Unreduced spore formation on the chimera pinnule induced in artificially produced haploid Athyrium niponicum

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Research Article

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Abstract

Haploid sporophytes of *Athyrium niponicum* with $2n = 40$, were produced artificially by induced apogamy *in vitro* for the first time. They were subsequently transplanted into pots and two of them have been cultivating for the investigation of sporogenesis and/or chimera induction of haploid sporophytes for more than 20 years since 2001. Haploid *A. niponicum* were sterile, however, an abnormal chimera pinnule was induced in the frond of one haploid plant and many sporangia with spores were produced on the pinnule in 2021. Approximately 32 spores per one sporangium were produced and they were almost same in size. Of 20 gametophytes arising from spores, five induced sporophytes with $2n = 40$. The ploidy levels between gametophytes and sporophytes produced were the same. Our study demonstrated that unreduced spores $x = 40$ are produced regularly on the chimera pinnule in haploid *A. niponicum*. It might consider from the results that the sporogenesis of unreduced spores in Braithwaite scheme occurs spontaneously by any gene mutation in a cell of the sporophyte with univalents and the unreduced spore formation might occur similarly by any gene mutation in the sterile triploid ferns with univalents. Our study is very important to know the origin of apogamous triploid hybrid ferns of the ABC type.

Introduction

Many haploid sporophytes of ferns were produced artificially by induced apogamy *in vitro* (Whittier and Steeves 1960, 1962, Palta and Mehra 1983, Kawakami et al. 1995, 1996, 1997, 2019). These haploid plants are very important materials to know the origin of ferns since ferns have developed by chromosome doubling and hybridization (Manton 1950, Wagner 1954, Lovis 1977). Dihaploid ferns produced were frequently used to know whether the donor plants are autotetraploid or alloplloid (Palta and Mehra 1983, Kawakami et al. 1995, 1996, 2019) and monoploid plants were used to clarify the precise base chromosome number by the behavior of meiotic chromosomes (Kawakami et al. 2019). At the same time, we consider that haploid plants become very important materials to know the origin of apogamous ferns, because the apogamous ferns, approximately 10% of all ferns (Walker 1979), produce unreduced spores and the sporogenesis of unreduced spores in Braithwaite scheme that was reported first in *Asplenium aethiopicum* (Braithwaite 1964), is carried out in the apogamous triploid hybrid ferns with all univalents such as haploids. Therefore, the occurrence of unreduced spore formation in the sterile haploid fern is considered to contribute to know the origin of apogamous triploid hybrid ferns of the ABC type. On the other hand, ploidy chimeras are reported to be induced in the fronds of haploid ferns such as haploid *Osmunda* (Kawakami et al. 2007), the unreduced spore formation might occur because of ploidy chimeras produced by chromosome doublings in the case of haploid *Athyrium niponicum*. In the present study, therefore, haploid sporophytes of *A niponicum* induced artificially *in vitro*, have been cultivating in pots for the investigation of sporogenesis and/or ploidy chimera induction for more than 20 years since 2001. Although haploid plants are sterile in general, many viable spores were obtained from the abnormal chimera pinnule of haploid *A. niponicum* in 2021. Then DNA contents were compared between gametophytes derived from spores and sporophytes induced from the gametophytes by using flow cytometry and the chromosome numbers of sporophytes were counted one by one to know the genome
content of spores and how the sporophytes were produced. In addition, the aposporous gametophyte induction was investigated here. The present study demonstrated that unreduced spores are produced regularly on the chimera pinnule of haploid plant. From the results it might suggest that apogamous triploid hybrid ferns of the ABC type also come from the sterile triploid ferns by any gene mutation such as haploid *A. niponicum*. Our study is very important to know the origin of apogamous triploid hybrid ferns of the ABC type.

**Materials and Methods**

Spores of *Athyrium niponicum* (Mett.) Hance collected in Gifu Pref., Japan, were used for axenic culture. Surface-sterilized spores of *A. niponicum* were sown on White’s (1963) inorganic salt medium supplemented with 2% glucose, 0.1% yeast extract solidified with 0.7% agar. Cultures were illuminated by a fluorescent lamp (NECFL15BR) for 12 h per day at 25 °C. After germination the cultures were kept in dark cabinet and when sporophytic shoots were observed they were exposed to light. The sporophytes were grown on agar until they developed roots. Then plants were transferred into pots. Two of them have been cultivating in pots for more than 20 years. Spores, gametophytes, and sporophytes of haploid *A. niponicum* were cultured on 1/4 strength MS (Murashige and Skoog 1962) medium supplemented with 1% sucrose and 0.7% agar. For apospory a piece of the sporophyte was culture on 1/2 strength MS medium supplemented with 3% sucrose, 0.1% casamino acid and 0.8% agar. Meiotic chromosomes were observed by fixing sporangia with 3:1 ethanol-acetic acid for 30 min at 5°C and squashing them in 2% aceto-orcein solution. For the observation mitotic chromosomes, root tips and/or young sporophytic leaves were harvested and pretreated in 0.002 M 8-hydroxyquinoline for 3 h at room temperature, fixed and hydrolyzed in the mixture of 1 M HCl and 45% acetic acid for 1 min at 60°C, and stained with 2% aceto-orcein solution. The DNA contents of nuclei in fronds were estimated by flow cytometry using a Partec Ploidy Analyzer PA (Partec Münster, Germany) (Kawakami et al. 2003).

**Results**

The chromosome number of donor *A. niponicum* was 2n = 80 (Fig. 1a). Spores of *A. niponicum* germinated in one week and sporophytic shoots were obtained after three months. The chromosome number of seven sporophytes grown from the shoots was 2n = 40 (Fig. 1b). They were haploids with half chromosome numbers of the donor sporophyte. Sporophytes with 2n = 40 were transplanted into pots after one year. Five were dead. Two have been cultivating in pots for more than 20 years since 2001. In spore mother cells of haploid sporophytes, 40 univalents were observed (Fig. 1c). Sporangia with many viable spores were not observed until 2020.

A frond with an abnormal chimera pinnule was induced in one haploid sporophyte in 2021 (Fig. 2a). Unlike other pinnules, sporangia with many spores were produced (Fig. 2b). Approximately 32 spores per one sporangium were produced and they were almost same in size (Fig. 2c). The guard cells in the chimera pinnule were not larger. They were almost same sizes as those of other pinnules in the same frond. Such a chimera pinnule was not induced in 2022.
Spores obtained from the chimera pinnule showed an ability of germination and they developed into gametophytes. The sporophytic shoots were produced from five gametophytes out of 20 after eight months (Fig. 3a) and they developed into juvenile sporophytes (Fig. 3b). The chromosome number of sporophytes was $2n = 40$. By culturing the piece of frond on agar, aposporous gametophytes were induced for approximately one month culture (Fig. 3c).

The relative DNA content of gametophytes developed from spores (Fig. 4a) and that of sporophytes induced from gametophytes (Fig. 4b) were almost same.

**Discussion**

The chromosome number of *A. niponicum* was reported to be $n = 40$ (Kurita 1960, Mitui 1968, Hirabayashi 1970) and $2n = 80$ (Tatuno and Okada, 1970). The mitotic chromosome number of the donor *A. niponicum* was coincident with the previous reports and that of apogamously produced sporophytes with $2n = 40$ was reported here for the first time. The base chromosome number of *A. niponicum* $x = 40$ was confirmed by the observation of univalent chromosomes. Apogamously produced sporophytes are monoploid with $2n = x = 40$. The methods of apogamous sporophyte formation used for *A. niponicum* followed the previous study of ferns such as *Dennstaedtia hirsute*, *Lepisorus thunbergianus* and *Onychium japonicum* (Kawakami et al. 1997).

It is generally considered that haploid plants are sterile because of irregular meiosis. The haploid sporophytes of *A. niponicum* produced artificially also showed to be sterile, however, the sporangia with many spores were produced spontaneously in the haploid sporophyte in 2021. Since we demonstrated in the previous study that haploid sporophytes such as *Osmunda claytoniana* and *O. japonica* occasionally yield ploidy chimeras (Kawakami et al. 2007), it was though first that the ploidy chimera might be induced in the frond of haploid sporophyte in the case of haploid *A. niponicum*. If so, the guard cell sizes, those are known to associated with ploidy levels (Takahashi 1962, Marinho et al. 2014, Kawakami et al. 2019), may be larger and then the spores may be produced by meiosis such a way of diploid sporophytes in the ploidy chimera. On the contrary, the guard cells of chimera pinnule were not larger and the number of spores produced in one sporangium was not 64 but approximately 32. Therefore, it was concluded that the chimera is not ploidy chimera.

Approximately 10% of all ferns (Walker 1979) and approximately 13% of Japanese pteridophyte taxa (Takamiya 1996) are apogamous, and then 73% of Japanese apogamous ferns are triploids (Kato 1997). Although the characteristics of apogamous ferns are unreduced spore formation and apogamous sporophyte formation, the comparative results were obtained from the haploid *A. niponicum*, i.e., ploidy levels between gametophytes and sporophytes are the same and spores obtained are unreduced. Besides aposporous gametophytes from the sporophyte were induced. Concerning the unreduced spore formation two types of sporogenesis are known. One is Döppe-Manton scheme and the other is Braithwaite scheme (Walker 1979), though 32 spores in the sporangium are produced either way. The sporogenesis of unreduced spores in haploid *A. niponicum* might followed the Braithwaite scheme (Braithwaite 1964)
from the observation of only univalent chromosomes at meiosis, though dyad spores were not observed. For the cause of unreduced spore formation, any genetic change of a single initial cell of pinnule in the haploid frond might be considered because the pinnule is produced from one initial cell in ferns (Kawakami et al 2007) and a chimera occurs by any genetic change (Marcotrigiano 1997). Similarly, in sterile ferns such as triploid hybrids of the ABC type having only univalents unreduced spore formation might occur by any gene change. Our study demonstrated that unreduced spores are produced on the chimera induced in haploid *A. niponicum*. That is very important information to know the origin of apogamous triploid hybrid ferns of the ABC type.

**Declarations**

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**References**


**Figures**
Figure 1

Mitotic and meiotic chromosomes in *Athyrium niponicum*. a Donor sporophyte with $2n = 80$. b Apogamously produced haploid sporophyte with $2n = 40$. c Apogamously produced haploid sporophyte with $n = 40$. Scale bars = 5

Figure 2

a Abnormal pinnule (marked by arrow) induced in the frond of haploid *Athyrium niponicum*. b Sporangia with spores produced on the chimera pinnule. Scale bar = 1 cm. c One sporangium with approximately 32 spores. They were almost same in size. Scale bar = 100
Figure 3

a Elongating sporophytic leaf from the gametophyte. Scale bar = 2 mm. b Juvenile sporophyte developed from the sporophytic leaf. Scale bar = 1 cm. c Aposporous gametophyes induced from the piece of the juvenile sporophyte. Scale bar = 1 mm

Figure 4

Relative DNA content of nuclei in haploid *Athyrium niponicum*. Histograms represent the number of nuclei per fluorescence intensity (arbitrary units). a Sample of gametophytes derived from spores. b Sample of sporophytes induced from the gametophytes