Cytological Study of Gender Conversion in Amur Grape

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ABSTRACT. Amur grape (*Vitis amurensis*) is a dioecious species. To elucidate the time of and reason for pistil abortion in male amur grape from the perspective of cytology, we observed the sections of pistil of a male line during its development using optical and transmission electron microscopes. The abnormality in the morphology of nucellar cell and the development of various organelles appeared before the abnormality of functional megaspore mitosis. Programmed cell death (PCD) of the nucellar cells might be an important reason for mitosis disorder, leading to the abortion of pistil in male flower. However, the abortion can be eliminated by forchlorfenuron treatment, resulting in the recovery of functional pistil in male amur grape. This study provides cytological information on the gender conversion mechanism in male amur grape, which can promote gender determination studies in *Vitis* species.

The wild amur grape is a dioecious species, mostly cultivated for wine production in China (Song et al., 2000). It is the hardest species within the genus *Vitis* and can tolerate temperatures as low as -40°C (Jiao et al., 2015; Ma et al., 2010). Therefore, it is a widely used species for grapevine cold-hardiness research (Dubrovina et al., 2015; Li et al., 2013) and for hardy cultivar breeding (Zhang et al., 2015). Furthermore, amur grape is resistant to various diseases incited by fungi, such as *Uncinula necator*, *Plasmopara viticola*, and *Coniothyrium diplodiella* (Li et al., 2008; Wan et al., 2007). Currently, several genes or quantitative trait loci that improve downy mildew resistance have been identified in amur grape (Blasi et al., 2011; He et al., 2017; Li et al., 2015a). Since the 1950s, China has been working on the collection, evaluation, and utilization of amur grape (Li et al., 2015b). In 1963, the first hermaphrodite plant, which was released later as a cultivar named Shuangqing in 1982, was discovered in this naturally dioecious species (Huang, 1980). Since then, the direction of research with amur

grape has turned from wild resource selection to sexual crossbreeding (Li et al., 2015b).

The gender of plants has long been a hotspot for plant researchers. They have mainly focused on model plants, such as *Silene latifolia* (Charlesworth, 2002; Lardon et al., 1999; Matsunaga et al., 1996; Scutt et al., 1997), *Zea mays* (Bensen et al., 1995; DeLong et al., 1993), and *Cucumis sativus* (Kamachi et al., 1997, 2000; Knopf and Trebitsh, 2006; Mibus and Tatlioglu, 2004; Trebitsh et al., 1997; Yamasaki et al., 2000, 2001). The dioecious amur grape is an ideal species for studies on the gender of perennial woody plants. In grapevine, it has been reported that not only cytokinin treatment (Hashizume and Iizuka, 1971; Iizuka and Hashizume, 1968; Moore, 1970; Negi and Olmo, 1966), but also ethephon treatment (Kender and Remaily, 1970) can convert male flowers to hermaphrodite flowers. Studies in northeast China have mainly focused on the effects of cytokinin on amur grape. For instance, Ai et al. (2002) compared different effects of 6-benzylaminopurine (6-BA) and N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea (CPPU) on gender conversion efficiency in male amur grape and found that CPPU was more efficient, resulting in a fruit setting rate of 96.7%. Xu et al. (2013) analyzed proteomic changes during the gender conversion of male amur grape and found a key protein that was upregulated by CPPU, which resulted in the ability to fruit at the end.

The gender conversion studies in amur grape have opened a new direction for germplasm innovation, providing some evidence on gender differentiation mechanism in *Vitis* species. In the present study, we 1) focused on the abortion of pistil and

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its recovery from the perspective of cytology and 2) aimed to provide a more theoretical basis for gender determination mechanism in amur grape.

Materials and Methods

PLANT MATERIALS AND CPPU TREATMENT. Male amur grape (accession no. 043) was used as the study material. The plants were grown in the National Field Gene Bank for Amur Grapevine, Zuojia, Jilin. The inflorescences were dipped in CPPU solution ($75 \text{ mg} \cdot \text{L}^{-1}$) for 5 s on 19 May 2016. The control inflorescences were treated with water. The flower buds were then collected on days 3, 6, 9, and 12 after treatment. The inflorescences developed to flowers on day 12 after treatment, 31 May 2016.

OBSERVATION OF PISTIL DEVELOPMENT. Ten flower buds were collected at each sampling time. After stripping the caps, the morphology of pistil was observed using a stereomicroscope (SZX7; Olympus, Tokyo, Japan), and the vertical and transverse diameters were measured by its self-contained measuring software. The statistical difference between the treated and control pistils collected on day 12 after treatment was analyzed by the *t* test using the analysis pack in Excel (Microsoft, Redmond, WA).

STATIONARY LIQUID PREPARATION AND SECTIONING. The flower bud samples were preserved in formaldehyde–alcohol–acetic acid stationary liquid [5 mL of formalin (38% formaldehyde), 5 mL of glacial acetic acid, and 90 mL of 50% ethyl alcohol] to prepare paraffin sections and in glutaraldehyde–paraformaldehyde stationary liquid [50 mL of $0.2 \text{ mol} \cdot \text{L}^{-1}$ phosphate buffer ($\text{pH} = 7.4$), 20 mL of 10% paraformaldehyde, 10 mL of 25% glutaraldehyde, and 20 mL of redistilled water] to prepare sections for transmission electron microscopy (TEM). The paraffin sections of $10\text{-}\mu\text{m}$ thickness were prepared following dehydration, transparency, waxdip, and embedment. The sections were then stained with safranin-fast green dye and sealed with neutral balsam. After desiccation, the sections were observed using an optical microscope (BA310; Motic, Xiamen, China). To prepare the sections for TEM, young flower buds were quickly dipped in glutaraldehyde–paraformaldehyde stationary liquid and incubated for more than 24 h. After incubation, the materials were washed with phosphate buffer and dehydrated with gradient concentrations of ethyl alcohol (70%, 85%, 95%, and 100%), and then sealed with resin. The sections of thickness 90 nm were obtained using an ultramicrotome (EM UC7; Leica, Wetzlar, Germany), double stained with uranyl acetate–lead citrate, and observed using a transmission electron microscope (HT7700; Hitachi, Tokyo, Japan).

Results

EFFECT OF CPPU TREATMENT ON THE DEVELOPMENT OF PISTIL. With the development of flower, the CPPU-treated pistil gradually expanded, forming an obviously inflated pistil on day 12 after treatment. However, there were no obvious changes in the size of control pistil during the development of flower (Fig. 1). The vertical and transverse diameters of the treated pistil were 2.322 and 1.319 mm, respectively, on day 12 after treatment, whereas, those of the control were only 0.895 and 0.759 mm, respectively (Fig. 2). Compared with those of the control, the vertical and transverse diameters of the CPPU-treated pistil increased significantly by 159% and 73.8%, respectively.

Fig. 1. Dissected buds of male amur grape, observed using a stereomicroscope, showing changes in the size of pistils in N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea-treated (right) and control (left) flowers: (A) day 3 after treatment, (B) day 6 after treatment, (C) day 9 after treatment, and (D) day 12 after treatment (magnification = $\times 2.5$).

EFFECT OF CPPU TREATMENT ON FRUIT PRODUCTION AND SEED FORMATION. Under natural condition, male amur grape cannot bear fruit, and its inflorescences wither and fall (Fig. 3A).

Fig. 2. Effects of N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea treatment vs. a control (CK) on the vertical and transverse diameters of pistils of male amur grape on day 12 after treatment. *Significant difference at $P \leq 0.05$.

Fig. 3. Withered inflorescence of control male amur grape (cluster, seeds, and fruit) produced by N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea-treated male amur grape: (A) withered inflorescence, (B) young fruit cluster, (C) green seeds, and (D) ripe fruit.

However, the treated inflorescence continued its development and formed a young fruit cluster (Fig. 3B). Furthermore, green seeds were revealed when the flesh of a pea-sized young fruit was stripped (Fig. 3C); eventually, ripe fruit were produced (Fig. 3D). These findings indicate that the reproductive function of pistil, which was supposed to have been aborted, can be recovered with CPPU treatment.

OBSERVATION OF THE OVULE MORPHOLOGY AND FUNCTIONAL MEGASPORE MITOSIS. The morphological differences in the ovule of treated and control flowers were observed at each development

stage. On day 3 after treatment, an anatropous ovule consisting of a near round-shaped nucellus tissue and double layer of integument was formed in the treated flowers. The inner integument was almost connected at the micropylar end, whereas the outer integument was not connected (Fig. 4B). However, in the control flower, an irregular-shaped ovule was formed (Fig. 4A). At this development stage, a single-nucleate embryo sac was formed in both treated and control flowers (Fig. 4A and B). On day 6 after treatment, a regular oval-shaped ovule was formed in the treated flower, and the outer cell layers of both the integument and nucellus tissue were darkly stained and orderly arranged, showing two distinguishable ovule structures, the integument and nucellus (Fig. 4H). However, in the control flower, only the outer layer of the integument was darkly stained, and the boundary of the integument and nucellus tissue was difficult to identify, leaving a gap outside the nucellus (Fig. 4C). At this stage of development, a double-nucleate embryo sac was formed in both treated and control flowers (Fig. 4C and D). On day 9 after treatment, the irregularity of nucellus shape intensified in the control flower. Furthermore, difference in mitosis appeared at this time. A normal four-nucleate embryo sac developed in the treated flower, whereas an abnormal three-nucleate embryo was observed in the control flower (Fig. 4E and F). On day 12 after treatment, a typical mature embryo sac containing eight nuclei was formed in the treated flower (Fig. 5A–C). However, mitosis stagnated in the control flower, still showing an abnormal three-nucleate embryo sac (Fig. 4G).

NUCELLAR CELL MORPHOLOGY AND ULTRAMICROSTRUCTURE OF ORGANELLES.

No obvious difference was observed in the nucellar cell between treated and control flowers on day 3 after treatment. The nucellar cells in both treated and control flowers exhibited a polyhedron shape and were orderly arranged (Fig. 6A and B). However, on day 6 after treatment, the cell morphology showed significant differences between the treated and control flowers. In the treated flower, the nucellar cell exhibited a regular shape with orderly arrangement (Fig. 6D); however, the shape of nucellar cell was highly irregular in the control flower (Fig. 6C). The morphological abnormality of nucellar cell in the control flower

Fig. 4. Optical micrographs of N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea-treated (right) and control (left) male amur grape embryo sacs, showing the process of functional megaspore mitosis: (A and B) day 3 after treatment, showing a single-nucleate embryo sac (arrowhead); (C) day 6 after treatment, showing a double-nucleate embryo sac (arrowhead) and a gap between the nucellus and integument (circle); (D) day 6 after treatment, showing a double-nucleate embryo sac (arrowhead); (E) day 9 after treatment, showing an abnormal three-nucleate embryo sac (arrowhead); (F) day 9 after treatment, showing a normal four-nucleate embryo sac (arrowhead); (G) day 12 after treatment, showing an abnormal three-nucleate embryo sac (arrowhead); and (H) day 12 after treatment, showing a complete ovule structure (scale bars: A–G = 20 μ m; H = 100 μ m).

Fig. 5. Optical micrographs of N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea-treated male amur grape embryo sac on day 12 after treatment, showing a mature eight-nucleate embryo sac: (A) embryo sac with three antipodal cells (arrowhead); (B) embryo sac with two polar nuclei (arrowhead); and (C) embryo sac with an egg cell and two synergids (arrowhead) (scale bars = 20 μ m).

might be an indication of the subsequent disorder of functional megaspore mitosis.

On day 6 after treatment, the organelle development in nucellar cell showed significant differences between the treated and control flowers. The cytoplasm of nucellar cell in the control flower was concentrated to a certain region and the organelles were also difficult to recognize (Fig. 7A). This might be attributed to the degradation of various organelles. However, in the nucellar cell of the treated flower, the organelles, including cell nucleus (Fig. 7B), chloroplasts (Fig. 7C), mitochondria (Fig. 7D), Golgi apparatus (Fig. 7E), and endoplasmic reticulum (Fig. 7F), were visible.

Discussion

GENDER DIFFERENTIATION MODEL OF AMUR GRAPE. The gender differentiation model of dioecious species can be divided into two types, models I and II (Mitchell and Diggle, 2005). In model I, both stamen and pistil primordia are formed during the bisexual stage. Subsequently, with the function of the gender determining gene either stamen or pistil primordium stops its development at a certain stage, whereas the other primordium continues its development to form a unisexual male or female flower (Aryal and Ming, 2014; Ming et al., 2011; Mitchell and Diggle, 2005). *Zea mays* (Cheng et al., 1983), *C. sativus* (Kater et al., 2001), *S. latifolia* (Farbos et al., 1997), *Rhaphis subtilis* (Dransfield et al., 2008), *Asparagus officinalis* (Park et al., 2003), *Swietenia macrophylla* (Gouvêa et al., 2008), *Rumex acetosa* (Ainsworth et al., 2005), and *Vitis vinifera* ssp. *silvestris* (Caporali et al., 2003) belong to model I. However, in model II, flower development bypasses the bisexual stage, and both the stamen and pistil primordium do not occur. *Spinacia oleracea* (Sherry et al., 1993), *Thalictrum dioicum* (Di Stilio et al., 2005), *Populus deltoides* (Kaul, 1995), and *Hedyosmum orientale* (Cui et al., 2011) belong to model II. In the present study, functional pistils were recovered in male flowers by

Fig. 6. Morphology of nucellar cells of N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea-treated (right) and control (left) male amur grape: (A and B) day 3 after treatment, showing polyhedron-shaped and orderly arranged nucellar cells; (C) day 6 after treatment, showing irregular-shaped nucellar cells; and (D) day 6 after treatment, showing regular-shaped nucellar cells (scale bars: A–C = 20 μ m; D = 10 μ m).

Fig. 7. A whole nucellar cell of N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea (CPPU)-treated (B) and control (A) male amur grape and the ultrastructure of nucellar organelles of CPPU-treated (C–F) male amur grape on day 6 after treatment: (A and B) a whole nucellar cell, (C) chloroplast (arrowhead), (D) mitochondrion (arrowhead), (E) Golgi apparatus (arrowhead), and (F) endoplasmic reticulum (arrowhead) (scale bars: A, B = 10 μ m; C–F = 1 μ m).

CPPU treatment, indicating the formation of pistil primordia. Therefore, according to Mitchell and Diggle (2005), gender differentiation in amur grape belongs to model I.

TIME OF PISTIL ABORTION IN MALE AMUR GRAPE. The stagnation of development of female organ occurs at different stages in different plant species. In male flowers of mahogany, the ovule primordia degenerate quickly as soon as they are formed (Gouvêa et al., 2008). However, in *Consolea spinosissima*, the ovules of the staminate flowers degenerate after complete maturation of the embryo sac (Strittmatter et al., 2002). In *Gymnocladus dioicus*, the pistil aborts when the megasporocyte is formed in the nucellus (Hou et al., 2005). In the present study, the stagnation of pistil development in male amur grape occurred before the formation of four-nucleate embryo sac, and this is consistent with the findings of a previous study in amur grape (Wang et al., 2013). According to the time of division reported by Diggle et al. (2011), the abortion of the female organ in amur grape happens at stage three (post-meiosis). However, in European grape, pistil growth is retarded at prophase of the first meiotic division (Caporali et al., 2003). Therefore, the abortion of the female organ happens at different stages in amur grape and *V. vinifera*. This suggests that these two species may have evolved the mechanism of gender differentiation independently, at least for male plants.

REASON FOR PISTIL ABORTION IN MALE AMUR GRAPE. The reasons of female organ abortion include cell death, PCD, parenchymatization, arrest of development or no development, and change in developmental time or lack of maturation (Diggle et al., 2011). Among these, cell death is an important strategy that arrests the development of sex organ in unisexual flowers. For instance, by cell death, maize ends gynecium development (Cheng et al., 1983) and *Thymelaea hirsuta* ends meiosis (Caporali et al., 2006). The sterility of pistil in the male flowers of European grape might arise due to the abnormal division of megaspore or female gametophyte,

and the subsequent PCD of external nucellar cells (Caporali et al., 2003). Sometimes cell death results in the formation of gaps in the floral organs, thus, acting as obstacles for nutrition transportation and information negotiation. For instance, Wetzstein et al. (2011) observed different types of arrested development of pistil in aborted *Punica granatum* flowers, one of which exhibited gaps between the inner and outer integuments. Rosati et al. (2011) found that the abnormality of integument formation breaks the negotiation between the integument and embryo sac, resulting in pistil abortion. In the present study, the organelles were not identified from day 6 after treatment, implying the occurrence of PCD of the nucellar cell. Furthermore, no cell layer in the outer edge of the nucellus tissue was stained with safranin-fast green dye, leaving a gap between the nucellus and integument. This is consistent with the findings of Wang et al. (2013).

Overall, pistil abortion in male amur grape might be caused by the PCD of nucellar cells that starts before the formation of the four-nucleate embryo sac. The successful gender conversion by CPPU treatment provides more opportunities to evaluate and use male amur grape. Herein, we have provided cytological evidence for gender conversion in amur grape. However, the exact mechanism remains unknown, and further studies should concentrate on mapping, cloning, and the expression of gender determining gene in amur grape.

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