Confirmation of Everbearing Mutants in the Everbearing Strawberry Cultivar ‘Natsuakari’ and the Effect of an Overwintering Condition on Inflorescence and Runner Development

Megumi Hamano1**, Masanori Honjo1**, Shiori Sato2, Atsushi Ito3, So Machita4, Uzuki Matsushima2, Masumi Okada5 and Kazuhisa Kato6*,**

1National Agricultural Research Center for Tohoku Region, Morioka 020-0198, Japan
2Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan
3Aomori Prefectural Industrial Technology Research Center, Agriculture Research Institute, Kuroishi 036-0522, Japan
4Aomori Prefectural Industrial Technology Research Center, Vegetable Research Institute, Rokunohe, Aomori 033-0071, Japan
5Organization of Revitalization for Sanriku Region and Regional Development, Iwate University, Morioka 020-8550, Japan
6Graduate School of Agricultural Science, Tohoku University, Sendai 980-8572, Japan

The everbearing strawberry cultivar ‘Natsuakari’ produces high-quality fruits and can be harvested in summer and autumn. However, flowering-defective individuals have been recently found at some farms in the Tohoku region. To solve this problem, we first investigated the behavior of flowering-defective individuals collected from three places in Aomori, Akita, and Miyagi prefectures and confirmed that the non-flowering phenomenon reproducibly occurred during summer and autumn. Next, we analyzed the genotypes of flowering-defective individuals using highly polymorphic simple sequence repeat (SSR) markers suitable for cultivar discrimination and SSR markers linked to the everbearing gene. As a result, flowering-defective individuals showed the same genotype as the original ‘Natsuakari’, and the fragment linked to the everbearing gene was not detected in flowering-defective individuals. It was suggested that cultivar contamination was not the cause of the non-flowering phenomenon, and that mutations may be present in flowering-defective individuals. To confirm the effects of previous history before transplanting on the flowering and runner development of normal flowering plants and the flowering-defective mutants, the influence of potting time of the daughter plant and overwintering conditions were investigated. The potting time had no effect on flowering or runner development. Inflorescences in the normal flowering plants continuously emerged, especially in summer and autumn under both heated and non-heated conditions during overwintering. The mutants hardly flowered in the summer and autumn under both conditions, although the inflorescences emerged in the early summer under the heated condition during overwintering. The number of runners observed in the mutants was higher than that in the normal plants throughout the study, especially in the summer, while in both heating during overwintering led to much less runner development in May through July. According to these results, although the mutants were suitable for plant propagation, defective flowering persisted in subsequent years. Therefore, to ensure stable production, it is important to observe flowering types in mother plants with adequate chilling and to select mother plants exhibiting flowering in summer and autumn.

Key Words: chilling, flowering-defective mutant, genotyping.

Introduction

June-bearing strawberry (Fragaria × ananassa Duch.) cultivars are primarily cultivated in Japan. They are mainly produced during December–May and the production of these cultivars is markedly reduced in summer and autumn. Most fresh strawberry fruits in Japan in the out-of-season production period are imported from the United States and other countries (alic, 2018). To supply demand, everbearing strawberry culti-
The number of produced runners per plant and inflorescences per plant varied among the strains. In general, the number of emerged inflorescences and runners produced by flowering-defective individuals was lower compared to normal flowering individuals. However, flowering-defective individuals from the B strain produced a similar number of emerged inflorescences and runners as normal flowering individuals from the Kuroishi and A strains. This suggests that flowering-defective individuals can still produce flowers and runners, but in lower numbers compared to normal flowering individuals.

Materials and Methods

1. Inflorescence and runner development in flowering-defective individuals (Test 1)

Normal flowering plants and flowering-defective individuals in the everbearing cultivar ‘Natsuakari’ were collected and cultivated as follows. The flowering-defective individuals were collected from Aomori Prefectural Industrial Technology Research Center, Agriculture Research Institute (Aomori ARI) in Aomori prefecture (Kuroishi strain) and from two farms, farm A in Miyagi prefecture (A strain) and farm B in Akita prefecture (B strain) (Fig. 1). Normal flowering plants were also obtained from Aomori ARI (Kuroishi-N strain). The Kuroishi and A strains were daughter plants potted in 2015 from mother plants that did not flower in the summer of 2015, and Kuroishi-N plants were daughters potted in 2015 from a normal flowering mother plant. Plants from the B strain were harvested plants that did not flower during the summer of 2015 and were dug up in early spring 2016. These plants were overwintered under unheated conditions at each place.

The plants were moved to NARCT (39° 46’ N, 141° 05’ E) in March 2016 and potted on April 20, 2016 in 21-cm-diameter plastic pots filled with commercial soil.

Fig. 1. The number of emerged inflorescences (A) and runners (B) in defective and normal flowering plants shown by respective individuals. Kuroishi 1–5, A1–5, and B1–5 were flowering-defective individuals from Aomori Prefectural Industrial Technology Research Center, Agriculture Research Institute (Aomori ARI) in Kuroishi, from farm A in Miyagi prefecture, and from farm B in Akita prefecture, respectively. Kuroishi-N 1–5 were normal flowering individuals from Aomori ARI.
mix for strawberry daughter plants (Ikubyo No. 2; Sankensoil, Hachimantai, Japan) with a pH of 5.8–6.0 and a nutrient composition of 220 mg/L N, 400 mg/L P2O5, and 150 mg/L K2O. All plants were grown under drip fertigation (Youekidokou solution No. 6; diluted 3,000 times; OAT Agrio Co., Ltd., Tokyo, Japan) in a plastic greenhouse in NARC under natural light and photoperiod. Temperature was maintained using a ventilating fan at > 25°C and side windows were opened at temperatures > 20°C.

Three branch crowns were maintained for each plant, and emerged inflorescences and runners were removed until one month after transplanting. The numbers of emerged inflorescences and runners were investigated monthly after the first month. The fruits were removed and discarded and were not recorded.

2. Cultivar discrimination and genotyping by SSR markers (Test 2)

Cultivar discrimination was conducted using the highly polymorphic SSR markers FxaHGA02P13 (Honjo et al., 2011) and EMFv104 (Govan et al., 2008). Genotype differences between the normal and defective flowering individuals were analyzed using SSR markers closely linked to the everbearing gene at 1.1–1.5 cM (Honjo et al., 2016).

3. Effects of the potting time and overwintering condition on the numbers of emerged inflorescences and runners in daughter plants from normal and flowering-defective mother plants (Test 3)

1) Plant materials

The normal flowering plants were potted in 10.5-cm diameter pots at NARC in the summer of 2015 then overwintered in an open field. Plants were later transferred into an unheated plastic greenhouse in mid-March then planted as mother plants in 21-cm diameter pots filled with Ikubyo No. 2. on April 20, 2016. For fertilizer application, two tablets of Big-one L (Jcam Agri Co., Ltd., Tokyo, Japan) with a nutrient composition of 200 mg N, 200 mg P2O5, and 200 mg K2O per tablet were applied per pot once a month. The mutant mother plants were the same plants used in Test 1 as flowering-defective individuals (Kuroishi, A and B strains) because the phenotype and genotype were the same. They were cultivated as described in Test 1.

The daughter plants from each of the mother plants were potted in plastic pots (10.5-cm diameter) filled with Ikubyo No. 2 in three periods: from the end of May to early June, from the beginning to the middle of September, and from the beginning to the middle of October. Daughter plants that began rooting with approximately two leaves were selected and directly rooted to the soil in 10.5-cm diameter pots, pressed with a runner clip, and then cut off after about two weeks.

A single Big-one L tablet was applied per pot once a month as additional fertilizer. The above plants were grown in an unheated plastic greenhouse in NARC, then moved to Iwate University (39° 42' N, 141° 07' E) and overwintered as follows.

2) Overwintering

The daughter plants potted and cultivated in NARC were overwintered at Iwate University from December 5, 2016 to April 20, 2017 under three conditions: in a glasshouse maintained at a minimum temperature of 10°C, an unheated plastic greenhouse, and an open field. Defoliation and manual irrigation were performed as required. Plants were covered with snow in the open field.

3) Cultivation

A high bed system made of non-woven fabric (20-cm wide) was used as the field in an unheated plastic greenhouse. The medium used for cultivation comprised rice hulls and akadama soil mixed at a volume ratio of 3:1 (Okada, 2016). Rice hulls exposed to rain for more than one year were used to minimize the water repellent effect. In addition, 17.9 g per plant of controlled-release fertilizer (Ecolong total 391 for 180 days; Jcam Agri) with a nutrient composition of 13% N, 9% P2O5, 11% K2O, 2% MgO, and micronutrients, and charcoal powder (3% of the medium volume) were mixed well with the medium, and the mixed medium was added to the high bed system (4 L per plant).

Overwintered daughter plants were transplanted on April 20, 2017 in staggered rows with 20 cm between the stocks and 10 cm between the streaks. Each treatment was replicated three times with four plants per replicate.

After transplanting, automatic irrigation was performed for 4–5 min per hour from 6:00 to 17:00 h with leaf removal and bactericide and insecticide application as required. Emerged inflorescences and runners during the overwintering period and in April were removed. The number of branch crowns was not adjusted.

4) Investigation

Temperature changes in three treatment conditions during overwintering were measured using a thermistor (RTR-501; T & D Corporation, Matsumoto, Japan) inside the shield and received chilling (< 5°C) during overwintering was calculated. The numbers of emerged inflorescences and runners per plant were determined once or twice a week between May 2017 and October 2017. Fruits that developed after transplantation were removed and discarded. Runners were also removed after the investigation.

5) Genotyping in daughter plants after overwintering

We confirmed the genotype of a total of 13 plants from normal and flowering-defective plants used in Test 3 using DNA markers linked to the everbearing gene as described in Test 2.

6) Statistical analysis

Each treatment was performed with three replicates using four plants per replicate for the number of emerged inflorescences and runners. Three-way analy-
sis of variance (ANOVA) was conducted for overwintering, potting time of daughter plants, and flowering type of mother plants using R (R Core Team, 2016). In addition, without considering potting time, the differences in the overwintering condition were examined for normal flowering plants and flowering-defective mutants using Tukey’s multiple comparison test in R. Each treatment was performed using nine replicates and four plants per replicate.

Results

1. Emerged inflorescences and runners in flowering-defective individuals (Test 1)

The number of inflorescences and runners that emerged from respective individuals are illustrated in Figure 1. Inflorescences in the normal flowering plants were continuously produced from May 20 through autumn. Conversely, no inflorescences were observed in the flowering-defective individuals for four months from June 21–October 20; however, a few inflorescences emerged after October 21. On the other hand, in comparison with normal plants, flowering-defective individuals produced runners much more vigorously during the test.

2. Cultivar discrimination and genotyping using SSR markers (Test 2)

Commercial strawberries are octoploid; therefore, numerous amplified fragments were detected after PCR analysis using the two SSR markers. Flowering-defective individuals showed the same genotypes at both markers as original ‘Natsuakari’ (FxaHGA02P13: 244, 262, 265, 282, 302; EMFv104: 102, 114, 122, 125, 131, 133, 134, 136, 138, 148, 152, 154).

Genotyping using the marker FxaACA02I08C, closely linked to the everbearing gene, revealed that all the normal flowering individuals had the same genotype (148, 151, 159, and 179) as original ‘Natsuakari’ (Fig. 2). Among these amplified fragments, a 151-bp fragment has been linked to the everbearing gene in coupling (Honjo et al., 2016). On the other hand, none of the flowering-defective individuals tested possessed the 151-bp fragment, indicating that some mutations occurred in the everbearing gene region.

3. Effects of the potting time and overwintering condition on the number of emerged inflorescences and runners in daughter plants from normal and flowering-defective mother plants (Test 3)

1) Chilling during overwintering

The total chilling (< 5°C) received at NARCT was for 250 h. After transfer to Iwate University, the daily average temperature variation from December 6, 2016 to April 19, 2017 is shown in Figure 3A. A total of 2404 and 1686 h of chilling were detected during overwintering in the open field and unheated plastic greenhouse, respectively, while no chilling was detected during overwintering in the heated glasshouse (Fig. 3B).

2) Effects of the potting time and overwintering condition on the number of emerged inflorescences in daughter plants from normal and flowering-defective mother plants

Daughter plants from the normal flowering plants and the flowering-defective individuals were designated...
as normal and mutant plants, respectively, in Test 3. Inflorescences emerged until April regardless of the flowering status of mother plants in the previous year or overwintering condition, and they were not counted because fasciation or dwarfing often occurred in the inflorescence development until April.

The monthly numbers of emerged inflorescences after May were analyzed using three-way ANOVA (data not shown), and no significant differences were observed among the potting times of daughter plants. The effect of overwintering conditions on the number of emerged inflorescences and the flowering status of the mother plants in the previous year without considering the potting time of daughter plants is shown in Figure 4. Inflorescences were continuously observed in the normal plants, and the levels were high, particularly from July to September. There were fewer emerged inflorescences in the mutant plants than in the normal plants, while most plants grown under unheated conditions during overwintering treatment had no flowers. The numbers of inflorescences that emerged in May and June in the mutants kept warm in winter were similar to those in the normal plants. The numbers of emerged inflorescences in the mutants were much lower than those in the normal plants.

3) Effects of the potting time and overwintering condition on the number of runners in daughter plants from normal and flowering-defective mother plants

Until April, runners were produced only under unheated conditions regardless of the flowering status of the mother plants in the previous year; however, few runners were produced under the heated condition during overwintering (data not shown). A three-way ANOVA was performed on the number of runners produced monthly from May (data not shown). Runner production was not affected by the potting time of daughter plants. Therefore, only the effect of overwintering conditions on the number of runners produced and each mother plant’s flowering status was investigated (Fig. 5). Production of runners in both plants was inhibited by heating during overwintering in May through July. The number of runners produced in the mutant plants gradually increased toward August and rapidly decreased after September. In contrast, the number of runners produced in the normal plants was the highest in the open field overwintering condition, followed by the unheated plastic greenhouse and the heated glasshouse in May and June. There were almost no runners produced in any overwintering conditions from July.

![Fig. 4. Effects of overwintering conditions on the number of emerged inflorescences. A: Daughter plant from a normal flowering mother plant; B: Daughter plant from a flowering-defective mother mutant. Different letters in the same month indicate significant difference at the 5% level based on Tukey's multiple comparison test (n = 9). Vertical bars indicate S. E.](image)

![Fig. 5. Effects of overwintering conditions on the number of runners produced. A: Daughter plant from a normal flowering mother plant; B: Daughter plant from a flowering-defective mother mutant. Different letters in the same month indicate significant difference at the 5% level based on Tukey’s multiple comparison test (n = 9). Vertical bars indicate S. E.](image)
4. Genotyping of the daughter plants after overwintering

All daughter plants that propagated from the normal mother plants showed the same genotypes as the normal plants. On the other hand, all daughter plants that propagated from the mutant mother plants showed the same genotype as the mother plants; they did not possess the 151-bp amplified fragment linked to the everbearing gene (data not shown).

Discussion

June-bearing strawberry cultivars can be harvested over a short period under natural conditions, and everbearing cultivars can be continuously harvested over a long period (Robertson, 1955). The difference in continuous flowering is mainly caused by different temperatures and day-length conditions that promote flower bud initiation. Flower bud initiation in June-bearing cultivars is induced by low temperature and short days (Bradford et al., 2010; Nishiyama and Kanahama, 2000; Sønsteby and Heide, 2007). Such flowering habits of everbearing cultivars are suitable for the harvesting of strawberries in summer and autumn. However, some mutants without emerged inflorescences in summer and autumn were recently found in some fields of the everbearing cultivar, ‘Natsuakari’. This has posed a major challenge; therefore, in this study we attempted to understand the current situation, investigate the cause, and establish countermeasures.

Test 1 revealed that the daughter plants from the flowering-defective mother plants in the previous year (Kuroishi and A strains) flowered in spring, but not in summer and autumn, as mother plants (Fig. 1). Hamano et al. (2017) reported on flowering-defective individuals of the Kuroishi strain separately cultivated at three research institutions: Iwate University, Aomori ARI and Aomori Prefectural Industrial Technology Research Center, and the Vegetable Research Institute. Individuals of the Kuroishi strain showed the same tendency for emergence of inflorescences and runners as in Test 1. Furthermore, some treatments that seemed to influence flowering (gibberellin spraying, defoliation, shading, or late transplanting) did not change the patterns of flowering and runnering.

For the B strain, which had been used as a harvesting plant in the previous year and had not flowered in the previous summer, did not have emerged inflorescences during the subsequent summer and autumn either. Taimatsu et al. (1991) reported that flower bud initiation of daughter plants in their second year in the everbearing cultivar ‘Summer-Berry’, which is the mother cultivar of ‘Natsuakari’ (Okimura et al., 2011), was more likely to produce flower buds than plants in their first year. The flowering behavior of the B strain was not consistent with the result of Taimatsu et al. (1991).

Based on the above results, the cultivation condition is unlikely to be related to the flowering-defective trait. Therefore, the flowering-defective phenomenon may be reproducible, and the trait can be propagated in daughter plants.

The two markers for cultivar discrimination used in Test 2 are highly polymorphic, and the possibility of matching genotypes of two different cultivars by chance is very low (Honjo et al., 2011). Therefore, we concluded that contamination of other June-bearing individuals was not the cause of the non-flowering phenomenon. On the other hand, none of the flowering-defective individuals possessed the 151-bp fragment at FxaACA02I08C linked to the everbearing gene in coupling. This strongly suggests that some structural variation, such as insertion or deletion, occurred near the everbearing gene, and this may explain the reproducibility of the flowering-defective trait under different cultivation conditions and propagation in daughter plants.

In Test 3, there were approximately 250 h of chilling at NARCT before overwintering at Iwate University; therefore, the plants could have been dormant at the start of the overwintering condition treatment. After that, there were 2404 and 1686 h of chilling during overwintering in the open field and in the heated plastic greenhouse, respectively; however, there was no chilling during overwintering in the heated glasshouse (Fig. 3). Consequently, the plants overwintered in the heated glasshouse did not satisfy the level of chilling required to break dormancy, > 1000 h (Hamano et al., 2011). It is well known that June-bearing cultivars have a continuous flowering pattern if chilling is avoided (Guttridge and Anderson, 1976; Voth and Bringhurst, 1958; Yanagi and Oda, 1992). In the everbearing cultivars, flowering also continues under heated conditions after low-temperature treatment (5°C) for a limited period during dormancy (Seyama and Imada, 1990). In the present study, the number of emerged inflorescences in the normal plants overwintered under heated conditions was higher in May than those under unheated conditions (Fig. 4). The number of inflorescences in the mutant plants overwintered under heated condition was particularly higher in May and June than those under unheated conditions. The results of the present study further confirmed that the everbearing cultivar has a continuous flowering pattern when chilling is reduced by heating during dormancy (June-bearing), and the mutant plants that lost the everbearing trait also maintained the same flowering characteristics.

Even in plants overwintered under the unheated condition, inflorescences differentiated before the overwintering emerged by April regardless of the flowering status of the mother plants in the previous year. However, the number of inflorescences that emerged in May...
in the unheated overwintering plants was lower than that in the heated overwintering plants (Fig. 4), as chilling delayed flower bud initiation (Guttridge, 1958; Smeets, 1982). In the normal plants, inflorescences were induced after a post-chilling physiological condition (Guttridge, 1958) and inflorescences appeared continuously after June. On the other hand, mutant plants may lack the ability to induce flowering under spring and summer environmental conditions, so they did not flower during summer through autumn. The environmental condition that can induce flowering in mutant plants is unclear. Studies to clarify this point would help to elucidate the difference in the flowering mechanism between June-bearing and everbearing strawberries.

The number of runners produced in the everbearing cultivars increased with an increase in the chilling time (Seyama, 2010). In the present study, the number of runners produced was also lower in the heated overwintering plants than those in the unheated overwintering condition. In addition, the longer the chilling, the more runners appeared in normal plants (Fig. 5).

Everbearing cultivars generally have fewer runners (Darrow, 1966; Downs and Piringer, 1955). In addition, the effect of chilling on the runner promotion of everbearing cultivars is lower than that on June-bearing cultivars (Yanagi and Oda, 1990). Here, the number of runners in the unheated overwintering mutant plants from May to July was higher than the number in the heated overwintering plants. The difference in the number of runners between heated and unheated mutant plants was higher than that in the normal flowering plants. These results suggest that the flowering-defective mutant lost its everbearing trait based on the number of runners produced.

The runners could have been produced robustly in the flowering-defective mutant. Runners are developed from axillary buds. In the crown of normal flowering plants, leaf initiation ceases when inflorescence is induced at the apical meristem, and a few axially buds develop into branch crowns (Guttridge, 1955; Neri et al., 2003). On the other hand, in flowering-defective mutants, leaf initiation is continued, and more axillary buds can develop into runners. Moreover, assimilation of nutrients may be utilized for runner growth instead of flowers or fruit. Therefore, the flowering-defective mutants proliferated and spread because the daughter plants were easily obtained. When a flowering-defective mutant is mixed with mother plants, the proportion of daughter plants from the flowering-defective mutant increases in the following year and the number of emerged inflorescences in summer and autumn considerably decrease.

It is critical to understand the flowering status of mother plants to avoid the use of mutants and to select plants with normal flowering. Such an approach could prevent a decrease in fruit yield in summer and autumn, while facilitating stable production.

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