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ORIGINAL PAPER

Construction of genetic linkage map and graphical genotyping of pseudo-backcrossed F_2 (BC'_2) progeny to introduce a CTV resistance from *Poncirus trifoliata* (L.) Raf. into *Citrus* by introgression breeding

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Abstract Most citrus tristeza virus (CTV) strains infect almost all *Citrus* species and have caused serious damage on citrus production in many countries. *Poncirus trifoliata* (trifoliate orange) has a single dominant resistance gene effective against a broad range of CTV strains. *P. trifoliata* is sexually compatible with *Citrus* species, and introgression breeding programs have been initiated to introduce the CTV resistance into *Citrus*. "Kankitsu Chukanbohon Nou 8 Gou" (Nou-8) was developed by crossing *Citrus sp.* "Kiyomi" with an F_1 hybrid derived from *Citrus hassaku* × *P. trifoliata*, but it still had undesirable eating traits. In aim to progress marker-assisted introgression breeding for CTV resistance efficiently, we selected 189 DNA markers which could be applied to genotyping for *Citrus* and *P. trifoliata* and constructed a genetic linkage map of Nou-8. The map included 189 DNA markers on 10 linkage groups and extended about 722.8 cM in total length. CTV resistance locus was located at 2.4 cM position in linkage group 02_(1), with the same location found when progeny were scored for resistance using an ELISA assay and for DNA marker for CTV resistant (CTV103). Graphical genotypes of Nou-8 and its progeny suggested that (1) 36 % of the chromosome in the BC'_2 population were inherited from Nou-8 without any recombination sites, (2) Nou-8 had been already completely substituted by *Citrus* genotype in two linkage groups, and (3) four of 93 progeny of Nou-8 had recombination sites in the flanking region of the CTV resistance gene locus.

Keywords *Citrus* · CTV resistance · Genetic linkage map · Graphical genotype · Introgression breeding · *Poncirus trifoliata*

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Introduction

Citrus tristeza virus (CTV) had injured over 100 million citrus trees in the world (Moreno et al. 2008). There are three major strain types characterized by symptom: seedling yellows, stem pitting, and quick decline. The last two types can cause significant damage for citrus trees. Virus-host interactions are clearly observed, and host ranges in *Citrus* species differ with CTV strains. For example, the isolate causing specific symptom in grapefruit did not cause any symptoms in other varieties (Dawson et al. 2013).

CTV is transmitted easily throughout the orchard by aphids so that eradication of this virus is an impossible task. A countermeasure using a mild viral strain for cross protection

has been applied in many countries (Lee and Keremane 2013). CTV resistance rootstock is also an important measure (Bordignon et al. 2007). These techniques have helped to suppress terrible damage in many countries, but it is still difficult to cultivate CTV-susceptible cultivars without yield decrease in CTV distribution areas. In Japan, it is difficult to produce a big and beautiful fruit without lesion on their surface from the trees infected with CTV on sweet orange (*Citrus sinensis* (L.) Osbeck), grape fruit (*Citrus paradisi* Macfad.), and yuzu (*Citrus junos* Seibold ex Tanaka). Therefore, the development of CTV-resistant scion cultivars would be a drastic measure to avert damage caused by CTV.

Some *Citrus* species, such as *Citrus grandis* (L.) Osbeck, *Citrus aurantium* L. "Afin Verna," *Citrus sunki* (Hayata) hort. ex Tanaka "200," *Citrus limonia* Osbeck "Limeira," are resistant to one or certain CTV strains (Garnsey et al. 1997; Fang and Roose 1999; Bernet et al. 2004; Bordignon et al. 2007). Moreover, *Poncirus trifoliata* (L.) Raf. (trifoliate orange) has high resistance to many strains (Yoshida et al. 1983; Garnsey et al. 1987; Gmitter et al. 1996; Mestre et al. 1997a). The CTV resistance of *P. trifoliata* is effective against almost all CTV strains except some CTV resistance-breaking strains found in New Zealand and Puerto Rico (Dawson and Mooney 2000; Harper et al. 2008; Roy et al. 2013). Fortunately, these resistance-breaking strains have not been found in most of citrus-producing countries and cause mild symptom if it infected to *P. trifoliata*. While other resistance locus could concern with CTV resistance (Mestre et al. 1997b; Asins et al. 2012), the CTV resistance of *P. trifoliata* was inherited as a single dominant gene (Yoshida 1993; Gmitter et al. 1996; Fang et al. 1998). This segregation pattern was also observed in our three breeding populations with no counterexample (Ohta et al. 2011). Therefore, *P. trifoliata* could be candidate materials for breeding CTV-resistant scion cultivars.

Breeding programs for introducing a CTV resistance gene from *P. trifoliata* using molecular marker have progressed in several countries (Garnsey et al. 1987; Gmitter et al. 1996; Mestre et al. 1997c). An intergeneric hybrid, "US119," has been developed in the USA (Hearn et al. 1993), and "Kankitsu Chukanbohon Nou 8 Gou" (described as Nou-8 in this manuscript, Fig. 1) has been released by our institute (Plant Variety Protection System in Japan; registration number 14544). Despite these efforts, both pseudo-backcrossed strains US119 and Nou-8 still have unfavorable traits originating from *P. trifoliata*, such as high acidity, acridity, and long thorns. Therefore, a further introgression breeding program with *Citrus* accessions was required to remove these unfavorable traits. In the present study, Nou-8 and *C. grandis* "Siamese Acidless" were crossed and a total of 110 BC'_2 individuals were obtained. In aim to progress generation and efficient selection of BC'_3 population, the development of marker-assisted selection system (MAS) is indispensable.

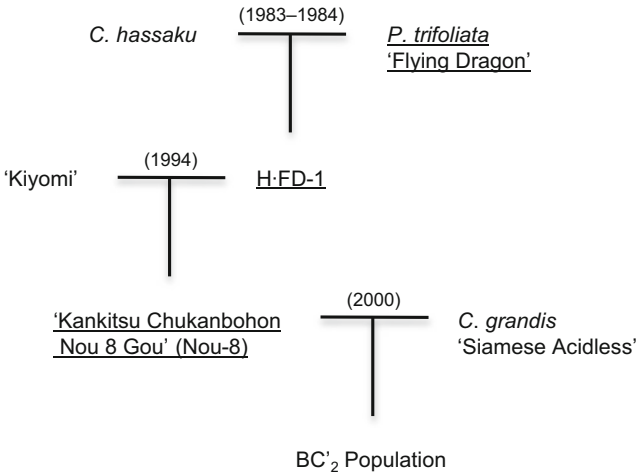

Fig. 1 Pedigree of Nou-8 and BC'_2 population for introducing CTV resistance into *Citrus*. *Underlined individuals* have CTV resistance

DNA markers improve the efficiency of introgression breeding by reducing the number of generations required for recovery of the recurrent parent genome (Frisch et al. 1999). Further, to aid in marker-assisted breeding for introducing the CTV resistance gene from *P. trifoliata* into *Citrus* cultivars, we developed three DNA markers linked to the CTV resistance and 46 cleaved amplified polymorphic sequence (CAPS) markers which can identify *P. trifoliata*-specific alleles (Ohta et al. 2011). We could estimate the positions of these DNA markers referencing to AG integration (AGI) map (Shimada et al. 2014), but a genetic linkage map could not be constructed, owing to the insufficient number of markers. Several genetic linkage maps of *P. trifoliata* have been developed by using random amplified polymorphic DNA (RAPD) markers (Gmitter et al. 1996; Cristofani et al. 1999), RAPD markers and 24 specific primer pairs (Weber et al. 2003), but these DNA markers were not applicable to our breeding populations because of less reproducibility, and the number of markers were not enough to construct a linkage map.

The concept of graphical genotypes has been proposed as a tool for marker-assisted backcrossing (Young and Tanksley 1989). Graphical genotypes express the origin of haplotypes and the position of recombination sites on chromosomes in each gamete. As the authors pointed out, it is thus possible to perform "whole genome selection" based on the graphical genotypes portrayed. In other words, comparison of graphical genotypes helps breeders to select a candidate mother plant that has the most desirable chromosomal regions and to identify chromosomal regions that should be replaced by those from the recurrent parent.

In this study, we selected 189 DNA markers which could be applied to genotyping for *Citrus* and *P. trifoliata* and to construct a genetic linkage map of Nou-8. The information of the genetic map was useful to understand the position of *P. trifoliata*-specific alleles and to direct further study for identifying the map positions of genes controlling undesirable

traits originating from *P. trifoliata*. We constructed graphical genotypes of Nou-8 and its offspring to identify chromosomal regions in which *P. trifoliata* alleles remained.

Materials and methods

Plant materials

Ninety-three individuals of the BC'$_2$ population and relatives in their pedigrees, *Citrus hassaku* hort. ex Tanaka, *P. trifoliata* "Flying Dragon," "Kiyomi" (*Citrus unshiu* Marcow. "Miyagawa" × *C. sinensis* "Trovita"), Nou-8, and *C. grandis* "Siamese Acidless" were used (Fig. 1). Three individuals were derived in F_1 generation, and only one of them had CTV resistance so that we use the one for next crossing. Nou-8 had been selected for a mother plant from 58 individuals of BC'$_1$ generation based mainly on fruit quality. Nou-8 had better fruit taste and appearance than the other BC'$_1$ individuals and it was monoembryonic. *C. grandis* "Siamese Acidless" was used as a pollen parent to reduce acidity, which Nou-8 shows 4–5 % in late December. Crossing between Nou-8 and *C. grandis* "Siamese Acidless" was conducted to develop CTV-resistant scion cultivars, and BC'$_2$ population was derived in 2000. Preselection of BC'$_2$ individuals was conducted mainly based on fruit taste because most of them had some amount of acridity or high acidity originating from *Poncirus*, and some individuals were eliminated by appearance and fruit size. All individuals were grown in the research field of the National Institute of Fruit Tree Science, Okitsu Citrus Research Station (Shizuoka, Japan). Only a few selected individuals as a mother plant were replicated on the second selection stage in our breeding system, so that we used the original tree for each individual.

DNA markers

A total of 216 primer sets reported in our studies (Omura et al. 2003; Ohta et al. 2011; Shimada et al. 2014) and new primer sets were used for CAPS analysis. These primer sets were screened to obtain DNA markers satisfying the following condition: (1) showing polymorphism between Nou-8 and *C. grandis* "Siamese Acidless," (2) heterozygous in Nou-8, and (3) identifiable which of alleles was inherited from Nou-8 to each BC'$_2$ individuals. If it was necessary to obtain polymorphism, restriction enzyme was changed. Genotypes of ancestors were also analyzed using selected markers in order to display graphical genotypes of Nou-8 and BC'$_2$ individuals. Primer sequences and restriction enzymes used in this study are listed in Online Resource 1.

Experimental methods for DNA typing and ELISA assay

Total DNA was extracted from young leaves following Dellaporta et al. (1983). PCR amplification was performed in a 10-μL reaction solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 0.2 mM each of dNTPs, 10 pmol of forward and reverse primers, 10 ng of genomic DNA, and 0.5 U of AmpliTaq Gold® (Applied Biosystems, Life Technologies, USA). PCR programs were as follows: 35 cycles at 94 °C for 1 min, annealing temperature (as shown in Online Resource 1) for 1 min, and 72 °C for 2 min. Depending on the primer sets, touchdown PCR was also applied for amplification as follows: initial denaturation for 5 min at 94 °C, 8 cycles of touchdown PCR, 35 cycles of general PCR, and a final extension of 7 min at 72 °C. The cycle of touchdown PCR consisted of 30 s at 94 °C, 30 s at an annealing temperature reduced by 2 °C every 2 cycles from 62 to 56 °C, and 1 min at 72 °C. The cycle of general PCR after touchdown PCR consisted of 30 s at 94 °C, 30 s at 54 °C, and 2 min at 72 °C. PCR products were digested with one restriction enzyme, but some markers required no digestion. PCR products (4 μL) were mixed with 1.0 μL of buffer and two to three units of restriction enzyme, and sterilized water was added to 10 μL. The reactions were incubated at 37 °C for 90 min before electrophoresis on a 1.5 % agarose gel.

An ELISA assay was performed to evaluate the CTV resistance of the BC'$_2$ population and its parental lines listed in Fig. 1. Rough lemon (*Citrus jambhiri* Lush.) infected with a CTV strain (CTV-SY) was grafted onto each individual for inoculation. This CTV-SY was isolated from the original tree of *P. trifoliata* "Kiyomi" planted in Okitsu Citrus Research Station, Japan. It caused severe stunting and yellowing in sour orange (*C. aurantium*) seedlings and severe dwarfing and pitting in sour lime (*Citrus aurantifolia* (Christm.) Swing.) seedlings (Yoshida 1996). CTV infection was assayed by ELISA using polyclonal antibodies against the virus 16 months after inoculation, following a method by Yoshida (1996).

Construction of genetic linkage map

The selected 189 DNA markers and the CTV resistance evaluated by ELISA assay were used to construct a linkage map of Nou-8 (Online Resource 1). Genotypes of all loci for all BC'$_2$ individuals were assigned to H or A types based on which allele was inherited from Nou-8. Linkage analysis was performed with JoinMap 3.0 (BC1 mode), and markers were grouped with a LOD threshold of 4.0. Genetic distances were evaluated using the Kosambi map function. Segregation was tested against the normal expectation ratios using the chi-square test. To display graphical genotypes of BC'$_2$ individuals, genotypes of ancestors (*C. hassaku*, *P. trifoliata* "Flying Dragon," "Kiyomi," Nou-8, and *C. grandis* "Siamese

Acidless") were examined, and the alleles derived from Nou-8 were traced and assigned as *Poncirus* or other *Citrus* species.

Results and discussion

Genetic linkage map of Nou-8

A total of 189 DNA markers were developed by screening 216 primer sets. These markers showed segregation in 93 individuals of the BC'$_2$ population (Fig. 2). Linkage analysis was performed with these 189 markers and the CTV resistance evaluated by ELISA assay, and these markers were distributed over 10 linkage groups (Fig. 3). Genetic linkage map of Nou-8 was compared with a map constructed from AG populations, A-254 × G-434 (Shimada et al. 2014). The total length of Nou-8 map was about 722.8 cM, which was 73.0 % of the length of the AGI map (990.9 cM, 706 markers). The two linkage groups 02_(1) and 02_(2) in the Nou-8 map were considered to be the same linkage group because these two linkage groups corresponded to linkage group 02 of the AGI map (Fig. 3). The size of the gap between linkage group 02_(1) and 02_(2) was estimated to be about 16.4–17.4 cM based on comparison to the AGI map. The marker on the nearly end of 02_(1), Al0037, was a sequence-tagged site (STS) to identify the locus Ciclev10026260m on scaffold_7 at the position 14310100 to 14311942 of Clementine genome, and the other marker on the end of 02_(2), Cp1736 was an STS to identify the locus Ciclev10026179m on the same scaffold_7 at the position 5640058 to 5643125 so that the physical length between two markers would be estimated as 8.7 Mb. The sizes of linkage groups in Nou-8 map ranged from 60.0 to 114.9 cM except the linkage group 02. The numbers of markers in each group ranged from 15 to 30 except for the 2 segments of linkage group 02 (14 and 11 markers). The distances between adjacent markers ranged from 0 to 29.9 cM, with a mean of 4.0 cM.

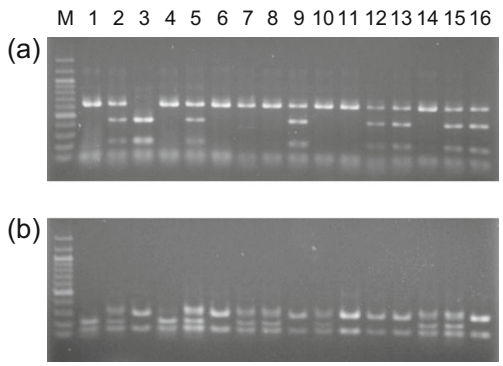

Fig. 2 CAPS patterns of *P. trifoliata* and its progeny. **a** Cp0632 digested with *Nde*II. **b** Cp0703 digested with *Rsa*I. *M*, 100-bp ladder marker; *1*, *P. trifoliata* "Flying Dragon"; *2*, *Citrus hassaku*; *3*, *P. trifoliata* "Kiyomi"; *4*, H FD-1; *5*, Nou-8; *6*, *C. grandis* "Siamese Acidless"; and *7–16*, individuals of BC'$_2$ population

Of the 189 markers mapped on the Nou-8 map, 111 corresponded to the AGI map. For 104 of these, the DNA marker organization of each linkage group was identical between the Nou-8 and AGI maps, although the positions were slightly different (Fig. 3). This high consistency between the two maps indicated their accuracy and high level preservation of genomic structure. A similar result was obtained from comparison with the *P. trifoliata* "Kiyomi" × "Miyagawa wase" map (Omura et al. 2003). DNA markers used in this study were gene-based markers, generated from genes expressed in *Citrus*. They facilitated the comparison of different maps and construction of the graphical genotype map.

Forty-two out of 93 BC'$_2$ individuals scored as CTV resistant using the ELISA assay carried the *Poncirus* resistance allele at CTV selection marker (CTV103), and CTV resistance locus was located at 2.4 cM position in the linkage group 02_(1). This result was reasonable because the CTV103 marker was developed from one ORF region included in the 282-kb region surrounding the CTV resistance gene of *Poncirus* which was analyzed by Yang et al. (2003). Three DNA markers, Gmom23, Lp0004, and Ks9005, were located around CTV resistance gene locus on the distance of 3.0, 3.0, and 1.3 cM, respectively. Therefore, these markers would be useful to evaluate the individual which have CTV resistance and genetic recombination site at the flanking region of CTV resistance gene. There are some candidate loci which concern CTV resistance (Mestre et al. 1997b; Asins et al. 2012), but any other locus for CTV resistance was not detected. This was suggested that one gene or one locus, which might be including some genes, concerning CTV resistance of *Poncirus* would be enough to prevent infection under the low inoculation pressure.

There were 32 markers showing significant distortion from the expected segregation ratio (χ^2>3.0), and these are indicated by asterisks in Fig. 3. Twelve of these markers were mapped together in terminal regions of linkage group 09, and six were mapped together in those of linkage group 04. These results showed that at least two regions were affected by segregation distortion. Many inviable seedlings were observed during the periods of sowing and raising plant materials so that we could not analyze every zygote derived by pollination. There may be a lethal gene or one essential for healthy growth in these two regions.

Graphical genotypes of Nou-8 and its progeny

Graphical genotypes of Nou-8 and individuals of BC'$_2$ population were visualized by comparing the genotypes of ancestors in the pedigree (Fig. 4). Of the 189 markers, 63 were derived from *P. trifoliata* alleles and identified *P. trifoliata* chromosomal regions in the graphical genotype map. These

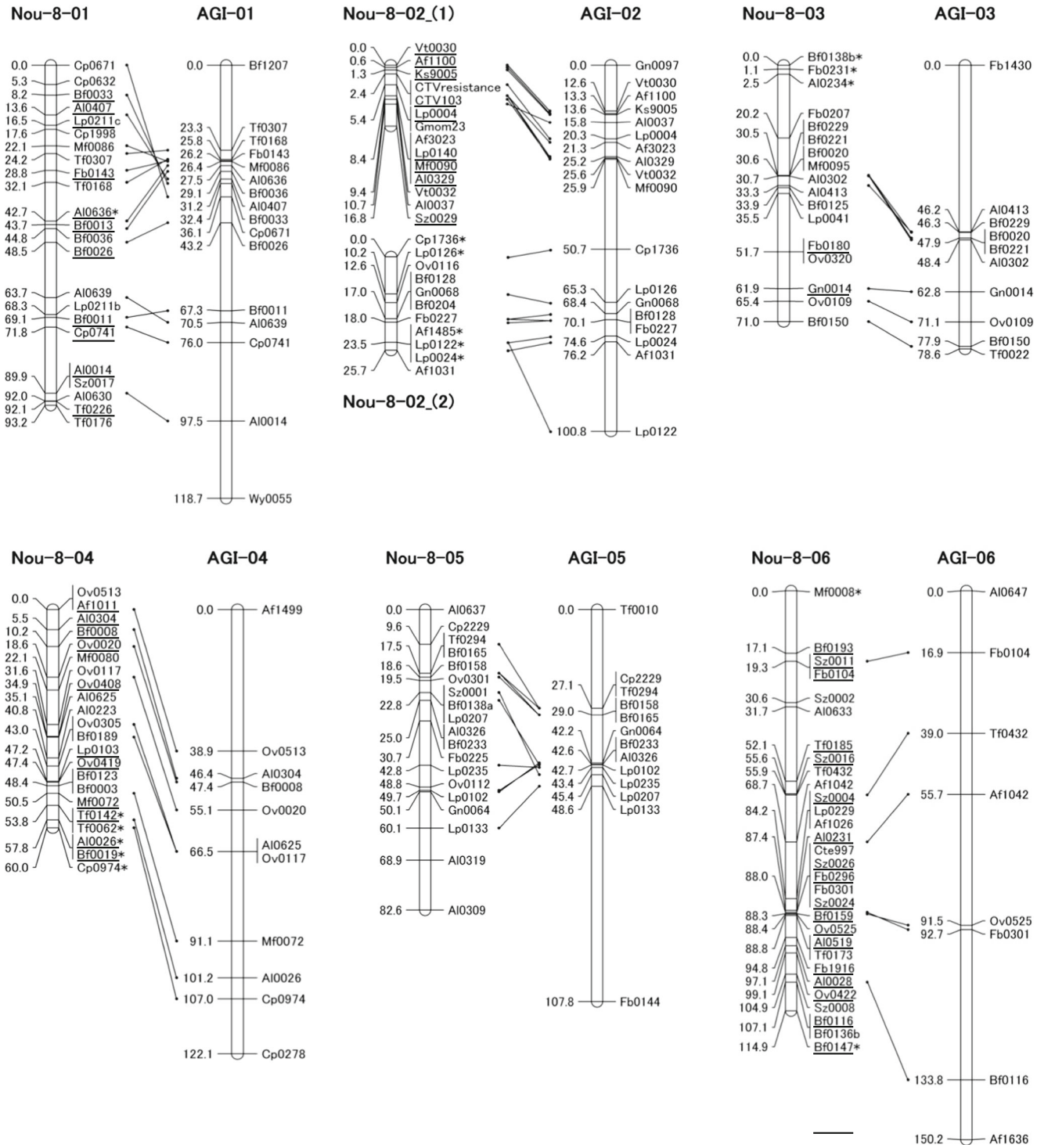

Fig. 3 Genetic linkage map of "Kankitsu Chukanbohon Nou 8 Gou" with comparison to AG integration map. The numbers of linkage groups were assigned by comparison with the AG integrated map (Shimada et al. 2014). Map distances (cM) are indicated on the *left side of each linkage map*, and DNA markers on the *right side*. *Asterisk* shows that the segregation ratio is significantly different from that expected (χ^2>3.0). *Underline* denotes that *P. trifoliata* allele was identified in Nou-8

markers, underlined in Fig. 4 and marked with asterisk in Online Resource 1, were located on linkage groups 01, 02_(1), 03, 04, 06, 07, and 08. On the other hand, *P. trifoliata* alleles on linkage groups 05 and 09 were not traced in Nou-8. This result indicated that Nou-8 had been already completely substituted with *Citrus* alleles on these two chromosomes. This method showed the origin of each chromosome, so that the recombination sites in each linkage group of each

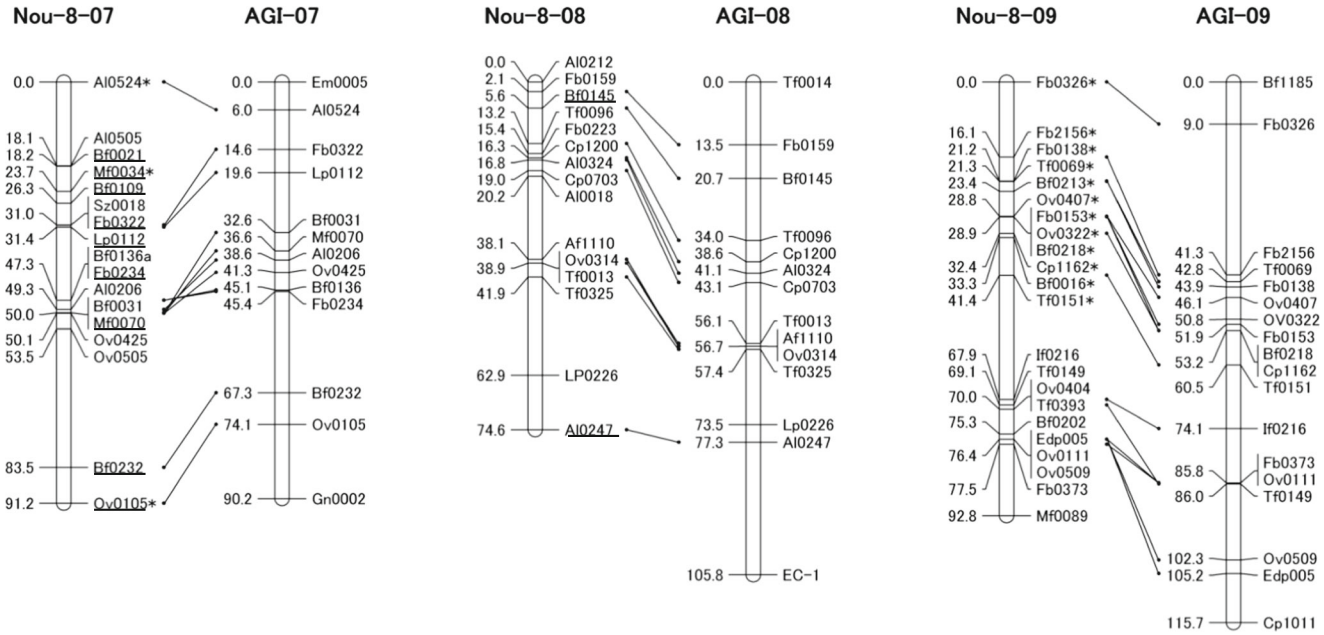

Fig. 4 Graphical genotypes of Nou-8 and TP-57 from the BC'$_2$ population. Numerals *1–9* denote linkage group numbers (01–09). Graphical genotypes were constructed by reference to the ancestors in the pedigree. *Intervals filled with black* indicate *P. trifoliata* alleles remaining, and *those in white* indicate that these regions have been completely substituted by *Citrus* alleles. The *star* shows the locus which correspond to the CTV resistance. The *bar* indicates 10 cM

individual would be estimated, except for linkage group 02, which was separated into two segments (Table 1), allowing only incomplete analysis of recombination sites. Of the chromosomes in the BC'$_2$ population, 64 % had at least one recombination site. However, 36 % of chromosomes had no recombination site, and approximately half of those chromosomes (20 %) were inherited from *P. trifoliata* "Kiyomi." These results also suggested that it would be possible to remove

Table 1 Number of individuals which showed each number of recombination sites in each linkage group

Linkage group	No. of recombination sites				
	0 (Km)[a]	0 (F1)[b]	1	2	>3
01	16	16	40	18	3
02	–[c]	–	–	–	–
03	24	16	36	13	4
04	23	18	51	1	0
05[d]	15	18	48	9	3
06	14	10	41	22	6
07	21	10	45	10	6
08	22	14	46	10	1
09[d]	14	16	47	15	1
Total (%)	20	16	48	13	3

[a] Chromosome of Kiyomi was inherited from Nou-8 to the BC2 individuals with no recombination sites

[b] Chromosome of H FD-1 was inherited from Nou-8 to the BC2 individuals with no recombination sites

[c] Not estimated because linkage group 02 was separated into two segments

[d] Nou-8 might have been completely substituted by *Citrus* alleles

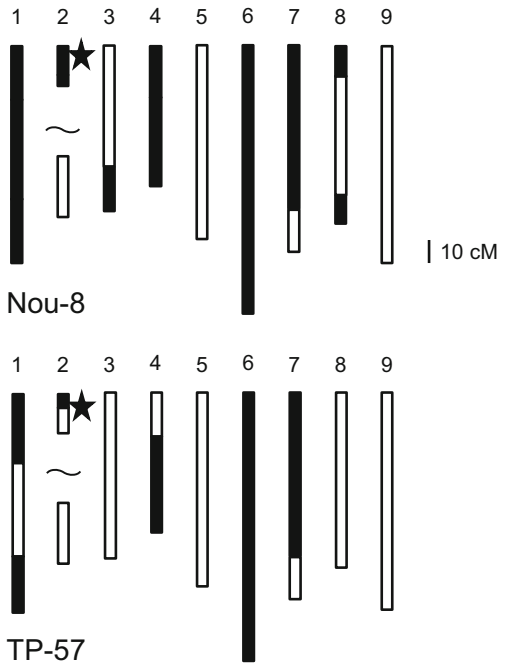

Fig. 5 Graphical genotypes of a CTV-resistant individual from the BC'$_2$ population on linkage group 02_(1) which contains the CTV resistance. *P*, allele from *P. trifoliata* "Flying Dragon"; *C*, allele from *Citrus* (*C. hassaku*, "Kiyomi" or *C. grandis* "Siamese Acidless"); +, CTV resistance originating from *P. trifoliata*

undesirable chromosome regions and entire undesirable chromosomes by recurrent pseudo-backcrossing with selection.

Substitution between flanking regions of a target gene is especially important in introgression breeding. In this study, most individuals showed no genetic recombination sites in either flanking region of the CTV resistance locus, but four individuals (TP-29, 57, 80, and 88) showed one genetic recombination site on one side of it (Fig. 5). According to our breeding selection, 4 individuals, TP-57, 74, 85, and 100, had better fruit taste but had less amount of acridity and unfavorable smell originating from *Poncirus* than the other individuals, so that they were preselected as mother plants for BC'_3 generation. TP-57 was supposed to be a major candidate of the mother plant among these four candidates because genotypes of all DNA markers in one side of the CTV resistance locus in linkage group 02_(1) were recombined to *Citrus* genotypes. The chromosomal region which had been substituted with *Citrus* alleles in TP-57 compared with Nou-8 was about 114 cM (Fig. 4). In backcross breeding in apple, five or more generation cycles were necessary to remove most of the unwanted alleles (Joshi et al. 2009). We consider that it takes less generation cycles to remove undesirable *P. trifoliata* loci in *Citrus* than in the case of apple because *Citrus* chromosome number ($n=9$) is lower than that of apple ($n=17$).

In conclusion, we developed a genetic linkage map of Nou-8 and a graphical genotype map and calculated the number of recombination sites in each linkage group in each individual. The information on graphical genotype of BC'_2 individuals pointed out the chromosomal regions requiring replacement by *Citrus* alleles. These results can lead to more efficient breeding of CTV-resistant cultivars. It can also be possible to map genes for undesirable traits originating from *P. trifoliata*, such as high acidity, long thorns, and ternate leaves, by investigating the characteristics of each individual in the BC'_2 population, a subject of our future research.

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Data archiving statement Genetic map is now under process of submission to Citrus Genome Database and will be completed during the review.

Ethical standards The experiments comply with the current laws of Japan where they were performed.

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