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Asynchronous male/female gametophyte development in facultative apomictic plants of *Cenchrus ciliaris* (Poaceae)

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ABSTRACT

Apomixis has been suggested to result from the asynchronous gene expression of duplicated genes governing plant reproduction. The similarity of embryological stages and gene expression patterns observed during sexual and apomictic processes, and appearance of apomictic stages (e.g., aposporous initials) at different stages of ovule development support this hypothesis. We evaluated this hypothesis by assessing temporal variation during microsporogenesis, microgametogenesis, megasporogenesis and megagametogenesis in *Cenchrus ciliaris* L. This study was conducted using individuals of *C. ciliaris* with two distinct modes of reproduction viz., facultative apomictic or sexual. Inflorescences were classified into five stages based on morphological indicators. Variation in the configuration and developmental timing of gametophytes of facultative sexual and apomictic plants was studied at these five stages using the high-throughput technique of whole-mount confocal microscopy. Asynchrony in development of early reproductive stages in apomictic plants, as observed by presence of greater number of embryological stages in florets of the same inflorescence, is prominent in comparison to sexual plants. Such inconsistency was greater in female than in male gametophyte development. Stages like 3-nucleated sexual embryo sac and aposporous proembryo could also be observed. This supports the hypothesis that apomixis could be the result of de-regulated sexual reproductive pathway.

1. Introduction

Sexual reproduction (amphimixis) and asexual reproduction are alternative modes of reproduction in flowering plants. Asexual reproduction may occur through vegetative propagation or apomixis (the asexual production of seeds). The absence of chromosome reduction in the pathway to embryo development is an essential feature of apomixis. All apomictic systems include three developmental components: apomeiosis, parthenogenesis, and the capacity to produce a functional endosperm with or without fertilization (Bicknell and Koltunow, 2004). Based on the absence or occurrence of an unreduced embryo sac stage (gametophyte) during embryo formation, apomixis is classified into sporophytic apomixis and gametophytic apomixis, respectively. Gametophytic apomixis can be further divided into apospory (where an unreduced embryo sac containing a parthenogenetically competent egg arises from a somatic cell that has also acquired the developmental program of a functional megaspore) and diplospory (where an unreduced embryo sac containing a parthenogenetically competent egg arises from a megaspore mother cell that exhibits a suppressed or modified meiosis) (Nogler, 1984a; Asker and Jerling, 1992; Koltunow, 1993; Allem, 2003; Richards, 2003; Bicknell and Koltunow, 2004). Apomixis has attracted great attention because it results in the production of clonal progeny through seed development and can therefore be employed to combine the advantages of fixation of hybrid vigor and propagation through seeds (Richards, 1997).

While apomixis is known to be genetically controlled, specific genes responsible for apomixis are yet to be identified. Apomixis is considered to be a result of genetic or epigenetic deregulations of sexual processes (Singh et al., 2011) or an ancient alternative pathway that modifies sexual processes in response to real or incorrectly perceived environmental signals (Carman et al., 2011). Departures from the sexual pathway in gametophyte development in apomicts may be characterized by the absence of particular stages of development, different configurations of embryo sacs, or temporal variation in the development of gametophytes and/or embryo. Heterochrony, an alteration in relative timing of developmental stages, leads to precocious development of embryo in several aposporous and diplosporous apomicts. This has been observed in grasses, e.g., *Brachiaria brizantha* (A. Rich.) Stapf (Alves et al., 2001), *Cenchrus ciliaris* L. (Vielle et al., 1995), *Panicum maximum* Jacq. (Savidan, 1982, 1989), *Paspalum notatum* Flüggé (Martínez et al., 1994), *Sorghum bicolor* (L.) Moench (Carman et al., 2011) and *Tripsacum* L. (Leblanc and Savidan, 1994; Grimanelli et al., 2003) and other taxa, e.g., *Ranunculus auricomus* L. (Nogler, 1984b), *Taraxacum officinale* F.H. Wigg. (Baarlen et al., 2002) and *Hypericum perforatum* L. (Barcaccia et al., 2006; Galla et al., 2011). Asynchrony, the variable relative timing of developmental events, is also common in apomicts, unlike the more

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synchronized developmental processes in sexual plants. For example, in diplosporous *Taraxacum*, megasporogenesis and megagametogenesis were found to proceed asynchronously among florets of a single capitulum of natural triploid apomicts but synchronously among florets within capitula of sexual plants (Baarlen et al., 2002). In contrast there are reports for other diplosporous systems, like *Tripsacum*, where ovules on a given ear were found at similar stages of development throughout reproductive development in both apomictic and sexual plants. On the other hand, male meiosis in *Tripsacum* apomicts was characterized by asynchrony of developmental stages unlike synchronized sexuals (Grimanelli et al., 2003). Relative timing of reproductive events in many well studied aposporous systems is known to be asynchronous, e.g., *Hieracium* L. (Koltunow et al., 1998), *H. perforatum* (Galla et al., 2011) and *Poa pratensis* L. (Yudakova and Shakina, 2007). In a facultatively apomictic population of *P. pratensis*, asynchronous maturation of ovules and gametophytes (with different timing of induction of aposporous initials) was observed within an inflorescence. Such asynchrony is thought to allow the population to simultaneously produce both sexual and apomictic progeny (Yudakova and Shakina, 2007).

Several hypotheses have been proposed to explain the complex phenomenon of apomixis. Apomictic events within ovules may be randomly determined, for instance in *Hieracium*, where it has been suggested that development involves a threshold response where a critical apomictic factor(s) is slightly limiting or in slight oversupply (Koltunow et al., 1998). Greater temporal variation in aposporic gametophyte development (relative to sexual ones) in *H. perforatum* is suggested to reflect stochasticity of gametophyte development due to disturbance of signaling pathways and accumulated mutations (Galla et al., 2011). Based on observations in 460 angiosperm families, a hybridization hypothesis of apomixis has been proposed (Carman, 1997). Different genome complements are known to follow different timelines for development, i.e., are heterochronic with respect to each other. Polyploidy or paleopolyploidy (diploidized polyploidy with chromatin from multiple genomes) leads to hybrids with duplicate sets of genes that control reproductive development. According to the hybridization hypothesis, partial to complete replacement of meiosis by embryo sac formation in apomictic species results from the heterochronous expression of duplicate genes. Competition between nearly complete sets of asynchronously-expressed duplicate genes leads to stochastic development and consequent precocious embryo sac initiation and embryogenesis at aberrant sites and times during reproduction (Carman, 1997; Tucker and Koltunow, 2009).

C. ciliaris (tribe Paniceae; family Poaceae), a model system for aposporous apomicts, is closely related to the economically important cereal *Pennisetum*. In this species, apospory and pseudogamy (requirement of fertilization of central cell for seed development) form the main mode of reproduction. As such, *C. ciliaris* is an important model system for aposporous apomicts; we are using the system to understand the molecular genetic basis of apomixis. As a first step, we wish to establish a calendar of developmental events that may be used as a baseline in further investigations. Most of the embryological studies in *C. ciliaris* to date have made comparative studies of apomixis and sexual reproduction without taking stages of floral development into account. However, we wish to study gene expression across varied ovule development events which should ideally be studied in relation to floral (morphological) stages of development across sexual and apomictic plants. Therefore, we need to establish a calendar of ovule developmental stages in relation to floral developmental stages. In this study, we use facultative apomictic and sexual F_2 individuals of *C. ciliaris* to establish calendars for apomicts and sexual plants. These F_2 individuals are expected to be genetically more similar to each other than to their sexual progenitor.

In apospory, a functional meiotically derived sexual female gametophyte may coexist with the aposporic embryo sac in the same or in different ovaries of the same plant. Hence, different degrees of facultative apomixis are possible at the individual level (Miles, 2007). Normal bipolar Polygonum-type gametophytes are formed in sexual genotypes of *C. ciliaris* while aposporous embryo sacs are monopolar and usually contain four nuclei in three to four cells at the micropylar end (Fisher et al., 1954; Synder et al., 1955; Bashaw, 1962; Sherwood et al., 1980; Vielle et al., 1995; Febulaus and Pullaiah, 1995). *C. ciliaris* shows *Panicum*-type apospory in which antipodals are invariably absent in the aposporous embryo-sacs, making them readily distinguishable from the eight-nucleate sexual embryo sac. There are many ways to identify apomicts, from morphological, cytological and embryological observation to molecular markers and techniques like flow cytometric seed screen (FCSS) (Carneiro et al., 2006). In the present study we use embryological observations of cleared embryo sacs and confocal microscopy (modified after Barrell and Grossniklaus, 2005) to identify the mode of reproduction. Unlike the traditional sectioning method, this high throughput procedure enables observation of many more ovules than otherwise possible.

2. Materials and methods

2.1. Plant material

A single sexual plant (IG-96-443) of *C. ciliaris* was open pollinated to obtain half-siblings, among which only obligate (all 4-nucleate mature gametophytes) or facultative (both 4- and 8-nucleate mature gametophytes) apomictic plants were observed (Dwivedi et al., 2007). Facultative apomictic half-siblings were selfed to get F_2 progeny. A facultative apomictic F_2 plant, A-7-18, and a facultative sexual F_2 plant, S-7-4, were used in the present study. These F_2 individuals were phenologically more similar to each other compared to their sexual progenitor. Both the plants were grown in the Botanical garden under similar microclimatic conditions.

2.2. Ovule clearing

Individual plants were examined by an ovule clearing technique modified after Young et al. (1979). Inflorescences showing 75–100% stigma exsertion were fixed in FAA (formaldehyde:glacial acetic acid: ethanol:water) for 24 h and then stored in 70% ethanol at 4 °C. The florets were dissected and passed through an ethanol series for 2 h each (1 × 85% ethanol; 2 × 100% ethanol; 100% ethanol overnight). The next day they were passed through an ethanol:methyl salicylate series (ethanol:methyl salicylate, 1:1 v/v; ethanol:methyl salicylate, 1:3 v/v; 100% methyl salicylate), again for 2 h each and finally stored in 100% methyl salicylate overnight. Cleared pistils were mounted in 100% methyl salicylate and observations made using differential interference contrast optics of a Leica DM 2500 microscope equipped with a Leica DF6360FX digital camera. Images were assembled into plates of figures using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Normal Polygonum-type embryo sacs containing egg apparatus (with an egg cell and two synergids), central cell with two closely associated polar nuclei, and a cluster of antipodal cells at the chalazal pole (Fig. 1A) were classified as sexual embryo sacs. Monopolar Panicum-type embryo sacs having no antipodals (Fig. 1B) were classified as aposporous embryo sacs. At least 50 ovaries collected from five inflorescences chosen at random were used to calculate the proportion of apomixis or sexuality. Observations were made in March–June season of flowering during 2006–2008.

2.3. Floral morphology

Easily identifiable floral morphological characters i.e. sheath opening, stigma and anther exsertion were used as markers to define five readily distinguishable floret phases. Spikelets were dissected under a stereomicroscope (Zeiss Trinocular Stereo Microzoom Microscope, STEMI 2000C). Dissected floral parts were measured using a ruler with a resolution of 0.5 mm. The length of anthers and ovaries (excluding