DNA Marker Linked to Everbearing Flowering Gene in Cultivated Strawberry, with High Applicability to Various Breeding Populations

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Everbearing cultivars of the octoploid cultivated strawberry (Fragaria × ananassa) enable the expansion of production. Because the identification of everbearing individuals by phenotypic observation is time- and labor-consuming, marker-assisted selection is useful. In this study, we developed a sequence-tagged site marker, s2430859, which specifically amplifies a DNA fragment linked to the everbearing gene from homoeologous chromosomes for genotyping by agarose gel electrophoresis. In the analysis of eight F₁ populations produced from various crosses of everbearing by June-bearing cultivars, the concordance between the phenotype and the marker genotype ranged from 94.4% in the ‘Hecker’ × ‘Sagahonoka’ population to 100.0% in the ‘Miyazaki-natsuharuka’ × ‘Karenberry’ population. The average match was 96.8%. These results suggest that s2430859 is closely linked to the everbearing gene and is applicable to various breeding populations. Because s2430859 is located on the opposite side of the gene from already reported markers, it will contribute to the detection of recombinant individuals by use in combination with these markers.

Key Words: flowering habit, Fragaria × ananassa, marker-assisted selection, octoploid, STS marker.

Introduction

The octoploid cultivated strawberry (Fragaria × ananassa Duch., 2n = 8x = 56) is economically and commercially important. Two types with distinct flowering habits are known: the June-bearing type and the everbearing type. Everbearing cultivars have attracted attention because they bear fruits from spring to autumn under natural conditions. This characteristic enables year-round production. Because the identification of everbearing individuals by phenotypic observation is time- and labor-consuming, marker-assisted selection would improve the efficiency of breeding.

Recent genomic studies indicate that the everbearing trait is regulated by a single dominant gene (locus), and several DNA markers linked to the gene have been developed (Castro et al., 2015; Gaston et al., 2013; Honjo et al., 2016; Perrotte et al., 2016; Sugimoto et al., 2005). However, these markers have some limitations in practical use. For example, Sugimoto et al. (2005) detected random amplified polymorphic DNA (RAPD) markers weakly linked (11.8–23.9 cM) to the everbearing gene in the cultivar ‘Ever-berry’. Castro et al. (2015) reported three SSR markers—ChFaM011 (Zorrilla-Fontanesi et al., 2011), ChFaM148 (Zorrilla-Fontanesi et al., 2011), and CX661225 (Spigler et al., 2008)—linked to the gene in the cultivar ‘Tribute’. Honjo et al. (2016) analyzed their applicability to Japanese cultivars and found that only ChFaM011 was linked, within 6.5–8.5 cM, in the F₁ populations of ‘Sagahonoka’ × ‘Summer-berry’, ‘Sagahonoka’ × ‘Ever-berry’, and ‘Hecker’ × ‘Sagahonoka’. Honjo et al. (2016) also developed a new SSR marker closely linked to the everbearing gene in the same three F₁ populations, mapping FxaACA02I08C at 1.1 cM in ‘Summer-berry’ and ‘Hecker’ and at 1.5 cM in ‘Ever-berry’. However, although it was applicable to more
than half of the 20 everbearing cultivars and 55 June-bearing cultivars tested, it was not applicable to the other cultivars owing to the existence of an unlinked SSR fragment of the same size as the linked fragment (Honjo et al., 2016). In addition, an auto-sequencer is needed to identify the target fragment for this marker and the three SSR markers reported by Castro et al. (2015) because several other fragments of similar size are also amplified from homoeologous chromosomes. Thus, it is necessary to develop new markers that are closely linked to the everbearing gene, are applicable to various breeding populations, and are easy to analyze.

In this study, we developed the sequence tagged-site (STS) marker s2430859, which specifically amplifies a DNA fragment linked to the everbearing gene among the homoeologous chromosomes. This marker is applicable to a diverse range of strawberry cultivars, needing only simple agarose gel electrophoresis to identify it.

**Materials and Methods**

**Mapping populations and characterization of the everbearing trait**

We used 147 F$_1$ individuals from a cross between everbearing ‘Miyazaki-natsuharu’ and June-bearing ‘Morioka 36’ to construct a linkage map and to detect loci related to the everbearing trait. The F$_1$ seeds were sown in June 2013, and the seedlings were planted 25 cm apart in 140 cm wide rows in a field at the NARO Tohoku Agricultural Research Center in Morioka City (39°46’ N, 141°8’ E), northern Japan, in April 2014. The presence of open inflorescences was recorded at weekly intervals from April to August 2014. A runner plant of each F$_1$ individual was planted as above in September 2014, and flowering was re-assessed in 2015. We considered a plant to be of an everbearing type if it flowered in both spring (under short days) and summer (under long days) (Honjo et al., 2016; Weebadde et al., 2008).

**Linkage map construction and QTL analysis**

We constructed a linkage map using next-generation sequencing and DNA microarray analysis as follows. Total genomic DNA from ‘Miyazaki-natsuharu’ and ‘Morioka 36’ was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and it was digested with PstI (New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h. Genomic DNA fragments were ligated with PstI adapters (5’-CACGATGGATCCAGTGCA-3’, 5’-CTGGATCCATCGTGCA-3’) using T4 DNA Ligase (New England Biolabs) at 16°C for 1 h, then at 37°C for 20 min, and at 37°C for 30 min. These samples were digested with BstNI (New England Biolabs) at 60°C for 1 h. Polymerase chain reaction (PCR) amplifications were performed with PrimeSTAR DNA polymerase (Takara Bio, Otsu, Japan). The PCR primer design was based on the PstI adapter sequence (5’-GATGGATC CAGTGCAG-3’). The PCR conditions were as follows: 30 cycles at 98°C for 10 s, 55°C for 15 s, and 72°C for 1 min, and finally, 72°C for 3 min. These steps amplify only the PstI–PstI DNA fragments but not the PstI–BstNI fragments. The DNA fragments obtained were sequenced on HiSeq 2000 and MiSeq sequencers (Illumina, San Diego, CA, USA). On the basis of the sequence data, we designed 291,041 probes with a length of 50–60 bp according to the procedures described by Iehisa et al. (2014), and constructed a microarray including the probes.

Genotypes of the parental cultivars and their 147 F$_1$ progeny were determined by microarray analysis. For each individual, DNA extraction, digestion of total DNA with PstI and BstNI, adapter ligation, PCR amplification, and purification of PCR products were conducted as described above, and then the sequences were labeled with Cy3 using a NimbleGen One-Color DNA Labeling Kit (Roche Diagnostics, Madison, WI, USA). Hybridization was performed with an Agilent Oligo CGH Microarray Kit (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. The microarrays were scanned with a NimbleGen MS 200 Microarray Scanner (Roche Diagnostics), and genotype calling based on the signal intensity was performed. Presence/absence calls of the probe sets reflected the presence/absence of DNA fragments hybridized to the probe sets. Thus, DNA polymorphisms including single nucleotide polymorphisms and insertions/deletions genotyped with this system were used as dominant markers. Linkage analysis was performed using a pseudo-testcross mapping strategy. Polymorphic markers segregated in a single-dose manner were analyzed using AntMap v. 1.2 software (Iwata and Ninomiya, 2006). Kosambi’s mapping function (Kosambi, 1943) was used to calculate map distances. Then, we conducted quantitative trait locus (QTL) analysis based on the linkage maps constructed and analysis of the flowering phenotype data in QTL Cartographer v. 2.5 software (Wang et al., 2012) with a composite interval mapping algorithm and an LOD threshold of 3.5 based on permutation testing (1000 permutations, $P = 0.05$).

**Verification of the developed marker**

We selected five markers (IB303507R, IB306953, IBA38559, IB204594R, and IB303642R) located at the same position as the QTL for the everbearing trait as candidates for STS markers. From the sequences of these markers, we designed five primer pairs for STS markers. Among them, the STS marker designated as “s2430859” (primer sequences are: forward, 5’-CGCC CATGTCTTGAATTC-3’, reverse, 5’-ATGAATTAT GCAGCAGGCT-3’) designed from the sequence of marker IBA38559 (patent application number: 2016-174602), was selected based on the stability of PCR amplification, correspondence between genotype and flowering habit, and its applicability among cultivars. The effectiveness of this marker was evaluated in
the following ways. First, we examined the correspondence between the genotype at s2430859 and the flowering habit in the mapping population of 147 F₁ individuals. Second, we investigated the correspondence in 22 to 177 F₁ individuals (602 in total, not including the mapping population; Table 1) derived from seven crosses between everbearing cultivars ('Miyazaki-natsuharuka', 'Summer-berry', 'Ever-berry', 'Hecker', and 'Summer-tiara') and June-bearing cultivars ('Morioka 36', 'Ohkimi', 'Karenberry', and 'Sagahonoka'). We grew these plants in a field at the NARO Tohoku Agricultural Research Center and assessed their flowering behavior from April to August under natural conditions from 2009 to 2016. Total DNA was extracted from each plant using a DNeasy Plant Mini Kit (Qiagen) or the modified PEG method (Rowland and Nguyen, 1993) with Plant DNAzol Reagent (Invitrogen, Carlsbad, CA, USA) as described by Sugimoto et al. (2005). PCR amplification was performed as follows: 1 min at 94°C, followed by 30 cycles of 10 s at 98°C, 15 s at 61°C, and 30 s at 68°C. The PCR reaction mix (10 μL) contained 0.25 U of Tks Gflex DNA polymerase (Takara Bio, Shiga, Japan), 0.2 μM of each primer, and 10 ng of template DNA. In addition, touchdown PCR (Sargent et al., 2003) with 60–55°C annealing temperature decreasing by 0.5°C per cycle was performed using 5.0 μL of GoTaq Colorless Master Mix (Promega, Madison, WI, USA). PCR products were separated by electrophoresis in 2–5% agarose gel in 0.5× TBE buffer and visualized under ultraviolet light. We also conducted linkage analysis of 137 'Sagahonoka' × 'Ever-berry' individuals and 177 'Hecker' × 'Sagahonoka' individuals using s2430859 and five previously reported SSR markers linked to the everbearing gene (Castro et al., 2015; Honjo et al., 2016) in JoinMap v. 4.1 software (van Ooijen, 2006) with an independence LOD score of 6.0. Markers within a linkage group (LG) were ordered using the maximum likelihood mapping algorithm. We assumed that the everbearing trait was controlled by a single dominant gene as in Honjo et al. (2016).

Third, we genotyped 82 additional cultivars and the 9 parents of the 7 above-mentioned crosses using s2430859 (27 everbearing, 64 June-bearing; Table 2). DNA extraction, PCR, and electrophoresis were performed as described above.

### Results and Discussion

In 147 F₁ individuals of ‘Miyazaki-natsuharuka’ × ‘Morioka 36’, we identified 70 everbearers and 77 June-bearers. The segregation ratio of everbearers and June-bearers fit the 1:1 ratio \( \chi^2 = 0.56 \) by chi-square test, suggesting that one major gene could be involved in everbearing trait segregation, as also indicated by previous studies (Ahmadi et al., 1990; Castro et al., 2015; Gaston et al., 2013; Honjo et al., 2016; Monna 2020).

| Cross                           | No. of F₁ individuals | s2430859 genotype | Flowering habit | Rate of match between genotype and phenotype (%) |
|---------------------------------|-----------------------|------------------|-----------------|-------------------------------------------------|
|                                 |                       |                  | Everbearing    | June-bearing                                    |
| Miyazaki-natsuharuka × Morioka36 | 147                   | +                | 70             | 3                                               | 98.0                                           |
|                                 |                       | -                | 0              | 74                                              |                                                |
| Miyazaki-natsuharuka × Morioka36 | 50                    | +                | 22             | 1                                               | 96.0                                           |
|                                 |                       | -                | 1              | 26                                              |                                                |
| Miyazaki-natsuharuka × Ohkimi   | 42                    | +                | 21             | 2                                               | 95.2                                           |
|                                 |                       | -                | 0              | 19                                              |                                                |
| Miyazaki-natsuharuka × Karenberry | 22                   | +                | 14             | 0                                               | 100.0                                          |
|                                 |                       | -                | 0              | 8                                               |                                                |
| Sagahonoka × Summer-berry       | 94                    | +                | 40             | 0                                               | 97.9                                           |
|                                 |                       | -                | 2              | 52                                              |                                                |
| Sagahonoka × Ever-berry         | 137                   | +                | 62             | 2                                               | 97.1                                           |
|                                 |                       | -                | 2              | 71                                              |                                                |
| Hecker × Sagahonoka             | 177                   | +                | 80             | 6                                               | 94.4                                           |
|                                 |                       | -                | 4              | 87                                              |                                                |
| Summer-tiara × Karenberry       | 80                    | +                | 40             | 1                                               | 98.8                                           |
|                                 |                       | -                | 0              | 39                                              |                                                |
| **Total**                       | **749**               | **+**            | **349**        | **15**                                          | **96.8**                                       |

\( ^a \) presence, \( ^b \) absence.

\( ^+ \) These individuals were used for the construction of linkage maps.

\( ^x \) These individuals are different from those used for the construction of linkage maps.

\( ^w \) Including reciprocal cross.
et al., 1990; Morishita et al., 2012; Perrotte et al., 2016; Salinas et al., 2017; Sugimoto et al., 2005). Two linkage maps were constructed from this mapping population. The ‘Miyazaki-natsuharuka’ map consists of 41 LGs with 8218 markers (1684.6 cM in total), and the ‘Morioka 36’ map consists of 39 LGs with 8039 markers (1110.5 cM in total). These markers and probe sequences are shown in Supplementary Tables S1 and S2. From the QTL analysis based on these linkage maps, we detected a significant QTL with LOD = 47.4 for the everbearing trait on LG 20 of the ‘Miyazaki-natsuharuka’ map (Fig. 1), but no QTLs on the ‘Morioka 36’ map. This QTL accounted for 74.8% of the phenotypic variance in flowering habit. Among the STS primers designed for the five markers within 5.4 cM of the everbearing locus, that for the STS marker designated as s2430859 derived from the sequence of marker IBA38559 amplified an approximately 150-bp fragment from the everbearing individuals. Genotype and flowering habit matched in 144 of 147 plants (98.0%; Table 1).

To evaluate the applicability of this marker in other populations, we determined the genotypes of F1 individuals from seven crosses. The correspondence between genotype and flowering habit ranged from 94.4% (167/177) in the ‘Hecker’ × ‘Sagahonoka’ population to 100.0% (22/22) in the ‘Miyazaki-natsuharuka’ × ‘Karenberry’ population (Table 1; Fig. 2). The average accuracy of the eight populations was 96.8% (725/749), slightly less than the 98.8% of the SSR marker FxaACA02108C, but higher than the 92.8% of the SSR marker ChFaM011 (as calculated from the data of Honjo et al., 2016). It is known that there are two major sources of everbearing cultivars: (1) old everbearing-

Table 2. Genotype at s2430859 of 91 strawberry cultivars.

| Cultivars                          | Genotype at s2430859* |
|------------------------------------|-----------------------|
| Everbearing cultivars              | + −                   |
| Awanatsuka, Dekoruju, Ever-berry,  | + −                   |
| Hecker, HS-138, Kurume64, Miyazaki- |                      |
| natsuharuka, Miyoshi, Morioka34,   |                      |
| Morioka37, Natsuakari, Natsunokayaki, |                      |
| Otishi-shikinari, Pechka, Selva,   |                      |
| ShindaiBS8-9, Summer-amigo, Summer- |                      |
| berry, Summer-candy, Summer-fairy, |                      |
| Summer-princess, Summer-tiara,     | + −                   |
| Tochihitomi, UC-Albion             |                       |
| Morioka30, Morioka33, Summer-drop  |                       |
| June-bearing cultivars             | + −                   |
| Elsanta, Florence, Hogyoku, Kentaro, |                       |
| Kitaekubo, Pegasus, Red Gauntlet    | + −                   |
| Aiko, Aistro, Akihime, Akitaberry, | + −                   |
| Amaekubo, Amaotome, Askawave,      | + −                   |
| Benihoppe, Beruruju, Bolero,       | + −                   |
| Cambridge Favourite, Cardinal,     | + −                   |
| Donner, Fukuba, Fukuoka56, Harumi, | + −                   |
| Haruyoi, Hatsuakuni, Hitachihime,  | + −                   |
| Hokowase, Ibarakiss, Ichigo-chukan- |                      |
| bohon-nou2gou, Kaorino, Karenberry, |                      |
| Kitanokagayaki, Koinorinori,       |                      |
| Koinoka, Komachiberry, Kurume62,   | + −                   |
| Kurume66, Kurume67, Mae, Marshall, | + −                   |
| Moikko, Morioka16, Morioka32, Morioka36, | + −               |
| Morioka37, Nohime, Nyoho,          | + −                   |
| Ohkimi, Oi-C-berry, Otemegokoro,   | + −                   |
| Ozeakarin, Pajaro, Pelican, Raiho,  | + −                   |
| Reiko, Sachinoka, Sagahonoka,       | + −                   |
| Sequoia, Tioga, Tochihihime,       | + −                   |
| Tochiotome, Toyonoka, Toyoyukihime, |                      |
| Tsuburoman, Yayoihime              | + −                   |

*+ presence, − absence.

Fig. 1. Putative QTL region for the everbearing trait on linkage group 20 of the everbearing strawberry cultivar ‘Miyazaki-natsuharuka’.
mutant cultivars of \( F \times ananassa \), and (2) an everbearing clone of \( F. virginiana \) ssp. \( glauca \) from the Wasatch Mountains of Utah, America (Hancock, 1999). Among the everbearing cultivars listed in Table 1, ‘Miyazaki-natsuharuka’, ‘Summer-berry’, and ‘Ever-berry’ are derived from the former, and ‘Hecker’ and ‘Summer-tiara’ from the latter. The high correspondence between genotype and flowering habit in both everbearing cultivars suggests that the same major gene controls flowering response in both lineages. This result corresponds to previous studies (Honjo et al., 2016; Salinas et al., 2017).

Furthermore, among the 27 everbearing cultivars, 24 had the amplified fragment at s2430859 (Table 2). Among the 64 June-bearing cultivars, in contrast, 57 lacked the amplified fragment. These results suggest that s2430859 may be applicable to more than 89% of the cultivars tested. In contrast, marker FxaACA02108C was applicable to only about half of the cultivars previously tested (Honjo et al., 2016) owing to the existence of an unlinked SSR fragment of the same size as the linked fragment. This unlinked fragment was probably amplified from homoeologous chromosomes. Because s2430859 specifically amplified the allele linked to the everbearing gene in coupling, its applicability is much wider. Ten cultivars for which s2430859 was not applicable encompassed several pedigrees. For example, ‘Kentaro’ is the offspring of ‘Kitaekubo’, and ‘Florence’ and ‘Pegasus’ are the offspring of ‘Red Gauntlet’. Among the three everbearing cultivars, the everbearing gene of ‘Morioka30’ is derived from ‘Selva’ (Table 2), and those of ‘Morioka33’ and ‘Summer-drop’ are inherited from each parental cultivar, ‘Summer-berry’ and ‘Miyoshi’ (Table 2), respectively. In addition, these three cultivars possessed the 151-bp fragment at the marker FxaACA02108C (Fig. 3), which was inferred to be linked to the everbearing gene (Honjo et al., 2016). Considering the pedigree and the genotype at FxaACA02108C, these three cultivars may have arisen from genetic recombination during meiosis or have mutated sequences at the primer sites. On the other hand, ‘Florence’, ‘Pegasus’, and ‘Red Gauntlet’

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**Fig. 2.** Agarose gel electrophoresis of PCR products amplified from strawberry cultivars ‘Miyazaki-natsuharuka’, ‘Ohkimi’, and their F\(_1\) progeny (see Table 1) using the marker s2430859. M, 200-bp ladder. Letters below the gel image indicate the flowering phenotype: e, everbearing; J, June-bearing. *Recombinant individual.

**Ad_Sagahonoka × Everberry**

**HS_Hecker × Sagahonoka**

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**Fig. 3.** Genetic linkage map of DNA markers linked to the everbearing gene (designated Evb) in F\(_1\) individuals from two crosses, ‘Sagahonoka’ × ‘Ever-berry’ (left) and ‘Hecker’ × ‘Sagahonoka’ (right). Markers with the prefix r\(_{c}\) are linked to Evb in repulsion.
did not possess the 151-bp fragment, corresponding to the phenotype. Therefore, the discrepancy between the genotype at s2430859 and phenotype may be caused by genetic recombination between the marker and gene. For the other four cultivars (‘Elsanta’, ‘Hogyoku’, ‘Kentarou’, and ‘Kitaekubo’), FxaACA02I08C was not applicable. Further investigation is needed to explore the cause of the discrepancy in these cultivars.

Linkage analysis of 137 individuals of the ‘Sagahonoka’ × ‘Ever-berry’ cross showed that s2430859 was linked to the everbearing gene in coupling and was mapped on the opposite side of the markers FxaACA02I08C and ChFaM011, at 3.0 cM from the everbearing gene (Fig. 3). In the F1 individuals of the ‘Hecker’ × ‘Sagahonoka’ cross, s2430859 was mapped at 6.0 cM from the everbearing gene (Fig. 3). Previous studies reported that the everbearing gene of F. × ananassa and linked markers, such as FxaACA02I08C and ChFaM011, are located on an LG in the homoeologous group IV, which corresponds to LG IV of F. vesca (Castro et al., 2015; Honjo et al., 2016; Perrotte et al., 2016). The marker s2430859 will contribute to the detection of recombinant individuals by use in combination with previously reported markers as described in the paragraph above.

In conclusion, we expect the newly developed marker s2430859 to be useful for screening of everbearing plants with high efficiency. Genotyping can be easily performed by agarose gel electrophoresis. The most significant merit of this marker is its applicability to a diverse range of cultivars.

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