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VARIATION OF ENDOGENOUS PHYTOHORMONE IN FUNCTIONAL MALE AND BISEXUAL FLOWERS OF POMEGRANATE (*PUNICA GRANATUM* L.) DURING DEVELOPMENT

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Abstract

Pomegranate have two types of flowers: bisexual flower and functional male flower. Flower development is a complex process which is regulated by an intricate transcriptional regulatory network. To understand the molecular mechanisms of regulating flower development and female sterility in pomegranate, we performed a comprehensive analysis of endogenous phytohormone changes during flower differentiation. Plant hormones, such as indole-3-acetic acid (IAA), zeatin riboside (ZR), gibberellin (GA), jasmonic acid (JA), and abscisic acid (ABA) contents in the pomegranate flowers were measured to expound the relationship between floral differentiation and endogenous hormones by ELISA. The results showed that the high accumulations of ABA, GA, and high ratio of ABA/ZR in bisexual flower primordium might stimulate the sterility of ovary and promote the development of stamen organ, which led to functional male flower. Moreover, low levels of IAA in functional male flower from S6 to S8 might accelerate the development of stamen and the abortion of pistil, and low levels of the ABA/GA ratio at S3 and S6 might also promote the development of the male organ. Also, ZR and JA had a similar accumulation tendency, low levels of ZR and JA might contribute to the male organ development in the late period of bud development. ZR had significant positive correlation with JA ($p<0.05$) in both bisexual and functional male flowers. With the above conclusions, ABA, GA and the ratio of ABA/ZR played determinant roles in the abortion of pistils of pomegranate. Under the premise of the bisexual stages which are identical in functional male flower and bisexual flower, specific hormones conditions are the key to the pomegranate flower sex determination.

Key words: Pomegranate flower; Endogenous phytohormone; Organ differentiation.

Introduction

Pomegranate is an ancient and favorite fruit tree belonging to the Lythraceae family (Yuan *et al*., 2018). It is considered to originate from central Asia areas around Iran. The pomegranate and its multi-utilization are found in ancient human culture. Pomegranate is highly adapted to various environment conditions, and it is mostly cultivated in tropics and subtropics regions, such as the Mediterranean basin, Asia, Australia, and North America (Holland *et al*., 2009). Recent scientific studies have indicated that pomegranate contains abundant polyphenols, tannins, anthocyanins, vitamins, and minerals, that are responsible to reduce blood pressure, and act against serious diseases such as cancer (Huang *et al*., 2005; Lansky & Newman, 2007; Basu & Penugonda, 2009). With such a variety of factors, these have led to an increasing demand for consumption of fresh fruit, juice, tea, and other pomegranate products.

Pomegranate is one of deciduous plant, while in the tropics, pomegranate blooms throughout a whole year, while it only blooms once a year followed by a dormant phase during winter in sub-tropical areas (Babu *et al*., 2011). Pomegranate flowers develop into two types on one tree: bisexual flowers (vase shape) and functional male flowers (bell shape) (Wetzstein *et al*., 2011). The functional male flower is underdeveloped, with rudimentary ovaries containing few ovules, so it is referred to as the functional male flower which will fall off after flowering. The bisexual flower is fertile, which will develop into fruit. Both types of the flower tend to have several hundred of stamens, and morphological characteristics of the stamen is no difference between the two types of pomegranate flowers (Hakan & Zeliha, 2017). Sometimes, three types of flowers may be found in the same tree, namely, hermaphrodite (bisexual), staminate (functional male) and intermediate forms (Babu *et al*., 2011). The intermediate type of flower is described with a short style and developed ovary which is fertile sometimes. We know that the quality and quantity of flower play essential roles in fruit production. Therefore, there is an excellent interest in different types of pomegranate flower differentiation.

The phytohormone play essential roles in regulating plant development from vegetative growth to reproductive growth. Many studies have revealed that various phytohormones are the important regulators in the development and sex differentiation of flowers (Golenberg & West, 2013; Aryal & Ming, 2014). It is well known that plant hormones have impact on flower organ differentiation including GA, auxin, and cytokinin, et al (Golenberg & West, 2013; Aryal & Ming, 2014; Sun *et al*., 2017). GA is considered one of male hormone (Irish & Nelson, 1989). Auxin is a regulator, and it is not only for flower organ differentiation but also helps in sex determination

impacting crosstalk with cytokinin in many plants (Irish & Nelson, 1989). The other regulators, such as ABA and JA have also been reported to contribute for sex-differentiation. ABA is a feminizing hormone (Solomon, 1985), and JA is the masculinizing hormone (Browse, 2009a). During reproductive growth in *Arabidopsis*, auxin response genes regulate the floral organ development (Tabata *et al*., 2010). In addition, Sun *et al*., (2017) reported that IAA, ZT, GA, JA and ABA are involved in regulating the formation of female and male floral buds of persimmons.

We conclude that the morphological differences of the two types of flowers in pomegranate may be closely related to endogenous hormone levels. Therefore, we measured phytohormone content in functional male flower and bisexual flower during development to investigate the underlying relationships between the endogenous hormone and pomegranate flower organ's morphological changes.

Materials and Methods

Plant material: Regularly managed six-year-old 'Taishanghong' pomegranate trees was used, which was grown in Baima Base orchard of Nanjing Forestry University, Jiangsu Province, China. The site has a subtropical monsoon climate. Annual precipitation was about 1037 mm, with most of the rain falling in summer. The average temperature was 15.5°C, and the average maximum and minimum temperature was 29°C in August and 2°C in December, respectively.

Flower development study was conducted during early-to-mid season flowering period. Buds and flowers were obtained from April to June in 2018. Functional male and bisexual flowers were divided according to the size of pistil, which is having a short style in functional male flowers (Holland *et al*., 2009; Wetzstein *et al*., 2011). According to different studies (Chen *et al*., 2017; Zhao *et al*., 2020) on the morphological analyses of ovule differentiation in pomegranate, the developmental processes of pomegranate floral buds were divided into 8 stages based on vertical bud diameter: 3.0-5.0 mm (S1), 5.1-8.0 mm (S2), 8.1-10.0 mm (S3), 10.1-12.0 mm (S4), 12.1-14.0 mm (S5), 14.1-16.0 mm (S6), 16.1-18.0 mm (S7), 18.1 ≤ mature flower (S8) (bisexual flowers and functionally male flowers). Samples were immediately taken back to the lab, weighted and then frozen in liquid nitrogen and stored at -80ºC until measured. Three biological replicates were analyzed per sample.

Phytohormone extraction and purification: The extraction and purification of endogenous ZR, IAA, ABA, GA, and JA levels were done by a direct ELISA technique, which was carried out as described by Yang *et al*., (2001) and Zhao *et al*., (2006). Fresh samples (0.5 g) were homogenized in liquid nitrogen and phytohormone were extracted in 10 mL precooled 80% methanol with Di-tert-butyl-p-cresol (1 mmol/L). The mixture was incubated for 4 h and centrifuged at 3500 r/min. Then supernatant was traversed a Sep-Pak Vac C18 Cartridge (Waters, Milford, MA) prewashed with 80% methanol. The extraction was eluted with absolute methanol and anhydrous ether. Hormones were dried by flowing nitrogen, then phytohormones were dissolved in phosphate buffer saline (1 mL, pH 7.5), finally analyzed by the method of ELISA.

Quantification of phytohormones by ELISA: ELISA was implemented on a 96-well microtitration plate. Every well was filled with buffer solution which contained antigens (0.25 μg/mL) to against hormone. Coated plates were incubated for endogenous hormone at 37°C. After washed four-time with PBS, every well was coated with 50 μL of extracts, and 50 μL antibodies (20.0 μg/mL) which respectively competed with IAA, ZR, GA, ABA and JA. The plate was incubated and washed as above. Then 100 μL coloration solution including 1.5 mg/mL 0-phenylenediamine and 30% hydrogen peroxide was added into every well. Finally, the reaction progress was terminated by adding sulfuric acid (2.0 mol/L) per well when the standard (50 ng/mL IAA and ABA; ZR and JA 10 ng/mL; GA 2 ng/mL) presented pale color, and the 0 ng/mL standard presented deep color. Coloration in every well was detected by ELISA Reader (model EL310, Bio-TEK) with A490. The enzyme-immunoassay data were calculated as described by Weiler *et al*., (1981).

Calculation of hormone concentration : The logit curve was used to calculate by ELISA in this study. The abscissa of the curve was represented by the natural logarithm of concentration (ng/ml) of the hormone sample, and the ordinate was represented by the logit value of the color rendering value of each concentration. The Logit value was calculated as follows Wu *et al*., (1988):

$$Logit\left(\frac{B}{B0}\right) = In\left(\frac{B}{B0 - B}\right)$$

where B0 is the color rendering value of the 0 ng/ml, and B is the value of other concentrations.

The natural logarithm of the hormone concentration (ng/ml) can be found according to the logit value of its color rendering value, and then the concentration of its hormone (ng/ml) can be known by the opposition logarithm. After the concentration of the hormone was obtained, the hormone content in the sample (ng/g. fw) was calculated. The logit curves for five hormones were shown in Fig. 1.

Statistical analysis: The data were statistical analysis using Microsoft Office Excel 2010 and the IBM SPSS Statistics 22.0, and means were separated according to Duncan's Test at $p<0.05$. The endogenous hormone contents in functional male flowers and bisexual flowers were analyzed by one-way analysis of variance (ANOVA). Pearson's correlation coefficients were calculated to analyze and determine the relationships between flower differentiation and hormones concentration of functional male flowers and bisexual flowers.

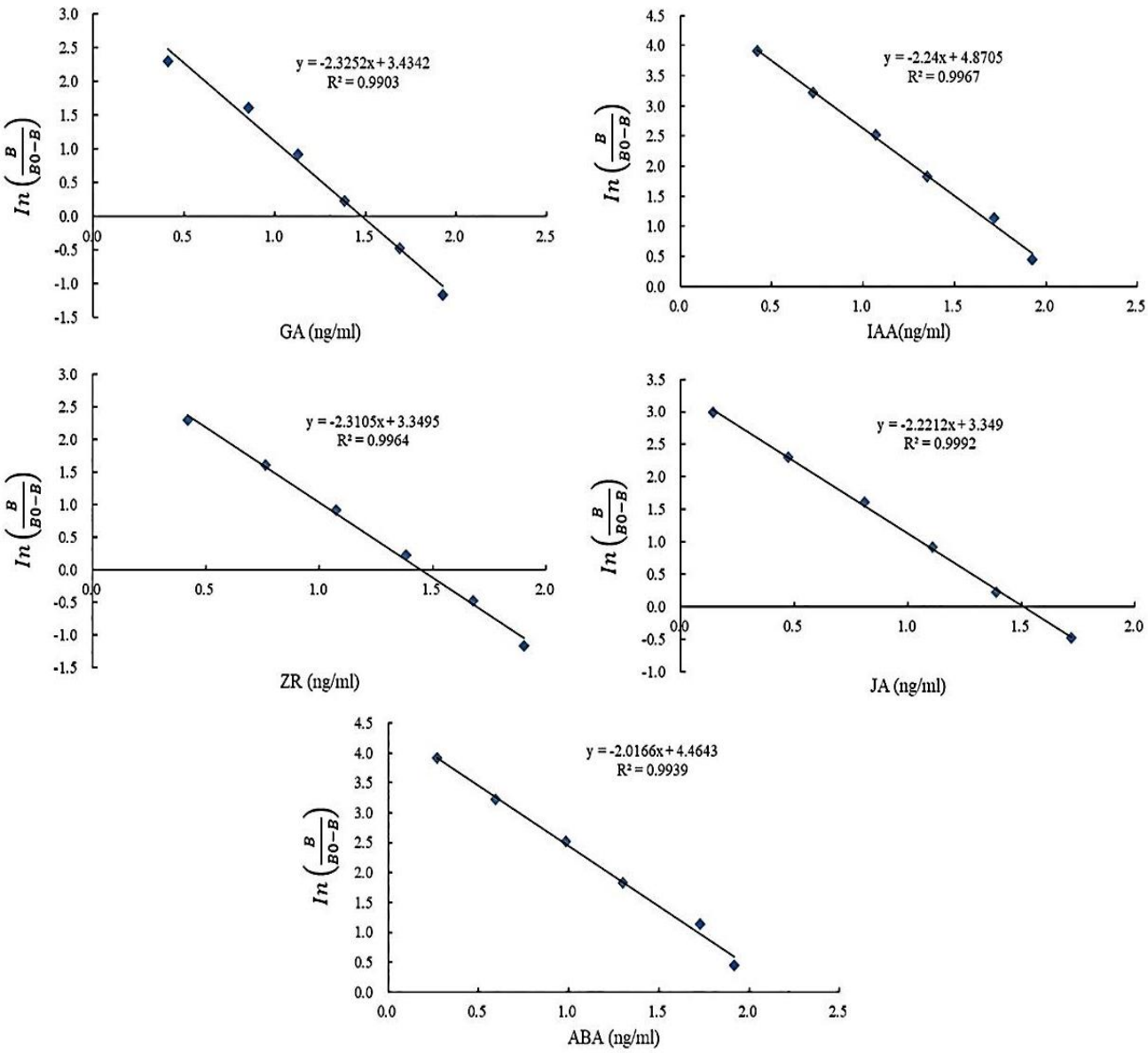

Fig. 1. The logit curves for GA, IAA, ZR, JA, and ABA

Results

Gibberellin: In functional male flower, GA content was the highest at S1 and it decreased sharply from S1 to S3, which resulted in GA content at S1 was approximately 3 times more than that S3. However, the GA content increased from S3 to S5, and it increased again from S7 to S8. The GA content at S8 was no-significant difference from that at S1, S2, S4, S5, and S6.

A similar tendency of changing GA was found in bisexual and functional male flower, and the GA content in functional male flower was higher than that in bisexual flower during whole developmental stages. Whereas, the GA content in bisexual flower was mildly lower than that in functional male flower at S6 and S8. These results implied that a high level of GA played crucial role in the development of functional male flower. Moreover, S3 with low level of GA may be the critical stage for the organ differentiation of both functional male and bisexual flower (Fig. 2A).

Indole-3-acetic acid: In bisexual flower (Fig. 2B), the IAA content decreased slightly from S1 to S3, then it increased from S3 to S7. The IAA content was the highest at S7, which was 3.8 times higher than that at S3.

In functional male flower, the IAA content at S3 was significantly lower than that at S1 and S2. After that, there was a sharp increase in IAA content from S3 to S5, which resulted in the content of S5 was 2.5 times as much as that S3. IAA levels were non-significant differences among S3, S6, and S7. At the S8, the IAA level in bisexual flower was differed significantly from that in functional male flower. IAA levels in the bisexual flowers were much higher than those in the functional male flowers from S6 to S8, which indicated that a high level of IAA in later stages might promote the development of the bisexual flower.

Zeatin riboside: In bisexual flower, the ZR level at S1 was markedly higher than that other stages, and ZR levels at S3, S7 and S8 were substantially lower than those at S1-S2 and S4-S6.

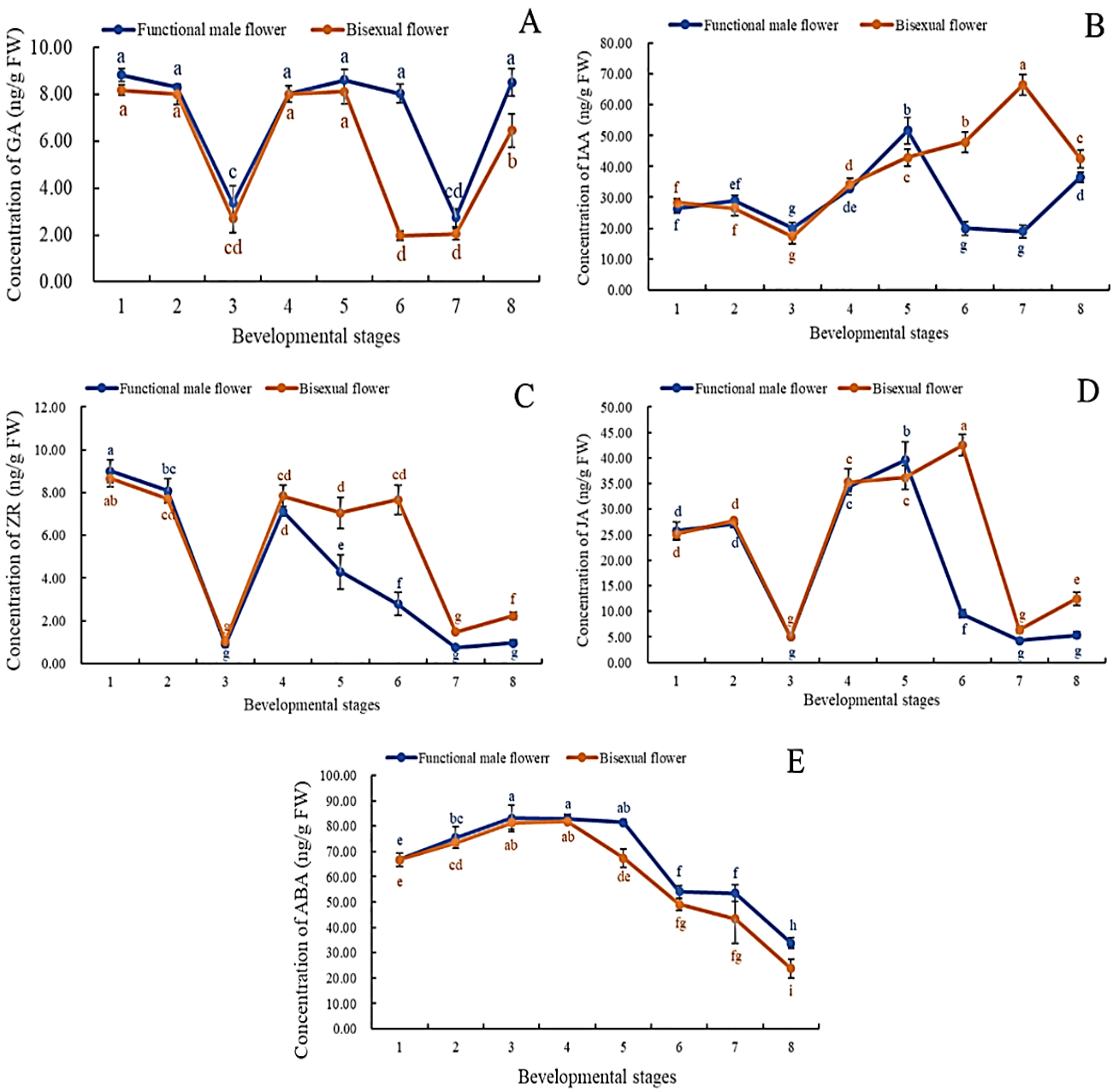

Fig. 2. (A) GA, (B) IAA, (C) ZR, (D) JA, and (E) ABA contents of functional male and bisexual pomegranate flower during the process of development; Data are expressed as the mean ± SE (n=3). Orange and Blue error bars with different letters are the significant difference among developmental stages for flower, based on Duncan's Test at the $p<0.05$ level.

ZR content in bisexual and functional male flower showed a similar trend at the first four stages. The ZR content at S1 was higher than any other stages for the development of functional male flower. There was a sharp reduction in ZR content from S4 to S7, as a result, that the content of ZR at S7 accounted for only a seventh of that at S4.

The ZR level in bisexual flower was mildly higher than that in the functional male flower at S5-S8, which suggested that higher level of ZR might be contributed to the development of bisexual flower (Fig. 2C).

Jasmonic acid: In pomegranate functional male flower, there were no-significant differences of JA content among the first four developmental stages (Fig. 2D). Whereas, the JA content at S5 was 9 times as much as that S3. The JA content started to decrease from S5 to S8, the JA level at S6 was a significant difference from that at S5 and S7. But, the JA level at S8 displayed no obvious changes for that at S7.

The JA changing trend in bisexual flower was discernible like to the JA level of functional male flower. The JA content of S6 was the highest compared with that at other stages in functional male flower. The JA content in bisexual flower was 4.5 times as much as that in functional male flower at S6, which was significantly the highest in the floral differentiation.

Abscisic acid: In functional male flower, ABA contents from S3 to S5 were significantly higher than that at other stages, and ABA contents from S6 to S8 were markedly lower than those from S1 to S5.

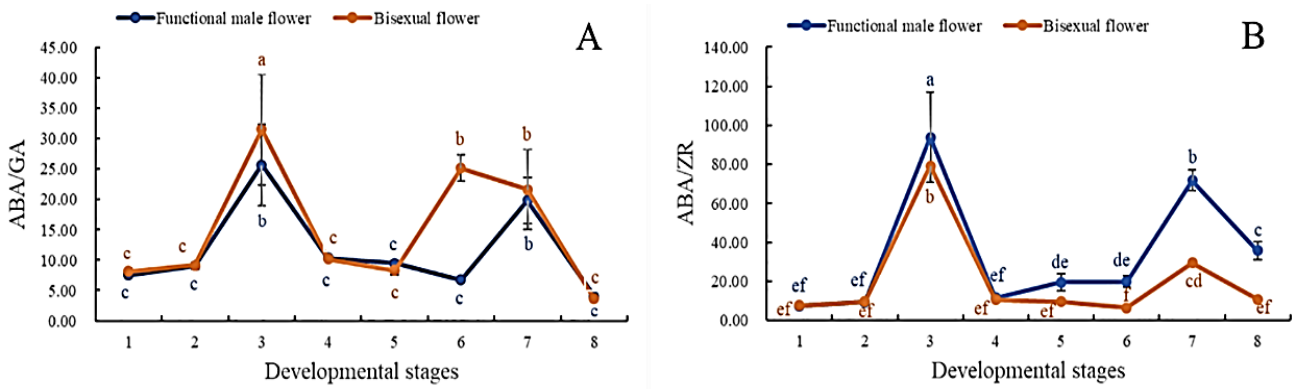

Fig. 3. (A) Ratio of abscisic acid to Gibberellin (ABA/GA) and (B) Ratio of abscisic acid to Zeatin Riboside (ABA/ZR) in functional male flower and bisexual flower during the process of development; the data processing method as above.

The ABA content of bisexual flower and functional male flower were non-significant differences from S1 to S4. Meanwhile, the ABA level in functional male flower was slightly higher than that in bisexual flower from S2 to S4. After S4, the ABA content in bisexual flower exhibited a continuous decline from S4 to S8, and it was significantly lower than that in functional male flower, which indicated that the high level of ABA in middle and later stages might contribute to the development of male flowers (Fig. 2E).

Ratio of ABA to GA: In bisexual flower, the ratios of ABA/GA were significantly increased from S2 to S3, and from S5 and S6. The changing pattern in male flower was the same as that in bisexual flower, except for an opposite change from S5 to S7. The ratio of ABA/GA in bisexual flower were higher than that in functional male flower at S3 and S6-S7, which suggested that a high ratio of ABA/GA was conducive to the formation of bisexual flower (Fig. 3A).

Ratio of ABA to ZR: In contrast with the ratio of ABA/GA level, the ratios of ABA/ZR in functional male flower were higher than those in bisexual flower from S3 to S8, especially at S3 and S7. These results implied that the high-level ratio of ABA/ZR might promote to functional male flower differentiation and flower dropping (Fig. 3B).

Correlation analysis: In functional male flower, the GA level made positive but non-significant correlations with IAA, JA and ZR, nevertheless, disclosed negative and non-significant association with ABA. The associations of IAA with JA, ZR and ABA were positive but non-significant. There was a significant positive correlation between ZR and JA (0.767, $p<0.05$). The ABA level revealed non-significant positive correlations with ZR and JA (Table 1).

At the development process of the bisexual flower, the GA level was negatively correlated with IAA, but it had positive and non-significant correlations with ZR, JA and ABA. The IAA level was correlated negatively and non-significantly with ABA, ZR and JA. The ZR level had a highly significant and positive correlation with JA (0.889, $p<0.01$), and it was positive but non-significantly correlated with ABA. The JA established positive but non-significant correlation with ABA (Table 2).

Table 1. The Pearson correlation analysis among hormones of the Functional male flower.

Male	GA	IAA	ZR	JA	ABA
GA	1	0.589	0.625	0.617	-0.045
IAA	-	1	0.202	0.686	0.18
ZR	-	-	1	0.767*	0.465
JA	-	-	-	1	0.64
ABA	-	-	-	-	1

Table 2. The Pearson correlation analysis among hormones of the bisexual flower.

Female	GA	IAA	ZR	JA	ABA
GA	1	-0.395	0.597	0.378	0.303
IAA	-	1	-0.209	-0.008	-0.667
ZR	-	-	1	0.889**	0.389
JA	-	-	-	1	0.245
ABA	-	-	-	-	1

Discussion

In most flowering plants, a hermaphroditic floral primordium is origin of flower, whereas selective development of the stamen or pistil result in unisexual flower. Recently, many studies showed that phytohormones are critical physiological regulators during the selectivity development in bisexual floral primordium (Aryal & Ming, 2014). Hence, the endogenous hormonal balance is vital for genital development in flowering plants.

The phytohormone have been shown similar masculinizing or feminizing effects on plant alternative or plastic sexual development (Golenberg & West, 2013); however, the phytohormone has different effects in various plants. GA involves in the regulation of flower organ development. It shows an apparent masculinizing function on populus (Song *et al*., 2013). Carpel is inducted by sprayed GA inhibitor in the male papaya (Kumar & Jaiswal, 1984). In our study, the trend of GA between the functional male flower and bisexual flower were similar, but the GA content was higher in functional male flower than that in bisexual flower, which indicated that the high level of GA in functional male flower was essential for pistil degeneration, stamen development, and the functional male flower formation. Our study on

pomegranate flower showed that the role of GA in maintaining male characteristics was consistent with the results of Yazici (2011).

Recent evidences show that, through a unique mechanism of perception and elicitation, the role of auxin is central to a plant structure differentiation (Teale *et al*., 2006). Auxin have been implicated in the regulation of various plant developmental processes, including inflorescence development, and leaf expansion and formation (Cheng *et al*., 2006; Gallavotti *et al*., 2008; Stepanova *et al*., 2008; Phillips *et al*., 2011; Kasprzewska *et al*., 2015; Kneuper *et al*., 2017). Auxin shows a feminizing effect on many species, such as *O. stenopetala* (Orozco-Arroyo *et al*., 2012), while masculinizing effect of auxin has also been reported (Hamdi *et al*., 1987). We found that the content of IAA of bisexual flower was significantly higher than that of functional male flower from S6-S8, and the functional male and bisexual flowers had a similar IAA level trend from S1-S5, suggesting the contribution of IAA to the later development of gynoecia in pomegranate.

Among plant developmental processes, JA regulates root growth, flower development and so on. (Floková *et al*., 2015). The mutants for studying JA pathway in *Arabidopsis* and maize are male abortion (Browse, 2009b; Browse, 2009c; Lunde *et al*., 2019), whereas the mutant of tomato *jai1* (jasmonic acid-insensitive 1) is female sterility (Li *et al*., 2004). JA content from S1-S4 showed similar trends between the functional male and bisexual flower, but the JA level of the functional male flower was a significant difference with the bisexual flower at S5-S6. The result suggested that high level of JA might promote differentiation of functional male and bisexual pomegranate flowers. The JA content of bisexual flower were slightly higher than functional male flower at S7 and S8, which implied that a high level of JA might promote bisexual flower blooming. The results are in contrast with previous research which showed that JA have vital function for male-sterile (Park *et al*., 2002; Browse, 2009a; Lunde *et al*., 2019). The distinction suggested that the function of JA has species-specific characteristics. Recent research reported that JA signaling do not work alone and mediated defence responses in plants, but it plays a part in diverse crosstalk network with other phytohormones such as auxin, GA, and BR (Hartwig *et al*., 2011; Dar *et al*., 2015). For instance, JA can act together with brassinosteroid (BR) to inhibit tassel development in maize (Acosta *et al*., 2009; Hartwig *et al*., 2011).

Cytokinins that are plant-specific hormones have been shown to be of pivotal importance in the cell cycle and developmental programs (Werner *et al*., 2001; Bartrinan *et al*., 2011). Auxins and cytokinins act together to regulate plant development. Male flowers show female characteristics via administering exogenous cytokinin, whereas auxin application produce the opposite effect in *Mercurialis annua* (Louis & Durand, 1978). In *Arabidopsis*, the distance between two ovule primordia and the final size of gynoecium is regulated by cytokinin; that is to say, cytokinin positively regulates the development of gynoecium (Bartrina *et al*., 2011). Our results were consistent with the report, which showed that ZT had higher levels in bisexual flower than that in functional male flower from S4-S8, suggested that the high level of ZT is necessary for pistil development. Male flowers of amur grape can be shifted to a hermaphrodite flower after treated with a synthetic cytokinin (CPPU) (Wang *et al*., 2013), which indicated that cytokinins display a similar function on pomegranate.

ABA implicate in the regulation of plant growth and development, including embryo maturation, seed dormancy, and so on (Zeevaart & Creelman, 1988; Pandey *et al*., 2003; Purty *et al*., 2005). ABA plays an important role in the downstream events of the autonomous floral pathway and the transition to flowering (Razem *et al*., 2006; Su *et al*., 2002). ABA contents gradually increased during development of flower (Domagalska *et al*., 2010). A high level of ABA inhibits the development of stamens specifically, so making flowers male-sterile in tomato (*Lycopersicon esculentum*) (Sekhar & Sawhney, 1991). In this study, the content of ABA was high in both functional male and bisexual flower from S1-S4, which implied that ABA might be a crucial factor for floral bud development in pomegranate. Then, the ABA content of female continued to decrease until flower booming. In functional male flower, the ABA content also showed a trend of decrease, but the level flattened from S3 to S4, and finally it decreased again. The results stated that a relatively high level of ABA might be crucial importance for functional male flower differentiation.

The contents of IAA and JA in bisexual flower were higher than that in functional male flower at S6-S8, and the content of ZR in bisexual flower were higher than that in functional male flower at S4-S8. Whereas the content of ABA and GA in functional male flower were higher than that in bisexual flower at the whole process, the ratio of ABA/GA in bisexual flower was significantly higher than that in functional male flower at S3 and S6. The ratio of ABA/ZR in bisexual flower was significantly lower than that in functional male flower at S3 and from S5-S8, this result was consistent with the report of Sun Peng (2017). These results suggested that a high ABA/GA ratio could promote the development of the bisexual flower, while a high ABA/ZR ratio was beneficial to functional male flower development.

Conclusion

It was indicated that high level of ABA, GA, and the ratio of ABA/ZR in functional male flower might stimulate sterility of ovary and promote stamen organ development, resulting in functional male flower. Bisexual stages were identical in functional male flower and bisexual flower, potentially providing a sex determination platform at the specific hormone conditions. We could spray exogenous hormones or hormone inhibitors as inducers to promote pistil or staminate development at a crucial stage. With the above conclusion, ABA and GA showed a determinant role in the abortion of pistils of pomegranate as well as in the ABA/ZR ratio. Hence, this study confirmed the importance of analyzing the hormonal balance in the floral organ of pomegranate. However, further studies are required for explicit explanation of the complex mechanism which leads to pistil sterility in pomegranate.

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