IDENTIFICATION AND MAPPING OF A DWARFNESS RELATED GENES IN WATERMELON (*Citrullus lanatus*)

NAMFON CHOMKAEO

DOCTOR OF PHILOSOPHY IN GENETICS

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT

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APPROVED BY   Advisory Committee

Chair ....

(Associate Professor Dr. Saengtong Pongjaroenkit)

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Committee ....

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..................../..................../ ....

Committee ....

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Program Chair, Doctor of Philosophy ....

in Genetics   (Assistant Professor Dr. Yuppayao Kophimai)

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ชื่อเรื่อง   การค้นหาและสร้างแผนที่ของยีนที่สัมพันธ์กับลักษณะต้นเตี้ยในแตงโม
                (*Citrullus lanatus*)
ชื่อผู้เขียน   นางน้ำฝน จอมแก้ว
ชื่อปริญญา   ปรัชญาดุษฎีบัณฑิต สาขาวิชาพันธุศาสตร์
อาจารย์ที่ปรึกษาหลัก   รองศาสตราจารย์ ดร.แสงทอง พงษ์เจริญกิต

บทคัดย่อ

          ต้นเตี้ยเป็นลักษณะทางการเกษตรที่สำคัญในแตงโม จากการทำแผนที่ยีนในประชากรรุ่นที่ 2 ซึ่งได้จากการผสมระหว่างแตงโมต้นสูงสายพันธุ์ KK-6939 และแตงโมต้นเตี้ยสายพันธุ์ TH-15974 พบการถ่ายทอดทางพันธุกรรมแบบยีนด้อย 1 ตำแหน่ง (dwarf, *dw*) สามารถระบุตำแหน่งของยีน *dw* ที่ปลายโครโมโซมแท่งที่ 9 ซึ่งอยู่ระหว่างเครื่องหมาย WMSNP-0002750 และ WMSNP-0002780 จากการค้นหายีนในบริเวณดังกล่าวในฐานข้อมูลจีโนมของแตงโม (watermelon reference genome "97103") พบว่ามียีนทั้งหมด 25 ยีน ซึ่งมีเพียง 2 ยีน ได้แก่ *Cla015407* และ *Cla015408* เป็นรหัสของเอนไซม์ gibberellin 3-beta-hydroxylase (GA3ox) จากการศึกษาลำดับเบสของยีนทั้งสองในแตงโมต้นเตี้ยพบการเปลี่ยนแปลงนิวคลีโอไทด์หนึ่งตำแหน่ง (single nucleotide polymorphism, SNP) ที่ตำแหน่ง 626 ของยีน *Cla015407* โดยเปลี่ยนจากนิวคลีโอไทด์ G เป็น A ทำให้ตำแหน่งจุดตัดอาร์เอ็นเอของแตงโมต้นเตี้ยขยับไปที่ตำแหน่ง 639 ส่งผลให้เกิดการขาดหายไป 13 นิวคลีโอไทด์ของเอกซอน 2 ส่งผลให้สร้างโปรตีนสั้นลง ถึงแม้ว่ายีน *Cla015408* จะเป็นรหัสของ GA3ox แต่จากการศึกษาการแสดงออกของยีน *Cla015408* ด้วย quantitative และ semi-quantitative RT-PCR ไม่พบการแสดงออกของยีน *Cla015408* ในระยะต้นกล้าดังนั้นยีน *Cla015408* จึงไม่สามารถทำหน้าที่ทดแทนยีน *Cla015407* ได้ จึงสรุปได้ว่ายีน *Cla015407* คือยีนที่ควบคุมความเตี้ย โดยจากการออกแบบเครื่องหมายดีเอ็นเอที่จำเพาะกับหน้าที่จาก SNP ตำแหน่ง 626 ชื่อ Cla015407-GA ที่สามารถจำแนกแตงโมกลุ่มต้นเตี้ยออกจากต้นสูงได้ ซึ่งจะสามารถนำไปใช้ช่วยคัดเลือกแตงโมต้นเตี้ยในการปรับปรุงพันธุ์แตงโมต่อไป

คำสำคัญ : แตงโม, ยีนควบคุมลักษณะต้นเตี้ย, การเปลี่ยนแปลงนิวคลีโอไทด์หนึ่งตำแหน่ง, จุดตัดอาร์เอ็นเอ, เอนไซม์ Gibberellin 3-beta-hydroxylase (GA3ox), เครื่องหมายดีเอ็นเอที่จำเพาะกับหน้าที่

Title	IDENTIFICATION AND MAPPING OF A DWARFNESS RELATED GENES IN WATERMELON (*Citrullus lanatus*)
Author	Mrs. Namfon Chomkaeo
Degree	Doctor of Philosophy in Genetics
Advisory Committee Chairperson	Associate Professor Dr. Saengtong Pongjaroenkit

ABSTRACT

Dwarfism is a desired agronomic trait in watermelon. Genetic analysis revealed that dwarf growth habit is controlled by a single recessive gene (dwarf; *dw*). Linkage analysis of F_2 derived from inbred watermelon KK-6939 (viny plant) and TH-15974 (dwarf plant) lines, positioned the *dw* locus at the terminal region of chromosome 9. Using the gene annotation data derived from watermelon reference genome "97103", 25 genes were located between WMSNP-0002750 and WMSNP-0002780 markers. Only 2 out of 25 candidate genes, *Cla015407* and *Cla015408* encode a gibberellin 3-beta-hydroxylase (GA3ox). Only the SNP (G/A) at the position 626th nucleotide of *Cla015407* could distinguish dwarf plants from viny plants. This point mutation is an acceptor splice site lead to altered splicing site happening moved from 626th to 639th nucleotide and then 13 bp were deleted in exon 2 resulting in truncated protein in dwarf plants. Even though *Cla015408* encoded the same protein as *Cla015407* but the expression of *Cla015408* could not be detected from quantitative and semi-quantitative RT-PCR in seedling stage. Therefore, *Cla015408* could not function instead of *Cla015407* in dwarf plants. These results suggested *Cla015407* should be the *dw* gene. The high throughput of functional marker, Cla015407-GA was developed from SNP 626th and validated in various inbred lines. This functional marker consisted completely with the phenotype. So this marker has high accuracy and high value to implement in a watermelon breeding program.

Keywords : Watermelon, Dwarf gene, Single nucleotide polymorphism (SNP), Acceptor splice site, Gibberellin 3-beta-hydroxylase (GA3ox), Functional marker

ACKNOWLEDGEMENTS

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CHAPTER ONE

Overview

1.1 General introductions

Watermelon (*Citrullus lanatus*) is a tropical fruit, belongs to family Cucurbitaceae is a flowering plant originally from South Africa. Watermelon fruit contains about 6% sugar and 92% water by weight. It is a source of vitamin C and many hidden nutrients. It is an important crop economically and is grown around the world. Based on statistical data from the (Food and Agriculture Organization of the United Nations, 2019), the total production of watermelon fruit worldwide reach to 100 million tons with the plant area recorded reaching 3 million hectares spread throughout the world. The center of watermelon production is Asia with a total harvest of 79 million tons or is about 79% of total worldwide production, followed by the Africa with 7.5 million tons or 7.5% of total worldwide production and the America with production is about 6.9 million tons or 6.9% of total worldwide production. China is the biggest producer and produced 60.86 million tons, followed by Turkey with produced about 3.87 million tons. Common watermelon has long vine and climbing habit. Plant must be grown at a wide spacing because of their long, trailing vines. Normal vine watermelon need a lot of space up to 20 squares feet per plant. If placed too close together, they will overlap and cause problems for the surrounding fruit plants. Therefore, the pruning watermelon is required to promote healthier vines, increase fruit size and decrease disease. The exception is for dwarf cultivars, which are natural mutation, can be grown at a tighter spacing. Dwarf watermelon has short vine plants with shorter internodes, which are highly suitable for high-density planting and can save land resources to a certain extent by improving yield per unit area. The crop may be established in the greenhouse by planting seeds or using containerized transplants

as well as decreasing of plant pests (weeds, insects, and diseases, including nematodes) during the production period. Therefore, dwarf plant is one of the most desirable and valuable traits in watermelon breeding program.

Dwarfism inheritance in watermelon has been identified from various studies and germplasm resources of mutant, which were assigned as different locus names. Even the dwarf mutants derived from different sources but all studies reported the inheritance of the dwarf was controlled by a single recessive gene.

The main causes of dwarfness in plants have been mutated in hormone biosynthesis pathway-related genes such as gibberellins (GAs), cytokinin, brassinosteroids and other key hormones influencing plant growth and development. Additionally, abnormally developed plant cell membranes or walls can also lead to dwarfness in plants. GAs is a crucial hormone to promote plant growth and stem elongation. The GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) catalyze the final two steps of GA biosynthesis pathway and lead to dwarf phenotype in rice and maize, respectively. The pumpkin (*Cucurbita moschata* Duchesne) bush mutant plant showed partial response to exogenous GAs hormones but not to indole acetic acid (IAA) and brassinosteroids (BR). Therefore the GAs hormones were corresponding to bushy or dwarf in pumpkin. Dwarfness related genes in watermelon have been identified and reported from various dwarf lines. Most of them are GA20ox and GA3ox genes.

Genetic mapping or linkage map analysis is one of the earliest methods used to develop molecular marker and map the genes on chromosomes. There are assignments of a specific gene to a particular region of a chromosome and relative distances between genes on the chromosome. Therefore, genetic mapping methods utilized marker technology and statistical methodology to localize the gene on chromosomes or identify tightly linked markers. The purposes of this study are to identify and characterize the gene that causes dwarf watermelon by QTL mapping

method and develop a high throughput genotyping marker for marker assisted selection (MAS) in watermelon breeding program.

1.2 Objectives

To identify the gene which regulated dwarfness in watermelon.

To characterize dwarf related genes in watermelon.

To develop functional marker which corresponding to dwarfness in watermelon.

1.3 Benefit

The functional marker which can distinguish dwarf from viny plant will be useful for breeding program because of functional marker was used as marker assisted selection with high accuracy and inbred lines independent.

CHAPTER TWO

Literature review

2.1 Taxonomy

Momordica lanata is bitter wooly melon and was described by Carl Peter Thunberg (Thunberg, 1794). Then Japanese botanists, Jinzu Mat sumuran and Takenpshin Nakai reassigned to the genus *Citrullus*. *Cucurbita citrullus* is sweet watermelon and was described by Carl Linnaeus (Linnaeus, 1753). Then German botanist, Heinrich Adof Schrader reassigned to the genus *Citrullus* in 1836 (Schrader, 1838). Watermelon has scientific name is *Citrullus lanatus*. The taxonomy of watermelon was classified as following.

Kingdom:	**Plantae**
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Rosids
Order:	Cucurbitales
Family:	Cucurbitaceae
Genus:	*Citrullus*
Species:	*C. lanatus*

Watermelon or *C. lanatus* includes three subspecies :

1. *C. lanatus* subtsp. *lanatus* : A group of ancient cultigens, the "tsamma" or "citron" watermelon, that naturally thrives in southern Africa.

2. *C. lanatus* subtsp. *mucosospermus :* The egusi watermelon group that has large edible seeds with a fleshy pericarp.

3. *C. lanatus* subtsp. *vulgaris* : The sweet watermelon group that gave rise to

the modern cultivated watermelon.

The population structure and relationships analysis was investigated by using two methods, a neighbor-joining tree and principal component analysis (PCA) by using SNP as the markers. Among three subspecies of *C. lanatus*, *C. lanatus* subtsp. *vulgaris* and *C. lanatus* subtsp. *mucosospermus* were clustered in the same group in both analyses, which indicated the close relationship between these two subspecies (Guo *et al.,* 2013).

2.2 Benefits and nutrition

Watermelon is one of the most delicious fruit that is low calories and rich in water is around 92%, which makes it useful for staying hydrated in the summer. Watermelon is a valued source of natural antioxidants with special reference to lycopene, ascorbic acid and citruline. These functional ingredients act as protection against chronic health problems like cancer insurgence and cardiovascular disorders (Fenko *et al.,* 2009; Omoni and Aluko, 2005; Zhang and Hamauzu, 2004). Considering the nutritional profile, consumption of 100 g watermelon provides 30 kcal. It contains almost 92 % water and 7.55 % of carbohydrates out of which 6.2 % are sugars and 0.4 % dietary fiber. It is enriched with carotenoid, vitamin C, citrulline, carotenoids and flavonoids and fat and cholesterol free, thus considered as low caloric fruit (Bruton *et al.,* 2009; Leskovar *et al.,* 2004). Additionally, watermelon is rich source of β-carotene acts as an antioxidant and precursor of vitamin A.

2.3 Watermelon production

The crop prefers a hot, dry climate with mean daily temperatures of 22 to 30℃. Maximum and minimum temperatures for growth are about 35 and 18 ℃ respectively. The optimum soil temperature for root growth is in the range of 20 to 35 ℃. Fruits grown under hot, dry conditions have a high sugar content of 11 percent in comparison

to 8 percent under cool, humid conditions. The crop is very sensitive to frost. The length of the total growing period ranges from 80 to 110 days, depending on climate (Dube *et al.,* 2021).

Watermelons were transplanted worldwide but Asia is the center production because of the climate. Based on statistical data from the Food and Agriculture Organization of the United Nations, 2019. China is the biggest country that produced watermelon. The ranking of countries that produce the most watermelon is presented in table 1.

Table 1 Total of top 20 countries that produce the most watermelon (Food and Agriculture Organization of the United Nations, 2019).

Rank	Countries	Pounds	%of top 20
1	China	134,175,909,133	67.78%
2	Turkey	8,533,014,779	4.31%
3	India	5,500,526,900	2.78%
4	Brazil	5,022,534,419	2.54%
5	Algeria	4,865,300,921	2.46%
6	Iran	4,256,442,197	2.15%
7	Russia	3,935,857,380	1.99%
8	United States	3,704,894,775	1.87%
9	Egypt	3,491,937,301	1.76%
10	Mexico	2,966,768,157	1.50%
11	Kazakhstan	2,956,379,988	1.49%
12	Uzbekistan	2,717,105,965	1.37%
13	Viet Nam	2,706,933,849	1.37%
14	Spain	2,645,742,416	1.34%

Rank	Countries	Pounds	%of top 20
15	Senegal	2,624,558,222	1.33%
16	Afghanistan	1,867,291,094	0.94%
17	Tajikistan	1,546,016,230	0.78%
18	Saudi Arabia	1,516,156,857	0.77%
19	Morocco	1,487,750,328	0.75%
20	Italy	1,433,928,940	0.72%

2.4 Growth habit of watermelon

Watermelon is normally seeded directly in the fields. In cool regions with short growing seasons, seeds are started indoors in paper or peat pots, two to four weeks before last frost date. Spacing between plants and rows varies from 0.6 x 0.9 to 1.8 x 2.4 m. Seeds are sometimes placed on hills spaced 1.8 x 2.4 m. In areas prone to frost, sowing time is dictated often by the occurrence of frost. Sometimes black plastic mulch is used for frost protection. Watermelons are usually planted in slightly mounded hills spaced 4 to 6 feet apart. These are large plants that need room to sprawl. Plant four to five seeds in the center of the hill, about 1 inch deep. When the seeds sprout, thin them out, leaving two or three plants per hill. If planting from nursery seedlings or seeds started indoors, plant two transplants per hill (Adekunle *et al.,* 2005).

Common watermelons have an indeterminate growth habit with normal internode length, thus allowing the vine to grow indefinitely under the normal conditions. The normal watermelon roots and vines spread up to 18 feet. Therefore normal watermelons need a lot of space up to 20 square feet per plant for transplant. The vines of normal watermelon should be trimmed to promote healthier vines, increasing the fruit size and decreasing diseases. Dwarf or bushy mutants have been

discovered in watermelon. Dwarf plant has short vine with shorter internodes. Dwarf habit or dwarfism is a desired agronomic trait in watermelon. As it allows higher plant density than is feasible for the standard indeterminate, trailing type, and thereby increases yield as well as the decreasing soil disease and labor required to cultivate.

2.5 The *dwarf* locus and the inheritance of dwarfness in watermelon

The dwarf of watermelon was natural mutation. However the sources of dwarf mutants had several sources and were assigned to different locus name depending on mutant lines. The dwarf inheritance was investigated in different dwarf sources by various studies. The morphological which represented to dwarf phenotype such as main vine length, internode length, and cell elongation were observed and evaluated. A chi-squared test was performed to check whether the observations were differing from the null hypothesis or not. Even though the source of dwarf mutants were different but the segregation ratio of the long vine and short vine was fit to single gene or Mendelian ratio. All studies reported the inheritance of the dwarf was controlled by a single recessive gene (Dyutin and AFANAS'EVA, 1987; Guner and Wehner, 2004; Hexun *et al.,* 1998; Liu and Loy, 1972; Mohr, 1956; Mohr and Sandhu, 1975) including *dw-1* in Bush Desert King cultivars (Mohr and Sandhu, 1975), *dw-1s* in Somali local (Dyutin and AFANAS'EVA, 1987), *dw-2* in WB-2 cultivar (Liu and Loy, 1972), *dw-3* in Dwarf Male-Sterile Watermelon (DMSW) (Hexun *et al.,* 1998), *dsh* in dsh (Dong *et al.,* 2018b), *Cldw-1* in WM102 (Zhu *et al.,* 2019b), *Cldf* in N21 (Wei *et al.,* 2019b), *Si* in w106 (Sun *et al.,* 2020) and *Cldw* in dwarf cultivar 812 (Zhang *et al.,* 2021a). The dwarf loci in various studies are presented in table 2.

Table 2 The dwarf locus which were reported from various studies.

Locus name	Dwarf source	Character	Reference
dw-1	Bush Desert King	Dwarf-1 plants have short internodes due to fewer and shorter cells than the normal plant type.	Mohr, 1956; Sandhu and Malik, 1975
dw-1s	Somali local	Plants with *dw-1s* have vine length intermediate between normal and dwarf, and the hypocotyls were somewhat longer than normal vine and considerably longer than dwarf The *dw-1s* is recessive to normal plant type.	Dyutin and AFANASIEVA, 1987
dw-2	WB-2 (*dw-2*)	Plants with *dw-2* have short internodes due to fewer cells than the normal type.	Liu, 1972
dw-3	Dwarf Male-Sterile Watermelon (DMSW)	Plants with *dw-3* have leaves with fewer lobes than the normal leaf.	Hexun *et al.*, 1998
dsh	dsh	The dsh watermelon plant is a bush with a short vine, short internodes, thin stems, numerous branches, and small leaves, flowers, and fruits.	Dong *et al.*, 2018
cldw-1	WM102	WM102 is a dwarf inbred line, which was selected from 'Bush Sugar Baby' [accession code: Grif15898; provided by USDA-ARS Germplasm Resources Information Network (GRIN). It exhibited short internodes in both the primary and secondary stems. *Cldw-1* was controlled by single recessive gene.	Zhu *et al.*, 2019
Cldf	N21	The N21 line had a short internode shows a dwarfism phenotype. The inheritance of dwarfism was controlled by single recessive gene.	Wei *et al.*, 2019

Locus name	Dwarf source	Character	Reference
Si	w106	Dwarf architecture of 'w106' was mainly caused by longitudinal cell length reduction and was controlled by a single recessive gene.	Sun *et al.,* 2020
Cldw	dwarf cultivar 812	812 is a dwarf-type bushy vine length and short internodes. The genetic inheritance of dwarfism was controlled by single recessive gene.	Zhang *et al.,* 2021

2.6 Plant hormones

Plant hormones are chemical compounds present in very low concentration in plants. These hormones are produced in almost all parts of the plant and are transmitted to various parts of the plant. The main function of plant hormones are control all the growth and development activities such as cell division, enlargement, flowering, seed formation, dormancy and abscission. Plant hormones include auxin (IAA), cytokinin (CK), gibberellin (GAs), abscisic acid (ABA), ethylene (ETH), brassinosteroids (BRs), and jasmonic acid (JA). The chemical structures of plant hormones are shown in Fig. 1. IAA is one of a key regulator of plant growth and development, orchestrating cell division, elongation and differentiation, embryonic development, root and stem tropisms, apical dominance, and transition to flowering. CK are a class of plant hormones that promote cell division, or cytokinesis, in plant roots and shoots. They are involved primarily in cell growth and differentiation, but also affect apical dominance, axillary bud growth, and leaf senescence. GAs are plant hormones that regulate various developmental processes, including stem elongation, germination, dormancy, flowering, flower development, and leaf and fruit senescence. ABA is a plant hormone that regulates numerous aspects of plant growth,

development, and stress responses. ABA-deficient mutants from various plant species display reduced seed dormancy and wilty phenotypes, highlighting that these crucial ABA functions are conserved in the plant kingdom. ETH induces diverse effects throughout the plant life cycle, including seed germination, flower senescence and fruit ripening. BRs are required for the development, growth, and productivity of plants which are involved in regulating the division, elongation, and differentiation of numerous cell types throughout the entire plant life cycle. JA is regulating plant responses to abiotic and biotic stresses as well as plant growth and development. The functional of all plant hormones are presented in table 3 (Gray, 2004).
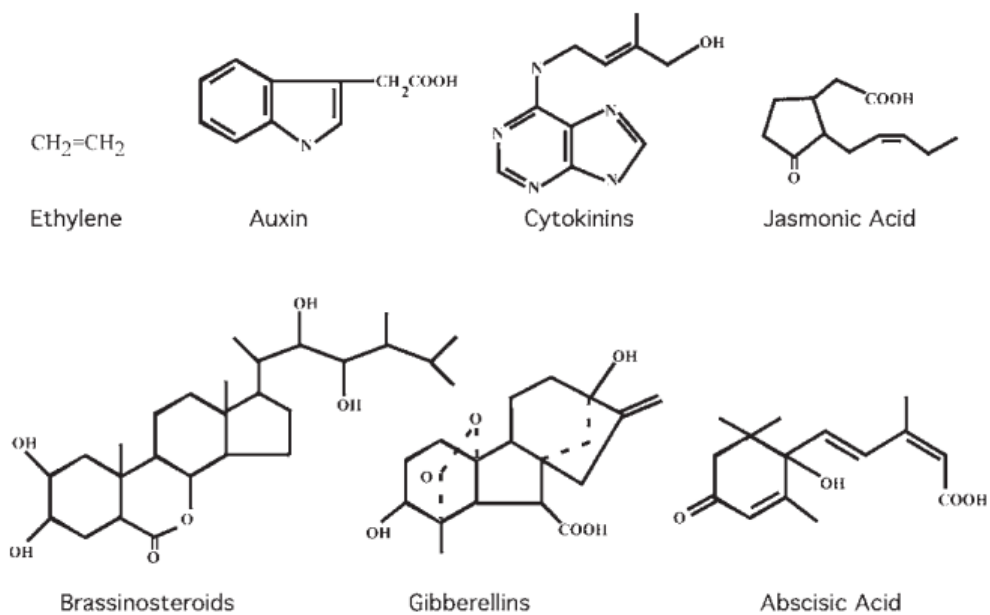
Figure 1 Chemical structure of the plant hormone (Gray, 2004).

Table 3 The function of plant hormones (Gray, 2004).

Plant hormone	Function
Ethylene (ETH)	Promotes fruit ripening, senescence, and responses to pathogens and abiotic stresses.
Auxin (IAA)	Regulates cell division and expansion, vascular differentiation, lateral root development, and apical dominance
Cytokinins (CK)	Cytokinins are adenine derivatives first identified by their ability to promote cytokinesis
Jasmonic Acid (JA)	JA is a volatile signal that modulates pollen development and responses to pathogen infection
Brassinosteroids (BRs)	The BRs regulate cell expansion and photomorphogenesis (light regulated development)
Gibberellins (GAs)	GAs are diterpenoid compounds that promote germination, stem elongation, and the induction of flowering
Abscisic Acid (ABA)	ABA promotes seed dormancy and is involved in several stress signaling pathways

2.7 Gibberellins (GAs) biosynthesis pathway

GAs are plant hormones that regulate numerous developmental processes in plants including stem elongation, germination, dormancy, flowering, leaf and fruit senescence (Hedden, 2016; Hedden and Thomas, 2012; Hooley, 1994). GAs are one of the longest-known classes of plant hormone which play important roles in many

aspects of plant growth and development. The main causes of dwarfness in plants have been mutated in hormone biosynthesis pathway-related genes such as gibberellins (GAs) (Ding *et al.,* 2021; Wu *et al.,* 2015; Zhang *et al.,* 2015). It is thought that the selective breeding of crop strains that were deficient GA synthesis was one of the key drivers of the green revolution in the 1960s.

GAs is a crucial hormone to promote plant growth and stem elongation (Sun, 2010). There are seven key catalyzing enzymes the GA biosynthesis pathway , including copalyl diphosphate synthase (CPS), kaurene synthase (KS), kaurene oxidase (KO), kaurenoic acid oxidase (KAO), GA 2-oxidase (GA2ox), GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) as presented in Fig. 2 (Hedden, 2016). GAs, which belong to the tetracyclic diterpenoid class of hormones, comprise a group of over 136 natural plant constituents (Hedden, 2016), but only some of them, e.g. GA1, GA3, GA4, GA5, GA6 and GA7 exhibit biological activity. Their biosynthesis is a multi-step process divided between plastids, reticulum and cytoplasm and effected by diverse enzyme families (Dill and Sun, 2001; Hedden and Thomas, 2012).
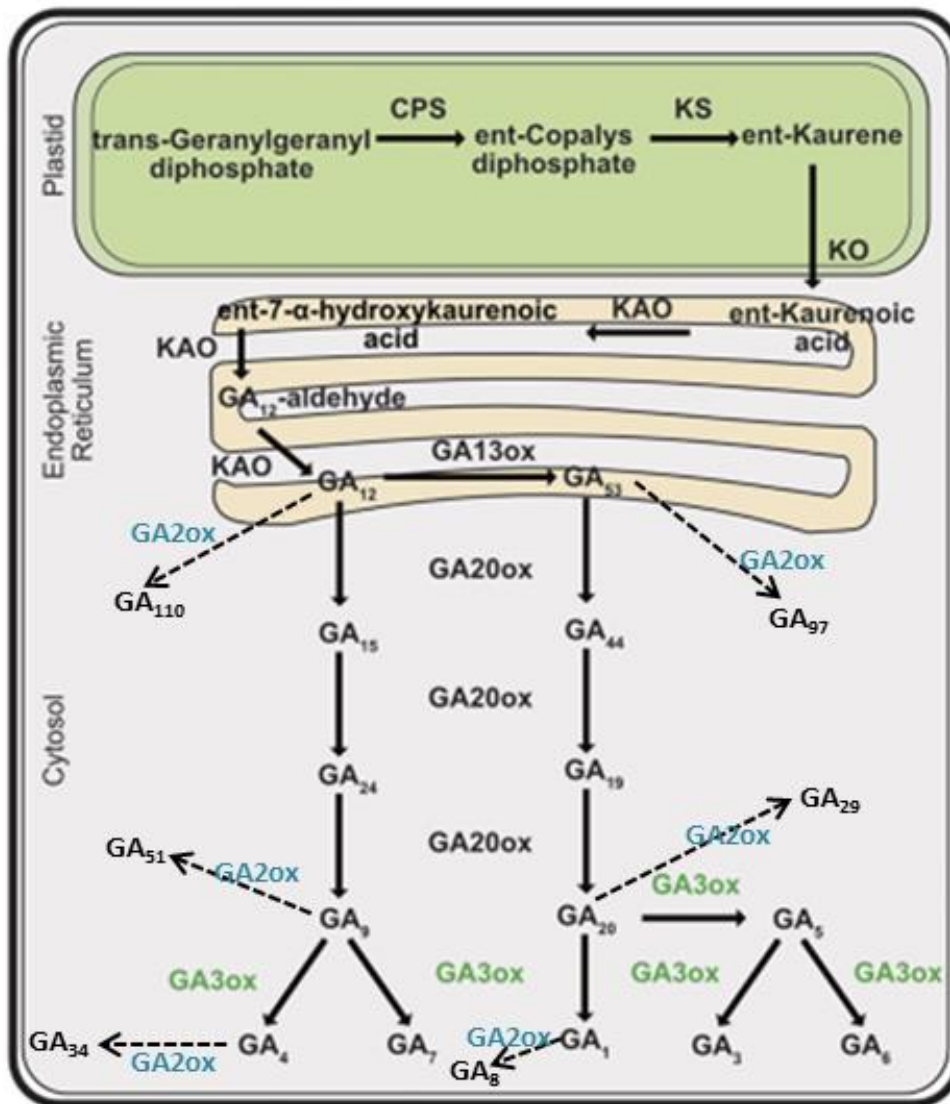
Figure 2 The gibberellin biosynthesis pathway in higher plants. The cellular localizations of metabolites are in plastids, endoplasmic reticulum and cytoplasm. Plant bioactive gibberellins are GA4, GA7, GA1, GA3, GA5 and GA6. Adapted from (Hedden, 2016; Igielski and Kępczyńska, 2017).

Among hundreds of plant GAs, only a few such as GA1, GA3, GA4, GA5, GA6 and GA7 are bioactive compounds (Hedden, 2016). The function of bioactive GAs generally depends on the concentration in a given tissue, and this is mainly affected by their biosynthesis and/or deactivation (Hedden and Phillips, 2000). GAs biosynthesis pathway can be divided into two main steps including early and late. The early steps are catalyzed by a series of enzymes, i.e., CPS, KS, KO, and KAO, which are all encoded by single genes. The enzymes catalyzing later steps (i.e., GA2 oxidase, GA20 oxidase, and GA3 oxidase) are encoded by small gene families. All the GA20ox, GA3ox and GA2ox are the key enzymes in a series of oxidation steps, which convert GA12 and GA53 to various GA intermediates and bioactive GAs (GA1 and GA4). Their sequences belonged to the 2-Oxoglutarate-dependent dioxygenase (2-ODDs) superfamily, which share high homology with the functional domains (de Carolis and de Luca, 1994; Phillips *et al.,* 1995; Schomburg *et al.,* 2003). CsGAox proteins from tea plants were performed phylogenetic analysis. GAox genes contain two conserved domains including DIOX_N and 2OG-FeII_Oxy domains (Fig. 3) (Pan *et al.,* 2017).
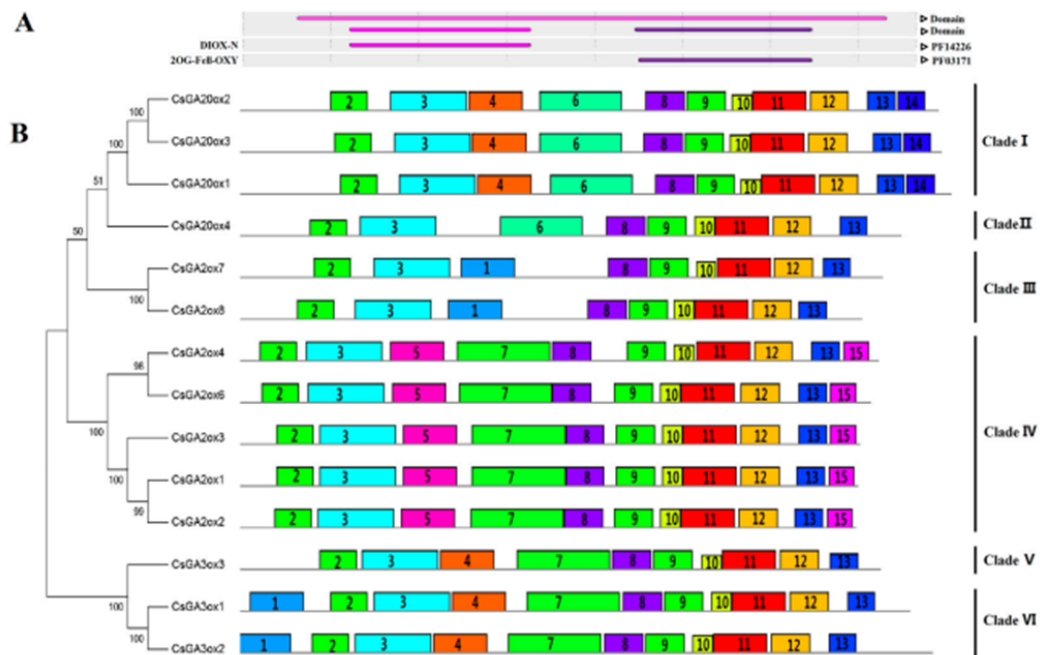
Figure 3 Phylogenetic analysis and conserved motif identification in CsGAox proteins from tea plants. (A) Interpro analysis revealed two types of domains as a part of the 2-oxoglutarate-dependent dioxygenase superfamily in previously defined GAox proteins; (B) De novo motif identification of GAox proteins. Motifs 2 and 3 show resemblance to the DIOX_N (PF14226) domain, while motifs 8–12 show resemblance to the 2OG-FeII_Oxy (PF03171) domain (Pan *et al.,* 2017).

2.8 Dwarf related genes

The enzymes in the late step play key roles in the regulation of bioactive GA levels. The loss-of-function in GA20ox and GA3ox can generate dwarf phenotypes, such as the well-known Green Revolution *sd-1* gene (Carrera *et al.,* 1999; Oikawa *et al.,* 2004; Sakamoto *et al.,* 2004; Spielmeyer *et al.,* 2002). Pumpkin (*Cucurbita moschata* Duchesne) bush mutant plants were designated as GA-related mutant plants because the internode elongation in pumpkin bush mutant plants was partially recovered by

GA4 together with GA7 and GA3 (Wu *et al.,* 2015). Dwarf gene of pumpkin (*Cucurbita maxima* Duch.) was detected by using genotyping-by-sequencing approach in F_2 population (n=186). A high-density pumpkin genetic map was constructed and three QTLs, which associated dwarf vine, were detected. One major QTL namely qCmB2 had the largest effect on vine length, explained 21.39 % of the phenotypic variation. The other two minor QTLs explained 7.65 and 9.95 % of the phenotypic variation. qCmB2 was predicted to be *Cma_004516*, encoding the GA20ox in the GA biosynthesis pathway, had a 1,249-bp deletion in its promoter in dwarf plant, and its expression level was significantly increased during the vine growth and higher in vine plants than dwarf plants, supporting *Cma_004516* as a possible candidate gene controlling vine growth in pumpkin (Zhang *et al.,* 2015). Two SNPs in exons together with several SNPs and InDels in promoter of Cp4.1LG10g05910.1 gene, which encoded GA2-oxidase, were revealed in the dwarf *Cucurbita pepo* L. line X10 (Ding *et al.,* 2021).

Hypocotyl and root elongation in a dwarf (dw-2) and a normal type of watermelon were investigated by gibberellin applied exogenously. The following GAs were employed: GA1, GA3, GA7, and GA4 together with GA7 (66% GA4 with 34% GA7) to dwarf and normal watermelon in light and dark growth condition. In both growth conditions, gibberellins were effective in stimulating hypocotyl elongation of dwarf type but normal type responded only slightly to the gibberellin employed. To comparison gibberellin specific, plants of the dwarf type are most sensitive to GA4 together with GA7 (Loy and Liu, 1974). Seedling growth in dark conditions is higher than in light conditions due to dark increased cell elongation. Root elongation was decreased when GA concentration increased, because GA inhibited root elongation (Loy and Liu, 1974).

Dwarfness related genes in watermelon have been identified and reported from various dwarf lines. The candidate gene, *Cla010726* on chromosome 7 was identified as dwarfism gene in dsh watermelon line by using BSA-seq and linkage analysis. The

SNP was detected in the promoter region of *Cla010726* gene, which encoded GA20ox and led to the expression level being significantly lower in the dwarf plants than viny plants (Dong *et al.,* 2018). The deletion of a single nucleotide in an ABC transporter B subfamily gene (*Cla010337*) on chromosome 9 was identified in dwarf line WM102 using BSA-Seq and linkage analysis. However, the regulation plant height mechanism of ABC transporter genes was unclear, probably crosstalk between auxins and other hormone pathways (Zhu *et al.,* 2019). The 13 bp deletion of *Cla015407* coding region (GA3ox) on chromosome 9 was detected in dwarf Duan 125 by molecular mapping (Gebremeskel *et al.,* 2020). A SNP at the 3′AG splice site of intron 1 and 13 bp deletion of exon 2 of *Cla015407* (GA3ox) on chromosome 9 was detected in dwarf N21 line and lead to truncated protein in dwarf watermelon (Wei *et al.,* 2019). A SNP at the 3′AG splice site of intron 1 of *Cla015407* (GA3ox) lead to generated two splicing isoforms, one splicing isoform retained the intron sequence while the other had a 13-bp deletion in exon 2, both isoforms lead to truncated protein in dwarf w106 line (Sun *et al.,* 2020). A splice site mutation at intron 1 was revealed in *Cla015407* (GA3ox) of dwarf cultivar 812 and result to the expression of *Cla015407* gene in dwarf cultivar 812 internode was significant lower than viny cultivar W1-1 internode at reproductive growth stage (Zhang *et al.,* 2021).

2.9 SNP genotyping

Single nucleotide polymorphisms (SNP) markers are the most abundant type of polymorphic marker found in eukaryotic genomes. SNP genotyping technology is rapidly progressing with the emergence of novel, faster and cheaper methods as well as improvements in the existing methods. Two components are necessary for SNP genotyping technologies including allele discrimination and signal detection. The principles of these methods were described (Twyman, 2005).

1. Allele discrimination, this method for determining the type of base present at a particular SNP locus. There are three general allele discrimination methods:

- Allele-Specific Hybridization using the simple method (allele-specific oligonucleotide probes) (Fig. 4a) which is a short piece of synthetic DNA complementary to the sequence of a variable target DNA. It acts as a probe for the presence of the target in a Southern blot assay or, more commonly, in the simpler Dot blot assay. And another method, modified method (allele-specific PCR) (Fig. 4b) is an application of the PCR that permits the direct detection of any point mutation DNA by analyzing the PCR products in an ethidium bromide-stained agarose or polyacrylamide gel.

- Allele-specific single-base extension (Fig. 4c) is a method for determining the identity of a nucleotide base at a specific position along a nucleic acid. The method is used to identify a SNP. In the method, an oligonucleotide primer hybridizes to a complementary region along the nucleic acid to form a duplex, with the primer's terminal 3'-end directly adjacent to the nucleotide base to be identified. Using a DNA polymerase, the oligonucleotide primer is enzymatically extended by a single base in the presence of all four nucleotide terminators; the nucleotide terminator complementary to the base in the template being interrogated is incorporated and identified. The presence of all four terminators suppresses misincorporation of non-complementary nucleotides.

- Allele-specific enzymatic cleavage. This method uses a restriction enzyme that cleaves only one of the alleles. The digested products are run on agarose gel electrophoresis, and the SNP genotype is determined based on the size and number of DNA fragments or using Invader assay. This assay uses two allele-specific probes with different dyes, reporter (R) and quencher (Q), at either end or one common invader probe. The allele-specific probe and invader probe hybridize with target DNA to form a three-dimensional structure at the SNP site which recognized by cleaves enzyme.

The allele-specific probe that complementary to the SNP is cleaved by the enzyme and releases its reporter dye in which SNP genotype can be discriminated by fluorescence analysis (Fig. 4d).
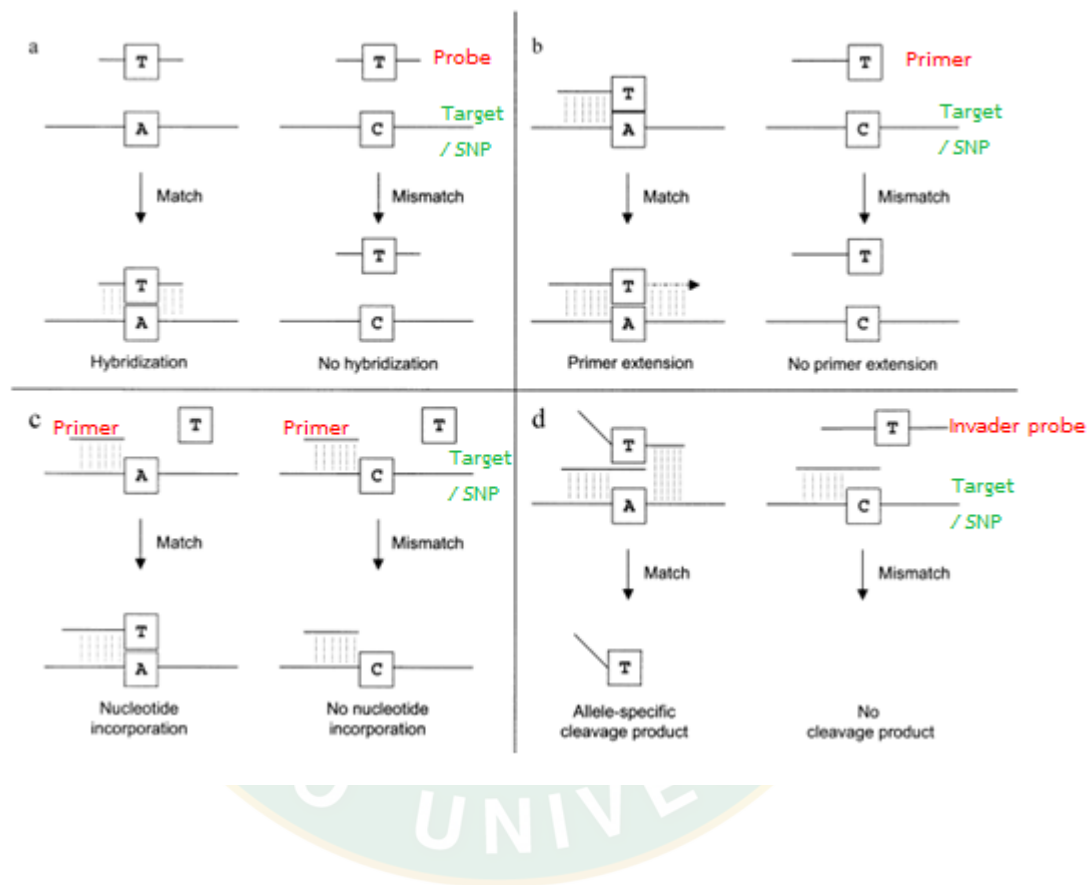
Figure 4 Principles of allele discrimination methods (Twyman, 2005). (a) Allele-specific hybridization. (b) Allele-specific PCR. (c) Allele-specific single base primer extension. (d) Allele-specific invasive cleavage.

2. Signal detection or detection of allele specific products, this method report the presence of the allele(s) by utilizing fluorescent labels. There are two most popular methods.

- Direct fluorescence detection (Fig. 5a) which refer to the TaqMan assay that involves prevention of fluorescence of a fluorophore by the close proximity with a quencher in a nucleotide probe. As the *Taq* polymerase reaches the probe, the 5′ to 3′ exonuclease activity of the polymerase degrades the probe, the fluorophore is released from the quencher, and fluorescence is detected. A typical TaqMan assay has two such probes that detect a single nucleotide difference at a specific location between two alleles of a given gene. Probes that are not 100 percent homologous with the DNA sequences do not bind sufficiently and the *Taq* polymerase cannot degrade the probe to release the fluorophore from the quencher.

- Fluorescence resonance energy transfer (FRET) (Fig. 5b) or molecular beacon probes, are oligonucleotide hybridization probes that can report the presence of specific nucleic acids in homogenous solutions. Molecular beacons are hairpin-shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid sequence.
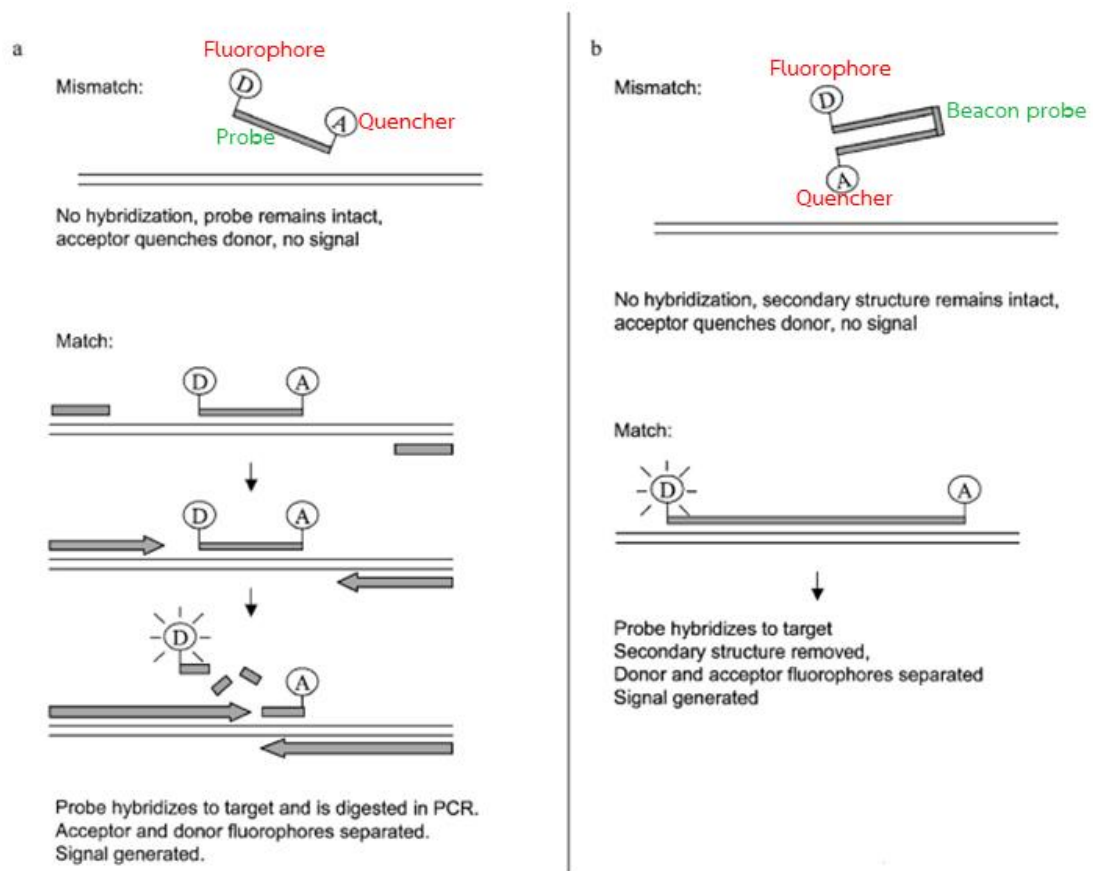
Figure 5 Principles of fluorescence-based homogenous genotyping methods: (a) TaqMan assay; (b) Molecular Beacon assay (Twyman, 2005).

2.10 KASPar or KASP SNP Genotyping System

KASPar SNP genotyping has been developed at KBiosciences by using a novel homogeneous fluorescent genotyping system. Their system relies on the discrimination power of a novel form of competitive allele specific PCR to determine the alleles at a specific locus within genomic DNA for SNP typing and combined with novel fluoresence resonance energy transfer (FRET) homogeneous format for reporter. They are three essential components in KASP genotyping assay (Fig. 6) (He *et al.,* 2014; Smith and Maughan, 2015).

1. DNA template contains a given SNP locus.

2. Primers consisted of two allele specific forward primers and common reverse primer. For allele specific primers, each primer contains a unique unlabelled tail sequence at the 5′ end.

3. KASPar master mix containing FRET cassette plus *Taq* polymerase in an optimised buffer solution. In the FRET cassette consisted of two 5′ fluor-labeled oligos, one labeled with FAM (F), one with HEX (H). These oligo sequences are designed to interact with the sequences of the tails of each allele specific primers.
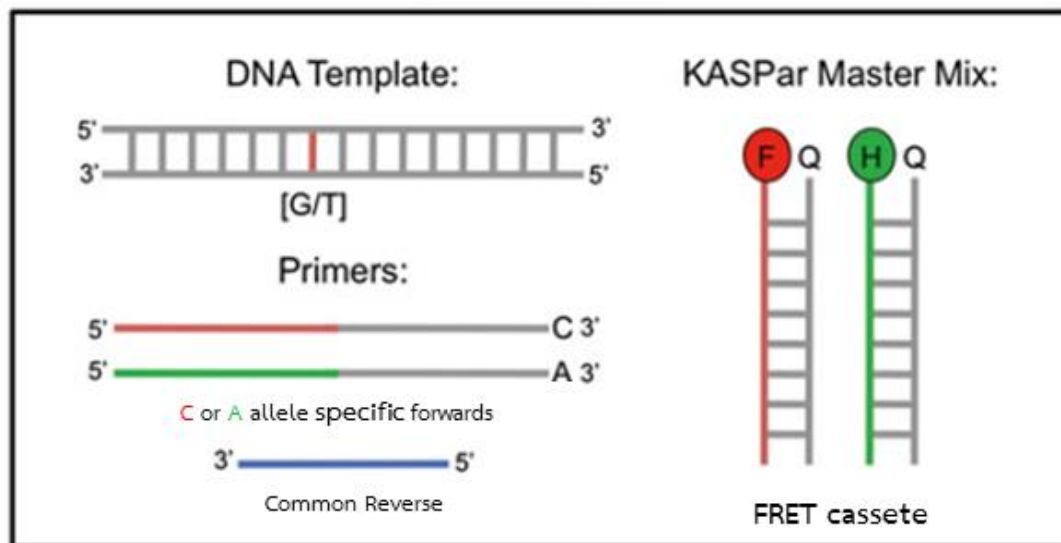
Figure 6 The KASPar genotyping chemistry consisted of three components (Smith and Maughan, 2015).

In the initial stage of PCR, the appropriate allele specific primer such as A-specific forward binds to its complementary region directly upstream of the SNP with the 3′ end of the primer positioned at the SNP nucleotide (Fig. 7). The common reverse primer also binds and PCR proceeds, with the allele specific primer becoming

incorporated into the template. During this phase, the fluor-labeled oligos remain bound to their quencher-labeled complementary oligos in the cassette, and no fluorescent signal is generated. As PCR proceeds further, one of the fluor-labeled oligos, corresponding to the amplified allele, is also incorporated into the template, and is hence no longer bound to its quencher-labeled complement. As the fluorescent signal is no longer quenched, the appropriate fluorescent signal is generated and detected by the proper light scanner and the detection methods require post-PCR laboratory time. If the genotype at a given SNP is homozygous, only one of the possible fluorescent signals will be generated. If the individual is heterozygous, the result will be a mixed fluorescent signal (Smith and Maughan, 2015).
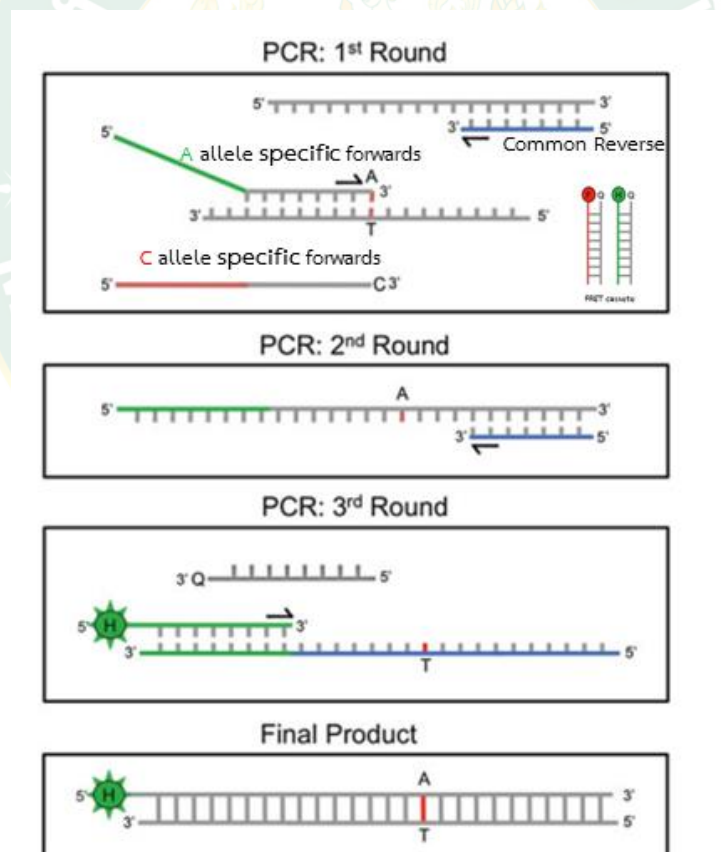
Figure 7 An overview of KASP mechanism of action (Smith and Maughan, 2015).

2.11 Genetic Mapping and QTL analysis

Genetic mapping or linkage map is one of the earliest methods used to develop molecular marker and map the genes on chromosomes. There are assigned of a specific gene to particular region of a chromosome and determining the location of and relative distances between genes on the chromosome. Therefore, genetic mapping method utilized marker technology and statistical methodology to localize the gene on chromosome or identify tightly linked marker. The principles and basic concepts of quantitative trait loci (QTLs) mapping or genetic mapping were described (Collard *et al.,* 2005). There are 5 major steps for process the genetic mapping (Fig. 8) including mapping population creating, phenotypic evaluation, genotypic assessment, linkage maps construction and QTL analysis. Finally, the significant region will be detected and the output of whole process is QTL identification.
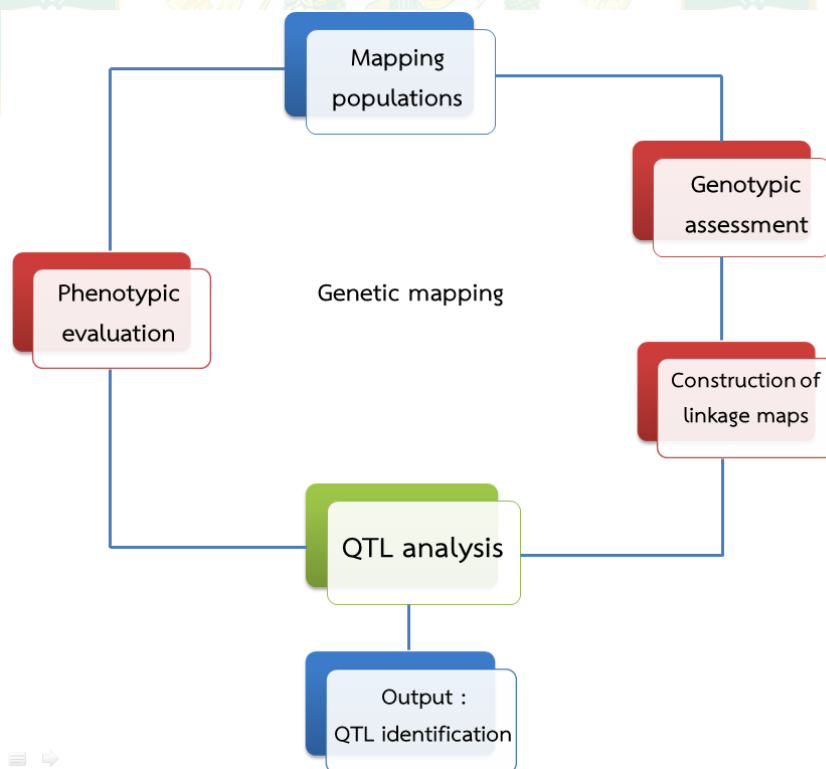
Figure 8 An overview of genetic mapping and QTL analysis consist of 5 major steps.

1. Mapping population

Genetic mapping requires segregating population which derived from a cross between two parental lines with contrasting phenotypes. Population size used generally range from 50 – 250 individuals. However, larger population sizes are the best but might not be viable because they increase the costs and require more labor, space and time for genetic mapping. The estimating the effects of population size and type on the accuracy of genetic maps were investigated. The results showed that in different populations a total of 200 individuals are enough for the construction of accuracy genetic map (Ferreira *et al.,* 2006). There are several types of segregating populations such as F_2, RILs, DH and BC etc. (Fig. 9) (Collard *et al.,* 2005). Two parental lines, which show contrasting of interested traits, are crossed to produce F_1 population. Individual F_1 plant is then selfed to produce an F_2 population. The backcross population (BC_1F_1) derived from Individual F_1 plant cross back to one parental line and individual BC_1F_1 plant is then selfed to produce an BC_1F_2 population. A recombinant inbred line (RIL) population is developed using single seed descent from the F_2 generation. Doubled haploids (DHs) are plants derived from a single pollen grain and doubled artificially to form homozygous diploids.
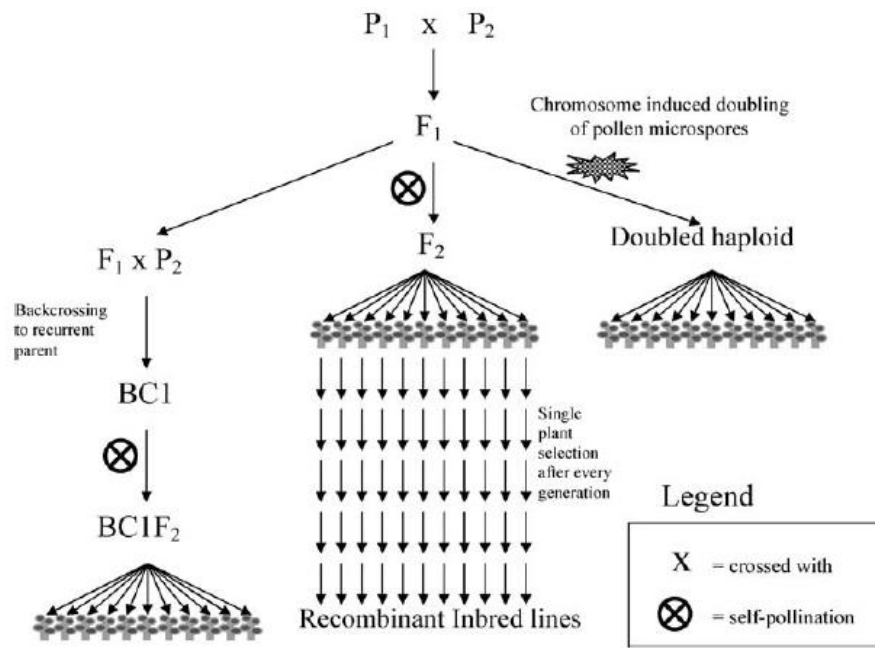
Figure 9 Diagram of main segregating population types for genetic mapping (Collard *et al.,* 2005)

2. Phenotypic evaluation

Genetic mapping requires the quality of phenotypic data set. Therefore, the measurement methods or tools used to monitor must be appropriate and accurate. All individuals in segregating population were collected phenotypic data in term of category or numeric data for analysis in further step.

3. Genotypic assessment

There are many types of molecular marker available. The higher numbers of marker are required for high resolution mapping, however based on resources and potential of each laboratory. The general step for genotypic assessment as following:

- **Identification of polymorphic markers**

  The polymorphic markers were surveyed between two parental lines. It is crucial that sufficient polymorphism exists between parents in order to construct genetic maps.

- **Genotyping in segregating population**

  Once polymorphic markers have been revealed, they must be screened across individuals of segregating population including parents and F_1. All genotypic data was collected (Fig. 10).
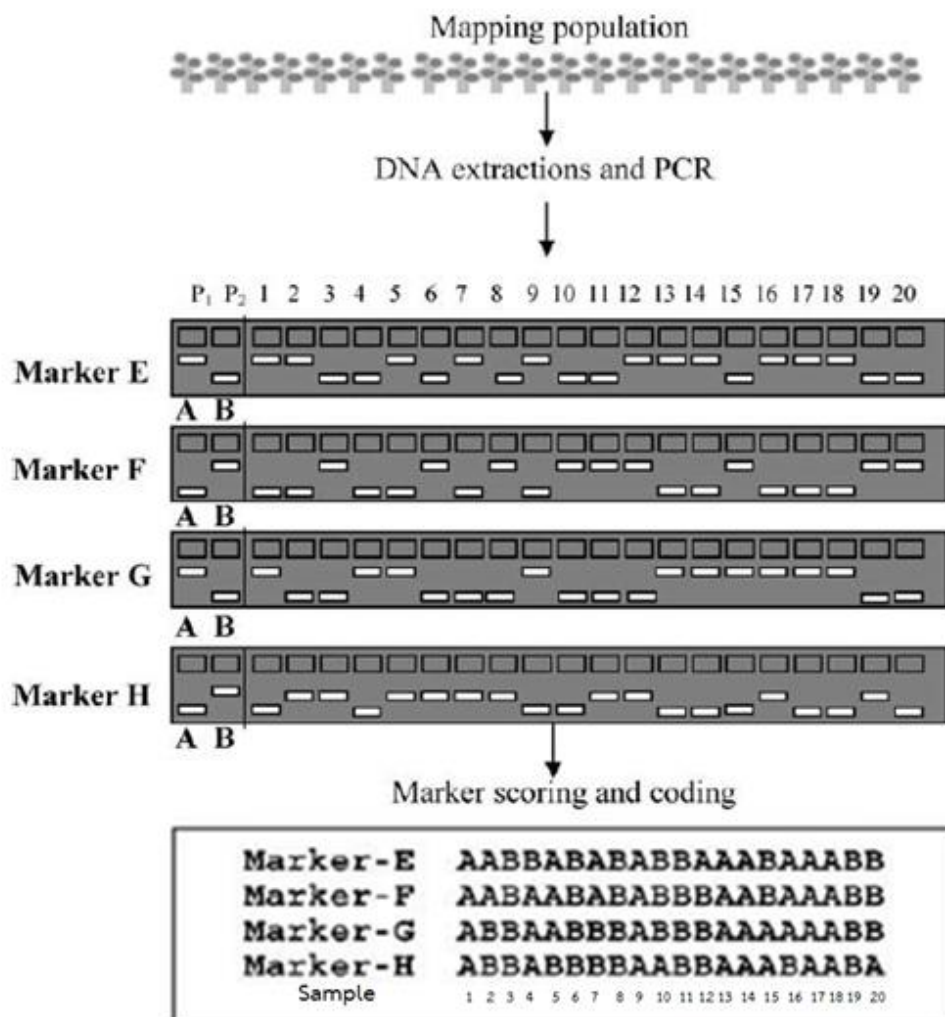
Figure 10 The diagram of genotyping across segregating population to construct linkage map (Collard *et al.,* 2005).

4. Construction of linkage maps

A linkage map or genetic map shows the relative distance of molecular markers along a chromosome that is determined by the recombination frequency between markers during crossing over of homologous chromosomes. A unit for measuring genetic linkage is centimorgan (cM) or map unit (m.u.). It is defined as the distance between markers or loci positions. The construction of genetic maps or linkage maps requires genotyping data and computer programs (Fig. 11). Commonly used software programs to construct linkage maps including JoinMap (Van Ooijen, 2006; Van Ooijen and Voorrips, 2006), R/qtl (Broman *et al.,* 2003), Mapmanager QTX (Meer *et al.,* 2004).
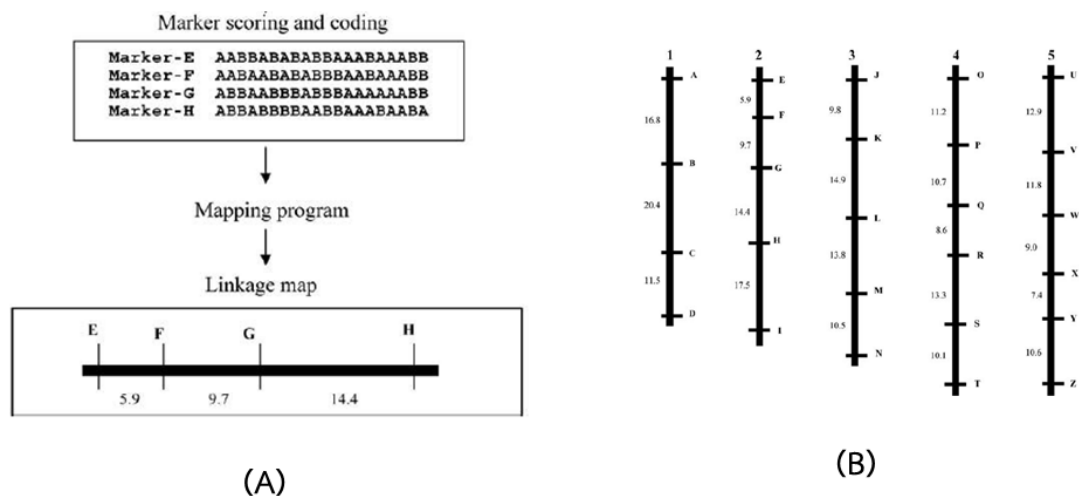
Figure 11 The linkage map was constructed by computer program using genotyping code (A). Five linkage groups could be represented each chromosomes (B) (Collard *et al.,* 2005).

5. QTL analysis

QTL analysis requires phenotypic data, genotypic data and powerful statistical methods to localize the particular region or gene on chromosome that controlling to phenotype or quantitative traits. There are three widely used methods for detecting QTLs, including single marker analysis, simple interval mapping and composite interval mapping (Fig. 12) (Collard *et al.,* 2005). There are many software programs for QTLs detection such as MapQTL6 (Van Ooijen and Kyazma, 2009), R/qtl (Broman *et al.,* 2003), WinQTLCart (Wang *et al.,* 2006).

- Single marker analysis or single point analysis (SMA), it is the simplest method such as t-tests, ANOVA, linear regression to detect QTL. The analysis can be performed using basic statistical software and does not require a complete linkage map.

- Simple interval mapping (SIM) method requires linkage maps and analyses interval between adjacent pairs of linked markers along chromosome.

- Composite interval mapping (CIM) method is a combination SIM and multiple regressions. CIM adds background loci to SIM. Background markers are usually 20-40 cM apart. CIM is more precise and effective compare to SMA and SIM.
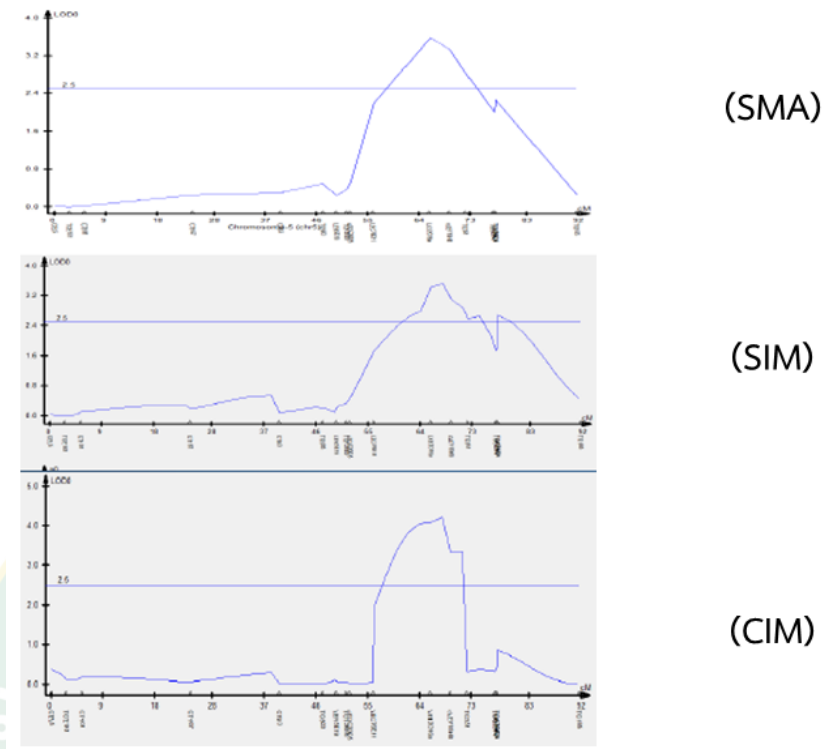
Figure 12 Comparison of three analysis methods, Single marker analysis (SMA), Simple interval mapping (SIM) and Composite interval mapping (CIM) (Van *et al.,* 2009).

2.12 Public watermelon reference genome

Knowledge of genome sequences is necessary for basic biological research and crop improvement. A high quality draft genome sequence of the East Asia watermelon cultivar 97103 ($2n = 2X = 22$) had been published (Guo *et al.,* 2013). Watermelon genomics databases (CuGenDB) have been available (http://cucurbitgenomics.org/) (Fig. 13). CuGenDB includes all resources of genomes, transcriptome profiles, annotations, genetic maps, markers, biochemical pathways and comparative genomic analysis (Zheng *et al.,* 2019; Zheng *et al.,* 2018). There are two watermelon genome databases, watermelon (97103) genome (Guo *et al.,* 2013), watermelon (Chaleston Gray) genome (Levi *et al.,* 2011) and one watermelon EST collection version 2.0 (Guo *et al.,* 2011; Levi *et al.,* 2006).

Figure 13 Watermelon genome databases (CuGenDB) (Zheng *et al.,* 2019).

Source: http://cucurbitgenomics.org/

2.13 Gene expression analysis

The expression studies are directed to detect and quantify messenger RNA (mRNA) levels of a specific gene. The development of the RNA-based gene expression studies began with the Northern Blot (Alwine *et al.,* 1977). Today, many of the techniques for quantification of RNA are deprecated because other new techniques provide more information, rapid, sensitive and reliable method. Currently the most widely used techniques are semi-quantitative RT-PCR, quantitative RT-PCR (qRT-PCR), expression microarrays, and RNAseq for the transcriptome analysis (Gachon *et al.,* 2004; Grosdidier *et al.,* 2017; Martiansyah *et al.,* 2018). Despite the greater accuracy of recently developed techniques such as real-time RT-PCR (qRT-PCR), semi-quantitative RT-PCR methods are still widely used and appropriate for many purposes in plant studies (Choquer *et al.,* 2003; Marone *et al.,* 2001; Tang *et al.,* 2007; Torp *et al.,* 2006).

Semi-quantitative RT-PCR

The semi-quantitative method provides a combination of traditional PCR and transcript quantification at a relatively low cost (Marone *et al.,* 2001). Firstly, the RNA isolation was performed under strict procedure to synthesize the best quality of cDNA. Secondly, the accumulation of gene transcript was determined by RT-PCR. Thirdly, the quantification of transcript expression was normalized using the expression of a reference gene such as housekeeping genes on a gel electrophoresis analyzer. It is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified, but also so that amplification is in the exponential range and has not reached a plateau yet. The optimal number of cycles on both internal control (housekeeping genes) and the target RNA has to be in the same range so that both can be measured on the same gel. Fourthly, data and statistical analysis were carried out.

The measurement resulted in band intensity as the expression unit. The ratio between the target sample RNA and housekeeping gene band intensity was calculated to normalize for initial variations in sample concentration resulting relative expression. Mean and standard deviation were calculated after normalization to housekeeping gene (Martiansyah *et al.,* 2018).

Quantitative RT-PCR (qRT-PCR)

The most quantitative and widely used technique is reverse transcription coupled to quantitative real time polymerase chain reaction (qRT-PCR). These assays are reproducible, quantitative, fast and can be adapted to study model and non-model plant species without the need to have whole genome or transcriptome sequence data available. qRT-PCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in each cycle (Gibson *et al.,* 1996). Real-time PCR (qPCR) uses fluorescent reporter dyes to combine the amplification and detection steps of the PCR reaction in a single tube. A SYBR Green-based fluorescence qRT-PCR assay is easy to establish even in non-specialist laboratories. SYBR Green fluorescence is measured after each cycle (real-time) and increasing levels of fluorescence during the qPCR are indicative of the amplification of double-stranded PCR fragments (Abdallah, 2016).

The two most commonly used methods to analyze data real-time qPCR experiments are absolute quantification and relative quantification. Relative quantification relates the PCR signal of the target transcript to internal control (housekeeping genes). The $2^{-\Delta\Delta C_T}$ method is a convenient way to analyze the relative changes in gene expression from real-time qPCR experiments. The C_T values provided from real-time PCR instrument. The data were analyzed using the equation:

$$\text{Where } \Delta\Delta C_T = (C_{T,Target} - C_{T,Actin})_{Time \ x} - (C_{T,Target} - C_{T,Actin})_{Time \ 0}$$

Time x is any time point and Time 0 represents the 1x expression of the target gene normalized to actin. The mean C_T values for both the target and internal control genes were determined at time zero. The relative expression of target, normalized to actin and relative to a calibrator, is given by Livak and Schmittgen (Livak and Schmittgen, 2001).

$$\text{Amount of target} = 2^{-\Delta\Delta C_T}$$

CHAPTER THREE

Materials and Methods

3.1 Materials

Instruments

1. High Throughput Homogenizers machine (VWR^{TM})

2. Incubator (Memmert)

3. Freezer -20 °C (Panasonic)

4. Micro benchtop centrifuges machine (Thermo fisher sciences)

5. Nano-Drop8000 (Thermo science)

6. Nexar® In-Line Liquid Handling And Assay Processing System (Douglas Scientific)

7. Soellex PCR Thermal Cyclers (Douglas Scientific)

8. Microarray centrifuges machine (Douglas Scientific)

9. Araya In line Fluorescence Detection System (Douglas Scientific)

10. Araya cluster analysis software (East-West Seed Company Limited)

11. QIAquant®real-time cycler (QIAGEN, USA)

12. Multichannel Pipettes 8 channels, P10, P50 and P300 (Labnet)

13. Multichannel Pipettes 12 channels, P10, P50 and P300 (Labnet)

14. Single Pipettes, P10, P20, P200 and P1000 (Eppendorf)

15. Microplate centrifuges machine (Thermo fisher sciences)

16. Agarose gel electrophoresis (Cleaver)

Consumables

1. Glass bead diameter 3 mm. (Sigma)

2. Tips (Axygen)

3. Barrier Tips (Neptune)

4. Nunc™ V96 MicroWell 96-Well x 300uL Assay Microplate (Thermo fisher sciences)

6. Microcentrifuge tube 1.5 ml. (Extragene)

7. Sealing tape (Douglas Scientific)

8. Array tapeTM, 384 wells, 2 ul (Douglas Scientific)

Chemicals and reagents

1. Allele specific forward 1 (F1) primers were synthesized by Integrated DNA Technologies Company.

2. Allele specific forward 2 (F2) primers were synthesized by Integrated DNA Technologies Company.

3. Allele of common reverse (R) primers were synthesized by Integrated DNA Technologies Company.

4. KASP Master mix (LGC)

5. 2x CTAB (cetyl trimethyl ammonium bromind extraction buffer)

6. Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)

7. 75% Ethanol

8. Isopropanol

9. Distilled water

10. 1X TE buffer

11. TRIzol Reagent (Invitrogen)

12. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Litthuania)

13. RapidOut DNA removal kit (Thermo Scientific, Litthuania)

14. 2X GoTaq$^®$ Master Mixes (Promega, USA)

15. SensiFAST^{TM}SYBR$^®$ No-ROX Kit (Bioline GmbH, GERMANY)

16. Tris base (Merck, Darmstadt, Germany)

17. Boric acid (Merck, Darmstadt, Germany)

18. Na$_2$*EDTA 2H$_2$O (Merck, Darmstadt, Germany)

19. NaOH (Ajax Finechem, New Zealand)

20. SYBR™ Safe DNA Gel Stain (Invitrogen, USA & Canada)

21. Agarose (Merck, Germany)

3.2 Plant materials

Viny parent (P$_1$)

KK-6939 or P$_1$ is inbred line (F$_9$ generation). It is a small fruit, dark green rind color with striped and normal long vine or trailing habit which was called viny type in this study. The internode length was about 70 - 90 mm and the average of vine length at flowering stage and harvesting stage were about 570 mm and 3,000 mm, respectively (Fig. 14).
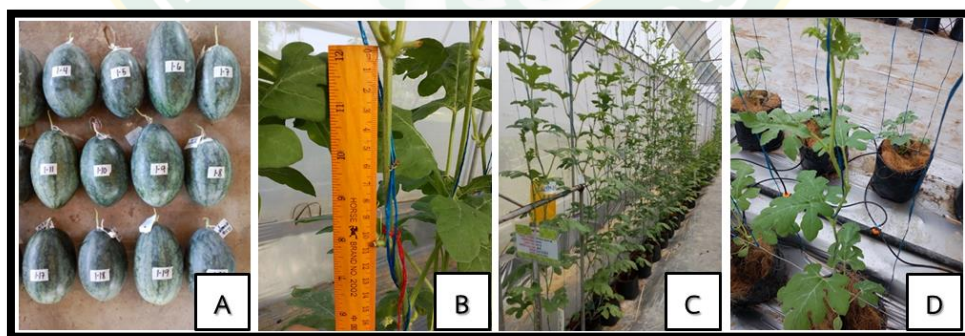
Figure 14 The morphological of KK-6939 or viny /non-dwarf parent with small fruit (A), normal internode length (B), normal long vine (C) and seedling morphology (D).

Dwarf parent (P₂)

TH-15974 or P_2 is inbred line (F_7 generation). It is a small fruit, light green rind color, non- striped and bushy or dwarf habit. The internode length was about 30 - 40 mm and the average of vine length at flowering stage and harvesting stage were about 250 mm and 1,000 mm, respectively (Fig. 15).
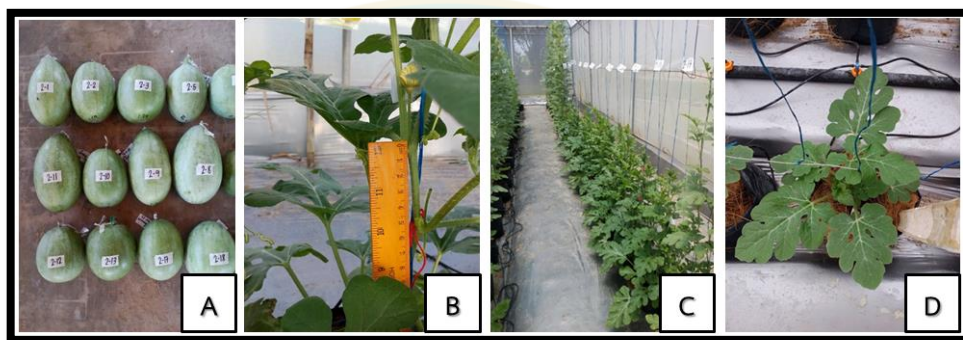
Figure 15 The morphological of TH-15974 or dwarf parent with small fruit (A), short internode length (B), short vine (C) and seedling morphology (D).

Segregating populations

F₂ population for gene inheritance study and genetic mapping

KK-6939 or P_1 (Viny) and TH-15974 or P_2 (dwarf) were crossed to get F_1 progenies. Individual F_1 plant was self to produce an F_2 population. An F_2 population was outcome that will use for gene inheritance study and mapping in the further step. The diagram for F_2 population creating was presented in diagram (Fig. 16).
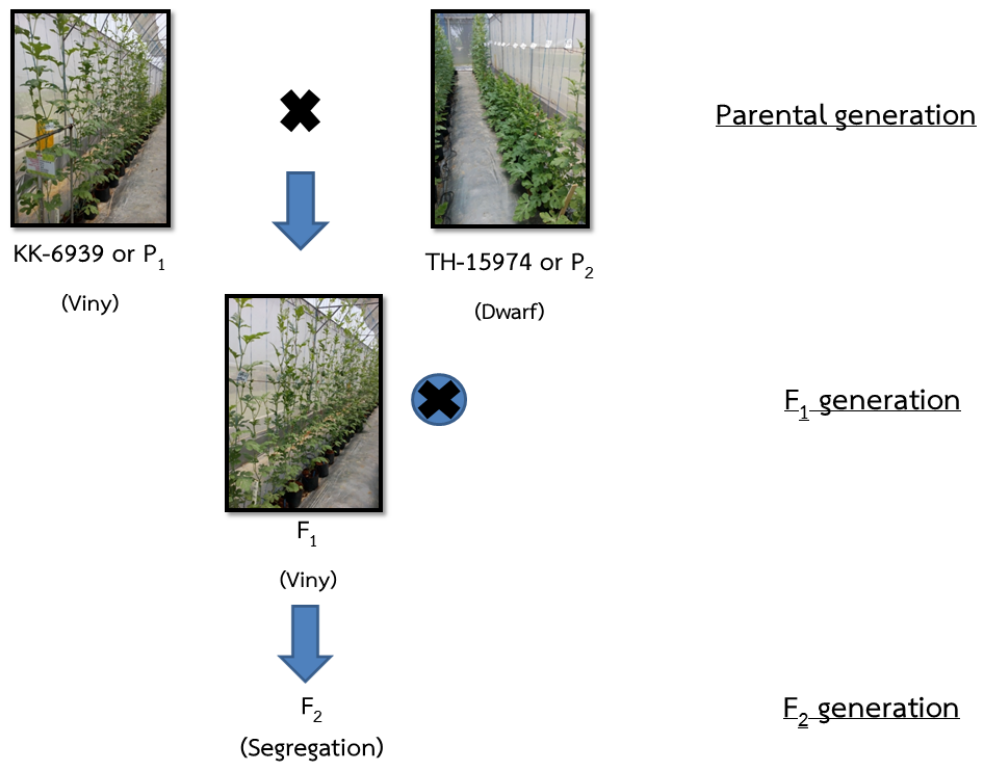
Figure 16 The diagram of an F_2 population was derived from a cross between viny and dwarf parents.

Backcross population for gene inheritance study and marker validation

KK-6939 or P_1 (Viny) and TH-15974 or P_2 (dwarf) are crossed to get F_1 progenies. A hybrid F_1 plant was back crossed to P_1 to generate BC_1P_1 population or back crossed to P_2 to generate BC_1P_2 population. BC_1P_1 and BC_1P_2 population will use for gene inheritance study and marker validation in the further step.

Watermelon inbred lines

A total of 31 various inbred lines that derived from different backgrounds were used for functional marker validation.

3.3 Methods

1. Phenotypic evaluation

1.1 Internode length measurement

1. The internode length of KK-6939 or P_1 (n=8), TH-15974 or P_2 (n=10), F_1 (n=20), F_2 (n=309), BC_1P_1 (n=114) and BC_1P_2 (n=117) from 10th node to 11th node were measured by ruler (Fig. 17).

2. The internode length (mm.) was recorded as numeric data.

3. The histogram was plotted to see the distribution of phenotype.
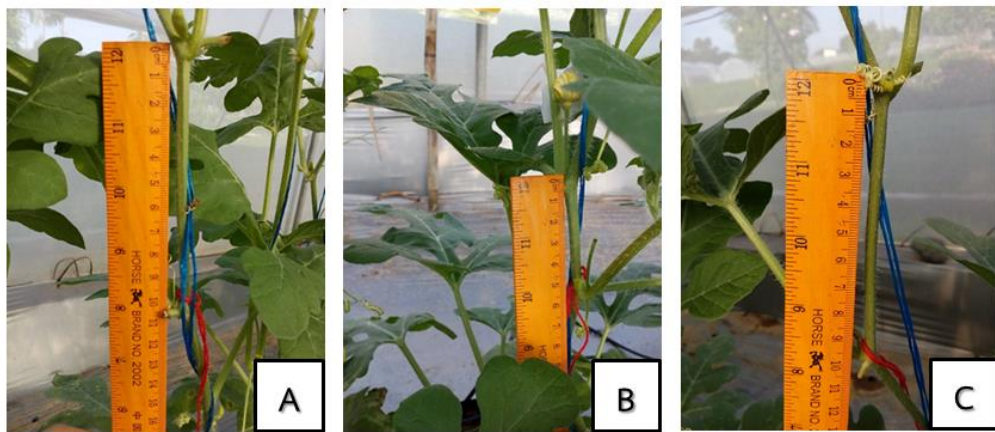
Figure 17 The internode length of KK-6939 or P_1 (Viny parent) (A), TH-15974 or P_2 (dwarf parent) (B) and F_1 (C).

1.2 Vine length measurement

Vine length was evaluated at two stage including flowering stage (measured from crown to 10th node) and harvesting stage (measured from crown to shoot) (Fig. 18).

1. The vine length of KK-6939 or P_1 (n=8), TH-15974 or P_2 (n=10), F_1 (n=20), F_2 (n=309), BC_1P_1 (n=114) and BC_1P_2 (n=117) were measured and recorded as numeric data.

2. The histogram was plotted to see the distribution of phenotype.

The correlation between internode length and two stage of vine length was analyzed by using Pearson correlation analysis.

Figure 18 The vine length of KK-6939 or P_1 (Viny parent) (A), TH-15974 or P_2 (dwarf parent) (B) and F_1 (C).

1.3 Determination of seedling morphological

1. The morphological of two parental lines at early vegetative stage were evaluated and recorded as category data.

2. KK-6939 or P_1 was recorded as "V", the morphological of seedling is Viny (Fig. 19A).

3. TH-15974 or P_2 was recorded as "dw", the morphological of seedling is dwarf (Fig. 19B).

4. The individual plant in populations, F_1 (n=20), F_2 (n=309), BC_1P_1 (n=114) and BC_1P_2 (n=117) were observed and determined the morphological types.

5. Seedling morphological types were recorded in term of "V" or "dw".

6. A chi-square test was used to test the goodness of fit.

KK-6939 or P$_1$
(Viny = "V")

TH-15974 or P$_2$
(Dwarf = "dw")
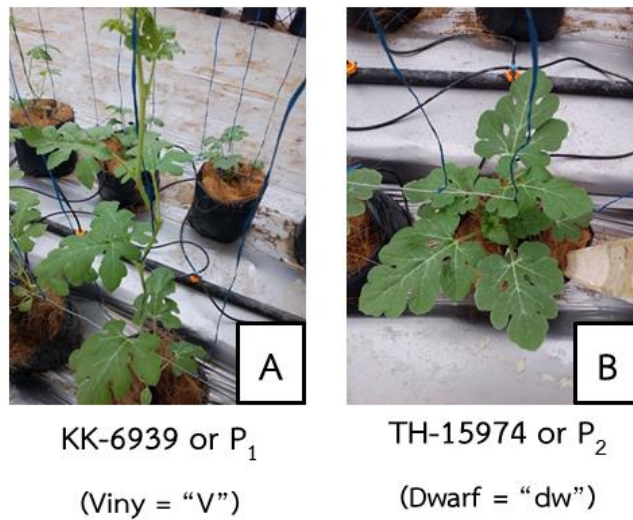
Figure 19 The seedling morphological of KK-6939 or P$_1$ (normal long vine parent) (A) and TH-15974 or P$_2$ (dwarf parent) (B).

2. DNA, RNA extraction and preparation

2.1 DNA extraction

1. One or two young leaves of watermelon were collected into 1.5 ml microcentrifuge tube and stored at -80 °C until use.

2. 3-4 glass beads were added in each sample tubes, then added 500 ul of 2X cetyl-trimethyl-ammonium bromide (CTAB) buffer. 2X CTAB extraction buffer consisting of 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% (w/v) CTAB and 1% (w/v) Polyvinylpyrrolidone (PVP).

3. The leaved samples were ground by using homogenizer machine until finely ground.

4. Mix by inversion and incubate in 60 °C for 30 minutes.

5. Remove from heat, and let cool to room temperature for 4 to 6 minutes.

6. Add 500 ul of Phenol:Chloroform:Isoamyl (25:24:1) and mix by inversion to form an emulsion.

7. After mixing thoroughly, spin at 12,000 rpm for 15 minutes in a centrifuge at room temperature.

8. Transfer 400 ul of top aqueous solution to new 1.5 ml microcentrifuge tubes.

9. Add equal volumes (400 ul) of cold isopropanol. Mix by inversion. If required, place in freezer -20 °C for 30 minutes to accentuate precipitation. The solution may be left at -20 °C to precipitate overnight.

10. Spin at 12,000 rpm in cold condition (4 °C) for 30 minutes.

11. Discard the supernatant, and then add 500 ul of cold 75% ethanol to wash DNA pellet.

12. Spin at 12,000 rpm in cold condition (4 °C) for 5 minutes.

13. Discard the supernatant, and then dry DNA pellet in room temperature for 1 hour or until dry.

14. Add 50 ul of distilled water to dissolve, leave overnight in refrigerator (4 °C) or store at -20 °C until use.

2.2 Total RNA extraction

1. Whole seedling samples were ground in liquid nitrogen to a fine powder and then transfer to 1.5 ml microcentrifuge tube.

2. Add 1 ml of TRIzol reagent and incubate for 5 min at room temperature.

3. Add 200 ul of chloroform and incubate for 5 min at room temperature.

4. Spin at 11,000 rpm in cold condition (4 °C) for 20 minutes.

5. Transfer about 500 - 600 ul of top aqueous solution to new 1.5 ml microcentrifuge tubes.

6. Add equal volumes (500 ul) of isopropanol and incubate for 10 min at room temperature.

7. Spin at 11,000 rpm in cold condition (4 °C) for 10 minutes.

8. Discard the supernatant, and then add 1 ml of cold 75% ethanol to wash RNA pellet.

9. Spin at 7,500 rpm in cold condition (4 °C) for 5 minutes.

10. Repeat wash step one more time (step 8).

11. Dry RNA pellet in room temperature.

12. Add 30 - 50 ul of DEPC treated dH$_2$O. Store at -20 °C until use.

2.3 Genomic DNA removal

The genomic DNA were removed from total RNA by RapidOut DNA removal kit (Thermo Scientific, Litthuania).

1. Mix the following components in RNase-free tubes (Table 4).

Table 4 The component of genomic DNA removal reaction.

Components	Amount
RNA sample	Up to 8.5 μl (5 pg – 2 μg)
10X DNase buffer with MgCl$_2$	1 μl
DNase I, RNase-free	0.5 μl (0.5 U)
Water, nuclease-free	Adjust to the final volume
Total	10 μl

2. Vortex gently or mix by pipetting and then incubate at 37 °C for 30 min.

3. Prior each use, vortex DNase Removal Reagent (DRR) until completely resuspended.

4. Add 2 µl of DRR for each unit of DNase I used.

5. Incubate at room temperature for 2 minutes gently mixing 2-3 times to resuspend the DRR. Avoid splashing.

6. Centrifuge the tube at ≥800 x g for 0.5 – 1 minutes to pellet the DRR.

7. Transfer the supernatant, containing DNA-free and DNase-free RNA into a new tube. Take care not transfer any of DRR.

2.4 First strand cDNA synthesis

The 1st strand cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Litthuania).

1. After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

2. Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order (Table 5).

Table 5 The component of first strand cDNA synthesis reaction.

Components	Amount
Total RNA	0.1 ng – 5 µg
Oligo (dT)$_{18}$ primer	1 µl
Water, nuclease-free	Adjust to the final volume
Total	12 µl

3. Mix gently, centrifuge briefly and incubate at 65 ºC for 5 minutes. Chill on ice, spin down and place the vial back on ice.

4. Add the following components in the indicated order (Table 6).

Table 6 The component of first strand cDNA synthesis reaction.

Components	Amount
5X Reaction buffer	4 µl
RiboLock RNase Inhibitor (20 U/ µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV RT (200 U/ µl)	1 µl
Total	20 µl

5. Mix gently and centrifuge briefly.

6. For oligo (dT)$_{18}$ primed DNA synthesis, incubate for 60 minutes at 42 °C.

7. Terminate the reaction by heating at 70 °C for 5 minutes.

The reverse transcription reaction product can be directly used in PCR applications or stored at -20 °C for less than one week. For longer storage, -70 °C is recommended.

3. QTL mapping

3.1 DNA preparation

The DNA was quantitated on a NanoDropTM 8000 Spectrophotometer to measure concentration and assess the purity of the DNA through standard A260/A280 and A260/A230 ratios. The KASP assay required final concentration 10 ng/ul of DNA. The DNA was diluted with distilled water for PCR reaction in the next step.

3.2 SNP primers of watermelon for QTL mapping

A total 346 SNP primers, which were distributed across the watermelon genome, were designed by East West Seed Company. Two lines of watermelon were selected to perform transcriptome sequencing by BGI Company. Then, short reads were assembled by using reference assembly. SNPs were called and subsequently were designed to SNP primers (Fig. 20).
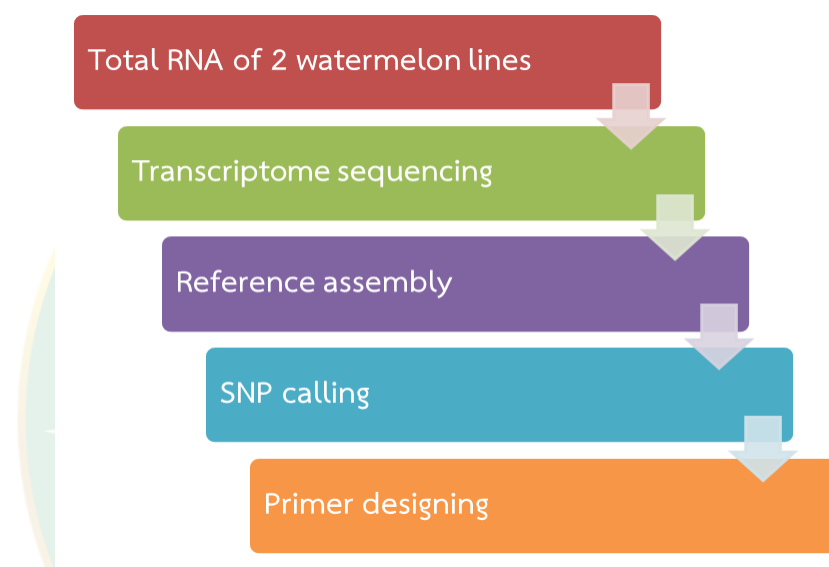
Figure 20 SNP primers development process.

3.3 KASP PCR reaction preparation

Stock and working SNP primers preparation

The dried oligos (primers) were dissolved by adding TE buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.0) to make a 100 μM of stock solution. Then, working primers of each SNP primer were prepared. Working primer consist of the mixture of allele specific forward 1 (F1), allele specific forward 2 (F2) and common primer (R) with optimize ratio (table 7) which called KASP assay mix.

Table 7 The preparation of working SNP primer KASP assay mix.

Primers	Volume (ul)	Final conc. (µM)
100 µM forward 1 (F1)	12	12
100 µM forward 2 (F2)	12	12
100 µM reverse (R)	30	30
Distilled water	46	-
Total volume	100	-

The KASP genotyping reaction

The KASP genotyping reaction comprises three components (Fig. 21).

- DNA template concentration about 10 ng/µl.
- KASP assay mix or working primers contain the allele specific primers.
- KASP master mix, which contains the two universal FRET cassettes (FAM and HEX), ROX™ passive reference dye, *Taq* polymerase, free nucleotides and $MgCl_2$ in an optimized buffer solution.
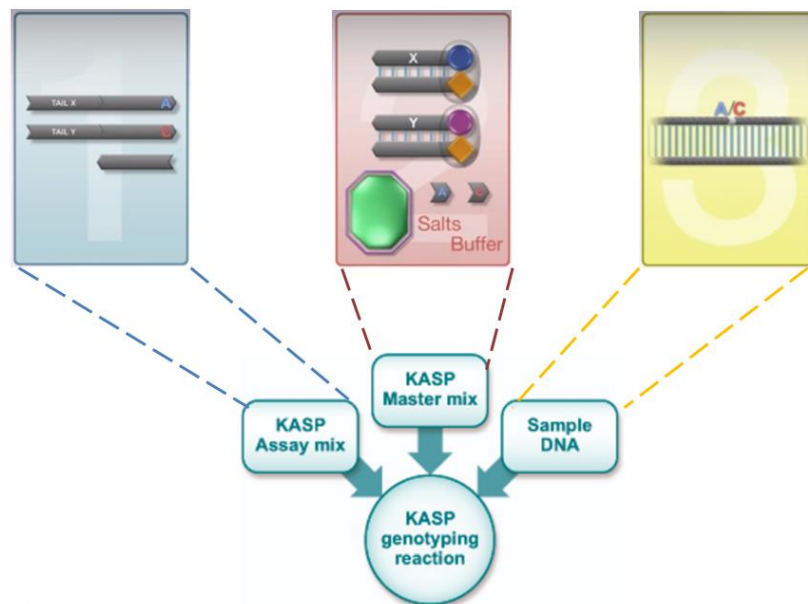
Figure 21 The KASP genotyping reaction.

Source: BIOSEARCH TECHNOLOGIES GENOMIC ANALYSIS BY LGC

The KASP genotyping

1. Assay plates were prepared by adding 2X KASP master mix and working primer in Nunc™ V96 MicroWells plate.

2. Sample plates were prepared by adding 10 ng/µl of DNA sample in Nunc™ V96 MicroWells plate.

3. Sample plates and Assay plates were took to Nexar® In-Line Liquid Handling to transfer DNA samples and assay mix to Array tape^{TM} , 384 wells, then PCR cocktails in Array tape^{TM} were sealed by sealing tape.

4. Sample array tape^{TM} were centrifuged at 5,000 rpm for 10 minutes.

5. Sample array tape^{TM} were put into Soellex PCR Thermal Cyclers.

PCR program

Step 1 94 °C for 15 minutes. (hot-start activation)

Step 2 94 °C for 20 seconds. (denature)

Step 3 65 - 57 °C for 60 seconds.

(Deceasing 0.8 °C /cycle)

Repeat step 2-3 for 10 cycles

Step 4 94 °C for 20 seconds. (denature)

Step 5 57 °C for 60 seconds. (for annealing and extension)

Repeat step 4-5 for 26 cycles.

6. After the PCR was done, array tapes[TM] were dried and briefly spin by Microarray centrifuges machine.

7. Array tapes[TM] were feed to Araya In line Fluorescence Detection machine for detect fluorescence signal of PCR product.

8. Numeric data from Araya In line Fluorescence Detection machine was analyzed and transformed to cluster plot by Araya cluster analysis software (East-West Seed Company Limited).

Whole process of KASP genotyping was presented in Fig. 22.

Nexar® In-Line Liquid Handling to transfer DNA samples and assay mix to Array tape

Array tape contains DNA sample and assay mix

Soellex PCR Thermal Cyclers

Araya In line Fluorescence Detection machine

Araya cluster analysis software
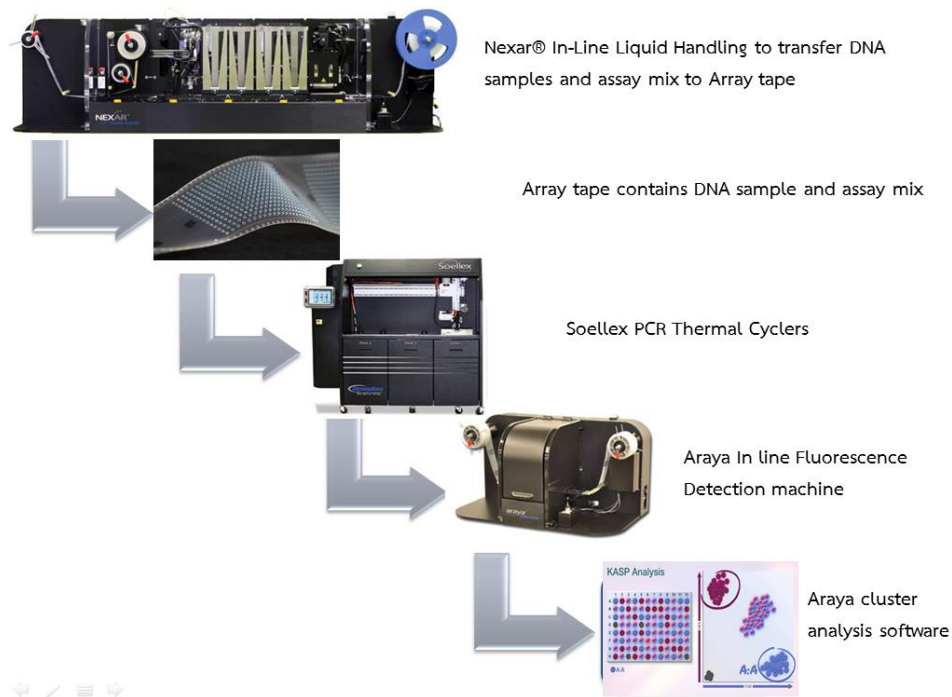
Figure 22 Whole process of KASP genotyping system.

3.4 Genotyping assessment for QTL mapping

Three bulk samples, KK-6939 (P_1), TH-15974 (P_2) and F_1 were screened with 346 watermelon SNP primers to detect the polymorphic primers between two parental lines. Then, those polymorphic SNPs were used for genotyping of 309 individuals of F_2 population. This genotypic data of F_2 population was used to construct genetic map and seedling morphology, which represented *dw* locus using JoinMap Version 4.0 software (Van Ooijen and Voorrips, 2006). Map construction excluded markers with obvious segregation distortion from the expected Mendelian segregation ratios of 1:2:1. Markers were positioned on linkage groups based on independence LOD threshold values of 6.0–10.0. Linkage analysis and marker order assignment were carried out using the regression mapping algorithm. Recombination fractions between markers were converted to map distances in cM using the Kosambi mapping function. Vine length and internode length traits were assumed as a quantitative trait locus (QTL) site and

then used QTL analysis to locate vine length and internode length QTL region. QTLs for two phenotypes were identified using the Interval Mapping program by MapQTL 6.0 software (Van Ooijen, 2009; Van Ooijen and Kyazma, 2009) to detect QTL region which correspond to dwarf.

3.5 Fine mapping and localize dw locus on watermelon genome

The initial genetic map was constructed with polymorphic SNP markers and then *dw* locus was identified on watermelon chromosome. Fine mapping was initiated in the target region by selecting more SNP markers which located surrounding target region by using the physical map information from watermelon genome database (Guo *et al.,* 2013). Those new SNPs were surveyed polymorphic between two parental lines and then those informative SNPs were conducted genotyping in F_2 population. The linkage map or genetic map and QTL analysis were re-calculated to generate high-resolution linkage and re-localized *dw* locus. Subsequently to minimize the genetic interval of the *dw* locus region and then candidate genes were annotated by using the 97103 watermelon reference genome (Guo *et al.,* 2013).

4. Dwarf gene identification and sequence analysis

Sequence of target genes were obtained using the specific primers, which were designed from the watermelon reference genome (Table 8). Full length sequences of target genes were amplified by using genomic DNA while coding region (CDs) sequences were obtained from total RNA. Genomic DNA and total RNA were extracted from the whole seedling plant using CTAB extraction as described in DNA extraction and TRIzol Reagent (Invitrogen) extraction as described in RNA extraction, respectively. First strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania) as described in first strand cDNA synthesis. The PCR was carried out in a reaction containing 1 × GoTaq® Master Mixes (Promega, USA), 0.67 μM of each

primer (07F with 07R1 and 08F1 with 08R1) and 50 ng genomic DNA or cDNA. Amplifications of candidate genes were performed under the following conditions: 95 ℃ for 5 minutes; 35 cycles of 30 seconds at 95 ℃, 45 seconds at 55 ℃ and 1.30 minutes at 72 ℃, followed by a final extension step at 72 ℃ for 10 minutes. Amplification products were analyzed on 1.5% agarose gel and sent to 1st BASE company for sequencing. The complete sequences *Cla015407* and *Cla015408* of five inbred lines were submitted to GenBank.

Table 8 Specific primer sequence of target genes and expected fragment sizes.

Genes	Primer sequence (5′ – 3′)		Expected size (bp)
	Forward	Reverse	
Cla015407	07F; ATGGGAAGCATCAAAATAACCGAA	07R1; TTAACCTACTTTAACCTGGCTGTG	1,257
		07R2; AAGGCATTGCCAATGAGATGA	235
Cla015408	08F1; ATGATTGGATTCATCCCTTCCTTCA	08R1; TTAGCCTACTTTCACCTGGCTA	1,623
	08F2; CCTCAAGACTACACCAAGCAC	08R2; ACACTGCCGAACTCTTTTCC	192
Cla007792 (actin)	F; CCATGTATGTTGCCATCCAG	R; GGATAGCATGGGGGTAGAGCA	220

5. Expression analysis of dwarf candidate genes by quantitative RT-PCR

The expression level of dwarf candidate genes were investigated by semi-quantitative PCR and quantitative RT-PCR (qRT-PCR) in five inbred lines using the dwarf candidate gene specific primers which were 07F with 07R2 and 08F2 with 08R2 together with *Cla007792* actin gene primer was used as internal control (Table 8). Semi-quantitative RT-PCR for dwarf candidate gene was performed for 28 cycles by using the dwarf candidate genes specific primer as mentioned previous with 1X Mytaq[TM] HS Red Mix (Bioline, UK). The reaction of semi-quantitative RT-PCR was performed for

28 cycles, with 30 seconds at 95 ℃, 45 seconds at 55 ℃ and 50 seconds at 72 ℃. The expression level of dwarf candidate gene was visualized by 1.5% agarose gel. Whereas quantitaive RT-PCR was used for analyzed transcript levels with the SensiFAST™ SYBR® No-ROX Kit (Bioline GmbH, GERMANY) and the QIAquant® real-time cyclers (QIAGEN, USA). The reactions were performed for 40 cycles, with 5 seconds at 95 ℃, 10 seconds at 60 ℃ and 20 seconds at 72 ℃. The melting curve analysis was performed to verify the specific transcript amplification. All reactions were performed in three times of triplicate and were normalized using the C_T values corresponding to the actin gene. A relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) against viny parent (KK-6939).

6. High throughput functional marker conferring dwarf in watermelon

High throughput functional markers for dwarf in watermelon, a KASP™ marker genotyping was developed based on mutation point detected from sequence alignment of dwarf candidate gene between vine plants and dwarf plants. To validate the functional marker, 31 various inbred lines of vine plant that derived from different backgrounds were conducted to confirm the accuracy of the functional markers.

CHAPTER FOUR

Results

4.1 Phenotypic distribution and correlation analysis

There were four phenotypic data sets including seedling morphological, internode length and two stages of vine length, which were represented growth habit trait. The seedling morphological was classified into two groups including viny and dwarf type depending on their feature appearance at seedling stage (Fig. 23). While the phenotypes of internode and vine lengths were collected in numeric data by measurement.
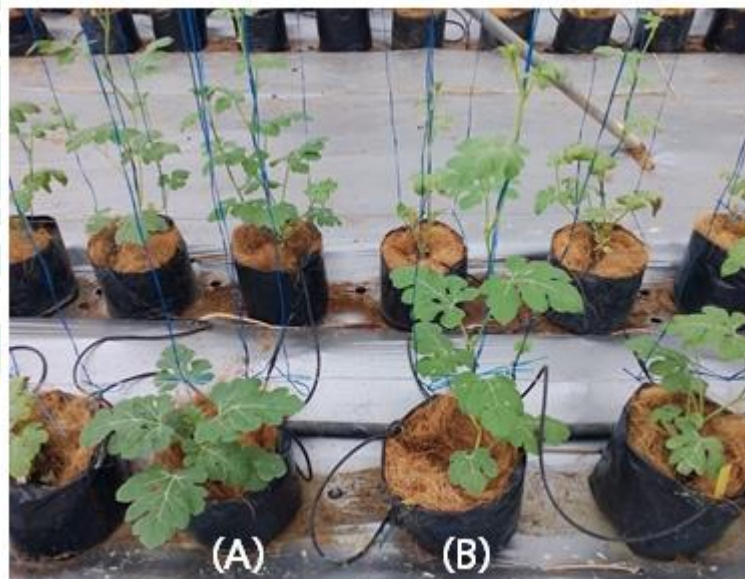
Figure 23 The seedling morphological in F_2 population consisting of two types (A) dwarf and (B) viny.

The histograms of internode and two stages of vine lengths in parents and F_2 population are presented in Fig. 24. The distribution of internode and two stages of vine lengths in F_2 populations showed continuous distribution and skewed to viny phenotype. These distributions suggested that the trait was controlled by one major QTL or single gene and viny phenotype dominant to dwarf phenotype. The correlation analysis of internode length and two stages of vine length by using Pearson correlation analysis showed positive relationship in all combinations. The correlation analysis between internode length and vine length at flowering stage showed highest of similarity value reach to 85%. The comparison of similarity value between internode length and two stage of vine length, the similarity of internode length and vine length at flowering stage was higher than internode length and vine length at harvesting stage because of the measurement of vine length at the flowering stage was measure at the same node as internode length. The correlation between internode length, vine length at flowering stage and vine length at harvesting stage with their similarity value was presented in Fig. 25.
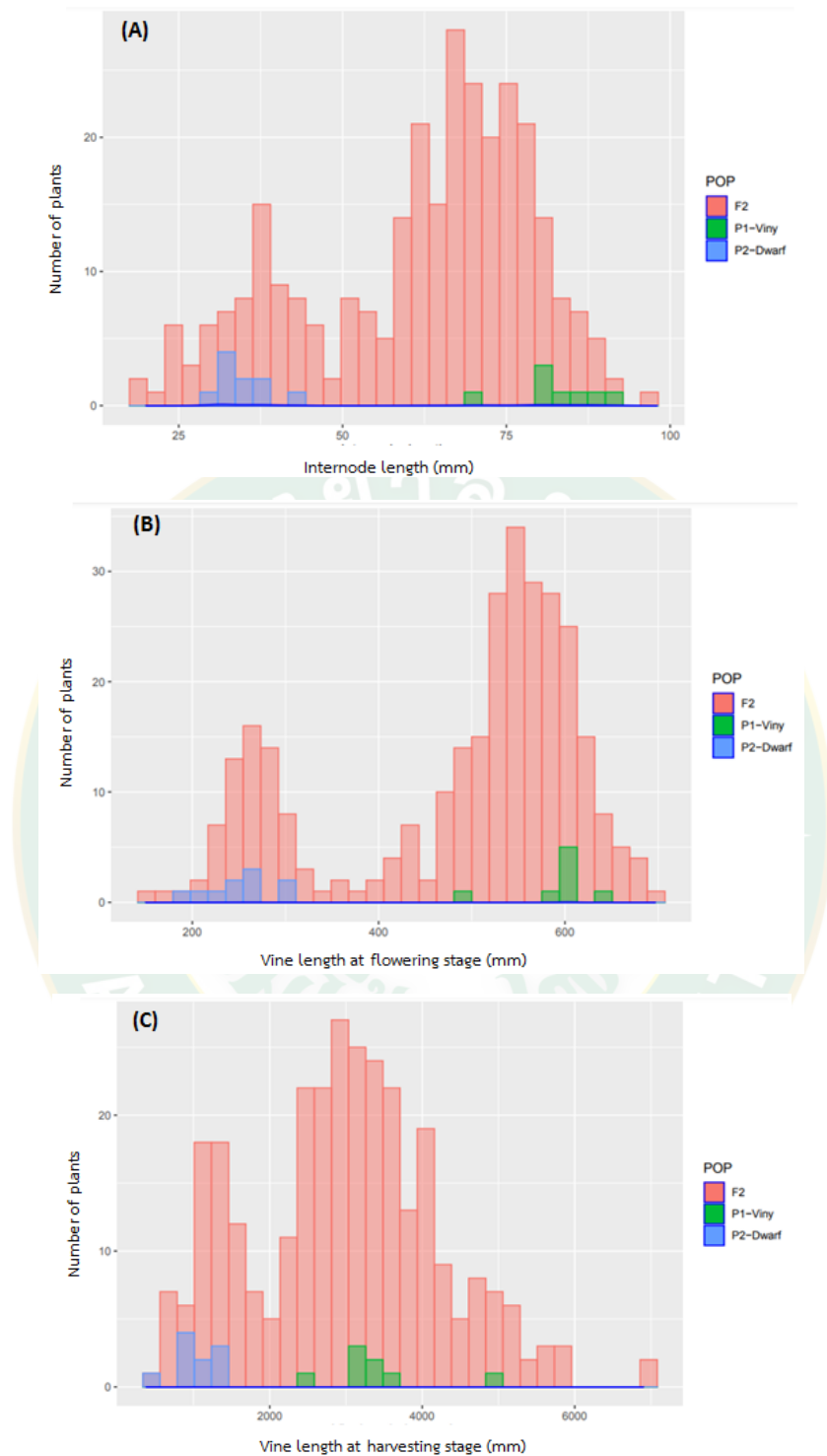
Figure 24 Histograms of internode length (A), vine length at flowering stage (B) and vine length at harvesting stage (C) in F_2 population (pink histogram), KK-6939 or P_1 viny parent (green histogram) and TH-15974 or P_2 dwarf parent (blue histogram).
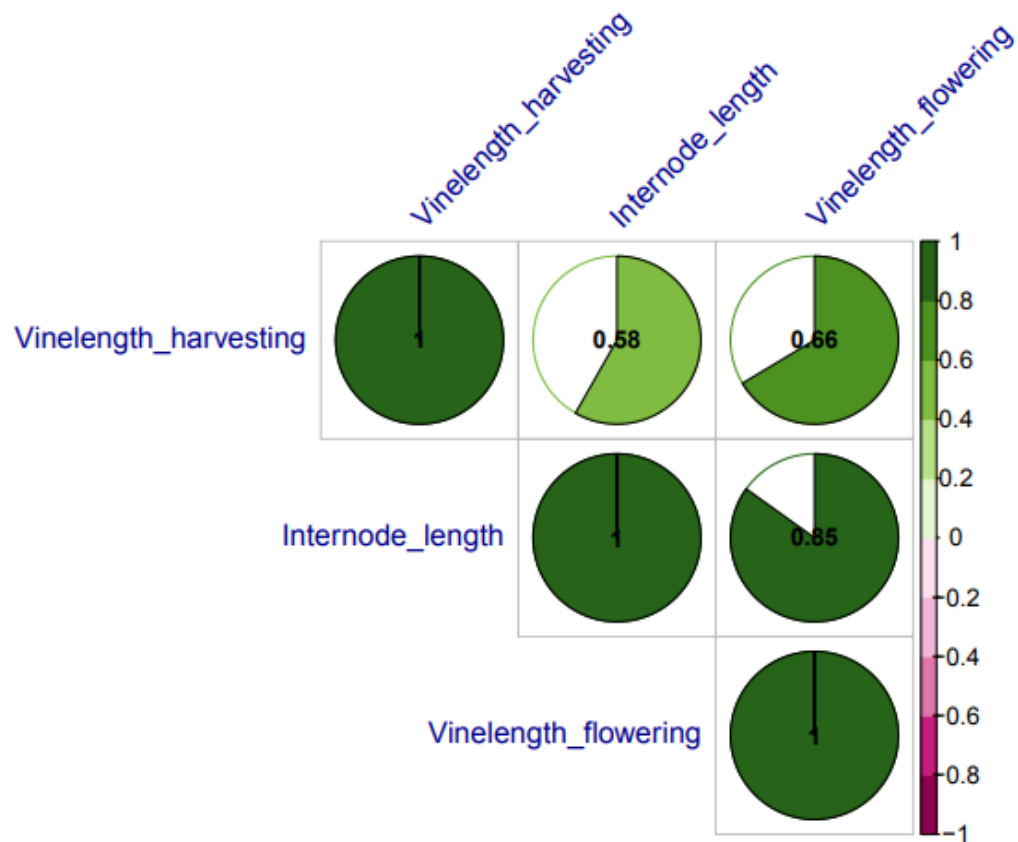
Figure 25 The correlation analysis between internode length and two stages of vine length of F_2 population by Pearson correlation analysis.

4.2 Phenotypic evaluation and dwarf gene inheritance study

The phenotype of F_1 plants exhibited completely viny (Table 9). The seedling morphology of individual plants in F_2 population (n = 309) were classified into two groups, consist of 239 viny and 70 dwarf. An average of internode length, vine length at flowering stage and vine length at harvesting stage in viny group were 69.16, 550.90 and 3,460.73 mm, respectively. While the internode length, vine length at flowering stage and vine length at harvesting stage in dwarf group were 35.19, 286.85 and 1,295.78 mm, respectively (Table 9). Chi-square tests were performed in F_2 population to determine the goodness of fit to the single gene model which is 3 : 1 (viny : dwarf) ratio. The χ^2 value was 0.907, which was lesser than 3.84 at the corresponding

confidence level is 95%, then the null hypothesis had been accepted and no significant deviation between expected and observed values (Table 10). Results indicated that the ratio of viny plants to dwarf plants was fitted to the 3 : 1 ratio. The single recessive inheritance results obtained from the F_2 population was confirmed by testing in BC_1P_1 population which derived from a cross between F_1 and KK-6939 which showed all viny and BC_1P_2 population which derived from F_1 and TH-15974. The phenotype BC_1P_2 was consistent with a 1 : 1 expected ratio (χ^2 = 0.214, P = 0.6439) as shown in table 10. These results suggested that a single recessive gene controlled dwarf trait.

Table 9 The phenotypic value summarization and pairwise (mean±SD) comparison of two parental lines and their progenies.

Samples	Observed (plants)	Phenotypes			
		Seedling morphology	Internode length (mm) (Mean ± SD*)	Vine length at flowering stage (mm) (Mean ± SD*)	Vine length at harvesting stage (mm) (Mean ± SD*)
Parent 1 (KK-6939)	8	Viny (V)	80.33 ± 8.34[a]	576.11 ± 53.68[a]	3,023.33 ± 1,048[a]
Parent 2 (TH-15974)	10	Dwarf (dw)	34.9 ± 4.23[b]	250.60 ± 39.26[b]	1,033.3 ± 260.73[b]
F_1	20	Viny (V)	75.00 ± 6.56 [a]	560.23 ± 50.61 [a]	3,100.52 ± 1,020[a]
F_2	239	Viny (V)	69.16 ± 11.01[a]	550.90 ± 63.67[a]	3,460.73 ± 934.46[a]
	70	Dwarf (dw)	35.19 ± 6.82[b]	286.85 ± 88.01[b]	1,295.78 ± 346.22[b]

* Means followed by the same letter in each column are not significantly different in Duncan's multiple range test (DMRT) at alpha 0.05.

Table 10 Genetic analysis of dwarf growth habit crosses between KK-6939 (viny growth habit) and TH-15974 (dwarf growth habit).

Generations	Number of plants	Observed (plants)		Expected ratio	Chi-square (χ^2)	P value
		Viny	Dwarf			
Parent 1 (KK-6939)	8	8	-	-	-	-
Parent 2 (TH-15974)	10	-	10	-	-	-
F_1	20	20	-	-	-	-
F_2	309	239	70	3:1	0.907	0.3409
BC_1P_1 (F_1 x KK-6939)	114	114	-	1:0	-	-
BC_1P_2 (F_1 x TH-15974)	117	56	61	1:1	0.214	0.6439

Significance limit of Chi-square (χ^2) (P = 0.05, df = 1) = 3.84.

$\chi^2 \geq 3.84$ and P value ≤ 0.05 are considered significant.

4.3 QTL analysis and fine mapping

Total of 346 SNP markers screened, 65 SNP markers were polymorphic marker between two parental lines which were distribute on 11 chromosomes (Fig. 26). These SNP markers were applied for initial linkage analysis, of which nine failed to be assigned to any linkage group (LG). Thus, 56 SNP markers together with seedling morphology, which represented *dw* locus, were mapped to eleven LGs and the *dw* locus was localized on a top of chromosome 9. For QTL analysis, only one major QTL on a top of chromosome 9 was detected for internode length and two stages of vine length. The SNP marker "WMSNP-9-88" showed highly significant co-segregation with phenotypes and was located 24.4 cM away from *dw* locus (Fig. 27A). Therefore, fine-mapping was performed to minimize *dw* locus region. A total 104 new SNP markers surrounding the QTL region were designed, only 20 SNP markers showed polymorphism between two parental lines and then those markers were genotyped

in the F_2 population and re-constructed the genetic map. Finally two flanking markers were detected; WMSNP-0002750 and WMSNP-0002780, with an interval map about 3.3 cM or 250 kb (Fig. 27B). As well as one major QTL, which located underlying *dw* lous, was detected in all phenotypes; internode length, vine length at flowering stage and vine length at harvesting stage with LOD score value 62.36 (R^2 = 62%), 96.55 (R^2 = 77.2%) and 51.02 (R^2 = 53.8%), respectively (Table 11, Figure 28).
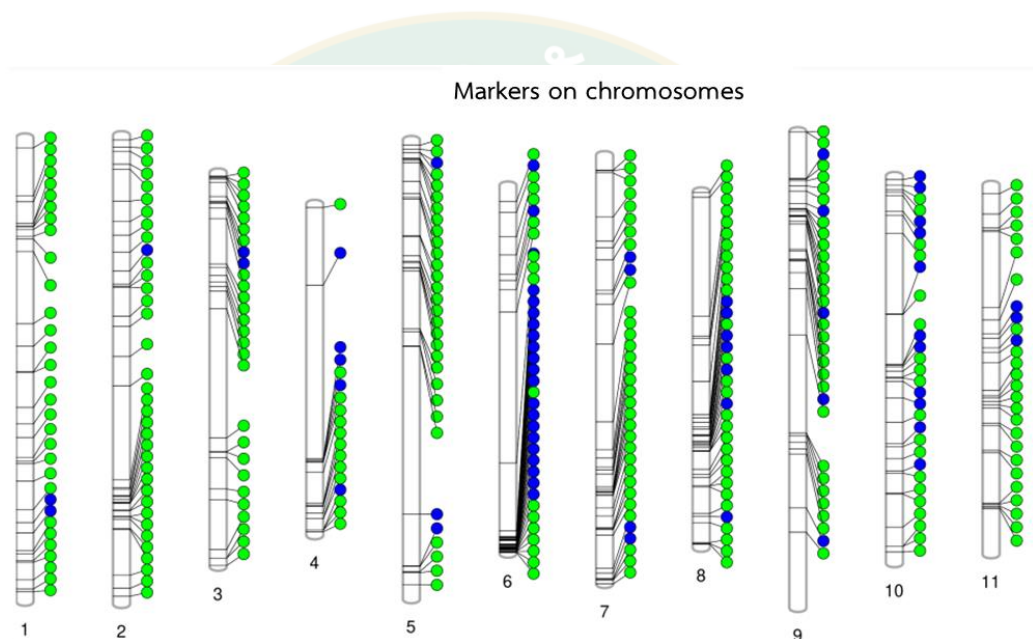
Figure 26 The 346 SNPs distributed in across watermelon chromosome. Green color represented monomorphic markers between two parental lines. Blue color represent polymorphic markers between two parental lines.

Table 11 Major QTL detected for internode and vine length in watermelon.

Trait/QTL	Chr.	Interval		LOD	%R^2
		Genetic map (cM)	Physical map (Mb)		
Internode length	9	0.8 – 4.1	1.65 – 1.90	62.36	62%
Vine length at flowering stage	9	0.8 – 4.1	1.65 – 1.90	96.55	77.2%
Vine length at harvesting stage	9	0.8 – 4.1	1.65 – 1.90	51.02	53.8%
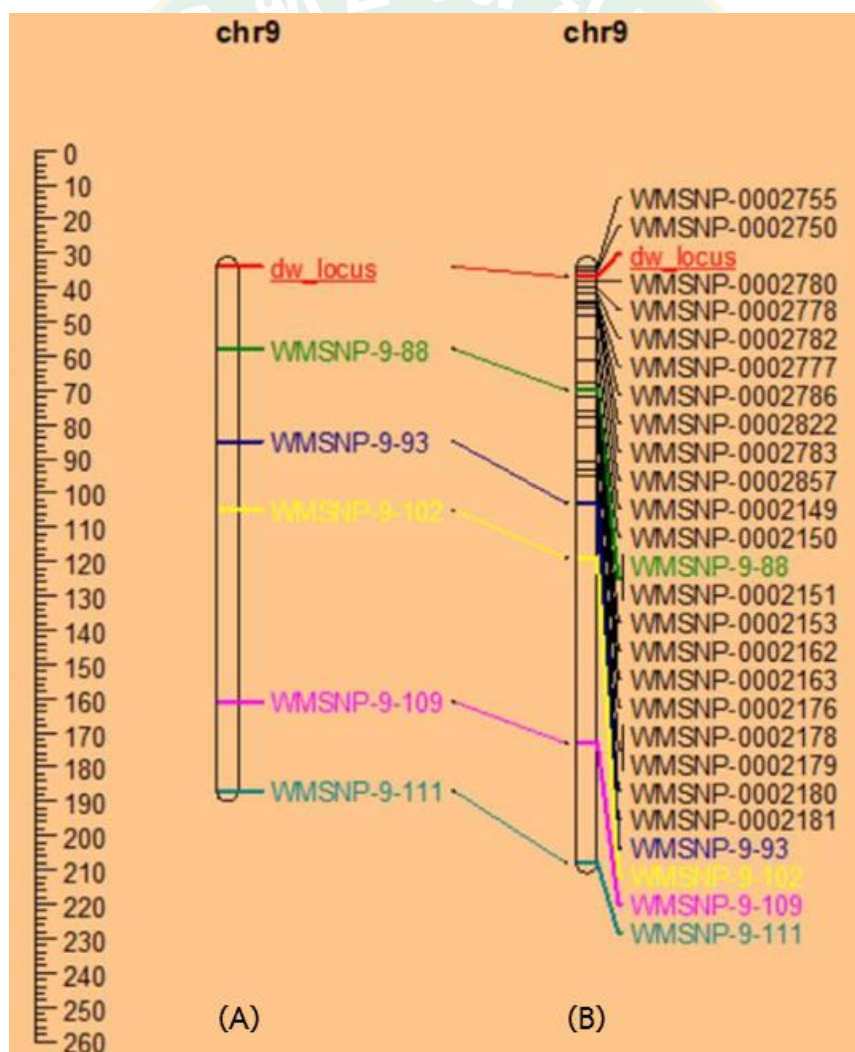
Figure 27 The initial genetic map of *dw* locus on chromosome 9 (A). The genetic map after fine mapped surrounding *dw* locus on chromosome 9 (B).
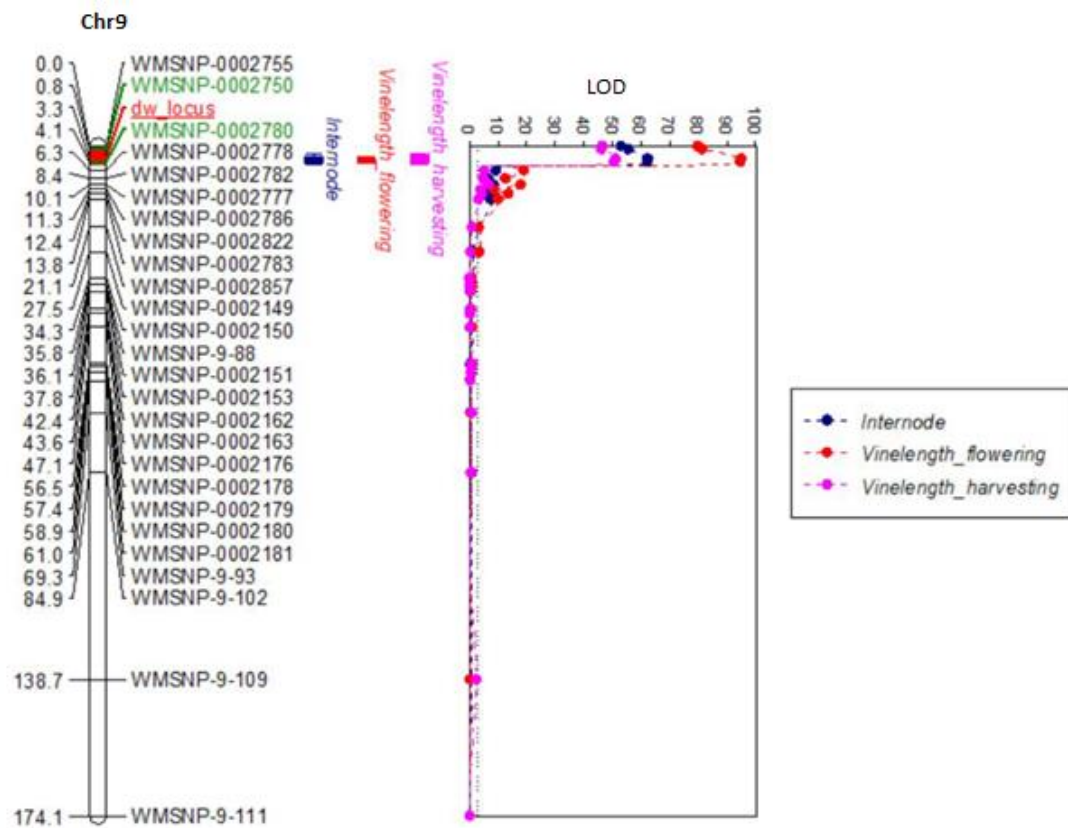
Figure 28 The genetic map of *dw* locus in watermelon chromosome 9, d*w* locus (red labeled) was mapped between WMSNP-0002750 and WMSNP-0002780 SNP markers (green labeled). Composite interval mapping analysis of quantitative trait loci associated with internode length (blue line), vine length at flowering stage (red line) and vine length at harvesting stage (pink line) on chromosome 9.

4.4 The candidate dwarf gene identification

Interval SNP markers were combined with the gene annotation within the major QTL regions to identify candidate genes associated with dwarf growth habit. Candidate genes were annotated by using the "97103" watermelon reference genome database (Guo *et al.,* 2013). According to the annotation of the watermelon reference genome, there were 25 predicted genes in the 250 kb major QTL region between WMSNP-0002750 and WMSNP-0002780 SNP markers (Fig. 29). Of these candidate genes, 20 genes had annotation information (Table 12). As dwarf growth habit is mainly determined by stem growth, the genes that encoded or related to growth hormone were selected which were *Cla015407* and *Cla015408*. Both genes encode a protein predicted to be a gibberellin 3-beta-dioxygenase enzyme (GA3ox), which is a catalyst in gibberellin biosynthesis III pathway that affects stem elongation in plants. The GA3ox enzyme converted GA9 to the bioactive GA4 and GA7 (Fig.2) that could be able recovered dwarf plant to normal plant (Loy and Liu, 1974; Wu *et al.,* 2015). Moreover, many previous studies identified the mutation in GAs related genes that effect to dwarfness in watermelon such as *Cla010726* (GA20ox), *Cla015407* (GA3ox) (Dong *et al.,* 2018; Gebremeskel *et al.,* 2020; Sun *et al.,* 2020; Wei *et al.,* 2019) while *Cla015403* (G subfamily) was not chosen because it was not the same subfamily with *Cla010337* (B subfamily) (Zhu *et al.,* 2019). However the *Cla015408* has not been well characterized in dwarfism. Therefore in this study *Cla015407* and *Cla015408* were potential candidate genes underlying *dw* locus.
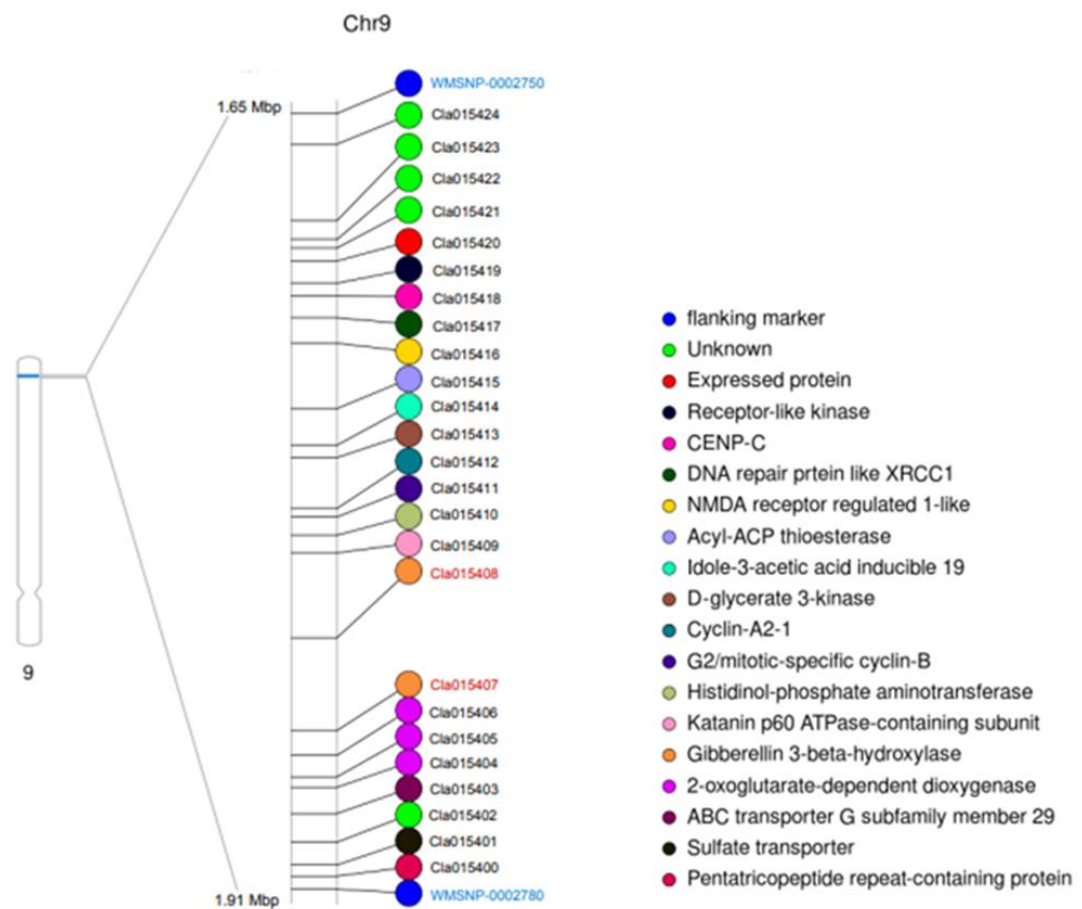
Figure 29 Physical map of watermelon chromosome 9. A total of 25 genes located between WMSNP-0002750 and WMSNP-0002780 SNP markers. Only two candidate genes, *Cla015407* and *Cla015408* (red labeled) are involved in the gibberellin biosynthesis pathway.

Table 12 The list of candidate genes which located between two flanking markers (WMSNP-0002750 - WMSNP-0002780).

Marker/ gene	position (bp)	Protein encoded
WMSNP-0002750	1.65 Mb	flanking marker
Cla015424	1660268..1660982	Unknown
Cla015423	1685975..1686560	Unknown
Cla015422	1692240..1692870	Unknown
Cla015421	1695059..1695678	Unknown
Cla015420	1699541..1703958	Expressed protein
Cla015419	1706846..1709473	Receptor-like kinase
Cla015418	1711170..1716626	CENP-C
Cla015417	1718518..1722713	DNA repair prtein like XRCC1
Cla015416	1727050..1746431	NMDA receptor regulated 1-like
Cla015415	1748873..1751565	Acyl-ACP thioesterase
Cla015414	1761209..1761589	Idole-3-acetic acid inducible 19
Cla015413	1765291..1767390	D-glycerate 3-kinase
Cla015412	1782514..1783426	Cyclin-A2-1
Cla015411	1785242..1787309	G2/mitotic-specific cyclin-B
Cla015410	1791454..1796416	Histidinol-phosphate aminotransferase
Cla015409	1797195..1801483	Katanin p60 ATPase-containing subunit
Cla015408	**1825632..1827254**	**Gibberellin 3-beta-hydroxylase**
Cla015407	**1856847..1858103**	**Gibberellin 3-beta-hydroxylase**
Cla015406	1865167..1867760	2-oxoglutarate-dependent dioxygenase
Cla015405	1872282..1874413	2-oxoglutarate-dependent dioxygenase
Cla015404	1875741..1877969	2-oxoglutarate-dependent dioxygenase
Cla015403	1884562..1891460	ABC transporter G subfamily member 29

Marker/ gene	position (bp)	Protein encoded
Cla015402	1894213..1897458	Unknown Protein (AHRD V1)
Cla015401	1901841..1903265	Sulfate transporter
Cla015400	1905671..1908064	Pentatricopeptide repeat-containing protein
WMSNP-0002780	1.91 Mb	flanking marker

4.5 Gene isolation and sequence comparison

Cla015408 was located upstream of *Cla015407* about 32.47 kb away Both DNA and mRNA were investigated to verify the sequence (Fig. 30). The gene size of *Cla015407* and *Cla015408* were 1,257 bp and 1,623 bp, respectively. The gene structure of *Cla015407* consisted of two exons as same as *Cla015408*. However, exon 1 size of *Cla015408* (377 bp) was shorter than *Cla015407* (503 bp) while the exon 2 of both genes are equal (631 bp). A complete DNA sequence of *Cla015407* gene from dwarf plants (GenBank accession number MZ568772 and MZ568773) were aligned with viny plants (GenBank accession number MZ568769, MZ568770 and MZ568771) was shown in Fig. 31. Whereas in *Cla015408* gene only full length DNA were obtained, then a complete sequence DNA of dwarf plants (GenBank accession number MZ568774 and MZ568775) were aligned with viny plants (GenBank accession number MZ568776, MZ568777 and MZ568778) as shown in Fig. 32. All full length sequences of *Cla015407* and *Cla015408* were shown in appendix A. In the DNA sequence level of the *Cla015407* gene, two SNP variants were detected among five accessions but only one SNP at the 626th nucleotide could distinguish dwarf plants from viny plants completely perfectly. The viny plants presented the "G" allele while dwarf plants presented the "A" allele at the 626th nucleotide. This G allele is an acceptor splice site in intron 1 of viny plant or normal type, whereas the 3'AG splice site of dwarf plants moved to the 639th nucleotide because of the SNP mutation event in dwarf plants (Fig. 31). Additionally,

a 13 bp deletion was detected in cDNA sequences of *Cla015407* gene that obtained from dwarf plants. This 13 bp deletion located at the beginning of the second exon of *Cla015407* gene, lead to frameshift translation and promoted the premature stop codon "TGA" at 656th-658th nucleotide resulting to produce a truncated protein in dwarf plants (Fig. 31). DNA sequence of *Cla015408* that obtained from five inbred lines showed one SNP at intron 1 that could not be classified dwarf plants from viny plants. Moreover, this detected SNP was not in the coding region and then had no effect on the amino acid sequence (Fig. 32).

Figure 30 Illustration of *Cla015408* and *Cla015407* gene map
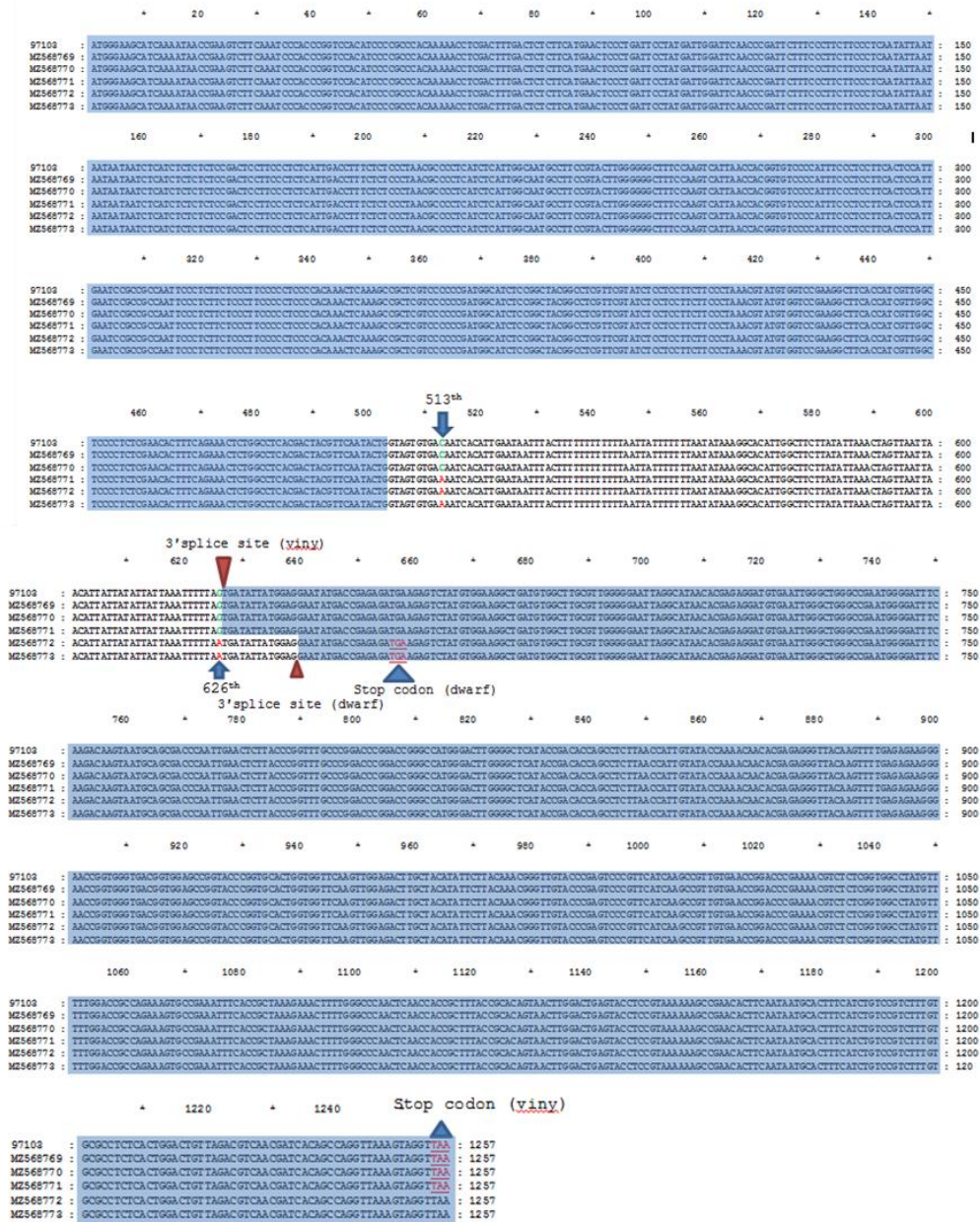
Figure 31 Multiple alignment of Cla015407 among viny lines (MZ568769, MZ568770 and MZ568771), dwarf lines (MZ568772 and MZ568773) and reference genome of watermelon (97103). Blue boxes represented exon regions. There were two SNPs at 513th and 626th nucleotide within intron 1 region. The TAA at 1,255th – 1,257th nucleotide was stop codon of viny plants whereas TGA at 656th – 658th nucleotide was stop codon of dwarf plants.

Figure 32 Multiple alignment of Cla015408 among viny lines (MZ568776, MZ568777 and MZ568778), dwarf lines (MZ568774 and MZ568775) and reference genome of watermelon (97103). Blue boxes represented exon regions. There was one SNP at 622th nucleotide within intron 1 region.

4.6 GA3ox amino acid sequence analysis

GA3ox protein had two crucial domains, which effect to GA3ox function, including non-haem dioxygenase N-terminal domain (DIOX_N domain; IPR026992) and oxoglutarate/iron-dependent dioxygenase domain (FE2OG_OXY domain; IPR005123). Full length amino acid of Cla015407_dwarf, Cla015407_viny were translated from cDNA sequences which were obtained from five inbred lines while *Cla015408* amino acid sequence was obtained from watermelon genome database because of mRNA fragments could not be obtained from five inbred lines. The amino acid of GA3ox that obtained from Cla015407_dwarf, Cla015407_viny and Cla015408 were predicted important domain by SMART (Simple Modular Architecture Research Tool) (Letunic and Bork, 2018). Two important domians, DIOX_N and FE2OG_OXY domain, were detected in Cla015407_viny and Cla015408 whereas Cla015407_dwarf lost FE2OG_OXY domain (Fig. 33). Moreover, full length amino acids of Cla015407_dwarf, Cla015407_viny and Cla015408 were conducted in multiple alignments. In normal plant, Cla015407_viny translated 377 amino acids while in mutant plant, Cla015407_dwarf had premature stop codon lead to translate only 173 amino acids and lost FE20G dioxygenase domain, which is crucial domain of GA3ox, lead to *Cla015407* gene produced nonfunctional enzyme. Whereas, the *Cla015408* amino acid was supposed to produce the functional enzyme and the similarity of amino acid alignment between Cla015407_viny and Cla015408 showed 79% similarity (Figure 34).
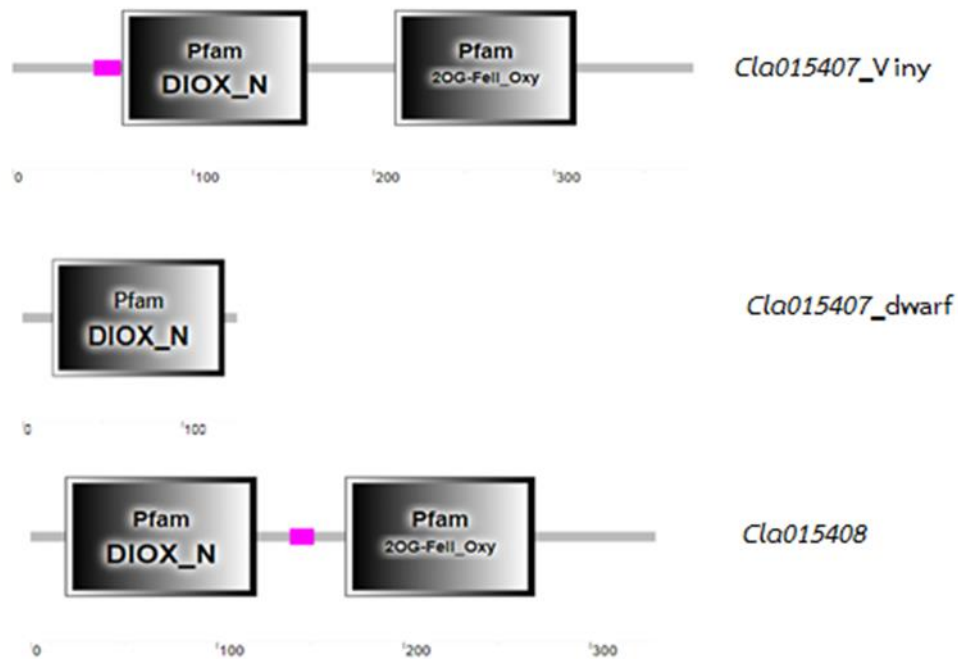
Figure 33 Domain structure of GA3ox protein in Cla015407_viny, Cla015407_dwarf and Cla015408.
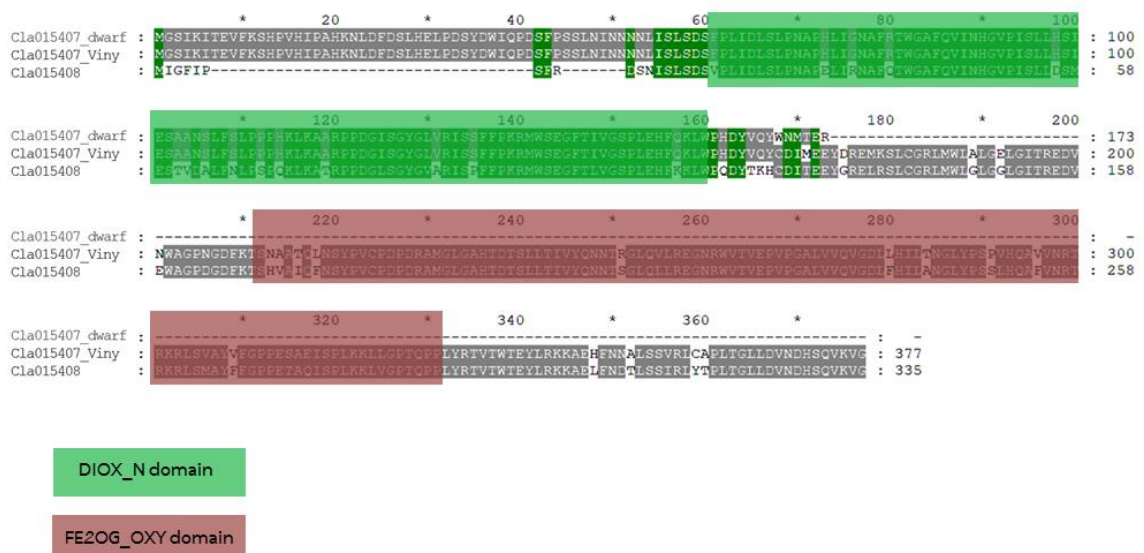
Figure 34 Illustration of two important domains of GA3ox protein and multiple alignment of GA3ox protein of Cla015407_dwarf, Cla015407_viny and Cla015408 amino acid sequences.

4.7 Expression analysis of dwarf candidate genes: *Cla015407* and *Cla015408* gene

There were two candidate genes, *Cla015407* and *Cla015408* that encoded the same protein GA3ox with 79% similarity. Based on the sequence analysis revealed that *Cla015407* in dwarf plants had the point mutation at an acceptor splice site in intron 1, then the alternative splicing happening and resulting in nonfunctional protein. While, in *Cla015408* only one SNP was detected at intron 1 region and could not affect the translation. Therefore, *Cla015408* could be translated as normal amino acid and produced functional protein. The expression analysis was investigated to prove the assumption why *Cla015408* could not function instead of *Cla015407* mutant. The semi-quantitative PCR and qRT-PCR was performed in whole seedling plants at seedling stage. Gene expression levels in *Cla015407* and *Cla015408* were normalized to the expression of actin gene. Consider the semi-quantitative RT-PCR and qRT-PCR, the expression of *Cla015408* was not detected that why *Cla015408* could not produce sufficient GA3ox and could not in place of *Cla015407* in *dw* plant (Fig. 35). The qRT-PCR revealed that relative of *Cla015407* expression was not significantly different among viny and dwarf plants. Moreover the viny plant "KK-28469" was clustered in the same group of dwarf plant (Table 13, Fig. 36). Therefore the expression level of *Cla015407* in this study did not relate to plant types at seedling stage. Although the expression of *Cla015407* could not affect to plant type in this study but the mutation of *Cla015407* lead to produce non-functional protein and result to dwarf phenotype. This result strongly supported *Cla015407* gene played an important role to control watermelon growth habit and suggested that *Cla015408* could not replace of *Cla015407* in dwarf plant at seedling stage. Therefore, the *Cla015407* gene should be a dwarf gene in the TH-15974 line.

Table 13 A relative expression value ($2^{-\Delta\Delta CT}$) of five inbred lines against viny parent (KK-6939) and their pairwise (mean±SD) comparison.

| Gene | Samples | Plant type | A relative expression value ($2^{-\Delta\Delta CT}$) | | | Mean* |
			Rep1	Rep2	Rep3	
Cla015407	KK6939	Viny	1.000	1.000	1.000	1.000 ± 0.0000[a]
	KK28334	Viny	0.862	0.775	0.814	0.817 ± 0.0436[b]
	KK28469	Viny	0.474	0.609	0.348	0.477 ± 0.1306[c]
	TH15974	dwarf	0.625	0.768	0.520	0.638 ± 0.1247[c]
	KK28413	dwarf	0.5334	0.504	0.404	0.480 ± 0.0678[c]

* Means followed by the same letter in each column are not significantly different in Duncan's multiple range test (DMRT) at alpha 0.05.
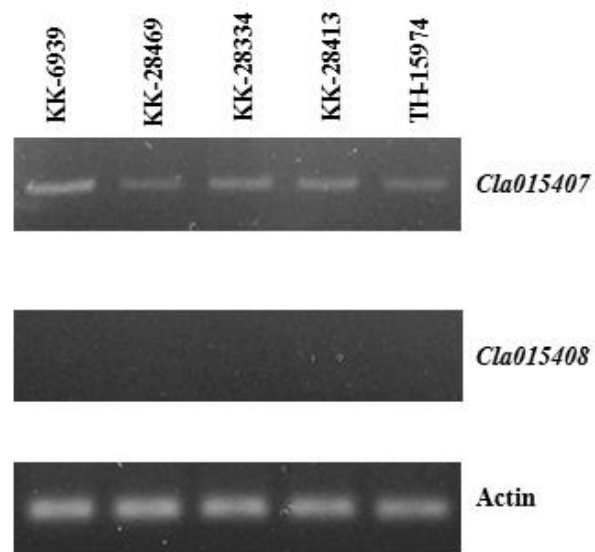
Figure 35 Gene expression analysis of GA3ox (*Cla015407* and *Cla015408*) transcripts by semi-quantitative RT-PCR from five independent watermelon inbred lines.
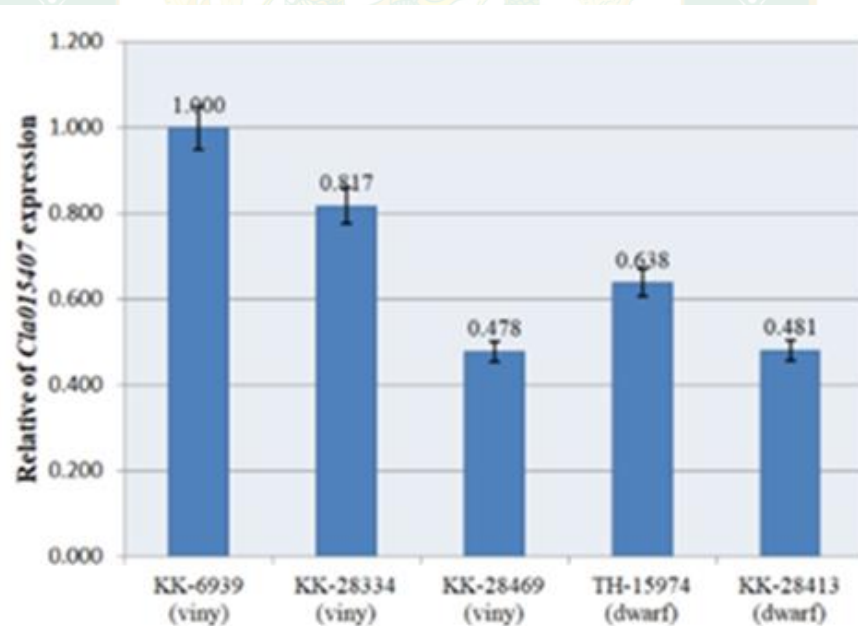
Figure 36 Relative of *Cla015407* expression of GA3ox (*Cla015407*) transcripts by qRT-PCR from five independent watermelon inbred lines. Error bar indicates the SD of the triplicate.

4.8 High throughput functional marker conferring dwarf in watermelon

KASP^{TM} genotyping marker was designed based on SNP at an acceptor splice site on intron 1 of *Cla015407* and named as "Cla015407-GA " marker. This functional marker was genotyped in F$_2$ population. The genotypic form was classified into three types including "G/G " genotype was presented viny plants with homozygous allele, "G/A" genotype was represented viny plants with heterozygous allele and "A/A" genotype was presented dwarf plants in homozygous allele. The genotype results of "Cla015407-GA" marker in F$_2$ were exactly perfect matched with seedling morphology phenotype. For internode length, the "A/A" genotype gave the internode length value rage from 20 to 48 mm and average was about 34 mm where as the "G/A" and "G/G" gave an average internode value was about 69 mm. For the vine length at flowering stage, the "A/A" genotype gave the vine length value rage from 150 to 360 mm and average was about 265 mm where as the "G/A" and "G/G" gave an average vine length value was about 550 mm. For the vine length at harvesting stage, the "A/A" genotype gave the vine length value rage from 380 to 2,150 mm and average was about 1,282 mm where as the "G/A" and "G/G" gave an average vine length value was about 3,300 mm. The genotype of "Cla015407-GA " marker that corresponding to internode and two stages of vine length value was presented in table 14. The genotype and phenotype correlation of F$_2$ were displayed in box plots (Fig. 37). The genotypic and phenotypic data of two parental lines and individual F$_2$ were presented in Appendix B. The LOD score and phenotypic variance explanation of functional marker "Cla015407-GA" was increased if compare to the flanking markers (table 11 and 15). Although the functional marker perfect match with seedling morphology but the phenotypic variance explanation of internode length and two stages of vine length could not reach to 100% because the internode or vine length had environment effect.

Table 14 Genotyping result of functional marker Cla015407-GA that corresponding to internode and vine length value in F_2 population

Phenotype	Genotype	Observed	Min (mm)	Max (mm)	Median (mm)	Mean (mm)*
	AA	70	20	48	36	34.62 ± 7^{a}
Internode	GA	165	24	98	71	69.09 ± 11.23^{b}
	GG	74	37	91	54	69.50 ± 10.58^{b}
Vine length at flowering stage	AA	70	150	360	270	265.22 ± 37.61^{a}
	GA	165	340	683	557	554.10 ± 62.06^{b}
	GG	74	260	697	553	545.47 ± 66.00^{b}
Vine length at harvesting stage	AA	70	380	2,150	1,300	$1,282.90 \pm 358.07^{a}$
	GA	165	1,040	6,900	3,350	$3,350 \pm 979.79^{b}$
	GG	74	2,080	5,520	3,440	$3,339.72 \pm 818.20^{b}$

* Means followed by the same letter in each column are not significantly different in Duncan's multiple range test (DMRT) at alpha 0.05

Table 15 The LOD score and phenotypic variance explanation of functional marker Cla015407-GA at all phenotypes.

Marker	Trait	LOD	$\%R^2$
	Internode length	67.29	65.1%
Cla015407-GA	Vine length at flowering stage	100.45	80.70%
	Vine length at harvesting stage	52.49	54.90%

This functional marker was validated in 31 inbred lines to confirm the accuracy of the marker. The "G/G " genotype was presented in all 29 viny plants whereas "A/A" genotype was presented in 2 dwarf plants. The genotyping result of this marker showed completely perfectly consistent with the phenotype (Table 16). Therefore this "Cla015407-GA " marker was functional marker conferring dwarf in watermelon.

Table 16 Genotyping result of functional marker Cla015407-GA in watermelon inbred lines

Entry	Acc.	Phenotype	Cla015407-GA
1	TH-15974	Dwarf	A/A
2	KK-28413	Dwarf	A/A
3	KK-6939	Viny	G/G
4	KK-28334	Viny	G/G
5	KK-28469	Viny	G/G
6	KK-43359	Viny	G/G
7	KK-13592	Viny	G/G
8	KK-33238	Viny	G/G
9	KK-43389	Viny	G/G
10	KK-44178	Viny	G/G
11	KK-43724	Viny	G/G
12	KK-44208	Viny	G/G
13	KK-43383	Viny	G/G
14	KK-31298	Viny	G/G
15	KK-31711	Viny	G/G
16	KK-43346	Viny	G/G
17	KK-24920	Viny	G/G

Entry	Acc.	Phenotype	Cla015407-GA
18	KK-34799	Viny	G/G
19	KK-34618	Viny	G/G
20	KK-34635	Viny	G/G
21	KK-34805	Viny	G/G
22	KK-35966	Viny	G/G
23	KK-41861	Viny	G/G
24	KK-40383	Viny	G/G
25	KK-34644	Viny	G/G
26	KK-31403	Viny	G/G
27	KK-34724	Viny	G/G
28	KK-33239	Viny	G/G
29	KK-44144	Viny	G/G
30	KK-43344	Viny	G/G
31	KK-44101	Viny	G/G
32	KK-44501	Viny	G/G
33	KK-44105	Viny	G/G
34	KK-44281	Viny	G/G
35	KK-44268	Viny	G/G
36	KK-44154	Viny	G/G
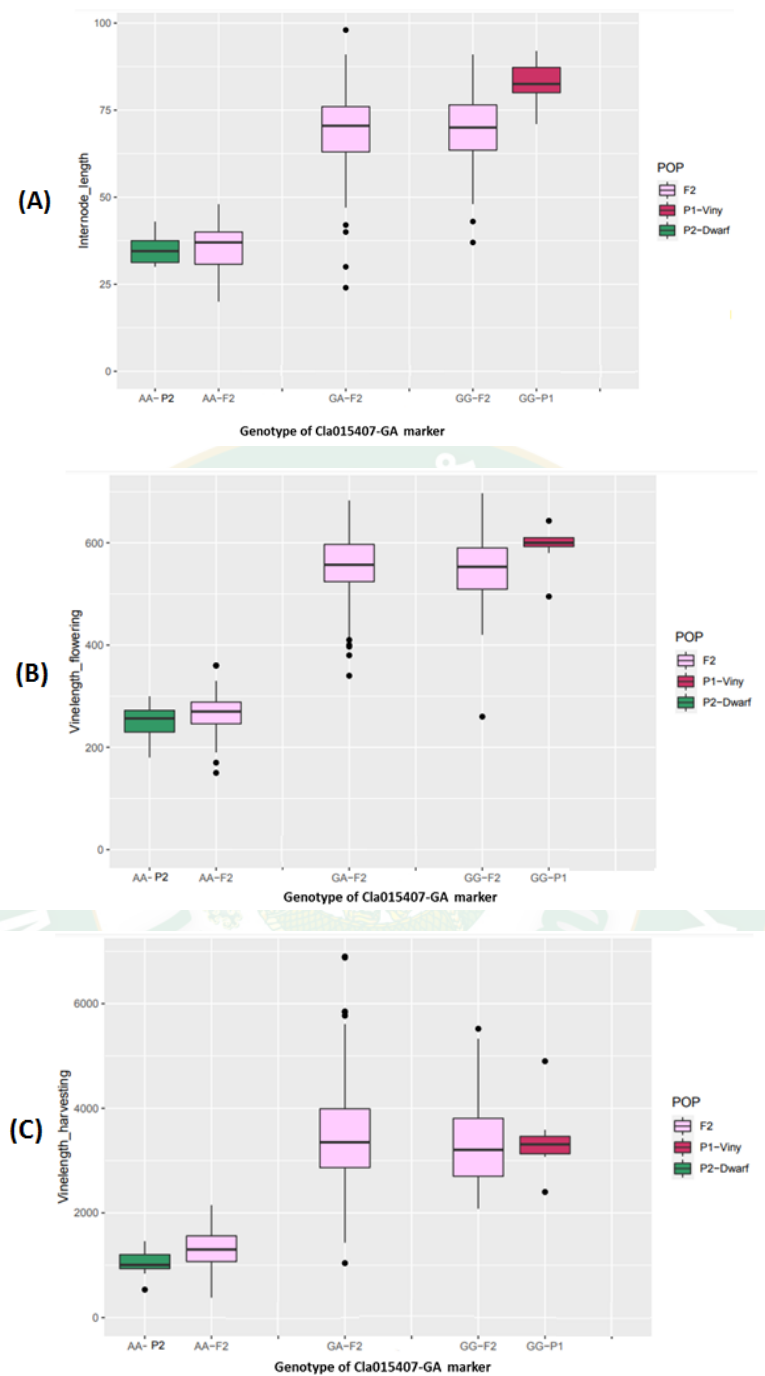
Figure 37 The validation of "Cla015407-GA " marker. The internode length (A), vine length at flowering stage (B) and vine length at harvesting stage (C) with the three different genotypes of KK-6939 viny parent (red), TH-15974 dwarf parent (green) and F$_2$ population (pink). G and A specific marker were represented viny and dwarf, respectively. The medians were indicated as solid horizontal lines in the box plot.

CHAPTER FIVE

Discussions

5.1 The *dw* gene inheritance

Dwarf growth habit is natural mutation and had been found in several germplasm such as Bush Desert King, Somali local, WB-2 (dw-2), Dwarf Male-Sterile Watermelon (DMSW), dsh, WM102, N21, w106, dwarf cultivar 812 and including TH-15974 in this study (Dong *et al.,* 2018; Dyutin and AFANASIEVA, 1987; Hexun *et al.,* 1998; Liu, 1972; Mohr, 1956; Mohr and Sandhu, 1975; Sun *et al.,* 2020; Wei *et al.,* 2019; Zhang *et al.,* 2021; Zhu *et al.*, 2019). Even though dwarf mutant derived from different sources, the dwarf inheritance of all studies showed a single recessive gene. In this study, the chi-square test (χ^2 test) was conducted in F_2, BC_1P_1 and BC_1P_2 to determine the goodness of fit to the single gene model. The result of viny : dwarf ratio in F_2, BC_1P_1 and BC_1P_2 populations was fitted to ratio 3:1, 1:0 and 1:1, respectively. The result confirmed that dwarf growth habit was controlled by a single recessive gene and supported the result from other studies (Dyutin and AFANASIEVA, 1987; Sun *et al.,* 2020; Wei *et al.,* 2019; Zhang *et al.,* 2021; Zhu *et al.,* 2019). However, the locus name was assigned in different name depending on dwarf sources such as *dw-1*, *dw-1s*, *dw-2*, *dw-3*, *dsh*, *cldw-1*, *Cldf*, *Si* and *Cldw* locus. The TH-15974 dwarf cultivar was assigned as *dw* locus.

5.2 QTL mapping and candidate dwarf gene identification

Now a day, there were many methods available to identify the QTLs such as linkage analysis, QTL mapping, next-generation sequencing from bulked segregant analysis (BSA-seq). All methods were capability to detect QTL region but the technique was selected to perform based on the resources available in laboratory. In this study, the linkage analysis and conventional QTL mapping were combined to identify the

QTL that corresponding dwarf growth habit. Two types of phenotypic data sets including category data (seedling morphology) and numeric data (internode, vine length at flowering stage and vine length at harvesting stage) were used for linkage analysis and QTL mapping analysis, respectively. The linkage analysis placed *dw* locus on the terminal of chromosome 9 by using seedling morphology data set coupled with genotypic data of F_2 population. Moreover, the internode length and two stages of vine length were performed QTL analysis and one major QTL was detected on the terminal region of chromosome 9 in all three phenotypes with the same region. However, the LOD score and phenotypic variance explanation ($\%R^2$) value which were obtained from three phenotypes were different based on the phenotypic value. For internode length, one major QTL was detected with LOD score and phenotypic variance explanation ($\%R^2$) was 62.36 and 62%, respectively. For vine length at flowering stage, one major QTL was detected with LOD score and phenotypic variance explanation ($\%R^2$) was 96.55 and 77.2%, respectively. While the vine length at harvesting stage, one major QTL was detected with LOD score and phenotypic variance explanation ($\%R^2$) was 51.02 and 53.8%, respectively. The flanking markers, WMSNP-0002750 and WMSNP-0002780 which were obtained from linkage analysis and the QTL mapping are the same. This result showed the confident on *dw* locus and QTL region detected. Moreover the locus, which corresponding to dwarf growth habit in many independent studies, was reported locating on the chromosome 9 (Gebremeskel et al., 2020; Sun et al., 2020; Wei et al., 2019a; Zhang et al., 2021b; Zhu et al., 2019a) and another one study reported chromosome 7 (Dong et al., 2018a). Even though many studies used different dwarf sources and different approach to detect the QTL, most of studies reported the significant region on terminal of chromosome 9.

The *dw* locus was minimized the interval region to about 3.3 cM or 250 kb and two flanking markers, WMSNP-0002750 and WMSNP-0002780 were identified and which were located at 1.65 Mb and 1.90 Mb on chromosome 9, respectively. A total 25 genes

located under *dw* locus and only two genes, *Cla015407* and *Cla015408* were encoded GA3ox, which was related to plant hormone and had high potential to related dwarf growth habit. Many previous studies reported *Cla015407* was the key gene that controlled dwarf growth habit in watermelon (Gebremeskel et al., 2020; Sun et al., 2020; Wei et al., 2019a; Zhang et al., 2019). Although *Cla015408* gene encoded GA3ox protein the same as *Cla015407*, the study of *Cla015408* gene still remain limited. Therefore two genes, *Cla015407* and *Cla015408* genes were focused and characterized in this study.

5.3 Gene isolation and characterization

Full length of *Cla015407* and *Cla015408* were re-sequenced in genomic DNA and mRNA levels by using five watermelon inbred lines consisting of two inbred lines represented dwarf plants and other three inbred lines represented viny plants. In the DNA sequence level of the *Cla015407* gene, two SNP variants were detected among five accessions but only one SNP at the 626th nucleotide, which located on 3′AG splice site of intron 1, could distinguish dwarf plants from viny plants completely perfectly. This point mutation in genomic DNA of dwarf plants lead to the 3′AG splice site of intron 1 of dwarf plants had been changed from 626th nucleotide to 639th nucleotide. This alternative splicing form was reported in dwarf N21, dwarf cultivar 812 and dwarf Duan 125 (Gebremeskel *et al.,* 2020; Wei *et al.,* 2019) whereas the another splicing isoform, the intron 1 still remaining were found in dwarf w106 (Sun *et al.,* 2020). Moreover the 13 bp deletion of *Cla015407* gene was detected in mRNA of dwarf plants. The 13 bp deletion in mRNA level caused by the 3′AG splice site of intron 1 of dwarf plants was changed. This 13 bp deletion located at the beginning of the second exon of *Cla015407* gene, lead to frameshift translation and promoted the premature stop codon "TGA" at 656th-658th nucleotide resulting to produce a truncated protein in dwarf plants. A truncated protein of *Cla015407* in dwarf plants had a high impact on

GA3ox function as shown in previous studies (Gebremeskel *et al.,* 2020; Sun *et al.,* 2020; Wei *et al.,* 2019).

Only one SNP at intron 1 of *Cla015408* was detected in DNA sequence among five inbred lines. However this SNP could not be classified dwarf plants from viny plants and this SNP was not in the coding region and then had no effect on the amino acid sequence. The full length of mRNA could not be obtained from all five inbred lines.

5.4 GA3ox amino acid sequence analysis

GAox proteins consisted of GA20ox, GA3ox and GA2ox, which were the key enzymes in the gibberellin biosynthesis pathway. GAox proteins contain two conserved domains including DIOX_N and 2OG-FeII_Oxy domains (Pan *et al.,* 2017). *Cla015407* and *Cla015408* genes of watermelon encoded GA3ox protein. *Cla015407* of viny plants was translated to complete amino acid GA3ox, which consisting of two importance domains; DIOX_N and 2OG-FeII_Oxy domains. Therefore, in viny plants had viny or trailing growth habit because of *Cla015407* gene able to produce enough GA3ox protein, which promoted the plant grow up as normal. *Cla015407* of dwarf plants had point mutation at 3′AG splice site of intron 1 and result to alternative splice site happen. The 3′AG splice site of dwarf was changed at the new position of exon2 which lead to a 13 bp deletion was happened in mRNA of dwarf plant. A 13bp deletion in mRNA lead to frameshift translation and promoted the premature stop codon. *Cla015407* of dwarf plants was translated to incomplete amino acid GA3ox, which lack of the 2OG-FeII_Oxy domains. Therefore, GA3ox protein, which was translated from *Cla015407* of dwarf, could not function and lead to the plant could not grow well and showed the dwarf phenotype. Even though *Cla015408* gene in both viny and dwarf plant translated the normal GA3ox protein, the expression of *Cla015408* gene of dwarf plant at seedling stage had not been detected. So the *Cla015408* could not be able

to produce enough GA3ox protein and could not function instead of *Cla015407* in seedling stage of dwarf plants.

5.5 Expression analysis of dwarf candidate genes: Cla015407 and Cla015408 gene

Cla015408 could not function instead of *Cla015407* mutant in dwarf plants despite of *Cla015408* had not been detected any mutation in coding region. Therefore expression analysis of two dwarf candidate genes was investigated. Gene expression levels in *Cla015407* and *Cla015408* were normalized to the expression of actin gene. Although *Cla015408* had no mutation in coding region, the expression of *Cla015408* had not been detected in all five inbred lines at seedling stage. Whereas the expression of *Cla015407* had been detected in all five inbred lines but expression was not significantly different among viny and dwarf plants. Therefore the expression level of *Cla015407* in this study did not relate to plant types at seedling stage while the expression of *Cla015407* was previously reported significantly different between viny and dwarf plants in internodes at reproductive growth stage (Zhang *et al.,* 2021). The different result was revealed because the plants were studied in different parts and stages. Although the expression of *Cla015407* could not affect to plant type in this study but the mutation of *Cla015407* lead to produce non-functional protein and result to dwarf phenotype. This result strongly supported *Cla015407* gene played an important role to control watermelon growth habit and suggested that *Cla015408* could not replace of *Cla015407* in dwarf plant at seedling stage. Therefore, the *Cla015407* gene should be a *dwarf* gene in the TH-15974 line which is similar to other watermelon dwarf lines.

5.6 Functional marker in breeding program

The 3′AG splice site at intron 1 of *Cla015407* had been proved that had an effect to dwarf growth habit in watermelon. This point mutation was selected to design the functional marker, Cla015407-GA, which promoted the high accuracy, precise selection and without background dependence in plant breeding programs (Salgotra and Stewart, 2020). The Cla015407-GA marker was validated in F_2 population and watermelon inbred lines which derived from different background. The genotyping result of Cla015407-GA marker showed perfectly match with seedling morphology. The LOD score and phenotypic variance explanation value of function marker was increased if compare to flanking markers from QTL mapping. Although the phenotypic variance explanation value of functional marker could not reach to 100% because of environment effect but the mean value of "A/A" genotype showed significant different from "G/A" and "G/G" genotype in both internode length and vine length value. Therefore, the functional marker, Cla015407-GA, was the value marker for marker assisted selection (MAS) in watermelon dwarf breeding program.

CHAPTER SIX

Conclusions

Dwarf growth habits in watermelons have several potential advantages in cultivation and agricultural management. The inheritance of dwarf growth habits in this study was controlled by a single recessive gene.

Linkage analysis and QTL mapping were performed to localize *dw* locus, which was mapped on a terminal of chromosome 9 and located in 250 kb interval between 1.65 Mb – 1.90 Mb. Two candidate genes, *Cla015407* and *Cla015408*, located within an interval and both genes encoded a gibberellin 3-beta-hydroxylase protein (GA3ox), which is a plant growth hormone related gene. The variants in two candidate genes were verified, full length sequence of genomic DNA and cDNA level were investigated and obtained from five inbred lines including viny and dwarf plants. There are three SNPs detected in full length of genomic DNA. One SNP was detected at intron 1 of *Cla015408* and could not be affected by translation. Another two SNPs were detected at intron 1 of *Cla015407*. Only a SNP at 626th nucleotide (from "G" changed to "A") of intron 1 *Cla015407* distinguished dwarf plants and viny plants completely perfectly. This SNP is 3′AG splice site of *Cla015407* and this mutation leading to altered splicing event, the 3′AG splice site of dwarf plant moves to 639th nucleotide. Therefore, a 13 bp deletion happened in cDNA level of dwarf plants. This deletion leads to frameshift translation and a premature stop codon, producing a truncated protein.

Amino acid sequences of both two genes were analyzed. The amino acid sequence of *Cla015407*_viny and *Cla015408* had two important domains consisting of DIOX_N domain and FE2OG_OXY domain while *Cla015407*_dwarf lost FE2OG_OXY domain and lead to *Cla015407* in dwarf could not function. Therefore, the gene expression analysis was attempted to prove the functional and expression level of *Cla015408* compared to *Cla015407*. Quantitative and semi-quantitative RT-PCR was

performed in both at seedling stage. The relative expression of *Cla015407* did not showed significantly relate to plant type while *Cla015408* could not be detected in all samples. Due to this, the *Cla015408* could not produce enough GA3ox enzyme and could not function instead of *Cla015407* in dwarf plant.

So far *Cla015407* is the key gene which encoded GA3ox and plays an important role to produce sufficient GA3ox protein in viny plants. The mutation at acceptor splice site in intron 1 lead to dwarfism in TH-15974, this mutation point is causal gene and we developed functional marker "Cla015407-GA " marker at SNP in 3'AG splice site of intron. The functional marker, Cla015407-GA, could identify viny plants and dwarf plants that had SNP mutation at 3'AG splice site of *Cla015407* gene without background dependence. Therefore, this marker could be implemented in breeding programs for dwarf selection.
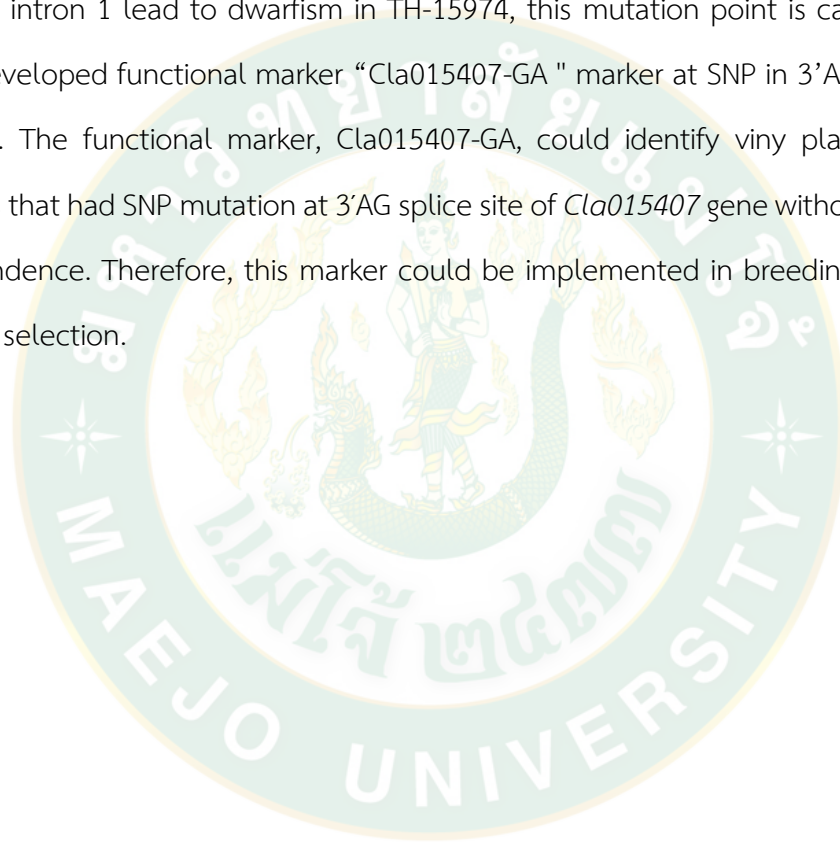
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Appendices

Appendix A

Gene Sequences

>MZ568772 [organism=Citrullus lanatus][cultivar=KK-28334] gibberellin 3-beta-dioxygenase homolog *Cla015407* gene, complete cds]

ATGGGAAGCATCAAAATAACCGAAGTCTTCAAATCCCACCCGGTCCACATCCCCGCCCACAAAA
ACCTCGACTTTGACTCTCTTCATGAACTCCCTGATTCCTATGATTGGATTCAACCCGATTCTTTC
CCTTCTTCCCTCAATATTAATAATAATAATCTCATCTCTCTCTCCGACTCCTTCCCTCTCATTGA
CCTTTCTCTCCCTAACGCCCCTCATCTCATTGGCAATGCCTTCCGTACTTGGGGGGCTTTCCAA
GTCATTAACCACGGTGTCCCCATTTCCCTCCTTCACTCCATTGAATCCGCCGCCAATTCCCTCTT
CTCCCTTCCCCCTCCCCACAAACTCAAAGCCGCTCGTCCCCCCGATGGCATCTCCGGCTACGGC
CTCGTTCGTATCTCCTCCTTCTTCCCTAAACGTATGTGGTCCGAAGGCTTCACCATCGTTGGCT
CCCCTCTCGAACACTTTCAGAAACTCTGGCCTCACGACTACGTTCAATACTGGTAGTGTGAAAA
TCACATTGAATAATTTACTTTTTTTTTTTTTAATTATTTTTTTTAATATAAAGGCACATTGGCTTCTT
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ACGAGAGGATGTGAATTGGGCTGGGCCGAATGGGGATTTCAAGACAAGTAATGCAGCGACCCA
ATTGAACTCTTACCCGGTTTGCCCGGACCCGGACCGGGCCATGGGACTTGGGGCTCATACCGAC
ACCAGCCTCTTAACCATTGTATACCAAAACAACACGAGAGGGTTACAAGTTTTGAGAGAAGGGA
ACCGGTGGGTGACGGTGGAGCCGGTACCCGGTGCACTGGTGGTTCAAGTTGGAGACTTGCTAC
ATATTCTTACAAACGGGTTGTACCCGAGTCCCGTTCATCAAGCCGTTGTGAACCGGACCCGAAA
ACGTCTCTCGGTGGCCTATGTTTTTGGACCGCCAGAAAGTGCCGAAATTTCACCGCTAAAGAAA
CTTTTGGGCCCAACTCAACCACCGCTTTACCGCACAGTAACTTGGACTGAGTACCTCCGTAAAA
AAGCCGAACACTTCAATAATGCACTTTCATCTGTCCGTCTTTGTGCGCCTCTCACTGGACTGTTA
GACGTCAACGATCACAGCCAGGTTAAAGTAGGTTAA

>MZ568773 [organism=Citrullus lanatus][cultivar=KK-28413] gibberellin 3-beta-dioxygenase homolog *Cla015407* gene, complete cds]

ATGGGAAGCATCAAAATAACCGAAGTCTTCAAATCCCACCCGGTCCACATCCCCGCCCACAAAA
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GTCATTAACCACGGTGTCCCCATTTCCCTCCTTCACTCCATTGAATCCGCCGCCAATTCCCTCTT
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ACCAGCCTCTTAACCATTGTATACCAAAACAACACGAGAGGGTTACAAGTTTTGAGAGAAGGGA
ACCGGTGGGTGACGGTGGAGCCGGTACCCGGTGCACTGGTGGTTCAAGTTGGAGACTTGCTAC
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GACGTCAACGATCACAGCCAGGTTAAAGTAGGTTAA

>MZ568769 [organism=Citrullus lanatus][cultivar=KK-28334] gibberellin 3-beta-dioxygenase homolog *Cla015407* gene, complete cds]

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ACGAGAGGATGTGAATTGGGCTGGGCCGAATGGGGATTTCAAGACAAGTAATGCAGCGACCCA
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ACCAGCCTCTTAACCATTGTATACCAAAACAACACGAGAGGGTTACAAGTTTTGAGAGAAGGGA
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GACGTCAACGATCACAGCCAGGTTAAAGTAGGTTAA

>MZ568770 [organism=Citrullus lanatus][cultivar=KK-6939] gibberellin 3-beta-dioxygenase homolog *Cla015407* gene, complete cds]

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>MZ568771 [organism=Citrullus lanatus][cultivar=KK-28469] gibberellin 3-beta-dioxygenase homolog *Cla015407* gene, complete cds]

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CTCCCTTCCCCCTCCCCACAAACTCAAAGCCGCTCGTCCCCCCGATGGCATCTCCGGCTACGGC
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>MZ568774 [organism=Citrullus lanatus][cultivar=TH-15974] gibberellin 3-beta-dioxygenase homolog *Cla015408* gene complete cds]

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CAAGTCATTAACCACGGCGTCCCTATTTCCCTCCTCGACTCCATGGAATCCACTGTCGATGCCC
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GCCTGATGGGGATTTCAAGACTAGTCATGTAGCAATTCAGTTCAACTCCTACCCGGTTTGCCCG
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>MZ568775 [organism=Citrullus lanatus][cultivar=KK-28413] gibberellin 3-beta-dioxygenase homolog *Cla015408* gene, complete cds

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>MZ568776 [organism=Citrullus lanatus][cultivar=KK-28334] gibberellin 3-beta-dioxygenase homolog *Cla015408* gene, complete cds]

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>MZ568777 [organism=Citrullus lanatus][cultivar=KK-6939] gibberellin 3-beta-dioxygenase homolog *Cla015408* gene, complete cds]

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>MZ568778 [organism=Citrullus lanatus][cultivar= KK-28469] gibberellin 3-beta-dioxygenase homolog *Cla015408* gene, complete cds]

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Appendix B

Phenotype data and genotype by Cla015407_GA of F_2 population

Table B1 Genotyping result of functional marker Cla015407-GA and phenotypic data in parental lines and F$_2$ population.

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
KK-6939-1	80	610	3,150	Viny	GG
KK-6939-2	92	580	3,380	Viny	GG
KK-6939-3	80	495	3,240	Viny	GG
KK-6939-4	81	643	4,900	Viny	GG
KK-6939-5	71	597	2,400	Viny	GG
KK-6939-6	87	600	3,420	Viny	GG
KK-6939-7	88	600	3,070	Viny	GG
KK-6939-8	84	610	3,590	Viny	GG
TH-15974-1	31	253	1,250	dwarf	AA
TH-15974-2	43	297	1,300	dwarf	AA
TH-15974-3	30	270	1,050	dwarf	AA
TH-15974-4	32	300	960	dwarf	AA
TH-15974-5	33	250	840	dwarf	AA
TH-15974-6	36	223	950	dwarf	AA
TH-15974-7	39	273	930	dwarf	AA
TH-15974-8	38	260	1,060	dwarf	AA
TH-15974-9	31	180	533	dwarf	AA
TH-15974-10	36	200	1,460	dwarf	AA
F2-1	84	620	3,330	Viny	GA
F2-2	72	610	3,420	Viny	GA
F2-3	73	655	4,100	Viny	GA
F2-4	68	570	2,860	Viny	GA
F2-5	63	583	4,040	Viny	GG
F2-6	75	633	3,400	Viny	GG
F2-7	73	530	4,670	Viny	GA
F2-8	76	583	3,190	Viny	GA
F2-9	70	540	2,700	Viny	GA
F2-10	63	570	2,960	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-11	67	530	2,550	Viny	GA
F2-12	75	567	2,110	Viny	GG
F2-13	75	550	4,260	Viny	GA
F2-14	68	560	3,090	Viny	GA
F2-15	67	553	3,020	Viny	GA
F2-16	70	555	5,190	Viny	GA
F2-17	78	573	2,470	Viny	GG
F2-18	47	247	1,600	dwarf	AA
F2-19	48	277	1,610	dwarf	AA
F2-20	42	323	1,220	dwarf	AA
F2-21	29	253	990	dwarf	AA
F2-22	28	257	1,350	dwarf	AA
F2-23	78	540	3,240	Viny	GA
F2-24	74	613	4,130	Viny	GG
F2-25	78	630	2,800	Viny	GA
F2-26	85	587	2,710	Viny	GA
F2-27	88	600	2,530	Viny	GA
F2-28	66	570	4,630	Viny	GG
F2-29	65	550	2,960	Viny	GG
F2-30	75	597	3,750	Viny	GA
F2-31	71	583	2,400	Viny	GA
F2-32	82	573	2,300	Viny	GA
F2-33	73	513	5,610	Viny	GA
F2-34	91	583	2,420	Viny	GA
F2-35	98	670	2,860	Viny	GA
F2-36	85	617	6,880	Viny	GA
F2-37	91	590	2,700	Viny	GG
F2-38	89	607	3,450	Viny	GG
F2-39	77	697	4,510	Viny	GG
F2-40	78	577	2,650	Viny	GA
F2-41	75	480	3,430	Viny	GA
F2-42	62	563	3,900	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-43	71	553	5,770	Viny	GA
F2-44	65	603	3,000	Viny	GG
F2-45	52	580	3,120	Viny	GA
F2-46	38	250	1,330	dwarf	AA
F2-47	37	320	1,550	dwarf	AA
F2-48	43	273	1,770	dwarf	AA
F2-49	40	290	1,420	dwarf	AA
F2-50	37	480	2,280	Viny	GG
F2-51	71	557	3,360	Viny	GA
F2-52	69	527	3,020	Viny	GA
F2-53	74	567	3,680	Viny	GA
F2-54	57	527	3,600	Viny	GA
F2-55	66	553	3,280	Viny	GG
F2-56	68	563	2,960	Viny	GG
F2-57	53	513	2,000	Viny	GA
F2-58	70	550	2,870	Viny	GA
F2-59	67	550	2,430	Viny	GA
F2-60	81	527	4,090	Viny	GA
F2-61	76	557	3,470	Viny	GA
F2-62	77	593	4,000	Viny	GG
F2-63	61	623	2,930	Viny	GA
F2-64	82	663	3,650	Viny	GA
F2-65	68	550	3,770	Viny	GA
F2-66	75	567	2,710	Viny	GA
F2-67	35	150	620	dwarf	AA
F2-68	74	500	3,100	Viny	GG
F2-69	38	310	1,010	dwarf	AA
F2-70	46	265	1,110	dwarf	AA
F2-71	28	330	1,120	dwarf	AA
F2-72	34	290	1,610	dwarf	AA
F2-73	73	640	3,480	Viny	GA
F2-74	71	610	4,650	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-75	71	590	3,180	Viny	GA
F2-76	73	660	2,950	Viny	GA
F2-77	62	500	2,490	Viny	GA
F2-78	78	607	4,920	Viny	GA
F2-79	80	650	5,840	Viny	GA
F2-80	38	250	990	dwarf	AA
F2-81	29	257	1,080	dwarf	AA
F2-82	37	270	1,370	dwarf	AA
F2-83	32	270	1,490	dwarf	AA
F2-84	36	213	1,610	dwarf	AA
F2-85	35	190	1,010	dwarf	AA
F2-86	63	637	3,220	Viny	GA
F2-87	84	623	4,780	Viny	GA
F2-88	81	603	3,240	Viny	GG
F2-89	81	580	3,290	Viny	GA
F2-90	86	600	3,050	Viny	GA
F2-91	80	627	3,650	Viny	GA
F2-92	78	640	2,540	Viny	GG
F2-93	64	620	3,190	Viny	GA
F2-94	85	577	2,820	Viny	GA
F2-95	75	643	3,000	Viny	GA
F2-96	37	293	1,330	dwarf	AA
F2-97	37	250	1,150	dwarf	AA
F2-98	37	237	1,980	dwarf	AA
F2-99	38	250	1,550	dwarf	AA
F2-100	41	277	1,720	dwarf	AA
F2-101	34	270	1,990	dwarf	AA
F2-102	65	577	2,480	Viny	GA
F2-103	75	603	2,750	Viny	GA
F2-104	74	535	1,490	Viny	GA
F2-105	80	590	3,370	Viny	GA
F2-106	72	640	2,650	Viny	GG

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-107	68	613	2,490	Viny	GG
F2-108	75	627	2,590	Viny	GA
F2-109	63	467	3,280	Viny	GA
F2-110	76	567	2,800	Viny	GA
F2-111	73	510	2,320	Viny	GA
F2-112	73	627	2,800	Viny	GG
F2-113	66	550	2,730	Viny	GG
F2-114	83	680	3,350	Viny	GA
F2-115	72	557	2,140	Viny	GG
F2-116	73	587	3,210	Viny	GA
F2-117	82	610	3,350	Viny	GG
F2-118	90	683	6,900	Viny	GA
F2-119	69	597	1,040	Viny	GA
F2-120	78	657	2,140	Viny	GA
F2-121	75	510	5,100	Viny	GA
F2-122	79	667	4,130	Viny	GA
F2-123	75	537	4,970	Viny	GA
F2-124	57	530	3,960	Viny	GA
F2-125	34	230	1,200	dwarf	AA
F2-126	68	643	2,550	Viny	GA
F2-127	31	247	750	dwarf	AA
F2-128	40	293	1,180	dwarf	AA
F2-129	26	280	1,070	dwarf	AA
F2-130	40	360	1,350	dwarf	AA
F2-131	76	580	2,710	Viny	GG
F2-132	52	530	4,400	Viny	GA
F2-133	65	533	4,100	Viny	GA
F2-134	71	607	2,540	Viny	GA
F2-135	76	617	2,440	Viny	GG
F2-136	82	627	2,080	Viny	GG
F2-137	74	610	2,690	Viny	GA
F2-138	63	547	2,880	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-139	78	617	3,100	Viny	GA
F2-140	85	610	3,650	Viny	GG
F2-141	78	587	3,500	Viny	GA
F2-142	64	523	5,110	Viny	GA
F2-143	65	543	2,800	Viny	GG
F2-144	89	530	2,980	Viny	GG
F2-145	68	440	4,900	Viny	GG
F2-146	68	573	3,000	Viny	GA
F2-147	54	577	3,320	Viny	GA
F2-148	77	597	2,490	Viny	GA
F2-149	70	553	3,740	Viny	GG
F2-150	42	253	1,250	dwarf	AA
F2-151	29	273	1,610	dwarf	AA
F2-152	44	240	1,240	dwarf	AA
F2-153	38	283	1,120	dwarf	AA
F2-154	25	200	690	dwarf	AA
F2-155	31	220	1,090	dwarf	AA
F2-156	71	583	3,150	Viny	GG
F2-157	67	547	2,700	Viny	GG
F2-158	78	547	3,940	Viny	GA
F2-159	60	495	5,090	Viny	GA
F2-160	72	543	2,730	Viny	GA
F2-161	58	550	3,760	Viny	GG
F2-162	61	600	3,560	Viny	GA
F2-163	68	617	2,390	Viny	GA
F2-164	-	-	-	Viny	GA
F2-165	52	397	2,280	Viny	GA
F2-166	61	440	4,240	Viny	GG
F2-167	-	-	-	Viny	GG
F2-168	62	437	4,080	Viny	GA
F2-169	37	230	1,700	dwarf	AA
F2-170	60	573	2,570	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-171	73	500	3,610	Viny	GA
F2-172	76	470	3,820	Viny	GA
F2-173	36	280	710	dwarf	AA
F2-174	-	360	380	dwarf	AA
F2-175	41	240	840	dwarf	AA
F2-176	31	257	1,110	dwarf	AA
F2-177	-	-	-	dwarf	AA
F2-178	63	507	3,670	Viny	GG
F2-179	71	490	3,310	Viny	GA
F2-180	88	557	3,400	Viny	GA
F2-181	50	440	4,900	Viny	GA
F2-182	58	490	4,200	Viny	GG
F2-183	72	493	2,470	Viny	GG
F2-184	61	530	3,720	Viny	GA
F2-185	69	487	2,880	Viny	GG
F2-186	58	400	3,050	Viny	GA
F2-187	58	423	2,800	Viny	GA
F2-188	81	533	3,590	Viny	GA
F2-189	68	527	3,700	Viny	GG
F2-190	84	487	3,640	Viny	GA
F2-191	50	420	2,360	Viny	GG
F2-192	48	440	2,470	Viny	GG
F2-193	63	513	3,770	Viny	GA
F2-194	70	530	4,750	Viny	GG
F2-195	41	273	1,300	dwarf	AA
F2-196	30	245	1,080	dwarf	AA
F2-197	77	560	3,630	Viny	GA
F2-198	73	583	3,210	Viny	GA
F2-199	52	440	3,560	Viny	GG
F2-200	80	600	3,080	Viny	GA
F2-201	78	540	2,860	Viny	GG
F2-202	65	557	3,980	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-203	66	553	4,110	Viny	GG
F2-204	63	560	4,680	Viny	GG
F2-205	78	600	3,950	Viny	GG
F2-206	50	380	2,400	Viny	GA
F2-207	72	497	4,880	Viny	GG
F2-208	-	-	-	Viny	GG
F2-209	79	590	4,460	Viny	GG
F2-210	74	567	4,270	Viny	GA
F2-211	72	513	3,600	Viny	GA
F2-212	75	540	5,030	Viny	GA
F2-213	-	-	-	Viny	GA
F2-214	58	475	3,520	Viny	GG
F2-215	64	543	2,960	Viny	GA
F2-216	86	550	4,360	Viny	GA
F2-217	78	577	2,930	Viny	GA
F2-218	83	560	4,490	Viny	GG
F2-219	42	283	1,700	dwarf	AA
F2-220	45	273	1,560	dwarf	AA
F2-221	35	293	1,450	dwarf	AA
F2-222	38	257	1,380	dwarf	AA
F2-223	20	303	720	dwarf	AA
F2-224	30	287	1,310	dwarf	AA
F2-225	20	170	710	dwarf	AA
F2-226	41	293	2,150	dwarf	AA
F2-227	71	543	4,720	Viny	GA
F2-228	55	540	3,620	Viny	GA
F2-229	64	513	3,000	Viny	GA
F2-230	70	510	2,740	Viny	GG
F2-231	78	600	2,940	Viny	GA
F2-232	68	563	3,300	Viny	GA
F2-233	68	577	3,950	Viny	GA
F2-234	80	533	2,680	Viny	GG

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-235	64	480	2,490	Viny	GG
F2-236	65	527	5,850	Viny	GA
F2-237	68	530	3,050	Viny	GA
F2-238	54	490	2,400	Viny	GG
F2-239	60	415	4,920	Viny	GA
F2-240	25	240	980	dwarf	AA
F2-241	37	277	1,140	dwarf	AA
F2-242	-	-	1,040	dwarf	AA
F2-243	67	-	2,820	Viny	GA
F2-244	43	290	1,410	dwarf	AA
F2-245	38	267	1,290	dwarf	AA
F2-246	32	310	1,620	dwarf	AA
F2-247	46	290	1,800	dwarf	AA
F2-248	-	235	1,020	dwarf	AA
F2-249	23	220	1,110	dwarf	AA
F2-250	78	553	4,100	Viny	GA
F2-251	60	487	3,740	Viny	GG
F2-252	67	530	3,220	Viny	GA
F2-253	58	463	3,260	Viny	GA
F2-254	71	567	3,070	Viny	GG
F2-255	65	450	3,130	Viny	GG
F2-256	60	580	3,400	Viny	GA
F2-257	83	580	3,600	Viny	GG
F2-258	67	533	5,280	Viny	GA
F2-259	68	260	2,310	Viny	GG
F2-260	24	613	4,260	Viny	GA
F2-261	68	597	4,330	Viny	GA
F2-262	-	553	3,500	Viny	GG
F2-263	58	543	3,580	Viny	GA
F2-264	60	410	2,400	Viny	GA
F2-265	65	480	4,000	Viny	GA
F2-266	54	680	5,250	Viny	GA

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-267	85	623	5,520	Viny	GG
F2-268	84	540	3,320	Viny	GA
F2-269	56	523	3,750	Viny	GA
F2-270	72	540	2,600	Viny	GG
F2-271	73	580	2,950	Viny	GA
F2-272	69	587	2,350	Viny	GA
F2-273	69	567	3,800	Viny	GA
F2-274	55	557	5,330	Viny	GG
F2-275	33	275	1,320	dwarf	AA
F2-276	40	270	1,370	dwarf	AA
F2-277	42	435	4,570	Viny	GA
F2-278	22	230	650	dwarf	AA
F2-279	25	230	1,060	dwarf	AA
F2-280	56	520	1,430	Viny	GA
F2-281	40	455	4,800	Viny	GA
F2-282	57	557	3,820	Viny	GA
F2-283	62	517	3,170	Viny	GG
F2-284	47	467	4,100	Viny	GA
F2-285	30	483	4,210	Viny	GA
F2-286	69	520	3,020	Viny	GG
F2-287	-	340	3,960	Viny	GA
F2-288	50	485	4,200	Viny	GA
F2-289	63	480	3,640	Viny	GA
F2-290	61	553	3,000	Viny	GA
F2-291	62	493	3,120	Viny	GA
F2-292	25	273	1,710	dwarf	AA
F2-293	32	283	1,620	dwarf	AA
F2-294	62	537	3,250	Viny	GG
F2-295	63	510	5,360	Viny	GA
F2-296	73	587	2,280	Viny	GA
F2-297	71	523	3,750	Viny	GA
F2-298	60	543	3,480	Viny	GG

Acc.	Internode length (mm.)	Vine length at flowering stage (mm.)	Vine length at harvesting stage (mm.)	Seedling morphology	Cla015407-GA
F2-299	82	603	2,780	Viny	GA
F2-300	68	500	3,620	Viny	GA
F2-301	47	530	3,360	Viny	GA
F2-302	83	617	2,850	Viny	GA
F2-303	54	550	5,520	Viny	GA
F2-304	68	493	3,100	Viny	GA
F2-305	70	523	4,010	Viny	GG
F2-306	64	493	3,400	Viny	GA
F2-307	43	560	3,300	Viny	GG
F2-308	-	-	1,720	dwarf	AA
F2-309	-	295	1,430	dwarf	AA

CURRICULUM VITAE

NAME	Namfon Chomkaeo
DATE OF BIRTH	23 September 1982
EDUCATION	1996 – 2001: High School, Mukdahan school, Mukdahan, Thailand
	2001 – 2004: Bachelor of Science Program in Microbiology, Chulalongkorn university, Bangkok, Thailand
	2012 -2015: Master of Science Program in Genetics, Maejo university, Chaingmai, Thailand
WORK EXPERIENCE	2004 -2005: Senior supervisor at Siam Ocean Frozen Foods CO., LTD., Sumutsakorn, Thailand.
	2005-2007: Scientist at Pan Rajdhevee group public company limited., Pathumthani, Thailand.
	2007 – present: Scientist at Hortigenetics Research S.E.Asia Ltd., Chaingmai, Thailand