Effective Chromosomal Preparation Protocol for the Dioecious Plant *Silene latifolia*

Taiki Kobayashi¹, Masako Takahashi², Ryo Nishijima¹, Ryuji Sugiyama³, Kotaro Ishii^{4†}, Shigeyuki Kawano^{2,5} and Yusuke Kazama^{1,4*}

¹ Faculty of Bioscience and Biotechnology, Fukui Prefectural University, 4–1–1 Kenjojima, Matsuoka, Eiheiji-Cho, Fukui 910–1195, Japan

² Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, FSB-601, 5–1–5 Kashiwanoha, Kashiwa, Chiba 277–8562, Japan

³ Botanical Garden, Faculty of Agriculture, Tokyo University of Agriculture, 1737 Funako, Atsugi, Kanagawa 243–0034, Japan

⁴ Nishina Center for Accelerator-Based Science, RIKEN, 2–1 Hirosawa, Wako, Saitama 351–0198, Japan

⁵ Future Center Initiative, The University of Tokyo, 178–4 Wakashiba, Kashiwa, Chiba 277–0871, Japan

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Summary Cytology requires chromosome specimens, thus, effective preparation methods are needed. Chromosome specimens are frequently prepared from plant root tips. Furthermore, cell cycle synchronization using chemical reagents is applied to obtain a large number of metaphase chromosome specimens. In this study, we focused on the timing of root tip sampling, which is optimal for the preparation of chromosome specimens of the dioecious plant *Silene latifolia*. The timing was determined from the time seeds were subjected to a germination treatment. Observation of metaphase chromosomes using microscopy revealed that the number of mitotic cells peaked 54h after the germination treatment. This trend was also observed when the DNA synthesis inhibitor aphidicolin was administered from 24 to 9h before sampling time points. We used ice-cold treatment for 8, 16, and 32h as a chromosome condensation method. The 16h treatment produced suitable chromosome specimens showing satellite ends of chromosomes, whereas the 32h treatment produced well-condensed chromosome specimens, which were suitable for counting chromosome numbers. Our findings suggest that the timing of root tip sampling is essential for effectively producing plant chromosome specimens.

Keywords *Silene latifolia*, Chromosome specimen, Cell cycle synchronization, Root tip.

Chromosome specimen preparation is fundamental for several aspects of cytology, including karyotyping, fluorescence *in situ* hybridization (FISH), and chromosome microdissection (Bhowmick Biplab and Jha 2019, Kono *et al.* 2019, Dash Chandan *et al.* 2020, Nam *et al.* 2020, Saensouk and Saensouk 2020, Samaropoulou *et al.* 2020, Santra *et al.* 2020, Shafiee *et al.* 2020, Sheng *et al.* 2020, Suto *et al.* 2020, Widarmi *et al.* 2020, Wang *et al.* 2021). These techniques revealed differences in chromosome architecture between species, chromosomal abnormalities associated with diseases, and the roles of chromosomes in sex determination. In many plants, root tip portions are typically used for preparing chromosome specimens because they are highly enriched in rapidly cycling cells with a steady state of growth (Torre *et al.* 1971). Synchronization of the cycle is typically achieved using DNA synthesis inhibitors such as hydroxyurea, aphidicolin, and 5-aminouracil

(Pan *et al.* 1993, Doležel *et al.* 1999) followed by treatment with agents inhibiting chromosome movement during mitosis, such as colchicine and 8-hydroxyquinoline (Dustin 1984). Although these preparation methods are well established, no detailed characterization of the growth stages of root tips optimal for chromosome preparation has been produced thus far.

The dioecious plant *Silene latifolia* has heteromorphic sex chromosomes (Matsunaga and Kawano 2001, Negrutiu *et al.* 2001, Vyskot and Hobza 2004, Kejnovsky and Vyskot 2010). The X and Y chromosomes are approximately 400Mb and 570Mb in size, respectively, and they can be observed under a microscope. The architecture of these sex chromosomes has been investigated using cytological techniques. Satellite DNAs have been observed on both ends of the X chromosome, but only on one end of the Y chromosome (Bůžek *et al.* 1997, Matsunaga *et al.* 1999, Kazama *et al.* 2003, 2006, Kazama and Matsunaga 2008). The q-arm of the Y chromosome and the p-arm of the X chromosome have the same satellite DNAs and consist of pseudo-autosomal regions (Lengerova *et al.* 2003, Kazama *et al.* 2006). The end of the X chromosome's q-arm may be translo-

* Corresponding author, e-mail: ykaze@fpu.ac.jp

† Present address: National Institutes for Quantum and Radiological Science and Technology, 4–9–1 Anagawa, Inage-ku, Chiba 263–8555, Japan

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cated from chromosome 7 (Ishii *et al.* 2010). Moreover, methylation patterns differ between two X chromosomes (Vyskot *et al.* 1993, Siroky 1998). While the tandem repeat TRAYC accumulated on the Y chromosome during sex chromosome evolution (Hobza *et al.* 2006), the retrotransposon *SlOgre1* accumulated on all chromosomes except for the Y chromosome (Filatov *et al.* 2009). In a related species, *S. diclinis*, a neo-Y chromosome was generated by combining the Y chromosome and an autosome (Howell *et al.* 2009). The development of X and Y chromosomes in *S. latifolia* and *S. dioica* involves multiple chromosome rearrangements (Bačovský *et al.* 2020). Furthermore, FISH has also been used to construct Y chromosome maps (Howell *et al.* 2011). Previously, we had made improvements to heavy-ion beam irradiation (Kazama *et al.* 2011, Hirano *et al.* 2015, Kazama *et al.* 2017; see the heavy ion beam feature in this issue) and developed a library of partial Y-chromosome deletion mutants using this method (Kazama *et al.* 2016). Thereafter, a Y-chromosome map was constructed using this library, and its accuracy was confirmed through FISH (Kazama *et al.* 2016). These results were obtained using chromosome specimens; however, the number of chromosome specimens per root tip portions is unstable.

Root tip portions of germinating seeds are frequently used for preparing chromosome specimens of *S. latifolia* and other plant species. After harvesting the root tip portions, cell cycles are synchronized using DNA synthesis inhibitors and microtubule polymerization inhibitors. These are routine procedures that have been used for the preparation of root tip portions containing a large proportion of cells in the metaphase; however, the timing for chromosome preparation has yet to be optimized. We hypothesized that the cell cycle was roughly synchronized during the first few days after rooting from the seeds. Under this premise, we assumed that there may be an optimal time for the observation of metaphase cells. In this study, we examined this hypothesis and explored the optimal timing of root harvesting, which may aid the preparation of chromosome specimens.

Materials and methods

Plant material and growth conditions

S. latifolia seeds were obtained from the inbred line (K line) (Kazama *et al.* 2003). One hundred seeds were placed in each sampling bottle and were incubated in water at 4°C for 4 days. The seeds were germinated in a bottle placed on an RT-50 rotator (Taitec, Tokyo, Japan) at 23°C in the dark.

Sampling and Fixation

Germinated seeds were classified into three classes based on the length of root tips (Fig. 1A). The number of roots produced during each stage was recorded every six hours. At the time when the ratio of class II was high [48 to 60 h after incubation started (HAIS)], the bottles were placed on ice. After 16 h, the root tips were fixed overnight at 4°C using ethanol:acetic acid (3:1) and incubated overnight at 4°C with 70% ethanol. The roots were stored in 70% ethanol at 4°C until further use.

Aphidicolin treatment

Roots were treated with 10 μ M aphidicolin in the dark at 23°C starting 24 h before the respective sampling points, and the treatment was terminated 9 h before the sampling.

Counting of metaphase cells

Fixed roots were stained using the Feulgen method (Feulgen and Rossenbeck 1924). Briefly, root tip portions were cut from the root tips, were treated with 1M HCl for 12 min, washed with water, and were stained with Schiff's reagent for 10 min. After staining, the root tip portions were washed thrice using distilled water. Each root tip was placed on a glass slide, crushed with a cover glass, and was observed using a microscope (BX53, Olympus, Tokyo, Japan). Thereafter, the total number of metaphase cells per root tip was counted.

Fig. 1. Overview of the experiment to determine the optimal incubation time. A; Classification of root elongation stages by root length. B; Numbers of seeds belonging to each stage are recorded every 6 h after the start of incubation at 23°C.

Observation of metaphase chromosomes

To observe the effect of the duration of ice-cold treatment on the extent of chromosome condensation, three different durations of ice-cold treatment, *i.e.*, 8, 16, and 32 h, were tested. After fixation, the root tip portions were cut from the root tips. Thereafter, the root tip portions were washed in distilled water for 10 min and were treated with 1.5% cellulase Onozuka RS (Yakult. Pharmaceutical Ind. Co. Ltd., Tokyo, Japan) and 1.0% pectolyase Y-23 (Kyowa Chemical Products, Osaka, Japan) in 0.1 M citric acid/0.1 M sodium citrate buffer (pH 4.0) at 37°C for 20 min. The portions were then carefully washed using distilled water. Two or three portions were transferred to a glass slide, macerated in a drop of acetic acid:ethanol (1:3) using fine forceps, and were air-dried. The prepared specimens were stained using DAPI (1 mg mL⁻¹) and were observed using a confocal laser-scanning microscope (Zeiss LSM 900 with Airyscan 2; Zeiss, Oberkochen, Germany).

Results and discussion

Germination treatment time showing root length suitable for chromosome preparation

A root length of approximately 2 mm is suitable for the preparation of chromosomes in *S. latifolia*. The roots shorter than 2 mm may contain large amounts of starch, which interferes with the observation of chromosomes, while those longer than 2 mm are narrow, which complicates handling. To trace root elongation during the incubation and to determine the optimal incubation time for chromosome preparation, the root lengths were classified into three classes, *i.e.*, class I: <2 mm; class II: approximately 2 mm; and class III: >2 mm (Fig. 1A). Every six hours after germination, the number of roots of each class was counted. The number of roots of class II peaked after 60 h of germination following incubation at 23°C (Fig. 1B), indicating that approximately 60 HAIS may be a suitable treatment time for chromosome preparation.

Germination treatment time showing the highest number of metaphase cells

The rough observation of root tips from 48 to 60 HAIS showed a high frequency of metaphase cells during 51 to 56 HAIS (data not shown). Therefore, root tip samples collected every hour from 51 to 56 HAIS were closely examined using the Feulgen method (See Material and methods section). The number of metaphase cells in the root tips reached a peak at 54 HAIS (mean=45.0; median=41; *n*=23) (Fig. 2; Fig. 3). The mean value of the metaphase cells 54 HAIS was over twice that of 51 HAIS and 56 HAIS. Significant differences were observed between these values. (Wilcoxon rank-sum test, *p*<0.01). This result indicates an optimal sampling time point after germination treatment, which, in the current

Fig. 2. Numbers of metaphase cells counted after the start of incubation at 23°C. Root tip portions were ice-cold treated for 16 h from each time point after the start of incubation at 23°C, without aphidicolin treatment (upper panel) and with aphidicolin treatment (lower panel). The aphidicolin treatment started 24 h before each time point and was released 9 h before each time point. In both cases, peaks in the number of metaphase cells were observed 54 h after the start of incubation.

study, is 54 HAIS.

DNA synthesis inhibition for metaphase chromosome preparation is typically achieved using aphidicolin. As the cell cycle in the roots was presumed to be roughly synchronized before 51–56 HAIS, the aphidicolin treatment was initiated 24 h before 51–56 HAIS and was terminated 9 h before 51–56 HAIS. Cell counting revealed a peak in the number of metaphase cells at 54 HAIS (Fig. 2). The mean value of the metaphase cells 54 HAIS was higher than that of 51 HAIS and 56 HAIS. Significant differences were observed between these values. (Wilcoxon rank-sum test, *p*<0.01). The shape of the peak with aphidicolin treatment was similar to that without the treatment. However, the number of metaphase cells in aphidicolin-treated specimens was higher than that in specimens without aphidicolin treatment. This result indicates that DNA synthesis inhibition at the appropriate time helps increase the number of metaphase cells, which facilitates chromosome examination.

Chromosome examination after DAPI staining

Chromosome preparations after cellulase and pectolyase treatments (see Material and methods) were

Fig. 3. Photographs of metaphase chromosomes prepared from germinating seeds 54 h after the start of incubation. A; metaphase chromosomes observed after 8 h ice-cold treatment. B; metaphase chromosomes observed after 16 h ice-cold treatment. C, D; metaphase chromosomes observed after 32 h ice-cold treatment. X and Y indicate the X and Y chromosome, respectively. Bars=5 μ m.

examined after DAPI staining. The root tip portions of specimens at 54 H AIS were observed. Furthermore, we assessed the effects of ice-cold treatments for 8, 16, and 32 h, which inhibit microtubule polymerization, thereby affecting the degree of chromosome cohesion (Kerr and Carter 1990, Bartolo and Carter 1991).

As expected, the degree of chromosome cohesion increased with the increasing duration of ice-cold treatment (Fig. 3). After the 16 h ice-cold treatment, chromosomal ends with satellite DNAs were visible, whereas, after the 32 h ice-cold treatment, highly condensed chromosomes were frequently observed. The X and Y chromosomes can be easily distinguished after the preparation of highly condensed chromosomes; therefore, controlling the duration of ice-cold treatment may be useful for selecting particular chromosomes of interest.

Currently used chromosome preparation methods from root tip portions are well established and can be applied to many plant species (Pan *et al.* 1993, Doležel *et al.* 1999); however, the timing of root tip sampling after the start of incubation has not been well documented. Here, we determined the optimal timing of root tip sampling for the preparation of chromosome specimens in

the dioecious plant *S. latifolia*. The number of metaphase cells peaked at 54 H AIS, and it was two-fold higher after treatment with aphidicolin. These observations suggest that the cell cycle is relatively synchronized at the early stages of root germination and that the aphidicolin treatment enhanced this synchronization. Therefore, the timing of aphidicolin or hydroxyurea treatment appears to be an important factor regarding the preparation of chromosome specimens. Recently, heavy-ion beam-induced *S. latifolia* mutants with partial Y-chromosome deletions revealed interesting characteristics associated with Y chromosome evolution, such as dosage compensation (Krasovec *et al.* 2019) and pollination systems (Aonuma *et al.* 2021). Physical mapping of these partial Y-chromosome deletion mutants and epigenomic characterization based on such a map may provide clues to elucidate these evolutionary mechanisms. An efficient method for the preparation of chromosome specimens may be useful for further studies.

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