Breeding of a New Aromatic Strawberry by Interspecific Hybridization

Fragaria x ananassa × F. nilgerrensis

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Summary

The wild, Asian diploid strawberry, Fragaria nilgerrensis has not been exploited as breeding material until now, although interspecific cross compatibility has been investigated with the cultivated strawberry. Breeding to introduce a new aroma from a diploid wild strawberry, F. nilgerrensis, was tried to expand the genetic variation in the cultivated strawberry, F. x ananassa. All lines, derived from interspecific hybridization between F. nilgerrensis var. Yunnan and F. x ananassa cv. Toyonoka, were completely sterile. By doubling the chromosome by in vitro colchicine treatment of the fertile lines, some superior fertile lines were obtained. From the results of RAPD analysis, 'TN13-125', one of these derived from the interspecific hybridization, had fragments specific to both parents. The morphological characters of 'TN13-125' almost resembled 'Toyonoka', but it had some characters from F. nilgerrensis such as numerous hairs on the petioles and peduncles. The fruits have almost the same size, brix and acidity levels as the cultivated strawberry, but they are very soft and pale pink in skin color. The interspecific hybrid resembles F. nilgerrensis in aroma components, with enriched ethyl acetate. The characteristic fragrance of the interspecific hybrid is peach-like. Moreover, it was possible to introduce the aroma from wild strawberry to cultivated types by back-crossing. We are using this hybrid line as the parental material to breed a cultivar with a special flavor.

Key Words: aroma, Fragaria x ananassa, F. nilgerrensis, interspecific hybrid, strawberry.

Introduction

The modern, cultivated strawberry (Fragaria x ananassa) originated from the interspecific hybrid between F. virginiana and F. chiloensis. As the first interspecific hybrids were produced accidentally in Europe from limited parental plants, the descendant do not have great genetic variation. Crosses with wild octoploid species have been tried to expand the genetic variation of the cultivated strawberry (Scott and Lawrence, 1975). In Japan and other East Asian countries, there are no indigenous octoploid wild strawberries that were progenitors of the cultivated strawberry, but some diploid and tetraploid species with a broad diversity of various characters exist. F. nilgerrensis is a wild diploid strawberry, native to southwestern China, where the climatic condition is comparatively similar to Japan. Though interspecific cross compatibility of F. nilgerrensis with cultivated strawberry has been studied, this species has not yet been exploited as a breeding material. In America, F. nilgerrensis has been rejected for breeding (Brinughst and Voth, 1984), because of its undesirable aroma and taste (Hancock and Luby, 1993). However, the mature fruits typically have aromas like banana (Staudt et al., 1975), melon or peach (Oda et al., 1990). Thus, the introduction of this interesting fragrance from F. nilgerrensis to cultivated strawberry was attempted. This is the first report of a fertile, fruit-setting strawberry from an interspecific hybrid of F. x ananassa and F. nilgerrensis.

Materials and Methods

Interspecific hybridization and chromosome doubling

Fragaria x ananassa cv. Toyonoka was pollinated with pollen from F. nilgerrensis var. Yunnan in May 1992. The achenes were sown on wet filter paper in petri dishes in June. The seedlings were planted on vermiculite; when the seedlings developed more than three leaves, they were transplanted to 12 cm polyethylene
pots in October, and raised for about one year in a glass house. They were transplanted to a plastic house in September 1993 and cultivated under forcing condition. 'TN13', which developed many runners, was treated with colchicine for chromosome doubling. Runner tips of 'TN13' were cultured on B5 medium (2% of sucrose, 0.2% of gellan gum), containing 100 mg·liter⁻¹ colchicine, 2.0 mg·liter⁻¹ 6-benzyladenine (BA) and 0.02 mg·liter⁻¹ 1-naphthalene acetic acid (NAA) under 25°C, 12-hr photoperiod (3000lx) in October 1993. After one month, the developed shoots from each runner tip were divided, and transplanted on B5 medium without growth regulators to produce plantlets. 152 plantlets, which originated from 'TN13', 'Toyonoka', 'Yunnan' and 'TN13' were cultivated in the plastic house under forcing condition from September 1994.

Randomly amplified polymorphic DNA (RAPD) analysis

'TN13' and two lines 'TN13-113', 'TN13-125', which regenerated from colchicine-treated apex of 'TN13', and their parents 'Toyonoka' and 'Yunnan' were prepared for RAPD analysis to confirm the hybridity. DNA was extracted from 5 g fresh weight of frozen young leaves of each line or variety and polymerase chain reactions (PCR) were performed in a volume of 20 μL, containing 2 μL reaction buffer (Pharmacia), 1.6 μL dNTP (Pharmacia, 2.5 mM), 0.8 μL (5 pmol·μL⁻¹) oligonucleotide primer and about 10 ng of template DNA. Eleven random primers (three from Operon Technologies and eight synthesized in NIVOT) were used to develop the RAPD markers. The reaction mixtures with one unit of Tag DNA Polymerase (Pharmacia) were overlaid with one drop of mineral oil; amplification was carried out with a thermal cycler (TSR-300, IWAKI), programmed for 50 sec preheating at 94°C, followed by 45 cycles, each consisting of 94°C / 30 sec, 38°C / 2 min and 72°C / 3 min for denaturation, annealing, and reaction extension steps, respectively. This was followed by a final soak at 72°C for 5 min. Amplified DNA was electrophoresed on 1.5% agarose gel. Each amplification was repeated at least twice and only the reproduced bands were considered for analysis.

Back-crossing to parental cultivars and fruit characters of back-crossed progenies

In the spring of 1996, 16 seedlings were obtained from back-crossing the interspecific hybrid line 'TN13-125' to F. x ananassa cv. Pajarro, which has excellent fruit appearance and firmness. Two lines, 'TNP-05' and 'TNP-14', which had good fruit appearance and aroma similar to 'Yunnan' by the organoleptic test, were selected under forcing culture in the spring of 1997. 'TNP-05' and 'TNP-14' were propagated and planted to a plastic house in September 1997 with 'TN13-125', 'Pajarro' and 'Toyonoka' as controls. Fruit weight, fruit length and the fruit diameter were measured. The flesh firmness was determined with a disk plunger (3 mm diam.) with a push-pull gage (Model 9501A, Aikoh Engineering). Each fruit was squeezed and the Brix determined with a refractometer (DBX55, Atago); the acidity was measured with an acidimeter (Acilyzer Model 6, Fujihira Kogyo). In addition, the aromatic volatile components of the mature fruit were analyzed by gas chromatograph – mass spectrometer (GC–MS, SHIMADZU QP-5000). The quadruplicate fruits were maintained at 60°C, and the headspace gas was introduced directly to the capillary column. The column flow rate was 0.9 ml·min⁻¹ and the column temperature was

<table>
<thead>
<tr>
<th>Groups and varieties</th>
<th>Flowering</th>
<th>Fruit - setting</th>
<th>No. of plants</th>
<th>Leaf area (cm²)</th>
<th>Petiole length (mm)</th>
<th>Flower character</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Petal color</td>
</tr>
<tr>
<td>Group I</td>
<td>W</td>
<td></td>
<td>15</td>
<td>9.0</td>
<td>8.3</td>
<td>W</td>
</tr>
<tr>
<td>Group II</td>
<td>+</td>
<td></td>
<td>28</td>
<td>28.3</td>
<td>16.8</td>
<td>W-S.P</td>
</tr>
<tr>
<td>Group III</td>
<td>+</td>
<td>+</td>
<td>109</td>
<td>41.5</td>
<td>20.9</td>
<td>W</td>
</tr>
<tr>
<td>Toyonoka</td>
<td>+</td>
<td>+</td>
<td></td>
<td>102.2</td>
<td>-</td>
<td>W</td>
</tr>
<tr>
<td>Yunnan</td>
<td>+</td>
<td>+</td>
<td></td>
<td>16.6</td>
<td>-</td>
<td>W</td>
</tr>
<tr>
<td>TN13</td>
<td>+</td>
<td>-</td>
<td></td>
<td>55.1</td>
<td>-</td>
<td>W</td>
</tr>
</tbody>
</table>

*: Not flowering or fruit - setting. +: Flowering or fruit - setting.
²: Leaf area=leaf length × leaf width × 2/3 × 3.
³: W: White, S.P: Salmon pink.
⁴: Group I was composed of plants without flowers.
⁵: Group II was composed of plants that bloom but do not set fruits.
⁶: Group III was composed of plants that bloom and set fruits.
⁷: 'TN13' was a sterile interspecific line between F. x ananassa cv. Toyonoka and F. nilgerrensis var. Yunnan.
raised linearly from 50°C to 230°C. The relative value of each peak area was estimated in the TIC charts.

**Results**

**Interspecific hybridization and chromosome doubling**

Out of 152 plants regenerated from colchicine treated apex of ‘TN13’, 137 bloomed and 109 set fruits by March 1995. Several plants showed typical higher polyploidy characters, such as thick leaflets and malformed fruits. The leaves of the fertile plants were bigger than those of the sterile plants (Table 1). Their petioles became thicker and shorter than those of the original line. Although the petals were white in most flowering plants, petals in some plants were light salmon-pink. The anthers of fertile plants which originated from ‘TN13’ were bigger than those of the sterile plants. Although many plants set conical fruits, some differences were recognized in fruit shape, from the cone shape that was similar to ‘Toyonoka’ to a short cone-like globular fruit.

**Randomly amplified polymorphic DNA (RAPD) analysis**

The electrophoretic pattern of the PCR products, using four kinds of primers (Table 2), indicated polymorphism. With primer OPAA - 17, two specific PCR products for ‘Yunnan’, about 2.1 kbp and 1.7 kbp, and also two specific PCR products for ‘Toyonoka’, about 1.35 kbp and 0.87 kbp, were recognized (Fig. 1). With primer RP - 15, a specific PCR product for ‘Yunnan’, about 4.3 kbp, and three specific PCR products for ‘Toyonoka’, about 0.87 kbp, 1.35 kbp and 2.50 kbp, were recognized. The lines derived from interspecific hybridization and colchicine treatment had all the PCR products that were specific for both parents. Based on plant morphology and the electrophoretic pattern of PCR - RAPD analysis, these two lines are undoubtedly interspecific hybrids between ‘Toyonoka’ and ‘Yunnan’.

**Back-crossing to parental cultivars and fruit characters of back-crossed progenies**

The harvest of ‘TN13 - 125’ began a little later than the parental cultivars but it was about one month earlier than back-crossed lines ‘TNP - 05’ and ‘TNP - 14’ (Table 3). The mean fruit weight of back-crossed lines is lighter than ‘Pajaro’ and ‘Toyonoka’, but heavier than ‘TN13 - 125’. The Brix of ‘TN13 - 125’ was 7.9% which is lower than ‘Toyonoka’; ‘Pajaro’ had the lowest value, whereas those of ‘TNP - 05’ and ‘TNP - 14’ were equal to their maternal parent ‘TN13 - 125’. Fruit of ‘TNP - 05’ and ‘TNP - 14’ were softer than ‘Pajaro’ but almost as soft as ‘Toyonoka’. The fruit of ‘TN13 - 125’ has a short cone shape with an inferior skin gloss and pale color (Fig. 2 - A). The fruit of ‘TNP - 05’ is conical with excellent skin gloss, shiny scarlet in color, and a superior overall appearance (Fig. 2 - B). The fruit of ‘TNP - 14’ is a short cone with moderately good skin.

![Graph](image)

**Fig. 1. RAPD profiles of F. x ananassa cv. Toyonoka, F. nilgerrensis var. Yunnan and interspecific hybrids (‘TN13’, ‘TN13 - 113’, ‘TN13 - 125’) generated by the primers OPAA - 17 and RP - 15. Amplified products were resolved on 1.5% agarose gel and stained with 10 mg liter −1 ethidium bromide. ‘TN13’ is a interspecific cross line between ‘Toyonoka’ and ‘Yunnan’. ‘TN13 - 113’ and ‘TN13 - 125’ are regenerated lines originated from ‘TN13’ after chromosome doubling treatment. Marker - 1 is λ / Hind III. Marker - 4 is φ / Hae III. White arrows ( ) indicate the bands specific to ‘Toyonoka’. Gray arrows ( ) indicate the bands specific to ‘Yunnan’.

**Table 2. The base sequence of primers that indicated polymorphism on F. x ananassa cv. Toyonoka, F. nilgerrensis var. Yunnan and their hybrids.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Base sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPAA - 17</td>
<td>5’ - GAG</td>
</tr>
<tr>
<td>RP - 8</td>
<td>5’ - CGC</td>
</tr>
<tr>
<td>RP - 12</td>
<td>5’ - GAC</td>
</tr>
<tr>
<td>RP - 15</td>
<td>5’ - GTG</td>
</tr>
</tbody>
</table>

* Operon Technologies.
* Synthesized in NIVOT.
Table 3. Fruit characters of interspecific hybrid line and the back-crossed lines.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Harvest starting date</th>
<th>Mean fruit weight (g)</th>
<th>Brix*</th>
<th>Firmness# (g/3 mm φ)</th>
<th>Acidity (g/100gFW)</th>
<th>Fruit shape</th>
<th>Shape* index</th>
<th>Skin gloss</th>
<th>Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN13 - 125(^{a})</td>
<td>1/21</td>
<td>13.0a</td>
<td>7.9b</td>
<td>-</td>
<td>0.399b</td>
<td>Short conic</td>
<td>1.2</td>
<td>inferior</td>
<td>medium</td>
</tr>
<tr>
<td>TNP - 05(^{a})</td>
<td>2/23</td>
<td>14.1a</td>
<td>7.2b</td>
<td>125.9a</td>
<td>0.418b</td>
<td>Conic</td>
<td>1.3</td>
<td>good</td>
<td>medium</td>
</tr>
<tr>
<td>TNP - 14(^{a})</td>
<td>2/23</td>
<td>13.5a</td>
<td>8.2b</td>
<td>127.8a</td>
<td>0.450b</td>
<td>Short conic</td>
<td>1.3</td>
<td>slightly good</td>
<td>slightly good</td>
</tr>
<tr>
<td>Pajaro</td>
<td>1/16</td>
<td>19.5b</td>
<td>5.8a</td>
<td>233.0b</td>
<td>0.217a</td>
<td>Long conic</td>
<td>1.7</td>
<td>excellent</td>
<td>slightly inferior</td>
</tr>
<tr>
<td>Toyonoka</td>
<td>1/12</td>
<td>16.9b</td>
<td>10.4c</td>
<td>126.0a</td>
<td>0.532b</td>
<td>Conic</td>
<td>1.3</td>
<td>good</td>
<td>good</td>
</tr>
</tbody>
</table>

*10 plants were used for the assessment.
* Brix of the fruit juice was determined as the sugar concentration.
# Pressure of the disk plunger diameter 3 mm into the flesh was determined as the firmness of fruit.
* Shape index = fruit length/fruit width.
\(^{a}\) 'TN13 - 125' was a line originated from 'TN13' after chromosome doubling treatment.
\(^{u}\) 'TNP - 05' and 'TNP - 14' were back-crossing lines of 'TN13 - 125' × 'Pajaro'.
\(^{i}\) Means indicated by different letters within a column are significantly different at 0.01 level by LSD.

Table 4. Comparison of the main aroma components of interspecific hybrids.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Relative values of peaks(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Components*</td>
</tr>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>TN13 - 125(^{a})</td>
<td>23.0</td>
</tr>
<tr>
<td>TNP - 05(^{a})</td>
<td>5.8</td>
</tr>
<tr>
<td>TNP - 14(^{a})</td>
<td>5.9</td>
</tr>
<tr>
<td>Toyonoka</td>
<td>1.9</td>
</tr>
<tr>
<td>Yunnan</td>
<td>19.5</td>
</tr>
</tbody>
</table>

* I : Ethyl acetate, II : Methyl n - butyrate, III : Etyl n - butyrate, IV : Butyl acetate, V : Formic acid, VI : Caproic acid, VII : 2, 5 - Dimethyl 4 - hydroxy (2H) furanone.
\(^{a}\) 'TN13 - 125' was a line originated from 'TN13' after chromosome doubling treatment.
\(^{a}\) 'TNP - 05' and 'TNP - 14' were back-crossed lines of 'TN13 - 125' × 'Pajaro'.

Fig. 2. Fruit appearance of the interspecific hybrid 'TN13 - 125' (A), and the back-crossed lines 'TNP - 05' (B) and 'TNP - 14' (C).

The highest Brix among the back-crossed lines with a better taste than 'TN13 - 125' and 'Pajaro'.

Many peaks were detected in the aromatic volatile components analysis by GC-MS (Table 4). Esters (ethyl acetate, ethyl butyrate, methyl butyrate, butyl acetate, etc.) were detected in the early retention times. Alcohols (hexanol, ethanol etc.), organic acids (butanoic acid, hexanoic acid, caproic acid, formic acid, etc.) appeared subsequently, and a furanone (2,5 - dimethyl 4 - hydroxy (2H) furanone) was detected finally. The relative value of the peak area in TIC of ethyl acetate was lower than that of ethyl n - butyrate in 'Toyonoka'. Nevertheless, the relative value of ethyl acetate was higher than that of ethyl n - butyrate in 'Yunnan', whose specific aroma was similar to peach. The aromatic volatile constituent of 'TN13 - 125', in which the relative value of ethyl acetate was higher than that of ethyl n - butyrate, is similar to 'Yunnan'. The relative value of ethyl acetate of the back-crossed lines 'TNP - 05' and 'TNP - 14' was higher than 'Toyonoka'. The relative value of ethyl n - butyrate
of 'TN13–125' and back-crossed lines was lower than 'Toyonoka'.

Discussion

Various polyploid species (diploid, tetraploid, hexaploid and octoploid) of genus *Fragaria* are distributed in the world, including many wild strawberries, such as the diploids *F. daumiana*, *F. iunumae*, *F. nilgerrensis*, *F. nipponica*, *F. nubicola*, *F. vesca*, *F. viridis*, *F. yezoensis*, and the tetraploids *F. moupinensis*, *F. orientaris*, which exist in Asia. Although the wild species native to Asia are not utilized for commercial purposes, probably they have adapted to the Asian climate and have some local resistances against the diseases and insects. Among them, *F. nilgerrensis*, which is native to the southwestern China, is recognized its specific aroma, similar to peach. It is desirable to utilize these characters for strawberry breeding.

Although cross-incompatibility has been recognized between the wild species and the cultivars in the genus *Fragaria*, it was reported that *F. vesca* has a comparatively high compatibility to many *Fragaria* species (Yarnel, 1931; Evans and Jones, 1967; Hancock and Luby, 1993). Some decaploid hybrids, which have been produced by crossing cultivars to *F. vesca*, have been released, such as 'Spadeka' (Bauer, 1979), 'Annelie' and 'Sara' (Trajkovski, 1996). However, cross compatibility of *F. nilgerrensis* to other species is comparatively low and the hybrid seedlings are dwarfish and weak in vigor (Hancock and Luby, 1993). In this study, some fasciculate plants having very small leaves were observed, although more than half of the interspecific hybrid seedlings were intermediate between both parents. These intermediate plants bloomed but they were completely sterile because they are considered to be pentaploid interspecific hybrids. Therefore, the chromosome doubling treatment by colchicine was attempted to restore the fertility.

There are many reports on the chromosome doubling by treating seeds or seedlings with colchicine in strawberry (Dermen and Darrow, 1938; Hull, 1960; Sébastiampillai and Jones, 1976; Morishita et al., 1996). However, these methods are not suitable as a chromosome doubling method of the pentaploid, which do not set any seed because of their complete sterility. The method of dipping the organs such as shoot apex in a colchicine solution was reported suitable for sterile materials (Niemierzowicz-Szczyttys et al., 1986; Morishita et al., 1996). In this study, colchicine treatment to the shoot tips in *vitro* according to Morishita et al., (1996) resulted in 71% of the regenerated plants set fruit, which confirms that the chromosome doubling treatment was effective. The interspecific hybrid line 'TN13–125' was selected and confirmed to be a decaploid by ploidy analysis using flow cytometer (unpublished).

Morishita et al. (1996) produced decaploid lines through back-crossing the interspecific hybrid between an octoploid cultivar and tetraploid *F. vesca*, to a cultivar. However, the pollen fertility of the decaploid hybrids was very poor, although the malformed fruits had an aroma similar to that of *F. vesca*. Inhibition of normal chromosome disjunction, caused by partial genomic homology of the octoploid cultivar and *F. vesca*, may have affected the fertility of the decaploid. Therefore, it might be possible to produce fertile interspecific hybrids with the wild species remote from the cultivated strawberry in the genetic relationship. Li et al. (1996) and Lei et al. (1999) estimated the relationship among the cultivated and wild strawberries, including native species in Asia by the PCR–RAPD. They reported that *F. vesca* was the closest to the cultivated strawberry among the diploid species, whereas *F. iunumae* and *F. nipponica* were more distant and the relationship of *F. nilgerrensis* with commercial cultivars was farthest. Arulsekar and Brinshurst (1983) reported that *F. nilgerrensis* was reproductively isolated from all other strawberry species on the basis of electrophoretic analysis using glucose-6-phosphate isomerase. Therefore, it was considered that the high fertility of the colchicine-doubled interspecific hybrid, was based on the low genomic homology between the cultivated strawberry and *F. nilgerrensis*.

'TN13–125', the interspecific hybrid between *F. x ananassa* cv. Toyonoka and *F. nilgerrensis* var. Yunnan, may have practical value as a cultivar, because fruit yield and quality are close to the *F. x ananassa* cultivars, and moreover it has a specific peach–like aroma. However, the shipping quality and appearance are inferior because the fruit is very soft and pale in color. We consider 'TN13–125' to be useful for processing and further breeding because it is possible to transmit the special aroma from the wild strawberry by back-crossing this hybrid.

Literature Cited


種間交雑（Fragaria x ananassa × F. nilgerrensis）による新芳香性イチゴ系統の育成

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摘　要

F. nilgerrensis を始めとするアジアに原生する 2 倍性野生種は、栽培イチゴとの交雑和性について調査されているが、育種素材としては利用されていない。そこで栽培イチゴの遺伝的変異選抜を目的として、2 倍性野生種 F. nilgerrensis から新しい香気特性を導入した育種素材系の育成を試みた。栽培品種 'とよのか' に F. nilgerrensis '雲南' を交配して得られた種間交雑系統は全て不稔であった。コルヒチンを添加した培地で培養する試験管内染色体倍加処理を行うことにより、稔性の回復した系統 'TN13－125' が得られた。RAPDによる雑種性検定の結果、'TN13－125' は両親の特異的バンドを併せ持つことから種間雑種であることが確認された。'TN13－125' の形態は 'とよのか' に類似したが、葉柄および花柄に毛茸が多いなど F. nilgerrensis 由来の特徴がみられた。'TN13－125' の果実は、平均一果重、Brix、酸度において栽培イチゴと同水準であったが、果実硬度が低く、果皮の着色が劣った。種間雑種系統の香気成分組成は F. nilgerrensis と類似し、ethyl acetateが豊富であり、モモ様の香りを持つことが特徴的であった。さらに、'TN13－125' は寒しき交雑により栽培品種に芳香性を導入することが可能であることから、芳香性育種素材としても有用であると考えられた。

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