Plant regeneration with maintenance of the endosperm ploidy level by endosperm culture in *Lonicera caerulea* var. *emphyllocalyx*

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Abstract

An endosperm culture of Haskap (*Lonicera caerulea* var. *emphyllocalyx*) was established to develop polyploid plants and investigate the regeneration ability of the endosperm. Based on histological analysis of embryo and endosperm development, endosperms at the globular to early torpedo-stages of developing embryos were used to initiate an endosperm culture. Formation of shoot primordia was observed on Murashige and Skoog (MS) medium (1962) containing benzyladenine (BA) and indole-3-butyric acid (IBA). Shoot primordium formation was confirmed in some genotypes with regeneration frequencies ranging between 1.9% to 10.0%. These proliferated on ½ MS medium containing 2.89 μM gibberellic acid (GA_3), and then elongated and rooted on MS medium containing 0.44 μM BA and 2.89 μM GA_3. These shoots developed into plantlets on ½ MS medium. Plantlets maintained ploidy of the

endosperm following flow cytometric analysis, thus confirming that these were derived from the endosperm. These results indicated that endosperms were capable of regeneration.

Abbreviations

BA	6- Benzyladenine
CH	Casein hydrolysate
DAP	Days after pollination
DAPI	4′, 6-Diamidino-2-phenylindole
GA_3	Gibberellic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog (1962) medium
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulator
TDZ	Thidiazuron

Introduction

The genus *Lonicera* belongs to the family Caprifoliaceae and comprises more than 200 species (Naugžemys et al. 2007). *L. caerulea* L. (blue honeysuckle) is an important species that is distributed in the northern regions of both Eurasia and North America (Thompson and Chaovanalikit 2003). In Japan, *L. caerulea* var. *emphyllocalyx* Nakai grows in cold-climate regions, especially Hokkaido, and has been cultivated as a commercial berry crop. *L. caerulea* var. *emphyllocalyx* is known as Haskap in the Ainu language used by the indigenous Ainu people of Hokkaido. Fruits of Haskap are sour to sweet in taste, and they are rich in nutrients such as anthocyanins, minerals, and vitamins (Terahara et al. 1993; Anetai et al. 1996; Tanaka et al. 1998). Haskap is known as a functional food, and food products such as juice, wine, and

jam made from Haskap are widely used. Recently, Haskap was introduced to North America as a new berry crop (Thompson 2006).

To breed this berry, Takada et al. (2003) evaluated the eating qualities and some horticultural characteristics of wild species in Japan, and made some elite selections. A critical problem in the cultivation of Haskap is that wild species bear small fruits with a thin pericarp. Therefore, harvesting these fruits is laborious, and the quantity of fresh fruits harvested barely meets market demands. Therefore, fruit yield and other traits must be improved to increase commercial production.

Polyploidy of *L. caerulea* has been induced by colchicine treatment, and several polyploid berry crops have yielded larger fruit size (Lyrene 1997; Sasnauskas et al. 2007). Suzuki et al. (2007) has induced tetraploidy in Haskap plants by *in vitro* chromosome doubling using colchicine treatment. On the other hand, triploid plants also produce large-sized fruits, and these have been developed from endosperm cultures in some plant species (reviewed by Thomas and Chaturvedi 2008), including fruit crops such as *Actinidia chinensis* (Gui et al. 1993), *Citrus* species (Gmitter et al. 1990), and *Morus alba* (Thomas et al. 2000). Triploidy has been commonly induced by crossing tetraploid with diploid counterpart plants. However, production of triploid plants by this method is time-consuming, especially for woody plants, since it is necessary to produce tetraploid plants in order to cross them with diploid plants. Alternatively, plant regeneration from endosperm cultures is a direct method for producing triploid plants as the endosperm is a triploid tissue.

Ammal and Saunders (1952) and Plekhanova et al. (1992) have reported that native *L. caerulea* L. plants are either diploid ($2n = 18$) or tetraploidy ($2n = 36$). In this study, tetraploid Haskap strains, collected from the Yufutsu plain in Hokkaido where the predominant Haskap community is located, are used. The overall goal of this study is to establish endosperm cultures of Haskap, and induce regeneration of polyploidy plant. Moreover, embryo and

endosperm development in Haskap are also characterized using histological analysis. Confirmation of the fidelity of the endosperm origin of regenerants is also confirmed using histological analysis.

Materials and Methods

Plant materials and sample preparation

Haskap (*L. caerulea* var. *emphyllocalyx*) plants grown at the Experiment Farms, Hokkaido University, were used. Yufutsu lines No. 5, 14, 20, 35, 47, and 51, and Tomatoh 96 lines No. 10, 25, 30, and 47, all having $2n = 4x = 36$, were used in this study. These plants were collected in 1995 from natural populations, and their ages at time of this study were estimated to be 20-30 years old. In addition, line 02Has1 ($2n = 4x = 36$), a 4 year- old seedling, was also used.

For histological observations of embryo and endosperm development, Yufutsu lines No. 20 and 51 were self-pollinated. In addition, crosses of lines Yufutsu No. 14 × Yufutsu No. 35 were made. Flowers were emasculated prior to anthesis and hand-pollinated with self or cross pollen. Ovaries were collected periodically, 1 to 35 days after pollination (DAP).

For endosperm culture, cross pollinations were performed in six combinations by using several Haskap lines as mentioned above. All ovaries were collected 21to 31 DAP.

Histological study of developing embryos and endosperms

For histological investigations of developing embryos and endosperms, ovaries were collected and prepared as described previously by Hoshino et al. (2000). Harvested ovaries were fixed in a formalin:acetic acid:50% ethanol (FAA, 5:5:90; v/v/v) solution for 2 d at room temperature. Then, samples were gradually dehydrated and cleared in a series of ethanol and n-butanol solutions as follows: 95% ethanol:n-butanol:DW (4:1:5; v/v/v) for 2 h, 95%

ethanol:n-butanol:DW (5:2:3; v/v/v) for 24 h, 95% ethanol:n-butanol:DW (10:7:3; v/v/v) for 2 h, 95% ethanol:n-butanol:DW (4:1:5; v/v/v) for 2 h, 95% ethanol:n-butanol (9:11; v/v) for 2 h, absolute ethanol:n-butanol (1:3; v/v) for 2 h, n-butanol for 2 h, and finally n-butanol for 24 h. Ovule tissues were embedded in paraffin, and cut into 10-μm thick sections with a microtome (HM315; Carl Zeiss, Oberkochen, Germany). Then, sections were stained with Mayer's haematoxylin solution. Embryo and endosperm development were histologically observed under an upright microscope (Nikon Optiphot; Nikon, Tokyo, Japan).

Endosperm culture

A total of 80 ovaries were used as explants to establish endosperm cultures, and each ovary contained approximately 15-20 ovules. Harvested ovaries were washed and sterilized with 1% sodium hypochlorite solution containing 1–2 drops of polyoxyethylene sorbitan monolaurate (Tween 20) for 10 min, and then rinsed three times with sterile-distilled water. Ovules were excised from ovaries under a microscope, longitudinally cut into two sections, and integument, nucellar tissue, and embryo were removed. Excised endosperms were incubated on a Murashige and Skoog (MS) basal medium (1962) containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum (Wako Pure Chemical Industries, Ltd., Tokyo, Japan). The basal medium was supplemented with various plant growth regulators (PGRs), including auxins, either indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA), and cytokinins, either benzyladenine (BA) or thidiazuron (TDZ). All PGR concentrations and combinations are shown in Table 1. The pH of the medium was adjusted to 5.8 prior to autoclaving at 120 C for 20 min. At least 30 explants were placed in 90 × 20-mm Petri dishes. A total of 5 replications per treatment or treatment combination was used. Plates were maintained in a controlled growth environment chamber at 20 C under 24 h photoperiod (35 μmol m^{-2} s^{-1}) provided by 40 watt fluorescent tubes.

Endosperm-derived calli with shoot primordia were transferred onto a ½ MS medium supplemented with 2.89 μM gibberellic acid (GA_3). For shoot elongation, proliferating shoot primordia were transferred onto a ½ MS medium supplemented with 0.44 μM BA and 2.89 μM GA_3. Elongated shoots, of 1–2 cm in length, were cultured onto either ½ MS medium or ¼ MS medium supplemented with either 1.45 μM or 2.89 μM GA_3 in combination with either 0.25 μM or 0.49 μM IBA. The latter medium, ¼ MS, contained 3 g l^{-1} gellan gum.

Cultures were placed in 120 × 25-mm test tubes and maintained at 20 C under 24 h illumination (35 μmol m^{-2} s^{-1}) provided by 40 watt fluorescent tubes. Subculturing was performed every 4–5 weeks.

Ploidy analysis using flow cytometry

The nuclear DNA contents of calli and leaves of plantlets derived from *in vitro* culture of endosperms were measured with a flow cytometer (Partec PA; Partec GmbH, Münster, Germany) using the protocol described by Mishiba et al. (2000). Leaves of Yufutsu No.47 (2*n* = 4*x* = 36) were used as an internal standard. Briefly, sample tissues were chopped with nuclei extraction buffer of CyStain UV precise P (Partec, Münster, Germany). Crude nuclear samples were stained with 4′, 6-diamidino-2-phenylindole (DAPI) solution, containing 10mM Tris, 50mM sodium citrate, 2mM $MgCl_2$, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100 and 2mgl^{-1} DAPI at pH 7.5. A total of 3 measurements were recorded per sample.

Chromosome counts

Root tips of plantlets derived from endosperm cultures were pretreated with ice water for 24 h and fixed with acetic acid:ethanol (1:3) at 4 C overnight. These root tips were treated with an enzyme mixture at 37 C for 20 min, according to the procedure of Shibata and Hizume (2002), but with several modifications. The enzyme mixture was prepared by replacing the citrate

buffer (0.01 M citric acid and 0.01 M trisodium citrate dehydrate) with 2× SSC and 10 mM EDTA. Root tips were rinsed with DW and squashed in 45% acetic acid. Cover slips were removed by freezing glass slides in liquid nitrogen, slides were dried at 37 C, and stained with DAPI solution (Sahara et al. 2003).

All slides were observed using a fluorescence microscope (Leica 6000E; Leica Microsystems AG, Wetzlar, Germany). A total of 5 measurements were recorded per sample.

Results

Histological observations of embryo and endosperm development

Based on histological observations of developing embryos of Haskap, the following observations were made (Fig. 1). Prior to pollination, the egg apparatus and polar nuclei were observed in the embryo sac; moreover, both synergids, with nucleus and cytoplasm, were located at the micropylar end. In contrast, chalazal ends of synergids were vacuolated (Fig. 1a), and no degenerated synergids were observed. The egg cell was located on the chalazal side of the synergids, and developed a vacuole at the synergid end (Fig. 1a, b). Fused and nonfused polar nuclei were observed in the central cells as shown in Fig. 1a and Fig. 1c, respectively. The fused polar nuclei were positioned near the egg cell (Fig. 1a). The fused polar nucleus was larger than the synergid as well as the egg cell. A number of cells developed at the chalazal end of the embryo sac. These were likely developing antipodals or nucellar tissue that entered the embryo sac. Haskap ovules had a single integument (unitegmic ovule), which was composed of one cell layer with a thick cell wall.

Division of the primary endosperm nucleus was initiated 3 DAP along with cell wall formation. At 5 DAP, the division was observed in most embryo sacs. Thin cell walls were formed between each endosperm nucleus at 3 and 5 DAP (Fig. 2a). Therefore, endosperm development was considered to be cellular type wherein cell wall formation follows the

division of endosperm nuclei. Moreover, at this time point, endosperm nuclei, ~10 μm in size, were positioned along the periphery of the embryo sac (Fig. 2a), the total number of nuclei was ~10, and nucellar tissues began to degenerate. At 7 DAP, cell walls were clearly formed among endosperm nuclei (Fig. 2b), and the endosperm was composed of 30 to 60 cells, uniformly distributed within the embryo sac. At 14 DAP, endosperm cells continued to divide (Fig. 2c), and the endosperm was composed of more than 100 cells. At 21 DAP, almost all nucellar tissues were degenerated, and the endosperm occupied the entire ovule (Fig. 2d). Starch accumulation was observed from this stage onwards (Fig. 2e). At 35 DAP, endosperm cells continued to divide and these were rich in starch grains (Fig. 2f).

In contrast to the endosperm, zygotes did not exhibit cell division activity until 7 DAP. However, at 14 DAP, embryos comprised of 5 to 9 cells were clearly observed (Fig. 3a). At 21 DAP, globular to heart-shaped embryos were formed (Fig. 3b, c); these globular embryos had flat suspensors, consisting of 3 to5 cells. Both suspensors and embryos were rich in cytoplasm. At 35 DAP, torpedo-shaped embryos were observed.

These morphological observations of both embryo and endosperm development were utilized to confirm the developmental stage of endosperms used as explants for in vitro culture.

Endosperm culture

Using the histological/morphological observations described above, endosperm formation was determined to begin ~3 DAP. Moreover, endosperm development at 3 to 7 DAP could not be easily distinguished from the embryo in excised ovules under a dissecting microscope. In ovules dissected at 14 DAP, small endosperms attached to nucellar tissues could not be separated; however, culture of segments of endosperms with nucellar tissues turned brownish in color in both solid and liquid media (data not shown). Endosperms could be detached from

nucellar tissues at 21 DAP; while, those at 35 DAP, endosperms were difficult to separate from the seed coat as most fruits were ripe and seed formation was almost complete at that time.

As endosperm development in the ovule is influenced by environmental conditions in the field, endosperms in ovules were at different stages of development. Therefore, endosperms at 21–31 DAP were used as explants for in vitro culture. Those endosperms with embryos at globular to early torpedo-shaped embryos were used as explants; whereas, those with torpedo-shaped embryos were discarded as it was difficult to separate them from seed coats.

Endosperms were ~1 mm in diameter in initial cultures (Fig. 4a); however, after 3 weeks, callus formation was observed on all media except for the medium lacking any PGRs. The frequency of callus formation varied from 6.7% to 88.9% after 15 weeks of culture (Table 1). High frequencies of callus formation were obtained on media supplemented with 4.44 μM BA and 0.54 μM NAA, 4.44 μM BA and 0.49 μM IBA as well as 2.22 μM BA and 4.92 μM IBA (Table 1). Overall, endosperm-derived calli on media containing BA showed a higher tendency to proliferate than those on media containing TDZ. Vigorously growing endosperm-derived calli that were > 1 cm in diameter were observed on media supplemented with BA in combination with either IBA or NAA.

After 8 weeks of culture, the number of brown-colored calli rapidly increased, regardless of PGR combinations in the medium. Calli induced on media supplemented with 2.27 μM TDZ and 2.22 μM BA or with 4.44 μM BA and 0.49 μM IBA showed more than 50% browning. Adding 3 g l^{-1} activated charcoal or 5 g l^{-1} polyvinylpolypyrrolidone (PVP) to the medium did not prevent browning of calli.

Shoot and plantlet regeneration from endosperm-derived calli

Endosperm-derived calli were continually subcultured every 4–5 weeks, without splitting them into smaller size segments, onto fresh media. After 10–15 weeks of culture, differentiation of calli into shoot primordia was induced on media supplemented with 2.22 μM BA and 0.49 μM IBA, 4.44 μM BA and 0.49 μM IBA, and 4.44 μM BA and 4.92 μM IBA (Table 1). The highest frequency of shoot formation was obtained on medium containing 2.22 μM BA and 0.49 μM IBA (Table 1).

To confirm the genotype-dependent shoot regeneration, endosperms derived from additional five different crosses were cultured on media supplemented with either 2.22 μM BA and 0.49 μM IBA or 4.44 μM BA and 4.92 μM IBA. In combination with the results of Table 1, the differences of genotype-dependent shoot regeneration were summarized in Table 2. Shoot primordium formation was observed on explants derived from four crosses and the frequencies were ranging between 1.9% to 10.0%. Endosperm-derived calli regenerating shoots were compact and either green or green with white segments in color (Fig. 4b). Shoot primordia were 1–2 mm in length (Fig. 4c). A total of 11 lines of shoot primordia were obtained.

Shoot primordia failed to elongate, and within three weeks, most regenerating calli began to turn brownish in color. Therefore, to promote shoot elongation, shoot primordia were excised and transferred to different elongation media. For shoot primodia transferred to MS medium supplemented with 4.44 μM BA alone or with 4.92 μM IBA in combination with 8.88 μM or 13.32 μM BA, no elongation was observed. Whereas, shoot primordia transferred to ½ MS medium supplemented with 2.89 μM GA_3 began to proliferate within one month (Fig. 4d), and subsequently when transferred to fresh medium of the same composition along with 0.44 μM BA, shoots began to elongate, 1-2 cm in length and occasionally developed roots (Fig. 4e).

When these shoots were transferred to a ½ MS medium without PGRs, most shoots

rooted and developed into plantlets (Fig. 4f). However, for those shoots that failed to develop roots, they were transferred to other media to induce rooting. Shoots transferred to medium consisting of ¼ MS supplemented with 0.25 μM IBA and 1.45 μM GA_3 was optimum to induce rooting (47%).

A total of five plantlets were obtained from 11 lines of shoot primordia. These five regenerated plantlets were propagated *in vitro*. However, these showed morphological abnormalities such as slow growth and vitrification, which might be attributed to ploidy levels and/or cultural environments. Thus far, these plantlets have not been acclimatized despite several attempts to do so.

Determination of ploidy of endosperm-derived calli and plantlets

Flow cytometric analysis showed that endosperm-derived calli as well as all five regenerated plantlets had 6C DNA content (Fig. 5a, b), indicating hexaploidy. One plantlet was used for chromosome count, and it revealed an aneuploid number of chromosomes ($2n = 6x + 1 = 55$) (Fig. 6).

Discussion

Endosperm-derived plantlets were produced from endosperm cultures on the basis of histological analysis of ovules and developing embryos. Endosperms at 21–31 DAP, at the globular to early torpedo-shaped embryo stages of development, were successfully induced to develop callus, and these in turn were capable of regenerating shoots. In addition, this developmental stage was suitable for isolation of endosperms as they were not attached to the embryo, nucellar tissues, or seed coat.

In previous studies, a high frequency of callus induction from endosperm cultures of various plant species required presence of both auxins and cytokinins (reviewed by Thomas

and Chaturvedi 2008). In this study, the frequency of callus induction was low on MS media supplemented with auxin (IBA or NAA) alone; however, in the presence of 4.44 μM BA and 0.54 μM NAA or 4.44 μM BA and 0.49 μM IBA, a high frequency of callus induction was obtained. Yang et al. (2002) reported that cell number and cell division activity in *Oryza sativa* were regulated by the levels of cytokinin in the endosperm. Therefore, in endosperm cultures, the presence of cytokinins (and appropriate concentration) in combination with auxins might play an important role in callus induction and subsequent organogenesis from the endosperm.

It was reported that regenerated plants derived from endosperm cultures of diploid plants exhibited variations in chromosome numbers. In *Juglans regia* (Tulecke et al. 1988), *Citrus* species (Gmitter et al. 1990), *Acacia nilotica* (Garg et al. 1996), *Passiflora foetida* (Mohamed et al. 1996), and *Morus alba* (Thomas et al. 2000), endosperm-derived plants were triploid, thus retaining ploidy of endosperms. In contrast, diploid and triploid plants were obtained from endosperm cultures of *Azadirachta indica* (Chaturvedi et al. 2003). Variations in number of chromosomes of endosperm-derived plants of rice were also observed (Bajaj et al. 1980). In addition to triploid plants, aneuploids and polyploids were alsoreported. In *A. chinensis*, most endosperm-derived plants were aneuploid, and only a few plants were either diploid or triploid (Gui et al. 1993). The recovery of diploid plants was attributed to incomplete fertilization, chromosome loss, or development from maternal tissues (Gui et al. 1993). In this study, endosperms derived from crosses between tetraploid ($2n = 4x = 36$) strains were utilized for culture. Flow cytometric analysis of regenerated plantlets revealed 6C DNA content, indicating maintenance of the ploidy level of the haskap endosperm. The chromosome numbers in root tip cells were confirmed, and an aneuploid ($2n = 6x + 1 = 55$) plant was also detected. Cytological observations of *Zephyranthes grandiflora* (Kapoor and Tandon 1963) and *Chrysanthemum carinatum* (Kapoor and Tandon 1964) revealed that in

general endosperms were triploid, but that nuclei with higher polyploidy and with aneuploidy also occurred in a small number of cells. It is likely that the aneuploid plantlet regenerated from the endosperm culture is derived from aneuploid cells present in the haskap endosperm.

The endosperm differentiates into a nourishing organ for embryo growth and degenerates at the time of seed maturation or germination. The programmed cell death during endosperm development is accompanied by an increase in nuclease activity and the degradation of nuclear DNA in maize and wheat (Young et al. 1997; Young and Gallie 1999). Huang et al. (2006) have suggested that callus formation from the endosperm is influenced by the programmed cell death during endosperm development in *Lycoris* spp. In *Lycoris* spp., endosperms at 25–30 DAP developmental stage readily produce calli; however, older endosperms showed dramatic reduction in callus formation. When endosperms derived from embryos 35 DAP, no callus formation is observed and endosperm cultures turned brown in color. Huang et al. (2006) have demonstrated that the decline or loss of the proliferation capacity of the endosperm is closely related to the nuclear DNA degradation caused by programmed cell death. They have also demonstrated that pre-programmed cell death in the endosperm is responsible for inducing nuclear DNA degradation in *in vitro* cultures. In this study, the number of brown-colored calli increased rapidly after 8 weeks of culture. This may indicate that programmed cell death could induce browning of endosperm cultures. Consequently, genetic control of programmed cell death may have been sustained during endosperm culture of haskap. Whereas, suppression of browning will lead to an increase in the likelihood of shoot regeneration from the endosperm culture of Haskap.

The endosperm is a unique tissue that is reported to be involved in genome imprinting in flowering plants. Genome imprinting is an epigenetic phenomenon that produces differential expression of maternal and paternal genes (reviewed by Kinoshita 2007). In *Arabidopsis*, the epigenetic regulation of imprinted genes plays an important role in the

reproductive barrier observed in the endosperm of interspecific and interploidy crosses (Josefsson et al. 2006). Although the importance of endosperm cells is recognized in the epigenetic regulation of imprinted genes, the genetic information in the endosperm is not transmitted to the next generation. Endosperm culture is a unique technique that provides an opportunity for plant regeneration from endosperm tissues. Analysis of plants regenerated from the endosperm may help devise a novel approach for elucidating the genome imprinting mechanism. Moreover, the findings of genome imprinting studies may contribute to the establishment of endosperm culture systems in a wide variety of plant species.

In this study, plants derived from endosperm tissues have been obtained. These plants can be used as breeding materials for further improvement of fruit traits of Haskap.

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Table 1. Effect of PGRs on callus and shoot primordium formation from endosperms of Haskap collected from Yufutsu No. 14 × Yufutsu No. 35 cross.

Plant growth regulators (μM)		No. of endosperm cultured (A)	No. of endosperm showing callus formation (B)	Frequency of callus formation (B/A × 100%)	No. of callus with shoot primordia (C)	Frequency of shoot primordium formation (C/A × 100%)
None		30	0	0	0	0
BA						
2.22		30	22	73.3	0	0
4.44		30	17	56.7	0	0
BA	IBA					
2.22	0.49	30	19	63.3	3	10.0
2.22	4.92	75	62	82.7	0	0
4.44	0.49	30	25	83.3	1	3.3
4.44	4.92	75	57	76.0	2	2.7
BA	NAA					
2.22	0.54	60	45	75.0	0	0
2.22	5.37	60	16	26.7	0	0
4.44	0.54	45	40	88.9	0	0
4.44	5.37	30	15	50.0	0	0
TDZ						
2.27		30	20	66.7	0	0
4.54		30	18	60.0	0	0
TDZ	IBA					
2.27	0.49	60	32	53.3	0	0
2.27	4.92	60	34	56.7	0	0
4.54	0.49	30	17	56.7	0	0
4.54	4.92	75	52	69.3	0	0
TDZ	NAA					
2.27	0.54	40	16	40.0	0	0
2.27	5.37	60	36	60.0	0	0
4.54	0.54	30	12	40.0	0	0
4.54	5.37	60	4	6.7	0	0

Endosperms were excised from ovaries harvested 21-31 DAP. A single callus was induced from single endosperm explants. Subculture was performed every 4-5 weeks without splitting. Data were recorded after 15 weeks of culturing.

Table 2. Shoot regeneration from callus cultures of endosperm derived from different crosses in Haskap.

Cross combination		No. of explants (A)	No. of explants with callus (B)	Frequency of callus formation (B/A × 100%)	No. of explants with shoot primordia (C)	Frequency of shoot primordia formation (C/A × 100%)
Female	Male					
Yufutsu No. 14	Yufutsu No. 35	30	19	63.3	3	10.0
Tomatoh 96 No. 47	Yufutsu No. 47	63	38	60.3	3	4.8
Yufutsu No. 14	Yufutsu No. 35	75	57	76.0	2	2.7
Tomatoh 96 No.10	Yufutsu No. 5	53	51	96.2	1	1.9
Tomatoh 96 No.10	Tomatoh 96 No.25	218	137	62.8	0	0
02Has1	Tomatoh 96 No.10	200	17	8.5	0	0
02Has1	Tomatoh 96 No.30	79	11	13.9	2	2.5

Endosperms were excised from ovaries harvested 21-31 DAP. A single callus was derived from a single endosperm explant. Subculture was made without dividing callus. Data were recorded after 15 weeks of culturing.

Excised endosperms of the first two crosses listed were incubated on MS basal medium supplemented with 2.22 μM BA and 0.49 μM IBA; while all excised endosperms of all remaining crosses were incubated on MS basal medium supplemented with 4.44 μM BA and 4.92 μM IBA.

Legends to figures

Fig. 1. Longitudinal sections of Haskap (Yufutsu No.20) ovules before pollination.

(a) The egg apparatus and fused polar nuclei (arrowhead) are in focus. Vacuoles (arrows) were observed in both synergids. The egg cell was located on the chalazal side of the synergids. Bar = 10 μm. (b) The nucleus of the egg cell (arrowhead) was observed. A vacuole (arrow) developed at the synergid end. Bar = 10 μm. (c) Two polar nuclei (arrowheads) were observed in the central cell. Bar = 10 μm.

EC, egg cell; Sy, synergid.

Fig. 2. Different stages of endosperm development in Haskap. Sections following (a) to(e) and (f) were obtained from self-pollinated Yufutsu No.20, and hybridization of Yufutsu No. 14 ×Yufutsu No. 35, respectively. (a) Endosperm tissues at 5 DAP. Thin cell walls (arrows) were formed between each endosperm nucleus (arrowheads). Bar = 20 μm. (b) Endosperm tissues at 7 DAP. Endosperm cells were uniformly distributed in the embryo sac. Bar = 20 μm. (c) Endosperm tissues without starch grains at 14 DAP. Bar = 100 μm. (d) Endosperm tissues accumulated starch grains at 21 DAP. Nucellar tissues were degenerated, and the endosperm occupied the entire ovule. Bar = 100 μm. (e) Starch grains in the endosperm cell at 21 DAP. Bar = 20 μm. (f) Endosperm tissues rich in starch grains at 35 DAP. Bar = 100 μm.

em, embryo; en, endosperm; and sg, starch grain.

Fig. 3. Longitudinal sections of embryos at different stages of development in Haskap (Yufutsu No.20). (a) Eight-celled embryo at 14 DAP. Bar = 10 μm. (b) Globular embryo at 21 DAP. Bar = 25 μm. (c) Heart-shaped embryo at 21 DAP. Bar = 25 μm.

em, embryo; en, endosperm.

Fig. 4. Plantlet regeneration from endosperm-derived calli in Haskap. Endosperms were

derived from the cross Yufutsu No. 14 × Yufutsu No. 35.

(a) Endosperm in the initial culture. Bar = 0.5 cm. (b) Endosperm-derived callus with shoot primordia on MS medium supplemented with 4.44 μM BA and 4.92 μM IBA. Bar = 0.5 cm. (c) Shoot primordia derived from endosperm callus incubated on MS medium supplemented 4.44 μM BA and 4.92 μM IBA. Bar = 0.5 mm. (d) Shoot primordia that proliferated on1/2 MS medium supplemented with 2.89 μM GA_3. Bar = 0.2 cm. (e) Shoots from shoot primordia on ½ MS medium supplemented with 0.44 μM BA and 2.89 μM GA_3. Bar = 0.5 cm. (f) Plantlets developed from shoots grown on ½ MS medium. Bar = 1 cm.

Fig. 5. Histograms of the relative fluorescence intensity of nuclei isolated from callus and plantlet derived from endosperms. (a) Endosperm-derived callus (6C) after 15 weeks of culturing on MS medium supplemented with 2.22 μM BA and 0.49 μM IBA. (b) Endosperm-derived plantlet (6C) from MS medium supplemented with 2.22 μM BA and 0.49 μM IBA. Leaves of Yufutsu No.47 ($2n = 4x = 36$) were used as an internal standard (4C).

Fig. 6. Chromosomes in root-tip cells of a plantlet derived from endosperm culture. An aneuploid ($2n = 6x + 1 = 55$) metaphase was observed. The plantlet was derived from MS medium supplemented with 2.22 μM BA and 0.49 μM IBA.

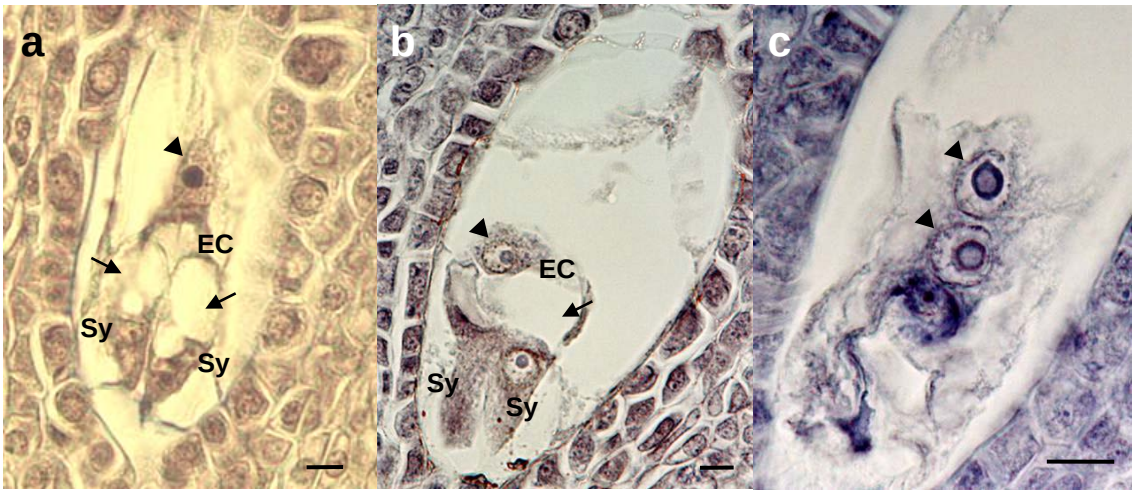


Fig. 1

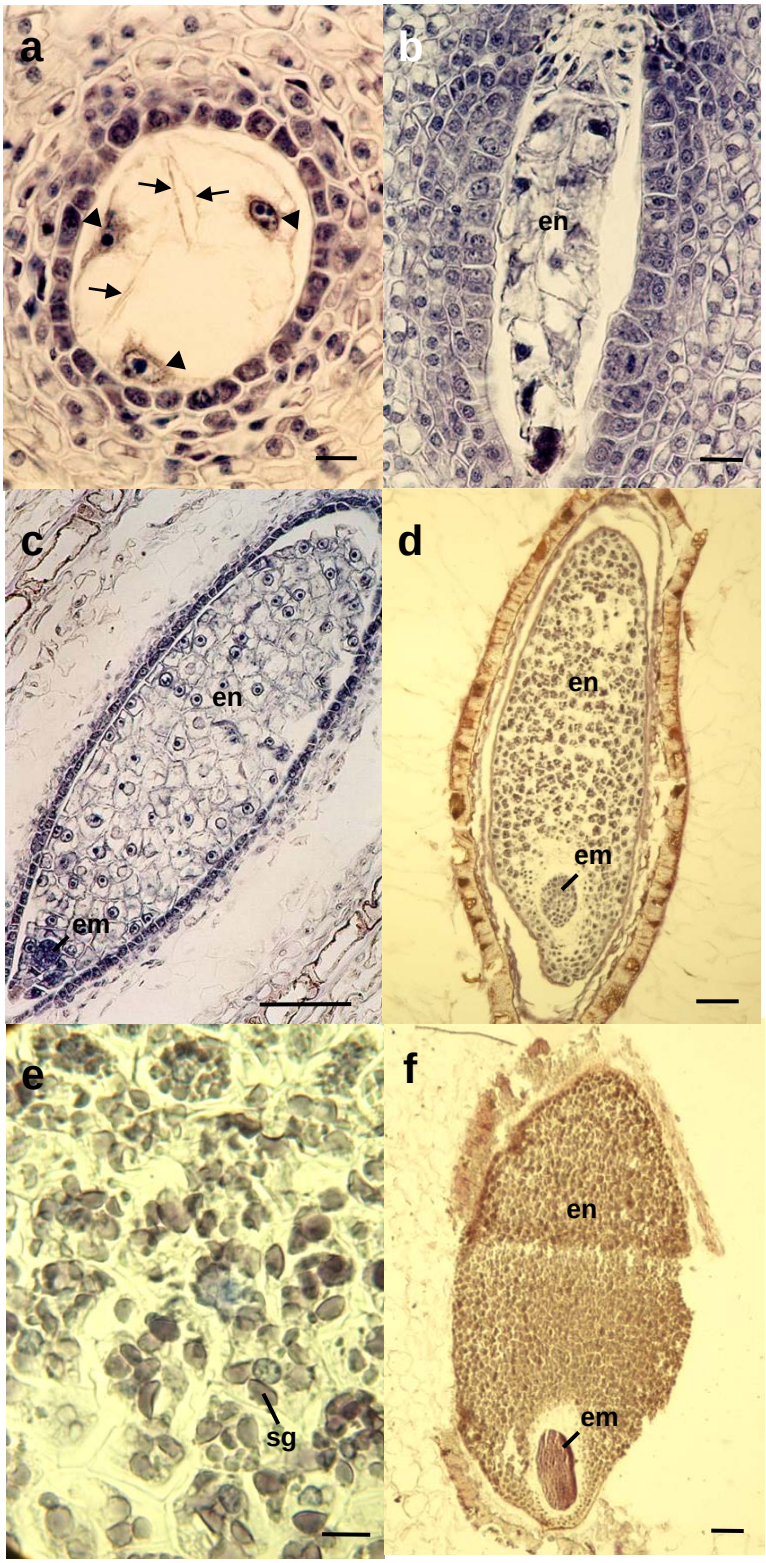
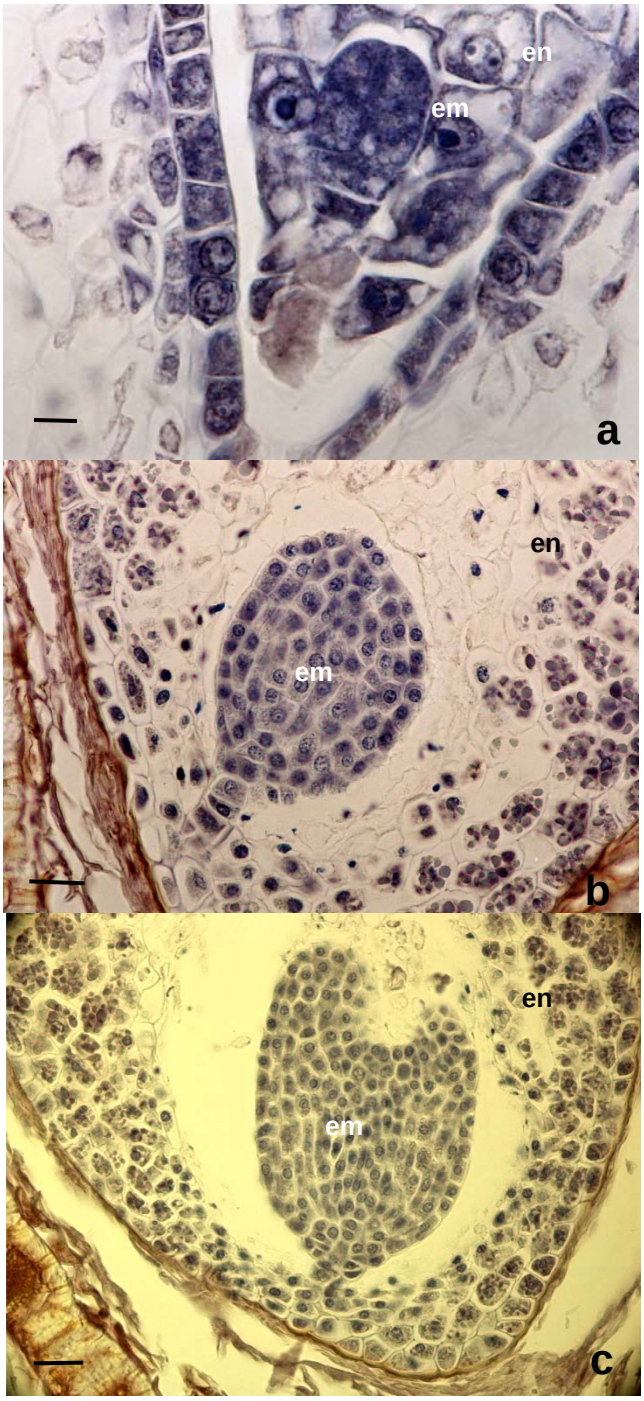


Fig. 2



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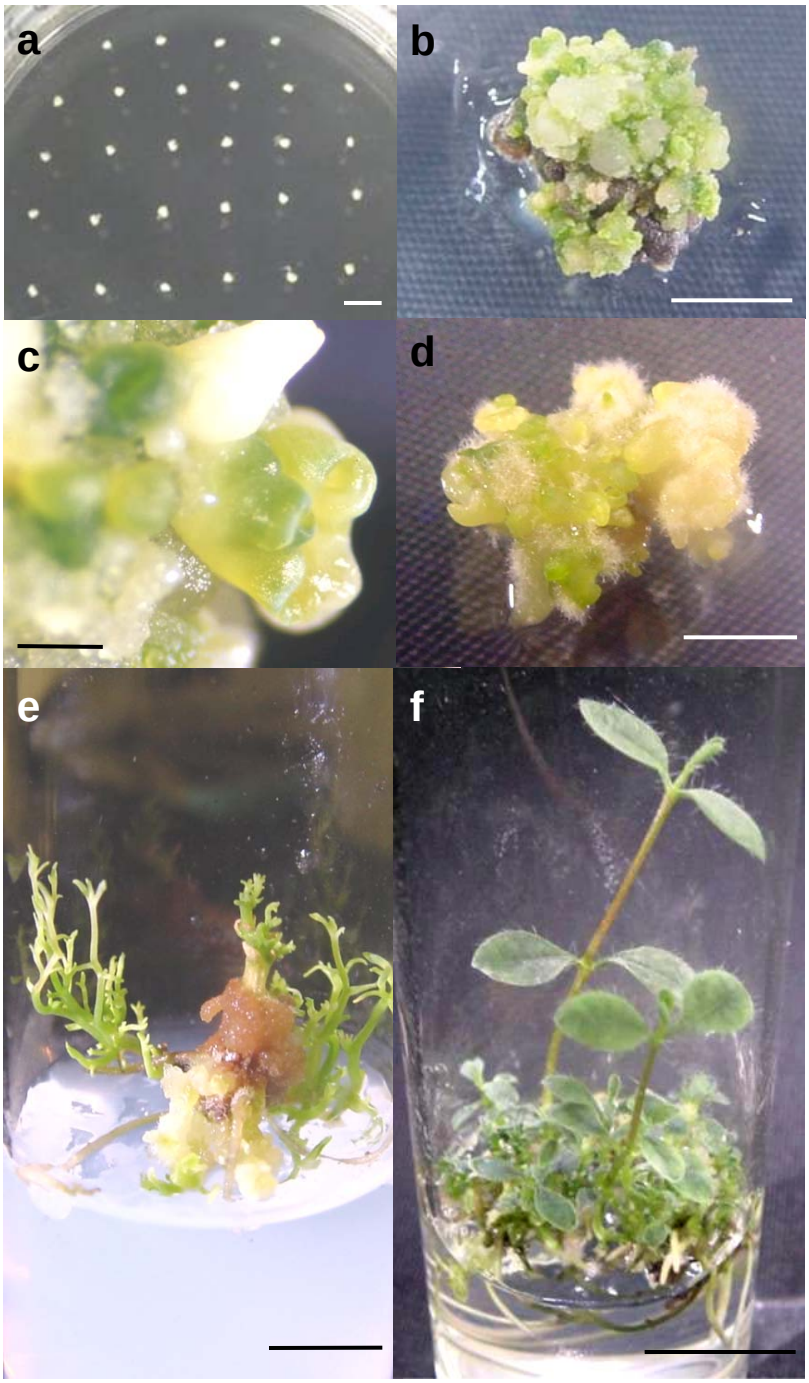
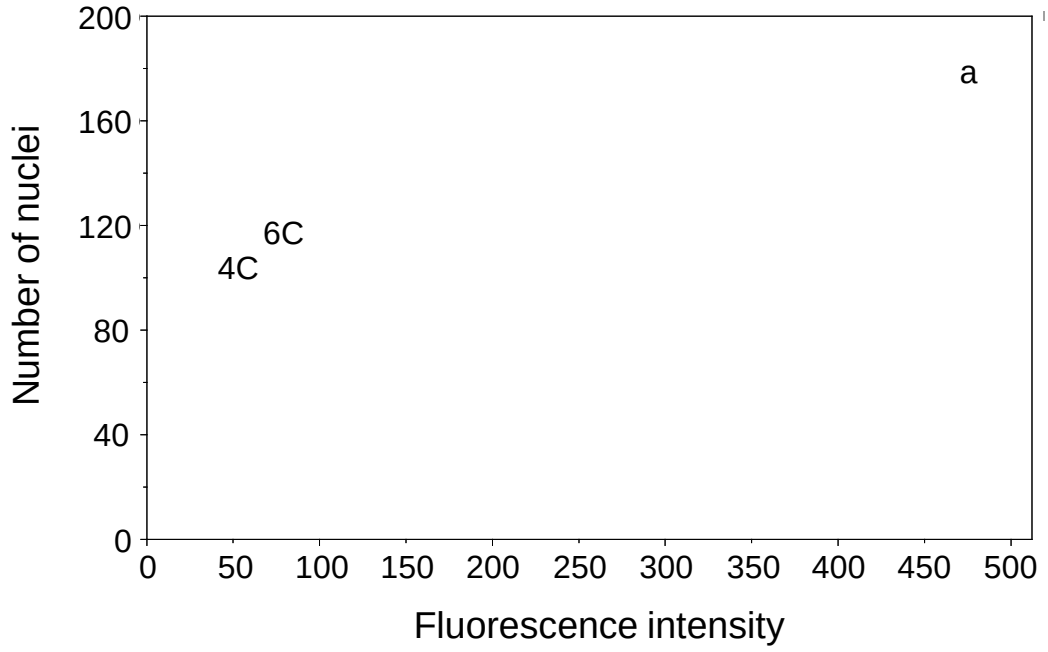


Fig. 4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6C
4C
Number of nuclei
Fluorescence intensity
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40
80
120
160
200
0
50
100
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300
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450
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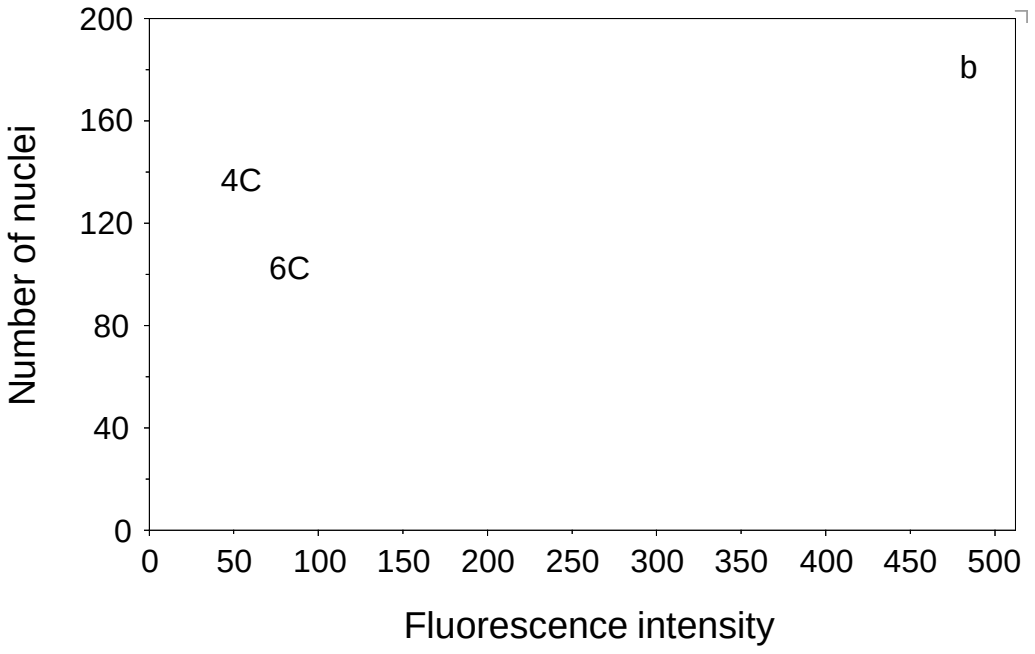
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4C
6C
Number of nuclei
Fluorescence intensity
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Fig. 5

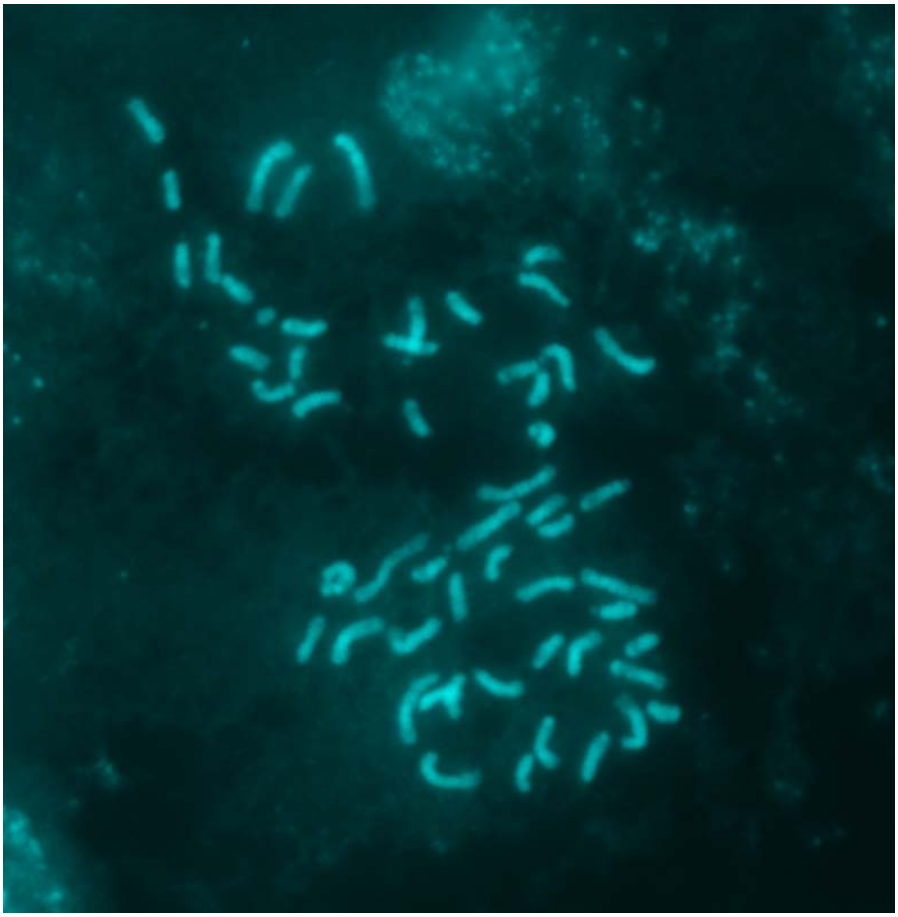

Fig. 6