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A CYTOLOGICAL STUDY ON THE MEIOSIS PROCESS OF *HUMULUS SCANDENS*

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Abstract

Humulus scandens is a common dioecious weed that possesses valuable forage and medicinal properties. It exhibits strong adaptability and a high seed yield, making it a subject of significant research interest. However, there is a lack of information concerning the meiosis process of *H. scandens* and the relevant methods used for studying it. In this study, we aimed to address this gap by utilizing intact *H. scandens* meiosis cells as research material. We employed a rapid labeling method to observe the meiotic chromosomes. Additionally, a single oligonucleotide probe of 5S rDNA was used to asertain that the number of 5S rDNA signals in the somatic cells of *H. scandens* was one pair. Using this probe, we conducted an inspection of homologous chromosome pairing in *H. scandens*. Our study provides a comprehensive description of the meiosis process in *H. scandens* and introduces a set of cytological methods suitable for the easy labeling of meiotic chromosomes and the monitoring of homologous chromosome pairing in *H. scandens*.

Key words: *Humulus scandens*; Meiosis; Chromosome; 5S rDNA; FISH; Cytogenetics.

Introduction

Meiosis is a special cell division involved in the maturation of reproductive cells in sexually reproductive organisms. During meiosis, DNA is replicated once, and the cell divides twice, resulting in the production of four gametes that contain half of the original amount of chromosomes. This process enables exchange of genetic information between parental chromosomes and successful segregation of homologous chromosomes. Meiosis plays a significant role in ensuring genetic stability and creating genetic variation. It is also the foundation of traditional crop breeding (Strelnikova and Komakhin, 2023). Interestingly, compared to animals and yeast, plants seem to be less affected by the mutation-induced death caused by meiotic checkpoints. This characteristic makes plants ideal subjects for studying meiotic progression (Wijnker & Schnittger, 2013; De Jaeger-Braet *et al*., 2022). With the completion of genome sequencing in model plants, the identification and functional research of meiosis-related genes in plants have been greatly accelerated (Osman *et al*., 2011). The study of meiotic recombination in the model organism *Arabidopsis thaliana* has revealed numerous factors associated with meiotic recombination, which has greatly improved the understanding of meiotic recombination mechanisms in other plants, such as rice, maize, wheat, and barley (Luo *et al*., 2014; Kianian *et al*., 2018; Wang *et al*., 2021; Higgins *et al*., 2022). Meiotic chromosomes are of fundamental interest to cytogeneticists (Higgins *et al*., 2014), therefore, an appropriate method for visualizing meiotic chromosomes is desired. Ross *et al*., (1996) provided a comprehensive and clear meiotic atlas of *Arabidopsis* using a chromosome spreading procedure combined with 4',6-diamidino-2-phenylindole (DAPI) staining. This technique is widely used to characterize the appearance of meiotic chromosomes and prepare chromosome samples for fluorescence *In situ* hybridization (FISH). Li *et al*., (2014) reported a rapid staining method for observing meiotic chromosomes, in which living meiosis cells were squashed into a high efficiency staining buffer, and the chromosomal phenotype was analyzed using fluorescence microscopy.

Humulus scandens is a perennial, twining dioecious plant that belongs to the Cannabaceae family. It exhibits strong adaptability and rapid growth, enabling it to quickly cover large areas. It is a traditional Chinese medicine widely used in the treatment of tuberculosis, dysentery, and chronic colitis (Feng *et al*., 2014). Furthermore, its medical value suggests its potential as an exogenous additive in animal diets, serving as a replacement for antibiotics and improving growth performance (Hao *et al*., 2022; Dang *et al*., 2023). Regarding the cytogenetic tools for *H. scandens*, Li *et al*., (2017) developed male-specific amplified fragment length polymorphism (AFLP) and sequence-characterized amplified regions (SCAR) markers. These markers can be used to identify the gender of *H. scandens* during early developmental stages. However, the understanding of chromosome behavior during meiosis is still incomplete in *H. scandens*. This knowledge gap hinders a more comprehensive understanding of *H. scandens* cytogenetics, especially when compared to well-studied species and model organisms.

In this study, we utilized fresh anthers to investigate meiosis in *H. scandens* using fluorescent microscopy. We have presented a comprehensive meiosis atlas of *H. scandens* and provided a brief description of the meiosis-related morphological features of this species, as well as the differences between *H. scandens* and *Arabidopsis*. Furthermore, we employed 5S rDNA probes to determine the number of signal foci on *H. scandens* chromosomes and monitored homologous chromosome pairing during *H. scandens* meiosis using these probes. This research contributes to the existing knowledge about *H. scandens* and offers valuable references for future studies in this field.

Material and Methods

Plant materials: The *H. scandens* plants were cultivated in the garden field of Henan Normal University, where they

were exposed to natural environmental conditions. Throughout the experiment, the average day and night temperatures were 35°C and 23°C, respectively. The *Arabidopsis* ecotype Columbia (Col-0) was used as the control. Seeds were sown on half peat and half vermiculite and imbibed at 4°C for 3 days in the dark. *Arabidopsis* plants were then grown for 3 to 4 weeks in long-day conditions (16 h light/8 h dark) at 22°C, 40%–60% relative humidity, and a light intensity of 63 $mE \cdot s^{-1} \cdot m^{-2}$(Yuan *et al*., 2020).

Reagents and supplies: A fast labeling buffer containing 5 μMSYTOX Green (Thermo Fisher Scientific, S7020), mounting medium containing 2 μg/ml DAPI (Merck, D9542), Carnoy's fixative, digestion enzyme solution, 20X Saline-sodium citrate (SSC), and 60% acetic acid were prepared as previously described (Li *et al*., 2022). Additionally, tweezers, disposable syringes, slides and cover glasses, a humid box, and a heating block were used.

Methods: Fresh inflorescence materials were collected either from 10:00-11:00 am or 3:00-4:00 pm. To preserve the moisture in the plant tissues during microscopic inspection, the fresh materials were temporarily stored in a petri dish with moist filter paper. For fixation using Carnoy's fixative, the tissue was placed in the fixative solution and kept at 4°C. After 3 hours, the fixative solution was replaced. The fixed material can be used for chromosome spreading after 3 days and can be stored at -20°C for several months. Fast labeling of meiocytes, chromosome spreading, and FISH assays a 5S rDNA oligonucleotide probe was used following the methods described in previous studies (Li *et al*., 2014; Li *et al*., 2022). The images of buds and anthers were captured using an MshotMD30 camera (Micro-shot Technology, Guangzhou, China). Fluorescent dyes were observed and captured using an Olympus BX63 fluorescence microscope with appropriate filters.

Results

Meiosis-related morphological features of *H. scandens*: An inflorescence of *H. scandens* and the development of its anthers are illustrated in (Fig. 1). The *H. scandens* inflorescence takes the form of a tower-shaped structure, with conical flower buds (Fig. 1A). Typically, each flower bud contains five scythe-shaped anthers, which have a smooth surface with flat epidermal cells (Fig. 1B). The size and color of the anther during meiosis stage are closely related. Generally, the larger the anther, the darker the color, and the later the meiosis stage. During meiosis, the anther appears transparent, as depicted in Fig. 1B (the left two anthers). These developmental features can be utilized as indicators for selecting anthers during the meiosis stage. In our study, we measured the correlation between the diameter of 40 *H. scandens* flower buds and their meiosis stage. The data revealed that the average diameter of flower buds at the meiosis stage was approximately 1.45 mm. However, it is important to note that this measurement can only serve as a rough guideline, as the development of meiosis may be influenced by plant growth conditions.

A cytogenetic atlas of *H. scandens* meiosis: After preparing the section, we initially located the anther sample using the bright field of the fluorescence microscope. Then we located a cell cluster shaped like a "worm," corresponding to the early stage of meiosis (Fig. 1C). Similar to other species, this "worm" structure disintegrated after progressing into a dyad or tetrad. We then switched to fluorescence mode to observe the morphology of the meiotic chromosomes of *H. scandens* (Fig. 1D).

The process of meiosis comprised of two divisions: meiosis I and meiosis II. Meiosis I consists of four stages: prophase I, metaphase I, anaphase I, and telophase I. Prophase I can be further divided into five substages based on chromosome morphology: leptotene, zygotene, pachytene, diplotene, and diakinesis. During leptotene, the chromosomes appear as thin thread-like structures, as they have not yet paired or undergone synapsis (Fig. 2A). The nucleus of the meiotic cell is disk-shaped and larger than that of a somatic cell, which helps to distinguish it from a somatic cell. In zygotene, some homologous chromosomes begin to pair (Fig. 2B). It has been reported in multiple species that chromosomes can migrate to one side of the nucleus during this stage (Sheehan & Pawlowski, 2009; Li *et al*., 2022). Consequently, sickle-shaped chromosome groups could be observed (Fig. 2C), which served as one of the typical characteristics for identifying the zygotene stage of meiosis in *H. scandens*. During the pachytene stage, chromosomes further condensed, and fully synapsed homologous chromosomes exhibited a thicker line structure than in leptotene (Fig. 2D). In diplotene, homologous chromosomes were separated, and there were contact points between them known as crossovers (Fig. 2E). As diplotene progressed, the length of chromosomes further shortened (Fig. 2F). In diakinesis, chromosomes became highly condensed, and eight highly condensed bivalents could be clearly observed (Fig. 2G). After crossover terminalization, local "X" or "O" shaped bivalents could be observed during diakinesis (Fig. 2H). In the metaphase I stage, the bivalent chromosomes aligned in the middle of the cell along the equatorial plate (Fig. 2I). In the anaphase I stage, the homologous chromosomes moved towards the two poles of the cell, guided by the spindle, resulting in the formation of two parallel genomes. Although most of the chromosomes clearly separated into two genomes, there was still some minimal connection between them (Fig. 2J). The final stage of meiosis I was telophase I, where the chromosomes completely disintegrated and formed two sets of chromosomes at the poles of the spindle (Fig. 2K). Following this, each set of chromosomes completely disintegrated to form a dyad (Fig. 2L), indicating the completion of the first division of meiosis.

The two divisions of meiosis had different durations within the cell, with meiosis II being shorter and less frequently observed (Li *et al*., 2015). During metaphase II, chromosomes condensed again and align along the equatorial plate of each nucleus (Fig. 2M). The sister chromatids of each chromosome separated into two chromosomes during anaphase II and migrated to the two spindle poles, breaking the cohesion between sister chromatids (Fig. 2N). In telophase II, four polar groups of haploid nuclei were formed (Fig. 2O), which then underwent cytokinesis to form four haploid cells, each surrounded by a new membrane, known as tetrad (Fig. 2P). Subsequently, the four haploid cells separated from each other and developed into mature pollen grains.

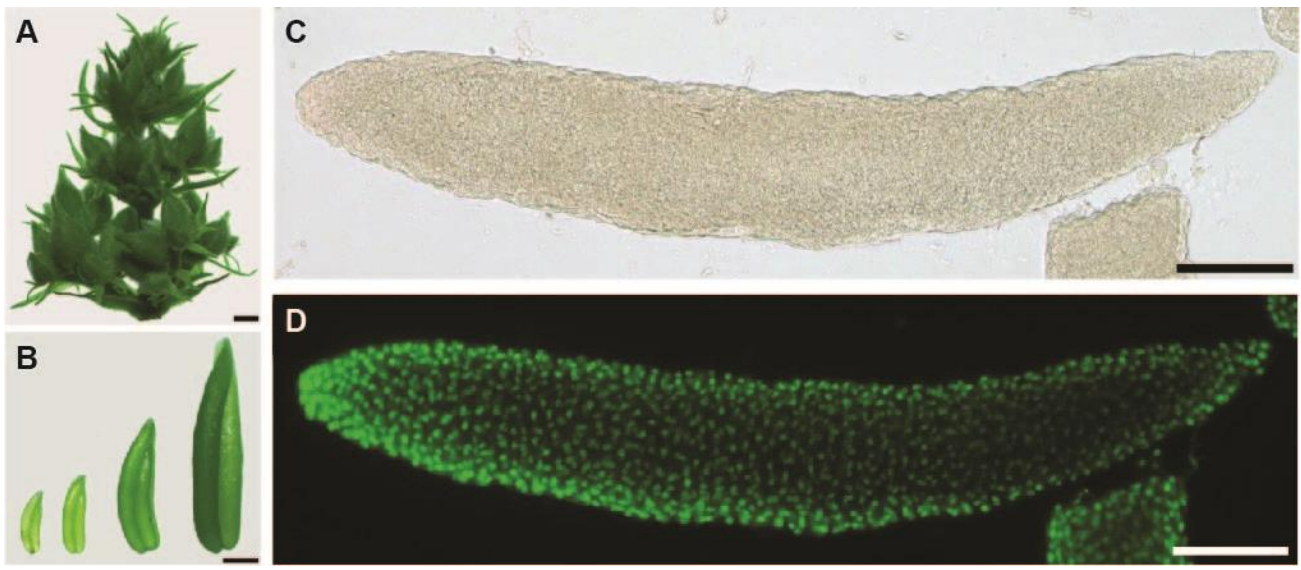

Fig. 1. Illustration depicting the materials utilized for visualizing the male meiotic chromosomes of *Humulus scandens*. A, a young inflorescence of *H. scandens*. B, dissected anthers of varying sizes, with the transparent appearance indicating active meiosis (left two anthers). C, isolated meiocytes exhibiting a worm-like shape. D, the corresponding labeling outcome of C achieved through the fast-staining method. Scale bars: 1 mm (A and B); 100 μm (C and D).

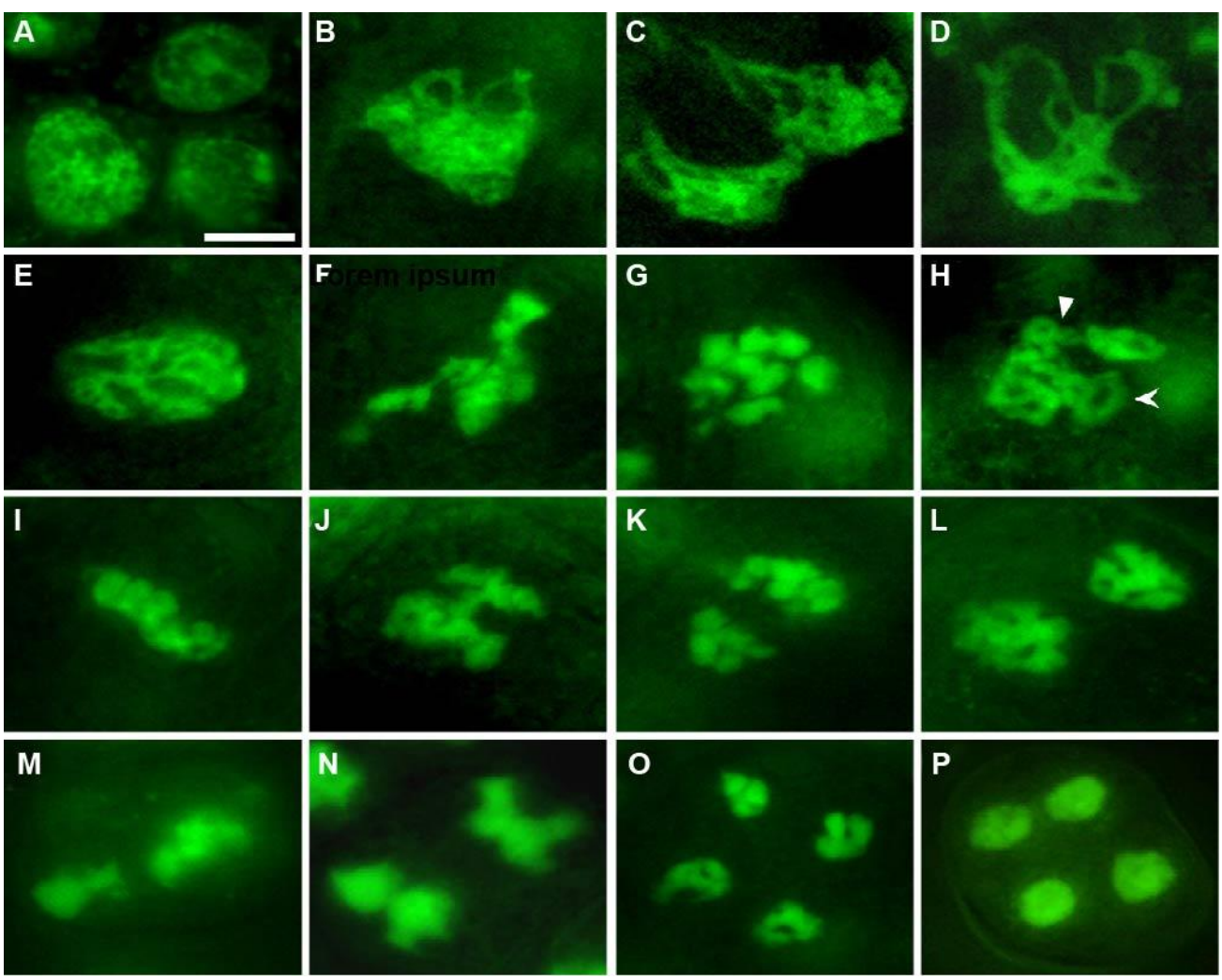

Fig. 2. A cytogenetic atlas of *H. scandens* meiosis. A, leptotene. B-C, zygotene. In C, sickle-shaped chromosome groups can be observed, formed after chromosomes moved to one side of the nucleus. D, pachytene. E, diplotene. F, late diplotene. G-H, diakinesis. In G, individual bivalents can be identified, while in H, an "X" shape (arrow) or an "O" shape (concave arrow) can be seen in bivalents due to crossover terminalization. I, metaphase I. J, anaphase I. K, telophase I. L, dyad stage. M, metaphase II. N, anaphase II. O, telophase II. P, tetrad stage. All figures are shown at the same magnification. Scale bar, 50 μm.

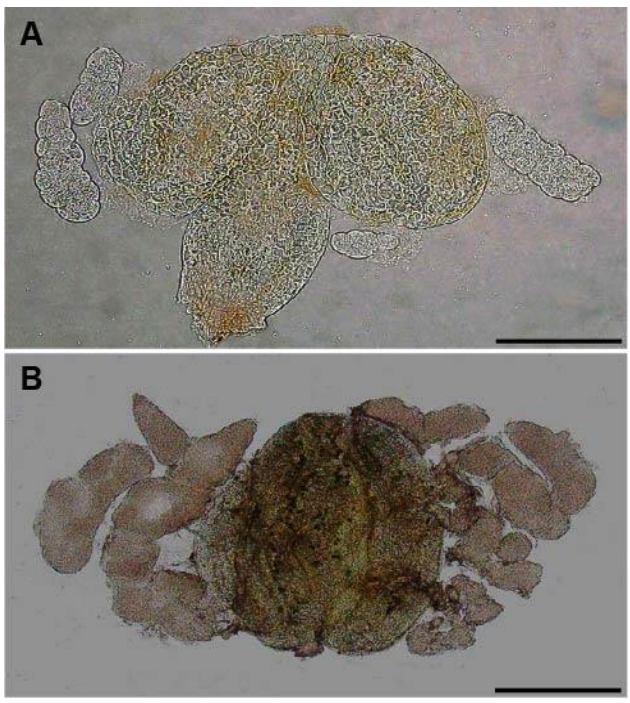

Fig. 3. A comparison of the size of meiocytes in *Arabidopsis* and *H. scandens*, and their production using the squash method. In *Arabidopsis* (A), the average production of meiocytes per anther is 2.96 ± 0.86, while in *H. scandens* it is 7.22 ± 2.85. Scale bars: 100 μm (A); 0.5 mm (B).

We have conducted a brief comparison of meiosis between *H. scandens* and *Arabidopsis*. During the squash step of sample preparation, we found that the "worm"-shaped cell clusters produced by a single *Arabidopsis* anther were relatively few (2.96 ± 0.86) and small and could only be seen under a microscope (Fig. 3A). On the other hand, the "worms" produced by a single *H. scandens* anther were larger and visible to the naked eye. The number of these clusters was also higher (7.22 ± 2.85) (Fig. 3B). This characteristic made it easier to observe the various stages of meiosis in *H. scandens*. Furthermore, in *Arabidopsis* meiotic cells, the daughter nuclei are clearly separated by DAPI-stained fluorescence spots. These spots formed a distinct band during the dyad stage and remained visible until anaphase II (Ross *et al*., 1996). A similar band was also observed in *Arabidopsis* during metaphase II and anaphase II using the quick labeling method (Li *et al*., 2014). In contrast, no fluorescence spot was observed throughout meiosis II in *H. scandens* (Fig. 2L-N).

Application of a single oligonucleotide probe for the 5S rDNA (Oligo-5S) in *H. scandens*: The 5S rDNA, typically arranged as repetitive sequences in eukaryotes, served as a valuable tool for identifying chromosomes and studying karyological evolution. To monitor chromosomal homologous pairing, researchers often employ fluorescence *In situ* hybridization (FISH) with 5S rDNA probes. This technique is especially useful when the genome contains a single 5S rRNA locus (Golubovskaya *et al*., 2002; Pawlowski *et al*., 2009; Ronceret *et al*., 2009). In our study, we utilized the FISH assay to investigate changes in the number and position of 5S rDNA on *H. scandens* chromosomes. By analyzing mitotic metaphase chromosomes from root tips, we observed a pair of 5S rDNA signal foci in the diploid *H. scandens* genome (Fig. 4A). During meiosis, we detected two 5S rDNA signal foci at leptotene (Fig. 4B), which merged into a single signal point following homologous chromosome pairing during pachytene (Fig. 4C). Consequently, this method offered a more precise analysis of homologous pairing in *H. scandens*.

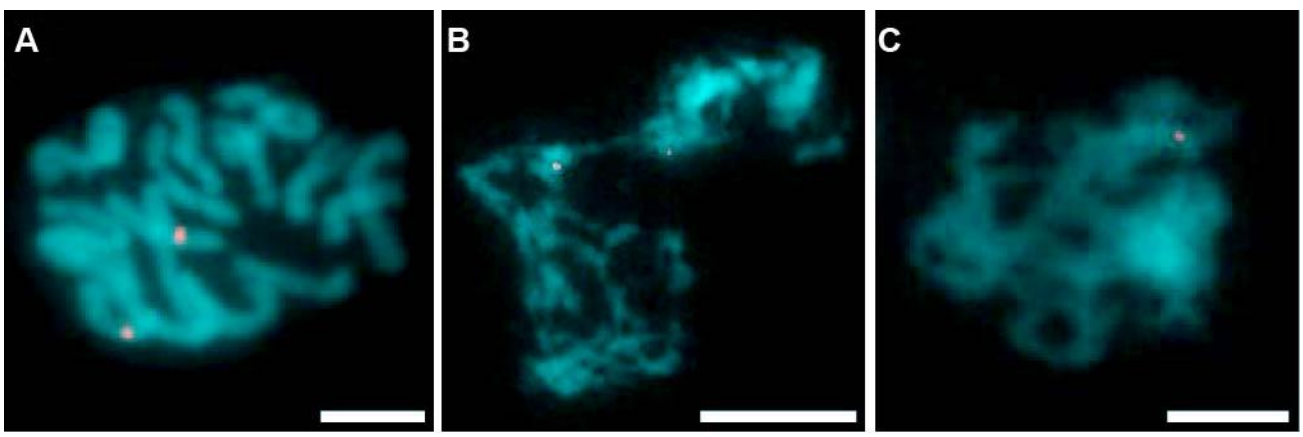

Fig. 4. Fluorescence *In situ* hybridization (FISH) assay utilizes an Oligo-5S rDNA probe. The image displays FISH signals (in red) within chromosomal spreads (in cyan) of a root-tip somatic cell during metaphase (A), a meiotic cell in zygotene (B), and a meiotic cell in pachytene (C). The Oligo-5S refers to a single 5S rDNA oligonucleotide probe that has been prelabeled with the Tamra fluorochrome. Scale bar, 5 μm.

Discussion

In this report, we studied the meiosis processes of *H. scandens*, using fresh anthers as the material. Ensuring that the material is taken from a period of vigorous meiosis is crucial for the meiosis experiment. The appropriate sampling time of *H. scandens* flower buds can be selected based on their morphological features. *H. scandens* inflorescence undergoes different stages of meiosis in a relatively short span of time, which are similar to that of *Asparagus officinalis* (Yuan *et al*., 2019), so sampling should be limited to this short period. However, since the inflorescences at different nodes on an indeterminately growing stem are in a wider developmental stages researchers can sample over a larger time span. Our provided developmental indicators, such as the bud

diameter and anther transparency during meiosis in *H. scandens*, can effectively assist in sampling.

We introduced the protocol for small-scale isolation of male meiocytes and subsequent labeling of meiotic chromosomes in *H. scandens*. This protocol was initially reported in *Arabidopsis* (Li *et al*., 2016). The meiocytes in the *H. scandens* anther were gently crushed and mixed with an appropriate amount of fast labeling buffer containing SYTOX Green nucleic acid stain. This allowed for the release of worm-like cell clusters or free meiocytes at different stages of meiosis, facilitating the timely and rapid identification of each meiotic stage. It is crucial to distribute the anthers evenly between the slides during squashing, and to apply appropriate pressure to ensure efficient observation of chromosomes at various meiotic stages. Excessive force should be avoided as it may disrupt the cells. Additionally, for ease of locating the next sample after inspecting meiocytes from an anther, it is recommended to arrange the anthers in order on the slide during the preparation process. To obtain different stages of meiosis in *H. scandens*, it is necessary to dissect flower buds of various sizes and observe the developmental features of *H. scandens* flowers during meiosis under specific growth conditions. If the desired meiosis stage is not observed, it is recommended to switch to a more appropriately sized bud on the same inflorescence based on information from the previous bud. This allows for the full utilization of the advantages of the rapid labeling method.

In this study, we observed all the main typical meiotic stages in *H. scandens*. Meiosis in *H. scandens* is generally similar to other species, as meiosis is highly conserved among sexually reproducing species (Tock & Henderson, 2018; Xin *et al*., 2021). The chromosome interaction events that occur during meiosis, such as pairing, synapsis, recombination, and segregation, are all associated with chromosome condensation and decondensation (Zickler & Kleckner, 2015; Zuo *et al*., 2021). Chromosome condensation facilitates the interaction between homologous chromosomes, while the high concentration of chromosomes aids in observing them during meiosis. In our experiments, the frequency of capturing meiotic chromosomes in *H. scandens* was significantly higher than in *Arabidopsis*. This can be attributed to several factors. Firstly, the flower buds of *H. scandens* are capable of generating a relatively greater number of male meiocytes than *Arabidopsis*, which aligns with the observation that *H. scandens* produces a substantial amount of allergenic pollen during its blooming season. Secondly, our experiments revealed that the anther walls of *H. scandens* are susceptible to rupturing under pressure, thereby facilitating the release of meiocytes. Furthermore, the meiotic cell clusters in *H. scandens* contain a higher concentration of meiocytes (Fig. 1D), particularly when compared to those of *Arabidopsis* and cucumber (Li *et al*., 2014; Li *et al*., 2022). Considering the low cost of obtaining *H. scandens* materials, *H. scandens* has the potential to be used as a scientific or teaching material for meiosis observation.

In summary, we have presented morphological images of all stages of meiosis in *H. scandens*, along with corresponding development indicators that can be utilized for sampling. Additionally, we have provided a method for accurately detecting homologous chromosome pairing in *H. scandens* through the use of FISH assay. Our squash protocol can be easily adapted for other downstream applications, such as reporter gene assays and callose staining (Li *et al*., 2016). These findings and methods can serve as a valuable reference for future studies on the genetic control mechanisms of homologous chromosome pairing and recombination in *H. scandens*.

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