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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

The accuracy evaluation of linkage map in cultivated strawberry (*Fragaria* × *ananassa* Duch.) by direct cycling-primed *in situ* hybridization technique
(直接リサイクリングPRINS法による栽培イチゴの連鎖地図における正確性の評価)

学位論文要約：
Dissertation Summary

Cultivated strawberry (*Fragaria* × *ananassa* Duch) is a member of the family Rosaceae, subfamily Rosoideae, genus *Fragaria* (Tanaka's cyclopedia of edible plants of the world, 1976). The cultivated strawberry plant is grown worldwide (FAO, 2014; Hummer and Hancock, 2009; Hummer *et al*., 2011). The production of cultivated strawberry became one of the most economically important crops in the world with the production of over 8.1 million tons in 2014 (FAO, 2014). The cultivated strawberry was proposed to be allopolyploid plant, having three kinds of complex genome compositions of either AABBBBCC (Fedorova, 1946), AAA'A'BBBB (Senanayake and Bringhurst, 1967) or AAA'A'BBB'B' (Bringhurst, 1990). Of these three models, the AAA'A'BBB'B' has been regarded as the most reliable candidate by the several studies of the DNA analysis (Kunihisa, 2011; Sargent *et al*., 2009 and 2012; Isobe *et al*., 2013). The allo-octoploidy is a serious obstacle for conducting a genetic analysis, because of the existence of similar sub-genomes which leaded to multiple alleles and complex segregation ratios (Van Dijk *et al*., 2012). The allo-octoploidy of cultivated strawberry made it difficult to conduct a theoretical genetic analysis based on the Mendel's law of inheritance. Several linkage maps have recently been constructed using various types of DNA markers for the purposes of conducting theoretical genetic analyses and efficient breeding in cultivated and wild octoploid strawberries. Therefore, physical chromosome mapping using DNA markers is required to evaluate the accuracy of the linkage groups. However, few studies have investigated physical chromosome mapping using DNA markers in cultivated strawberry. Fluorescent *in situ* hybridization (FISH), primed *in situ* (PRINS) hybridization, and indirect and direct cycling PRINS (C-PRINS) hybridization techniques enable the location of labeled sequences on the chromosomes to be detected under a fluorescent microscope. It has previously been shown that the direct C-PRINS

technique is superior to the FISH, PRINS, and indirect C-PRINS techniques (Pellestor, 2006). The direct C-PRINS hybridization technique became the powerful tool for cytogenetic study. Verification of the linkage map can be accomplished by the detection of fluorescence signal from DNA markers which located near both ends of each linkage group on the same chromosome. The aim of this study was to develop the PRINS hybridization technique for chromosome labelling in cultivated strawberry, examine the availability of a PRINS hybridization technique for physical chromosome mapping, evaluate the accuracy of the linkage map of cultivated strawberry by using PRINS hybridization technique. Three papers were published and accepted by the data in this study (Tantivit *et al*., 2016; Tantivit *et al*., 2017a; Tantivit *et al*., 2017b)

Development of the PRINS hybridization technique for chromosome labeling in cultivated strawberry

Chromosome observations were carried out using meristematic cells from the root tips of the Japanese strawberry cultivar 'Sachinoka'. Pretreatment and fixation were conducted using the modified method described by Iwatsubo and Naruhashi (1989, 1991) and Nathewet *et al.* (2007, 2009). The new root tips (2–3 mm) were collected from the plants at 5:00 p.m., pretreated for 1 h with 2 mM 8-hydroxyquinoline solution at room temperature, and subsequently kept at 4℃ for 15 h. The roots were then fixed in a 1 : 3 acetic acid and ethanol solution for 40 min at room temperature, and preserved in a 70% ethanol solution at -20℃.

The fixed root tips were softened using an enzyme mixture that included 4% cellulase 'Onozuka' RS (Yakult Co., Ltd., Tokyo, Japan), 0.3% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan), 2.1% macerozyme R10 (Yakult Co., Ltd., Tokyo, Japan), and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 4.2, at 37℃. To determine the most appropriate enzymatic treatment time, the root tips were dipped in the solution for 20, 25, 30, and 35 min (Experiment 1). Following maceration, the root tips were short-rinsed in distilled water and individually placed onto a glass slide. They were then treated with 10 μL acetic acid solution for 2 min and mangled into invisible particles using forceps. To determine the most appropriate concentration of acetic acid, the root tips were treated with 30%, 45%, and 60% solutions (Experiment 2). A cover slip was then placed over the root tip particles, tapped gently with a chopstick to spread the chromosomes across the glass slide, heated for about one second with a spirit lamp, and pressed with a thumb for a few seconds. To elucidate the effects of maceration time and acetic acid concentration on chromosome

image, the chromosomes on the slides were stained by a 2.0 mg mL^{-1} 4′,6-diamidino-2-phenylindole solution (DAPI; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and covered by a cover slip. The slide sample was observed under a fluorescent microscope (Olympus BX51; Olympus Co., Ltd., Tokyo, Japan) equipped with a WU filter set (excitation=372 nm, emission=456 nm, DAPI), a 3CCD camera (DP72; Olympus Co., Ltd., Tokyo, Japan), and a computer running the DP2-BSW software (Olympus Co., Ltd., Tokyo, Japan). In Experiment 1, the number of chromosomes in one cell was counted in each of 40 chromosome images. In Experiment 2, 10 chromosome images were observed per treatment to detect differences in the size and color of the chromosomes and cytoplasm. The color difference was analyzed using the method described by Nathewet *et al.* (2009), and the RGB values at five positions within the chromosomes and cytoplasm of each image were obtained using the software. The RGB values were then converted to theoretical CIE L* a* b* values using the method described by Connolly and Fliess (1997). In Experiment 1, the 45% acetic acid solution was used, while in Experiment 2, a 25-min maceration time was used. The sample slides that had been treated with a 25-min maceration time and a 45% acetic acid solution during slide preparation were used in the following experiments. The slides were frozen at -80°C for a minimum of 5 min and the cover slip was then removed using a razor blade. The specimens were then dehydrated for 5 min through an alcohol series (75%, 90%, and 99.5%). To determine the effect of storage duration on the number of chromosomes and fluorescent signals, the fully prepared sample slides were stored at 37°C for 0, 24, 72, 120, and 168 h (Experiment 3). Following storage, the sample slides were soaked in a denaturing solution (70% formamide with 2×standard saline citrate (SSC)) at 72°C for 2.5 min and dehydrated in an alcohol series (75%, 90% and 99.5%) at -20°C for 5 min per concentration. They were then air-dried at room temperature. Subsequently, the specimens were moistened with a 12.5-μL PCR mix and covered with a cover slip that had aluminum tape stuck over the entire upper surface. The cover slip was then sealed using paper cement (Mitsuwa paper cement S-coat; Kogyo Co., Ltd., Fukuoka, Japan) to prevent the specimen from drying out for the duration of the PCR. In total, 50 µL of PCR mixture was made for four slides, which included five units of 5 µL Taq DNA polymerase (KOD FX Neo; Toyobo Co., Ltd., Osaka, Japan), 4 µL Taq-polymerase buffer (0.5 µL of 1 M Tris–HCl, pH 8.0, 2.5 µL of 1 M KCl, 0.5 µL of 0.4 M MgCl2, and 0.5 µL of 1% Triton X), 1.5 µL of deoxynucleotide (dNTP) [10 mM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP); TaKaRa Bio Inc., Otsu, Japan], 1.28 µL of 25 mM

fluorescein-12-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) and deoxythymidine triphosphate (dTTP), and 4 mM of the primer FVES2524 (5’-AAG CCC ATC TCC ATC AAA TG-3’; 20 bp), and made up to 50 μL with deionized distilled water. A thermal cycler (GeneAtlas S100; Astec Co., Ltd., Japan) that was equipped with a heat block for four slides at the same time was used to conduct the PCR, which consisted of the following steps: a first cycle of 4 min at 55°C and 10 min at 72°C, followed by a main cycle of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C. To determine the effect of the number of cycles on the number of chromosomes and fluorescent signals detected, 1, 5, 10, and 20 cycles were performed (Experiment 4). Following the PCR, the sample slides were washed by dipping them in a jar filled with 2×SSC solution for 5 min at 42°C. The cover slip and paper bond were then removed, following which the same washing procedure was repeated three times using fresh 2×SSC solution for 5 min at 42°C. The slides were then soaked in 1×phosphate buffered saline (PBS) at room temperature for 5 min, and treated with DAPI and DABCO, using the same method as for the chromosome observations. In Experiment 3, 20 PCR cycles were performed, while in Experiment 4, a 72-h incubation time was used. Ten chromosome images were used for each PCR cycle treatment to detect color differences between the fluorescent signals and chromosomes, which were analyzed using the method outlined above. In addition, to determine the best method for ensuring an accurate temperature during PCR, a glass slide was moistened with 12.5 μL water and covered with either a standard cover slip or a cover slip that had aluminum tape stuck over its entire upper surface (Experiment 5). The temperature of each glass slide was then measured using an ultra-fine thermocouple (0.05-mm-diameter wire) with a digital temperature indicator (TX1003; Yokokawa Co.) that was fixed to the center of the cover slip with either aluminum tape or transparent mending tape. Statistical analyses were conducted using one-way analysis of variance and Tukey’s multiple comparison tests with the Statistical Package for the Social Sciences (SPSS) version 16.0 software (SPSS Inc., U.S.A.).

In experiment 1, the largest number of cells containing 56 chromosomes was obtained with a 25-min maceration time (Table 1). Few images with 56 chromosomes were observed following a maceration time of 20 min because many of the chromosomes overlapped in the somatic cells. A larger percentage of the images contained lower numbers of chromosomes following maceration times of 30 and 35 min than 25 min.

Table 1 Effect of maceration time on the number of chromosomes in the somatic cells of cultivated strawberry. The different letters indicate significant differences (α = 0.05). Slide samples were stained with DAPI without PRINS hybridization, and observed under fluorescence microscope.

The number of chromosomes in a somatic cell	Maceration time (min)			
	20	25	30	35
	The number of somatic cells			
56	1[b]	10[a]	7[ab]	6[ab]
50-55	14	10	13	15
45-49	11	5	6	7
Less than 45	14	15	14	12
Total number of cells	40	40	40	40

* Cultivated strawberry has 56 chromosomes in an ordinary somatic cell.

In this experiment, 25 min was found to be the most suitable maceration time for the root tips of octoploid strawberries. With the 25-min treatment, the middle lamella and cell wall were sufficiently weakened for them to be separated, and the chromosomes in the somatic cell appeared to be spread close to each other without overlapping.

In experiment 2, the average length of the chromosomes was significantly shorter with the 45% and 60% acetic acid treatments than with the 30% acetic acid treatment (Fig. 1A). However, the color difference between the chromosomes and background was significantly higher with the 45% and 60% acetic acid treatments than with the 30% acetic acid treatment (Fig. 1B). The 30% acetic acid treatment was not high enough to make the cytoplasm clear as shown by the lack of difference in color between the chromosomes and cytoplasm after stained with DAPI solution. The treatment of 45% and 60% acetic acid was found to provided clear chromosome images. And the chromosomes that were treated with 45% acetic acid appeared to be healthier than those treated with 60% acetic acid.

In experiment 3, the highest percentages of cells containing 56 chromosomes were obtained with 72-h and 120-h incubation times. However, ghost-like chromosomes that exhibited a pale blue color in and around them were found in the images of cells that had been incubated for 120 h and 168 h. Fluorescent signals were observed in 93.3% and 100% of the images of cells that had been incubated for 24 h and 72 h, respectively. Our study clearly demonstrated that fluorescence signals were obtained from chromosome samples that were incubated at 37°C for 24 h and 72 h. Furthermore, the shape of the chromosomes could be observed clearly when the slide samples were incubated for 72 h.

In experiment 4, there had no the detection of fluorescence signals on the sample slides that had been treated with one cycle of PRINS hybridization, and the number of fluorescence signals on the chromosomes increased with an increase in the number of PCR cycles (Table 2), which is similar to the findings of previous studies (Kaczmarek, *et al*. 2007).

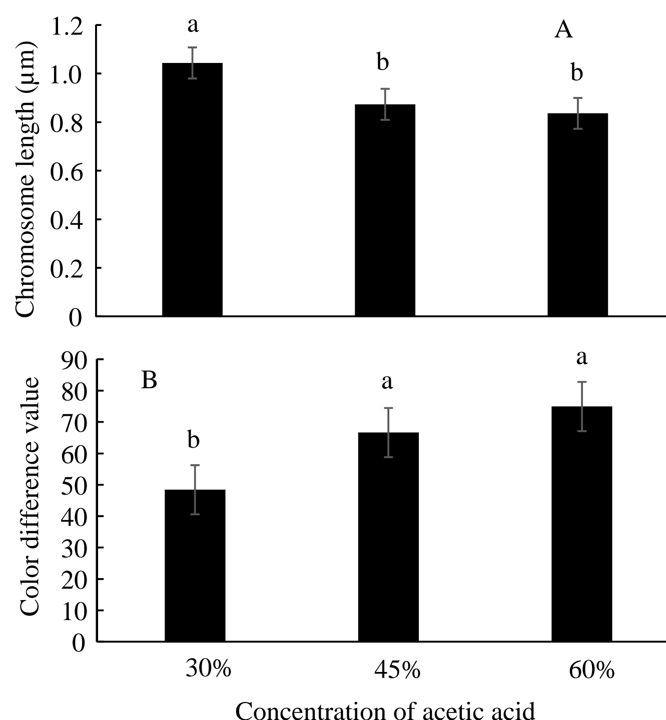

Fig. 1 Effect of 30%, 45%, and 60% acetic acid solutions on the chromosomes of cultivated strawberry (Fragaria×ananassa). (A) Differences in chromosome length and (B) differences in color between the chromosomes and the background. Vertical bars indicate S.E. and different letters indicate significant differences (p= 0.05) within each figure. Slide samples were stained by DAPI without PRINS labeling and observed under fluorescent microscope.

Table 2 Effect of PCR cycles on the number of chromosomes which had fluorescence signals and the color difference between fluorescence singles and chromosomes. The different letters indicate significant differences ($\alpha = 0.05$). After finishing PRINS hybridization, the slide samples were stained with DAPI, and observed under fluorescence microscope.

	The number of PCR cycles			
	1	**5**	**10**	**20**
Average number of chromosomes which had signals in one image	0^{b}	1.75^{b}	3^{b}	13^{a}
Color difference values between signal and chromosome	0^{c}	13.7^{b}	14.6^{b}	27.9^{a}

In experiment 5, when measured at a set value of 94°C for denaturation, the cover slip with aluminum tape was almost the same temperature as the set value, whereas the standard cover slip was approximately 2°C lower. Similarly, when the temperature was set to 55°C for hybridization and 72°C for extension, the cover slips with aluminum tape were almost the same temperature as the set values,

whereas the standard cover slips were approximately 1°C and 1.5°C lower, respectively. Temperature accuracy is important for the PRINS hybridization technique. However, few studies appear to have examined the temperature of the sample slides during the PCR. The temperature of the standard cover slip was slightly lower than the set values. With an ordinary thermal cycler, the temperature of the lid is maintained at around 100°C. However, the lid temperature of the thermal cycler for the PRINS hybridization was not controlled, and so the heat may have been lost from the glass into the air. Therefore, we tested whether the temperature accuracy could be improved by using aluminum tape, since aluminum is widely used for thermal insulation. The temperatures of the cover slips that were covered with aluminum tape during PCR were almost the same as the set value, indicating an improved temperature accuracy.

The appropriated method of direct C-PRINS hybridization for the chromosome of cultivated strawberry was developed. Treatments of 25 min enzymatic maceration and 45% acetic acid were found to provide the best results of chromosome preparation for all *in situ* hybridization techniques. For PRINS hybridization technique, the treatment of 72 h 37°C incubation and 20 PCR cycles were found to produce strong and clear hybridization signals of SSR markers on the chromosomes of cultivated strawberry. In addition, aluminum tape can use to improve the temperature accuracy of sample on the glass slide during conduct PCR.

CAPS DNA marker labeling using direct C-PRINS hybridization technique in cultivated strawberry

The technique of PRINS hybridization was developed to use for identifying the position of specific DNA sequences on the chromosome(s) by the DNA marker and fluorescence labeling (Vega *et al.*,1997). In previous study, fluorescence signals were detected after the chromosomes of cultivated strawberry were labeled with a SSR marker by C-PRINS hybridization technique. However, there is a possibility that the fluorescence labeling can be detected on the wrong position or wrong chromosomes (non-specific labeling) in case of the C-PRINS hybridization. For this reason, it was necessary to confirm the correctness of C-PRINS hybridization for the physical chromosome mapping in cultivated strawberry. The chromosome-specific markers and the metaphase spreads can be used to confirm the preciseness of PRINS hybridization technique. Kunihisa *et al.* (2003, 2005, and 2009) developed various cleavage amplified polymorphic sequence (CAPS) DNA markers. The CAPS markers include

a pair of forward and reverse primers and a restriction enzyme with which the primers are able to clip single allelic pairs. Results of CAPS marker analysis show that individual plants can be genotyped into either two types of homozygotes or a heterozygote by electrophoresis. Furthermore, if one assumes that the cultivated strawberry is a disomic polyploid, then genotypes of F1 progenies can be expected to fit disomic Mendelian inheritance. Furthermore, it is conceivable that a one-to-one relationship existed between a CAPS marker and a homologous chromosome in the cultivated strawberry. Such a characteristic of DNA markers was defined as chromosome specificity. Therefore, the correctness of C-PRINS hybridization for physical chromosome mapping in cultivated strawberry can be confirmed if direct C-PRINS hybridization with a chromosome-specific CAPS marker was conducted, and the two homologues chromosomes having a single signal were observed in the set of somatic chromosomes. Then, the DNA analysis and chromosome labeling using CAPS DNA marker were conductd on the cultivated strawberry. A DNA marker could not be regarded as having chromosome specificity if it amplified more than two sizes of the DNA segments by PCR. To confirm it in the three CAPS, which were used for the physical chromosome mapping, the PCR analysis was first conducted. As a result of electrophoresis, two bands were detected in every CAPS marker. The thin upper band, which had lower molecular weight of less than 100 bp in each lane, seemed to be primer dimer and not genomic DNA. However, the remaining one band in each lane, which had molecular weight more than 100 bp, was the genomic DNA amplified by PCR. These results indicated that it was highly probable that the CAPS markers had chromosome specificity.

As shown in the three examples of the chromosome image, a small but obviously labeled signal on the two chromosomes of the ‘Sachinoka’ was observed among the 56 somatic ones in every CAPS marker (Fig. 2). When detailed images were obtained, two separate paranemic signals were detected at one chromosome in APX2 and two in F3H3. These signals were apparently produced from both sister chromatids, although a small number of chromosome images, which had such signals, were obtained. Other labeled chromosomes had one signal on each chromosome because of overlapping with two signals produced by the sister chromatids. Results showed that these chromosome images clarified that these CAPS markers had true chromosome specificity, and the direct C-PRINS technique with CAPS marker is applicable to physical chromosome mapping of the cultivated strawberry. Furthermore, these chromosome images indicated that the two labeled chromosomes are a pair of homologues chromosomes. Therefore, the direct C-PRINS with a chromosome-specific DNA

marker(s) is expected to be useful to identify not only the physical location(s) on the chromosomes, but also homologous chromosome pairs.

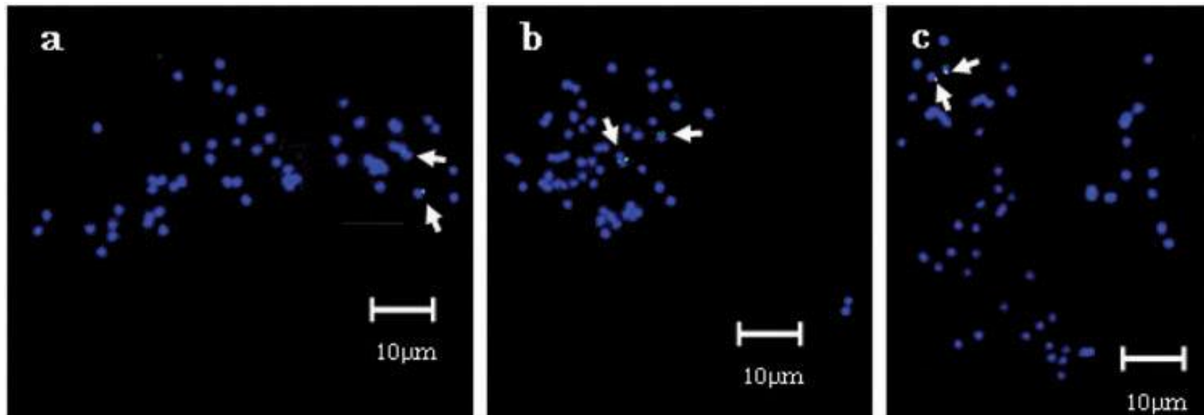

Fig. 2 Three examples of the chromosome image (2n = 56). Chromosomes of the strawberry cultivar Sachinoka were counterstained by DAPI (blue). The green signals were localized on the chromosomes and which portions seemed to be hybridized with CAPS primer by the direct C-PRINS technique (signals marked with arrowheads). The CAPS primers of (a) APX2, (b) F3H3, and (c) OLP were used for labeling on the chromosomes.

Kunihisa *et al.* (2009), the conflicting results were obtained from the experiments using the APX2, F3H3, and OLP and the inbred progenies of 'Sachinoka', because they indicated that these three markers were highly linked on one occasion, but ambiguous in another case. To make things clear, these three markers were used in the present study. Also, before conducting the experiments using the direct C-PRINS with two pairs of the primers, it was expected that two separate signals on a single chromosome would be detectable on a pair of homologues chromosomes among the 56 somatic chromosomes if the positions, which were matched by two primers on the chromosome, had a large interval. Results show that not two separate signals but a single signal can be observed on each of two chromosomes in both cases (Fig. 3). From these results, two possible reasons can be considered. One was that PCR amplification failed in one of the two pairs. The other was that the two signals on the chromosome had overlapped because their physical distance was extremely close. We observed more than three slides and five somatic cells that had complete sets of metaphase chromosomes. However, no chromosome images that had two separate signals on the two chromosomes were obtained. Therefore, it is more likely that these CAPS markers, which belonged in that same linkage group, were closely situated on the same chromosomes.

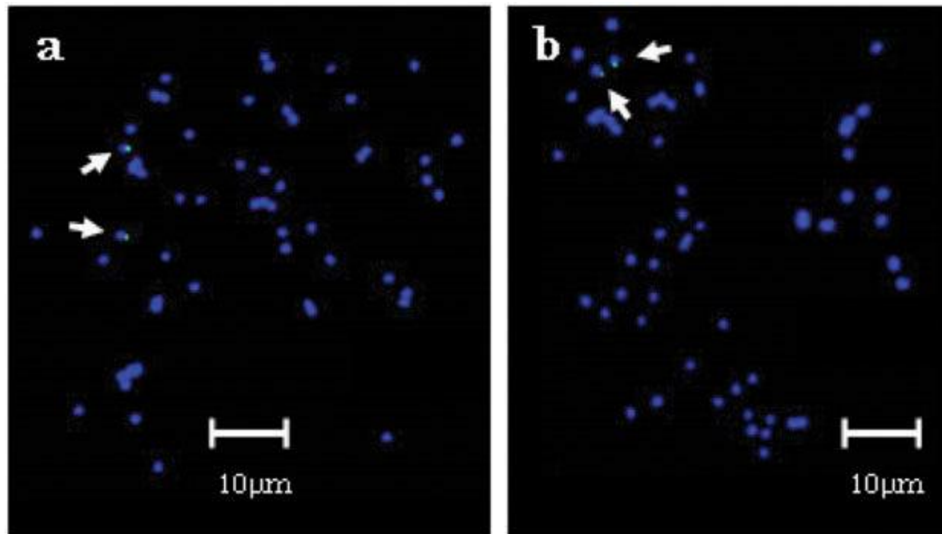

Fig. 3 Two sets of CAPS markers were labeled using PRINS technique on the chromosomes in the strawberry cultivar Sachinoka (2n = 56). Both primers were belonging in the same linkage group (Kunihisa et al., 2005). The chromosomes were counterstained with DAPI (blue). The CAPS primers of (a) APX2 with F3H3 and (b) F3H3 with OLP were used for labeling the chromosomes. The PRINS signals are a green color (signals marked with arrowheads).

Accuracy evaluation of linkage map in cultivated strawberry using a physical chromosome mapping by direct C-PRINS labeling technique

Isobe *et al*. (2013) reported the linkage map of cultivated strawberry using many SSR markers. The linkage map was used for the present study, because many SSR markers converged with 28 linkage groups that was similar to the chromosome number of haplotype in cultivated strawberry. The linkage map was desirable to evaluate the accuracy. The accuracy of a linkage group could be confirmed, if chromosomes were fluorescently labeled with SSR markers that are located close to both ends on the same linkage group, and their signals were detected close to both ends on a pair of the homologues chromosomes. In addition, it was expected that the fluorescent signals were detected on only one pair of homologous chromosomes.

The verification of linkage map using PRINS labeling technique was conducted. In case of example results of linkage group 1A, red and green signals were observed on 16 and 13 chromosomes, respectively (Fig. 4A). Two of 56 chromosomes had both signals on the both ends. The same tendency was observed in the example results of linkage group 2A (Fig 4B). From these results of the linkage group 1A and 2A, only two chromosomes in one cell showed both fluorescence signals on both ends, the 2 chromosomes which showed red and green fluorescence signals seemed like a pair of

homologues chromosomes. It was consistent with the expected results, indicated that the linkage group 1A and 2A correct.

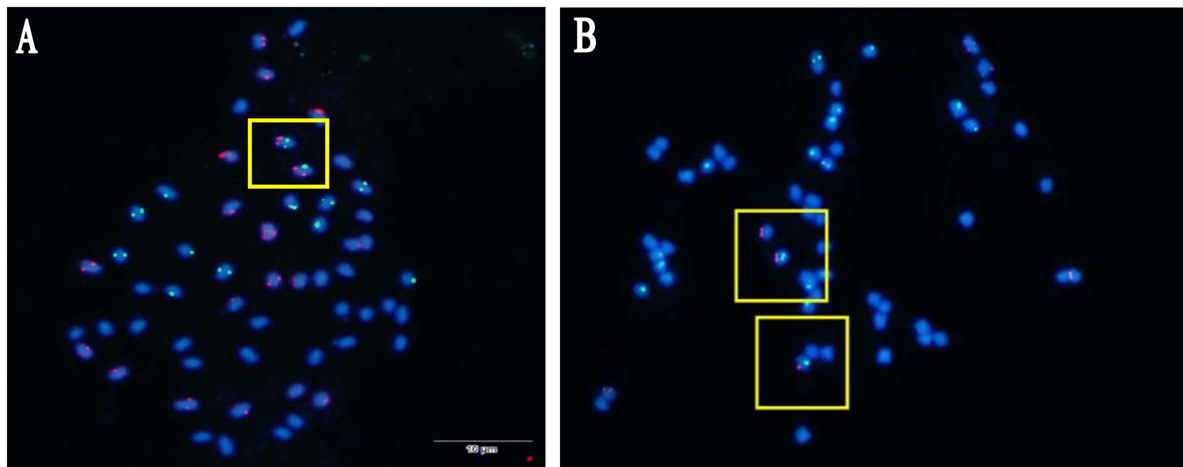

Fig. 4 The example images of the correct linkage groups which were confirmed by PRINS hybridization. Two chromosomes in each image showed all (red and green) signals from the DNA marker which locate near both end in linkage group 1A (A) and 2A (B). (Scale bar = 10 µm.)

When labeled the chromosomes with two SSR markers on the two ends of linkage group 1B. the 39 red and 21 green fluorescence signals were observed within the 56 chromosomes of one somatic cell, respectively (Fig. 5). More than three chromosomes in the cell showed both fluorescence signals on both ends. It was difficult to identify whether the 1B linkage group was correct or not.

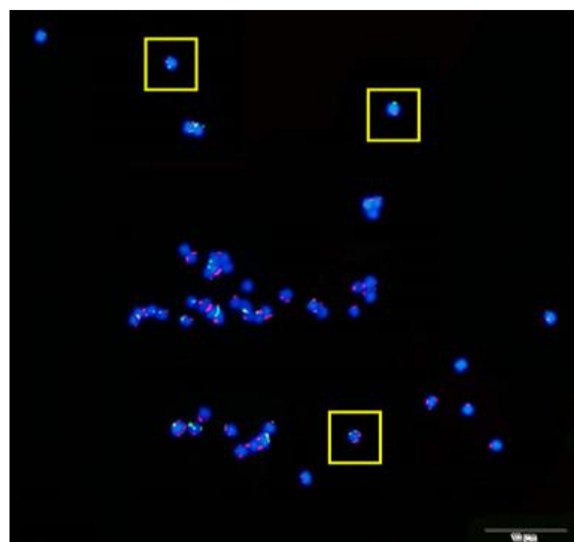

Fig. 5 The example image of the location of FVES2524 marker (red), FVES3197 marker (green) on the chromosomes of cultivated strawberry (blue). More than two chromosomes in this image showed all (red and green) signals. (Scale bar = 10 µm.)

The linkage map of cultivated strawberry made by Isobe *et al*. (2013) was evaluated for the accuracy using a physical chromosome mapping by direct C-PRINS hybridization technique. Total of 14 linkage groups of 1A, 1C, 1D, 2A, 2C, 2D, 4A, 4C, 5A, 5B, 5C, 6A, 6C, and 7A were confirmed as having been correctly assigned. In case of linkage group 3D, there are no SSR markers for cultivated strawberry in the cultivar 'Sachinoka'.

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