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ARTICLE

VvAGAMOUS Affect Development of Four Different Grape Species Ovary

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ABSTRACT

Grape pistil has an important influence on fruit size and quality. However, there were few studies on grape ovary, and the development process of the ovary is still unclear. Therefore, in this paper, four different grape varieties with different lengths of small inflorescences, namely 'Musct Hambourg' grape (*Vitis vinifera*), 'Concord' grape (*Vitis labrusca*), 'ShanPuTao' grape (*Vitis amurensis*) and 'GongNiang2Hao' grape (*Vitis amurensis* × *Vitis vinifera*) were used as test materials. Four varieties ovary were significant differences by means of stereomicroscope, paraffin section. The expression of ovary determining gene *VvAGAMOUS* (*VvAG*) and its development related genes *VvCRABS CLAW* (*VvCRC*) and *VvAGAMOUS-LIKE 11* (*VvAGL11*) with similar functions during the development of different grape varieties were preliminarily explored using fluorescence quantitative test. The relationship between *VvAG* and *VvCRC*, *VvAG* and *VvAGL11* were analyzed using Y1H assay. Our results showed that there were obvious abdominal sutures on the surface of expect for 'Musct Hambourg' grape, and existing poly carpels. The ovary development of 'ShanPuTao' and 'GongNiang2Hao' grape was completed when the inflorescence length was less than 1 cm, while the 'Concord' and 'Musct Hambourg' grape were fully developed when the length of inflorescence was 3–4 and 4–5 cm, respectively. *VvAG* and *VvCRC* began to express in large quantities after the formation of stamen primordia, while *VvAGL11* during the forming of ovule primordia. Therefore, *VvAG* and *VvCRC* mainly regulated the development of stamens and carpels and also promote the development of ovules, while *VvAGL11* major regulated the development of ovules. The promoters of *VvCRC* and *VvAGL11* were bound by *VvAG*. This study provides an important theoretical basis for further research on the molecular mechanism of grape ovary development.

KEYWORDS

Grape; carpel; ovule; *VvAGAMOUS*; *VvCRABS CLAW*; *VvAGAMOUS-LIKE 11*; flower development process

1 Introduction

Vitis genus (*Vitis* L.) is a woody vine, that contains multiple grape species, which have various characteristics. *V. vinifera* grapes are the main cultivars in the world, mostly used for fresh food and vintage, but the cold and disease resistance are poor. *V. labrusca* grapes are mostly used for rootstocks due to their strong disease resistance. The cold resistance of *V. amurensis* grapes is the strongest among all the grape varieties, often cultivated in cold regions [1,2]. *V. amurensis* × *V. vinifera* grapes are mainly used for wine

making, juice making and inherits the characteristics of strong cold resistance of *V. amurensis* grapes. Grape ovary, as a precursor to fruit, usually forms an internal space in the flower organ by folding and curling, in which the ovule was wrapped for development and finally forms the seed [3–6]. The ovary is divided into monocarpellary ovary and multicarpellary ovary, of which the multicarpellary is divided into li-berate ovary and coalescent ovary. Most grapes are fruits with 2 carpels and 4 ovules, and contain up to 4 seeds, but a few varieties form multiple carpels in the process [7]. Ovary characters affect fruit size and quality [8,9].

At present, the development of ovary had been extensively studied in several model plants, however there was little research in grapes. *AGAMOUS* (*AG*) is a class C gene in the ABCDE model of plant flower organs development, which is involved in the third and fourth rounds of flower organs development [10–12]. *AG* is a transcription regulator that regulates the development of stamens and carpels primordia, participates in the formation of reproductive flower organs and the decisive control of meristems, and is the earliest cloned flower development regulatory gene [13,14]. In tomato, when *TAG1* gene was inhibited, stamens of tomato became petal-like organs, and carpels development abnormal [15,16]. During the formation and development of rose stamens, *RhAG* was significantly and massively expressed. Silencing *RhAG* could increase the number of rose petals, indicating that *RhAG*, like *AG* in *Arabidopsis*, was mainly involved in stamen development [17].

CRABS CLAW (*CRC*), a member of YABBY family gene, is one of the regulators, as downstream genes of *AG*, involved in the carpel development of *Arabidopsis* [18]. It was expressed in the abaxial side of the carpel and participates in the development of the carpel primordium abaxial tissue and pistil, key developmental processes for ensuring successful plant reproduction and crop production [19,20]. Studies had shown that *CRC* and *SPATULA (SPT)* genes have similar functions to class C genes and can promote the development of carpels and ovules [21]. For example, in the process of termination regulation of *Arabidopsis* flower meristem, *AG* gene could directly activate the expression of its downstream target gene *CRC* and participate in pistil formation [22].

AGAMOUS-LIKE 11 (*AGL11*), also known as *SEEDSTICK* (*STK*), is classified as class D according to its gene function. It was highly expressed in the central tissue of seeds and young fruits, and plays a crucial role in ovule development and seed formation [23–26]. *AGL11* not only controlled *Arabidopsis* ovule morphogenesis, but also controlled the development of embryo stalk and seed shedding [27,28]. Silencing *SlyAGL11* gene in tomato produced a seedless fruit, which indicated that *SlyAGL11* plays a direct and important role in the seed development of fleshy fruits [29]. In the process of studying the molecular mechanism of controlling seedless grapes found *VvAGL11* transcription was essential for seed morphogenesis in grapevine during berry development [30]. On the formation process of ovule primordia of 'Xiangfei' grape, it was found that *VvAG2* and *VvAGL11* were involved in the formation of grape ovules [31]. In 'Muscadine' grape, *VroAGL11* was found that the gene was most expressed in the berries with the highest seed content (weight) and almost not in seedless berries, indicating that *VroAGL11* gene played an important role in controlling grape seed morphogenesis [32].

In this study, the inflorescences of four different grape varieties with different lengths were used as test materials. The development process of four grape varieties ovaries were studied by means of stereoscope and paraffin section. At the same time, to clarify the expression of *VvAG*, *VvCRC* and *VvAGL11*, related to carpel and ovule development, during the development of grape ovary, quantitatively were detected by fluorescence. The interactions between *VvAG* and *VvCRC, VvAG* and *VvAGL11* were further proved by Y1H assay.

2 Materials and Methods

2.1 Plant Materials

The experimental materials were collected from the national "Grape Germplasm Resource nursery" Pomology Institute, Shanxi Agricultural University/Shanxi Academy of Agricultural Sciences on the

morning of May 01, 2021. The resource garden is located at (37 °N, 112 °E) with temperate continental climate and four distinct seasons. The average sunshine duration in this area is 2500~2600 h, and the annual average temperature and precipitation are about 5°C~10°C and 458 mm, respectively. The cultivation mode and growth environment of the four different grape varieties were the same, which would not affect the phenotype. Inflorescences of four different grape varieties, 'Musct Hambourg' grape (*V. vinifera*), 'Concord' grape (*V. labrusca*), 'ShanPuTao' grape (*V. amurensis*) and 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*), were collected (Table S1). Sampling was performed at different developmental stages, according to the length of inflorescences: <1, 1–2, 2–3, 3–4, 4–5 cm (Fig. 1) [33]. Basal position small inflorescences of samples were taken, one of which was immediately frozen in liquid nitrogen and taken back to the laboratory for storage in an ultra-low temperature refrigerator at –80°C; one copy was kept in FAA [50% (v/v) ethanol, 5% (v/v) glacial acetic acid and 5% (v/v) formaldehyde] fixative.

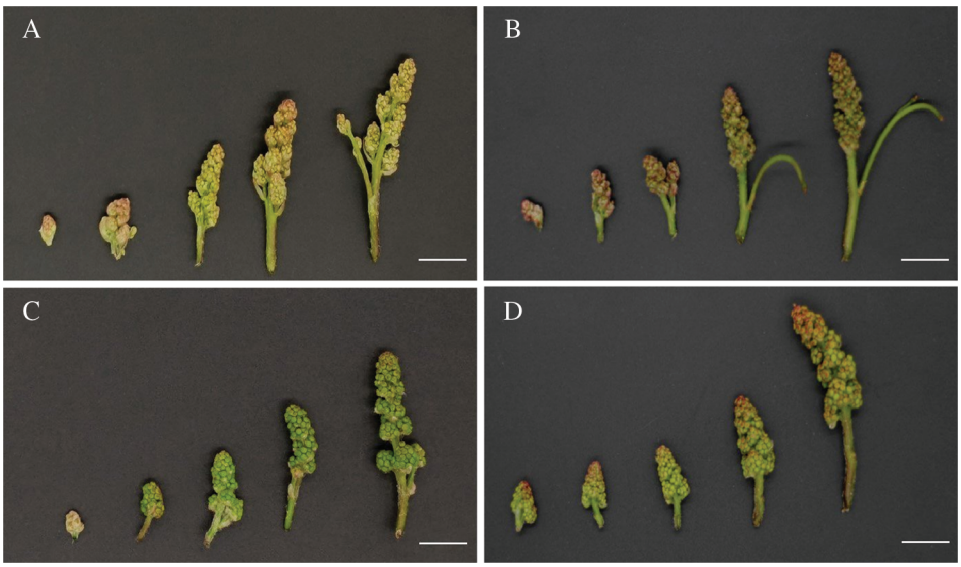

Figure 1: Inflorescences from four grape varieties with different lengths. A. The 'Musct Hambourg' grape inflorescences (*V. vinifera*). B. The 'Concord' grape inflorescences (*V. labrusca*). C. The 'ShanPuTao' grape inflorescences (*V. amurensis*). D. The 'GongNiang2Hao' grape inflorescences (*V. amurensis* × *V. vinifera*). White bars indicate the scale = 1 cm

2.2 Phenotypic Observation

At the full flowering stage, 30–50 small fruits of four different grape varieties were selected. The appearance characters were observed by stereomicroscope, and then the young fruits were crosscut to observe and determine the young fruits phenotypic characters, carpels and ovules number.

2.3 Paraffin Section

The florets of basal of inflorescences with four varieties grape with different lengths were selected for paraffin sectioning [34]. Materials were fixed in FAA, dehydrated with different ethanol concentration gradients, then place in a mixture of different proportions ethanol and n-butanol gradients. Afterwards dipped with pure paraffin and embedded in paraffin. The samples were cut into 8 μm sections using a microtome, after which they were stained with Safranin O-Fast Green. Finally, used a microscope to observe and take pictures.

2.4 Cloning of VvAG Gene

Total RNA was extracted from the florets occupying the basal positions of the inflorescences of four grape varieties with different lengths. RNA was extracted by modified CTAB method [35], the concentration was detected, and the clarity and integrity of band was checked using 1.2% agarose gel electrophoresis. RNA was reverse transcribed into cDNA by using reverse transcription Kit (Takara Bio, Beijing, China). The primers for amplification of the *VvAG* gene full-length cDNA of the coding region were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). (AGF-TGGGAAGGGGGAAGATCGAG; AGR-TTACACTAATTGAAGAGCTGGTTGG). PCR reaction conditions were: heat denaturation at 94°C for 5 min; 40 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1.5 min; extension at 72°C for 5 min. The obtained cDNA fragments were recovered with the gel recovery kit (Real-Times Biotechnology Co., Ltd., Beijing, China) and then connected to the pMD-19T vector (Takara Bio, Beijing, China), then transformed into *Escherichia coli* Trans5α (TransGen Biotech, Beijing, China), plated and cultured, strains were selected, positive clones were identified by PCR, and sent to Sangon Biotech Co., Ltd. (Shanghai, China) sequencing.

2.5 RNA Extraction and qRT-PCR

RNA from roots, tendrils, young leaves, mature leaves, flowers and young fruits of 'Musct Hambourg' grapes were extracted for tissue-specific analysis. Using cDNA as template, the expression levels of *VvAG*, *VvCRC* and *VvAGL11* in different grape tissues and the expression levels of four different grape varieties with different inflorescence lengths were detected by qRT-PCR. Real time qRT-PCR reaction was carried out with SYBR Green PCR Master Mix Kit (Mei5 Biotechnology Co., Ltd., Beijing, China). Fluorescent quantitative primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Table S2). 20 μL reaction system: 2× realtime PCR super mix 10 μL + primers 1.0 μL + 1.0 μL sample cDNA + 8.0 μL ddH_2O. qRT-PCR reaction conditions: predenaturation at 95°C for 60 s; denaturation at 95°C for 15 s, annealing at 58°C for 15 s, extension at 72°C for 60 s, 40 cycles; 72°C for 5 min. Using *VvUBQ* as the internal reference gene, the relative expression of the corresponding gene was calculated by $2^{-\overline{\Delta}\Delta CT}$ method. Each sample was repeated 3 times.

2.6 Yeast One-Hybrid Assay

The *VvAG*-coding sequences were cloned into the pGADT7 vector. The cis-acting elements of promoter *VvCRC* and *VvAGL11* were predicted according to PlantCARE online website. The fragments containing CArG-box elements were inserted into pAbAi vector to construct pAbAi-*VvCRC* and pAbAi-*VvAGL11* vectors. The mutation-related vectors pAbAi-*Vvcrc* and pAbAi-*Vvagl11* were constructed by Sangon Biotech Co., Ltd. (Shanghai, China). The resultant constructs were transformed into the yeast Y1H Gold strain and selected by optimal AbA (Aureobasidin A) concentration on the SD/-LEU (Synthetic Dropout Medium/-Leucine) medium. The pGADT7-*VvAG* + pAbAi-*VvCRC*, pGADT7-*VvAG* + pAbAi-*VvAGL11* were experimental group and pGADT7-*VvAG* + pAbAi-*Vvcrc*, pGADT7-*VvAG* + pAbAi-*Vvagl11* were negative control (Table S3) [36].

3 Results

3.1 Differences of Ovary Morphology among Four Grape Varieties

The young fruits of four grape varieties were observed and photographed under the stereomicroscope. It was found that the 'Musct Hambourg' grape had different appearances compared with the 'Concord', 'ShanPuTao' and the 'GongNiang2Hao'. The 'Musct Hambourg' grape fruits had an irregular appearance, with obvious abdominal sutures on the surface, and the more abdominal sutures, the more carpels and ovules. As shown in Fig. 2 A1, A3 and A5, there were two, three and four abdominal sutures, respectively. The observation of the corresponding young fruits crosscut were shown in Fig. 2 A2, A4 and A6, in which are two carpels four ovules, three carpels six ovules and four carpels eight ovules,

respectively. The remaining three grape varieties are round or oval in appearance, with smooth pericarp surface and no abdominal sutures. Two ovaries were observed after cross cutting, and each ovary contains two ovules.

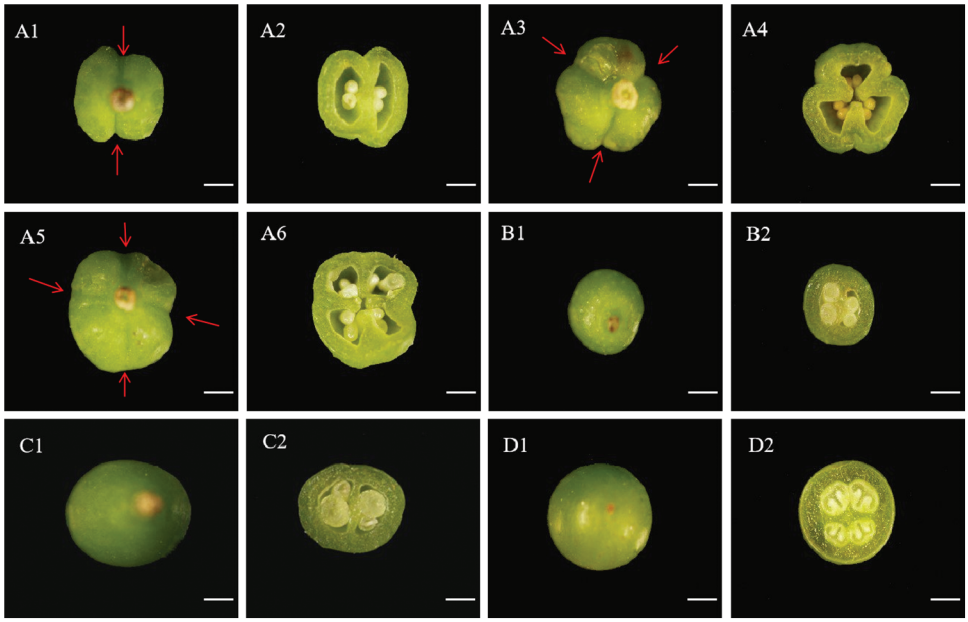

Figure 2: Observation on young fruits appearance and ovary morphology of four grape varieties. A1 and A2. 2 abdominal sutures and 2 carpels of 'Musct Hambourg' grape (*V. vinifera*). A3 and A4. 3 abdominal sutures and 3 carpels of 'Musct Hambourg' grape (*V. vinifera*). A5 and A6. 4 abdominal sutures and 4 carpels of 'Musct Hambourg' grape (*V. vinifera*). B1 and B2. 2 abdominal sutures and 2 carpels of 'Concord' grape (*V. labrusca*). C1 and C2. 2 abdominal sutures and 2 carpels of 'ShanPuTao' grape (*V. amurensis*). D1 and D2. 2 abdominal sutures and 2 carpels of 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*). Red arrow indicates abdominal suture. White bars indicate the scale = 100 μm

3.2 Anatomical Observation on Florets of Four Grape Varieties at Different Stages

To determine the key period of grape ovary formation, four grape varieties inflorescences with different lengths were observed through paraffin sections (Fig. 3). The flower development of 'Musct Hambourg' grape (*V. vinifera*) was the slowest. At the early stage of inflorescence development, when the inflorescence length was less than 1 cm, the sepals and flower caps had been fully developed and the stamen primordia began to form (Fig. 3 A1). When the inflorescence length was 1–2 cm, the stamens were completed development and the carpel primordia began to form (Fig. 3 A2). In inflorescences 2–3 cm in length, stamens had entered the stage of sporogenous tissue differentiation, and the two carpel primordia were crescent shaped and high protuberant (Fig. 3 A3). The carpel primordia continued to grow and the ovule primordia began to form in the 3–4 cm inflorescences (Fig. 3 A4). Ovule primordia further differentiated when the inflorescences were 4–5 cm long (Fig. 3 A5).

At the early stage of inflorescences development, the 'Concord' grape (*V. labrusca*) formed sepals, flower caps, stamens and the two carpel primordia began to form (Fig. 3 B1). Carpel primordia was further developed, extending around and widening at the bottom, when the inflorescences were 1–2 cm long. The two symmetrical segments form the carpel marginal primary meristem, and the stamens undergo protoplast differentiation (Fig. 3 B2). The primary meristems on both sides were close to the center, and the carpels gradually fused in the 2–3 cm inflorescences, and the ovule primordia began to

form (Fig. 3 B3). In inflorescences 3–4 cm in length, the ovule primordia further differentiated, carpels fully fused, and stigma cells began to develop (Fig. 3 B4). The stigma form and the ovules entered the inversion stage, when the inflorescences were 4–5 cm long (Fig. 3 B5).

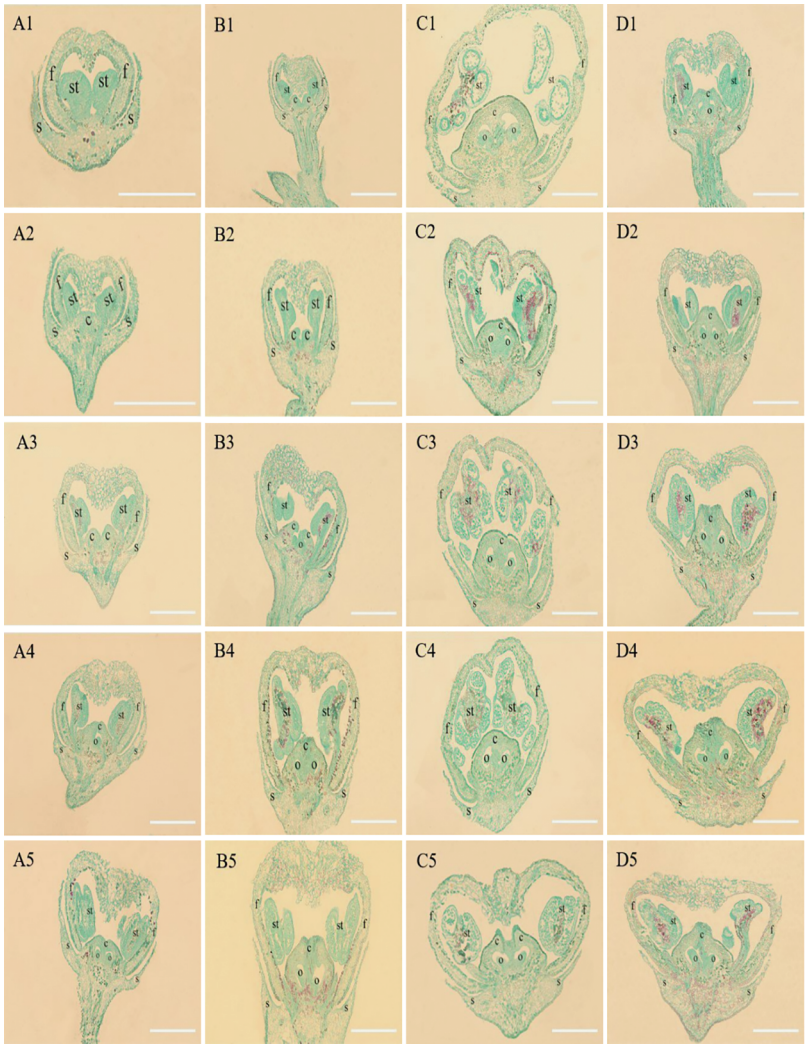

Figure 3: Anatomical structure of four grape varieties at different developmental stages. A1–A5. The 'Musct Hambourg' grape (*V. vinifera*). B1–B5. The 'Concord' grape (*V. labrusca*). C1–C5. The 'ShanPuTao' grape (*V. amurensis*). D1–D5. The 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*). 1. < 1 cm; 2. 1–2 cm; 3. 2–3 cm; 4. 3–4 cm; 5. 4–5 cm. s. sepals; f. flower caps; st. stamens; c. carpels; o. ovules. White bars indicate the scale = 100 μm

The flower development of 'ShanPuTao' grape (*V. amurensis*) was the fastest, the carpels and ovules both fully development in inflorescence lengths less than 1 cm (Fig. 3 C1).

The sepals, flower caps, stamens and carpels of 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*) were formed when the inflorescences length was 0–1 cm, in which the ovule primordia began to form (Fig. 3 D1). Ovules were completed development in the 1–2 cm inflorescences (Fig. 3 D2). In

inflorescences 2–3 cm in length, the ovules entered the inversion stage and the stigma cells began to develop (Fig. 3 D3). The stigma had formed, in the small florets of the 3–4 cm inflorescences. (Fig. 3 D4).

3.3 Sequence Analysis of VvAG Gene

The results showed that bands with a molecular size of 680 bp were amplified, respectively, which were consistent with the sequence size published by NCBI. There was only one amino acid difference in the non-conserved domain of the *VvAG* genes of the four grape varieties, which will not affect the function of the gene. The encoded protein contains a MADS domain of 57 amino acids, a K domain of 67 amino acids, and a C domain of 75 oxyacids (AGI and AGII). The M region is highly conserved, K regions are relatively conserved, and the C-terminal specificity is strong. They had highly similar to the amino acid sequences of *Arabidopsis*, *Nicotiana* and *Malus domestic* (Fig. 4).

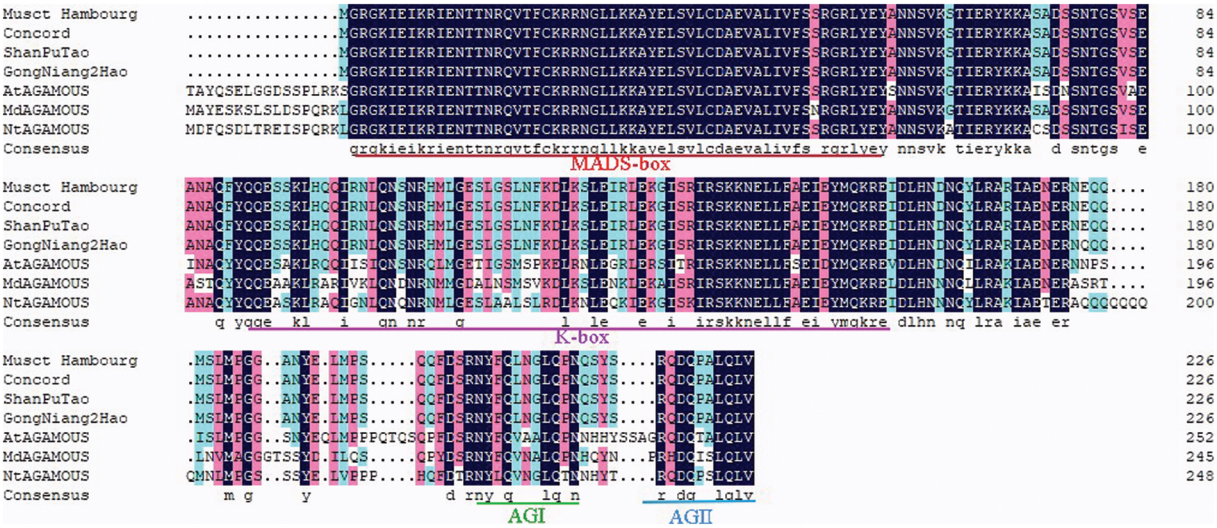

Figure 4: Analysis of *VvAG* gene amino acid sequences of four different grape varieties and other representative species

3.4 VvAG, VvCRC and VvAGL11 Tissue Specificity

The expression of *VvAG*, *VvCRC* and *VvAGL11* in different tissues of grapes was detected with qRT-PCR (Fig. 5). It was found that the expression of *VvAG* and *VvCRC* was the highest in flowers, followed by young fruits, a small amount of expression in young leaves and mature leaves, and almost no expression in stems and tendrils. *VvAGL11* was highly expressed in young fruits, followed by flowers, but not in stems, tendrils, young leaves and mature leaves.

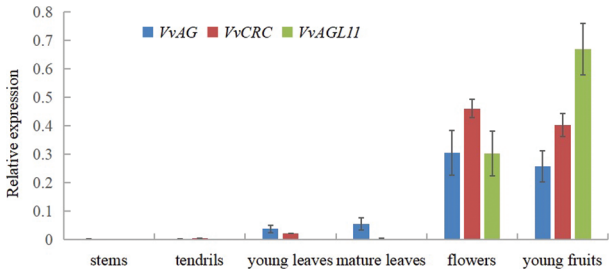

Figure 5: The relative expression levels of *VvAG*, *VvCRC* and *VvAGL11* in different grape tissues

3.5 Expression Differences of VvAG, VvCRC and VvAGL11 in Florets at Different Developmental Stages

The expression levels of *VvAG*, *VvCRC* and *VvAGL11* genes were measured at different inflorescences length of grapes in different varieties (Fig. 6). *VvAG* was expressed when the 'Musct Hambourg' grape inflorescence length was 0–1 cm, and the stamens forming. In inflorescences 1–2 and 2–3 cm in length, *VvAG* began to express in large quantities, and the carpel primordia began to form and rapidly formation, respectively. The expression of *VvCRC* gene increased followed the increase of *VvAG* gene expression. The expression of *VvAGL11* gene was low between inflorescences 0–1 and 1–2 cm in length. However, the expression of *VvAGL11* gene was increased quickly in inflorescences length 2–3 and 3–4 cm, in which ovule primordia began to form and gradually developed, respectively (Fig. 6A).

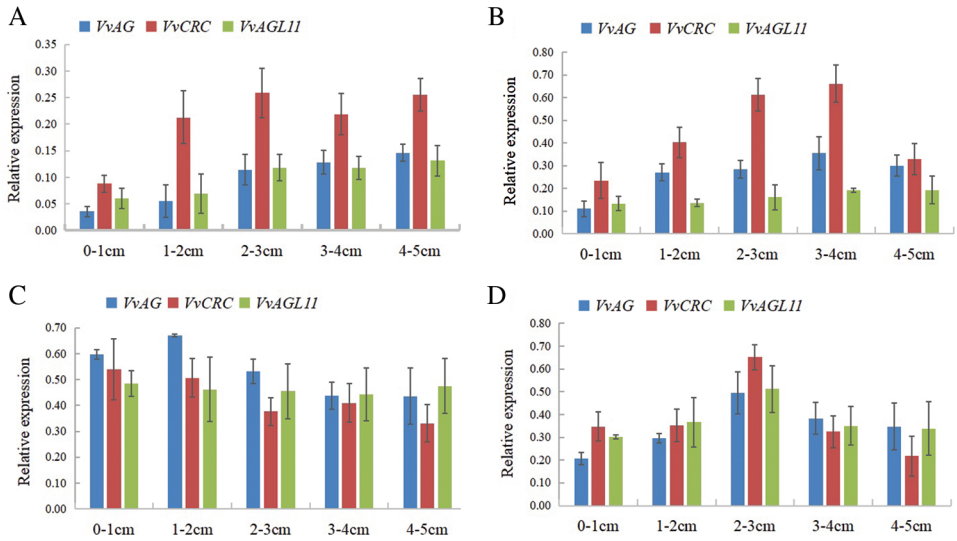

Figure 6: The relative expression levels of *VvAG*, *VvCRC*, *VvAGL11* at different inflorescence developmental stages. A. The 'Musct Hambourg' grape (*V. vinifera*). B. The 'Concord' grape (*V. labrusca*). C. The 'ShanPuTao' grape (*V. amurensis*). D. The 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*)

The expression of *VvAG* and *VvCRC* genes were increasing in 'Concord' grape (*V. labrusca*) inflorescences 0–1 cm length, which the formation of carpel primordia. The carpel was completely fused, and the expression reached the highest level, when the inflorescences were 3–4 cm long. The expression of *VvAGL11* was low in inflorescences 0–1 and 1–2 cm in length, slightly increased in inflorescences 2–3 cm long, in which the ovule primordia began to form. The expression increased significantly in inflorescences 3–4 cm in length, in which the ovule primordia began to develop (Fig. 6B).

The expression of *VvAG* gene was reached the highest when the 'ShanPuTao' grape (*V. amurensis*) inflorescence length was 1–2 cm. The expression of *VvCRC* and *VvAGL11* reached the maximum in the 0–1 cm inflorescence, in which the carpels and ovules were fully developed (Fig. 6C).

The expression of *VvAG*, *VvCRC* and *VvAGL11* genes in the inflorescence of 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*) reached the highest when the inflorescence were 2–3 cm long. During this period, the carpels and ovules were fully developed. After that, the expression began to decline, and the genes regulating the development of subsequent flower organs began to express (Fig. 6D).

3.6 Interaction of VvAG with VvCRC and VvAGL11 Promoters

Specific binding sites (CArG-boxes) of *VvAG* were found in promoters of *VvCRC* and *VvAGL11*. Protein-DNA interactions were examined with Y1H assays. Our data showed that both the empty vector and mutant control successfully inhibited the growth on SD/−leu+50 ng AbA plate, while the experimental group grew on SD/−leu+100 ng AbA plate, indicating that *VvAG* can directly bind to the CArG-boxes acting elements of *VvCRC* and *VvAGL11* promoters (Fig. 7).

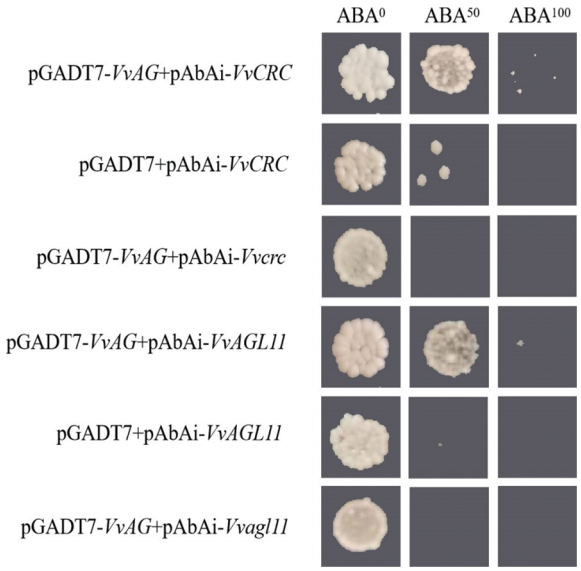

Figure 7: The interaction between *VvAG* and the promoters of *VvCRC* and *VvAGL11* was analyzed by Y1H assay

4 Discussion

4.1 Ovary Phenotypic Differences of Four Grape Varieties from Different Species

The formation and development of ovaries are very important to the number of grape seeds, fruit size and quality. However, there are few studies on grape ovaries, mainly focusing on *V. vinifera* species. In this study, four grapes varieties from different species were used to provide a theoretical basis for the follow-up studies. The experiment found that the 'Musct Hambourg' grape (*V. vinifera*) had multiple carpels, while the other three grape varieties only had two carpels (Fig. 2). *V. vinifera* species had abdominal sutures obvious, while that of other species is not. A researcher had investigated the carpel number of seven different grape cultivars for three consecutive years and found that six grape cultivars have two carpels or little three carpels. Only the 'Xiangfei' grape (*V. vinifrea*) has a high ratio of multi carpels, and its multi carpel ratio is stable at more than 50% annually [33]. Therefore, the polycarpels grape could be selected to cultivate large fruit grains grape varieties, but there is no in-depth study on this aspect.

4.2 Ovary Formation of Four Grape Varieties from Different Species

This study found that 'ShanPuTao' had the fastest ovary development, then 'GongNiang2Hao' also had a faster ovary development, next was 'Concord', and 'Musct Hambourg' was the slowest. Grapes need to be buried into the soil to spend the cold winter in northern of China. They will not be dug out again until the weather warms up around April of the next year. However, 'ShanPuTao' and 'GongNiang2Hao' grapes need not to be buried into the soil due to their strong cold resistance. Therefore, they have begun to carry out

physiological activities to form flower primordial when other grapes are still in dormancy. That is why the rapid ovary development of *V. amurensis* and *V. amurensis* × *V. vinifera* grapes.

The carpels and ovules of the 'Musct Hambourg' were developed in inflorescences 3–4 and 4–5 cm in length, respectively (Fig. 3 A4 A5). Inflorescences of different lengths of 'Xiangfei' (*V. vinifrea*) were observed by paraffin slices and found that the carpels were fully fused when the inflorescences were 4–5 cm long, in which the ovule primordia were begun to form [33]. It showed that the ovary fully developed in the 4–5 cm long inflorescence of *V. vinifrea* grapes, and slightly differences may be existed in different varieties.

There were little studies on ovary development of *V. labrusca* grapes. When the inflorescences of the 'Concord' were 2–3 and 3–4 cm length, the carpels and ovules were fully developed (Fig. 3 B3 B4). Its ovaries developed faster than *V. vinifrea*.

4.3 VvAG, VvCRC and VvAGL11 Participate in Grape Ovary Development

Some studies had found that the expression of *VvAG2* was the highest in flowers, instead low in roots, mature leaves and young leaves [31]. There were some studies indicated that *VviAGL11* gene highly expressed in flower and fruit tissues of grapes, but inhibited in roots, branches, leaves, buds and tendrils [26]. These results consistent with the tissue-specific expression test of *VvAG* and *VvAGL11* genes in this study. *VvCRC* had been rarely studied on grapes, but *CRC* was mainly expressed at the distal axis of the carpel and nectary in *Arabidopsis* [19]. This result was consistent with the expression of *VvCRC* in this study.

At the same time, the fluorescence quantitative results of 'Musct Hambourg' showed that *VvAG* gene began to express in large quantities during the inflorescences 2–3 cm length, in which carpel primordia began to develop (Fig. 5 A). The expression signal of *VvAG* gene had been found in 1–2 cm stamen primordium using *in situ* hybridization, the signal could still be observed when the inflorescences were 5–6 cm long [33]. Similarly, other study also found that *VvAG2* was expressed on carpel and ovule primordia in 4–5 cm inflorescences, which had strong hybridization signal on the ovule primordia [31]. Therefore, *VvAG* not only regulates carpel development, but also ovule development.

In this study, we found that *VvAGL11* began to express abundantly from the development of ovule primordia. A weak *VvAGL11* gene signal could be observed in the ovule primordia in the 4–5 cm inflorescences at the stage of ovule primordia formation through *in situ* hybridization [31]. Reduced expression of *VvAGL11* is responsible for stenospermocarpic seedlessness in bunch grapes [32]. The *VroAGL11* gene controlled the seed morphogenesis after ovule formation in 'Muscadine' grapes by analyzed its divergence from other plants of molecular level [32]. *VvAGL11* transcripts exhibited a high accumulation in seeds after 2 and 4 weeks of development in 'Chardonnay' using *in situ* hybridization [30]. Therefore, *VvAGL11* might be a major gene for regulating ovule development.

In *Arabidopsis*, *CRC* gene is essential for carpel and nectary development [37]. *PfCRC* was found that it was mainly expressed in the carpel, and the loss of *PfCRC* function would change the determination of carpel meristem, carpel closure and ovule number [38]. The strong expression of *EcCRC* was detected at the flower development stage after the beginning of pistil using *in situ* hybridization. VIGS test proved that *EcCRC* gene participated in the initiation of ovule [39]. This is consistent with the results that *VvCRC* gene mainly regulated pistil development. In tomato, *in situ* hybridization analysis of flowers at different stages found that *SlCRCa* began to accumulate uniformly when the carpel primordia was initiated, and persisted in carpel growth and primordium of placenta emerged [40].

4.4 The Interactions between VvAG and VvCRC, VvAG and VvAGL11

We observed that *VvAG* can directly activate *VvCRC* and *VvAGL11* promoters by Y1H assay. There had less research on the interaction of *VvAG* and *VvCRC* in grapes. But Some studies found that the grape

MADS-box family transcription factor complexes *VvAG2/VvSEP3* and *VvAGL11/VvSEP*3 form tetramers that may be involved in the formation of ovules [31].

5 Conclusions

This study found that the multi carpel existed in 'Musct Hambourg' grape (*V. vinifera*), instead, other varieties not. The speed of ovary development was 'ShanPuTao' grape (*V. amurensis*) > 'GongNiang2Hao' grape (*V. amurensis* × *V. vinifera*) > 'Concord' grape (*V. labrusca*) > 'Musct Hambourg' grape (*V. vinifera*). *VvAG* and *VvCRC* genes mainly regulated the development of stamens and carpels, but also promoted the development of ovules, while *VvAGL11* might be a major gene for regulating ovule development. The CArG-boxes acting elements of *VvCRC* and *VvAGL11* promoters were bound by *VvAG*.

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Appendix

Table S1: Experiment material

Variety name	Source	Species	Use
Musct Hambourg	England	*Vitis vinifera*	Fresh food
Concord	American	*Vitis labrusca*	Juice
ShanPuTao	China	*Vitis amurensis*	Rootstock
GongNiang2Hao	China	*Vitis amurensis* × *Vitis vinifera*	Brew

Table S2: Primers used for qRT-PCR analysis

Gene	Forward primer	Reverse primer
VvAG	CAAGAGCCTGGAGATTCGG	CATTCTCGGCTATCCTTGC
VvCRC	CTTTCTTAGCACCAGACCTCCA	GATGCGTTGTATTTCCTCCTTC
VvAGL11	GTTGCCCTCATCGTCTTCTC	CAAGGAAGCCAAGGAATCAC
VvUBQ	GCTCGCTGTTTTGCAGTTCTAC	AACATAGGTGAGGCCGCACTT

Table S3: Primers used for Y1H analysis

Gene	Forward primer	Reverse primer
VvAG	CCCATATGATGGGAAGGGG GAAGATCGAG	CGAGCTCTTACACTAAT TGAAGAGCTGGTTGG
VvCRC	GCGTCGACGCATGGAAACA AATTGAAATCCATA	CCAAGCTTTTCTTCCA GTTCTGAGACATC
VvAGL11	GCGTCGACGTTTTTTAA TAATTGGAGATACCAT	CCAAGCTTCTGCAAAC TTTCTTCTCTTC