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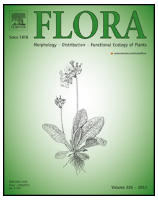

Anther and ovule development in *Camellia japonica* (Naidong) in relation to winter dormancy: Climatic evolution considerations

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ABSTRACT

The *Camellia japonica* (Naidong) population in Qingdao, Shandong province is the only one in temperate areas in China. While this population has been present in this area since the tertiary, its has evolved independently after that. To date, there are no reports on its embryology, and no research on the connection between embryology and climate. Here, we studied anther and ovule development of *C. japonica* (Naidong) using light and scanning electron microscopy, in order to determine the characteristics of early flower development, microsporogenesis, microgametogenesis, megasporogenesis and macrogametogenesis in this plant. We observed basic anther wall formation, glandular tapetum and successive microsporocyte cytokinesis. Moreover, microspores tetrads were mostly tetrahedral and occasionally symmetrical, we did not observe pseudopollen grains as previously reported for *Camellia.* The ovary was superior and presented axial placentation and anatropous, bitegmic ovule, allium type of embryo-sac, integumentary tapetum and hypostate which may provide winter cold temperature resistance. The early flower development sequence placed *C. japonica* (Naidong) in a most primitive branch of the phylogenetic tree compared to other species. The embryo characteristics and the development of *C. japonica* (Naidong) are significant for phylogeny research of Theoideae, as the taxonomy of this family is currently problematic at all levels. Moreover, this information is also useful flowering induction as a possible horticultural application. Flower buds went in dormancy during winter as gametophyte state, a characteristic evolved in *C. japonica* (Naidong) in response to the climates changes.

1. Introduction

Species of genus the *Camellia* of the Theaceae family are largely distributed in southeastern and eastern Asia. *Camellia japonica* is mainly located in eastern coastal areas of China, on the Japanese islands of Shikoku and Honshu, and in the southern Korean peninsula (Gao et al., 2005; Lin et al., 2012), The town of Qingdao in Shandong province is the northernmost point where the plants is present in China. The species in this area are unique due to the distinct climatic characteristics in Qingdao. In particular, the climate is different from that of the Zhoushan archipelago, considered the distribution center of *C. japonica* (Wang et al., 2009a). *C. japonica* (Naidong) (hereafter called Naidong), a Tertiary relict species, with a unique biological and cultural importance (Lin et al., 2013; Matsuda, 1915), grows on the islands of Qingdao (Zang and Wang, 1994). With a gradual climate change, Qingdao got colder, and its climate shifted from subtropics to temperate (Dolezal et al., 2012; Lee et al., 2008). Faced with colder temperatures, most thermophyilic species extincted or migrated to warmer regions. In contrast, Naidong adapted to temperate climate (Zang et al., 2001). While we know that climate can influence vital rates that shape life history strategies, it is also critical to understand the effects of climate change on rapid life history evolution, which might modify the influence of climate change on population dynamics (Williams et al., 2015).

Naidong displays a good cold-resistance, as shown by physiological indexes and leaf structure (Wang et al., 2009b; Wang et al., 2007). Naidong not only adapted to cold temperature, but is also able to reproduce stably (Jia, 2015). Previous papers the distribution of *C. japonica* in temperate regions focused mainly on physiological and ecological indexes. Research on *C. japonica* focuses on photosynthesis in different seasons under different canopy (Miyazawa and Kikuzawa, 2005; Miyazawa and Kikuzawa, 2006), or on the relationship between warm temperature and growth (He et al., 2012; Nakashima et al., 2002). In particular, Tojo studied *C. japonica* in the context of coevolutionary research in the temperate climate

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and typical island environment of Yakushima (Toju, 2008). To our knowledge, there are no reports on Naidong flower bud development in winter, which is important to temperate woody perennials. Dormancy is a prerequisite for adequate flowering and plays a role in adapting species and cultivars to climatic areas (Julian et al., 2011). Dormancy is a genetically controlled phenomenon common in woody perennial species adapted to temperate climates and indicating an overwintering mechanism (Chouard, 2003). A previous study on flowering times of numerous plant species over years indicate that flowering patterns are related to seasonal and climatic changes (Tooke and Battey, 2010). Cold winter temperature is a key element that can influence plants reproductions (Julian et al., 2011; Mercado et al., 1997). Therefore, here, we studied the development state of anthers and ovules in Naidong during winter dormancy.

Reproduction studies, and particular embryology, represent basic information for any plant. The embryology of Naidong in specific has not been studied, but twelve *Camellia*, and other *Pyrenaria* and *Tutcheria* species of the Theaceae family has been previously investigated (Cao, 1965; Feng, 2011; Hu, 2014; Kapil and Sethi, 1963; Li and Cao, 1986; Liu and Zhang, 1983; Luo et al., 2010; Luo and Zhao, 1989; Mathew, 1978; Yang and Ming, 1995; Ye, 2015; Zou, 2010). While these previous works describe the embryological characteristics of different species and place them in an evolutionary context, this information cannot be used for comparative embryology as it is not always complete. The two clades *Camellia* and *Franklinia-Schima* are phylogenetically defined by the contrasting expression of five embryological markers (Tsou, 1997). The phenetic and leaf anatomy of *Camellia* have broad variations (Erxu et al., 2009; Su et al., 2012). Generally, the embryological characteristics of species within the same genus are similar (Tsou, 1997; Yang et al., 2002). However, insufficient ontogenetic studies resulted in confusing terminology and misunderstanding when discussing the embryology of *Camellia* and other Theaceae genera; therefore more embryological studies are needed.

Embryonic and early floral development are commonly used to compare evolution and phylogeny (Hardy et al., 2000; Tsou and Mori, 2007). Tsou described twelve species from ten Theaceae genera, distinguishing two major evolutionary lineages (Tsou, 1998). Here, we report for the first time the development of sporophytes and gametophytes of Naidong in detail, and at the same time observed the early floral development of this species. On the basis of these observations, we evaluated the evolutionary position of Naidong in the Theaceae family. Additionally, Naidong has the same embryo development progression as other *Camellia* species present in southern China that experienced the climate change form Tertiary to Quaternary and survived to 21 st century. Naidong entered winter dormancy in a most temperature-sensitive state that may be a specific Naidong characteristic, which evolved in response to the temperature in Qingdao. This study is significant for the research of Camellia phylogeny and its historical distribution. Moreover, this work stands as a theoretical basis for the development of new techniques for camellia flowing induction.

2. Materials and methods

2.1. Plant material

Buds at various stages of development were collected from 12 plants cultivated at 1950s in Qingdao Botanical Garden and May Fourth Square of Qingdao (36°05′N, 120°05′E Qingdao city, Shandong, China) where the annual mean temperature was around 12.7 °C. Floral buds of different sizes and open flowers were harvested from 12 plants from three Naidong populations between May and December in 2014 and 2015. The average basal diameter of the 12 plants are 14.24 ± 0.55 and all in good growth conditions.

2.2. Sectioning and microscopy

Every 4–5 days inflorescences were collected and fixed in FAA (formalin: acetic acid: 50% ethyl alcohol, 1:1:18, v/v/v) immediately. Floral buds of 12 plants from three populations were carefully dissected under a dissecting microscope and flushed in 50% ethyl alcohol, dehydrated through in an ethyl alcohol: isoamyl acetate series, and then dried with a critical point dryer (QUORUM, K850). Samples were mounted on aluminum stubs with double adhesive tape, coated with gold in a sputter coater (JEOL, JFC-1600), and then observed with a scanning electron microscope under 2 KV electric current (JEOL, JSM-7500F) (Sajo et al., 2012). A second sample set was embedded in paraffins and sectioned using a microtome (Leica RM-2145). Ovary blocks were longitudinal sectioned with a thickness of 10 μm were made, anther blocks were used for transverse sections with a thickness of 8 μm. Sections were stained with Ehrlich's haematoxylin and eosin and imaged using a CCD camera (Nikon D70, Japan) attached to a light microscope (Olympus BX51, Japan) (French, 1985).

3. Results

3.1. Overview of inflorescence and floral morphology

Naidong flowering initiated from mid May to October. Flower development completed all flower bud differentiation and right before entering into tardy growth state, to bloom between March and May next year. The course of development can divide into four phases: early differential, bract initiation, petals initiation, stamen initiation, carpel initiation (Fig. 1A–L).

In early differentiation, primary flower buds were between 0.3 and 0.4 cm in width and presented several bud scales. Development entered this phase when annual branches differentiated five to six leaves. The central zone in the apical meristem of each primary flower bud presented light ridges (Fig. 1A).

The perianth was the first organ to be formed on the floral apex (Fig. 1 B and C). As this organ continued to grow, its diameter enlarged. The bottom of primordia gradually developed from linear to circular. Five sepals were initiated individually and asynchronously in all plants. Sepals differentiated one by one with the next sepal appearing only after the previous one completely differentiated and curved to cover the central zone. The floral apex was rounded, elliptic, or tetragonal in polar view at sepal initiation. Then, the differentiation of five to six petals initiated individually and asynchronously at the borders of the nearly circular-shaped floral apex, after sepals had grown to almost envelop the floral apex (Fig. 1D). As petals appear one by one and the floral apex became broadly concave. When all petals had appeared, the floral apex developed into a slightly concave pentagon-shape.

During stamen initiation, the inner-androecium first appeared as an androecial ring primordium demarcated around the central apex (Fig. 1E–I). The middle circumference of the ring meristem was visible shortly after the inner circle and three carpel primordia appeared. After the inner-androecium had developed into a hemisphere, the outer-androecium appeared. Finally, the central apex formed a three-circle androecium. Stamens began to differentiate expanding into filaments and anthers. Pollen sacs differentiated forming median and lateral grooves. The filament bases presented inner-androecial adnations with ovaries. Filament bases of other two-circle androecium displayed connation. Androecia had a centrifugal development pattern.

At carpel initiation, three carpel primordia appeared synchronously in inner androecium, (Fig. 1F-I). The gynoecium was composed of three congenitally fused carpels, grown as an ovary