Endogenous Hormones Levels and *Csexpansin* 10 Gene Expression in the Fruit Set and Early Development of Cucumber

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Summary: Fruit set and early development depends on endogenous hormone levels and gene expression related to fruit growth and development in cucumber (*Cucumis sativus*). In this study, the growth and development of cucumber ovaries and young fruits, endogenous hormones levels, and *Csexpansin* 10 (*CsEXP* 10) gene expressions in pollinated fruits and unpollinated ovaries were studied from -2 to 6 days post anthesis (DPA). The results showed that the fruit diameter and length, single fruit weight, endogenous hormone levels, and *CsEXP* 10 gene expression levels were all higher in pollinated fruits than in unpollinated ovaries, and *CsEXP* 10 gene expression levels were positively correlated with indole-3-acetic acid (IAA), but negatively correlated with gibberellic acid (GA_3) and abscisic acid (ABA). This suggests that pollination may stimulate the fruit set and development by inducing increases in endogenous hormone levels and *CsEXP* 10 gene expression, and the *CsEXP* 10 gene may be downregulated by GA_3 and ABA.

Keywords: Fruit set; Growth and Development; Endogenous hormone; *CsEXP* 10 gene; Cucumber (*Cucumis sativus* L.)

Introduction

Cucumber (*Cucumis sativus* L.) is one of the main vegetable crops in the world. It plays a more important role in China′s vegetable supply and national economy [1]. However, a low fruit set is a serious problem in agricultural production, especially under protected cultivation conditions because of low temperature, low light, and the lack of male flowers, which seriously affects the high yield and good quality of cucumber. Therefore, it is necessary to stimulate the fruit set and early development of cucumber. Fruit set and early development is the most critical stage in fruit growth and development, and is fundamental in agricultural production. But until recently, little was known of the physiological and molecular mechanisms of fruit set and early development [2-4]. It has been demonstrated that fruit set and early development are mainly induced by endogenous hormones produced in the ovaries, such as auxins and gibberellins (GAs), which have been described in the ovaries of pollinated or parthenocarpic fruits [5], or the exogenous application of hormones to unpollinated ovaries [6-7]. Some of the genes related to the biosynthesis and signal transduction pathways of these hormones during fruit development have recently been identified. For example, auxins, GAs, and abscisic acid (ABA) are the key regulators in tomato fruit set and early development, and parthenocarpic fruit development has been induced by the genes related to these regulators in the signal transduction pathway [8-10].

Fruit set and early development is accompanied by the expansion of plant cell walls and cell growth. Expansins are the cell wall-loosening proteins inducing extension of plant cell walls and cell growth, which are widespread in the growing organization and ripening fruits. Many reports have shown the important roles of expansins in plant growth and development [11-12]. Expansin genes have also been isolated and characterized in the fruits of tomatoes [13], strawberries [14], peaches [15-16], bananas [17], cucumbers [18] and litchis [19]. In cucumbers, ten expansin genes were isolated and cloned from differential tissues: *CsEXP* 1 and *CsEXP* 2 were isolated from growing cucumber hypocotyls [20]; *CsEXP* 3-*CsEXP* 9 were found in cucumber root tissues; and *CsEXP* 10 was isolated from young cucumber fruits after pollination, which showed differential expression patterns in roots, stems, leaves, and young fruit of cucumbers [21]. In a previous report, we also found that *CsEXP*10 could be involved in the extension of cell walls related to fruit set and early development [18]. However, its role in the fruit set and early development of cucumbers, and whether it was involved in the signal transduction pathways of hormones during fruit development were unclear. Thus, in the present study, we examined the correlation between *CsEXP*10 gene expression and the fruit diameter and length, single fruit weight, and the correlation between *CsEXP*10 gene expression and levels of endogenous hormones including

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indole-3-acetic acid (IAA), gibberellic acid (GA_3), ABA, and zeatin riboside (ZR).

Experimental

Plant Material and Experimental Design

Cucumber cultivar Cs0601 was used in the work. Cucumber seedlings were grown in a large plastic house at Henan Institute of Science and Technology in China. Flowers were clamped 2 d before anthesis to prevent self-pollination. Experiments included two treatments: (1) pollination, and (2) no pollination. Ovaries and young fruits from cucumber plants of the two treatments were collected at -2, 0, 1, 2, 3, 4, 5, and 6 days post anthesis (DPA), immediately frozen in liquid nitrogen, and stored at −80°C.

Extraction and Analysis of Endogenous Hormones

The ovaries and young fruits of approximately 0.5 g were separately ground with 5 mL 80% (v/v) methanol extraction medium containing 0.1% butylated hydroxytoluene and 0.06% polyvinylpyrrolidone (PVP) per gram fresh material in an ice-cooled mortar. The homogenate was used to purify the endogenous hormones (IAA, GA_3, ABA, and ZR) using the methods described by Wang *et al*. (2015) [22]. Final quantification of hormone levels were analyzed using the ELISA kits (China Agricultural University, Beijing, China). Three biological replicates were analyzed for endogenous hormones levels.

Quantitative RT- PCR analysisi of CsEXP10 gene

Total RNA was isolated from ovaries and fruits of cucumber plantings using a total RNA extractor (Sangon, Shanghai, China), according to manufacturer's instructions. Total RNA was subjected to DNase I (Fermentas, UK) treatment for 30 min at 25°C according to manufacturer's protocol. Purified RNA was used to synthesize first-strand cDNA using a M-MuLV First Strand cDNA Reverse Transcription Synthesis Reagent Kit (Sangon, Shanghai, China), following the manufacturer's instructions. Expression levels of *CsEXP*10 were determined using real-time RT-PCR. The primer sequences of *CsEXP*10 and *CsActin* are listed in Table-1. The PCR was performed using the program described by Sun *et al.* (2015) [23]. Relative mRNA expression data was calculated using the $2^{-\Delta\Delta Ct}$ formula according to the method described by Audran–Delalande *et al*. (2012) [24].

Statistical Analysis

Statistical analysis was performed using SPSS 17.0 software. Analysis of variance (ANOVA) was followed by Tukey's pair wise comparison tests, at a level of $P<0.05$, in order to determine the significant differences between means.

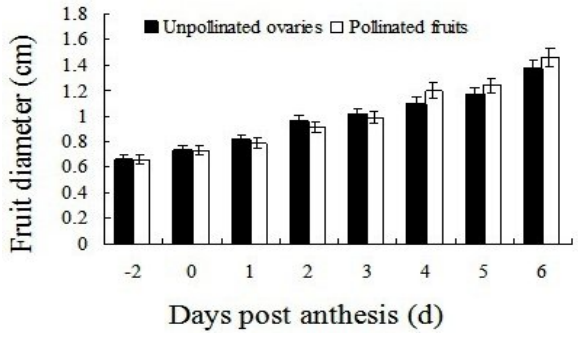
Results and Discussion

Characteristics of Growth and Development in Cucumber Fruits

To investigate the characteristics of growth and development of ovaries and young fruits in cucumber plants, the fruit diameter and length, and single fruit weight from the ovaries and young fruit of pollinated and unpollinated treatments at -2, 0, 1, 2, 3, 4, 5 and 6 DPA were analyzed. The fruit diameter and length, and single fruit weight significantly increased with growth and development, which indicated that the ovaries of cucumbers could initiate growth in the absence of pollination. The growth rates of ovaries and young fruits in pollinated and unpollinated treatments were very similar from -2 to 3 DPA. However, the unpollinated ovaries showed a significantly slower growth rate than the young pollinated fruits from 3 to 6 DPA. At 6 DPA, the fruit diameter and length and single fruit weight were the highest in pollinated fruits and unpollinated ovaries (Fig. 1). These results indicated that pollination stimulated fruit growth and development.

Table-1: The primer sequences of *CsEXP10* and *CsActin.*

Primer	Primer sequences	Description
***CsEXP10*-F**	CCGTAACTGGGGCCAAAATT	qRT-PCR
***CsEXP10*-R**	TCCGGTGAA TGTCTGACCAA	qRT-PCR
***CsActin*-F**	CCACGAAACTACTTACAACTCCATC	qRT-PCR
***CsActin*- R**	GGGCTGTGATTTCCTTGCTC	qRT-PCR



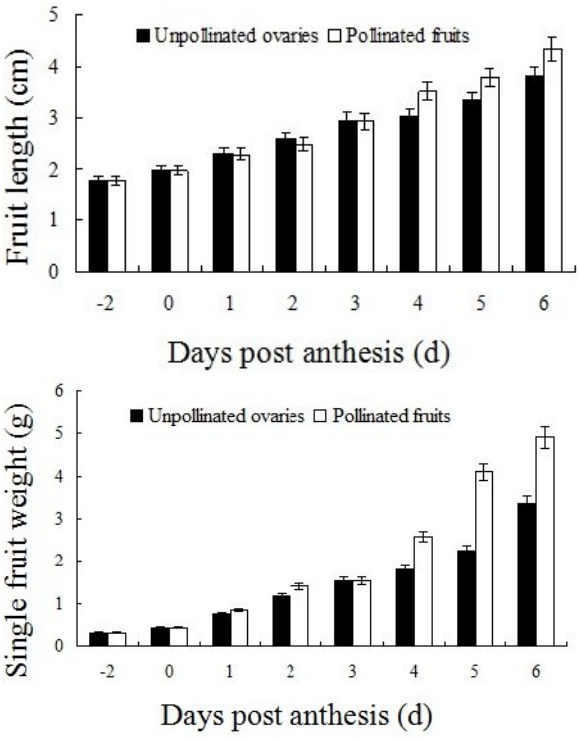


Fig. 1: Changes of fruit shape index in fruit set and early development of cucumber.

Endogenous Hormone Levels

Fruit development is associated with complex hormonal regulation. To determine the physiological mechanisms of the endogenous hormones in fruit set and early development of cucumber plants, endogenous hormones levels were quantified in pollinated fruits and unpollinated ovaries at -2, 0, 1, 2, 3, 4, 5 and 6 DPA. Auxins play an important role in fruit set and growth, and the genes related to the biosynthesis, transport and response of auxin are associated with fruit development. In our study, in the same period (from -2 to 6 DPA), the IAA levels showed similar patterns in pollinated fruits and unpollinated ovaries, but the IAA levels in pollinated fruits were higher than those in unpollinated ovaries. The IAA levels in pollinated fruits and unpollinated ovaries dramatically increased from 0 DPA to 3 DPA, and were the highest at 3 DPA. The endogenous IAA levels decreased after 3 DPA, and dropped to relatively low values at 5 DPA. At 6 DPA, the IAA levels increased again (Fig. 2). The results indicated that the higher IAA levels induced higher growth rates of fruit, and promoted fruit development, which supports that the role of IAA in fruit fruit set and growth. The levels of endogenous GA_3 in pollinated fruits and unpollinated ovaries sharply decreased during fruit growth and early development (from -2 to 5 DPA) and then increased at 6 DPA. However, GA_3 levels in unpollinated ovaries were lower than in pollinated fruits (Fig. 2), which is consistent with the findings of Serrani *et al*. [7]. The GA_3 levels were significantly negatively correlated with the fruit diameter and length, and single fruit weight in pollinated fruits and unpollinated ovaries (Table-2). The ABA levels in unpollinated ovaries sharply decreased from -2 to 6 DPA. However, the ABA levels in pollinated fruit gradually decreased from -2 to 2 DPA, increased at 3 DPA, decreased from 4 to 5 DPA, and increased again at 6 DPA (Fig. 2). The ABA levels in pollinated fruits and unpollinated ovaries were significantly negatively correlated with the fruit diameter and length, and single fruit weight (Table-2). The ZR levels in pollinated fruits and unpollinated ovaries showed a bimodal variation pattern during the growth and early development of cucumber fruits. In pollinated fruits, peaks occurred at 0, 3, and 5 DPA. However, in unpollinated ovaries, peaks occurred at 1, 3, and 6 DPA (Fig. 2).

Table-2: The correlation between endogenous hormones and fruit shape index in fruit set and early development of cucumber.

	Pollinated fruits			Unpollinated ovaries		
	Fruit diameter	**Fruit length**	**Single fruit weight**	**Fruit diameter**	**Fruit length**	**Single fruit weight**
IAA	**0.615**	**0.613**	**0.475**	**0.537**	**0.553**	**0.52**
GA_3	**-0.914****	**-0.92****	**-0.861**	**-0.851****	**-0.879****	**-0.782***
ABA	**-0.911****	**-0.915****	**-0.907**	**-0.947****	**-0.963****	**-0.903****
ZR	**0.344**	**0.334**	**0.329**	**-0.425**	**-0.376**	**-0.329**

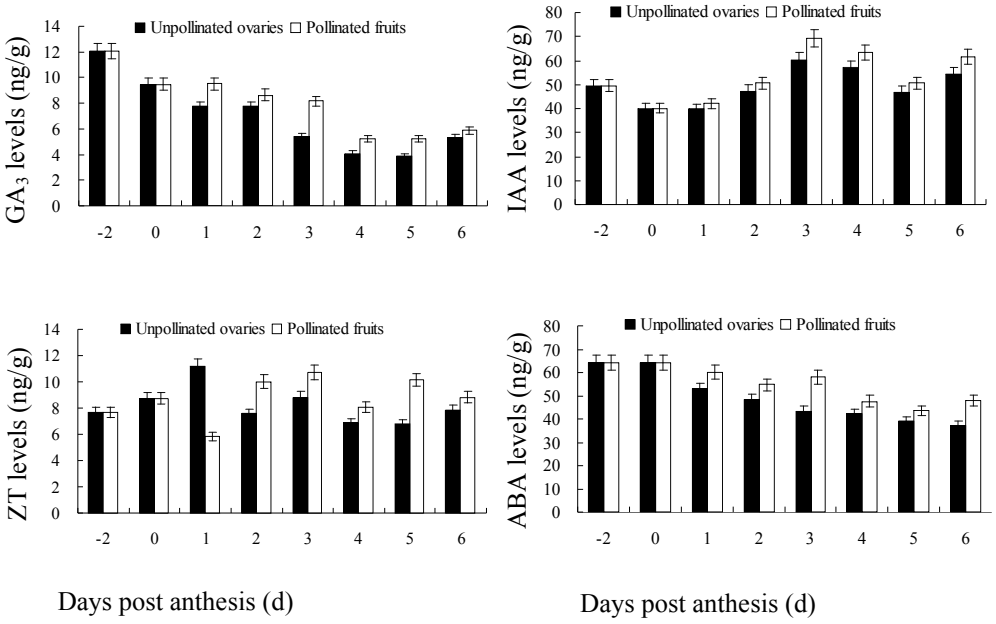


Fig. 2: Changes of endogenous hormones in fruit set and early development of cucumber.

To investigate the role of endogenous hormones during cucumber fruit growth and development, we focused our study on the levels of endogenous hormones in pollinated fruits and unpollinated ovaries during the early stages of fruit growth and development in cucumbers. Our results showed that the levels of IAA, GA, ABA, and ZR in pollinated fruits were higher than in unpollinated ovaries, which confirmed that pollination induced an increase in the levels of endogenous hormones of fruit, and stimulated fruit set and development by signal transduction pathway of hormones regulatory network [25-27].

CsEXP 10 *gene expression*

Expansins has different expression in each stage during fruit growth and development. The high expression levels of expansin genes were obtained in growing vegetative tissues such as tomato [13], pear [28], cucumber [21], banana [29] and longan [30]. To assess whether the *CsEXP* 10 gene genetically regulated fruit set and early fruit development in cucumbers, the relative *CsEXP* 10 gene expression was studied in pollinated fruit and unpollinated ovaries at -2, 0, 1, 2, 3, 4, 5 and 6 DPA. *CsEXP* 10 gene expression patterns in pollinated fruits and unpollinated ovaries were consistent from -2 to 6 DPA. The *CsEXP* 10 gene expression levels were gradually downregulated from -2 to 1 DPA, and then upregulated from 1 to 6 DPA. The highest *CsEXP* 10 gene expression level in pollinated fruits and unpollinated ovaries was at 6 DPA, and expression levels in pollinated fruits were higher than in unpollinated ovaries in all cases (Fig. 3). Furthermore, *CsEXP* 10 gene expression levels during the early developmental stages of cucumbers (-2～6 DPA) were significantly correlated with the fruit diameter and length, and single fruit weight (Table-3). It is possible that *CsEXP* 10 gene expression is associated with fruit growth and development, and pollination can stimulate the *CsEXP* 10 gene expression and promote fruit development. The similar results were obtained in longan fruit [30], which indicated that *EXP* mRNAs were correlated with the fruit development and exhibited different expression patterns at different fruit developmental stages. To determine whether *CsEXP* 10 gene expression is regulated by plant hormones, the correlation between *CsEXP* 10 gene expression and the levels of hormones were evaluated. The results showed that *CsEXP* 10 gene expressions was positively correlated with IAA, and negatively correlated with GA_3 and ABA, while there was no significant correlation between *EXP*10 and endogenous hormone levels (Table-4). This suggests that the *CsEXP* 10 gene may be upregulated by IAA, and downregulated by GA_3 and ABA.

Table-3: The correlation between *CsEXP* 10 gene expression and fruit shape index in fruit set and early development of cucumber

	Pollinated fruits			Unpollinated ovaries		
	Fruit diameter	Fruit length	Single fruit weight	Fruit diameter	Fruit length	Single fruit weight
CsEXP 10	0.888**	0.878**	0.916**	0.828*	0.801*	0.896*

Table-4: The correlation between *CsEXP* 10 gene expression and endogenous hormones in fruit set and early development of cucumber

	Pollinated fruits				Unpollinated ovaries			
	IAA	GA	ABA	ZA	IAA	GA	ABA	ZA
CsEXP 10	0.448	-0.651	-0.683	0.163	0.354	-0.453	-0.653	-0.227

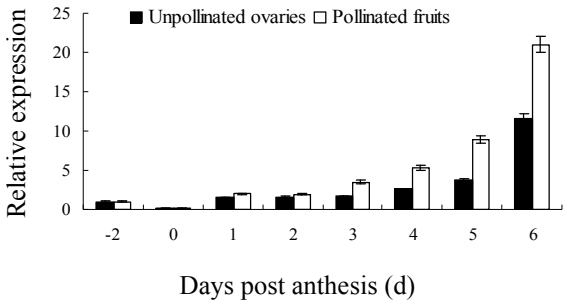


Fig. 3: Expression of *CsEXP* 10 gene in fruit set and early development of cucumber.

Conclusion

In this paper, the characteristics of growth and development, endogenous hormones levels, and *CsEXP* 10 gene expression in cucumber fruits from differential developmental stages. The results showed that the fruit diameter and length, single fruit weight, endogenous hormone levels, and *CsEXP* 10 gene expression levels were all higher in pollinated fruit than in unpollinated ovaries, and *CsEXP* 10 gene expression levels were positively correlated with IAA, but negatively correlated with GA_3 and ABA. This suggests that pollination may stimulate the fruit set and development by inducing increases in endogenous hormone levels and *CsEXP* 10 gene expression, and the *CsEXP* 10 gene may be downregulated by GA_3 and ABA. This study would provide valuable information for the molecular regulatory mechanisms of fruit development in cucumber.

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