Inheritance of Resistance to Cowpea Aphid (*Aphis craccivora* Koch.) in Yardlong Bean and Cowpea by Microsatellite Markers

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ชื่อวิทยานิพนธ์ การถ่ายทอดทางพันธุกรรมลักษณะความต้านทานเพลี้ยอ่อนถั่ว (*Aphis craccivora* Koch.) ในถั่วฝักยาวและถั่วพุ่มโดยใช้เครื่องหมายไมโครแซตเทลไลต์

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บทคัดย่อ

เพลี้ยอ่อนถั่ว (*Aphis craccivora* Koch.) เป็นแมลงศัตรูพืชที่สำคัญข องการผลิตถั่วฝักยาวในหลายประเทศ ปัจจุบันมีการใช้สารเคมีฆ่าแมลง เพื่อควบคุมเพลี้ยอ่อนถั่ว การปรับปรุงพันธุ์ต้านทานต่อการเข้าทำลายของ เพลี้ยอ่อนถั่ว จึงมีความสำคัญ วัตถุประสงค์ ของการศึกษาครั้งนี้ เพื่อ วิเคราะห์ อัตราพันธุกร รมลักษณะ ความต้านทานเพลี้ ยอ่อนถั่ว และ หาเครื่องหมายไมโครแซ ตเทลไลต์ที่สัมพันธ์กับ ลักษณะความต้านทานเพลี้ยอ่อนถั่ว โดยทำการผสมทำการผสมข้ามระหว่า งถั่วฝักยาว พันธุ์คัด -ม.อ. ที่อ่อนแอต่อ เพลี้ยอ่อนถั่ว กับพันธุ์ต้านทานคือถั่วพุ่มพันธุ์ IT82E-16 เพื่อสร้างประชากร F_1 และ F_2 ปลูกทดสอบ 4 กลุ่มประชากร ประกอบด้วยพันธุ์แม่ (P_1) พันธุ์พ่อ (P_2) ลูกผสมชั่วที่ 1 (F_1) และลูกผสมชั่วที่ 2 (F_2) ในโรงเรือนตาข่ายปิด โดยวางแผนการทดลองแบบสุ่มในบล็อกสมบูรณ์ จำนวนซ้ำไม่เท่ากัน เมื่อต้นพืชอายุ 30 วันหลังเพาะเมล็ด ปล่อยเพลี้ยอ่อนถั่วจำนวน 5 ตัวต่อต้น เพื่อศึกษาจำนวนเพลี้ยอ่อนถั่วและระดับความรุนแรงการเข้าทำลาย โดยให้คะแนนเป็น 5 ระดับคือ 1-5 คะแนน ปลูก ภายใต้สภาพแปลงปลูกในโรงเรื อนตาข่ายปิด พบว่า ลูกผสมชั่วที่ 1 เกือบทั้งหมด ต้านทานต่อการเข้าทำลายของเพลี้ยอ่อนถั่ว ส่วนลู กผสมชั่วที่ 2 มีจำนวนต้นต้านทานต่อเพลี้ยอ่อนถั่ว 177 ต้น อ่อนแอ 63 ต้นมีค่าสอดคล้องกับอัตราส่วน 3:1 เมื่อทดสอบโดยไคสแควร์ แสดงว่า ยีนควบคุมความต้านทานเพลี้ยอ่อนถั่วเป็นยีนคู่เดียว มีการแสดงออกของยีนแบบข่มสมบูรณ์ จากการทดสอบใช้คู่ไพรเมอร์ เครื่องหมายไมโคร แซตเทลไลต์ 11 คู่แยกความแตกต่างระหว่างพันธุ์พ่อและแม่ มีเพียง 5 ไพรเมอร์ คือ VM 31, VM 34, VM 35, VM 37 และVM 78 สามารถแยกความแตกต่างระหว่างพันธุ์ต้านทานและอ่อนแอได้ เมื่อใช้ ไพรเมอร์ดังกล่าวตรวจสอบในชั่ว F_1 และ F_2 พบว่า เครื่องหมาย 220 bp จากไพร์เมอร์ VM 34 มีแนวโน้มว่ามีความใกล้ชิดกับยีนต้านทานเพลี้ยอ่อนถั่ว

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ABSTRACT

The cowpea aphid (*Aphis craccivora* Koch.) is an insect that causes considerable damage to the production of yardlong bean in many countries. Nowadays, chemical insecticides is used to control the cowpea aphid. Breeding for resistance to this insect is important. The objectives of this study were to determine the inheritance of resistance to cowpea aphid and to identify microsatellite markers linked to cowpea aphid resistance. In this study, the cross between a susceptible variety of yardlong bean (Selected-PSU) and a resistant variety of cowpea (IT82E-16) was made to produce F_1 and F_2 progenies. All plants in each generation were screened for aphid resistance in the field using 1-5 damage scores. Four generations, P_1, P_2, F_1 and F_2 were evaluated in a Completely Randomized Design with unequal replications under the screenhouse condition. Five apterous adult cowpea aphids were released on each plant at 4 weeks after planting. Then, the number of aphids and the visual scores damage were analyzed. The distribution of damage score was recorded. The results showed that most of F_1 plants were resistant to cowpea aphids, while F_2 populations segregated 177 resistances to 63 susceptible: fit a 3:1 ratio. Results indicated that there was a single gene controlling resistance in IT82E-16. The molecular microsatellite markers were applied to investigate cowpea aphid resistant gene in the F_2 populations. A selected of 11 primer pairs were first screened and five primers pairs were chosen to analyze genetic resistance variation of 30 F_1 and 237 F_2 individual plants. After the results from microsatellite analysis were studied, we found that markers VM 34-220 bp are most associated to aphid resistant gene in cowpea IT82E-16.

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Somsak Potarot

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Chapter 1

Introduction

Background

Yardlong bean, *Vigna unguiculata* subspecies *sesquipedalis*, is a common vegetable in Asian markets. It originated from central west africa and is now cultivated extensively in many countries in southeast Asia such as Taiwan, Philippines, Indonesia and Thailand. This crop is also widely grown in Southern China and Southern Asia (India, Pakistan and Bangladesh) (Bounnhong, 1997). In Thailand, production area of yardlong bean was estimated at 18,560-20,160 ha annually (Sarutayophat *et al*., 2007). The major constraint to production of the crop is severe infestation and damages caused by insect pests in the field. Yield reduction caused by insects can reach as high as 95%, depending on location, year and cultivars (Carlos, 2000). Cowpea aphid (*Aphis craccivora* Koch.) is a serious pest of yardlong bean and cowpea in most growing area (Quan, 1996; Singh and Jackai, 1985). This insect is a major constraint to cowpea and yardlong bean production. The cowpea aphid feeding by sucking terminal shoots, flowers and pods. Several aphid infestations can cause leaf distortion, stunning and reduced pod set in the plant. Cowpea aphid caused an estimated 40% yield losses (Jayappa and Lingappa, 1988). In extreme cases, the infested plant dies. Cowpea aphids are also capable of transmitting a large of virus diseases (Atiri *et al*., 1984).The use of resistance cultivar is the cheapest and most affective ways to control insect pests in the production area.

Many cowpea accessions from the cowpea germplasm at the International Institute of Tropical Agriculture have been identified as resistance to *A. craccivora* Koch. Aphid resistance in cowpea is inherited as a monogenic dominant trait (Bata *et al.*, 1987; Pathak, 1988). In Thailand, Benchasri *et al.* (2007) evaluated 24 yardlong bean and cowpea genotypes for cowpea aphid resistance, and they reported that the cowpea IT82E-16 displayed a high level of resistance. This finding offers the promising for development of aphid resistance in yardlong bean cultivars.

Identification gene or markers linked to the gene of interest making it increasingly possible to detect genetic differences for traits among individual plants, there by assisting in the selection of desired traits. The molecular markers can then be used to assist breeder track that specific gene. Several molecular marker techniques have been used such as RFLP (restriction fragments length polymorphism), AFLP (amplified fragments length polymorphism), RAPD (random amplified polymorphic DNA) and Microsatellite. Microsatellite or simple sequence repeat (SSR) is tandem repeats of one to five base pair repeated units (Hayden and Sharp, 2001). The advantage of microsatellite marker is co-dominant markers; it can be separated between homozygous and heterozygous. Variations in the number of repeats can be detected by polymerase chain reaction (PCR) technique. Microsatellite markers have been used in several leguminous plants such as common bean (Yu *et al*., 2000), soybean (Mian *et al*., 2008) and cowpea (Li *et al*., 2001).The purpose of study were to confirm single gene segregation of cowpea aphid resistance in IT82E-16 and identify microsatellite markers related to cowpea aphid resistant gene.

Literature Review

1. Importance of *Vigna*

The genus *Vigna* is a tropical crop and comprises approximately 150 species, most of which are found in Africa and Asia (Verdcourt, 1970). According to Jain and Mehra (1978) among *Vigna* species, *V*. *radiata*, *V. mungo*, *V. aconitifolia* and *V. umbellate* are believed to have originate in the Indian center of origin and *V. unguiculata* in Africa. The economic *Vigna* species can be grown in extreme environments such as high temperatures, low rain fall, and poor soils. And all of *vigna* produce inexpensive protein in several edible products (tender green shoots, leave, mature pods, green pod and dry seed). Many of these species are also valued as fodder for animal, cover crop and green manure crops (Fery, 2002). One of the most importances in Asia is *vigna unguiculata*. It widely used to produce multiple edible products.

2. Taxonomy and morphology of cowpea and yardlong bean

2.1 Taxonomy

Yardlong bean and cowpea belongs to *Vigna unguiculata*, the Leguminosae family. It is one of several species that widely grown in many regions of the world. Three cultivated subspecies of *Vigna unguiculata* are as following: cowpea (*V. unguiculata* subspecies *unguiculata, formerly V. sinensis* (L) Savi ex Hassk), Catjang (*V. unguiculata* subspecies *cylindrical or catjang*), which is characterized by small erect pods and yardlong bean (*V. unguiculata* subspecies *sesquipedalis*, formerly *V. sesquipedalis* (L) Fruw (Verdcourt, 1970). However, Ehlers and Hall (1997) did not consider Verdcourt three cultivated subspecies as being distinct, but considered the subspecies *unguiculata* and *sesquipedalis* as cultigroups of cowpea, recognized as *unguiculata*. All the subspecies have the same chromosome number, 2n = 2x = 22 (Ehlers and Hall, 1997). Yardlong bean is also known as asparagus bean, snake bean, string bean (Purseglove, 1974). The origin of yardlong bean is possibly in the middle

west of Africa or in the northeastern part of Yunnan province in Southern China (Purseglove, 1974; Quan, 1996). Yardlong bean exhibits a wide range of growing habits (Ehlers and Hall, 1997). It is short day plants with many accessions that are photoperiod sensitive (Ehlers and Hall, 1997). Cowpea is believed of originate in Africa and India (Menendes *et al.,* 1997). Cowpea is also known as black-eyed peas, cowpea, crowder pea, southern pea, field pea, china pea and Indian pea (Roy and Guste, 2001). It is an importance grain legume widely cultivated in the tropics and subtropics. Cowpea serves various purposes such as it is the main sources of protein in Africa (IITA, 1984). Young green pods are eaten as vegetable and also as cover crop in the area with poor soil (Bittenbender *et al.,* 1984; Okigbo, 1978). One of the most reasonable things about cowpea is its tolerate to dry environment (Van Rij, 1999).

2.2 Morphological characteristics

Yardlong bean and cowpea are an annual plant that requires more sunshine during its growth and the roots are vigorous taproot system. Yardlong bean has a strong climbing vine, twinning counterclockwise. It has a height of 2-4 m. Cowpea has determinate, semi-determinate; prostrate (trailing). Leaves, both yardlong bean and cowpea are trifoliate leaf, which variation 7-12 cm long, shape can be ovate or lanceolate and they are usually dark green, but cowpea leaves have wide range of shapes, with long terminal leaflets. Flowers of yardlong bean are white or yellowish color with length 2-2.5 cm, with usually two flowers per inflorescence. But Flowers of cowpea is purple, with in multiple racemes on flower. The style and stigma are surrounded by anthers tightly enclosed in a straight keel, open in the early day and close at approximately mid day after blooming. The cowpea and yardlong bean are a self-pollinated crop and easily crossed (Phansak *et al*., 2005). Out crossing rates as high as 5% have been recorded. (Singh and Jackai, 1985; Ehlers and Hall, 1997). Pods of yardlong bean vary in size, 30-120 cm in length. Pod of cowpea can be smooth, cylindrical and general curved, but only 7.5 to 45 cm long, pod color and texture, they may be green and yellow when ripe (Fery, 2002). Seed is large with 8-12 mm in length of yardlong bean and cowpea is 4-8 mm in length, color including white, red, brown and black. It is exhibition vigorous growth in warm climate. Optimum average temperature

during the growing growth period is 20°C to 30°C (Santipracha and Santipracha, 1994). It can be grown in various soil types, from sandy loam to clay, but loam and sandy loam with pH 6.2-7 are the best for yardlong bean production (Bounnhong, 1997).

3. Cowpea aphid

The cowpea aphid (*Aphis craccivora* Koch.) (Figure 1) belongs to the order Hemiptera, Aphididae family (Dixon, 1985). The characteristic of *Aphis craccivora* is a small soft-bodieds, being 1.5 to 2 mm long. In Thailand, it has been identified as one of the major pests for yardlong bean production. The body of cowpea aphid is generally pear-shaped and divided into head, thorax and abdomen. The leg and antennae are slender and hind end of the abdomen bears a pair of tubular wax glands, the cornicles are black (Ofuya, 1997). Adult may be wangle (alate) or non-winged (apaterous). In the tropic, aphids reproduce without mating and colonies consist entirely of females (Schreiner, 2000). A female in general produce 100 nymphs in 30 days. The characteristic of nymph is that they look like adult, which are smaller and yellow or brown. The length of nymphs is around 0.5 mm. The leg and antennae are usually white and cornicles are usually black. The nymph stage is about 5-7 days, 4 times for molting. Adult length is around 1 mm and the adult stage has for a period of 11days (Miyazaki, 1997).

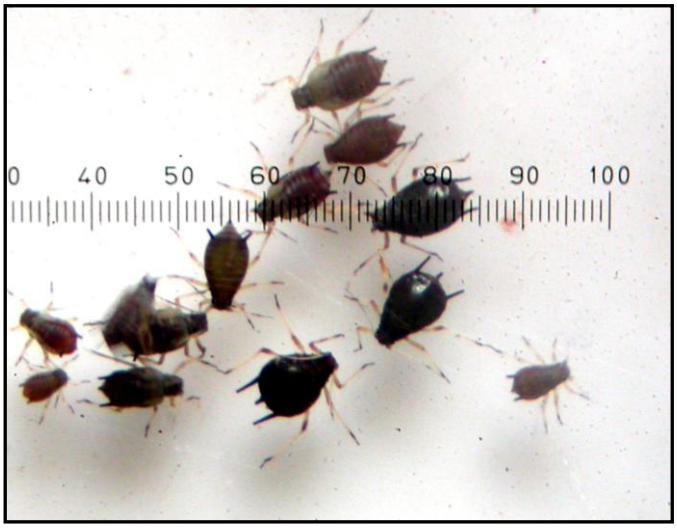

Figure 1 Morphology of cowpea aphid (*Aphis craccivora* Koch.)

3.1 Feeding damage

Aphis craccivora Koch. is the main pest on yardlong bean and cowpea. Adult and nymphs of cowpea aphid feed by suckling fruit from stem, terminal shoot, flowers and pods (Ofuya, 1997). Immediately after infestation, aphids start sucking the juice from cowpea leaves, stem, flowers and pods of the plant reducing their growth and development and causing severe reduction in yield (Singh, 2009). In most cases the cowpea aphid infestation in pre-flowering stage, can caused severe damage and yield can loss up to 40% in the field condition (Jayappa and Lingappa, 1988). Other effect, the growth of black sooty mold fungus are aphid honeydew that sticks on the surface of leaves, where turns the leaves black that effects photosynthetic activity of plant (Ofuya, 1997). During the feeding process, adults and nymphs not only causing severe damage on yardlong bean plant, but they are also through the vectoring of cowpea virus such as Cowpea Aphid-Born Mosaic Virus (CABMV), Cowpea Mosaic Viruses (CMV), Broad Bean Leaf Roll Viruses (BBLRV) and Bean Yellow Mosaic Viruses (BYMV) (Atiri *et al*., 1984). It can cause severe damage and yield can loss up to 80% in the field condition (Bashir *et al.*, 1989). In Thailand, the application of chemical insecticide is the most common method for controlling *Aphis crccivora* Koch. Use of the insecticides poses major health, environmental and economic problems. However, the nature enemies may provide biological control, such as the *coccinelidae beetl*, *Menochillus sexmaculatus* Fabr., but *A*. *craccivora* populations in the fields are very high (Schreiner, 2000). Developing cowpeas with resistance to insect pests will not be easy but probably can be done. To use of resistant varieties to reduces the application of insecticides and increases the number of nature enemies on the field need to be done. Several methods, they used for breeding such as host plant resistance or mutations to resistance (Dogimont *et al.*, 2010). Some accessions of the cowpea appear to have strong resistance to cowpea aphid. First step for breeding resistant to cowpea aphid can be performed by screen accessions from a germplasm collection for cowpea aphid resistance. Second, attempts should be made to cross cowpea aphid resistance line with commercial varieties, to produce F_1 seeds. Third step, selfing of the F_1 hybrid to produces F_2 seeds and the greatest range of variability can be seen in the F_2 generation where selection begin.

4. Host plant resistance

Mechanism of resistance is the plant ability to live, grow when destroyed by insect. Mechanism of resistance is classified into three mechanism including antixenosis (non-preference), antibiosis and tolerance (Painter, 1968).

4.1 Antixenosis: antixenosis is also known as non-preference and is defined as the plant ability to affects the behavior of insect caused by chemical and physical factor such as tissue thickened, waxy on leaves or stem, leaf size and width. Webter *et al*. (1994) noticed that high levels of leaf pubescence in wheat are resistant to the yellow sugarcane aphid. PI37739 and PI 225245 varieties in wheat were antixenotic to Russian wheat aphid based on the host choice test, aphid probing behavior and examination of leaf surface structure (Ni and Quisenberry, 1997). Islam and Karim (1997) indicated that longer and tougher leaf blades might contribute to resistance in rice. It appeared that rice leaf blade morphology, i.e. width length and toughness may play a vital role in resistant against rice leaf-folder. In cowpea, Joseph and Peter (2007) reported that non-preference mechanism of aphid resistance was evidenced, for example, densely pubescent and trichome at the shoot tips were presented in resistance lines. The resistant line also had more reducing sugars than susceptible lines.

4.2 Antibiosis: antibiosis is a direct effect on insect life cycle which refers to affect their biotic potential such as chemical or morphological factors. Some toxins and inhibiters level of essential nutrients was decreases that result in an insect weight, reduced metabolic and death. Annan *et al.* (1996) reported that antibiosis was governing modality of aphid resistance in cowpea ICV-12. Cowpea variety IT835-720-20 had adverse effect on reproductive performance of *A. craccivora*, indicating some level of host plant resistance (Obopile and Ositite, 2010).

4.3 Tolerance: tolerance is defined as the plant ability or the ability to withstand attack or recover from damage without appreciable loss of vigor, growth, or crop yield. However, this resistance is usually overlapped between antixenisis and antibiosis.

5. Genetic resistance to cowpea aphid

There are several studies on genetic resistance to cowpea aphid in yardlong bean and cowpea. Pathak (1988) studied the genetic resistance to cowpea aphid and reported that the cowpea aphid resistance was conferred by a single dominant gene, designated as *Rac*$_1$ and *Rac*$_2$. These two genes are non-allelic. Ombakho *et al.,* (1987) studied in F_1 and F_2 generation of cowpea (TVU310, ICV10, ICV11) and reported that resistant gene in TVU310 and ICV10 is designated as *Ac*$_1$, while resistant gene in ICV11 is *Ac*$_2$. Ofuya (1995) evaluated 12 cowpea varieties (TVu 36, TVu 408, TVu 801, TVu2876, TVu 3000, TVu 36, TVu 9930, TVu 9944, IT84S-2246-4, Ife Brown, Vita 7, IT84S-2231-15 and found that only IT84S-2246-4 resistant to cowpea aphid. Myers *et al.* (1996) identified IT84S-2246-4 cowpea line as a resistant variety to cowpea aphid and NI936 as susceptible cultivar. Systematic genetic studies indicated that the cowpea aphid resistance gene (*Rac1)* located in chromosome 1 that linked with one RFLP marker (*bg*4D9b). Githiri *et al.* (1996) studied inheritance of aphid resistance and allelic relationships among sources of resistance in 8 cowpea genotypes (ICV 10, ICV 11, ICV 12, IT82E-25, TVU 310, IT87S-1394, IT67S-1459 and IT 84S-2246) and one susceptible variety, TVU946. Five populations included F_1, F_2, F_3, backcrosses (BC_1 and BC_2) of crosses were investigated. The segregation data in F_2 and BC_2 from those eight crosses between resistant and susceptible cultivars indicated that the ratio of aphid resistant and susceptible progenies were fit 3:1 and 1:1 ratios, respectively. In Thailand, Benchasri *et al.* (2007) evaluated 24 yardlong bean and cowpea genotypes for cowpea aphid resistance, and they reported that the cowpea IT82E-16 displayed a high level of resistance. Benchasri and Nualsri (2009) also studies the genetic inheritance of cowpea aphid resistant gene by crossing among yardlong bean (Selected-PSU) and cowpea (IT82E-16). They reported that resistance to cowpea aphid is controlled by dominant gene.

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6. Molecular markers in plant breeding

Nowadays, plant breeders can directly employ molecular markers closely linked to insect resistance gene in breeding program. The interaction between plants and insect has been used in different molecular marker techniques. In the application, it includes the analysis of segregation population, selection for resistance to pest, gene tagging, gene mapping and located insect resistance genes. Molecular markers have been developed and measuring genetic analysis, they are including Restriction Fragment Length Polymorphism: RFLP (Myers *et al*., 1996), Random Amplified Polymorphic DNA: RAPD (Ogundiwin *et al*., 2005), Amplification Fragment Length polymorphic: AFLP (Ourdraogo *et al*., 2001), Cleaved Amplified Polymorphic Sequence: CAPs and Microsatellite or Simple Sequence Repeats (SSR) (Li *et al.,* 2001) etc.

6.1 Microsatellite markers

Microsatellites or simple sequence repeats (SSR) are tandem repeats consisting of one to five base pair repeated units that are dispersal throughout eukaryotic genomes (Hayden and Sharp, 2001). Variations in the number of repeats can be detected by polymerase chain reaction (PCR). Microsatellite marker has been used in different plant species such as soybean, barley, wheat, rice and cowpea. Advantages of microsatellite marker are: highly polymorphism between individuals within populations, co-dominant marker that can be separated between homozygous and heterozygous and easily to perform. Moreover, microsatellite marker has been recognized as useful molecular markers in marker-assisted selection (MAS) in many traits of various plant species. Liu *et al.* (2001) identified microsatellite markers closely linked to the russian wheat aphid resistance gene in wheat and reported that GWM111 (Xgwn111) is tightly linked to *Dn1* and *Dn5* gene. Liu *et al.* (2002) studied in wheat and reported two microsatellite markers Xgwn106 and Xgwn337 are linked in coupling with chromosome 1D at 7.4 cM and 12.9 cM and Xgwn 44 and Xgwn11 are linked to *Dn6* with independent of *Dn1*, *Dn2* and *Dn5.* In rice, Sun *et al.* (2006) crossed two rice varieties namely ASD7 and C418 to selected gene *bph 2* that linked to brown plant hopper in 134 F_2 individual. From this result, RM7102 and RM 463 located in

chromosome 12 linked to brown plant hopper at the distance 7.6 cM and 7.2 cM repectively. In soybean, Mian *et al.* (2008) crossed between PI 243540 (resistant) and Wyandot (susceptible) to produce F_2 to look for fine microsatellite marker linked to aphid resistance gene from PI 243540 and reported that resistance in PI 243540 is positioned between SSR markers satt334 and Sct_033 linkage group F, namely *Rac2* . In cowpea, Li *et al.* (2001) constructed two microsatellite-enriched libraries of cowpea and 46 microsatellite primer pairs were used to investigate genetic relationship among 90 accessions of cowpeas. They reported 27 primer pairs amplified clear polymorphic bands with 2-7 alleles/primer. Preliminary study of microsatellite markers linked to cowpea resistance gene was carried out by Benchasri (2009). Crossing between Selected-PSU and IT82E-16 was made and some SSR primer pairs from Li *et al*. (2001) were screened. He reported that SSR marker 1080 base pair from primer VM 37 is probably associated with cowpea aphid resistant gene in yardlong bean and cowpea. However, F_2 population in his experiment was too small.

Objectives

The objectives of the present study are:

1. To determine the inheritance of cowpea aphid resistance
2. To identify microsatellite markers closely linked to the cowpea aphid resistance gene in cowpea IT82E-16

Chapter 2

Materials and Methods

Materials

1. Plant materials

Two varieties of yardlong bean (Selected-PSU) and cowpea (IT82E-16) were use as susceptible and resistant varieties, respectively.

2. Laboratory materials

2.1 Chemicals

2.1.1 Chemicals for DNA extraction

- CTAB (Hexadecyl Trimethyl-Ammonium Bromide)
- β- mercaptoethanol
- Polyvinyl pyrrolidon (PVP-40)
- Sodium chloride (NaCl)
- Disodium ethylene diaminetetraacetate (Na_2EDTA)
- Potassium acetate (KA_c)
- Tris-HCl pH 8.0
- Chloroform
- Isopropanol
- TE buffer
- Ethanol

2.1.2 Chemicals for electrophoresis

Agarose gel electrophoresis

- LE agarose (FMC Bioproduct, USA)
- Seakem agarose (FMC Bioproduct, USA)
- Glacial acetic acid
- Boric acid
- Tris-base
- Ethidium bromide
- Loading buffer
- Lamda DNA (λ DNA)
- 100 bp DNA Ladder (Operon, USA)

Denaturing polyacrylamide gel electrophoresis

- Acrylamide : bis-acrylamide solution (29:1)
- Bind silane
- Repel silane
- Formamind
- Formaldehyde
- Urea
- TEMED (N,N,N',N'-tetramethyethyenediamine)
- Ammonium per sulfate
- Sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$)
- Sodium carbonate (Na_2CO_3)
- Silver nitrate ($AgNO_3$)

2.1.3 Chemicals for PCR

- dNTP (dATP, dTTP, dCTP and dGTP) (Promega, USA)
- Primer pairs for Microsatellite (VM 5, VM 9, VM 10, VM 22, VM 21, VM 34, VM 35, VM 36, VM 37, VM 75 and VM 78)
- dH$_2$O
- 10X *Taq* buffer (Promega, USA)
- *Taq* DNA Polymerase B (Promega, USA)

3. Agricultural materials

3.1 Field materials

- Pots (12 cm in diameter)
- Fertilizer
- Plastic cover conversion
- Water drops
- Hoe
- Bamboo stake
- Nets 22 mesh
- Forceps
- Paper bags
- Clip
- Tag
- 70% Alcohol

3.2 Laboratory equipments

- Freezer -30^{o}C
- Microcentrifuge

- Electrophoresis equipment
- Vortex mixer
- Autoclave
- PCR Machine (DNA Thermal Cycler)
- Mortar and pestle
- Microwave
- Gel documentation

Methods

1. Hybridization

Crossing between Selected-PSU and IT82E-16, was made by hand. Crossing scheme is shown in Figure 2. Hands and forceps were sterilized with 70% alcohol. Emasculation was performed daily between 16.00 and 18.30. Stamens from female flowers were removed and bagged to prevent the contamination by insects. In the morning between 7.00-8.00 am., pollen was collected from male flowers and the hand pollination was made. Pollens from male plant were taken with paintbrush and smeared on emasculated flower's stigma, to produce F_1 seeds. About 21 days after pollination pods were collected and seed were removed from pod. F_1 seed were grown in the field and self pollination was made to produce F_2.

X

Selected-PSU
(*V. unguiculata* subspecies *sesquipedalis*)
(susceptible parent)

IT82E-16
(*V. unguiculata* supspecies *sinensis*)
(resistant parent)

F_1 (Selected-PSU x IT82E-16)

⊗

F_2 (Selected-PSU x IT82E-16)

Figure 2 Crossing scheme between Selected-PSU (*V. unguiculata* subspecies *sesquipedalis*) and IT82E-16 to study the inheritance of resistance to cowpea aphid

2. Cowpea aphid population

Cowpea aphid population used for evaluation in this study was collected from a yardlong bean field at Prince of Songkla University and maintained on susceptible plants in a small screenhouse to increase their population.

3. Screening populations for cowpea aphid resistance

Four generations of cross between Selected-PSU x IT82E-16 (P_1, P_2, F_1 and F_2) were screened for cowpea aphid resistance in the screenhouse in the field at Faculty of Natural Resources, Prince of Songkla University, Hat Yai Campus. The experiment was arranged in a Completely Randomized Design (CRD) with unequal replications. Each parental line and F_1 were planted in 3 replications while F_2 plants were planted in 24 replications, 10 plants/plot/replication. Plants were grown in 11 rows (30 plants per row), with spacings 0.5 m within row and 0.75 m. between rows. Five adult aphids were released in second trifoliate stage on each plant at 30 days after seed emergence. Regular watering and weed cleaning were done as necessary and fertilizer was applied twice a month.

Figure 3 Nursery for screening aphid resistance under screenhouse condition

4. Assessment of cowpea aphid damage

Damage of individual plants was scored for aphid resistance and the number of aphids was recorded every week after artificial infestation. Visual damage scores were assessed for each generation at five levels based on the scales suggested by Smith *et al.* (1994) (Table 1). Grouping of infestation index into resistance to aphids are identified based on score ≤ 3 for resistant to aphids and 4-5 score for susceptibility.

Table 1 Scores of the symptom from on leaves and flower buds on each plant

Score	Damage Descriptions	level of Resistance
1	visual damage on leaves and flower buds < 10%	R
2	visual damage on leaves and flower buds 11-25%	R
3	visual damage on leaves and flower buds 26-50%	R
4	visual damage on leaves and flower buds 51-75%	S
5	visual damage on leaves and flower buds 76-100%	S

Note R: resistance; S: susceptible

5. Data collection

- Number of aphids on individual plants from 1st week to 4th week after artificial infestation.
- Visual score damages on each plant were made 1st week to 4th week after artificial infestation.

6. Data analysis

The segregation ratio of resistance to susceptible was analysis by chi-square. Chi-square (χ^2) to test for the phenotypic ratio was calculated by the following formula,

$$\chi^2 = \frac{(O - E)^2}{E}$$

Where O = on observed value, E = an expected value. Chi-square value was considered significant ($P \leq 0.05$) if it was greater than 3.84.

7. Microsatellite analysis

7.1 DNA extraction

Young leaves of F_2 progeny were freshly harvested from 240 individual plants, along with both parents and their F_1 hybrid. DNA was extracted from approximately 200 mg of young leaf with CTAB extraction buffer [2% CTAB, PVP-40, NaCl, Na_2EDTA 0.5 M, pH 8.0, 2% B-mercaptoethanal] modified from Doyle and Doyle (1990). DNA with extraction buffer were then incubated at 60^{o}C for 1 hour and equal volume of chloroform: isoamyl alcohol (24:1) was added. The tubes were inverted repeatedly and centrifuged at 10,000 rpm for 20 min. The upper layer was transferred into a new centrifuge tube and 750 µl of isopropanal was added to precipitate DNA. The DNA was washed with 70% ethanol and air-dried. After complete drying, 10 of TE buffer [10nM Tris-HCl, pH 8] was added to dissolve DNA. DNA was then stored at -20^{o}C until use. DNA quantity was estimated by spectrophotometry and comparing the intensity of ethidium bromide-stained DNA bands on 0.8% agarose gel with those of known concentration for use in PCR analysis.

7.2 PCR amplification

The PCR reaction was carried out in 10 µl final volumes containing 20 ng of genomic DNA, 10X *Taq* buffer, 0.2 µM each of the forward and reverse primers, 200 µM dNTPs and 0.7 unit of *Taq* polymerase. A total of 11 SSR primers (VM 5, VM 9, VM 10, VM 22, VM 21, VM 35, VM 37, VM 34, VM 36, VM 75 and VM 78) were used for PCRs according to Li *et al*. (2001). Sequences of SSR primer used were shown in Table 2. The reaction was performed by denaturizing step at 94^{o}C for 2 min followed by 94^{o}C for 1 min, 64^{o}C for 30 sec (declining 0.5^{o}C/cycle), and 72^{o}C for 1 min 18 times. These cycles were followed by 30 times at 94^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 1 min and final extension at 72^{o}C for 10 min.

Table 2 The sequences of SSR primers which used to screen the parental lines and their hybrids (Li *et al*., 2001)

Primers	Forward Sequence (5' to 3')	Reverse Sequence (3' to 5')
VM 5	AGC GAC GGC AAC AAC GAT	TTC CCT GCA ACA AAA ATA CA
VM 9	ACC GCA CCC GAT TTA TTT CAT	ATC AGC AGA CAG GCA AGA CCA
VM 24	TCA ACA ACA CCT AGG AGC CAA	ATC GTG ACC TAG TGC CCA CC
VM 31	CGC TCT TCG TTG ATG GTT ATG	GTG TTC TAG AGG GTG TGA TGG TA
VM 34	AGC TCC CCT AAC CTG AAT	TAA CCC AAT AAT AAG ACA CAT A
VM 35	GGT CAA TAG AAT AAT GGA AAG TGT	ATG GCT GAA ATA GGT GTC TGA
VM 36	ACT TTC TGT TTT ACT CGA CAA CTC	GTC GCT GGG GGT GGC TTA TT
VM 37	TGT CCG CGT TCT ATA AAT CAG C	CGA GGA TGA AGT AAC AGA TGA TC
VM 39	GAT GGT TGT AAT GGG AGA GTC	AAA AGG ATG AAA TTA GGA GAG CA
VM 71	TCG TGG CAG AGA ATC AAA GAC AC	TGG GTG GAG GCA AAA ACA AAA C
VM 78	GGA TAC CCA CCG CTA AAC	ACA TCA ATG CCT CCA CAG TAT CT

7.3 Electrophoresis

Polyacrylamide gel electrophoresis was used to separate amplified PCR products in a vertical system descried by Antti and Matti (1993). The glass plates were washed with the detergent and rinsed thoroughly with tap water then was cleaned with 95% ethanol and wiped out with a tower paper two times. The long glass plate was wiped with binding solution (bind saline, 1 ml 95% ethanol, 5 ml acetic acid) in a fume hood. The solution wiped out through using a tissue paper till the binding tissue was dry for 5 min. The shorter plate was also wiped with 500 µl of 5% Dimethyl dichlorosilane and wiped with tower paper. Both plates were clipped and sealed by placing spacer inside. Polyacrylamide gel (6%) containing acryl amine 60 µl, 7 M urea 114 g, 5X TBE buffer, 10% ammonium per sulfate, 1000 µl and 50 µl TEMED Tetra methylethylenediamine was added just before pouring. The gel was then left for solidify solid for two hours and pre-run at 50 Watts for 30 min.

7.4 Denaturing polyacrylamide gel electrophoresis

Ten microliter of each sample with 2 µl of 2X loading solution were added to each PCR sample. The samples were denatured by heating at 95 ^{o}C for 5 min and immediately chilled on ice. Each sample (5 µl) was loaded into the respective well. DNA ladder was used as marker. The gel was run at 50 W for 150 min. After finished, the gel was covered with 10% acetic acid for 20 min and rinsed 2 times in deionizer water. The gel was immersed in staining solution (2 g silver nitrate dissolved in 2 liters of deionizer water) for 30 min. The gel was then washed with deionizer water for 5 seconds and was immediately placed in 1 liter of cold developing solution (25 g sodium carbonate dissolved in 2 liter of deionizer water: 500 µl of 37% formaldehyde and 50 µl of 1% sodium thiosulphate was just added before use). The gel was agitated until the bands started to appear. Development of gel was stopped by 1 liter of fixative solution. The gel was then air-dried and DNA profiles were scanned using scanner.

7.5 Microsatellite data analysis

Genotype: phenotype associations were established by sorting the rating score for resistance to cowpea aphid. The allele size of microsatellite primer from each plant were then determined for each microsatellite marker. The cowpea aphid resistance associated microsatellite markers were tested by linear regression analysis.

Chapter 3

Results

1. Evaluation for resistance to cowpea aphid

1.1 Number of cowpea aphid population

Cowpea aphid damaged of yardlong bean and cowpea by sucking fluid from stem and terminal shoots, flowers and pods. The data for the number of cowpea aphid of adult and nymphs were recorded every week under the screenhouse condition. In the first two weeks, the number of cowpea aphids remained the same as it was first deposited. Three weeks after the artificial infestation, the number of cowpea aphids in all crop populations increased rapidly. The number of observed on F_1 (Selected-PSU x IT82E-16) almost the same as on their resistant parent, IT82E-16 (3,206 and 3,115 respectively) and the highest number was recorded on F_2 (5,306) followed by that on Selected-PSU (4,496). The data was shown in Figure 4.

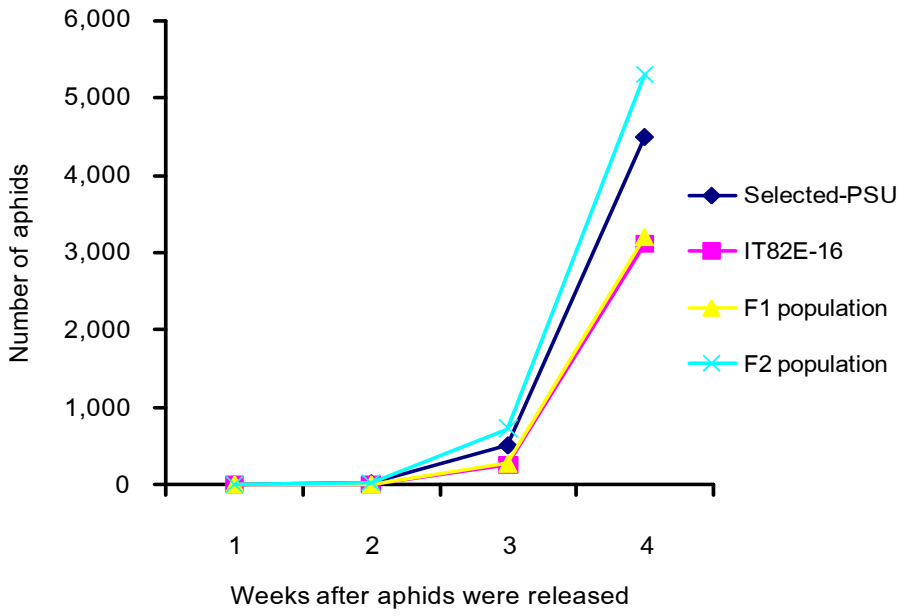

Figure 4 Average aphid number recorded weekly on F_1, F_2 and their parents of yardlong bean x cowpea cross

1.2 Damage score

To assess the resistance to cowpea aphid, three weeks after 5 adults were deposited on each plant; damages were recorded with a 1-5 score. Data regarding damage scores in F_1 and F_2 were given in Figure 5. It was found that all 30 plants of Selected-PSU were susceptible (rating score=5) and rating score for IT82E-16 was 2. Almost all F_1 plants appeared to be resistant to cowpea aphid (29 from 30 plants showed resistance with the rating score of 2-3). The individual F_2 progenies segregated for cowpea aphid resistance. The scores were ranging from a 2 to 5. The results of F_2 individuals scored for cowpea aphid resistance are shown in Table 3. The phenotypic data for F_2 were tested by using chi-square for the hypothesis of 3:1 resistant to susceptible ratio. The total 240 F_2 individuals were classified into two categories: 177 resistances (R) and 63 susceptible (S). The probability for the expected 3 resistant: 1 susceptible segregation in F_2 was not significant. The result indicated that the F_2 segregation in the ratio of 3:1 which fit the inheritance model of a single dominant gene. Thus the cowpea aphid resistance to IT82E-16 is controlled by a single dominant gene.

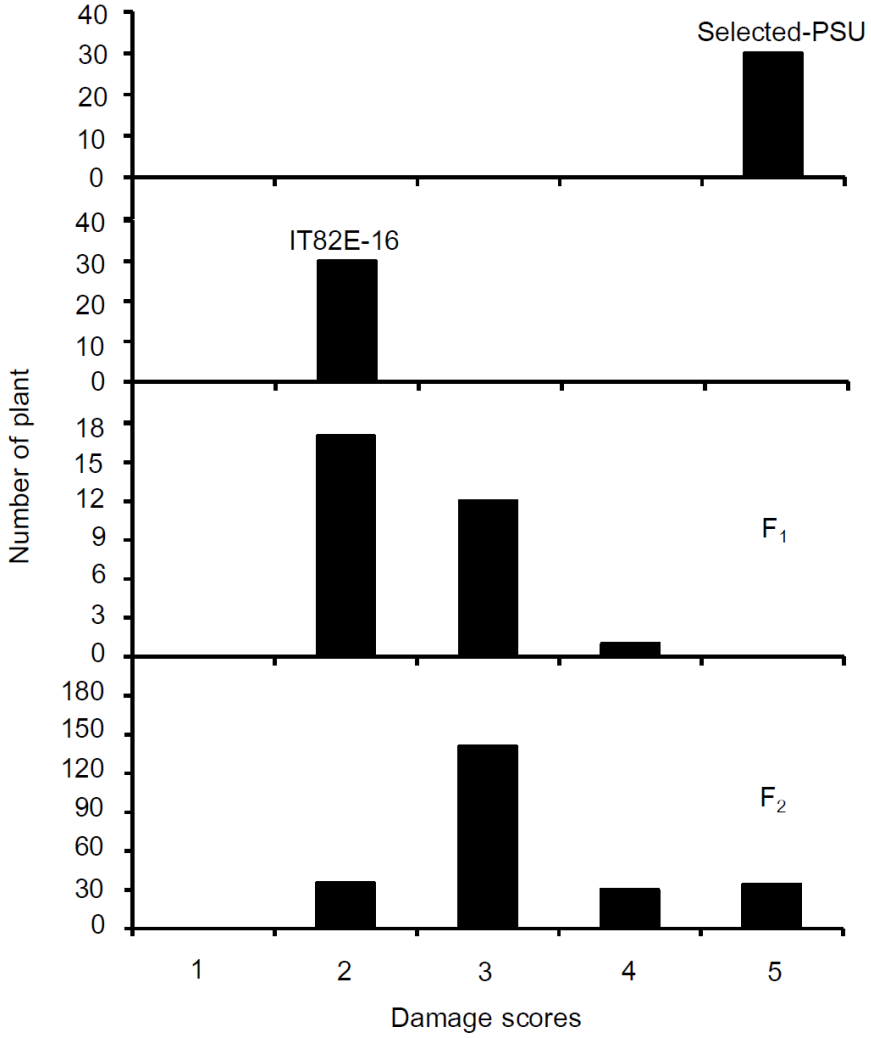

Figure 5 The frequency distribution of severity score for damage caused by insect pest in F_1 and F_2 populations of Selected-PSU x IT82E-16 cross

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Table 3 Phenotypic segregation for responses of cross between Selected-PSU x IT82E-16 to cowpea aphid resistance in parents, F_1 and F_2 populations evaluated by Chi-squared test to fit a 3:1 single gene model

Population	No. of plants	Frequency Observed		χ^2	P
		R	S		
Selected-PSU	30	0	30	-	-
IT82E-16	30	30	0	-	-
F_1(Selected-PSU x IT82E16)	30	29	1	-	-
F_2(Selected-PSU x IT82E16)	240	177	63	0.200	0.655

2. Screening aphid resistance in cowpea and yardlong bean using microsatellite markers

2.1 Identification of DNA marker linked to cowpea aphid resistant gene

Microsatellite was used to identify marker linked to cowpea aphid resistant gene in IT82E-16. Total of 11 microsatellite primers pairs based on an experiment of Li *et al*. (2001) were screened for polymorphism between parental lines and their F_1 and F_2 progenies. From 11 primers used, five primer pairs (VM 31, VM 34, VM 35, VM 37 and VM 78) (Table 6) were able to detect polymorphism between Selected-PSU and IT82E-16. These primer pairs were then used for further investigation in 30 F_1 and 237 F_2 populations of cross Selected-PSU x IT82E-16**.** Based on the five primers which gave polymorphism, one to three alleles was observed. The result concerning the polymorphic fragment found in particularly IT82E-16 (resistant) and segregating for resistance in F_2 generation are shown in Table 4. The VM 31, VM 34, VM 35, VM 37 and VM 78 markers highlighted polymorphic markers 200, 220, 450, 1020 and 460 bp, respectively, at IT82E-16 and not present in Selected-PSU. The result is shown in Figure 6-10.

Table 4 Distribution of cowpea aphid resistant score by genotype of VM 31, VM 34, VM 35, VM 37 and VM 78 in F_2 population derived from cross Selected-PSU and IT82E-16

Primers	Specific fragment found in resistant parent (bp)	Number of plant genotype		
		Homozygous resistant	Segregating heterozygous	Homozygous susceptible
VM 31	200	74	103	60
VM 34	220	112	76	49
VM 35	450	74	103	60
VM 37	1020	53	137	47
VM 78	460	58	119	60

2.2 Microsatellite markers associated with cowpea aphid resistant gene

The segregation of genotypes from five primer pairs (VM 31, VM 34, VM 35, VM 37 and VM 78) generated resistance and susceptible. The association between segregating markers in the F_2 population and phenotypic trait in cowpea aphid resistance was tested by linear regression analysis. In this study, the result showed that only VM 34 primer pairs revealed significantly relation (Table 5).

Table 5 Correlation values (*r*) and *p*-value between cowpea aphid resistant and microsatellite markers in F_2 individuals

Primers	No. of plants	*r*	*p*-value
VM 31	237	0.0533	0.7167
VM 34	237	0.1610	0.0462*
VM 35	237	0.1323	0.1265
VM 37	237	0.0533	0.9964
VM 78	237	0.0256	0.9292

* = significant differences at P = 0.05

Table 6 Sequences of microsatellite primers selected to test in the present

Primers	Forward Sequence (5' to 3')	Reverse Sequence (3' to 5')
VM 31	CGC TCT TCG TTG ATG GTT ATG	GTG TTC TAG AGG GTG TGA TGG TA
VM 34	AGC TCC CCT AAC CTG AAT	TAA CCC AAT AAT AAG ACA CAT A
VM 35	GGT CAA TAG AAT AAT GGA AAG TGT	ATG GCT GAA ATA GGT GTC TGA
VM 37	TGT CCG CGT TCT ATA AAT CAG C	CGA GGA TGA AGT AAC AGA TGA TC
VM 78	GGA TAC CCA TAC CCG CTA AAC	ACA TCA ATG CCT CCA CAG TAT CT

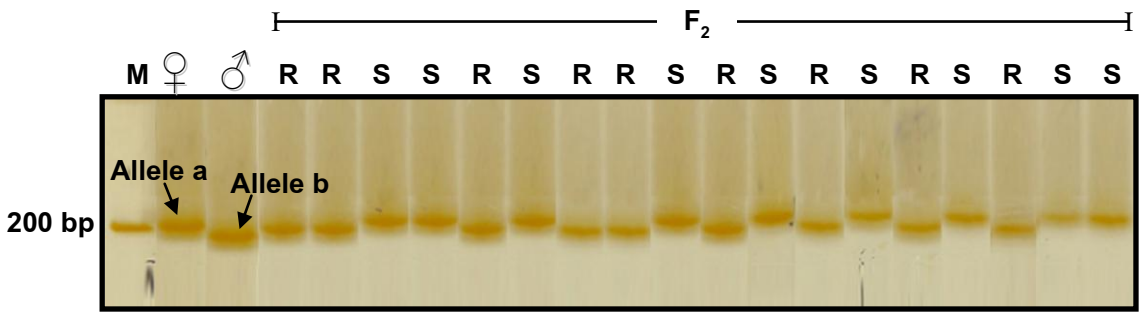

Figure 6 The microsatellite profile, using VM 31 primer pair in some F_2 plants of cross Selected-PSU x IT82E-16. M is 100 bp ladder, ♀ and ♂ are Selected-PSU and IT82E-16, respectively, R and S are resistance and susceptible F_2. Arrow heads (a and b) on the picture indicate the position of polymorphic alleles

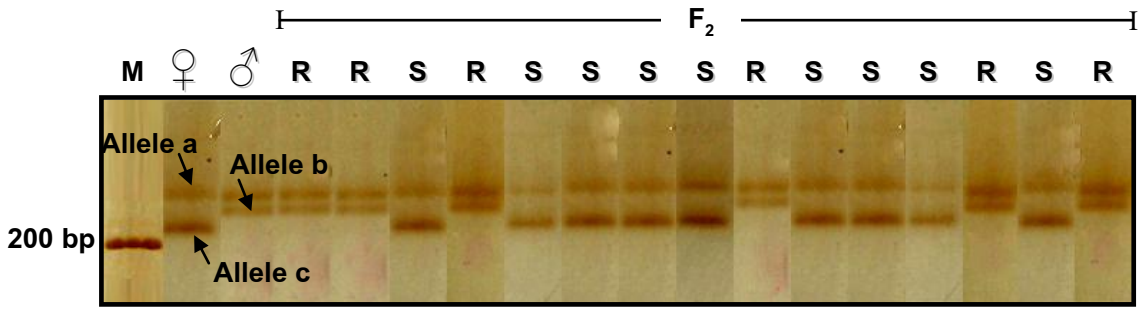

Figure 7 The microsatellite profile, using VM 34 primer pair in some F_2 plant of cross Selected-PSU x IT82E-16. M is 100 bp ladder, ♀ and ♂ are Selected-PSU and IT82E-16, respectively, R and S are resistance and susceptible F_2. Arrow heads (a, b and c) on the picture indicate the position of polymorphic alleles

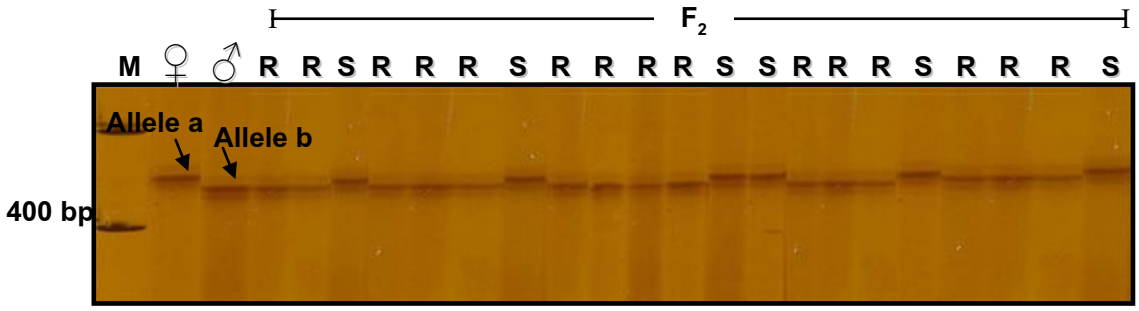

Figure 8 The microsatellite profile, using VM 35 primer pair in some F_2 plant of cross Selected-PSU x IT82E-16. M is 100 bp ladder, ♀ and ♂ are Selected-PSU and IT82E-16, respectively, R and S are resistance and susceptible F_2. Arrow heads (a and b) on the picture indicate the position of polymorphic alleles

Figure 9 The microsatellite profile, using VM 37 primer pair in some F_2 plant of cross Selected-PSU x IT82E-16. M is 100 bp ladder, ♀ and ♂ are Selected-PSU and IT82E-16, respectively, R and S are resistance and susceptible F_2. Arrow heads (a, b and c) on the picture indicate the position of polymorphic alleles

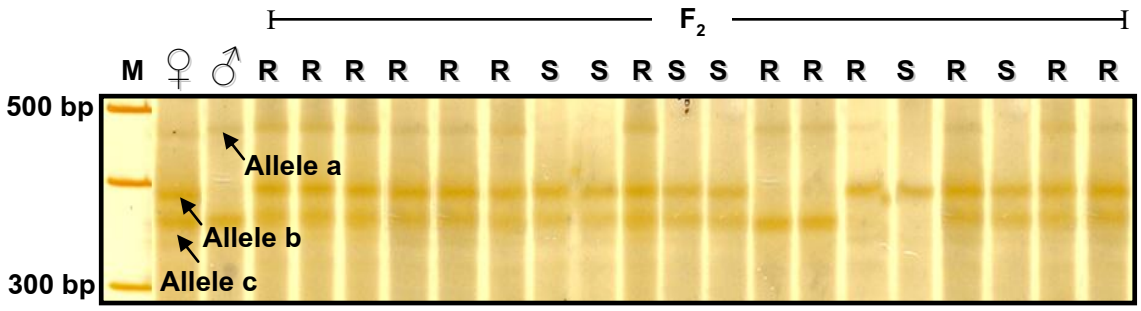

Figure 10 The microsatellite profile, using VM 78 primer pair in some F_2 plant of cross Selected-PSU x IT82E-16. M is 100 bp ladder, ♀ and ♂ are Selected-PSU and IT82E-16, respectively, R and S are resistance and susceptible F_2. Arrow heads (a, b and c) on the picture indicate the position of polymorphic alleles

Chapter 4

Discussion

Cowpea aphid resistant varieties of yardlong bean could be a potential means of pest control in this species. Until now, there has been no report of genetic resistance to cowpea aphid in any yardlong bean varieties in Thailand. Resistant varieties must be developed to provide an environmentally safe mean of controlling aphid that promotes the production of healthy products (Dogimont *et al*., 2010). To achieve that goal, the understanding of plant aphid interaction and the genetic basis of the resistance has to be understood. Crossed between Selected-PSU, a susceptible line and IT82E-16 was made to investigate genetic inheritance of cowpea aphid resistance.

1. Inheritance of cowpea aphid resistant gene in IT82E-16

From the present studies, resistance and susceptibility were classified according to the number of cowpea aphid on each plant and visual damages rating. Initially, five cowpea aphids were released in each plant one month after planting. Three weeks after the artificial infestation, the number of cowpea aphid rapidly increased in all plant populations. The highest number of cowpea aphid population found in F_2 progenies and Selected-PSU. The number of cowpea aphid in F_2 varied from 100-8000. Cowpea aphid started sucking the juice from leaves, flowers and pods resulting in reducing growth and development of plants. It was noted that number of cowpea aphids / plant positively associated with damage rating score level 5. High damage rating indicated by leaf distortion, defoliation and chlorosis. In observation, F_2 plants with narrow leaves displayed higher level of resistance than the wider leaves. Wuttiwong *et al.* (2010) reported cowpea aphids tended to be more attracted to large leaves than to narrow leaves. The same finding was also reported by Laamari *et al*. (2008) who studied in broad bean.

Segregation analysis of the F_2 progenies obtained from Selected-PSU x IT82E-16 cross in the present study indicated a single dominant gene conferred resistance to cowpea aphid in IT82E-16. In our study, the segregation in F_2 population

fit a 3:1 ratio for resistance and susceptibility while all F_1 were scored as resistant except one plant. This finding confirmed the earliest work of Benchasri and Nualsri (2009) who worked with 4 crosses between cowpea and yardlong bean, Selected-PSU x IT82E-16 was included. Bata *et al*. (1987) and Pathak (1988) proposed a symbol of a single dominant resistant gene in cowpea as *Rac1*. In the meantime Ombakho *et al*. (1987) identified a second dominant gene for aphid resistant as *Rac2* and concluded that *Rac1* and *Rac2* genes are not linked. Entomologists have distinguished two mechanisms of resistance that affected insects: antixenoxis which affects the behavior of insects and antibiosis which affects their biotic potential such as growth, development and reproduction (Painter, 1951). Ombakho *et al.* (1987) concluded that at least 3 distinct biotypes (A, B and K) of cowpea aphid may occur in Africa and Asia and they all require different resistant genes. Unfortunately, we have no information of cowpea aphid biotype in this study. Bata *et al.* (1987) reported that gene resistance to cowpea aphid involved antibiosis and is conferred by a single dominant. However, Wuttiwong *et al.* (2010) reported that length and density of leaf pubescent in IT82E-16 affect the number of cowpea aphid indicating that IT82E-16 gave strong antixenosis against the cowpea aphid. Leaf pubescent or leaf shape that mentioned before were included in direct defense mechanism. Direct defense also include repellent effect on attacking aphids (Smith and Clement, 2012). Although IT82E-16 did not showed very strong resistance to cowpea aphid colonization, it appeared to be of some of promising levels of tolerance to cowpea aphid that could be used in breeding program.

2. Microsatellite analysis among cowpea and yardlong bean to cowpea aphid

The high correlation between molecular markers and phenotypic traits is the best tool for breeder to track specific genes. In cowpea, one RFLP marker (*bg4D9b*) was found to be tightly linked with aphid resistant gene (*Rac1*) (Myers *et al.*, 1996). Microsatellite markers are one of molecular markers recommended for marker assisted selection. Barkley *et al*. (2007) have shown that microsatellite can detect more polymorphisms than RFLP. In this study, we looked for microsatellite marker associated with cowpea aphid resistance that can be used in selecting plants with resistant gene. We found that 5 markers (VM 31, VM 34, VM 35, VM 37 and VM 78) could identify

polymorphic fragments between the parental genotypes. For example, primer VM 31 and VM 35 highlighted polymorphic fragment of 200 and 450 bp of the resistant variety IT82E-16 which were not present in Selected-PSU, a susceptible variety. The given scores for each plant in the field were then determined the association between markers presented in resistant genotypes and phenotypes in the field.

Results from microsatellite analysis indicated that 1,020 bp fragment primer from VM 37, 220 bp fragments from VM 34 and 460 bp fragments from VM 78 seem to associate with cowpea aphid resistance in the F_2 generation derived from Selected-PSU x IT82E-16. However, the best result was obtained from VM 34 microsatellite primer. Microsatellite marker from VM 34 (220 bp) showed 74% accuracy predicting cowpea aphid resistant gene. Thus, this fragment could be applied for marker assist selection (MAS) of cowpea aphid resistance in yardlong bean and cowpea. Sun *et al.* (2006) found that microsatellite markers linked with brown plant hopper (BPH) resistance (*bph2*) in rice. He reported that RM 7102 and RM 413 were used to detected gene *bph2* with the average of 89.9% and 91.2%, respectively. Chang-Chao *et al.* (2006) found that the SSR marker RM 403 and RM 5341 linked with gene resistant with to brown plant hopper gene *Bph9* in rice breeding program. Liu *et al*. (2002) found that microsatellite Xgwn106, Xgwm44, Xgwn106, Xgwm337 marker linked to the russian wheat aphid (RWA) resistant gene in wheat. The marker can be used as marker assisted selection for the identification of *DN4* and *DN6* genes. Sutam *et al*. (2008) reported the primer OPG16 generated polymorphism between the two parental lines and the two bulks in peanut population. The susceptible group showed 850 bp DNA fragment and this particular band was absence in the resistance group. The result indicated that the $OPG16_{850}$ marker linked with peanut bean necrosis disease (PBND) which is susceptible cultivar of peanut.

In summary, studies on genetic analysis will be used as the information in breeding program. In this study, the resistance to cowpea aphid in IT82E-16 was controlled by single dominant gene. Therefore, the resistant gene of IT82E-16 can be used as a source to transfer the resistant gene from cowpea to yardlong bean. Based on the present study, the 220 base pair fragment from primer VM 34 is likely linked with aphid resistant gene in IT82E-16 can be benefit to *V. unguiculata* breeding program.

Chapter 5

Conclusion

1. Inheritance of resistance to cowpea aphid (*Aphis craccivora* Koch.) in IT82E-16

The evaluation of cowpea aphid resistance in IT82E-16 was made in F_1 and F_2 obtained from Selected-PSU x IT82E-16 cross. The visual damage scores of F_1 was similar to the resistant parent, IT82E-16 where as the segregation ratio of resistant and susceptible individuals in the F_2 was found to fit the 3:1 ratio. This indicated that the resistance of yardlong bean to cowpea was controlled by one gene pair.

2. Microsatellite markers associated with the cowpea aphid resistance gene in IT82E-16

Among 11 microsatellite markers tested, only 5 primers (VM 31, VM 34, VM 35, VM 37 and VM 78) showed polymorphism between the parental lines, a resistant variety (IT82E-16) susceptible variety (Selected-PSU). All these markers were tested in the F_1 and 237 individual plants of F_2 population and correlation between molecular marker and cowpea aphid resistance was analyzed. Based on correlation analysis, 220 bp from VM 34 was found to link with cowpea aphid resistant gene in IT82E-16.

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Appendix

Chemical for DNA extraction

1) CTAB buffer (Hexadecyl trimethyl-ammonium bromide), 100 ml

PVP-40	1	g
$NaCl_2$	8.12	g
0.5M Na_2EDTA (pH 8.0)	4	ml
1.0M Tris-HCl (pH 8.0)	10	ml
CTAB	2	g

Deionized water was added volume to 100 ml. Add 2 g of CTAB and put on the water bath at 60 $^{\circ}$C for 20 min. Sterilize using an autoclave. Added β-mercaptoethanol 2% in the buffer.

2) TE buffer, 500 ml

1.0 M Tris-HCl (pH 7.5)	500	µl
0.25M Na_2EDTA (pH 7.0)	200	µl

Deionizer water was added to make the final volume to 500 ml and sterilize using an autoclave.

Chemical for denaturing polyacrylamide gel electrophoresis

1) 30% Acrylamide Bis-Acrylamide solution 29:1

2) 5X TBE

Tris Base	216	g
Boric acid	110	g
0.5M Na_2EDTA (pH 8.0)	80	ml

Deionizer water was added to make the final volume to 4 L and sterilize using autoclave.

3) 10% (w/v) Ammonium persulfate (APS)

Ammonium persulfate	1	g

Deionizer water was added to make the final volume to 10 ml and stored at 4°C.

4) 6X gel loading

Formamide	950	µl
5% Bromophenol blue	10	µl

5) Bind silane

Bind silane	1	µl
Glacial acetic acid	2.5	µl
95% Ethanol	500	µl

Chemical for silver staining

1) Fixative and Stop solution (10% Acetic acid)

Glacial acetic acid	100	ml

Deionizer water was added to make the final volume to 1,000 ml

2) 0.2% Silver nitrate

Silver nitrate	2	g

Deionizer water was added to make the final volume to 1,000 ml

3) Develop solution

Sodium carbonates	25	g
40% Formaldehyde	500	µl
Sodium thiosulfate ($Na_2S_2O_3$)	40	µl

Deionizer water was added to make the final volume to 1000 ml and stored at 4^{o}C.

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