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การพัฒนาระบบสืบพันธุ์เพศเมียในมะละกอและอิทธิพลต่อคุณภาพเมล็ด

Female Gametophyte Development of *Carica papaya* L. and Its Possible Role in Seed Quality

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บทคัดย่อ

มะละกอ (*Carica papaya* L.) เป็นไม้ผลซึ่งสามารถนำไปบริโภค และแปรรูปเป็นผลิตภัณฑ์ได้อย่างหลากหลาย มะละกอมีถิ่นกำเนิดในแถบอเมริกากลาง ปัจจุบันมีการปลูกอย่างกว้างขวางในประเทศเขตร้อน และกึ่งร้อน มะละกอได้รับเลือกเป็นพืชต้นแบบสำหรับไม้ผลเขตร้อน และใช้ในการศึกษาการแสดงเพศ เนื่องจากมีความสำคัญทางเศรษฐกิจ จีโนมขนาดเล็ก และมี Y โครโมโซม มะละกอแขกดำเป็นพันธุ์ที่นิยมปลูกมากที่สุดในประเทศไทย มะละกอแขกดำ ระยะผลดิบสามารถนำมาบริโภคเป็นผัก ในขณะที่ผลสุกรับประทานเป็นผลไม้ อย่างไรก็ดีการผลิตมะละกอแขกดำ พบปัญหาด้านความไม่สม่ำเสมอของคุณภาพเมล็ด โดยเฉพาะปริมาณเมล็ดสมบูรณ์ และอัตราการงอกของเมล็ด คณะผู้วิจัยได้ศึกษาเปรียบเทียบรูปร่าง และขนาดของระบบสืบพันธุ์เพศเมียกับคุณภาพของเมล็ดของมะละกอแขกดำ เปรียบเทียบกับปลักไม้ลาย ซึ่งเป็นพันธุ์ที่มีคุณภาพเมล็ดดี ผลการศึกษาชี้ว่ารูปร่างของยอดเพศตัวเมียส่งผลต่อคุณภาพของเมล็ดอย่างมีนัยสำคัญทางสถิติ โดยยอดเกสรเพศเมียสั้นที่พบในพันธุ์ปลักไม้ลาย ส่งเสริมการผสม (fertilization) ทำให้มีจำนวนเมล็ดที่สมบูรณ์มากกว่า ในขณะที่มะละกอพันธุ์แขกดำ ซึ่งมียอดเกสรเพศเมียที่ยาวกว่า มีเมล็ดที่สมบูรณ์น้อยกว่า

คำสำคัญ : กล้องจุลทรรศน์แสง ; ผลไม้ ; โครงสร้างเซลล์ ; มะละกอ

Abstract

Papaya (*Carica papaya* L.) is a fruit crop with versatility for consumption and manufacturing into products. Originated in Central America, it is currently cultivated in many tropical and subtropical countries. Due to its economic importance, small genome size and the presence of Y-chromosome, papaya is considered to be a plant model for tropical fruits and for the sex determination study. *Carica papaya* cv. *Khak Dum* is the most widely grown papaya cultivar in Thailand. The fruit can be consumed as vegetable at mature green stage and as fruit at ripe stage. The inconsistency of seed quality including seed number and its germination rate however is occurred frequently. This causes difficulty in seed collection and plantlet preparation. In order to investigate the basis of such irregularity, the morphology of female gametophytes and seed numbers and maturity from *Khak Dum* fruit were determined. The data was compared with that of *Carica papaya* cv. *Pluk Mai Lie*, a cultivar known for its consistency in seed quality. The outcome showed that stigma morphology significantly impacted seed quality in papaya fruit. Papaya cv. *Pluk Mai Lie* with short stigma was likely promoted high fertilization and consequently produced large amount of good quality papaya seeds. On the other hand, Papaya cv. *Khak Dum* with significantly longer stigma produced lower numbers of good quality seeds.

Keywords : light microscopy ; fruit ; cellular structure ; papaya

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Introduction

Papaya (*Carica papaya* L.) is a fruit crop originated in Central America from Mexico to Costa Rica (Medina *et al*., 2004). It is later introduced to other parts of the world and widely grown in tropical and sub-tropical countries such as India, Australia, Malaysia and Thailand. It was in the world 4th major tropical fruit crop after mango, pineapple and avocado. Its global demand and production are still climbing (OECD-FAO, 2020). Papaya is a fast-growing tree that produces fruits within 6-9 months. With good maintenance, papaya fruit can be harvested up to 2-3 years. In 2020, Thailand papaya plantation areas were 23,198.66 Rai and produced 115,559.8 tons. The cultivated areas were mainly in the West and East regions. *Carica papaya* cv. *Khak Dum* was the most popular cultivar (Department of Agriculture Extension, 2020). Though papaya has three types of sex; male, female and hermaphrodite, only hermaphrodites produce desirable fruit shape. The hermaphrodites produce 3 types of flowers; pentandria, reduced elongata and elongata. The elongata flowers with complete 10 anthers produce fruits with desirable cylindrical shape. While the fruit from *Pluk Mai Lie* cultivar is commonly consumed at ripe stage, *Khak Dum* cultivar can be consumed at both green and ripe stages. This adds more income to papaya growers.

Because of its significance, papaya is regarded as a plant model for tropical fruit (Moore and Ming, 2008). It is widely studied in various disciplines including nutrition, manufacturing, genetics and breeding. The ripe papaya fruit is high in vitamin A and C and fiber content. It is an important raw material for food, cosmetics and pharmaceutical products (Karunamoorthi *et al.*, 2014). Papaya Y chromosome was discovered and used as a model for plant sex chromosome (Liu *et al.*, 2004; Ma *et al.*, 2004). Papaya is susceptible to many diseases and several International programs were established to produce disease resistant papaya using both genetic engineering and molecular markers combining with conventional breeding (Drew, 2016; Gonsalves, 2006). Although papaya is generally adapted well for growing condition in Thailand, inconsistency of seed and plantlet quality occurs. These problems increase the starting cost and often the delay in papaya plantation. Iamjud *et al* (2014) reported variation of seed production and its quality among papaya cultivars. Previous studies in papaya indicated that both male (through pollen) and female gametophytes contributed to seed quality (Tamaki *et al*., 2011; de Macedo *et al*., 2013). In other plants such as Arabidopsis, female gametophytes also reportedly impacted seed development. The lack of fertilization independent seed (FIS) protein complex in Arabidopsis female gametophytes resulted in defects in endosperm and consequently arrested of embryo development and seed abortion (Leroy *et al.*, 2007). Female gametophytes also controlled Arabidopsis seed coat development (Ingouff *et al.*, 2006). In *Brassica napus*, number of seeds per silique was correlated with female gametophyte formation (Li *et al*., 2015). In this study, morphology of female gametophytes was investigated via physical measurement and microscopic techniques. Both fresh and fix tissues were used. The results were analyzed and compared with those of papaya cv. *Pluk Mai Lie* which is known for its consistency in seed quality.

Methods

Physical measurement and statistical analysis

The elongata flower buds (0.5-1 cm in length) from papaya cvs. *Khak Dum* and *Pluk Mai Lie* were tagged on the same day. At 7, 14, 21 and 28 days after tagging, the flowers were collected. The 28-day old flowers were used for pollen study. At 28 days after tagging, the flowers and female gametophyte were fully developed but not yet blooming (Figure 1). Hence, pollens within anthers were still resided within the flowers. Anthers from elongata-type flowers of papaya cvs. *Khak Dum* and *Pluk Mai Lie* were placed into glass plates and acetocarmine solution was added. The anthers were crusted with glass rods and the mixture was transferred to glass slide. The total numbers and viable pollens (stained with acetocarmine solution) were counted under light microscope at 10X magnification. The fruits developed from elongata-type flowers of papaya cvs. *Khak Dum* and *Pluk Mai Lie* were harvested after 4 months. Total seeds per fruits and well-developed seeds numbers were counted. The data were statistically analyzed using Analysis of Variance (ANOVA).

Papaya female gametophyte preparation

The elongata flower buds (0.5-1 cm in length) from papaya cvs. *Khak Dum* and *Pluk Mai Lie* were tagged on the same day. At 7, 14, 21 and 28 days after tagging, the flowers were collected. The flower at 28 days were used for determination of ovule numbers. The ovules were gently removed from ovaries, placed on glass slides and counted under light microscope at 10 X magnification. The flowers at 7, 14, 21, 28 and 30 days after tagging were used for determination of female gametophyte development. Papaya ovaries were excised into 1x2x10 mm and washed with sterilized distilled water 3 times. The samples were fixed with 5% acetic acid solution in 0.1 M phosphate buffer pH 7.0, degassed and kept at 4 °C for 3 h. The samples were washed 3 times with 0.1 M phosphate buffer pH 7.0. The samples were suspended in 1% OsO_4 solution in 0.1 M phosphate buffer pH 7.0. The ethanol replacement in papaya tissues was done using ethanol series (30, 50, 70, 80, 90, and 100%) twice for each concentration. Afterward, the tissues were suspended in ethanol: n-butylglycidyl ether (QY-1) ratio 1:1 for 30 min and the process was repeated once. The tissues were incubated in QY-1: Spurr's resin ratio 1:1 for 30 min and poured into the mold. The mixture was polymerized at 60 °C for 24 h. The polymerized resin block was sliced into pieces of 2.5 μm thick. The tissues were stained with toluidine blue solution at 70°C for 5 min and washed with sterilized distilled water. The papaya female gametophyte was observed under light microscope (Nikon YS 100, Japan).

Results

The comparative study on physical and microscopic structures of two papaya cultivars was investigated. Visual observation indicated that the anthers was in closer proximity to stigma in flowers of papaya cv. *Pluk Mai Lie* than in papaya cv. *Khak Dum*. In addition, papaya cv. *Pluk Mai Lie* flowers have curvier, thinner and more branches stigmas than those of cv. *Khak Dum* (Figure 2). The female gametophyte development in two papaya cultivars; *Khak Dum* and *Pluk Mai Lie* were compared (Figure 4). While stage 1 was observed in seven-day old flowers, stage 2 and 3 were observed in 14-day old flowers. After 21 days, the development was mostly completed. In stage 1, tightly pack cells surrounded by outer layers of cells were observed. Though the development was noticed in both cultivars, it was slightly advanced in *Pluk Mai Lie*. In stage 2, finger-like structures made up of funiculus, chalaza and nucellus were formed. The nucellus is megasporogenesis, initiating cell division through mitosis to form a megaspore at the nucellus (Pillitteri *et al.*, 2007; Yamada *et al.*, 2016). Only in *Pluk Mai Lie*, the outer integument was formed. In stage 3, the development of magagametogenesis, a globular ovule developed from a mono-nuclear is found in the embryo sac was observed (Schneitz *et al.*, 1995). The components such as the nucellus, inner integument, and outer integument were observed in *Pluk Mai Lie* while inner integument was not yet detected in *Khak Dum*. After the blooming, the development of ovules in cv. *Khak Dum* was found to be uneven (Figure 3) comparing with cv. *Pluk Mai Lie* (Buathongjan *et al*., 2020). Among measured morphological characters of fruits from two papaya cultivars, three parameters including the length of ovary and stigma and numbers of well-developed seeds showed significantly different (Table 1). The outcome supported the visual observation. The data was further analyzed for correlation using Pearson's correlation coefficient (*rp*) (Table 2). The results showed high correlation between the cultivars and three physical characters including the length of ovary and stigma and well-developed seeds per fruit. In addition, the length of ovary was highly correlated with the length of stigma.

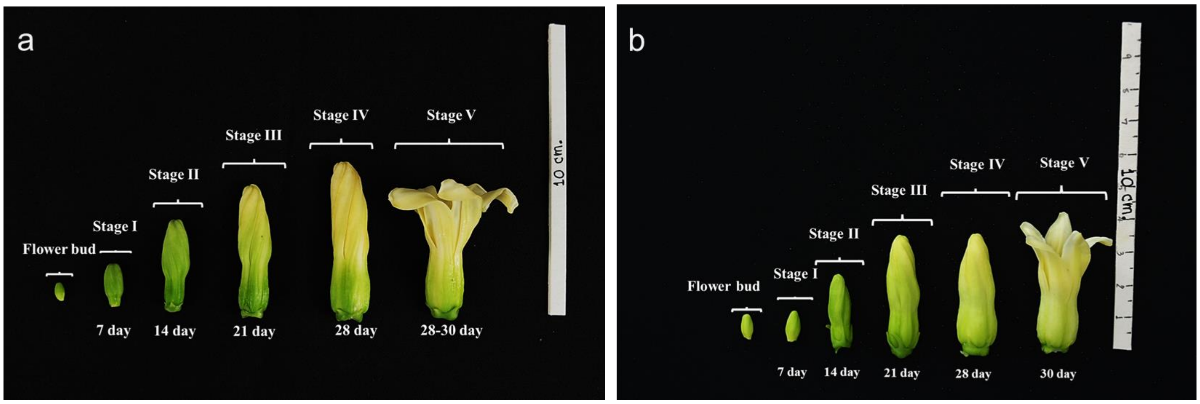

Figure 1 The development of elongate flowers from cvs. *Khak Dum* (a) and *Pluk Mai Lie* (b) at 7,14,21,28 and 30 days after tagging, respectively.

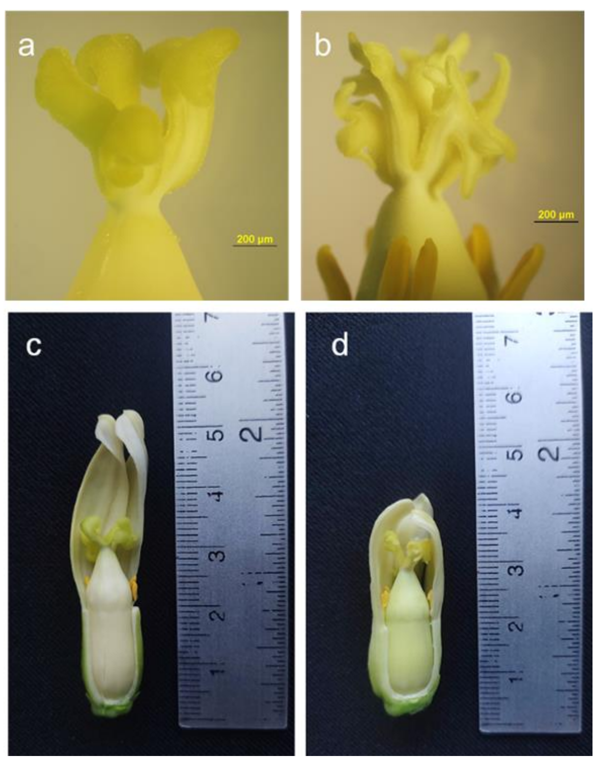

Figure 2 The closed-up stigma and longitudinal flowers from cv. *Khak Dum* (a and c) and cv. *Pluk Mai Lie* (b and d).

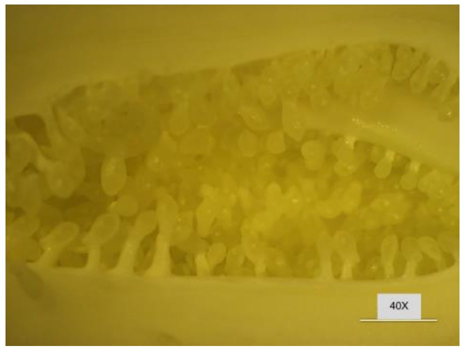

Figure 3 Longitudinal section of fertilized ovary showing young ovules in cv. *Khak Dum* with uneven development. The magnification was 40X.

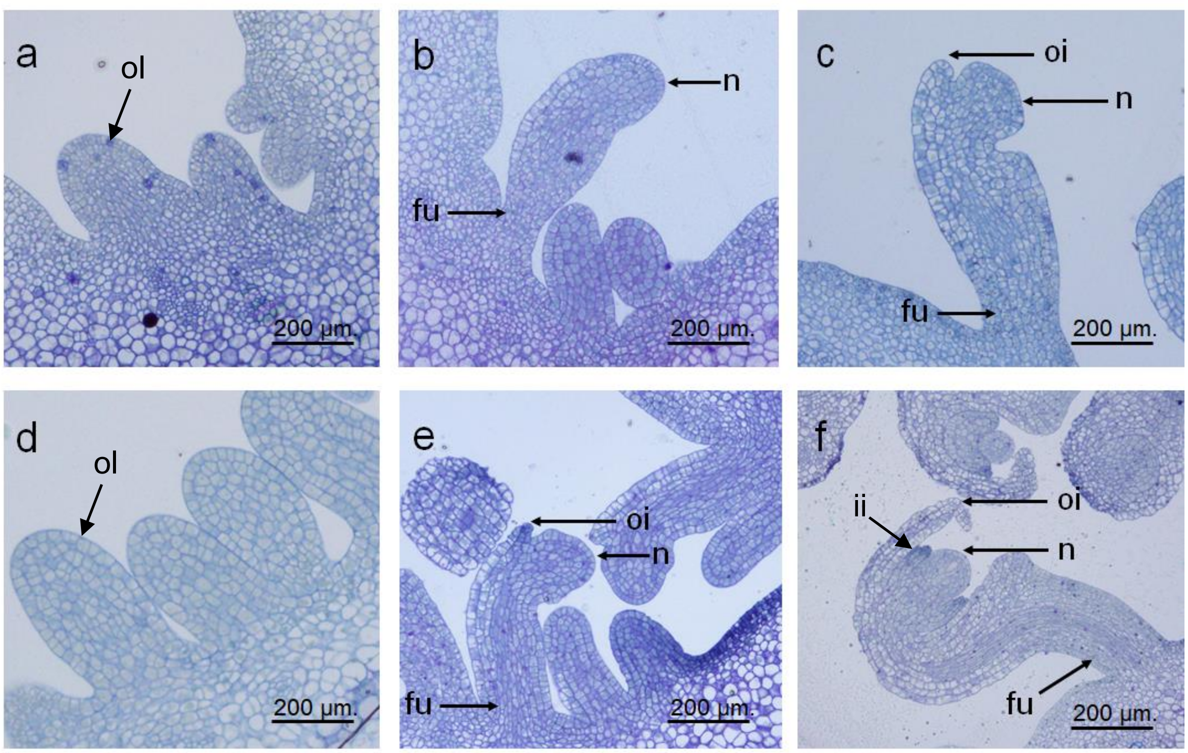

Figure 4 Female gametophyte development stage 1-3 in *Carica papaya* cvs. *Khak Dum* (a-c) and *Pluk Mai Lie* (d-f). ol = outer layer cells, n = nucellus, oi = outer integument, ii = inner integument, fu = funiculus.

Table 1 Comparison of morphological characters of flowers and fruits from *Carica* papaya cv. *Khak Dum* and C. papaya cv. *Pluk Mai Lie*. The data was analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT). Data within the same column followed by different letters are significantly different at P = 0.05.

Cultivars	Dimension of Ovary		Number Ovule	Viable Pollen	Dimension of Stigma		Total seeds per fruit	Well-developed seeds per fruit
	Length (cm)	Width (cm)			Length (cm)	Width (cm)		
KD	2.33 ± 0.1 A	0.90±0.00 B	1,230.33 ± 477.3 A	49,322 ± 20301 A	1.20±0.10 A	0.93±0.15 A	526.58 ± 189.4 A	287.63 ± 117.5 A
PML	2.07 ± 0.1 B	1.00±0.00 A	1,220.00 ± 87.7 A	40,883 ± 10467 A	0.87±0.06 B	0.93±0.15 A	642.52 ± 271.8 A	520.24 ± 227.4 B

Table 2 Pearson's Correlation between papaya morphological characters

		Cultivars	Length of Ovary	Width of Ovary	Length of Stigma	Width of Stigma	Total seeds
Length of Ovary	Pearson's r	-0.943	-	-0.943	0.919	-0.189	-
	p-value	0.005	-	0.005	0.010	0.720	-
Width of Ovary	Pearson's r	1	-	-	-0.928	0	-
	p-value	*	-	-	0.007	1	-
Length of Stigma	Pearson's r	-0.928	-	-0.005	-	-	-
	p-value	0.007	-	0.926	-	-	-
Width of Stigma	Pearson's r	0.000	-	-	-	-0.050	-
	p-value	1.000	-	-	-	0.926	-
Number Ovule	Pearson's r	-0.018	-0.090	-	-	-	-
	p-value	0.972	0.865	-	-	-	-
Total seeds	Pearson's r	0.200	-	-	-	-	-
	p-value	0.057	-	-	-	-	-
Well-developed seeds	Pearson's r	0.452	-	-	-	-	0.941
	p-value	0.000	-	-	-	-	0.000

Discussion

Flower architecture has profound impacted on the success of fertilization either self-, cross-pollination and even for insect pollination. In most fruit crops, fertilization is essential for fruit development and seed production. Although papaya can develop fruits without fertilization, the fruits are small, with little flesh and no viable seeds. The flowers from papaya cv. *Pluk Mai Lie* were more compacted with shorter ovary and stigma comparing with those of papaya cv. *Khak Dum.* Despite similarity in numbers of viable pollens and ovules, papaya cv. *Pluk Mai Lie* had significantly more well-developed seeds. This suggested high fertilization rate. Tristylous flowers consist of 3 types of styles; short, medium and long. The positions affected stigma receptivity, pollination and consequently seed numbers (Waites and Gren, 2006). Investigation into the effects of stigma position in radish showed that the flowers with large or lower stigma were mostly self-pollinated while those with small or high stigma were cross-pollinated. These morphological differences impacted seed productivity (Kobayashi *et al.*, 2009). Inserted stigma (stigma is beside or very close proximity to anthers) is an established phenotype in cultivated tomato to ensure good fertility. Exserted stigma that caused reduced fertility is found in some landraces and vintage varieties. It is highly possible that the stigma positon in papaya flower also affected fertilization and consequently seed production. Riccini *et al.* (2021) reported that stigma position is regulated by cell wall related genes and elongation enhance hormone, auxin. Previous studies indicated the importance of stigma shape to the efficiency of fertilization. The Y-shaped of stigma of *Dianthus* spp, had the highest pollen germination rate and significantly improved seed development. Even after 5-6 days of blooming, the stigmas were still functional because of its curved horn shapes. On the other, column shape stigma had lower pollen germination rate and lesser seed numbers (Fu *et al.*, 2011). This scenario could also apply to papaya.

Conclusion

Integration of physical measurement and microscopy techniques were used to investigate relationship between morphology of female gametophytes and seed quality in papaya fruit. It is suggested that the results indicated that morphological differences likely impacted fertilization rate. That occurrence combined with well-developed female gametophytes boosted high seed quality and quantity.

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