Scientia Horticulturae 177 (2014) 85–91

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Reproductive barriers in interspecific hybridizations among *Chimonanthus praecox* (L.) Link, *C. salicifolius* S. Y. Hu, and *C. nitens* Oliver from pollen–pistil interaction and hybrid embryo development

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ARTICLE INFO

Article history:
Received 28 April 2014
Received in revised form 24 July 2014
Accepted 28 July 2014
Available online 17 August 2014

Keywords:
Chimonanthus praecox
C. salicifolius
C. nitens
Interspecific hybridization
Pollen–pistil interaction
Hybrid embryo development

ABSTRACT

To investigate interspecific cross-compatibility among *Chimonanthus praecox* (L.) Link, *C. salicifolius* S. Y. Hu, and *C. nitens* Oliver, pollen–pistil interaction by fluorescence microscopy and hybrid embryo development by whole mount clearing technique were observed. The results indicated that for all reciprocal crosses among the three species, pollen grains of the male parents could adhere to and germinate in the stigmas of female parents. For any cross combination, pollen tube growth could reach the embryo sac, and double fertilization could be completed. These findings indicated that no obvious barriers to interspecific hybridization existed at the stages of pollen germination and pollen tube elongation until double fertilization. Although certain proportion (29.6% at 9 d and 36.3% at 15 d after pollination) of abortions (e.g., non-fertilization and retarded or non-existent endosperm development) in normally pollinated embryos of *C. praecox* at different stages were also observed, a greatly increased proportion (66.1% at 8 d and 76.7% at 15 d after pollination in *C. praecox* × *C. nitens*, and 60.0% at 8 d and 75.0% at 15 d after pollination in *C. praecox* × *C. salicifolius*) of hybrid embryos did not complete double fertilization or completed double fertilization but underwent abortion due to the retardation of endosperm development. Comparing to normally pollinated embryos of *C. praecox*, the retardation of endosperm development in hybridizations occurred earlier. Moreover, the occurrence of abortions among different cross combinations was basically consistent. Thus, the retardation of the endosperm development of hybrid embryos after double fertilization (after the globular embryo stage) is an important factor leading to the failure of distant hybridization between *Chimonanthus* species.

1. Introduction

Calycanthaceae is a small and relatively primitive family that includes a total of three genera (*Chimonanthus* Lindley, *Calycanthus* L., and *Sinocalycanthus* Cheng et S.Y. Chang) and 7–9 species. This family has a disjunct distribution across East Asia and North America. In spite of a small number of species in the family, these species are highly diverse with respect to relevant traits, such as growth and ecological habits, flowering period, floral structure, odor, floral display, leaf structure, and leaf content, especially abundant diversity in the floral traits. *Chimonanthus* is endemic to China and the tertiary relict taxa, which include 6 species, mostly grow in mixed evergreen and deciduous broad-leaved forests and evergreen broad-leaved forests in warm temperate and subtropical regions (Zhang et al., 1998; Li and Li, 2000). The plants in this genus are widely distributed and are most abundant in the Yangtze River basin.

Chimonanthus praecox (L.) Link is a traditionally ornamental fragrant plant that flowers in winter (from December to February) in China (Zhang et al., 1998; Kozomara et al., 2008). The flowers have an elegant aroma and flavor, with abundant variations in floral traits. *C. nitens* Oliver is a fragrant evergreen shrub that has leathery leaves with smooth surfaces, white tepals with a flowering period from September to November. *C. salicifolius* S. Y. Hu is a semi-evergreen shrub with narrow, willow-like leaves that have a rough upper surface and a lower surface covered in white powder. This plant's flowers are similar to the flowers of *C. nitens* and also have a flowering period from September to November. These three species exhibit rich variations in growth habits, floral traits, flowering period, and stress resistance. In particular, the leaves of *C. nitens* and *C. salicifolius* are rich in alkaloids and flavonoids

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http://dx.doi.org/10.1016/j.scienta.2014.07.040
0304-4238/

(Xu et al., 2006; Ouyang et al., 2010; Shu et al., 2010), which not only possess extremely high medicinal value but also confer good insect resistance.

The use of distant hybridization to introduce heterologous genes and thereby create novel germplasm with advantages from both parents to expand genetic pools is currently a primary method (Eeckhau et al., 2007). With respect to the distant hybridization in Calycanthaceae, Lasseigne et al. (2001), first reported obtaining intergeneric hybrids of *S. chinensis* × *Calycanthus floridus*, and Yao et al. (2007), have also reported obtaining intergeneric hybrids of these two species (*S. chinensis* × *C. floridus*). We have utilized conventional hybridization approaches to obtain *C. floridus* var. *oblongifolius* × *S. chinensis* hybrids, which first flowered in 2013 (unpublished work). Although these intergeneric hybrids have been obtained, the observed seed sets in hybridizations have been extremely low, indicating the presence of certain reproductive barriers in the intergeneric hybridization. Therefore, Wang et al. (2013), examined the underlying mechanisms of barriers to the hybridization of *S. chinensis* var. *oblongifolius* and *C. floridus* and found that reciprocal crosses involved different incompatibility mechanisms. When *S. chinensis* was the female parent, the main barrier to successful hybridization was the abnormal disintegration of the early hybrid embryo after fertilization. When *C. floridus* var. *oblongifolius* was the female parent, the main barriers to hybridization were due to the combination effects of high proportion of developmental abnormalities of female pistils and the abortion of early hybrid embryos.

No prior studies have reported on the cross-compatibility of interspecific hybridizations within *Chimonanthus*. Interspecific hybridization may represent a method of successfully transferring or aggregating desirable traits to create new varieties with high ornamental value and strong stress resistance. However, in numerous tests of interspecific hybridization, conventional hybridization experiments involving the aforementioned these three species could produce the enlargement of fruit during the early post-pollination stages but could not successfully obtain hybrid seeds. Therefore, in this paper, the reproductive barrier of interspecific hybridization within *Chimonanthus* is investigated from the perspectives of pollen–pistil interaction (pollen germination, pollen tube elongation, and fertilization), and hybrid embryo development after pollination. Using this approach, the present study clarified the underlying mechanisms of reproductive barriers to hybridization, thereby providing a reference for future breeding efforts involving the distant hybridization of *Chimonanthus*.

2. Materials and methods

2.1. Materials

The materials of *C. praecox* 'CHg08' (Fig. 1a), *C. nitens* (Fig. 1b), and *C. salicifolius* (Fig. 1c) were from the germplasm resource of Zhejiang Agriculture and Forestry University. *C. praecox* 'CHg08' is an early-blooming germplasm whose anthesis is from the end of October to the beginning of December. *C. nitens* and *C. salicifolius* are introduced from the field respectively in Lishui, Zhejiang and Qiyunshan, Anhui. These plants had been cultivated for above 8 years and were capable of normal flowering. Hybridization pollinations were conducted during the October–December flowering periods in 2010–2012.

2.2. Cross-pollination and sample fixation

Reciprocal crosses was conducted for each pairwise combination of the three aforementioned *Chimonanthus* species according to Wang et al. (2013), and a normal pollination (natural pollination) of *C. praecox* 'CHg08' using the conspecific pollen from other strains was as a control. The following specific operating procedures were employed. On a clear day, well-developed flower buds on a female parent were selected. Emasculation was performed on the relaxed flower buds by entirely removing the upcoming inner and outer tepals as well as the stamens (without damaging the stigma). Anthers just before dehiscence from the male parents were collected and dehydrated under incandescent lamp for 2 h, and then pollen were picked up and used to pollinate the stigmas of female parents. After pollination, flowers were promptly bagged and labeled. Pistils were collected at 1, 2, 6, 12, 24, 36, 48, 72, and 96 h after pollination, fixed with FAA (formalin: acetic acid: 50% alcohol, 1:1:8) in a 5 mL centrifuge tube for more than 24 h, and observed by fluorescence microscopy. In addition, to observe hybrid embryo development, for 3–10 d after pollination, sampling was performed once per day; for 10–20 d after pollination, sampling was performed at 11 d, 13 d, 15 d, 17 d and 20 d; beyond 20 d after pollination, sampling was performed every 5 d until shedding occurred.

2.3. Fluorescence microscopy

A slightly modified version of the fluorescence microscopy method described by Zhou et al. (2006), was utilized in this study. The developing fruits were gently cut with a razor blade, and the whole pistils including stigmas and ovaries were separated from fruits and collected with tweezers. Each sample was softened and bleached with 2 mol L^{-1} NaOH for 2 h and then soaked in 0.1% aniline blue staining solution for 10 h (overnight). Samples were mounted in glycerol, and sections were prepared. The adhesion to and germination of the pollen on the stigma and pollen tube elongation was observed under the fluorescence microscope (Leica DM4000).

2.4. The observation of embryo development using the whole mount clearing technique

The whole mount clearing technique allows for the rapid observation of internal organization and structural development within certain materials that are small in size, difficult to prepare by section, or inefficient to observe by section (Jana et al., 2006; Hao and Qiang, 2007). The following specific procedures were employed in this process. (1) Samples (developing fruits) were fixed for more than 24 h was gently cut with a razor blade, and the pistils were separated with tweezers and placed into a centrifuge tube; (2) the pistils were successively treated with a 75–85–95–100% I–100% II ethanol gradient for 2 h per ethanol solution, (3) were treated with an ethanol and methyl salicylate mixture (1:1 by volume) for 3 h, (4) and were cleared three times with methyl salicylate for 12 h for each of the first two clearing treatments and for 24 h for the final clearing treatment. (5) Then samples were placed on a concave glass slide and observed and photographed under the differential interference microscope (Nikon Eclipse 80i).

3. Results

3.1. Pollen germination and pollen tube elongation of hybridization between C. praecox and C. salicifolius

In *C. praecox* × *C. salicifolius*, pollen grains adhered to the stigma by 6 h after pollination (Fig. 2a); pollen germination had begun at 12 h after pollination (Fig. 2b); the pollen tube was continuing to grow downward at 24 h after pollination (Fig. 2c); and the pollen tube had reached the embryo sac at 48 h after pollination (Fig. 2d). When *C. salicifolius* was the female parent, observations again