Variation in Ploidy Level and Morphological Traits in the Progeny of the Triploid Apple Variety Jonagold

PING HE*, LINGUANG LI*, LAIIANG CHENG, HAIBO WANG and YUANSHENG CHANG

Shandong Institute of Pomology, Taian, Shandong, P.R. China
*Corresponding author: heping024@163.com, llg6536@163.com

Abstract


Variation with respect to both ploidy level and morphology was characterized for a set of 690 seedlings of the triploid apple variety Jonagold, of which 481 were obtained via in vitro culture of mature embryos, and 209 via conventional germination. Their ploidy level was determined by a combination of flow cytometry and root tip chromosome counting. The assessed morphological traits were leaf length, width and shape, stomatal density, guard cell length and chloroplast width and number. A total of 452 seedlings were aneuploid, 225 diploid, nine triploid and four tetraploid. All four tetraploid seedlings were derived by in vitro culture. When the triploid seedlings were genotyped at the S-locus and at selected microsatellite loci, we found that the Jonagold stigma was compatible with pollen which shared some of the maternal parent S-locus alleles.

Keywords: flow cytometry; karyotypic analysis; S-alleles; SSR markers

The majority of commercial apple (Malus domestica Borkh.) varieties are diploid (2n = 2x = 34), but triploid (2n = 3x = 51) forms are also known (Lespinasse et al. 1976); the latter types tend to be more vigorous and to form larger fruits. Triploid varieties are largely self-sterile, but typically produce a small number of viable gametes carrying either n = 17, 34 or 51. Poly-ploid variants of many plant species typically differ from the diploid ones at the level of morphology, so that morphology can be used to predict the ploidy level: for example, leaf shape, stomatal size and the number of chloroplasts harboured by guard cells have all been used as predictors of ploidy level in alfalfa (Bingham 1968), clover (Najcevska & Speckmann 1968) and sugar beet (Moehizuki & Sueoka 1955). The most reliable method for establishing the ploidy level is, however, to perform a mitotic chromosome count in either the root tip or the shoot tip, although this is a rather labour-intensive and time-consuming procedure (Michaelson et al. 1991). An attractive alternative is to exploit the capacity of flow cytometry to quantify nuclear DNA content in non-dividing cells (Riccardi & Nicoletti 2006).

As many as 50% of angiosperm species are self-incompatible. Rosaceae species achieve this property via gametophytic self-incompatibility (GSI), in which the pollen phenotype is determined by its haploid genotype. The genetic basis of GSI in apple is based on the allelic constitution of the S-locus, which harbours at least two genes, one encoding an S-RNase (SLF) and the other an F-box protein (SFB). These genes control pistil and pollen specificity, respectively (Minamikawa et al. 2014).

Here, the ploidy level of seedlings of the triploid apple cultivar Jonagold was determined. Some of the seeds, derived by open-pollination, were germinated conventionally, while others were used to provide mature embryos as the explant for in vitro culture. The seedlings were subsequently characterized with respect to a panel of morphological traits, namely leaf length and shape, stomatal density, guard cell length and chloroplast width and number. A small number of triploid seedlings was genotyped with respect to the S-locus and a set of genome-wide microsatellite (SSR; simple sequence repeat) loci. The objectives were to (1) determine whether the in vitro culture of
mature embryos represented an effective means of multiplying germplasm and obtaining novel germplasm, (2) identify morphological traits associated with ploidy level to provide a simple method for ploidy selection, (3) evaluate the variation (if any) in the S-locus genotype between the maternal donor plant and its triploid progeny and (4) provide polymorphisms for the Jonagold seedlings from in vitro mature embryo culture which had the same ploidy level and S-alleles as its maternal material.

MATERIAL AND METHODS

Plant material. Open-pollinated fruits were harvested from a Jonagold tree grown in an orchard where most of the trees were Jonagold. In all, 2000 seeds were obtained; the embryos were aseptically removed from 843 plump and 157 shrivelled seeds, and subjected to in vitro culture, while the remainder (809 plump and 191 shrivelled seeds) was subsequently after-ripening stratified and sown.

In vitro culture of mature embryos. The seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 30 s, then in 0.1% (w/v) aqueous mercuric chloride for 10 min, and were finally rinsed four times in sterile distilled water. After stripping off the testa, the embryos were excised and placed on Murashige and Skoog (1962) medium (MS medium) solidified with 6 g/l agar and supplemented with 3% (w/v) sucrose, 83 mM activated carbon, 400 mg/l lactalbumin hydrolysate, 2.2 μM 6-benzyladenine (BA), 1.2 μM α-naphthaleneacetic acid and 0.57 mM ascorbic acid. Each 100 ml bottle, containing 40 ml of the medium, housed seven embryos, and was exposed to a constant temperature of 25 ± 2°C with a 14 h photoperiod provided by white fluorescent tubes providing 50 μmol m⁻²/s of light. The regeneration percentage after 15 days was calculated from the number of regenerants/the number of embryo explants, while the mortality percentage was calculated after 30 days from the number of dead regenerants/the number of initial regenerants.

Multiplication, rooting and transplantation. To vegetatively multiply each regenerant, 1 cm stem sections were cut and set vertically in MS medium containing 1.1 μM 6-BA and 0.1 mg/l indole-3-butyric acid to encourage root growth. Rooted plantlets were planted into trays containing a growing medium, maintained at 15–24°C under a 12 h photoperiod and watered regularly.

Seed common stratification and germination. The seeds were surface-sterilized by immersion in 10% v/v commercial bleach (4.5% NaOCl) for 5 min, then rinsed three times in sterile distilled water. The seed was then mixed with three parts of autoclaved white sand, and left to stratify in sterile boxes covered with polyethylene film at 0–15°C room. After about 12 weeks, when the radicle had emerged from > 50% of the seed, the seedlings were planted into a growing medium and maintained in a greenhouse delivering 10–24°C and a 12 h photoperiod. Percentage of germination was calculated after further 30 days, along with percentage of mortality.

Morphological traits. Fifty leaves were selected, the third or fifth leaf from the every plantlet bottom. Measurements were taken of leaf length and width, and a leaf shape index was calculated. Stomatal density of 125 per μm² was made statistic and transformed into the quantity of 1 mm², by choosing 20 pieces per shoot from each leaf. Stomatal size was estimated using an ocular measuring tiny ruler and the number of chloroplasts per guard cell was counted.

Root-tip chromosome counts. Chromosome numbers were determined from root tips prepared following a protocol modified from that described by Kondo and Lavara (1984). Excised root tips were incubated for ca. 8 h in p-dichlorobenzene saturated water solution at 18°C, then fixed for 15 min in 3:1 95% ethanol/glacial acetic acid at room temperature. Fixed root tips were hydrolyzed in 5M HCL for 3 min at 20°C, rinsed three times in distilled water, stained in carbol fuchsin for 4 min and finally squashed under a micro cover glass. A minimum of 20 well-spread metaphase plates per root tip were sampled for chromosome counting.

Leaf tissue flow cytometry. Leaf tissue was chopped with a scalpel, then homogenized in nuclear isolation buffer [10 mM MgSO₄, 7 H₂O, 50 mM KCl, 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 3 mM dithiothreitol (Sigma, St. Louis, USA), 100 μm/l propidium iodide, 0.25% (v/v) Triton X-100, pH 8.0] After passing the homogenate through a 30 μm nylon mesh, the material was centrifuged (15 000 rpm, 30 s), the supernatant was discarded, and the pellet was treated with 1 μl 1 mg/ml RNase A (dH₂O) for a final concentration of 1.25 μg/ml for 15 min at 37°C. Nuclear DNA contents were measured with a
Beckman Coulter EPICS Altra flow cytometer (www.beckmancoulter.com/). Nuclei extracted from leaves of the Golden Delicious variety were treated as the internal standard, and a minimum of 10^4 nuclei per sample was analysed. Detection followed excitation with an argon laser (488 nm). The mean fluorescence of each seedling G1 phase peak was divided by the fluorescence reading of the internal control. Each sample was run in triplicate. An estimate of the relative nuclear DNA content was derived from the ratio between the sample G1 and the internal standard G1.

**PCR-based genotyping.** DNA was extracted from young leaves of the triploid in vitro mature embryo culture-derived progenies using the method given by Koller et al. (2000), and diluted to 50 ng/μl. For S-locus (on LG17) genotyping, each 20 μl PCR comprised 1× PCR buffer (Promega, Shanghai, P.R. China), 1.75 mM MgCl$_2$, 200 μM each dNTP, 1 μM each primer (sequences given in Table 1), 0.6 U Taq DNA polymerase (Promega, Shanghai, China) and 100 ng template. The reactions were first denatured (94°C/3 min), then subjected to 30 cycles of 94°C/30 s, 60°C/60 s and 72°C/60 s, and finally to 72°C/10 min. The amplicons were electrophoresed through a 1.5% TBE/agarose gel and visualized by EtBr staining. For the five SSR assays (CH01b12 on LG4, LG12 or LG13, CH01e12 on LG8, CH01f02 on LG12, CH01f09 on LG8 and CH02d12 on LG11), the PCR volume was reduced to 15 μl, containing 50 ng genomic DNA, 10 mM Tris-HCl pH 9.0, 0.2 mM each dNTP, 1.5 mM MgCl$_2$, 50 mM KCl, 0.2 μM each primer (Table 1) and 1 U Taq polymerase (Tianwei, Beijing, P.R. China). The amplification profile included an initial denaturation (94°C/3 min), followed by four cycles of 94°C/30 s, 65°C/60 s, 72°C/60 s, with the annealing temperature falling by 1°C per cycle; further 30 cycles of 94°C/30 s, 60°C/60 s, 72°C/60 s were then given, and the reaction was completed by a final extension of 72°C/5 min. The amplicons were denatured by the addition of 1 volume of denaturing gel loading buffer and holding at 94°C for 5 min; they were then electrophoresed through a 6% denaturing polyacrylamide gel. Fragments were visualized by silver staining, following Bassam et al. (1991).

**Experimental design and data analysis.** All data were subjected to the analysis of variance for a randomized complete block design prepared and analysed by the SPSS program (version 10.0, 1999). The data were expressed as the mean and standard deviation (SD).

**RESULTS**

**Gaining seedlings from in vitro culture and common stratification and germination.** A comparison of the recovery rate of seedlings following in vitro culture or common stratification and germination is shown in Table 2. For the former, 632 out of the 843 plump seeds (73.9%), but only 89 out of the 157 shrivelled seeds (56.7%) developed into a regenerant. The equivalent frequencies following common stratification and germination were 32.9% (266/809) and 17.8% (34/191). Not all of the seedlings/regenerants were viable: the mortality rate of plants recovered from shrivelled seeds was higher than that of plants recovered from plump seeds. In all, 690 viable plants were obtained, of which 481 were the outcomes of in vitro culture and 209 of common stratification and germination, suggesting a twofold efficiency advantage of the tissue culture approach.

**Variation in mitotic chromosome number in the Jonagold progeny.** The euploid seedlings carried either 2n = 2x = 34, 2n = 3x = 51 or 2n = 4x = 68, while a large number of aneuploids was also detected (Figure 1, Table 3). Of the 690 seedlings, four (0.6%) were not euploid, and the chromosome number ranged from 2x = 28 to 4x = 63. The most frequent aneuploid was CH02D12, which was found in 14% of the samples. The chromosome number of the remaining 686 euploid and aneuploid seedlings was 34, 51, or 68.

**Table 1. Sequences of PCR primers in Jonagold**

<table>
<thead>
<tr>
<th>SSR locus name</th>
<th>Primer sequence 5’–3’</th>
<th>S-allele name</th>
<th>Primer name</th>
<th>Primer sequence 5’–3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH01B12</td>
<td>cgcatctgcatgttgaat</td>
<td>S1</td>
<td>FTC168</td>
<td>atattgtaaggacgccatatcat</td>
</tr>
<tr>
<td></td>
<td>cgg tgtgaccctttagtga</td>
<td></td>
<td>FTC169</td>
<td>ggttgtgtgagggagagcaca</td>
</tr>
<tr>
<td>CH01E12</td>
<td>aaattgtaaggacagggc</td>
<td>S2</td>
<td>OWB122</td>
<td>gttcaaagctagttctgc</td>
</tr>
<tr>
<td></td>
<td>tttcaattcactagcgt</td>
<td></td>
<td>OWB123</td>
<td>gttggtctctcattcatg</td>
</tr>
<tr>
<td>CH01F02</td>
<td>accaattagacaggttggag</td>
<td>S3</td>
<td>FTC177</td>
<td>caaagtttaaaacetccac</td>
</tr>
<tr>
<td></td>
<td>ctgtgttctgccttcagc</td>
<td></td>
<td>FTC226</td>
<td>tatagtaacactacgctt</td>
</tr>
<tr>
<td>CH01F09</td>
<td>atgatatcataagttggtgattttggttcttcagc</td>
<td>S5</td>
<td>FTC10</td>
<td>caaagtttagctagctgctttcc</td>
</tr>
<tr>
<td></td>
<td>gcgctgctgctactacagc</td>
<td></td>
<td>FTC111</td>
<td>taatatagctatcagctg</td>
</tr>
<tr>
<td>CH02D12</td>
<td>aacccagtggctgatgaccatc</td>
<td>S9</td>
<td>FTC154</td>
<td>cagcgggcttgccacactt</td>
</tr>
<tr>
<td></td>
<td>cttggtgtaagctgagttg</td>
<td></td>
<td>FTC155</td>
<td>cgggtcgctgagacttgg</td>
</tr>
</tbody>
</table>

---


Original Paper

https://doi.org/10.17221/201/2016-CJGPB
were tetraploid, nine (1.3%) triploid, 225 (32.6%) diploid and 452 (65.5%) aneuploid. Although euploid individuals were recovered following both *in vitro* culture and conventional germination, the representation of diploid individuals was fivefold greater in the former than in the latter progeny set. All four tetraploids recovered emerged from *in vitro* culture.

**Variation in nuclear DNA content among the Jonagold progeny.** The relative nuclear DNA content of young leaf cells was tested using flow cytometry for a set of 100 karyotyped seedlings: of these, 13 were tetraploid/triploid, 30 were diploid and 57 were aneuploid. The test was based on an internal standard provided by the G1 phase peak value of an established ploidy species within the cultivar Golden Delicious, which is diploid and the nuclear DNA content of Golden Delicious was 47.2. Four representative outcomes are illustrated in Figure 2: here,

<table>
<thead>
<tr>
<th>Culture method</th>
<th>Source of plantlet</th>
<th>No. of cultured seed</th>
<th>No. of plantlet</th>
<th>Plantlet rate (%)</th>
<th>No. of death plantlet</th>
<th>Death rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> culture</td>
<td>plump-seeds</td>
<td>843</td>
<td>623</td>
<td>73.9</td>
<td>187</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>shriveled-seeds</td>
<td>157</td>
<td>89</td>
<td>56.7</td>
<td>44</td>
<td>49.4</td>
</tr>
<tr>
<td>Common stratification and germination</td>
<td>plump-seeds</td>
<td>809</td>
<td>266</td>
<td>32.9</td>
<td>74</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>shriveled-seeds</td>
<td>191</td>
<td>34</td>
<td>17.8</td>
<td>17</td>
<td>50</td>
</tr>
</tbody>
</table>

**Table 2. The production and mortality of apple regenerants/seedlings following either *in vitro* culture or conventional germination**

Figure 1. Variation of chromosome number in different ploidy levels of Jonagold seedlings: $2n = 34$ (a), $2n = 51$ (b), $2n = 68$ (c), $2n = 40$ (d); bar: 10 µm

**Figure 2.** Relative nuclear DNA contents inferred from the flow cytometry-based analysis of nuclei isolated from young leaves of Jonagold seedlings: diploid (a), triploid (b), tetraploid (c), aneuploid (d)

**Table 3. Ploidy level variation in Jonagold progeny based on karyotypic analysis**

<table>
<thead>
<tr>
<th>Source of plantlet</th>
<th>No. of plantlet</th>
<th>No. of the same ploidy level for plantlets</th>
<th>diploid</th>
<th>triploid</th>
<th>tetraploid</th>
<th>aneuploid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vitro</em> and plump-seeds</td>
<td>436</td>
<td>179</td>
<td>5</td>
<td>3</td>
<td>249</td>
<td></td>
</tr>
<tr>
<td><em>In vitro</em> and shriveled-seeds</td>
<td>45</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Common stratification and germination and plump-seeds</td>
<td>192</td>
<td>29</td>
<td>2</td>
<td></td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>Common stratification and germination and shriveled-seeds</td>
<td>17</td>
<td>5</td>
<td></td>
<td></td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
the areas marked C, D and E defined the presence of diploid, triploid and tetraploid nuclei, respectively. The DNA content of diploid nuclei was estimated to be 50.9 ± 0.08, of triploid ones 72.8 ± 0.12 and of tetraploid ones 103.8 ± 0.11. The aneuploid nuclei were identified as those harbouring an intermediate DNA content (67.2 ± 0.21) (Table 4).

### Variation in leaf shape and number of stomata among the Jonagold progeny

Variation in leaf shape and in the number of stomata present in the guard cells was characterized for 39 seedlings (13 euploids, 13 diploids and 13 aneuploids). The euploid individuals formed a larger and thicker leaf lamina, which was more intensely green and tended to be rounder than in the diploids (Figure 3a, b); their mean leaf length was 7.12 cm, width 5.04 cm and shape index 1.51 (Table 5). In contrast, the aneuploid seedlings produced small, thin leaves (Figure 3d), which were on average 3 cm shorter and 3 cm narrower than the euploid ones, and had a shape index > 2 (Table 5). The leaves of the diploids were intermediate (Figure 3c).

Stomatal density ranged from 19.7 in the polyploids to 38.5 in the aneuploids (Table 6), while the length and width of the guard cells were 26.3 and 32.6 cm (euploids), 14.8 and 20.5 cm (diploids), and 11.0 and 17.0 cm (aneuploids). Chloroplast numbers varied in the same direction as the guard cell size. Stomatal density in the euploid seedlings was relatively low, although their size was larger; the number of chloroplasts harboured by guard cells was greater than that of either diploids or aneuploids (Figure 4).

### S-locus and SSR genotyping

A sample of seven triploid seedlings was genotyped with respect to the S-locus. The parental Jonagold S-locus genotype was defined by the amplification of a fragment when the DNA template was primed by pairs S2, S3 and S9.
and this genotype was shared by progenies No. 1, 5 and 7 (lanes 2, 4 and 8 in Figure 5). The templates prepared from the other four seedlings (No. 2, 3, 4 and 6), when amplified with primers FTC168 and FTC169 (S1 allele), each seedling provided a fragment which was not amplified in Jonagold. The S2 profiles of progenies No. 3 and 6 lacked the fragment amplified in Jonagold. Similarly progenies No. 2 and 4 lacked the Jonagold’ S3 fragment. All seven seedlings, along with Jonagold, provided a fragment when the template was amplified with primers FTC154 and FTC155 (S9 allele). When seedlings No. 3, 5 and 7 were subsequently genotyped at the five selected SSR loci, a polymorphism was identified in the CH01F09 locus, but not in any of the other four ones (Figure 6).

DISCUSSION

Polyploidy has played a major role in the evolution of the angiosperms (Soltis et al. 2003). It has long been understood that higher ploidy levels can induce an increase to the overall size of the plant as well as to its constituent organs. A relationship has been established in certain species between the ploidy level and both the size of the stomata and the number of chloroplasts harboured by guard cells (Rensing 2014; Soltis et al. 2015). Here, triploidy and tetraploidy in apple were shown to be associated with the formation of larger, thicker leaves which are darker green than those formed by diploids; they also

Figure 4. Variation of stomatal size in Jonagold seedlings with different ploidy levels: tetraploid (a), triploid (b), diploid (c), aneuploid (d); bar: 50 µm

Figure 5. Polymorphism in the S-locus based on amplicons using primers for alleles S1 (a), S2 (b), S3 (c), S5 (d) and S9 (e); M – DNA weight marker (100 bp ladder); lanes: 1 – Jonagold, 2–8 – triploid seedlings No. 1 through No. 7; 9 – control without DNA

Figure 6. Polymorphism among selected Jonagold seedlings in the SSR locus CH01f09; lanes: 1 – Jonagold; 2 – seedling No. 3; 3 – seedling No. 5; 4 – seedling No. 7
develop a rounder leaf, a smaller leaf shape index, smaller stomata and larger guard cells. Aneuploid plants, however, tended to be weak.

Both triploid and tetraploid offspring can be generated from a triploid mother plant by pollinating with a diploid donor. The number of viable seeds produced by these crosses tends to be limited, with most of the progeny being aneuploid. In a survey of 884 progenies of a triploid mother plant harvested in an orchard containing many diploid trees, only eight proved to be tetraploid (Laubscher & Hurter 1960); similarly, Einset (1952) showed that the open-pollinated progeny of triploids included a very low (0.6%) proportion of haploids and only a few euploids (1.8% diploids, 1.8% triploids and 3.0% tetraploids), with an overwhelming proportion (92.8%) of progeny being aneuploid. Later research by the same author suggested that not all triploids respond in this way, as some are able to produce a higher proportion of polyploids (Einset 1952). Here, the proportion of polyploid (triploid and tetraploid) progeny obtained from the triploid parent was just 1.9%, but the proportion of diploids was as high as 32.6%.

The chromosome number of the offspring of a plant characterized by non-diploid-like meiosis is unpredictable. When one parent of an apple cross is triploid, the female gamete chromosome complement can be highly variable, including the three possible complete complements of \( n = 17 \), 34 or 51. The fusion of euploid gametes with a haploid gamete \( (n = 17) \) will produce a diploid, triploid and tetraploid hybrid. Other possible routes to polyploidy include the formation of unreduced pollen \( (n = 2x = 34) \) or apomixis in the mother plant. The former route has been observed in a range of fruit species (Sanford 1983; Passvetaeva 1985; Jackson and Chen 2010; Jiao et al. 2011), while apomixis is known to occur in 300 plant species at least (Levin 2002). Among the seven triploid progenies of Jonagold, three shared the same \( S \)-locus genotype as Jonagold and existed different in codomain. The implication is that for these progenies, the maternal stigma supported the growth of pollen carrying some of the same \( S \)-locus alleles. The genotypic analysis of the \( S \)-locus and the selected SSR loci suggested that the triploid progeny must have originated from the fusion of an \( n = 2x = 34 \) female gamete with an \( n = x = 17 \) male gamete, or vice versa, rather than via apomixis.

**Acknowledgments.** The authors thank the Changli Institute of Pomology (Hebei Academy of Agricultural and Forestry Sciences of China) for providing access to flow cytometry. We acknowledge the financial support of the Fruit Germplasm Resources Collection and New Germplasm Innovation (Lu No. 2014-96), National Science and Technology Plans Program (2013BAD02B01) and China Agriculture Research System (CARS-27).

**References**


Received for publication December 27, 2016

Accepted after corrections February 9, 2018

Published online April 23, 2018