Aneuploids without Bulbils Segregated in F₁ Hybrids Derived from Triploid Lilium lancifolium and Diploid L. leichtlinii Crosses

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Lilium leichtlinii (2n = 2x = 24) is the most commercially cultivated edible lily in Japan, although viral and fungal diseases are severe problems. Triploid L. lancifolium (2n = 3x = 36), the bulbs of which are also edible, adapts well to the climate in Japan, and feral triploid L. lancifolium plants are often seen along roadsides and in croplands. Thus, triploid L. lancifolium is an important genetic resource for edible lilies, but it develops many bulbils (aerial bulbs) on leaf axils. Bulbil formation is undesirable for edible lilies because bulbils can compete for photosynthate with bulbs, which limits the use of triploid L. lancifolium in commercial production. Edible lily cultivars have been bred by intraspecific crosses within L. leichtlinii, although interspecific hybridization, which increases the level of genetic variation, is the major technique used to breed floricultural lily cultivars. In this study, interspecific-hybrid plants were developed by crossing triploid L. lancifolium and L. leichtlinii, and their characteristics, including bulbil formation ability, were evaluated. The crosses of triploid L. lancifolium × L. leichtlinii developed aneuploid plants, of which the chromosome number ranged from 26 to 34. Bulbil formation ability was continuously distributed in the F₁ offspring, although 86% of F₁ plants did not develop bulbils, indicating that many aneuploid plants without bulbils are developed by this cross combination. The plants harboring abnormal anthers were segregated in the hybrids. In addition, pollen germination in the F₁ hybrids was lower than 20%, and 85% of the hybrids exhibited no pollen germination. However, abnormal anther morphology and low pollen fertility should not be major problems for edible lily production because bulbs are the main commercial output. These results indicate that interspecific hybridization between triploid L. lancifolium and L. leichtlinii has the potential to develop elite edible lily cultivars.

Key Words: bulbil formation ability, chromosome number, edible lily, interspecific hybridization, pollen sterility.

Introduction

The genus Lilium comprises > 90 species and is distributed in the temperate zone of the Northern Hemisphere (Asano, 1989). Lilium species are classified into sections (Comber, 1949; Smyth et al., 1989). Species that belong to the same section generally have high interspecific crossing ability, and hybrid lilies used as floricultural crops have been developed primarily by crosses between species belonging to the same section.

Received; July 10, 2015. Accepted; October 26, 2015.
First Published Online in J-STAGE on December 10, 2015.
Part of this paper was presented at the 2014 Spring Meeting of the Japanese Society for Horticultural Science.
This work was partially supported by a Grant-in-Aid for Scientific Research (no. 24380014) from the Japan Society for the Promotion of Science.
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belongs to the same section as *L. leichlinii* (the Sinomartagon section). *Lilium lancifolium* is a polyplloid species complex consisting of diploid (*2n = 2x = 24*) and triploid (*2n = 3x = 36*) forms, although most lily species are cytologically diploid (*2n = 2x = 24*; Noda, 1986). On the basis of classical cytogenetic (karyotype) analyses, Noda (1966, 1986) hypothesized that the triploid form is an allotriploid consisting of the *L. lanciforium* and *L. leichlinii* genomes. However, recent studies using genomic in situ hybridization and the allele compositions of molecular markers indicate that the triploid form is an autotriploid originating from the diploid form (Chung et al., 2015; Sultana et al., 2010). *Lilium lancifolium* develops many bulbils (aerial bulbs) on the leaf axils of stems, and the detached bulbils germinate and grow into mature plants; the triploid form propagates asexually mainly via the bulbils. The original habitats of *L. lancifolium* are thought to be relatively restricted areas, including the western to southern coastal areas and islands of the Korean Peninsula, Jeju Island (South Korea), and the Tsushima Islands (Japan), where both diploid and triploid forms exist, while the triploid form has a wider distribution in East Asia, including China, inland areas of the Korean Peninsula, the main islands of Japan, and the Russian Far East (Chung et al., 2015; Kim et al., 2006; Noda, 1986). Triploid *L. lancifolium* has been cultivated and transported due to human activities, and has subsequently naturalized in many habitats by escaping from cultivation (Chung et al., 2015; Noda, 1986). Indeed, feral triploid *L. lancifolium* plants are often seen along roadsides and in croplands, parks, and gardens in Japan. The wide distribution of this species indicates that the triploid *L. lancifolium* has a robust constitution and adapts well to the climate in East Asia; thus, triploid *L. lancifolium* is an attractive genetic resource for edible lily breeding. However, *L. lancifolium* develops many bulbils, whereas *L. leichlinii* rarely produces bulbils (Noda, 1966). Bulbil formation ability is an undesirable trait for edible lilies because bulbils can compete for photosynthate with bulbs and prevent bulb enlargement. Thus, triploid *L. lancifolium* is not presently used for commercial edible lily production.

Cultivars of edible lilies have been bred by intraspecific crosses within *L. leichlinii* var. *maximowiczii* (Miki, 1988), although interspecific hybridization has not been applied to edible lily breeding. Interspecific hybridization, which increases the level of genetic variation, is the major technique used to breed floricultural lily cultivars (Lim et al., 2008). *Lilium lancifolium* was involved in the breeding of Asiatic hybrid lilies; e.g., *L. lancifolium* was used to develop ‘Enchantment’ (one of the mid-century hybrids), which has a vigorous habit, tall stems, and bulbils, and has been used as a parent for many Asiatic hybrid lily cultivars (Merae, 1998; Shimizu, 1987). Noda (1986) showed that crosses of diploid *L. lancifolium × L. leichlinii* and triploid *L. lancifolium × L. leichlinii* produced hybrid seeds, and that the latter cross combination developed aneuploid plants. These results prompted us to apply interspecific hybridization between triploid *L. lancifolium* and *L. leichlinii* in edible lily breeding. If robust *F*₁ plants with few bulbils are segregated, these can be candidates for new edible lily cultivars. Before using *L. lancifolium* for edible lily breeding, it is necessary to evaluate the genetic transmission of bulbil formation ability in its offspring. In this study, interspecific hybrids were produced by artificial hybridization between triploid *L. lancifolium* and *L. leichlinii*. Chromosome number, ability to produce bulbils, pollen fertility, and other phenotypic characteristics in the *F*₁ hybrid plants were evaluated and compared with those in interspecific hybrids from diploid *L. lancifolium* and *L. leichlinii* crosses. We then discuss the possibility of selecting for elite edible lily cultivars with few bulbils.

**Materials and Methods**

*Plant materials*

*Lilium lancifolium* var. *flaviflorum* (*2n = 2x = 24*, hereafter, diploid *L. lancifolium*), *L. lancifolium* var. *flaviflorum* ‘Pure Gold’ (*2n = 2x = 24*, hereafter, diploid *L. lancifolium* ‘Pure Gold’), triploid *L. lancifolium* (*2n = 3x = 36*), *L. leichlinii* var. *maximowiczii* cv. ‘Hakugin’ (*2n = 2x = 24*, hereafter, *L. leichlinii*), and *L. leichlinii* (it is called Kihiratoyuri in Japan, *2n = 2x = 24*, hereafter, *L. leichlinii* Kihiratoyuri) were used. Triploid *L. lancifolium* plants were collected in Hokkaido, Japan, and other plant materials were purchased from a local market. Diploid *L. lancifolium* and triploid *L. lancifolium* were each crossed with *L. leichlinii*, and the parental and *F*₁ plants (20 and 60 plants, respectively) were grown at the experimental farm of Hokkaido University. To analyze which cross combinations could produce fertile seeds, additional cross-fertilizations were carried out among the plant materials described above (Table 1). In the crosses of triploid *L. lancifolium × diploid L. lancifolium* and triploid *L. lancifolium × diploid L. lancifolium* ‘Pure Gold’, a cut-style pollination technique (Asano and Myodo, 1977) was additionally used.

*Chromosome observations*

The root tips of the parents and *F*₁ plants were collected in the morning and immediately placed into cold deionized water. These were fixed in a mixture of ethanol and acetic acid (3:1, v/v) overnight at 4°C and stored in 80% ethanol at 4°C until used. After two 15-min washes in deionized water, the root tips were placed into 0.3% pectolyase Y enzyme solution (Seishin Pharmaceutical Co., Tokyo, Japan) for 20 min at 37°C and then placed into 2 M HCl solution for 40 s at 69.5°C to macerate them. They were stained with acetocarmine solution (Wako Pure Chemical Industries, Osaka, Japan) for 1 h at approximately 22°C.
Deeply stained parts of the root tips were cut off and placed on glass slides with deionized water. Cover glasses were mounted, and the root tips were squashed by light tapping using a soft stick. Chromosome number was counted using a light microscope (BX50; Olympus Co., Tokyo, Japan).

**Bulbil formation**

The number of bulbils per leaf axil was evaluated.

**Anther morphology**

Anther morphology varied among the F₁ plants, and was recorded as follows: level 4 anthers (normal anthers) were plump and smooth, and pollen grains appeared after anther dehiscence; level 3 anthers were thin and unsmooth; level 2 anthers were partly cracked, producing few pollen grains; and level 1 anthers did not crack (Fig. 5). When anthers with different levels were included in a single flower, average anther levels were used to indicate anther morphology. Sometimes petaloid stamens appeared on the F₁ plants, which were excluded from the average calculation.

**Pollen germination**

Pollen fertility of the parents and F₁ plants was examined in vitro. Pollen grains were floated on a liquid medium containing 0.01% CaCl₂, 0.01% H₃BO₃, 0.0007% KH₂PO₄, 10% sucrose, and 0.01% yeast extract (w/v) at pH 5.7 (Hirano and Hoshino, 2009) at 22°C for 9 h. The percentage of germinated pollen was recorded using a light microscope (DM IRB; Leica Microsystems GmbH, Wetzlar, Germany).

**Statistical analysis**

Regression analysis was performed using Microsoft Excel 2008 software.

**Results**

**Seed production by artificial crossing**

In order to determine which cross combinations produced fertile seeds, artificial crossing among triploid *L. lancifolium*, diploid *L. lancifolium*, and *L. leichtlinii* was carried out (Fig. 1; Table 1). Capsules enlarged resulting from the crosses of triploid *L. lancifolium* × *L. leichtlinii* (Fig. 1A), diploid *L. lancifolium* × *L. leichtlinii* (Fig. 1B), and diploid *L. lancifolium* × *L. leichtlinii* Kihiratoyuri. The crosses between triploid *L. lancifolium* × *L. leichtlinii* produced more seeds within the capsule, with 260, 266, 253, and 258 seeds,

<table>
<thead>
<tr>
<th>♂️</th>
<th>L. leichtlinii⁵</th>
<th>L. leichtlinii Kihiratoyuri</th>
<th>Diploid L. lancifolium</th>
<th>Diploid L. lancifolium 'Pure Gold'</th>
<th>Triploid L. lancifolium</th>
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<tbody>
<tr>
<td>Triploid L. lancifolium</td>
<td>4 (5)</td>
<td>nt</td>
<td>0 (18) [5 (8)]⁷</td>
<td>nt [7 (8)]⁷</td>
<td>0 (10)</td>
</tr>
<tr>
<td>Diploid L. lancifolium</td>
<td>4 (4)</td>
<td>4 (5)</td>
<td>0 (2)</td>
<td>3 (4)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>Diploid <em>L. lancifolium</em> 'Pure Gold'</td>
<td>nt</td>
<td>nt</td>
<td>15 (16)</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>L. leichtlinii⁵</td>
<td>0 (12)</td>
<td>0 (16)</td>
<td>0 (8)</td>
<td>0 (5)</td>
<td>0 (10)</td>
</tr>
<tr>
<td>L. leichtlinii Kihiratoyuri</td>
<td>7 (8)</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
</tbody>
</table>

⁵ L. leichtlinii var. maximowiczii 'Hakugin'.
⁷ Results of cut-style pollination.
nt: not tested.
than the diploid *L. lancifolium × L. leichtlinii* cross, which produced 110, 124, 65, and 52 seeds. The diploid *L. lancifolium × L. leichtlinii* Kihiratoyuri cross produced 146, 143, 136, and 107 seeds. Capsules did not enlarge sufficiently in the crosses of *L. leichtlinii × diploid L. lancifolium* and *L. leichtlinii × diploid L. lancifolium* ‘Pure Gold’. The crosses of triploid *L. lancifolium × diploid L. lancifolium* did not produce seeds by normal pollination, but the same crosses set seeds when a cut-style pollination method was applied.

By self- and cross-fertilizations within *L. leichtlinii*, *L. leichtlinii Kihiratoyuri × L. leichtlinii* produced seeds but the other two combinations failed to produce seeds. Similarly, by pollinations within *L. lancifolium*, diploid *L. lancifolium × L. lancifolium* ‘Pure Gold’ and its reciprocal crosses produced seeds but diploid *L. lancifolium × diploid L. lancifolium* failed to produce seeds, indicating that success in intraspecific hybridization depends on the combination. When triploid *L. lancifolium* was used as a pollen parent, no capsules enlarged.

### Chromosome numbers in the F₁ plants

Because triploid and diploid parents were used for artificial crossing, chromosomes were evaluated in the F₁ hybrids derived from the crosses of the triploid *L. lancifolium × L. leichtlinii* and diploid *L. lancifolium × L. leichtlinii*. The chromosome number of parental species was 36 in triploid *L. lancifolium* (Fig. 2A), 24 in diploid *L. lancifolium* (Fig. 2B), and 24 in *L. leichtlinii* (Fig. 2C). All of the 4 F₁ plants derived from crosses of diploid *L. lancifolium × L. leichtlinii* had 24 chromosomes (Fig. 2D), while 29 F₁ plants derived from crosses between triploid *L. lancifolium* and *L. leichtlinii* showed varied chromosome numbers, ranging from 26 to 34 (Figs. 2E, F and 3), indicating that aneuploidy is generated by this cross combination.

### Bulbil formation

As *L. lancifolium* develops bulbils but *L. leichtlinii* does not, the ability to produce bulbils is predicted to be segregated among the F₁ plants derived from the crosses between these species. Lily bulbils develop on leaf axils, and bulbil number increases as the number of leaf axils increases; thus, the number of bulbils per leaf axil was used to indicate bulbil formation ability. Triploid *L. lancifolium* and diploid *L. lancifolium* developed 0.52 and 1.48 bulbils per leaf axil, respectively. Among the two offspring populations, the number of bulbils per leaf axil showed a continuous distribution, and 9 (45%) and 49 (86%) F₁ plants derived from diploid *L. lancifolium × L. leichtlinii* crosses and triploid *L. lancifolium × L. leichtlinii* crosses, respectively, did not form bulbils (Fig. 4). To clarify whether chromosome number influenced bulbil formation ability, regression analysis was carried out, and no association between chromosome number and bulbil formation ability was observed ($R^2 = 0.001, P = 0.88$) in the progeny of triploid *L. lancifolium × L. leichtlinii* crosses.

### Anther morphology

Among the F₁ plants derived from the crosses be-
between triploid *L. lancifolium* and *L. leichtlinii*, abnormal anthers were frequently observed, which were categorized from level 1 to 4 (Fig. 5A): 22% of the F₁ plants exhibited level 4 anthers (normal anthers), whereas 46%, 10%, and 22% generated 3, 2, and 1 level anthers, respectively (Fig. 5B). The correlation of chromosome number with anther morphology level was not significant (\(R^2 = 0.15, P = 0.08\)) among the F₁ plants. All of the F₁ plants from diploid *L. lancifolium* × *L. leichtlinii* crosses and the parental species of triploid *L. lancifolium*, diploid *L. lancifolium*, and *L. leichtlinii* developed level 4 (normal) anthers (Fig. 5B). Apart from anther abnormality, no other morphological defects were observed in the F₁ offspring.

Pollen germination

Pollen fertility in the hybrid plants was evaluated. The pollen grains of triploid *L. lancifolium* did not germinate (Fig. 6A), whereas 65% and 51% of pollen grains germinated in diploid *L. lancifolium* and *L. leichtlinii*, respectively (Figs. 6B, C and 7). Pollen germination in the F₁ plants from diploid *L. lancifolium* × *L. leichtlinii* crosses varied from 14% to 94% (Figs. 6D and 7). In the progeny of triploid *L. lancifolium* × *L. leichtlinii* crosses, pollen germination in 27 F₁ plants was evaluated, of which 31%, 35%, 19%, and 15% had level 4, 3, 2, and 1 anthers, respectively. Pollen grains in 22 of 27 F₁ plants (81%) did not germinate and the remaining 5 F₁ plants (19%) exhibited < 20% pollen germination (Figs. 6E, F and 7), indicating that pollen fertility in these F₁ plants was very low. There were no correlations between chromosome number and pollen germination (\(R^2 = 0.16, P = 0.20\)) or between anther morphology and pollen germination (\(R^2 = 0.10, P = 0.11\)) in the progeny.

**Discussion**

Interspecific hybridization has been predominantly used to breed floricultural lily cultivars but rarely applied to edible lily breeding. Bulbs of both *L. lancifolium* and *L. leichtlinii* are edible, and if robust plants with few bulbils are segregated among the F₁ plants derived from these species, interspecific hybridization between them should be an attractive method for breeding new edible lily cultivars. In this study, we evaluated some properties, including bulbil formation ability and chromosome number, in the hybrid plants from *L. lancifolium* and *L. leichtlinii* crosses.

The crosses of triploid *L. lancifolium* × *L. leichtlinii* produced more seeds than diploid *L. lancifolium* × *L. leichtlinii* crosses, and our results are in agreement with Noda (1986). However, reciprocal crosses between them did not produce seeds in this study or in the study...
high pollen fertility (65%), it is not clear why the crosses of *L. leichtlinii* × diploid *L. lancifolium* could not produce seeds. Different seed fertilities between reciprocal crosses have often been reported in the interspecific hybridization of lilies (Asano, 1987; Fernández et al., 1996), although the reason for this has not been clarified. Crosses between triploid *L. lancifolium* and *L. leichtlinii* produced seeds, indicating that triploid *L. lancifolium* can generate fertile female gametes, although it cannot develop fertile male gametes. Triploid *L. lancifolium* × diploid *L. lancifolium* did not produce seeds in this study or in the study by Noda (1986). *Lilium* species often show gametophytic self-incompatibility (Ichimura and Yamamoto, 1992; Sakazono et al., 2012), and self-incompatibility is one possible reason for the sterility in this cross combination, which was supported by the results that fertile seeds were obtained by a cut-style pollination. The crosses between *L. leichtlinii* and *L. leichtlinii* did not produce seeds, but those between *L. leichtlinii* Kihiratoyuri and *L. leichtlinii* did (Table 1), indicating that seed production ability depends on the cross combinations, in which self-incompatibility is involved. Similarly, self-pollination of diploid *L. lancifolium* did not produce seeds, whereas diploid *L. lancifolium* × diploid *L. lancifolium* ‘Pure Gold’ and its reciprocal crosses did (Table 1), indicating that gametophytic self-incompatibility prevents self-fertilization in diploid *L. lancifolium*.

Crosses between triploid *L. lancifolium* and *L. leichtlinii* generated aneuploidy, and chromosome numbers varied from 26 to 34 (Fig. 3). Similar results have been reported previously; somatic chromosome numbers in the *F*₁ hybrids from the crosses between triploid *L. lancifolium* and *L. leichtlinii* ranged from 25 to 33 (Noda, 1986). In interspecific hybridization of ornamental lilies, cross combinations between triploids and diploids usually produce aneuploids (Lim et al., 2003; Barba-Gonzalez et al., 2006; Zhou et al., 2011). Different results have been reported in other plant species. For example, interspecific hybridization between triploid and diploid *Lycoris* species produced tetraploid hybrids, where unreduced gametes (2n-gametes) in the triploid species were involved in tetraploid production (Ogawa et al., 2006). Unreduced gametes in triploid plants have also contributed to the development of tetraploid cultivars in *Narcissus* (Lifante et al., 2009) and pentaploid hybrid progenies in the potato (Adiwilaga and Brown, 1991).

*Lilium lanciflorum*, *L. bulbiferum* var. *bulbiferum* (section Sinomartagon), and some Asiatic hybrid lily cultivars (developed by crossing species belonging to section Sinomartagon) develop many bulbils on their leaf axils (Bird, 1991; Suh and Roh, 2014), while other wild species of section Sinomartagon and many Asiatic hybrid lily cultivars rarely develop bulbils under normal
conditions. In this study, bulbil formation ability showed a continuous distribution in both of the F₁ populations (Fig. 4), indicating that this ability is a quantitative trait. Eighty-six percent and 45% F₁ plants derived from the crosses of triploid *L. lancifolium × L. leichtlinii* and diploid *L. lancifolium × L. leichtlinii*, respectively, did not form bulbils, indicating that the distribution was skewed to the ability of *L. leichtlinii*. The frequency of F₁ plants without bulbils was higher in the former cross combination, which should be mainly due to the lower bulbil formation ability of triploid *L. lancifolium* (0.52 bulbils per leaf axil) than that of diploid *L. lancifolium* (1.48 bulbils per leaf axil). No F₁ plants exceeded the *L. lancifolium* parents in bulbul formation, i.e., transgressive segregation was not observed, suggesting that *L. leichtlinii* does not have loci that are positively correlated with bulbul formation. To the best of our knowledge, genetic analysis of bulbul formation ability has not been reported previously. The result that many F₁ plants derived from triploid *L. lancifolium × L. leichtlinii* crosses did not form bulbils is advantageous to edible lily breeding because bulbul formation is considered to be undesirable for edible lily production.

The F₁ offspring derived from the triploid *L. lancifolium × L. leichtlinii* crosses often developed anthers with abnormal morphologies (Fig. 5). Because such anthers did not appear in the F₁ plants derived from the crosses between diploid *L. lancifolium* and *L. leichtlinii* or in the parents of triploid *L. lancifolium*, aneuploidy could be involved in abnormal anther development. No correlation was found between anther morphology level and chromosome number. This suggests that the combination of extra chromosomes has a greater influence on anther development than chromosome number, although the mechanisms underlying abnormal anther development are not clear. Although the appearance of flowers harboring abnormal anthers is undesirable, this is not a major problem for edible lilies because the bulbs are the main commercial output.

Pollen germination in the F₁ plants from diploid *L. lancifolium × L. leichtlinii* crosses showed a continuous distribution, ranging from 14% to 94% (Fig. 7). In interspecific hybridization of plants, genes such as gametophytic selection genes often reduce pollen fertility in F₁ plants (Matsushita et al., 2003; Sanó, 1990), which should be one of the major reasons for the segregation of the F₁ plants with low pollen fertility. In the F₁ progeny plants of triploid *L. lancifolium × L. leichtlinii* crosses, pollen germination was very low, ranging from 0% to 19%, which might be mainly due to their aneuploid properties.

To select new edible lily cultivars, resistance to botrytis and viral diseases must be evaluated in the offspring of triploid *L. lancifolium × L. leichtlinii* crosses in future studies. The evaluation of bulb qualities will also be important: a pure-white color and no-bitter taste are desirable (*L. leichtlinii* ‘Hakugin’ has such properties; Miki, 1988). Although one plant was sufficient to evaluate chromosome number and bulbul formation ability, several plants are necessary to evaluate disease resistance traits and bulb quality; therefore, it will take at least 2 years to select the final candidates for new edible lily cultivars.

In conclusion, this study showed that interspecific hybridization of *L. lancifolium × L. leichtlinii* can produce hybrid plants with few bulbils. The hybrids from triploid *L. lancifolium × L. leichtlinii* crosses often exhibited abnormal anther morphology and pollen sterility, which are probably caused by their aneuploidy properties. However, these defects are not predicted to be major problems in edible lilies. Therefore, this study indicates that interspecific hybridization is a useful method for breeding, not only for floricultural lilies but also edible lilies.

**Literature Cited**


