 72 h of air treatment. GA_3 treatment induced the expression of pathogenesis-related (PR) proteins and genes that function in photosynthesis, chloroplast biogenesis, resistance, defense and stress. Also, GA_3 treatment reduced the transcription of several ethylene-inducible genes, such as carotenoid metabolic genes, which are associated with fruit ripening. Contrasting effects between GA_3 and ethylene were observed. The endogenous GA_3 level might be important for the endogenous regulation of maturation and senescence in mature citrus fruit.

2. An algorithm and computer program for the identification of minimal sets of discriminating DNA markers for efficient cultivar identification

DNA markers are frequently used to analyze crop varieties, with the coded marker data summarized in a computer-generated table. Such summary tables often provide extraneous data about individual crop genotypes, needlessly complicating and prolonging DNA-based differentiation between crop varieties. At present, it is difficult to identify minimal marker sets—the smallest sets that can distinguish between all crop varieties listed in a large marker-summary table—because of the absence of algorithms capable of such characterization. Here, we describe the development of just such an algorithm based on combinatorial optimization and the computer program, named MinimalMarker. MinimalMarker is available for use with both dominant and co-dominant markers regardless of the number of alleles, including simple sequence repeat (SSR) markers with numeric notation.

3. High-throughput genotyping in citrus accessions using an SNP genotyping array

A 384 multiplexed single nucleotide polymorphism (SNP) genotyping array, named *CitSGA-1*, for the genotyping of citrus cultivars, was developed, and the performance and reliability of the genotyping were evaluated. SNPs were detected in cultivars representing the genetic diversity of citrus currently bred in Japan. The assay using *CitSGA-1* was applied to a hybrid population of 88 progeny and 103 citrus accessions for breeding in Japan. A total of 351 SNPs could call different genotypes among the DNA samples. To confirm the reliability of SNP genotype calls, parentage analysis was used, which indicated that the numbers of reliable SNPs were 276. Using 7 SNPs that were identified by MininalMarker, all germplasm accession could be discriminated from each other. The SNP genotyping array reported here will be useful for the efficiently constructing linkage maps, for the detection of markers for marker-assisted breeding and for the identifying of cultivars.

By developing two genomic tools in this study, comprehensive and

high-throughput gene expression analysis and genotyping have become accessible. In addition, the bioinformatics tool has developed to use the genomic tools thoughtfully. These tools are available as the research base for detection of regulatory genes which control trait, linkage mapping and maker aided selection.

摘　　要

カンキツは世界的に経済上，最も重要な果樹の１つであり，果実の色や香り，味，健康機能性を持つ二次代謝成分などの多様性に基づいた新品種の育成が進められている．しかし，カンキツには多胚性や雄性不稔性，開花までの年数が長いといった性質があり，交雑育種や品種特性の生理学的な解析を困難にしている．近年，急速に進展しているゲノム科学の成果を利用して，カンキツの育種や生理学的な解明が進められている．例えば，発現遺伝子配列断片（EST）解析や DNA マーカー，連鎖地図，量的形質遺伝子座（QTL）解析等が育種の効率化や果実における遺伝子発現の解明に利用されてきている．また，2011 年以降に３つのカンキツゲノムの全塩基配列解析結果が公開された．こうした研究にもかかわらず，カンキツの重要形質に関する遺伝子レベルの解明は十分ではない．重要形質の解明のためには，形質に関わる多数の遺伝子の発現解析や多数の品種についてのゲノムワイドな遺伝子型情報解析及び，それらを組み合わせた解析が不可欠である．本研究は，急速に蓄積が進んでいるカンキツゲノム情報を総合的に利用するための基盤を構築するために、一連の研究を行ったもので，以下の課題により構成される．

１．オリゴアレイによるカンキツ成熟果実における遺伝子発現解析

1)オリゴアレイによるカンキツ成熟果実におけるエチレン応答遺伝子のプロファイリング

ウンシュウミカン（*Citrus unshiu* Marc.）の成熟果実におけるエチレン応答遺伝子の特定とその転写制御の解明のために，カンキツの 22K オリゴマイクロアレイ（オリゴアレイ）を作成し，遺伝子発現解析を行った．エチレン処理 72 時間後に３倍以上の発現変化があった 1493 個の遺伝子をエチレン応答遺伝子と特定した．エチレン応答遺伝子の半分以上はエチレン処理により発現が抑制さ

れていた．このことは，エチレンが多くの生物学的なプロセスを低下させ，果実の成熟と老化に関して重要な役割を果たしていることを示していた．エチレンは光合成や葉緑体生合成，糖代謝等の遺伝子の転写を抑制した一方，病害抵抗性や防御，ストレス，アミノ酸合成，タンパク質分解，2 次代謝に関連する遺伝子の転写を誘導した．

2) オリゴアレイによるカンキツ成熟果実におけるジベレリン応答遺伝子のプロファイリング

ウンシュウミカンの成熟果実でのジベレリン 3（GA_3）による転写制御を知るために，オリゴアレイを用いて GA_3 応答遺伝子を調査した．GA_3 処理 72 時間後のウンシュウミカン成熟果実と対照区との遺伝子発現を比較したところ，3 倍以上の発現変化を示す 213 個の GA_3 応答遺伝子が特定された．GA_3 処理は，生体防御タンパク質や光合成，葉緑体生合成，病害抵抗性，防御，ストレスに関連する遺伝子の発現を誘導した．また，果実の成熟に関連するカロテノイド代謝遺伝子等のエチレンに誘導される遺伝子の転写を低下させた．その効果はエチレンと対照的で，内性の GA_3 のレベルが果実の成熟と老化の制御に重要であると推測された．

２．DNA マーカーによる効率的な品種識別のためのソフトウエアの開発

DNA マーカーを様々な品種に適用した結果は，DNA マーカー型を記号に置き換えた表に整理され，品種識別に利用される．膨大な 2 次元表から最も少ない数で全ての品種を識別することができる最少マーカーセットを求めることは簡単ではない．そこで，最少マーカーセットを求めるための組み合わせ最適化理論に基づくアルゴリズムとその計算を実現するソフトウエア MinimalMarker を開発した．MinimalMarker は共優性マーカーにも優性マーカーにも適用可能であり，アリルの数を問わない．また，単純反復配列（SSR）マーカーのような数値表記のマーカーにも適用できる．

３．SNP アレイを用いたカンキツ品種のハイスループットなジェノタイピング

カンキツのハイスループットなジェノタイピングを実現するために，*CitSGA-1* と名付けた 384 個の一塩基多型（SNP）を搭載する SNP ジェノタイピングアレイ（SNP アレイ）を開発し，その性能と信頼性を評価した．搭載した SNP はわが国のカンキツ育種の遺伝的な多様性を代表する品種から収集した．*CitSGA-1* を 88 個体の交雑集団とわが国のカンキツ育種に関連する 103 種類の品種・系統に適用した結果，351 個の SNP で遺伝子型を識別できた．SNP アレイ解析による遺伝子型の信頼性を確認するために親子分析を行った結果，276 個の SNP が信頼できると判定された．これらの SNP に MinimalMarker を適用したところ，7 つの SNP を用いることにより，すべての品種・系統を識別できることがわかった．

これらの研究を通じて開発したオリゴアレイと SNP アレイの２つのゲノム解析ツールにより，網羅的かつハイスループットな遺伝子発現解析と品種ジェノタイピングを可能になり，これらの情報を使いこなすバイオインフォマティクスツールの開発とあわせて，形質制御遺伝子の探索，連鎖地図の効率的な作成や，マーカー支援育種などの研究基盤としての利用されるものと期待される．

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