Pak. J. Bot., 53(5): 1743-1752, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-5(34)

THE RELATIONSHIP BETWEEN NUTRIENTS AND GENDER CONVERSION IN MALE AMUR GRAPE

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Abstract

Amur grape (*Vitis amurensis* Rupr.) is a dioecious species. To elucidate the cause of pistil abortion in male amur grape from the perspective of nutrition, we performed a metabolic pathway analysis based on its transcriptome data and used optical microscopes to observe slices of the pistil of a male line during its development. Abnormality was found in its ovary shape and structure, and the expression of some genes in the glucose metabolism pathway changed, and the content of starch and protein in the ovule decreased during pistil development. These abnormities may thus serve as important contributors to pistil abortion in male flower. However, abortion could be eliminated by N_1-(2-chloro-4-pyridyl)-N_3-phenylurea (CPPU) treatment, which caused the recovery of functional pistil in male amur grape. Altogether, this study provides information from the perspective of nutrition regarding sex conversion mechanism in male amur grape and may promote sex determination studies in *Vitis* species.

Key words: *Vitis amurensis*; Gender; Pistil; Starch; Protein; Glucose metabolism.

Introduction

Most amur grapes are dioecious and are important raw materials for brewing. Amur grapes are widely distributed in Northeast China (He, 1999; Sha *et al*., 2016; Tang *et al*., 2008; Wang *et al*., 1999) and are known as the hardiest species within the genus *Vitis*. They can tolerate a temperature as low as -40°C (Ma *et al*., 2010; Jiao *et al*., 2015), and are highly resistant to various fungal diseases, such as powdery mildew, downy mildew, and white rot (Li *et al*., 2008; Wan *et al*., 2007). Amur grapes have thus become an important resource for grapevine cold hardiness and disease resistance breeding. In natural field environment, male amur grapes can flower but cannot produce fruit. Through treatment with exogenous cytokinin, gender conversion of the male amur grape can be achieved and fruit can be produced (Guo *et al*., 1995; Negi *et al*., 1971, 1972). N_1-(2-chloro-4-pyridyl)-N_3-phenylurea (CPPU) is a plant growth regulator with high cytokinin activity. As CPPU promotes cell division, organogenesis, and fruit enlargement, it is widely used in crops, fruit trees, vegetables, and flowers (Ben *et al*., 1997; Cruz-Castillo *et al*., 2002; Lewis *et al*., 1996; Watanabe *et al*., 1989; Kefford *et al*., 1968). Furthermore, it is significantly better than the 6-benzylaminopurine (6-BA) used for gender conversion in male grapes (Ai *et al*., 2002).

Gender differentiation of plants is a special organogenesis phenomenon, and is an important research hotspot in plant developmental biology. However, previous studies have mainly focused on model plants, such as *Silene conoidea* (Matsunaga *et al*., 1996; Scutt *et al*., 1997; Lardon *et al*., 1999; Charlesworth, 2002), *Zea mays* (Delong *et al*., 1993; Bensen *et al*., 1995), and *Cucumis sativus* (Kamachi *et al*., 1997, 2000; Trebitsh *et al*., 1997; Yamasaki *et al*., 2000, 2001; Mibus and Tatlioglu, 2004; Knopf and Trebitsh, 2006). The formation of parthenocarpic flowers in most higher plants is a transition process from bisexual to monosexual. First, the process of bisexuality must occur; thereafter, under the action of gender-determining genes, the stamen primordia or pistil primordium will sequentially differentiate (Bai *et al*., 2004; Delong *et al*., 1993; Akagi *et al*., 2014). The differentiation of the unisexual flower of the amur grape also experiences this process. The male amur grape is a bisexual flower in the early stage; however, as its flower develops, the pistil is aborted and a male flower eventually forms (Ai *et al*., 2002; Wang *et al*., 1994; Jiao *et al*., 2015; Shen *et al*., 2018).

Nutrients such as starch, polysaccharides, and proteins play important role in plant growth and development. In fact, the development of the plant female gametophyte, megasporogenesis, and the development of embryo and endosperm are closely related to the content of nutrients such as polysaccharides, starch, and protein in the tissues of multiple species (Meng, 1997). An insufficient nutrition amount in flower organs can seriously affect the normal development of pistils, causing abortion of pistil in *Boehmeria nivea* (Liu *et al*., 2006) and *Prunus salicina* (Yao *et al*., 2000). To date, there has been no report on the effect of changes in the nutrient content of pistil on gender differentiation during pistil abortion in male grape. However, gender conversion studies with amur grape have revealed a new direction for germplasm innovation, providing some evidence on the gender differentiation mechanism in the *Vitis* species. In the present study, we focused on the abortion of pistil and its recovery from the perspective of nutrition, and aimed to provide a more theoretical basis for the gender determination mechanism in amur grape.

Materials and Methods

Plant materials and CPPU treatment: Male amur grape (accession No. 043, No. 75134, No. 75017, No. 73061, No. 1-3, and No. 75047) was used as the study materials. The plants were grown in the National Field Gene Bank for Amur Grapewine, Zuojia, Jilin. The inflorescences were dipped in CPPU solution (75 $mg \cdot L^{-1}$) for 5 s on May 17, 2014. The control inflorescences were treated with water. The inflorescences developed into flowers on day 12 after treatment (May 30, 2014).

Comparison of inflorescence, flower bud, and pistil size: After 12 days of treatment, the floral organ morphology of male plants (No. 043, No. 75134, No. 75017, No. 73061, No. 1-3 and No. 75047) and the controls was investigated. Ten inflorescence, ten flower buds, and ten pistils were collected at each sampling time. The inflorescence size was measured with a ruler and the flower buds and pistils size were measured with a slide caliper rule. Statistical analysis was conducted with Microsoft Office Excel. The difference between treated and control plants was analyzed by SPSS 22.0.

Stationary liquid preparation and sectioning: The flower bud samples were preserved in formaldehyde-alcohol-acetic acid (FAA) 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. Paraffin sections (10-mm thick) were prepared following dehydration, transparency, wax dip, and embedment. The sections were then stained with safranin-fast green dye and sealed with neutral balsam. After desiccation, the sections were observed with an optical microscope (BA310; Motic, Xiamen, Fujian, China).

Transcriptome analysis of the flower buds: Twenty flower buds in the middle of inflorescence of the treated and control male grape (No. 043) were collected at each stage. Sampling was performed at 3 h, 6 h, 12 h, 1 d, 3 d, 6 d, 9 d, and 12 d after treatment. Flower buds were mixed as a singel sample at each sampling time. Transcriptome sequencing and analysis were relegated to Biomarker Technologies (Beijing, China).

Observation of the chemically-stained ovule tissue: Ten flower buds in the middle of inflorescence of the treated and control male grape (No. 043) were collected at each stage. Sampling was performed at 3 h, 6 h, 12 h, 1 d, 3 d, 6 d, 9 d, and 12 d after treatment. The pistil was cut vertically to create paraffin sections, and the starch grains and proteins in the ovule tissue were stained by the Schiff- phenol yellow counter staining technique. Observations were performed with an optical microscope (BA310, Motic, Xiamen, Fujian, China). The following steps were employed for Schiff-phenol yellow counter staining: the paraffin section was first dewaxed in xylene for 5 min, and then dewaxed in a mixture of 1/2 xylene and 1/2 ethanol for 2 min. Thereafter, a gradient ethanol dehydration was initiated. The order of ethanol concentration was 100%, 95%, 85%, 70%, and 50% ethanol, respectively. Each concentration was used for 2 min for dehydration. Following dehydration, the section was soaked in distilled water for 2 min, and then transferred to a 0.5% potassium periodate solution for 10 min. The section was washed carefully with water for 5 min, and then with distilled water for 2 min. Thereafter, the Schiff reagent was applied for 20 min. After three rounds of washing (2 min each), the section was washed with tap water for 5 min and then with distilled water for 2 min. The washed section was then placed in a 0.02% phenol yellow acetic acid dye solution for 10 min, and washed with distilled water for 2 min, and dehydrated with tert-butyl alcohol twice (2 min each). The dehydrated sections were treated with xylene transparent for 5 min, and then sealed with a gum.

Results

Effect of CPPU treatment on flower organ development: CPPU had a minor effect on inflorescence size of male amur grape (Fig. 1). Although the length of inflorescence of the treated No. 1-3 was significantly higher than that of the control, other treated male amur grapes did not significantly differ from their corresponding controls. In addition, no significant difference was found in inflorescence width between treatment and control. However, CPPU significantly promoted the development of male flower bud and pistil size (Fig. 2). After treatment, the length of the flower bud increased by 26.3%-45.8% (average increase, 32.3%), and the width of the flower bud increased by 46.6%-75.9% (average increase, 61.4%); the length of the pistil increased by 102.9%-188.9% (average increase, 143.8%), and the width of the pistil increased by 57.1%-121.1% (average increase, 83.5%).

Effect of CPPU treatment on the development of pistil morphology and structure: The entire pistil shape of the control was small, and its placenta was not evident, and its ovary wall was irregular and appeared locally convex. Although some male amur grapes had either no style (Figs. 3A, Fig. 3C, Fig. 3E, Fig. 3F) or a short style (Fig. 3B), others had a certain style length, but the junction between the style base and the ovary almost appeared as a right angle (Fig. 3D). The stigmas of most male amur grapes were abnormal, being very small, or almost absent (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3E, Fig. 3F). 12 days after CPPU treatment, the placenta, ovary, style, and stigma of male plants were evidently expanded. Additionally, their shape was regular and the pollen tube channel could be evidently observed (Fig. 3A, Fig. 3B, Fig. 3C, Fig. 3D, Fig. 3E, Fig. 3F).

Analysis of the metabolic pathway annotated by KEGG for the differentially expressed genes: A total of 19.32 Gb clean data were obtained by sequencing quality control. In the treated and control samples, the percentage of G and C content in the total base was 47.74% and 47.96%, while the percentage of Q30 content was 93.42% and 92.89%, respectively (Table 1).

A total of 90,028 unigenes were obtained. The N50 length of unigene was 1,204 bpand displayed high assembly integrity. The 200-300 nt length had the most unigenes (37,455; 41.60%) while the 300-500 nt length had the second highest number of unigenes (22,011; 24.45%). The 500-1000 nt, 1000-2000 nt, and 2000+ nt lengths had 14,243 (15.82%), 9,964 (11.07%), and 6,355 (7.06%) unigenes, respectively (Table 2).

According to the results of KEGG classification (Fig. 4), a total of 161 DEGs were annotated in the KEGG metabolism map. These included genes involved in metabolism, organic system, genetic information processing, cell process, and environmental information processing. As a result, 50 metabolic pathways were obtained. There were 14 DEGs involved in plant hormone signal transduction, 13 in the pentose glucuronic acid conversion pathway, 13 in the starch sucrose metabolism pathway, 10 in the carbon metabolism pathway, and 10 in the plant and pathogen interaction pathway, etc. These findings suggest that gender conversion of male amur grape was closely related to glucose metabolism.

Changes in the starch content of different parts of the ovule: Three days after treatment, some starch-stained red granules were recognized in the nucellus of the control ovule; however, in the inner and outer integuments, starch granules were almost completely absent (Fig. 5A). Starch content in the treated ovule was significantly higher than that in the control, and starch grains were abundantly distributed in the inner and outer integument and nucellus. In particular, the inner side of the outer integument, inner integument, micropyle end, and the chalazal end of the nucellus tissue had a dense distribution (Fig. 5B). Six days after treatment, the starch content in the control ovule increased. With the exception of some starch distribution in the nucellus area, starch grains were mainly distributed in the inner and outer integuments (Fig. 5C); in this period, the starch content in the treated ovule remained higher; a significantly higher content than that in the control (Fig. 5D). Nine days after treatment, starch content in the control ovule decreased rapidly (Fig. 5E). Meanwhile, starch content in the treated ovule in this period remained high, and the content change was not evident compared to the previous period (Fig. 5F). Twenty days after treatment, the starch content in the nucellus sharply decreased in the control, and starch granules were rarely observed in the entire ovule (Fig. 5G). Similar to the control, starch content of treated ovules in this period decreased sharply (Fig. 5H). Overall, throughout the entire ovule development process, starch content in the control was significantly lower than that in the treated ovule, and was mainly concentrated in the inner integument.

Changes in the protein content of different parts of the ovule: Three days after treatment, protein content in the treated ovule was significantly higher than that in the control. In addition, the protein almost covered the entire ovule, displaying a relatively uniform yellow color. The nucellus was found to be slightly deeper than the integument while the chalazal end was slightly deeper than the micropyle end (Fig. 5B). The protein in the control was solely distributed in the nucellus, with a small amount existing in the integument (Fig. 5A). Six days after treatment, the protein content in the treated ovule was significantly lower than that found in the previous period. In addition, it was mainly distributed in the chalazal end of the nucellar tissue (Fig. 5D). Conversely, the protein content in the control was significantly increased. Furthermore, most of the protein almost filled the entire ovule, thereby possessing a relatively uniform yellow color (Fig. 5C). Nine days after treatment, the protein content in the treated ovule significantly increased relative to that of the previous period. In addition, its distribution was mainly found in the chalazal end of the nucellus (Fig. 5F). The protein content in the control decreased rapidly, and was predominantly found in the nucellus; distribution in the integument was seldom (Fig. 5E). Twenty days after treatment, the protein content in the treated ovule and control significantly decreased relative to that of the previous period, and the distribution of the protein changed; it was found to be concentrated in the region of the micropyle end of the nucellus (Fig. 5G, Fig. 5H). Throughout the entire process of ovule development, the protein content in the control was significantly lower than that in the treated ovule, and the distribution area changed. In the early stage, the protein was concentrated in the chalazal end, but in the later stage, it was transferred to the micropyle end.

Fig. 1. Effect of CPPU treatment on the size of the flower of male amur grape.
A: Indicates the effect of CPPU treatment on the growth of male inflorescence length; **B**: Indicates the effect of CPPU treatment on the growth of male inflorescence width; **C**: Indicates the effect of CPPU treatment on the growth of male inflorescence length; **D**: Indicates the effect of CPPU treatment on the growth of male inflorescence width; **E**: Indicates the effect of CPPU treatment on the growth of male inflorescence length; **F**: Indicates the effect of CPPU treatment on the growth of male inflorescence width.

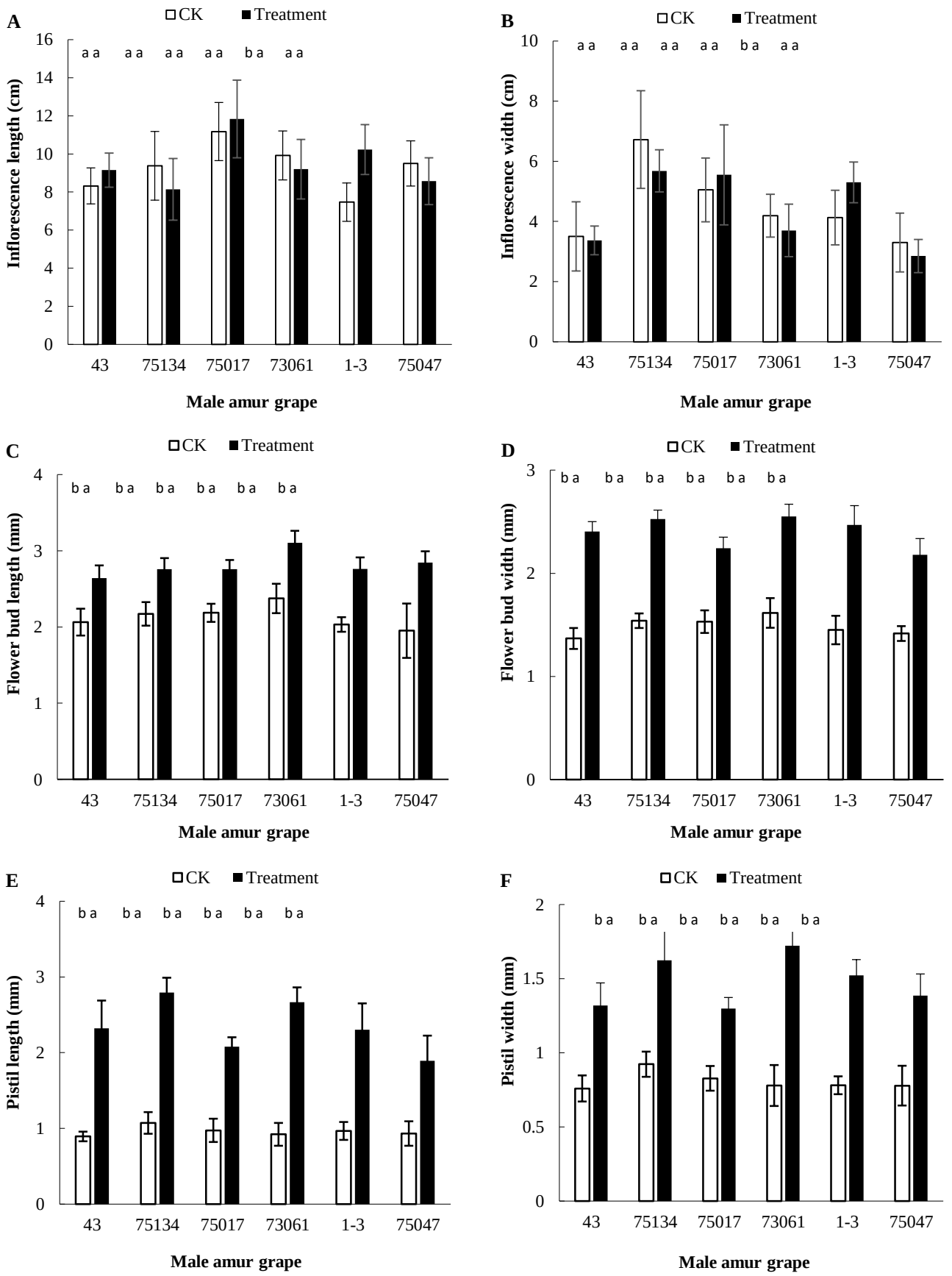

Fig. 2. Comparison of inflorescence between treated and control plants following 12 days of CPPU treatment.
A: Displays inflorescence of treated (left) and control (right) male 73061; **B**: Displays inflorescence of treated (left) and control (right) male 75047; **C**: Displays inflorescence of treated (left) and control (right) male 1-3; **D**: Displays inflorescence of treated (left) and control (right) male 75017; **E**: Displays inflorescence of treated (left) and control (right) male 75134; **F**: Displays inflorescence of treated (left) and control (right) male 043.

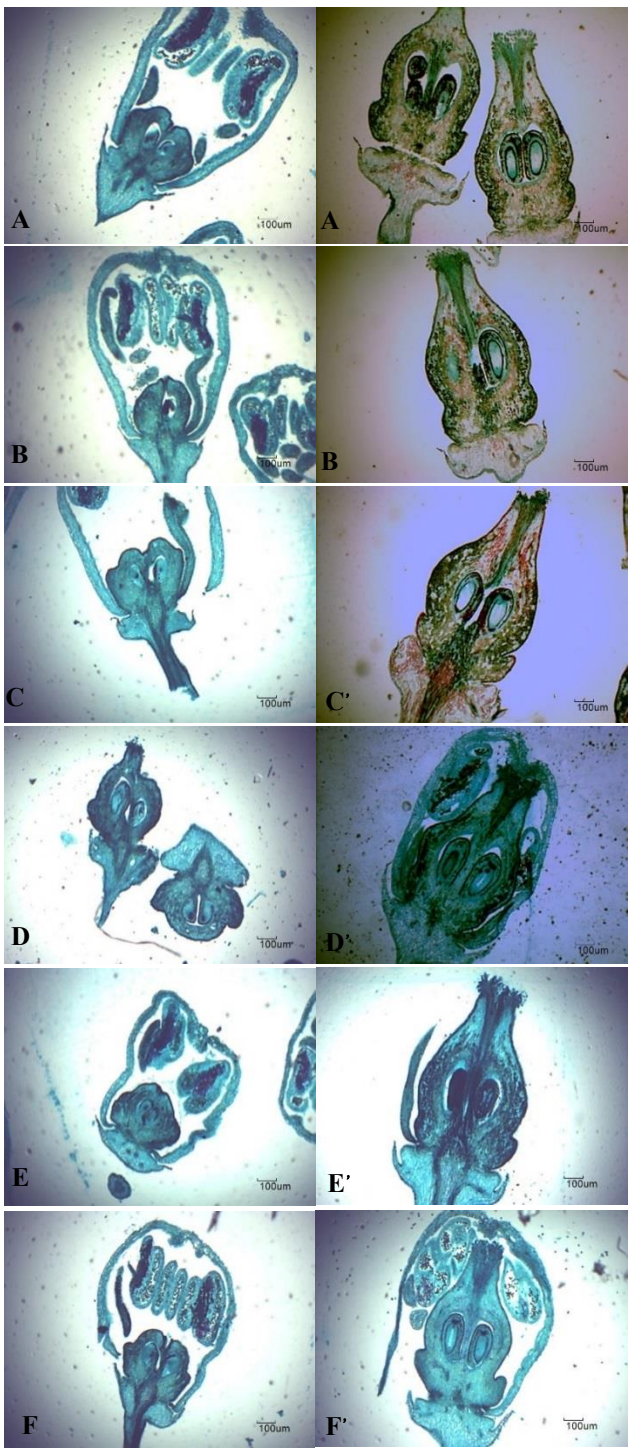

Fig. 3. Comparison of pistil morphology between the treated and control 12 days after treatment with CPPU.
A, B, C, D, E, and F represent sections from the control pistil of male 043, 75134, 75017, 73061, 1-3, and 75047, respectively; A', B', C', D', E', and F'' represent sections of the treated pistil of male 043, 75134, 75017, 73061, 1-3, and 75047, respectively. Magnification: 4x.

Discussion

Morphological changes of flower organs during pistil abortion in amur grape: The unisexual flowers of some plants must first proceed through a bisexual stage, and then successively form a stamen primordium or pistil primordium via sex determining genes. The pistil primordium and stamen primordium co-exist in the flower bud at the bisexual flower stage in cucumber. Although the carpel primordium cells of some flower buds continue to divide at this development stage, changes in the appearance of these carpels are not evident (i.e., there is no normal stigma, style, and enlarged ovary) (Bai *et al*., 2004). Because normal ovule primordium cannot be produced after the formation of flower bud ventricles, pistil abortion occurs in male *Actinidia chinensis* (Yang *et al*., 2011). Generally, the formation types of unisexual flowers are divided into two types. The first type has flowers that are hermaphroditic at the early stage of development, but gradually become unisexual as the flowers develop. As a result, they are considered to be degenerative organs without function, such as *Fragaria ananassa*, *Carica papaya*, *Silene conoidea*, *Rumex acetosa*, etc. The second type has sex differentiation occurring before the development of stamens or carpels, with no organ residues found in the mature flowers, such as *Thalictrum aquilegifolium*, *Cannabis sativa*, *Populus deltoides*, and *Mercurialis annua* (Mitchell and Diggle, 2005). According to the morphology of pistil abortion in amur grape, its gender differentiation belongs to the first category. However, the abortion forms of pistils in the mature flowers of different male lines display differences, which indicate that the specific time, stage, or degree of abortion differs in different amur grape lines.

CPPU is a plant growth regulator with high cytokinin activity that can promote cell division, organogenesis, fruit expansion, and gender differentiation (Ben-Arie *et al*., 1997; Cruz-Castillo *et al*., 2002; Lewis *et al*., 1996). By treating wild grapes (sylvestris) with different hormones, Negi *et al*., (1966) found that SD8339, which has cytokinin activity, could transform male flowers into hermaphroditic flowers three weeks before flowering. However, GA_3, IBA, chlorpromazine, 2-naphthylacetic acid, and IAA could not induce this sex conversion. Guo *et al*., (1995) demonstrated that treating amur grape with 1000 $\mu g \cdot g^{-1}$ 6-BA had a good effect on sex conversion. Furthermore, through a comparison of the effect of CPPU and 6-BA on sex conversion in male amur grape, Ai *et al*., (2002) found that the former was more stable and reliable than the latter. When the male plants were treated with CPPU in the present study, the placentation, ovary, style, and stigma displayed an evident expansion, the shape appeared regular, the pollen tube channel could be evidently observed, and finally, fruit was produced. Under natural conditions, pistil development in male amur grape is abnormal; however, plant growth regulators can cause sex transformation of flower organs, promote the expansion of pistil morphology, and form a normal morphological structure, which may be related to the production or distribution of nutrients controlled by plant growth regulators.

Sugar metabolism pathway and gender conversion of amur grape: Sugar signals affect photosynthesis, cell cycle, carbon and nitrogen metabolism, reproductive development, and plant senescence by activating or inhibiting gene expression (Smeekens, 2000). Sugar can regulate and exert multiple effects on flower transformation based on its concentration, the nutritional stage, and genetic background (Corbesier *et al*., 1998; Zhou *et al*., 1998;

Roldan *et al*., 1999; Ohto *et al*., 2001). By studying the promoter function of the *LeFRK4* in tomato, German *et al*., (2002) found that *LeFRK4* was specifically expressed in the stamen of tomato. Moreover, Pawelkowicz *et al*., (2019) revealed that the DEGs involved in glucose metabolism play an important role in the sex differentiation of *Cucumis sativus*. In *Asparagus officinalis*, a certain relationship was found between the differential expression of male and female and the pathway of glucose metabolism (Chen *et al*., 2016). In the present study, we found that CPPU promoted normal development of the pistil of male amur grape, thereby demonstrating gender conversion. We also found that most of the DEGs were involved in the glucose metabolism pathway, which indicates that gender conversion in amur grape is closely related to this pathway.

Starch content and gender conversion of amur grape: The development of the female gametophyte, megaspore, embryo, and endosperm is closely related to the content of polysaccharide, starch, protein, and other nutrients (Meng, 1997). CPPU can regulate carbohydrate production and affect fruit development in *Actinidia chinensis* (Antognozzi *et al*. 1996). In fact, Liu *et al*., (2006) revealed that normal development of the pistil of *Boehmeria nivea* would be seriously affected by a deficiency in nutrients, such as starch. Pistil abortion was found to be related to substance metabolism or the transportation obstacle. Furthermore, Yao *et al*., (2000) showed that malnutrition is an important cause of the high rate of embryo sac dysplasia in *Prunus salicina*. In *Litchi chinensis*, the abortion of pistil is accompanied by programmed cell death and starch degradation (Xiao *et al*., 2002).

In the present study, CPPU treatment enabled the production of more starch, which ensured there was sufficient energy and nutrition for nuclear division and cell proliferation. Because of the abnormality in different organelles of the nucellus and the gap between the nucellus and the integument in the process of ovule abortion in the middle and late stage of ovule development, starch content in the inner integument of the control ovule was greater than that in the nucellus. The existing gap may also affect substance exchange and transport between the nucellus and the integument (the findings of our study on the organelles of the nucellus and the formation of the gap in male amur grape were published in *Journal American Society Horticultural Science* in June 2018).

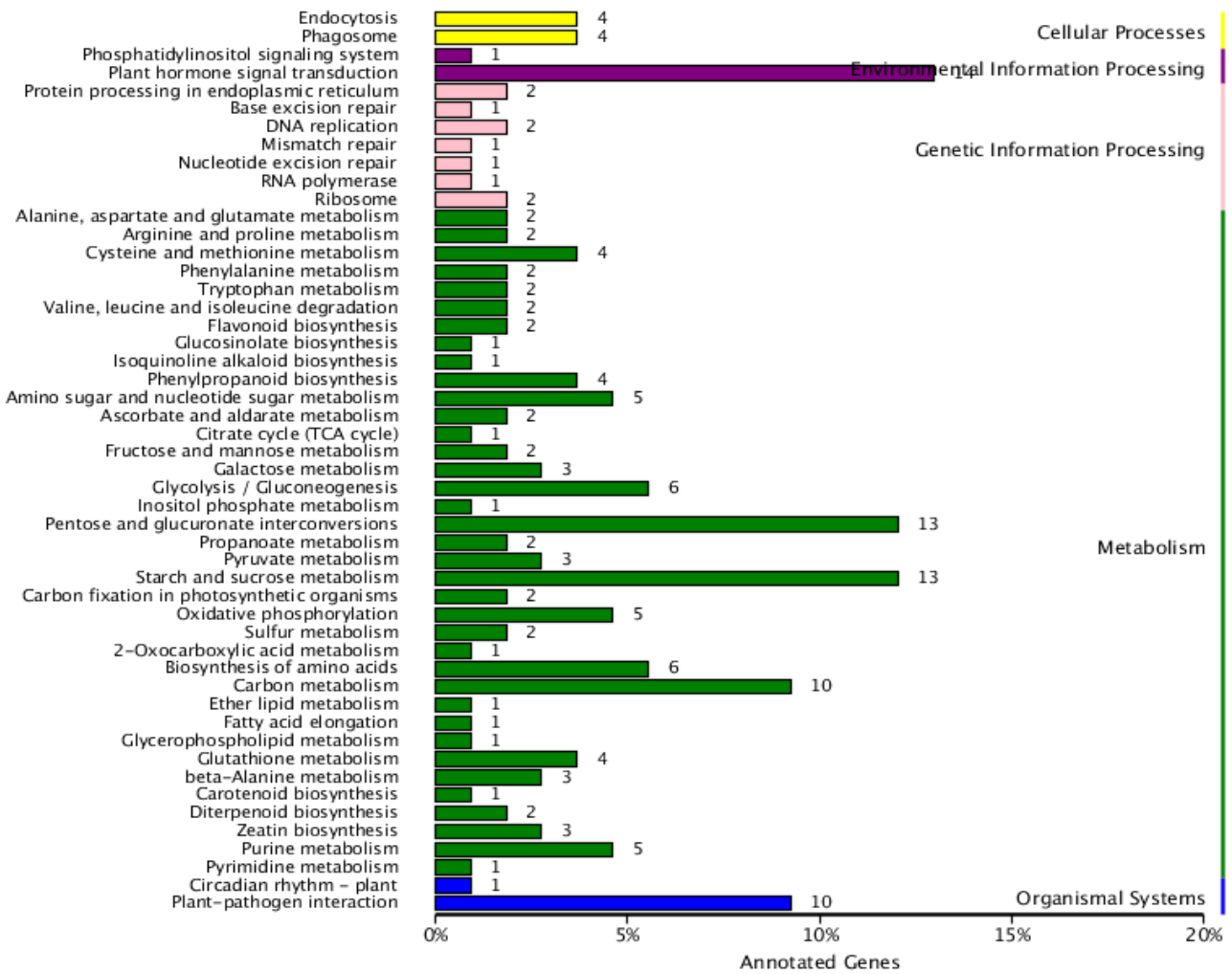

Fig. 4. KEGG classification of the differentially expressed genes.
Note: the vertical coordinate represents the metabolic pathway of KEGG, and the horizontal coordinate represents the number of genes annotated to the pathway and the ratio of the number of genes annotated to the total number of genes annotated.

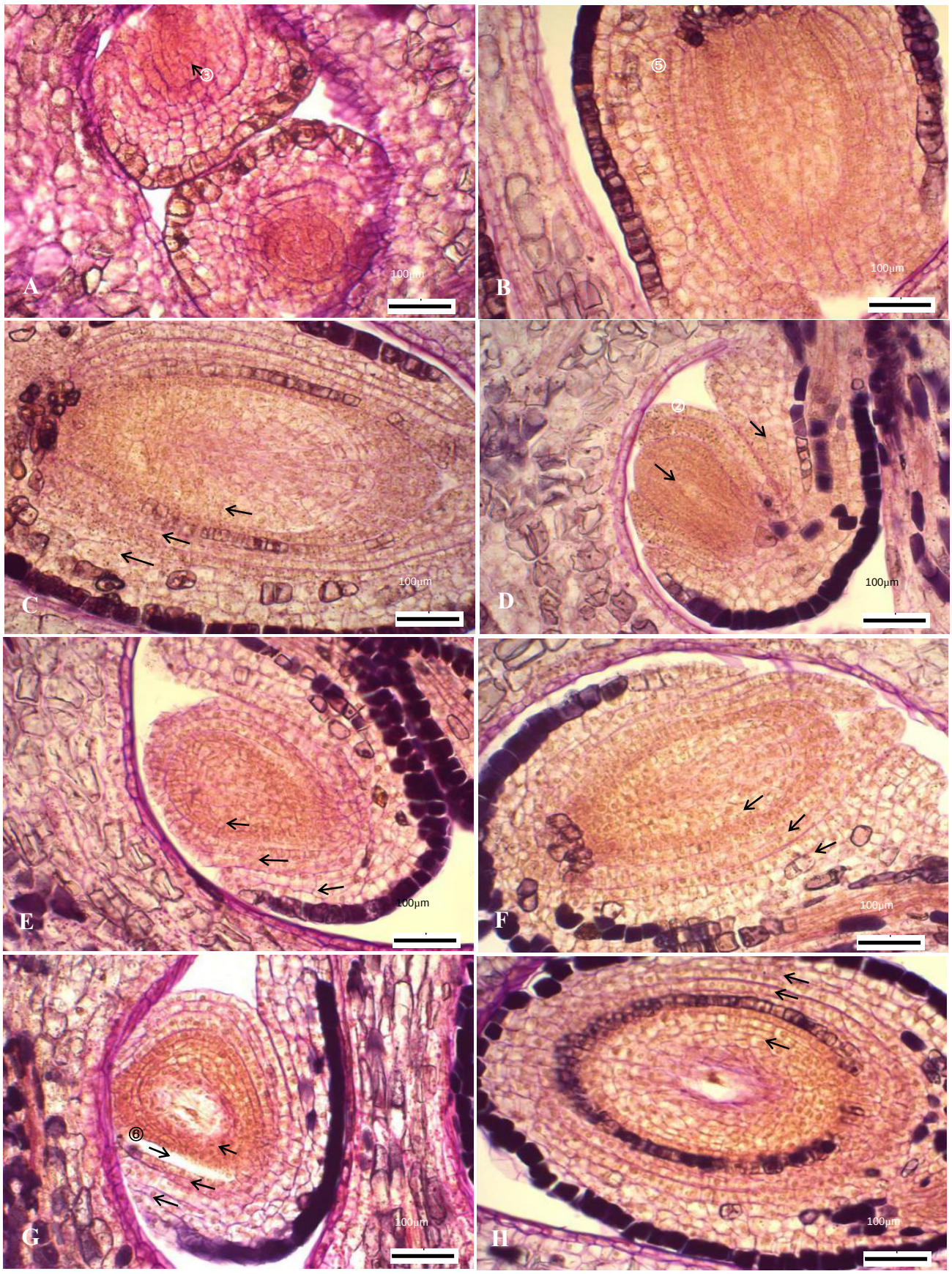

Fig. 5. Images of the treated and control ovule tissues (male 75134) after staining.
A: Control ovule 3 d after treatment; B: Treated ovule 3 d after treatment; **C**: Control ovule 6 d after treatment; D: Treated ovule 6 d after treatment; **E**: Control ovule 9 d after treatment; F: Treated ovule 9 d after treatment; **G**: Control ovule 12 d after treatment; **H**: Treated ovule 12 d after treatment. Arrow (1) indicates the outer bead quilt, (2) indicates the inner bead quilt, arrow (3) indicates the center of bead, arrow (4) indicates the empty end of bead, arrow (5) indicates the closing point end, and arrow (6) indicates the gap.

Table 1. Statistics table of the sequencing data.

Samples	Read number	Base number	Percentage of GC content	Percentage of Q30 content
Treatment	32,155,092	9,445,721,324	47.74%	93.42%
Control	33,480,442	9,876,697,134	47.96%	92.89%

Table 2. Statistics table of the assembly results.

Length range (nt)	Transcript	Unigene
200-300	44,172(27.04%)	37,455(41.60%)
300-500	30,759(18.83%)	22,011(24.45%)
500-1000	29,165(17.85%)	14,243(15.82%)
1000-2000	32,280(19.76%)	9,964(11.07%)
2000+	26,977(16.51%)	6,355(7.06%)
Total number	163,353	90,028
Total length	174,345,970	60,769,797
N50 length	1,934	1,204
Mean length	1067.30	675.01

Protein content and gender conversion of amur grape: The differential expression of specific proteins encoded in pistil abortion at different times and space are critical in this process. If the specific genes that determine female development cannot be expressed and translated in the normal manner, pistil abortion will ensue (Hennig *et al*., 2003). In addition, the loss or mutation of genes that control meiosis can lead to pistil abortion (Stacey *et al*., 2006). In *Asparagus officinalis*, most target genes related to gender expression were found to be involved in nucleic acid, protein binding, and protein dimerization (Chen *et al*., 2016). However, in amur grape (Xu *et al*., 2013) and *Xanthoceras sorbifolia* (Hu *et al*., 2004), some specific proteins appeared and disappeared in the processes of pistil abortion and sex differentiation. In the present study, protein content in the control ovule was significantly lower than that in the treated ovule. Furthermore, we found that more protein was induced in the early stage (3 days after CPPU treatment), which may be related to the expression of sex determining genes in this period. The increase in protein content at the middle stage (9 days after CPPU treatment) may be related to mitosis of the embryo sac nucleus. However, as the protein was concentrated in the micropyle at the late stage (12 days after CPPU treatment), such occurrence may be related to the existence of oocytes, fertilization, and the development of zygotes at the later stage of ovule development.

Conclusions

In the present study, we found that the sugar metabolism pathway is closely related to sex differentiation of *Vitis amurensis* and the change in starch and protein content is an important nutrient basis for sex differentiation. By using CPPU, the structure and function of the pistil of male amur grape could be restored and gender conversion could be achieved. We also recognized that gender reversal by exogenous plant growth regulators is not only related to nutrient supply, but is also closely related to the change in endogenous hormone content and specific gene expression. Therefore, analyzing the mechanism of sex differentiation in amur grape, the changes in nucleic acids and proteins, and the relationship between hormones and these biological macromolecules are of great significance in further understanding the spatiotemporal expression process of female and male flowers. In addition, separating and identifying the genes specifically expressed in male and female flowers are crucial in gaining a better understanding of this process.

Acknowledgments

The present study was financially supported by the Agricultural Science and Technology Innovation Program of Jilin Province (grant number CXGC2017ZY008), National Natural Science Funds of China (grant number 31501722), China Agriculture Research System (grant numbers CRAS-30-yz-6 and nytycx-29), and Liaoning Province Doctor Startup Fund (grant number 20141060). We thank Institute of Special Wild Economic Animal and Plant Science，National Field Gene Bank for Amur Grapevine (Zuojia, China) for providing the plant material used in the present study.

References

Ai, J., A.M. Li, C.Y. Li, J. Wang and Y.J. Shen. 2002. The effect of cytokinin on sex conversion of male plants of *Vitis amurensis* Rupr. *Acta Hort*., 29: 163-164.

Akagi, T., I.M. Henry, R. Tao, and L. Comai. 2014. A Y-chromosome-encoded small RNA acts as a sex determinant in persimmons. *Science,* 346: 646-650.

Antognozzi, E., A. Battistelli, F. Famiani, S. Moscatello, F. Stanica and A. Tombesi. 1996. Influence of CPPU on carbohydrate accumulation and metabolism in fruits of *Actinidia deliciosa* (A. Chev.). *Sci. Hort*., 65: 37-47.

Bai, S.L., Y.B. Peng , J.X. Cui , H.T. Gu, L.Y. Xu, Y.Q. Li, Z.H. Xu and S.N. Bai. 2004. Developmental analyses reveal early arrests of the spore-bearing parts of reproductive organs in unisexual flowers of cucumber (*Cucumis sativus* L.). *Planta*, 220: 230-240.

Ben-Arie, R., P. Sarig, Y. Cohen-Ahdut, Y. Zutkhi, L. Sonego, T. Kapulonov and N. Lisker. 1997. CPPU and GA_3 effects on pre- and post-harvest quality of seedless and seeded grapes. *Acta Hort*., 463: 349-357.

Bensen, R.J., G.S. Johal, V.C. Crane, J.T. Tossberg, P.S. Schnable, R.B. Meeley and S.P. Briggs. 1995. Cloning and characterization of the maize *An1* gene. *Plant Cell*, 7: 75-84.

Charlesworth, D. 2002. Plant sex determination and sex chromosomes. *Heredity*, 88: 94-101.

Chen, J., Y. Zheng, L. Qin, Y. Wang, L. Chen, Y. He, Z. Fei and G. Lu. 2016. Identification of miRNAs and their targets through high-throughput sequencing and degradome analysis in male and female *Asparagus officinalis*. *BMC Plant Biol*., 16: 80. DOI: 10.1186/s12870-016-0770-z.

Corbesier, L., P. Lejeune and G. Bernier. 1998. The role of carbohydrates in the induction of flowering in Arabidopsis thaliana: comparison between the wild type and a starchless mutant. *Planta*, 206: 131-137.

Cruz-Castillo, J.G., D.J. Woolley and G.S. Lawes. 2002. Kiwifruit size and CPPU response are influenced by the time of anthesis. *Sci. Hort*., 95: 23-30.

Delong, A., A. Calderon-Urrea and S.L. Dellaporta. 1993. Sex determination gene *TASSELSEED2* of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. *Cell*, 74: 757-768.

German, M.A., N. Dai, I. Chmelnitsky, I. Sobolev, Y. Salts, R. Barg, A.A. Schaffer and D. Granot. 2002. *LeFRK4*, a novel tomato (*Lycopersicon esculentum* Mill.) fructokinase specifically expressed in stamens. *Plant Sci*., 163: 607-613.

Guo, X.W., S.X. Jing and X.G. Lin. 1995. Studies on germplasm resources of *V. amurensis*. *J. Shenyang Agric. Uni*., 26: 271-276.

He, P.C. 1999. Enology. Beijing: China Agriculture Press 20.

Hennig, L., P. Taranto, M. Walser, N. Sehonroek and W. Gruissem. 2003. Arabidopsis MSl1 is required for epigenetic maintenance of reproductive development. *Development*, 130: 2555-2565.

Hu, Q., S.M. Gao and F.L. Li. 2004. Pistil Development in 2 Types of Flowers of *Xanthoceras sorbifolia*. *Forest. Stud. China*, 6: 13-16.

Jiao, L., Y.L. Zhang, J. Wu, H.Q. Zhang and J. Lu. 2015. A novel U-Box protein gene from "Zuoshanyi" Grapevine (*Vitis amurensis* Rupr. cv.) involved in cold responsive gene expression in *Arabidopsis thanliana*. *Plant Mol. Biol. Rep*., 33: 557-568.

Kamachi, S., H. Mizusawa, S. Matsuura and S. Sakai. 2000. Expression of two 1-aminocyclopropalle-1-carboxylate synthase genes, *CS-ACS1* and *CS-ACS2*, correlated with sex phenotypes in cucumber plants (*Cucumis sativus* L.). *Plant Biotechnol*. *Nar*., 17: 69-74.

Kamachi, S., H. Sekimoto, N. Kondo and S. Sakai. 1997. Cloning of a cDNA for a 1-aminocyclopropane-1-carboxylate synthase that is expressed during development of female flowers at the apices of *Cucumis sativus* L. *Plant Cell. Physiol*., 38: 1197-1206.

Kefford, N.P., J.A. Zwar and M.I. Bruce. 1968. Antagohism of benzylurea, In: *Biochemstry and physiology of plant growth substances*. Ottawa: Runge press, Ltd., Ottawa. pp. 61-69.

Knopf, R.R. and T. Trebitsh. 2006. The Female-specific *CS-ACS1G* gene of cucumber. A case of gene duplication and recombination between the non-sex-specific 1-Aminocyclopropane-1-Carboxylate synthase gene and a branched-chain amino acid transaminase gene. *Plant Cell Physiol*., 47: 1217-1228.

Lardon, A., S. Georgiev, A. Aghmir, G. Le Merrer and I. Negrutiu. 1999. Sexual dimorphism in white campion: Complex control of carpel number is revealed by Y chromosome deletions. *Genetics,* 151: 1173-1185.

Lewis, D.H., G.K. Burge, M.E. Hopping and P.E. Jameson. 1996. Cytokinins and fruit development in the kiwifruit (*Actinidia deliciosa*) II. Effects of reduced pollination and CPPU application. *Physiol. Plant*., 98: 187-195.

Li, D., Y. Wan and P. He. 2008. Relatedness of resistance to anthracnose and to white rot in Chinese wild grapes. *Vitis,* 47: 213-215.

Liu, H.W. and R.Y. Zhou. 2006. Effect of red light and far-red light dark-breaking on substance metabolism of photoperiod-sensitive female sterile ramie. *J. Basic Sci. Eng*., 14: 167-172.

Ma, Y.Y., Y.L. Zhang, H. Shao and J. Lu. 2010. Differential physio-biochemical responses to cold stress of cold-tolerant and non-tolerant grapes (*Vitis* L.) from China. *J. Agron. Crop Sci*., 196: 212-219.

Matsunaga, S., S. Kawano, H. Takano, H. Uchida, A. Sakai and T. Kuroiwa. 1996. Isolation and developmental expression of male reproductive organ-specific genes in a dioecious campion, Melandrium album (*Silene latifolia*). *Plant J*., 10: 679-689.

Meng, J.L. 1997. Plant reproductive genetics. Beijing, Science Press 1-32.

Mibus, H. and T. Tatlioglu. 2004. Molecular characterization and isolation of the *F/f* gene for femaleness in cucumber (*Cucumis sativus* L.). *Theor. Appl. Genet*., 109: 1669-1676.

Mitchell, C.H. and P.K. Diggle. 2005. The evolution of unisexual flowers: morphological and functional convergence results from diverse developmental transitions. *Amer. J. Bot*., 92: 1068-1076.

Negi, S.S. and H.P. Olmo. 1966. Sex Conversion in a Male *Vitis vinifera* L. by a Kinin. *Science*, 152: 1624.

Negi, S.S. and H.P. Olmo. 1971. Conversion and determination of sex in *Vitis vinifera* L. (sylvestris). *Vitis*, 9: 265-279.

Negi, S.S. and H.P. Olmo. 1972. Certain embryological and biochemical aspects of cytokinin SD8339 in conversion sex of a male *Vitis Vinifera* (Sylvestris). *Amer. J. Bot*., 59: 851-857.

Ohto, M., K. Onai, Y. Furukawa, E. Aoki, T. Araki and K. Nakamura. 2001. Effects of sugar on vegetative development and floral transition in *Arabidopsis thaliana*. *Plant Physiol*., 127: 252-261.

Pawelkowicz, M., L. Pryszcz, A. Skarzynska, R.K. Woycicki, K. Posyniak, J. Rymuszka, Z. Przybeckiand and W. Plader. 2019. Comparative transcriptome analysis reveals new molecular pathways for cucumber genes related to sex determination. *Plant Reprod*., 32: 193-216.

Roldan, M., C. Gomez-Mena, L. Ruiz-Garcia, J. Salinas and J.M. Martinez-Zapater. 1999. Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of Arabidopsis in the dark. *Plant J*., 20: 581-890.

Scutt, C.P., Y. Li, S.E. Robertson, M.E. Willis and P.M. Gilmartin. 1997. Sex determination in dioecious *Silene latifolia*. Effects of the Y chromosome and the parasitic smut fungus (*Ustilago violacea*) on gene expression during flowers development. *Plant Physiol*., 114: 969-979.

Sha, Y.F., Q.L. Zheng, C.J. Zhang, S.J. Fan and M.L. Tang. 2016. Two MADS-box gene expression analysis in male and female floral development of *Vitis amurensis* Rupr. *Chinese Agric. Sci. Bull*., 32: 122-126.

Shen, H.L., Z.D. Liu, K. Yan, L.R. Zou, J.H. Wen, Y.S. Guo, K. Li and X.W. Guo. 2018. Cytological study of sex conversion in amur grape. *J. Amer. Soc. Hort. Sci*., 143: 289-295.

Smeekens, S. 2000. Sugar-induced signal transduction in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol*., 51: 49-81.

Stacey, N.J., T. Kuromori, Y. Azumi, G. Roberts, C. Breuer, T. Wada, A. Maxwell, K. Roberts and K. Sugimoto-Shirasu. 2006. Arabidopsis SP011-2 functions with SP011-1 in meiotic recombination. *Plant J*., 48: 206-216.

Tang, M.L., J. Kong, X.F. Xu, Y.J. Shen, Y. Wang, T.Z. Li and Z.H. Han. 2008. Screening of the AFLP markers associated to the sex locus of *Vitis amurensis* Rupr. and its conversion to SCAR marker. *Acta Hort*., 35: 195-200.

Trebitsh, T., J.E. Staub and S.D. O'Neill. 1997. Identification of a 1-aminocyclopropane-1-carboxylic acid synthase gene linked to the female (F) locus that enhances female sex in cucumber. *Plant Physiol*., 113: 987-995.

Wan, Y., H. Schwaninger, P. He and Y. Wang. 2007. Comparison of resistance to powdery mildew and downy mildew in Chinese wild grapes. *Vitis*, 46: 132-136.

Wang, J. and L. R Yin. 1994. Study on the flower bud differentiation of *Vitis amurensis* Rupr. *Spec. Wild Econ. Ani. & Plant Res*., (3): 6-9.

Wang, J., P.C. He, R.Q. Li and Y.J. Shen. 1999. Study on RAPD makers of male *Vitis amurensis* Rupr. *J. Gansu Agric. Uni*., 34: 320-322.

Watanabe, H. 1989. The use of growth regulators applied to stem cuttings of lilies. *Bull. Agri. Exp. Stud*., 20: 67-71.

Xiao, H.S., L.X. Lv, X.P. Wang and P. Wang. 2002. Observation on cell ultrastructural of flower bud differentiation in Litchi (*Litchi chinensis* Sonn.). *Journal of Fujian Teachers of University (Natural Science)*, 18(2): 57-60.

Xu, P.L., Z.Q. Jiao, Z.X. Wang, J. Ai, Y.M. Yang, Q.T. Zhang, H.Y. Qin, Y. X. Liu, Y. Zhao and S.T. Fan. 2013. Proteomic analysis of sex conversion induced by CPPU in male grapevine of *Vitis amur*ensis. *Vitis*, 52: 177-184.

Yamasaki, S., N. Fujii and H. Takahashi. 2000. The ethylene-regulated expression of *CS-ETR2* and *CS-ERS* genes in cucumber plants and their possible involvement with sex expression in flowers. *Plant Cell Physiol*., 41: 608-616.

Yamasaki, S., N. Fujii, S. Matsuura, H. Mizusawa and H. Takahashi. 2001. The M locus and ethylene-controlled sex determination in andromonoecious cucumber plants. *Plant Cell Physiol*., 42: 608-619.

Yang, M.X., D.X. Xiao, H. Liang and W. Liu. 2011. Morphological observation on of sex differentiation of *Actinidia chinensis*. *Acta Hort*., 38: 257-264.

Yao, J.L. and C.H. Fu. 2000. Abnormal phenomena during embryo development in *Prunus salicina*. *J. Huazhong Agric. Uni*., 19: 71-73.

Zhou, L., J.C. Jang, T.L. Jones and J. Sheen. 1998. Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. *Proceed. Natl. Acad. Sci. U. S. A*., 95: 10294-10299.

(Received for publication 26 November 2019)