Title:

Interploid and intraploid hybridizations to produce polyploid Haskap (*Lonicera caerulea* var. *emphyllocalyx*) plants

Interploid and intraploid hybridization in Haskap (*Lonicera caerulea* var. *emphyllocalyx*)

A concise title: Interploid and intraploid hybridizations in *Lonicera caerulea*

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Abstract

We produced polyploid Haskap (*Lonicera caerulea* var. *emphyllocalyx*) plants by performing interploid and intraploid crosses of wild accessions. Embryo rescue in a tetraploid (4*x*) × diploid (2*x*) cross produced triploid plants; reciprocal 2*x* × 4*x* cross failed to produce viable seeds. Intraploidy crosses of 4*x* × 4*x* produced mostly tetraploids but also several hexaploid (6*x*) and octoploid (8*x*) plants. Using hexaploids obtained from this cross, we examined reciprocal 4*x*–6*x* crosses and found that both produced pentaploid plants. An octoploid was produced by applying colchicine to a tetraploid; a 4*x* × 8*x* cross using this plant and aided by embryo rescue culture produced three hexaploid plants, with an aneuploid number of chromosomes. Several plants obtained in this study flowered and set fruits. We discusse the overall efficiency of producing polyploid plants in inerploid and intraploid crosses.

Keywords: Caprifoliaceae, Interploid cross, Intraploid cross, *Lonicera caerulea*, Haskap, Polyploid

1. Introduction

More than 200 species comprise the genus *Lonicera*, which belongs to the family Caprifoliaceae (Poyarkova 2000). Blue honeysuckle (*Lonicera caerulea* L.) belongs to the section *Isika*, subsection Caeruleae (Rehder 1903). It is a deciduous shrub with edible fruits that is found in the northern regions of Eurasia and North America (Rehder 1903). In Japan, blue honeysuckle grows in cold regions, from the alpine areas of the middle island to the entire Hokkaido (Hara 1983; Sato 1985). Japanese blue honeysuckle is known as Haskap in the Ainu language used by the indigenous Ainu people of Hokkaido. The fruits of blue honeysuckle are sour to sweet or bitter in taste, and are known as functional food because they are high in nutritional value, containing anthocyanins, polyphenolics, minerals, vitamins, and loganin (Anikina et al. 1989; Terahara et al. 1993; Machida et al. 1995; Anetai et al. 1996; Tanaka and Tanaka 1998; Plekhanova 2000; Thompson and Chaovanalikit 2003; Chaovanalikit et al. 2004; Svarcova et al. 2007). Haskap has been cultivated as a berry crop in Hokkaido; Haskap juice, wine, and jam are popular. For breeding, interspefic hybrids have been produced between *L. caerulea* and *L. gracilipes* (Miyashita and Hoshino 2010). Recently, Haskap was introduced to North America as a new berry crop (Thompson 2006).

The size and taste of the wild Haskap fruit vary. To increase commercial production, the

Hokkaido Research Organization evaluated the botanical and agricultural traits of wild Haskap. Some elite strains were selected and the first cultivar, 'Yufutsu,' was released in 1992 (Tanaka et al. 1994). Takada et al. (2003) evaluated the eating qualities and some horticultural characteristics of wild Haskap species, and made some elite selections. Local agricultural cooperatives also selected several elite strains from wild plants. Thompson and Barney (2007) performed evaluation and breeding of Haskap in North America. However, further improvement of the plant is needed to increase its commercial production. A major issue for Haskap growers is that wild plants have small fruits with thin pericarp. Harvest is laborious, and the harvest volume barely meets market demand. Therefore, fruit yield and other traits must be improved.

In general, polyploid plants have larger plant parts and greater adaptability than do diploid plants (Lewis 1980). Polyploids have larger fruits (Notsuka et al. 2000; Wakana et al. 2005; Sasnauskas et al. 2007; Zhang et al. 2010), many polyploid ornamentals have larger flowers, and others have higher quantities of effective components (Gao et al. 1996; Zhang et al. 2010). Triploid plants are usually sterile and in such species as banana, grape, watermelon, and many others triploidy is used for seedless fruit production. Polyploid variation is important because of the phenotypic variation it introduces and because it may enhance the effectiveness of plant breeding.

Diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 36$) varieties of *L. caerulea* have been found in Eurasia and North America (Ammal and Saunders 1952; Plekhanova et al. 1992; Solovyeva and Plehanova 2003; Plekhanova 2007). Both diploids and tetraploids were found in wild populations of Haskap (Miyashita et al. 2011). In order to prepare for ploidy breeding in this plant species, we examined the production of octoploids from tetraploids by colchicine, oryzalin, and trifluralin treatments (data not published). Suzuki et al. (2007) developed a method of *in vitro* colchicine treatment using the nodal segment. Miyashita et al. (2009) examined the production of hexaploid plants by endosperm culture.

The aim of the present study was to produce polyploid Haskap plants to increase the genetic variation of the species. Intraploid and interploid crosses using diploid, tetraploid, hexaploid, and octoploid variants were investigated. Lateral, compatibility or incompatibility in interploid hybridization is discussed from the viewpoints of paternal and maternal genome ratios in the embryo and endosperm.

2. Materials and Methods

2.1. Plant materials

Haskap (*L. caerulea* var. *emphyllocalyx*) plants grown at the Experiment Farms of Hokkaido University were used. Two diploid ($2n = 2x = 18$) strains, Di-K8 and Di-BeY, were collected from Kiritappu mire and Betsukai in Hokkaido, respectively, and six tetraploid ($2n = 4x = 36$) strains, Tet-Y2, Tet-Y16, Tet-Y27, Tet-Y37, Tet-Y0049, and Tet-Has1 were collected from Yufutsu mire in Hokkaido. One octoploid strain ($2n = 8x = 72$). Oct-T1, was produced by colchicine treatment of a tetraploid plant in our laboratory.

2.2. Hybridization patterns

Intraploid or interploid hybridization was performed using diploid, tetraploid, hexaploid, and octoploid plants. The cross combinations tested were $2x \times 2x$, $4x \times 4x$, $2x \times 4x$, $4x \times 2x$, $4x \times 6x$, $6x \times 4x$, and $4x \times 8x$. Flowers were emasculated prior to anthesis and hand-pollinated. To prevent cross pollination all pollinated flowers were covered with paper bags.

Fruits were harvested at 40–45 days after pollination (DAP), when mature seeds are generally present in Haskap. Seeds germinate and develop into seedlings more quickly when cultured on a artificial media and so seed media culture was utilized in intraploid and interploid crosses. All seeds were disinfected with 1% sodium hypochlorite solution containing 1–2 drops of polyoxyethylene sorbitan monolaurate (Tween 20) for 20 min, rinsed three times with sterile-distilled water and cultured on a half-strength Murashige and Skoog (1962) basal medium containing 30 g L^{-1} sucrose and 2 g L^{-1} gellan gum (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). The pH of the medium was adjusted to 5.8 and it was autoclaved at 121 °C for 20 min. The seeds cultures in 90 × 20-mm petri dishes were maintained in a growth chamber at 20 °C under continuous illumination 24 h photoperiod (35 μmol m^{-2} s^{-1}) provided by 40W fluorescent tubes.

Immature seed culture was also performed in $4x$–$4x$, $2x$–$4x$ and $4x$–$8x$ hybridization. Immature fruits were harvested at 14–28 DAP, disinfected with 1% sodium hypochlorite solution containing 1–2 drops of Tween 20 for 10 min and rinsed, immature seeds were collected and cultured under the same conditions as above.

2.3. Ploidy analysis using flow cytometry

The relative DNA contents of hybrids from intraploid or interploid crosses were determined using flow cytometry (Partec PA; Partec GmbH, Münster, Germany). Fresh leaves (ca. 0.5 cm × 0.5 cm) were chopped in 0.2 mL of nuclei extraction buffer (CyStain UV precise P; Partec, Münster, Germany). After filtration through a 30-μm nylon mesh, crude nuclear samples were stained with 0.8 mL 4', 6-diamidino-2-phenylindole (DAPI) solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100, and 2 mg L^{-1} DAPI (pH 7.5) (Mishiba et al. 2000), incubated for 5 minutes at room temperature and relative DNA contents were measured with fresh leaves of *Capsicum annuum* L. (cv. 'Kyonami') were used as the internal standard.

2.4. Chromosome analysis

Chromosome numbers were counted in the actively growing root tips. The root tips were pretreated in ice water for 24 h and fixed in a mixture of acetic acid:ethanol (1:3) at 4 °C overnight. Fixed root tips were treated with a mixture of 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Co., Ltd., Japan) and 0.5% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan) (Shibata and Hizume, 2002) in the citrate buffer (0.01 M citric acid and 0.01 M trisodium citrate dehydrate) at pH 4.5, at 37 °C, for 20 min, rinsed in distilled water and squashed with forceps in a drop of 45% acetic acid on a glass slide, covered with another slide, and squashed again (I do not understand this procedure? Double Squashing? Two slides?). Covers slips were removed by freezing in liquid nitrogen, and slides were dried at 37 °C. A drop of DAPI solution [0.233 g 1,4-diazabicyclo(2.2.2)-octane, 1 mL 0.2 M Tris-HCl, pH 8.0, 9 mL glycerol, 0.5 μg ml^{-1} of DAPI] (Sahara et al. 2003) was added for staining and preparations were observed under a fluorescence microscope at ×1000 magnification (Axio Imager M1; Carl Zeiss, Oberkochen, Germany). For each sample, 10 measurements were recorded.

2.5. Characterization of polyploid plants

Corolla length, pollen diameter, pollen germination rate, the size of guard cells, and fresh fruit weight were measured in polyploids and their parents. The corolla length is an average of seven flowers. Pollen diameter (n = 20 per plant) was measured following staining of fresh pollen in aceto-carmine. The pollen germination ability was tested in a pollen culture medium, following Brewbaker and Kwack (1963), with

minor modifications. The culture medium was liquid, containing 100 g L^{-1} sucrose, 100 mg L^{-1} boric acid, 300 mg L^{-1} calcium nitrate, 200 mg L^{-1} magnesium sulfate, and 100 mg L^{-1} potassium nitrate in water. For each plant, three replicates were performed. The size of the guard cells (n = 30 per plant) was measured using the replica method. Microscopic observations were performed using Primo Star (Carl Zeiss, Oberkochen, Germany) at × 40 magnification.

Statistical tests were performed using the SPSS 16.0 J program. The differences were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test, with $p < 0.05$ as the level of statistical significance.

2. 6. *Evaluation of intraploid and interploid hybridization*

To evaluate interploid hybridization, seed development in 4*x* × 2*x* and 2*x* × 4*x* was observed. In total, the relationship between the embryo/endosperm genome ration and germination efficiency (the number of seed germination / the number of seeds obtained) was analyzed.

3. Results

3.1. 2x × 2x crosses

The results are shown in Table 1. The seed germination rate of 2*x* × 2*x* (Di-K8 × Di-BeY) crosses was 100%. Flow cytometry showed that all progeny obtained from 2*x* × 2*x* crosses were diploid.

3.2. 2x–4x crosses

The results of reciprocal 2*x*–4*x* crosses are shown in Table 2. The 2*x*–4*x* crosses produced many shriveled seeds compared with the 2*x*–2*x*, 4*x*–4*x*, and 4*x*–6*x* crosses. The seeds from the 2*x* × 2*x* and 4*x* × 4*x* crosses contained embryos at the torpedo-shape stage, and the endosperm almost filled the seed at 40 DAP. In contrast, in 2*x* × 4*x* crosses at 40 DAP, the embryos were at the globular stage, and the endosperm degenerated. In 4*x* × 2*x* crosses, most of the embryo and endosperm were underdeveloped.

In reciprocal crosses between the diploids and tetraploids, only 4*x* × 2*x* crosses were successful. The reciprocals failed to produce viable seeds and no germination was observed. Only a small percentage of the 4*x* × 2*x* crosses succeeded in setting fruit. Nearly all of these produced shriveled seeds (97−100%).

However, several shriveled seeds germinated in seed culture and grew into plants. The germination rate varied from 0% to 14.7%. A total of 9 (7 + 2) plants survived out of 13 (10 + 3) germinated seeds. Flow cytometric analysis showed that they were triploid (Fig. 1a) and in five individual seedlings the chromosome number was confirmed at $2n = 3x = 27$ (Fig. 2a). These triploid seedlings were acclimated and grown in pots (Fig. 3a) and were still growing normally three months after acclimation.

The effect of the immature seed culture was confirmed in reciprocal $2x$–$4x$ crosses using the strains Di-K8 and Tet-Y27. The results of immature seed culture are shown in Table 3. A total of 107 (31 + 49 + 27) immature seeds from $2x \times 4x$ crosses were cultured but no germination was observed at any of the harvested stages. In contrast, triploids were obtained by immature seed culture in $4x \times 2x$ crosses. The germination rate (19% to 37.6%) of immature seeds in culture was higher than that of seed culture in $4x \times 2x$ crosses. The highest germination rate was observed in immature seeds harvested at 21 DAP. A total of 69 (18 + 27 + 24) plants survived out of 93 (24 + 38 + 31) germinated immature seeds. Progeny obtained from immature seed culture were triploid.

3.3. 4x – 4x crosses

The results of $4x \times 4x$ crosses are shown in Table 4. The germination rate of seeds from $4x \times 4x$ crosses (Tet-Y27 × Tet-Y37 and Tet-Y37 × Tet-Y27) ranged from 84.5% to 94.4%. The germination rate in immature seed culture (Tet-Y27 × Tet-Y37, Tet-Y37 × Tet-Y27, and Tet-Has1 × Tet-Y16) was 13.6% to 29.2%. A total of 382 progeny were obtained from $4x \times 4x$ crosses. Flow cytometry analysis showed that almost all the progeny (378 seedlings) were tetraploid. The exceptions were two hexaploids and two octoploid (Fig. 3e, f, j). The chromosome number of both hexaploids was $2n = 6x = 54$ (Fig. 2b). One hexaploid plant (strain: Hex-1, $2n = 6x = 54$) grew normally and set fruit. This individual was used for hybridization listed below. Vigor of the octoploids was strongly suppressed; they were very small compared to the plants of other ploidy levels.

3.4. 4x–6x crosses

A hexaploid plant (strain: Hex-1) obtained from the $4x \times 4x$ cross was used in reciprocal $4x$–$6x$ crosses (Table 5). Similar levels of seed shriveling was observed in both crosses, 52% for $4x \times 6x$ 58% for $6x \times 4x$. However, these seeds were able to germinate in culture and grew into plants. The germination rates were

75.4% (for 4*x* × 6*x* crosses) and 89.5% (for 6*x* × 4*x* crosses). Flow cytometry showed that progeny obtained from 4*x* × 6*x* crosses and 6*x* × 4*x* crosses were pentaploids (Fig. 1b). The chromosome number was confirmed for 17 plants at $2n = 5x = 45$ (Fig. 2c) from 17. These pentaploid plants were acclimated and grown in pots (Fig. 3b, c, d). Three of them bloomed and set fruit at the age of two.

3.5. 4x × 8x crosses

An octoploid strain (Oct-T1) produced by colchicine treatment of a tetraploid was used for 4*x* × 8*x* crosses. In 4*x* × 8*x* direction all crosses were successful and set fruits (Table 6). The total 131 (37 + 84 + 10) immature seeds were cultured and three seedlings were obtained. The germination rates were 2.4% to 10%. Flow cytometry analysis showed that these progeny were hexaploid (Fig. 1c) but chromosome counts revealed aneuploid chromosome numbers of chromosomes at $2n = 6x-4 = 50$ (Fig. 2d), $2n = 6x-3 = 51$ (Fig. 2e), and $2n = 6x-2 = 52$ (Fig. 2f). Aneuploid plants obtained from 4*x* × 8*x* crosses grew normally (Fig. 3g, h), except for one plant with $2n = 6x-2 = 52$ form (Fig. 3i) showing growth suppression.

3.6. Characterization of polyploids hybrids

Triploid, tetraploid, pentaploid, hexaploid, and octoploid hybrids were obtained from intraploidy and interploidy crosses. Flowering was observed in three pentaploid plants and one hexaploid plant (strain: Hex-1).

Corolla length, pollen diameter, pollen germination, the size of guard cells, and the fresh weight of fruit were investigated in several individuals and their parents (Table 7). The corolla lengths of tetraploid, hexaploid, and octoploid plants were larger than those of diploids. Octoploid plant Oct-T1 had the largest flowers of all plants evaluated (larger by 43% compared to diploid), but the octoploids showed reduced growth and set no fruit. Pollen diameter tended to increase with the ploidy level (Fig. 4). Octoploid pollen diameter was 1.6× lager than that of diploid. A pollen germination rate of more than 70% was observed in diploid (75.5%), tetraploid (72.7% to 87.3%), and hexaploid (81.4%) plants. In contrast, the pollen germination rate of pentaploid (15.4% to 24%) and octoploid plants (3.5%) was low. The size of guard cells tended to increase with the ploidy level (Fig. 5) being 1.63× larger in the octoploid than in thediploid. In external appearance, thick leaves were observed in pentaploid, hexaploid,

and particularly octoploid plants. Three pentaploid and one hexaploid plant set fruit (Fig. 6a, b). The fresh weight of the fruit was 1.4 g in the pentaploid (strain: Pen-No.12) and 0.8 g in the hexaploid (strain: Hex-1) plant.

3. 7. *Evaluation of intraploid and interploid hybridization*

Seeds from the 4*x* × 2*x* (seed parent: 4*x*, pollen parent: 2*x*) cross contained developed endosperm (Fig. 7a). In contrast, the 2*x* × 4*x* cross (seed parent: 2*x*, pollen parent: 4*x*) contained undeveloped endosperm (Fig. 7b) and failed to produce viable seeds.

In the present study, the 2*x* × 2*x* and 4*x* × 4*x* crosses that produced with 2m:1p (m: maternal genome; p: paternal genome) endosperm resulted in successful seed development. On the other hand, the 2*x* × 4*x*, 4*x* × 2*x*, 4*x* × 6*x*, and 6*x* × 4*x* crosses that produced 1m, 4m:1p, 4m:3p, and 3m:1p endosperm, respectively, showed abnormal seed formation. The relationship between the embryo:endosperm genome ratio and germination efficiency in interploidy crosses is shown in Figure 8.

4. Discussion

Various interploidy cross combinations were tested and progeny was obtained from all except for the 2*x* × 4*x*, which produced no viable progeny. The reciprocal cross with the tetraploid as female was successful. The 2*x* by 4*x* combination set few seeds and the seeds exhibited abnormal growth. Crosses between diploid and tetraploid plants often fail in other plant species because seeds develop abnormally and/or are nonviable (Haig and Westoby 1991; Ramsey and Schemske 1998). Low rates of seed set from crosses between diploid and tetraploid species of *L. caerulea* were also reported by Plekhanova (2000). In the present study, although the germination rate was substantially lower than that of other cross combinations, several triploid seedlings were obtained from the 4*x* × 2*x* cross. The seeds had endosperm (Fig. 7a), however, the 2*x* × 4*x* cross could not produce viable seeds and contained undeveloped endosperm (Fig. 7b). The direction of the cross affects both the endosperm development and viability of progeny. Embryo rescue by immature seed culture produced triploid seedlings only in the 4*x* × 2*x* combination. In other words, production of triploid plants in reciprocal 2*x*–4*x* crosses appeared to be successful only in one direction in Haskap, when the seed parent is 4*x*.

Interploidy crosses often lead to abnormal seed development, followed by seed abortion (Scott et al. 1998). In *Solanum*, a 2:1 ratio of maternal to paternal genomes in the endosperm is necessary for normal endosperm development in intraspecific interploidy crosses (Johnston et al. 1980). In maize, Lin (1984) observed that normal seed development required a 2:1 maternal to paternal genome ratio in the endosperm, and suggested that the involvement of parentally imprinted genes is required in a 2m (m; maternal genome) :1p (p; paternal genome) ratio. The results of interploidy crosses in *Arabidopsis thaliana* also suggested that different ratios of maternally and paternally expressed imprinted loci affect endosperm development (Scott et al. 1998).

In the present study, the $2x \times 2x$ and $4x \times 4x$ crosses that produced 2m:1p endosperm resulted in successful seed development. On the other hand, combinations $2x \times 4x$, $4x \times 2x$, $4x \times 6x$, and $6x \times 4x$ crosses that resulted in 1m:1p, 4m:1p, 4m:3p, and 3m:1p endosperm genome ratios, respectively, showed abnormal seed formation. Haskap, similarly to other species also appears to require a 2m:1p ratio in the endosperm for normal seed development. In particular, the $2x \times 4x$ cross might be highly influenced by genetic regulation because germination was not observed despite embryo rescue by immature seed culture. Tiwari et al. (2010) reported that a paternal genomic imbalance particularly affects proliferation of the endosperm. The results obtained in the present study might indicate genomic imbalance in interploidy crosses as shown in Figure 8 The aberration from 2:3 (embryo:endosperm genome ratio) reduced germination frequency, confirming previous results.

In our study, the majority of plants (99%) obtained from the $4x \times 4x$ cross were tetraploid, but a small proportion of hexaploid (0.5%) and octoploid (0.5%) plants were obtained. Similar results were previously reported in other plant species. For example, production of tetraploid (98.7–99.0%), hexaploid (0.3–0.7%), and octoploid (0.2–0.7%) progeny from a $4x \times 4x$ cross were reported in intersectional crosses of *Primula* (Hayashi et al. 2007) due to the fertilization of unreduced gametes. In *Allium tuberosum*, hexaploid plants naturally occurred in open-pollinated seedlings of tetraploid plants (Sharma and Gohil 2013). These authors suggested that the hexaploid plant was produced from the fusion of reduced male and unreduced female gametes. Production of unreduced gametes is one mechanism of spontaneous polyploid formation, which has been identified in many plant species (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Other mechanisms of spontaneous polyploid formation in plants are somatic doubling of meristem tissue, zygotes, or young embryos; and polyembryonic seeds

283 (Ramsey and Schemske 1998). The hexaploid and octoploid plants from the 4*x* × 4*x* cross in the present
284 study may have resulted from one of these mechanisms.
285 The 4*x* × 8*x* cross produced hexaploid plants with aneuploid chromosome numbers (6*x*−2, 6*x*−3,
286 and 6*x*−3). These aneuploids might have originated via aneuploid pollen from the octoploid parent. The
287 octoploid plant used in this study was produced from a colchicine-treated tetraploid (Takada 2001).
288 Generally, autopolyploid plants have the potential to produce aneuploid gametes through irregular meiosis
289 (Ramsey and Schemske 2002; Comai 2005).
290 In the present study, triploid, tetraploid, pentaploid, hexaploid, and octoploid plants were
291 obtained from 2*x*–4*x*, 4*x* × 4*x*, 4*x*–6*x*, and 4*x* × 8*x* crosses. Polyploid seedlings were acclimated and
292 grown in pots. Several pentaploid and hexaploid plants flowered and set fruits. Polyploid variation can
293 provide phenotypic variation and introduce new perspectives in the breeding of Haskap. These different
294 ploidy progenies will be utilized for analyses of sexual events during interploidy hybridization and
295 provide new material for breeding.
296
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304
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Table 1 Seed production, germination, and ploidy level of progeny in 2*x* × 2*x* cross

Cross combination (seed parent × pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds obtained	No. of seeds germinated	% of seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
2*x* × 2*x*							
Di-K8 × Di-BeY	3	3	39	39	100.0	38	2*x* (38)

Seeds harvested at 40–45 days after pollination were cultured.

Table 2 Seed production, germination, and ploidy level of progeny in reciprocal 2*x*–4*x* crosses

Cross combination (seed parent × pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds obtained	No. of seeds germinated	% of seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
2*x* × 4*x*							
Di-K8 × Tet-Y16	10	9	84	0	0.0	–	–
Di-K8 × Tet-Y27	5	5	35	0	0.0	–	–
Di-K8 × Tet-Y37	10	9	81	0	0.0	–	–
4*x* × 2*x*							
Tet-Y16 × Di-K8	10	6	68	10	14.7	7	3*x* (5)
Tet-Y27 × Di-K8	5	3	45	0	0.0	–	–
Tet-Y37 × Di-K8	10	3	36	3	8.3	2	3*x* (2)

Seeds harvested at 40–45 days after pollination were cultured.

Dashes indicate no data.

418 **Table 3** Immature seed culture in reciprocal 2*x*–4*x* crosses

Cross combination (seed parent × pollen parent)	Days after pollination (DAP)	No. of fruits used	No. of immature seeds obtained	No. of immature seeds that germinated	% of immature seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
2*x* × 4*x*							
Di-K8 × Tet-Y27	14 DAP	5	31	0	0.0	–	–
	21 DAP	4	49	0	0.0	–	–
	28 DAP	4	27	0	0.0	–	–
4*x* × 2*x*							
Tet-Y27 × Di-K8	14 DAP	5	126	24	19.0	18	3*x* (11)
	21 DAP	4	101	38	37.6	27	3*x* (18)
	28 DAP	4	103	31	30.1	24	3*x* (12)

419 Dashes indicate no data.

Table 4 Seed production, germination, and ploidy level of progeny in 4*x* × 4*x* cross

Cross combination (seed parent × pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds (immature seeds) obtained	No. of seeds (immature seeds) that germinated	% of immature seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
4*x* × 4*x*							
Seed culture [a]							
Tet-Y27 × Tet-Y37	5	5	148	125	84.5	125	4*x* (125)
Tet-Y37 × Tet-Y27	5	5	71	67	94.4	67	4*x* (67)
Immature seed culture [b]							
Tet-Y27 × Tet-Y37	21	21	497	74	14.9	74	4*x* (72), 8*x* (2)
Tet-Y37 × Tet-Y27	15	15	250	73	29.2	73	4*x* (73)
Tet-Has1 × Tet-Y16	14	14	317	43	13.6	43	4*x* (41), 6*x* (2)

[a] Seeds harvested at 40–45 days after pollination (DAP) were cultured.

[b] Immature seeds harvested at 14 to 28 DAP were cultured.

Table 5 Seed production, germination, and ploidy level of progeny in reciprocal 4*x*–6*x* crosses

Cross combination (Seed parent × Pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of seeds obtained	No. of seeds that germinated	% of seeds that germinated	No. of seedlings that survived	Ploidy level (no. of seedlings examined)
4*x* × 6*x*							
Tet-Y27 × Hex-1	4	4	65	49	75.4	43	5*x* (30)
6*x* × 4*x*							
Hex-1 × Tet-Y27	2	2	19	17	89.5	13	5*x* (13)

Seeds harvested at 40–45 DAP were cultured.

Dashes indicate no data.

Table 6 Seed production, germination, and ploidy level of progenies in 4*x*–8*x* cross by immature seed culture

Cross combination (seed parent × pollen parent)	No. of flowers pollinated	No. of flowers that set fruits	No. of immature seeds obtained	No. of immature seeds that germinated	% of germination	No. of seedlings that survived	Ploidy level
4*x* × 8*x*							
Tet-Y2 × Oct-T1	5	5	37	0	0.0	–	–
Tet-Y27 × Oct-T1	8	8	84	2	2.4	2	$2n = 6x-3 = 51$, $2n = 6x-2 = 52$
Tet-Y0049 × Oct-T1	1	1	10	1	10.0	1	$2n = 6x-4 = 50$

Dashes indicate no data.

Immature seeds harvested at 14 to 21 days after pollination were cultured.

Table 7 Characterization of diploid, triploid, tetraploid, pentaploid, hexaploid, and octoploid plants

Strain	Corolla length (mm)	Pollen diameter (μm)	Pollen germination (%)	Length of guard cell (μm)	Fresh weight of fruit (g)
Diploid (2*x*)					
Di-K8	14.4 ± 0.3 c	55.4 ± 1.5 e	75.5 ± 8.8 a	21.0 ± 1.0 e	0.7 ± 0.1 (*n* = 10)
Triploid (3*x*)					
Tri-No.2	–	–	–	21.9 ± 1.3 e	–
Tri-No.3	–	–	–	21.7 ± 1.8 e	–
Tetraploid (4*x*)					
Tet-Y16	18.7 ± 0.5 b	67.0 ± 3.1 c	83.7 ± 2.3 a	23.8 ± 1.4 d	0.8 ± 0.1 (*n* = 10)
Tet-Y27	18.2 ± 0.7 b	60.1 ± 2.7 d	72.7 ± 12.5 a	23.7 ± 1.3 d	1.1 ± 0.1 (*n* = 10)
Tet-Has1	17.6 ± 0.4 b	61.4 ± 3.6 d	87.3 ± 7.7 a	23.3 ± 0.8 d	0.7 ± 0.2 (*n* = 10)
Pentaploid (5*x*)					
Pen-No.12	–	67.9 ± 4.5 c	24.0 ± 5.9 b	27.2 ± 1.1 c	1.4 ± 0.1 (*n* = 3)
Pen-No.21	–	66.5 ± 2.6 c	15.4 ± 4.1 bc	28.6 ± 1.4 b	–
Hexaploid (6*x*)					
Hex-1	17.7 ± 1.6 b	75.8 ± 4.1 b	81.4 ± 1.9 a	28.7 ± 1.9 b	0.8 ± 0.2 (*n* = 6)
Octoploid (8*x*)					
Oct-T1	20.6 ± 1.0 a	89.0 ± 9.2 a	3.5 ± 1.9 c	34.3 ± 2.5 a	–

Triploid plants were from the 4*x* × 2*x* cross (Tet-Y27 × Di-K8). Pentaploid plants were from the 4*x* × 6*x* cross (Tet-Y27 × Hex-1). Hexaploid plant was from the 4*x* × 4*x* cross (Tet-Has1 ×Tet-Y16).

Means ± SD followed by the same letter are not significantly different (Bonferroni's test, $p < 0.05$). Dashes indicate no data.

Figure captions

Fig. 1. Histograms of the relative fluorescence intensity of nuclei isolated from seedlings of interploidy crosses. (a) Seedling (3Cx) obtained from 4*x* × 2*x* cross. (b) Seedling (5Cx) obtained from 4*x* × 6*x* cross. (c) Seedling (6Cx) obtained from 4*x* × 8*x* cross.

Fig. 2. Chromosomes in root-tip cells of plants derived from interploidy crosses. (a) Triploid (2*n* = 3*x* = 27). (b) Hexaploid (2*n* = 6*x* = 54). (c) Pentaploid (2*n* = 5*x* = 45). (d) Aneuploid (2*n* = 6*x* – 4 = 50). (e) Aneuploid (2*n* = 6*x* – 3 = 51). (f) Aneuploid (2*n* = 6*x* – 2 = 52). Bar = 5 μm.

Fig. 3. Triploid, pentaploid, hexaploid, and octoploid plants of *Lonicera caerulea*. (a) Triploid plants obtained from 4*x* × 2*x* cross, 3 months after acclimation. (b–d) Pentaploid plants from 4*x* × 6*x* cross (strain: Pen-No.9, Pen-No.12, and Pen-No.21), 2 years old. (e) Hexaploid plant (strain: Hex-1), 4 years old. (f) Hexaploid plant (strain: Hex-2), 4 years old. (g) Aneuploid (2*n* = 6*x* – 4 = 50) plant from 4*x* × 8*x* cross. (h) Aneuploid (2*n* = 6*x* – 3 = 51) plant from 4*x* × 8*x* cross. (i) Aneuploid (2*n* = 6*x* – 2 = 52) plant from 4*x* × 8*x* cross showing growth suppression. All aneuploid plants are 3 years old. (j) Octoploid plant from 4*x* × 4*x* cross, 1 year old. Bar = 10 cm.

Fig. 4. Pollen grains of diploid, tetraploid, pentaploid, hexaploid, and octoploid plants. (a) Diploid (strain: Di-K8). (b) Tetraploid (strain: Tet-Y27). (c) Pentaploid (strain: Pen-No.12) from 4*x* × 6*x* cross (Tet-Y27 × Hex-1). (d) Hexaploid (strain: Hex-1). (e) Octoploid (strain: Oct-T1). Bar = 50 μm.

Fig. 5. Guard cells of diploid, triploid, tetraploid, pentaploid, hexaploid, and octoploid plants. (a) Diploid (strain: Di-K8). (b) Triploid (strain: Tri-No. 3) from 4*x* × 2*x* cross (Tet-Y27 × Di-K8). (c) Tetraploid (strain: Tet-Y27). (d) Pentaploid (strain: Pen-No.12) from 4*x* × 6*x* cross (Tet-Y27 × Hex-1). (e) Hexaploid (strain: Hex-1). (f) Octoploid (strain: Oct-T1). Bar = 20μm.

Fig. 6. Fruits of pentaploid and hexaploid plants. (a) Pentaploid (strain: Pen-No.9). (b) Hexaploid (strain: Hex-1). Bar = 1cm.

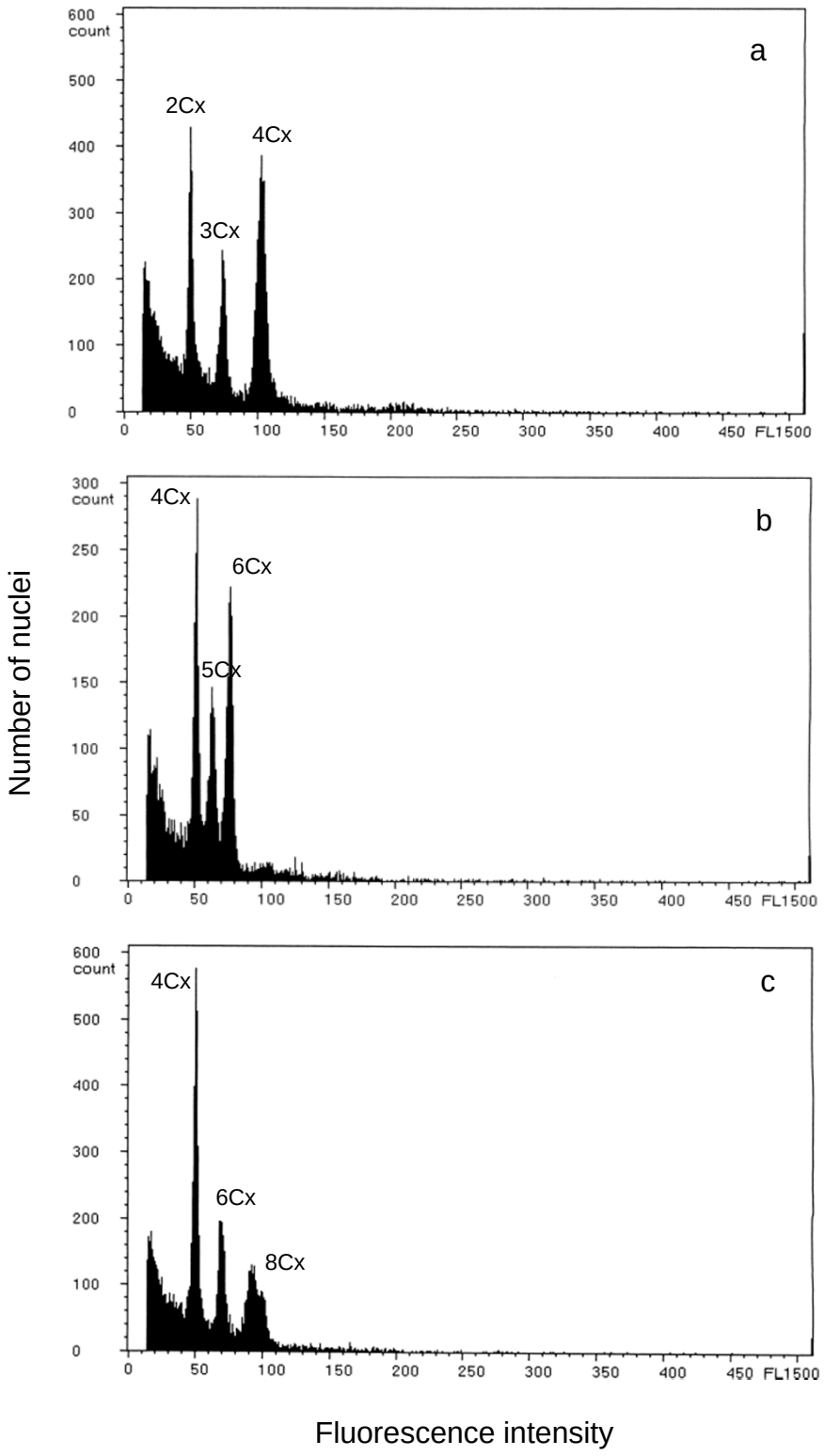
Fig. 7. Seed development in 4*x* × 2*x* and 2*x* × 4*x*. (a) A seed from Tet-Y27 (4*x*) × Di-K8 (2*x*) 40 days after pollination. A seed coat is removed for observation. Developing endosperm (arrow) is exposed. (b) A seed from

463 Di-K8 (2*x*) × Tet-Y27 (4*x*) 40 days after pollination. No endosperm development is observed. Bars = 1 mm.

464

465 Fig. 8. The relationship between the embryo/endosperm genome ratio and germination efficiency in interploidy

466 crosses.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count
500
400
300
200
100
0
2Cx
3Cx
4Cx
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b
300
count
250
200
150
100
50
0
4Cx
5Cx
6Cx
0 50 100 150 200 250 300 350 400 450 FL1500
c
600
count
500
400
300
200
100
0
4Cx
6Cx
8Cx
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Number of nuclei
Fluorescence intensity

Figure 1

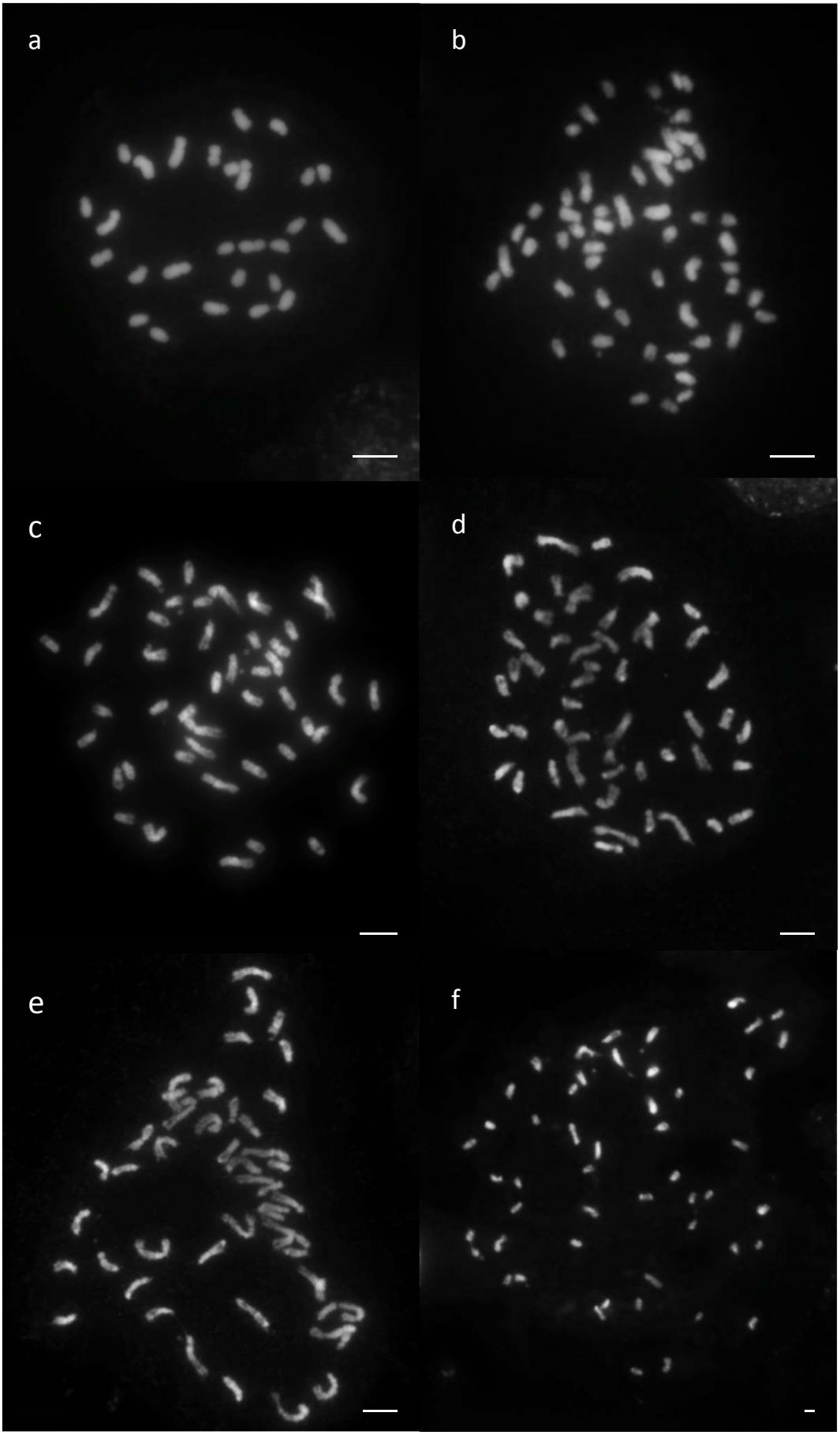


Figure 2

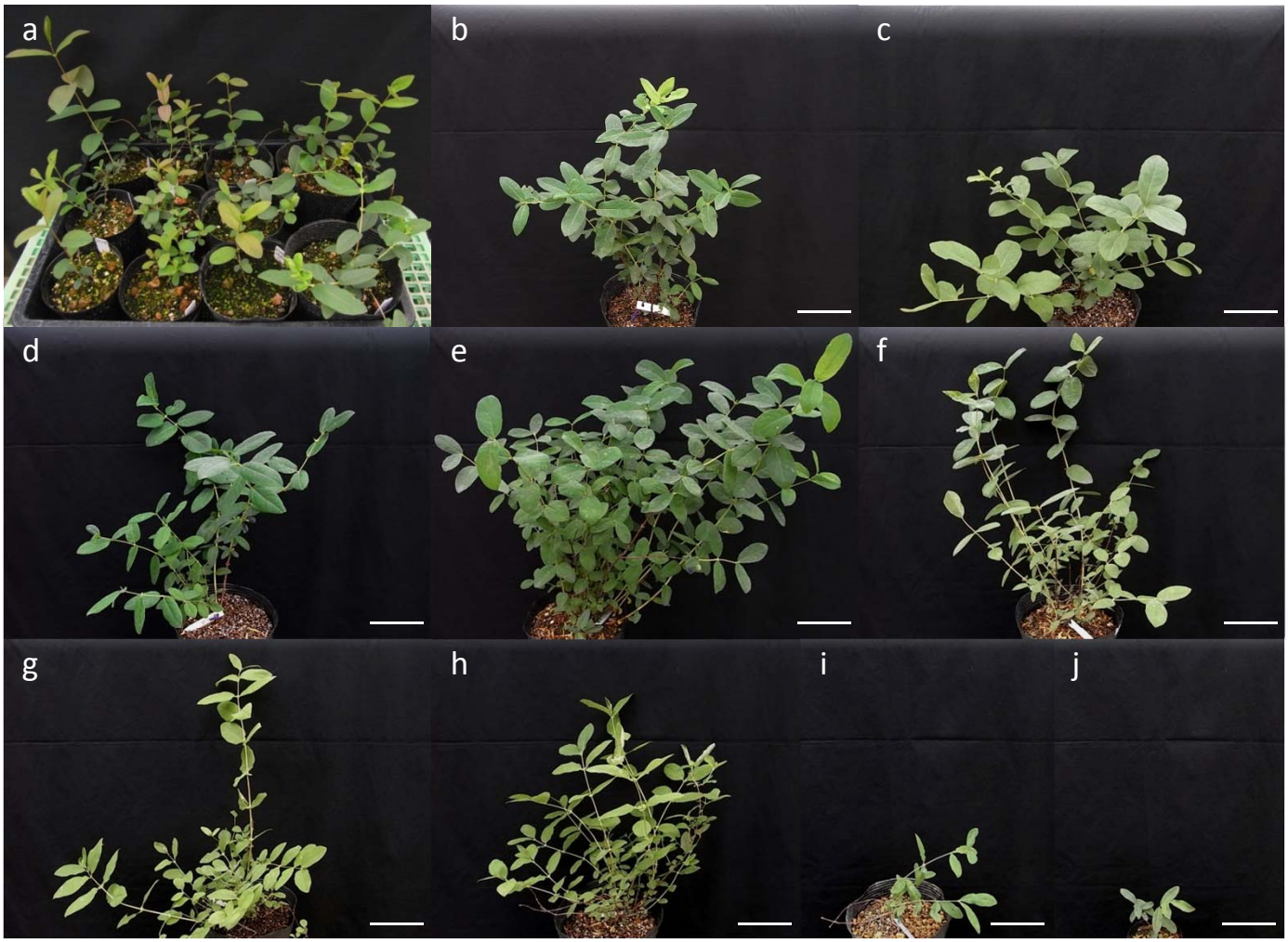


Figure 3

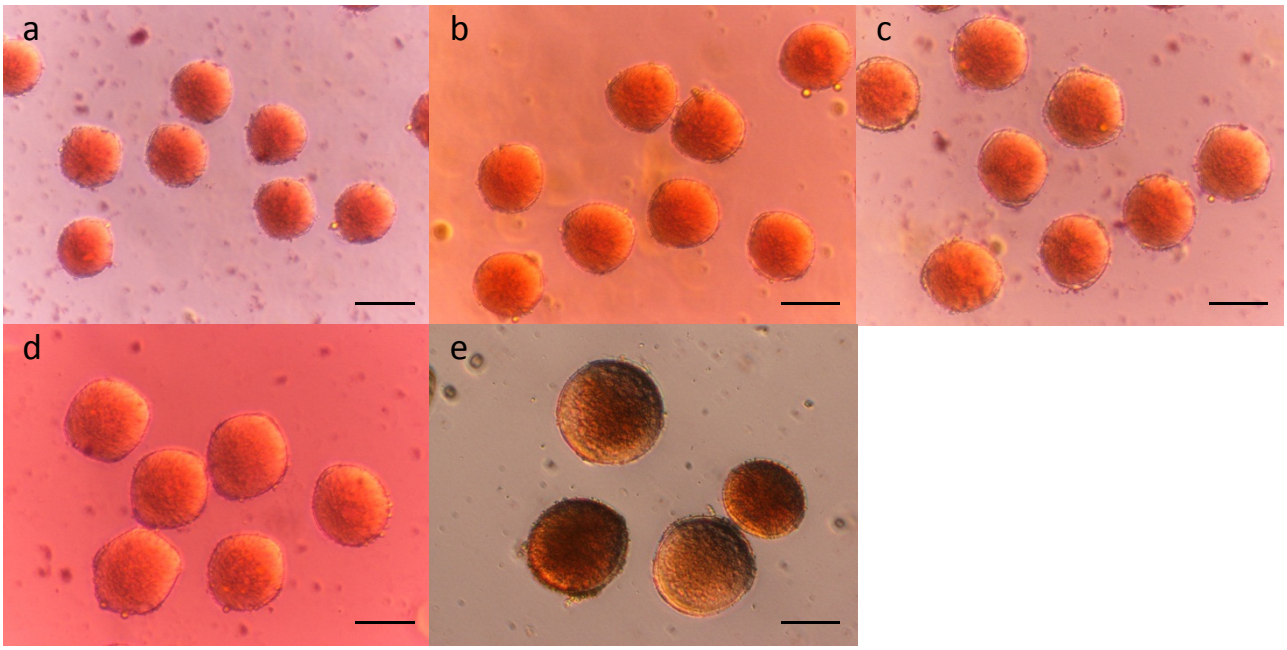


Figure 4

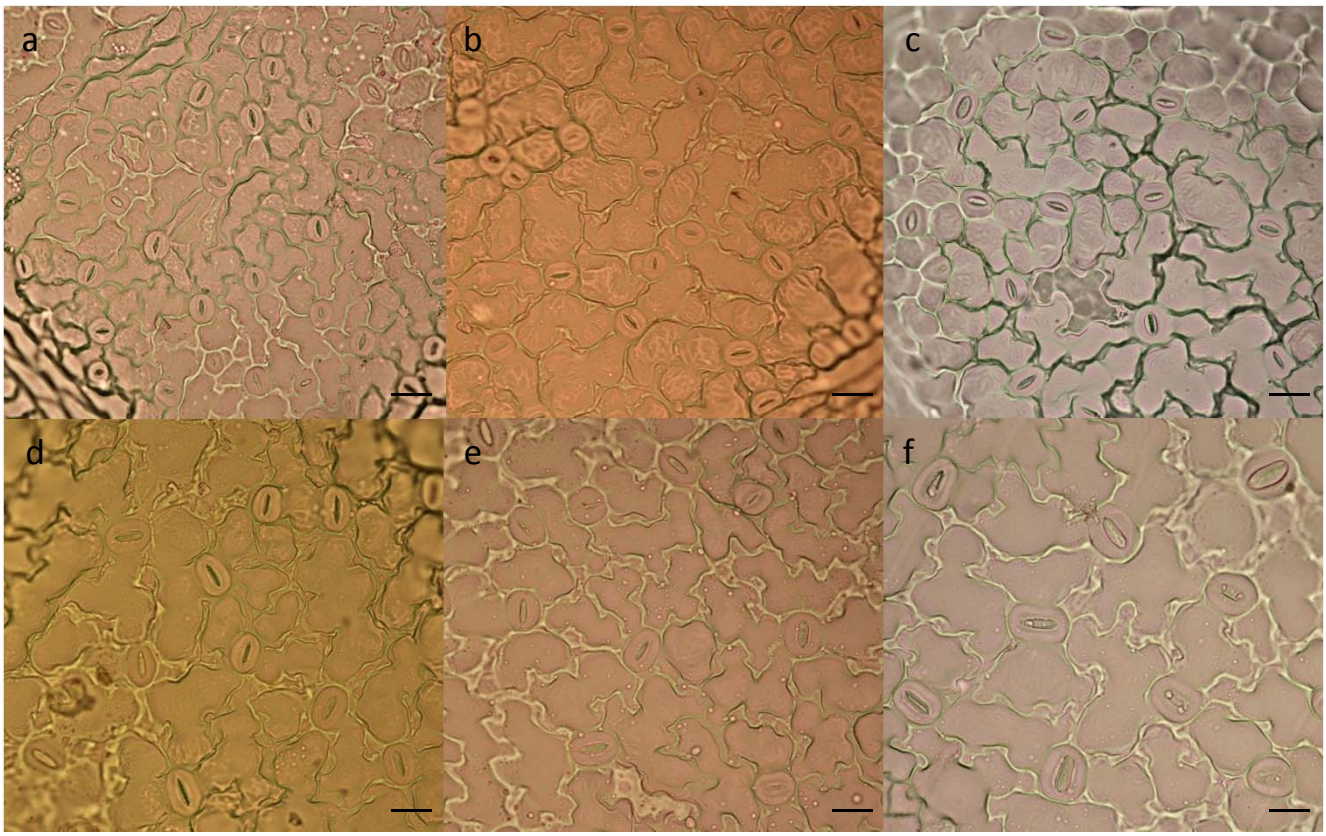


Figure 5

Figure 6

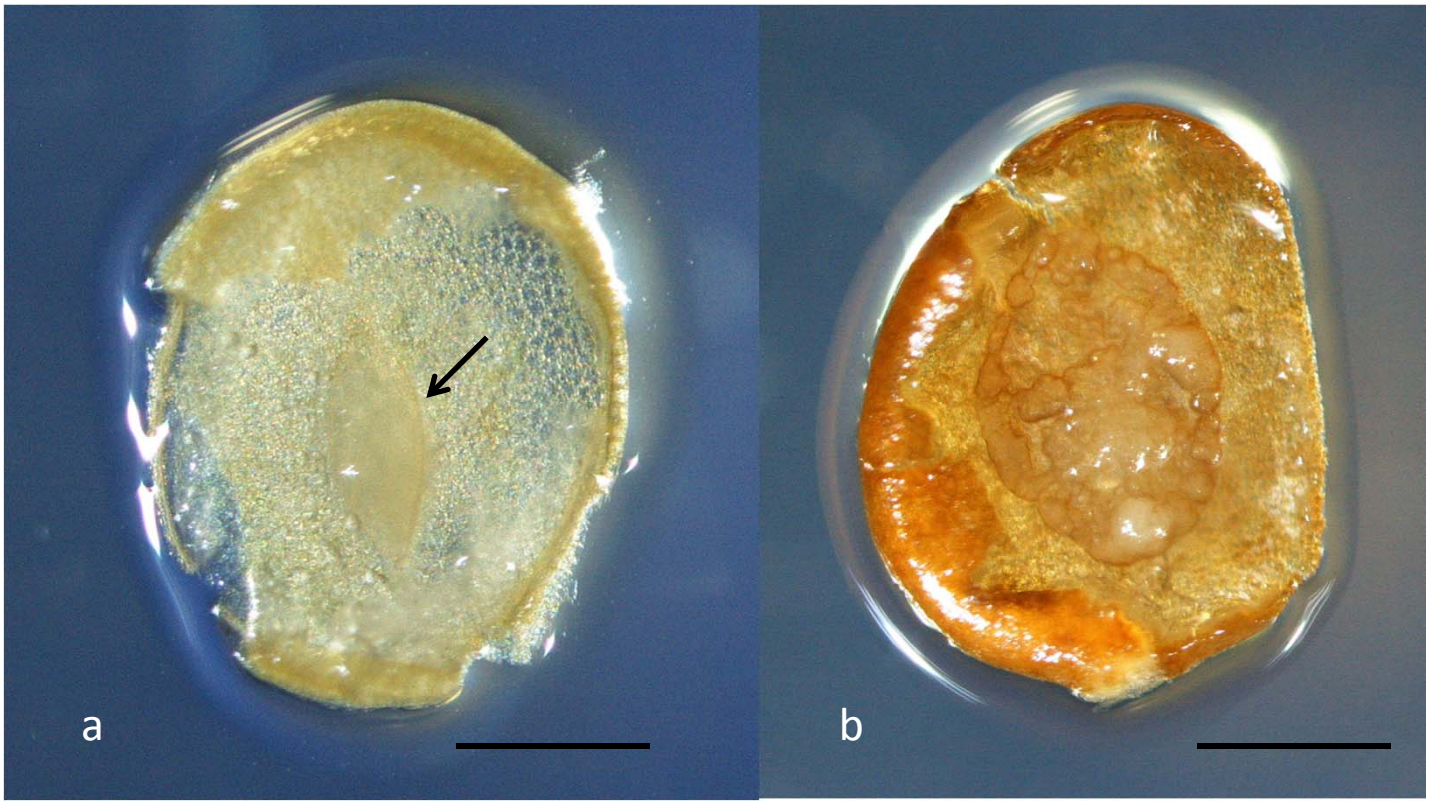
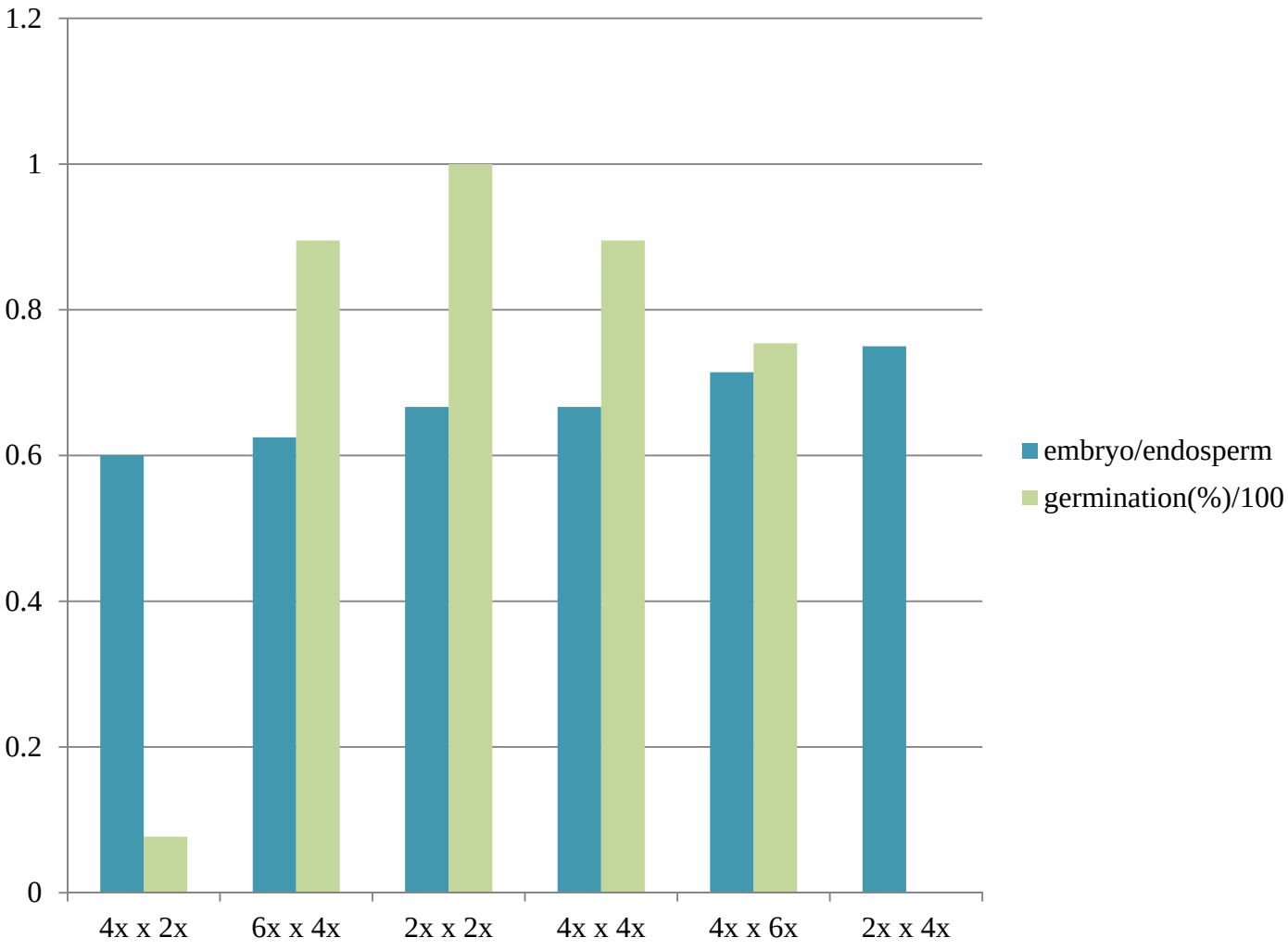


Figure 7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0.6
0.4
0.2
0
4x x 2x
6x x 4x
2x x 2x
4x x 4x
4x x 6x
2x x 4x
embryo/endosperm
germination(%)/100

Figure 8