ปริมาณสารออกฤทธิ์ทางชีวภาพในต้นทานตะวันงอกสายพันธุ์ต่าง ๆ และยีนที่เกี่ยวข้องในชีวสังเคราะห์ของสารอนุพันธ์กรดคาฟีโออิลควินิค

นางสาวพัทธ์วิรา บุญจริง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUND CONTENTS IN DIFFERENT VARIETIES OF SUNFLOWER SPROUTS AND GENES INVOLVED IN CAFFEOYL QUINIC ACID DERIVATIVE BIOSYNTHESIS

Miss Patwira Boonjing

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	BIOACTIVE COMPOUND CONTENTS IN DIFFERENT VARIETIES OF SUNFLOWER SPROUTS AND GENES INVOLVED IN CAFFEOYL QUINIC ACID DERIVATIVE BIOSYNTHESIS
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ในปัจจุบัน ต้นทานตะวันงอก (*Helianthus annuus*) เป็นที่นิยมในการรับประทาน เนื่องจากมีการสะสมของสารอาหารที่มีประโยชน์ รวมถึงมีสารออกฤทธิ์ทางชีวภาพหลายชนิด ในงานวิจัยนี้มีการเปรียบเทียบปริมาณสารออกฤทธิ์ทางชีวภาพระหว่างต้นทานตะวันงอก 6 สายพันธุ์ ได้แก่ 5A 10A S473 S475 สายพันธุ์ไม่ทราบชื่อจากอินเตอร์เน็ต "ไร่คุณลุงท๊อป ต้นอ่อนทานตะวันออแกนิค" (UNK) และจากบริษัทเจียไต๋ (CH) ผลการทดลองพบว่า สายพันธุ์ UNK มีปริมาณสารต้านอนุมูลอิสระและปริมาณสารฟีนอลิกรวม รวมทั้งไซนารินมากที่สุด เมื่อเปรียบเทียบที่ระยะการเจริญเติบโตต่าง ๆ กันของสายพันธุ์ 5A และ CH พบว่าปริมาณของกรดคลอโรจีนิคและไซนารินในสายพันธุ์ 5A ไม่มีความสอดคล้องกับปริมาณสารต้านอนุมูลอิสระและปริมาณสารฟีนอลิกรวมที่มีมากที่สุดในวันที่ 3 ของการเจริญเติบโต ปริมาณของกรดคลอโรจีนิคและไซนารินในต้นทานตะวันงอกที่ใช้ไคโตซานเป็นตัวกระตุ้นส่วนใหญ่คงที่ในสายพันธุ์ 5A และลดลงในสายพันธุ์ CH ขณะที่ปริมาณสารต้านอนุมูลอิสระและปริมาณสารฟีนอลิกรวมลดลงเล็กน้อย ส่วนการใช้เมทิลจัสโมเนตพบว่าเป็นตัวกระตุ้นที่สามารถเพิ่มปริมาณสารต้านอนุมูลอิสระและปริมาณสารฟีนอลิกรวมได้ในปริมาณมาก อีกทั้งยังพบว่ากรดคลอโรจีนิคและไซนารินเป็นสารประกอบหลักที่มีฤทธิ์ต้านการต่อหมู่น้ำตาล และจากการหายีนที่เกี่ยวข้องในชีวสังเคราะห์ของสารอนุพันธ์กรดคาฟีโออิลควินิค พบยีน hydroxycinnamoyl-CoA: quinate hydroxyl cinnamoyltransferase (*HQT*) ที่น่าจะเกี่ยวข้องทั้งหมด 3 ยีน ได้แก่ *HaHQT1 HaHQT2* และ *HaHQT3* เมื่อวัดระดับการแสดงออกของยีน โดยวิธี qRT-PCR พบว่ายีน *HaHQT1* เป็นยีนที่มีการแสดงออกสูงสุด จึงน่าจะเป็นยีนที่เกี่ยวข้องกับการสังเคราะห์กรดคลอโรจีนิคและไซนารินในต้นทานตะวันงอก งานวิจัยนี้สามารถนำไปใช้ประโยชน์โดยเป็นข้อมูลในการคัดเลือกสายพันธุ์เพื่อใช้ในการปรับปรุงพันธุ์ของต้นทานตะวันงอกเพื่อเพิ่มปริมาณสารออกฤทธิ์ทางชีวภาพได้ในอนาคต

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PATWIRA BOONJING: BIOACTIVE COMPOUND CONTENTS IN DIFFERENT VARIETIES OF SUNFLOWER SPROUTS AND GENES INVOLVED IN CAFFEOYL QUINIC ACID DERIVATIVE BIOSYNTHESIS. ADVISOR: ASST. PROF. SUPAART SIRIKANTARAMAS, Ph.D., 130 pp.

At present, sunflower sprouts (*Helianthus annuus*) are popularly consumed because they accumulate several kinds of nutrients and bioactive compounds. In this study, six varieties of sunflower sprouts, namely, 5A, 10A, S473, S475, unknown variety from internet shop "Lungtoporganic" (UNK), and unknown variety from Chiatai company (CH) were compared for bioactive compound contents. The results demonstrated that UNK contains the highest amounts of antioxidants and total phenolic compounds including cynarin. At different germination times, 5A and CH varieties showed that the antioxidant activities and total phenolic contents were highest at day 3. However, the chlorogenic acid and cynarin amounts in 5A were not associated with those results. Chlorogenic acid and cynarin levels in chitosan-elicited sunflower sprouts mostly remained stable in 5A and decreased in CH, while, antioxidant and total phenolic contents slightly increased. Methyl jasmonate application strongly induced the amounts of antioxidants and total phenolics in sunflower sprouts. This study also suggests that chlorogenic acid and cynarin are the major compounds having antiglycative activity. From database searching for genes involved in caffeoyl quinic acid derivative biosynthesis, three candidate genes including hydroxycinnamoyl-CoA:quinate hydroxyl cinnamoyltransferase (*HQT*), namely, *HaHQT1*, *HaHQT2*, and *HaHQT3* were found. The *HaHQT1* shows the highest gene expression level, therefore, it supposed to be a gene responsible for chlorogenic acid and cynarin biosyntheses in sunflower sprouts. Content analysis of bioactive compounds can be performed to select the most suitable variety for further breeding to increase the amounts of bioactive compound in the future.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2015 Advisor's Signature .....

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CHAPTER I

INTRODUCTION

Nowadays, sunflower sprouts are popularly consumed because they accumulate several kinds of nutrients and bioactive compounds (Marton et al., 2010). Sunflower seeds have already had a high contents of vitamin E, vitamin B complex, essential fatty acids, proteins, magnesium, selenium and zinc. The contents of β-carotene, chlorophyll and vitamin C in sprouts are higher than those found in seeds (Alda et al., 2011). Therefore, it is interesting to analyze the bioactive compound contents in sunflower sprouts to select the most suitable variety for further breeding to increase the amounts of bioactive compound in the future.

The objectives of this study were to compare antioxidant capacities, total phenolic contents, chlorogenic acid, and cynarin in different varieties of Thai sunflower sprouts including 5A, 10A, Suranaree 473 (S473), Suranaree 475 (S475), the unknown seeds that purchased from the internet (UNK) and Chiatai company (CH), to find out which germination time has the highest bioactive compound contents, to increase the content of bioactive compounds, to determine antiglycative activity of chlorogenic acid and cynarin, and to study related genes that involved in the biosynthesis of these compounds. This data could be an alternative way to provide to people who would like to consume sunflower sprouts for their health benefit. In this chapter, other information on related topic was reviewed.

1.1 Sprouts

Sprouts have been firstly consumed by the Chinese for nearly 5,000 years ago which germinated mung beans (*Vigna radiata* L.) were the most well-known and oldest among all sprouts (Larimore, 1975). In the past, sprouts that people usually consumed mainly came from Leguminosae family. By the way, cereal sprouts are mainly used as animal feed (Waginger, 1984). The nutritive value and the health qualities of food in natural way can be increased by sprouting the seeds (Bau et al., 1997). Firstly, the seed comes out of the dormant stage which appropriate environmental conditions (moisture content, temperature, oxygen) are necessary. Oligo- and monosaccharides were synthesized by the polysaccharides degradation. The fats and proteins degrade to free fatty acids and free amino acids, respectively. During germination, the biochemical mechanisms in plant organism were supported by these processes. The protein-, carbohydrate-, and fatty acid-decomposing enzymes efficiency were improved. Some compounds such as glucosinolates and natural antioxidants have health-maintaining effects and phytochemical properties, especially an important role in the prevention of many kind of diseases (Sangronis & Machado, 2007). The sprouts have a higher nutritional value, higher protein quality, more favorable composition of amino acid, higher polyunsaturated fatty acid content, higher essential minerals and vitamin contents comparing to seeds. During sprouting the amount of such antinutritive materials as haemagglutinins, trypsin inhibitor activity, phytic acid, pentosans, tannins decreases. The sprouts are also a good source of pantothenic acid, tocopherol, thiamin, ascorbic acid, choline, and riboflavin (Lintschinger et al., 1997). Sprouting of seeds is inexpensive and convenient to cultivate, currently, there are different kinds of seeds that could be sprouted for the consumption of human such as all legumes (mung bean

(Savage, 1990), pea (Obizoba, 1991), lentil (Bhatty, 1988), soybean (Mostafa et al., 1987)), grains (rye (Centeno et al., 2001), wheat (Harmuth-Hoene et al., 1987), barley (Peer & Leeson, 1985), oats (Tian et al., 2010)) and recently, some vegetables (alfalfa (Plaza et al., 2003) and radish (Martinez-Villaluenga et al., 2010)). More recently, there was a previous study showed that germination time of sunflower sprouts affected on the contents of oil, sugars and nitrogenous compounds (Balasaraswathi & Sadasivam, 1997). The activity of antioxidant in sprouts is also higher than that in seeds (Cho et al., 2008).

In this study, six different varieties of *H. annuus* L. were used in the experiment (Figure 1) which were separated into two groups including the varieties which were developed by Sunflower Breeding Program of Suranaree University of Technology for improving oil contents in sunflower seeds and the unknown varieties purchased from the market. For the former group, the inbred lines are 5A and 10A. The Suranaree 473 (S473) and Suranaree 475 (S475) are synthetic varieties. These varieties of sunflower sprouts gave high uniformity of plant height, days to flowering, seed yield and oil content. The different between inbred line and synthetic variety was reported that synthetic varieties were drought tolerant, whereas inbred lines were drought sensitive. Seed yields and oil contents also depend on water stress, therefore, inbred line contain lower seed yields and oil contents (Saensee et al., 2012). The other two varieties in the latter group consisting the unknown varieties namely CH and UNK which are purchased from chiatai company and the internet (https://www.facebook.com/ lungtoporganic), respectively.

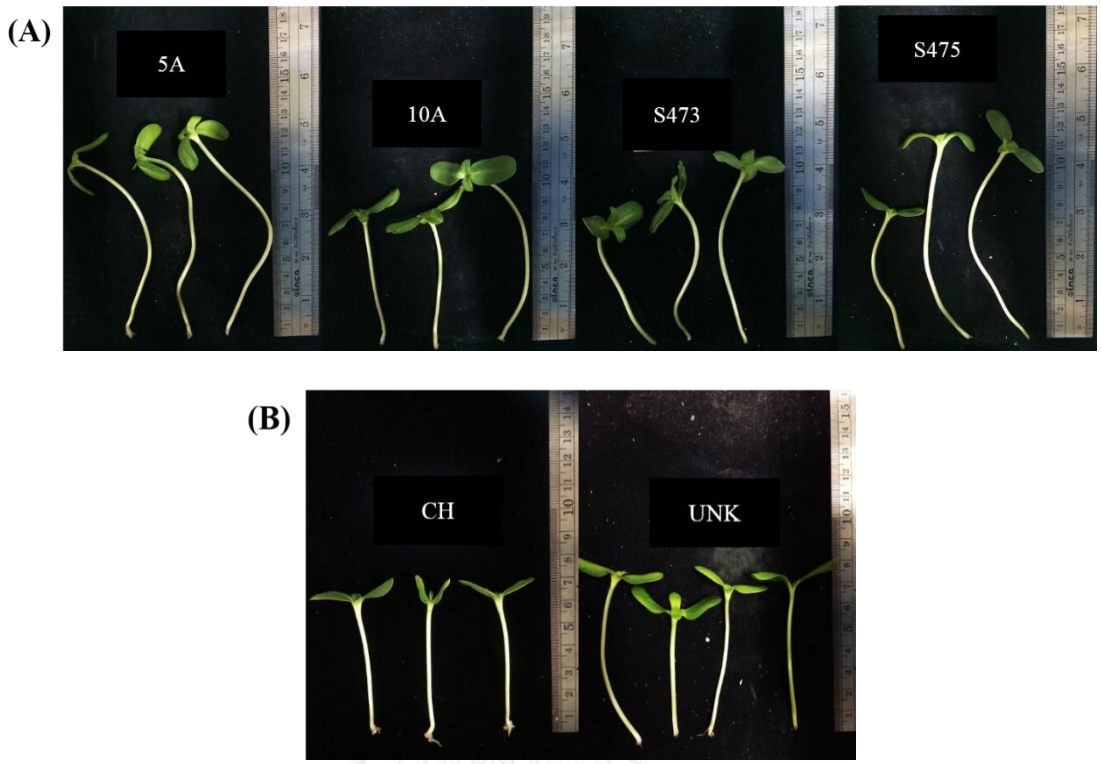


Figure 1. Six varieties of sunflower sprouts using in this experiment. (A) the varieties which were developed at Suranaree University of Technology (5A, 10A, S473, and S475), and (B) the varieties which available on the market (CH and UNK).

1.2 Bioactive compounds in sunflower sprouts

1.2.1 Antioxidants

Antioxidants can be defined in many ways. The definition from dictionary is “a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances (as the tocopherols) being used as preservatives in various products (as in fats, oils, food products, and soaps for retarding the development of rancidity, in gasoline and other petroleum products for retarding gum formation and other undesirable changes, and in rubber for retarding aging)” (Merriam-Webster, 2002). In other meaning is “synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or *β*-carotene, that are capable of counteracting the damaging effects of oxidation in animal tissues” (Huang et al., 2005). Antioxidants have a broader scope in food science. They contain components which prevent fats in food to become rancid as well as dietary antioxidants “a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans”(Monsen, 2000). Halliwell defined biological antioxidants as “molecules which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules” (Halliwell, 1990). The scope of biological antioxidants is divided into two groups containing enzymatic antioxidants (such as superoxide dismutase, glutathione peroxidase, and catalase) and nonenzymatic antioxidants such as oxidative enzyme (such as cyclooxygenase) inhibitors, transition metal chelators, ROS/RNS scavengers, and antioxidant enzyme cofactors (Figure 2). Depending on chemical

reactions, there are two categories of antioxidant activity assays including single electron transfer (ET) reaction based assays and hydrogen atom transfer (HAT) reaction based assays (Table 1). The ET-based assays involve the oxidant (also as the probe for monitoring the reaction) with one redox reaction as the reaction endpoint indicator. Most HAT-based assays control competitive reaction kinetics. The quantitation is derived from the kinetic curves. HAT-based methods commonly are included an antioxidant, an oxidizable molecular probe, and a synthetic free radical generator. ET- and HAT-based assays are intended to measure the oxidant or radical scavenging activity, instead of a sample preventive antioxidant activity. In this study, ET-based assays were mainly focused because 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH assay), ferric reducing antioxidant power (FRAP assay), and Folin–Ciocalteu −Total phenolic compound assay which were used in this experiment are classified into ET-based assays.

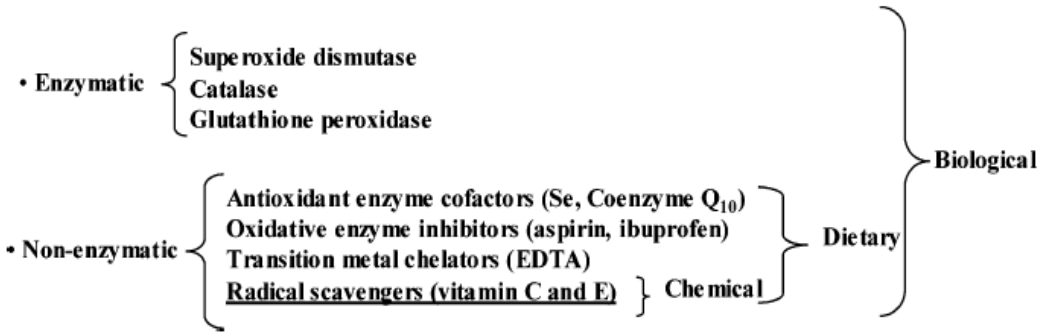


Figure 2. Broad scope of antioxidants (taken from Huang et al., 2005)

Table 1. In Vitro antioxidant activity assays (taken from Huang et al., 2005)

Type of assays	Examples
assays involving hydrogen atom transfer reactions	ORAC (oxygen radical absorbance capacity)
$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$ $ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$	TRAP (total radical trapping antioxidant parameter) Crocin bleaching assay IOU (inhibited oxygen uptake) inhibition of linoleic acid oxidation inhibition of LDL oxidation
assays by electron-transfer reaction	TEAC (Trolox equivalent antioxidant capacity)
$M(n) + e$ (from AH) $\rightarrow$ $AH^{\bullet+} + M(n-1)$	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (diphenyl-1-picrylhydrazyl) copper(II) reduction capacity total phenols assay by Folin–Ciocalteu reagent
other assays	TOSC (total oxidant scavenging capacity) inhibition of Briggs–Rauscher oscillation reaction chemiluminescence electrochemiluminescence

1.2.1.1 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH assay)

The DPPH assay has popularly been used for measuring antioxidant capacity of compounds for acting as hydrogen donors or free radical scavengers (Figure 3). This assay is one of a few stable assay. It commercially has long-lived organic nitrogen radicals with a deep purple color which has the maximum UV-visible absorption at 515 nm. The solution color fades from purple to colorless of the corresponding hydrazine when an antioxidant compound reduced DPPH radical. The mixture reducing ability is measured at 515 nm for 30 min or until the absorbance is stable. The antioxidant kinetic behavior is divided as follows: <5 min (rapid), 5-30 min (intermediate), and >30 min (slow)

(Jiménez-Escrig et al., 2000). This assay was supposed to involve in HAT reaction for long times ago, nevertheless, the transfer of initial electron occurs very fast and the transfer of subsequent hydrogen slowly occurs. This depends on the hydrogen bond accepting solvent used such as ethanol and methanol. Therefore, it was recommended that the behavior of the reaction is similar to an ET reaction more than HAT reaction (Huang et al., 2005).

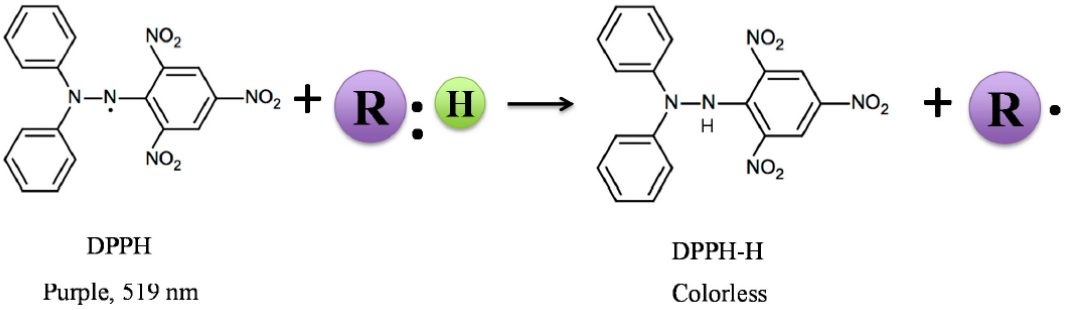


Figure 3. Reaction mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. R:H = antioxidant radical scavenger; R = antioxidant radical. (Taken from Liang & Kitts, 2014).

1.2.1.2 Ferric reducing antioxidant power (FRAP assay)

The ferric ion reducing antioxidant power (FRAP) assay is a non-specific, colorimetric, and redox-linked assay which is associated with the molar concentration of the available antioxidant. Because FRAP measures the ferric reducing activity of the sample, a low pH buffer is required to reduce the ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to a ferrous (Fe^{2+}-TPTZ) form. It is measured at a maximum absorption, 593 nm (Vignoli et al., 2011) (Figure 4).

The condition of the assay mostly supports the Fe^{3+}-TPTZ complex reduction which color is developed to the degree of which antioxidant activity is displayed. There is an excess amount of Fe^{3+} used in the assay, then, the rate-limiting factor for the Fe^{2+}-TPTZ is the sample reducing activity. Using the change in absorbance (ΔA593nm) from a reagent blank, FRAP has been automated to give results within 10 min. The final calculation for each sample was related to a (ΔA593nm) of a Fe^{2+} standard. Although the FRAP assay was originally developed to measure the antioxidant power of plasma, this assay has also been used to evaluate the total antioxidant activity of different food systems since it is a highly reproducible and inexpensive assay (Liang & Kitts, 2014).

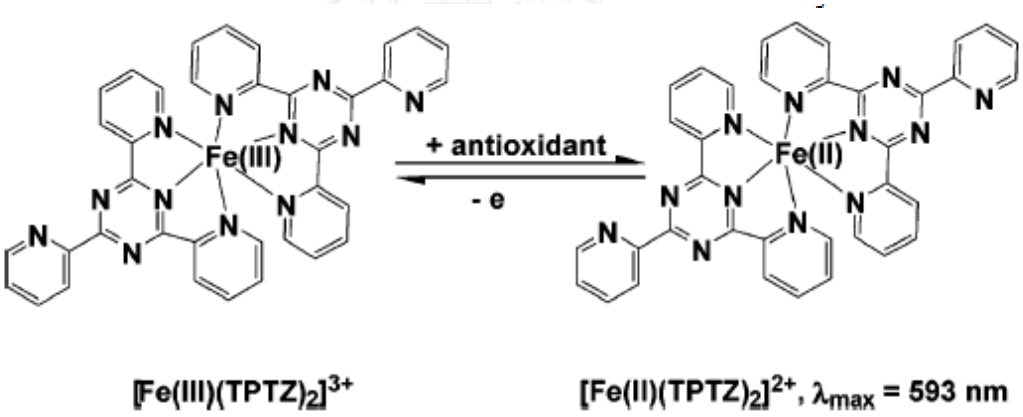


Figure 4. Reaction mechanism of ferric reducing antioxidant power (FRAP), with antioxidant (Taken from Huang et al., 2005).

1.2.1.3 Folin–Ciocalteu −Total phenolic compounds assay

The Folin-Ciocalteu method is used for measuring the total concentration of phenolic groups in the plant extract which react with Folin-Ciocalteu reagent. A blue chromophore was formed by a phosphotungstic-phosphomolybdenum complex which lithium sulfate and bromine were added to prevent the turbidity. This reagent is used together with a saturated sodium carbonate solution to turn the reaction into base condition (pH 10-12) which it generates the phenolate anion. Then, the color of the solution changes from yellow to blue after about 60 min approximately in the dark at room temperature because a redox reaction occurs between the phenolate anion and Folin-Ciocalteu reagent (yellow). The development of blue metallic complex [$(PMoW_{11}O_4)^{4-}$] involving with the total phenolic contents of the sample can cause the shift in color. The maximum absorption of the blue complex is 765 nm (Figure 5).

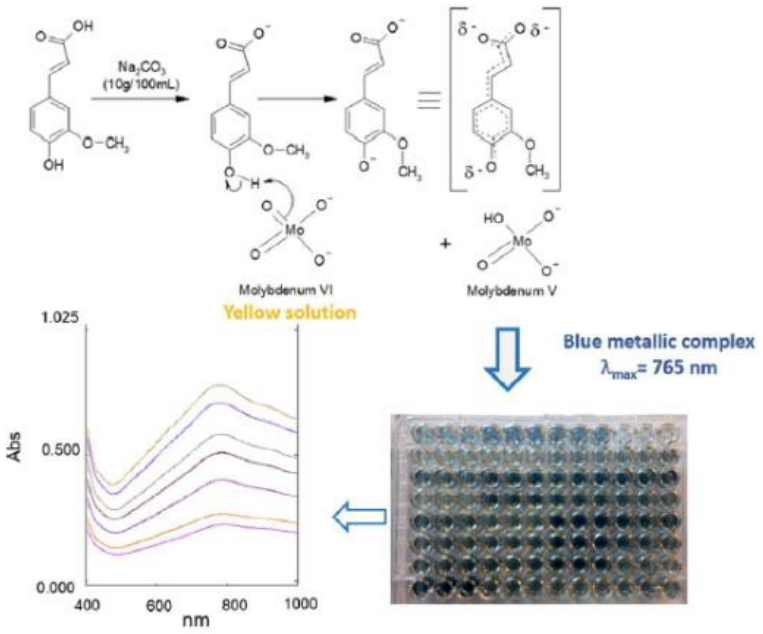


Figure 5. Chemical reaction and UV/VIS spectra of total phenolic content of plant extracts at different concentrations measured by the Folin-Ciocalteu method (Taken from Granato et al., 2016).

1.2.2 Phenolic compounds in sunflower

The major phenolic compounds identified in sunflower seeds are chlorogenic acid (55%) (Figure 6) and cynarin (1,5-di-O-caffeoylquinic acid) (30%) (Figure 7) (Pedrosa et al., 2000). These compounds have a chloresterol lowering activity (Gebhardt, 1998). Moreover, chlorogenic acid can eliminate particularly toxic reactive species by scavenging alkylperoxyl radicals and prevent carcinogenesis by reducing the DNA damage (Sawa et al., 1999). Cynarin also have an anti-inflammatory activity (Peluso et al., 1995), a selective inhibition of HIV replication (McDougall et al., 1998), a suppression of melanogenesis (Kaul & Khanduja, 1998), an antihepatotoxic activity (Choi et

al., 2005), and an antiglycation activity (Sun et al., 2012). Because of the large determination by the number of hydroxyl groups presenting on the aromatic rings, the physiological effects of dicaffeoylquinic acids such as cynarin are commonly greater than those of monocaffeoylquinic acids like chlorogenic acid (Wang et al., 2003).

Chlorogenic acid is an significant antioxidant in plants (Tamagnone et al., 1998) and is a major soluble phenolic compound in Solanaceae family (Clifford, 1999). The biosynthetic pathway of chlorogenic acid is proposed into two different routes. The former route is usually occurred in tomato, tobacco and potato which the enzyme hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) catalyzes the formation of chlorogenic acid from caffeoyl CoA and quinic acid (Ulbrich & Zenk, 1979) (route 1 in Figure 8). The latter involves the synthesis of *p*-coumaroyl quinate by an acyl transferase and subsequent hydroxylation by *p*-coumarate 3′′-hydroxylase (C3′H) to synthesize chlorogenic acid (route 2 in Figure 8). However, this enzyme is active in *Arabidopsis thaliana* which does not accumulate chlorogenic acid, so this route may not accumulate the amount of chlorogenic acid in plants (Schoch et al., 2001). On the other hand, route 1 is the most possible pathway for chlorogenic acid biosynthesis in Solanaceous species because there is a close correlation between the induction of HQT activity and the chlorogenic acid synthesis in developing tomato cotyledon (Strack & Gross, 1990). Moreover, the synthesis of chlorogenic acid have been occurred by transesterification of caffeoyl-CoA with quinic acid at neutral pH by HQT catalysis in the tomato cytosol (Figure 9) (Moglia et al., 2014).

Beside chlorogenic acid, the formation of 3,5- dicaffeoylquinic acid or cynarin is observed by a coffee hydroxycinnamoyl-coenzyme A shikimate / quinate hydroxycinnamoyl transferases (HCT/HQT) after prolonged incubation of protein extracts with quinate and caffeoyl-CoA (Lallemand et al., 2012). The cynarin formation is biosynthesized from chlorogenic acid at low pH by HQT catalysis which depend on chlorogenate:chlorogenate hydroxycinnamoyl transferase (CCT) in the vacuole of tomato (Figure 9) (Moglia et al., 2014).

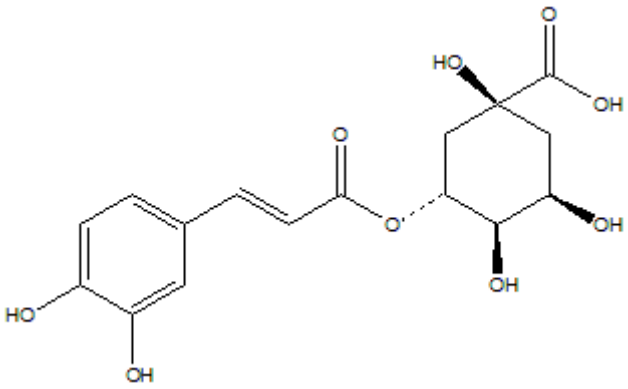


Figure 6. The structure of chlorogenic acid

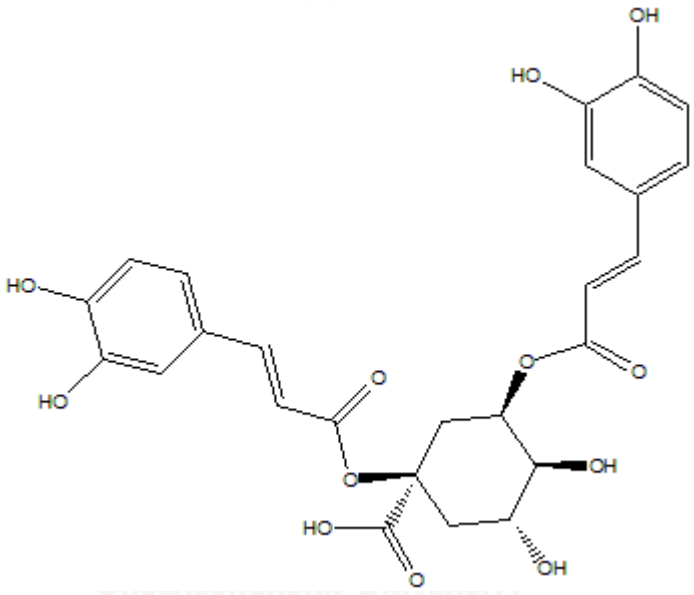


Figure 7. The structure of cynarin

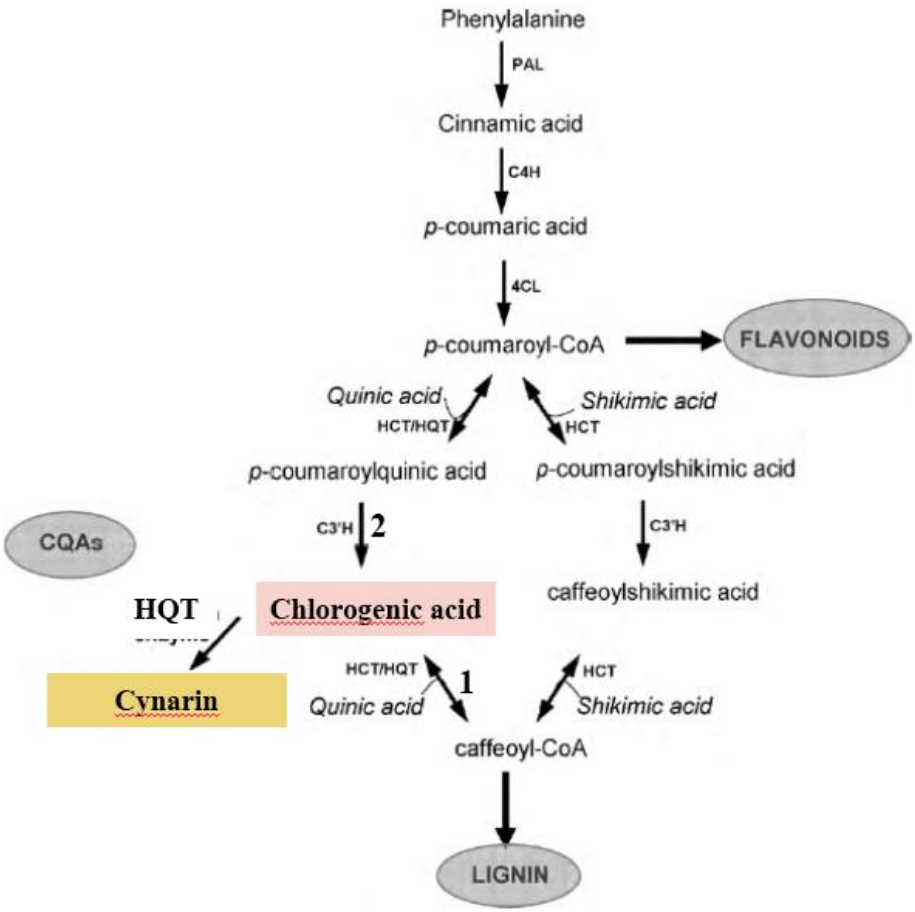


Figure 8. Proposed pathways for the biosynthesis of chlorogenic acid and cynarin in plants. PAL, phenylalanineammonia-lyase; C4H, cinnamate4-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl-transferase; HQT, hydroxycinnamoyl-CoA quinatehydroxyl cinnamoyl-transferase; C3′H, p-coumaroyl ester 3′-hydroxylase (Adapted from Menin et al., 2010).

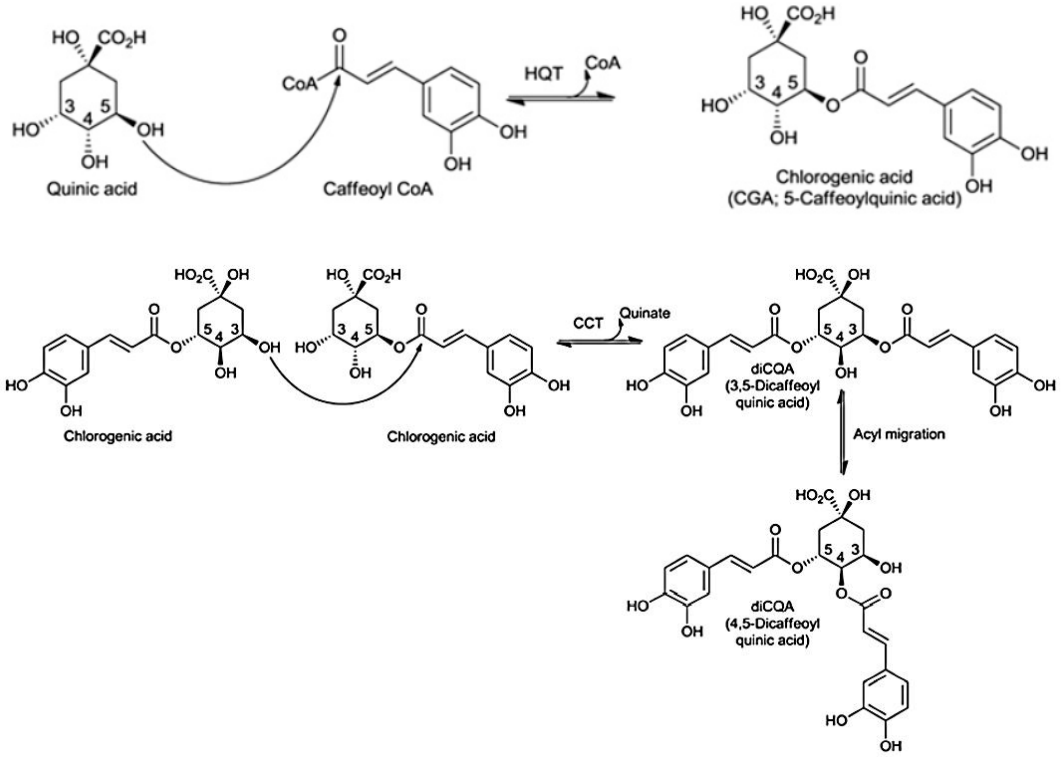


Figure 9. Proposed reaction scheme for 3,5- dicaffeoylquinic acid or cynarin synthesis by CCT using chlorogenic acid as the acyl donor as well as the acyl acceptor compared with the BAHD activity (D'Auria, 2006) of hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) using caffeoyl-CoA as the acyl donor and quinate as the acyl acceptor, respectively (Taken from Moglia et al., 2014)

1.3 Compounds with antiglycative activity

Non-enzymatic glycation is involved in the diabetes development (Vlassara & Palace, 2002) which is one of the most common chronic diseases and can cause kidney failure and blindness (Nathan, 1993). Protein glycation is formed by a nucleophilic addition reaction between a carbonyl group from a reducing sugar and a free amino group from a protein and to synthesize a Schiff base which is freely reversible, then, it rearranges to form a more stable ketoamine or Amadori product. This reaction results in the formation of advanced glycation endproducts (AGEs) (Figure 10) (Ahmed, 2005). AGEs can cause protein cross-linking in the basement membrane of the extracellular matrix, generating fluorescence, and exhibiting browning. They lead to impair cellular function and modify the structure of protein. Moreover, AGEs can cause the interaction with their cellular receptors (RAGE) which play an important role in the diabetic pathogenesis complications (Stern et al., 2002). RAGE usually found on macrophages, astrocytes, endothelial cells and smooth muscle cells. It is a member of the immunoglobulin superfamily of cell surface molecules and a multiligand receptor. RAGE also interacts with AGEs on macrophages which brings about oxidative stress and activation of nuclear factor-kB (NF-kB) via activation of the $p21^{ras}$ and the mitogen-activated protein (MAP) kinase signalling pathway which upregulates the expression of several genes relevant for coagulation, vasoconstriction, and inflammation (Figure 11) (Yan et al., 1994). Although, many chemicals are proved to have inhibitory capacities against the formation of AGEs, some side effects in people are occurred. For example, due to the high toxicity for diabetic patients, aminoguanidine which is an effective AGE-inhibitor in both in vitro and in vivo studies was found to fail in the phase III clinical trials therapy (Thornalley, 2003). Currently,

natural products are more considerable to consume by people. The previous report showed that there is an antiglycation in vitro in plant derived flavonoids such as kaempferol, rutin, and quercetin with antioxidant activity (Asgary et al., 1999). Recently, sunflower sprouts was proved to have the strongest inhibitory effects against the formation of AGEs comparing among four different sprouts which are popularly consumed by the Chinese (Sun et al., 2012).

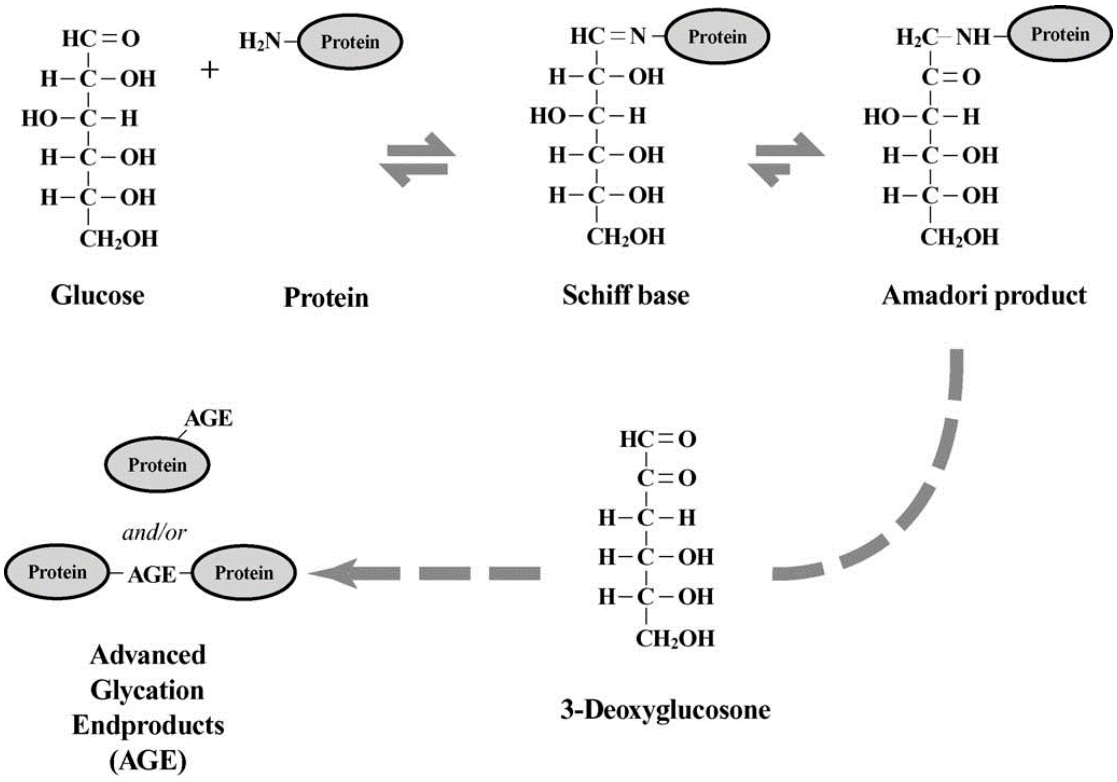


Figure 10. Glycation of a protein by glucose and the subsequent formation of AGEs. The initial reaction between glucose and protein amino groups forms a reversible Schiff base that rearranges to a ketoamine or Amadori product.With time, these Amadori products form AGEs via dicarbonyl intermediates such as 3-DG. (Taken from Ahmed, 2005).

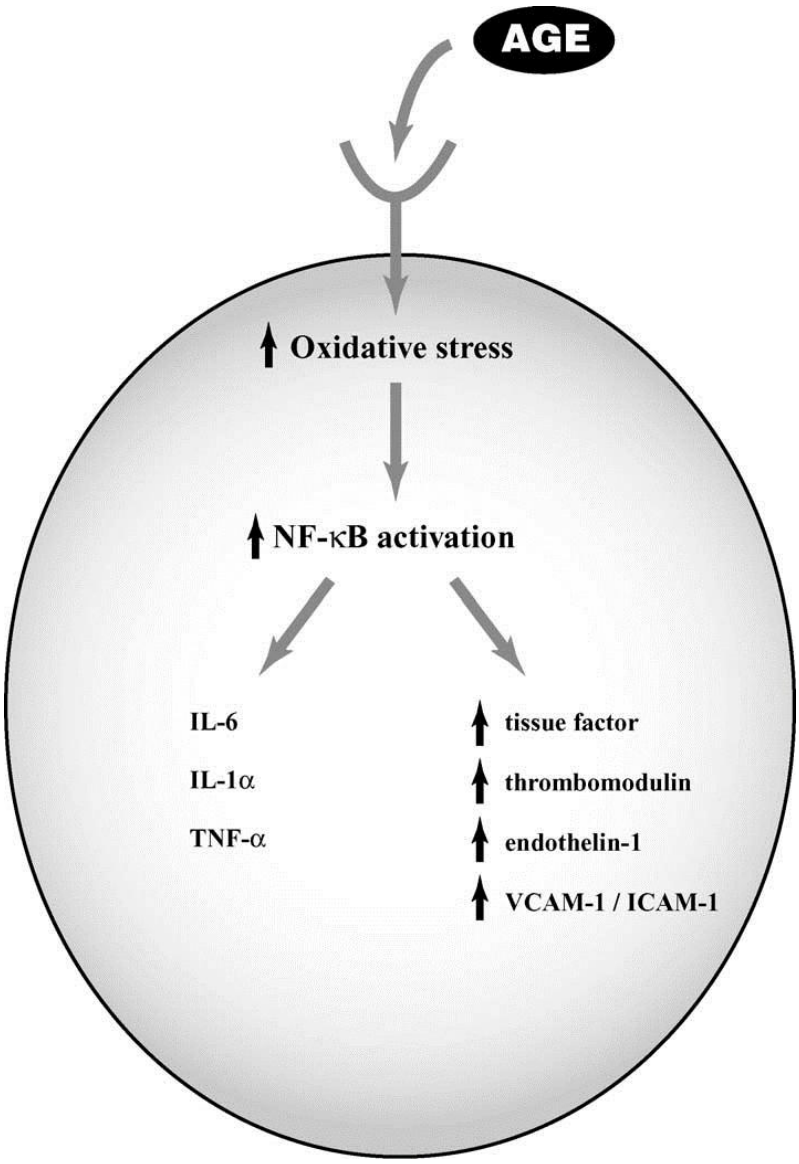


Figure 11. Interaction of advanced glycation endproducts (**AGEs) with receptor of advanced glycation endproducts (RAGEs) on macrophages induces oxidative stress and activation of intracellular signaling causing secretion of cytokines and mediators of inflammation, vasoconstriction and coagulation** (Taken from Ahmed, 2005).

1.4 Bioactive compounds enhancement by elicitation

Currently, elicitors are useful tools to enhance plant secondary metabolites. The meaning of elicitor is a compound that can induce the response in living organisms which leads to the secondary metabolites accumulation. Commonly, the attack of herbivores, insects, pathogens, or other biotic and abiotic stresses are responded by plants. The reaction occurs by activating an array of defense mechanisms including structural defensive barriers such as lignin deposition on cell wall, hypersensitive responses, and induction of biosynthesis of secondary metabolites as phytoalexins (Hutcheson, 1998). Elicitors are divided into two groups consisting of biotic or abiotic depending on the nature of the elicitors. The biotic elicitors derived from the pathogen or from the plant itself (endogenous elicitor). In contrast, abiotic elicitors are not originally from biological sources which they are classified into physical factors and chemical compounds. In addition, it can also be divided into two groups depending on the interaction of plant elicitors including general elicitors and race specific elicitors. General elicitors can activate defense responses both in host and non-host plants. Race specific elicitors can stimulate responses resulting in disease resistance only in specific host cultivars, according to the simultaneous presence of resistance genes in plants and avirulence genes in pathogens (Staskawicz et al., 1995). The crucial events by the elicitor activation have been proposed (Figure 12). Initially, elicitor binding to receptors localized in the plasma membrane resulting in G-protein activation is occurred. Then, G-protein-mediated stimulates adenylyl cyclase (AC) and phospholipase C (PLC) leading to second messenger levels (cAMP, DAG, IP3) increases coupled to the stimulation of their target kinases (PKA, PKC). After that, cytoplasmic Ca^{2+} concentrations are altered resulting in involvement of Ca^{2+} fluxes through plasma

membrane or intracellular reservoirs. The protein kinase cascades are rapidly activated which induce changes in their translocation into the nucleus and in phosphorylation of MAPKs. Finally, transcription of enzymes involving in synthetic pathways of secondary metabolites is activated (Vasconsuelo & Boland, 2007).

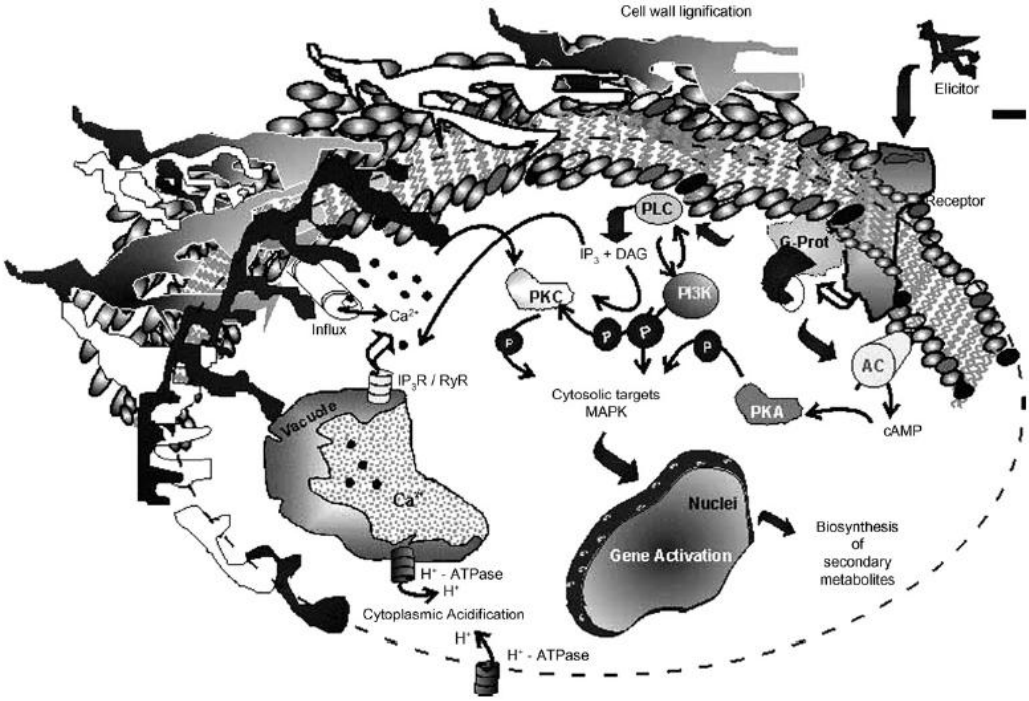


Figure 12. Key signalling events triggered by the elicitation process (Taken from Vasconsuelo & Boland, 2007).

1.4.1 Chitosan

Chitosan (deacetylated chitin) is a biotic elicitor which is extracted from exoskeleton of the crustaceans. It is a linear polymer which D-glucosamine residues are connected by β-1,4 linkages. N-acetylglucosamine residues are randomly distributed along the whole polymer chain (Figure 13) (Chirkov, 2002). Recently, due to their unique biological activities consisting antimicrobial (Kendra & Hadwiger, 1984), antitumor (Suzuki et al., 1986), hypocholesterolemic functions (Sugano et al., 1992), and antioxidative (Youn et al., 2001). Furthermore, treating plants with chitosan has been shown to improve postharvest vegetables and fruits storability (Ghaouth et al., 1991) and to induce plant development (Kim, 1998). Previous studies found that chitosan treatment can increase the growth rate and decrease the rot of soybean sprouts (Lee et al., 1999). Soybean sprouts treated with high molecular weight chitosan showed a significant increase in respiration, in the growth of the sprouts, especially, in fresh weight (Lee et al., 2005). More recently, chitosan treatment was studied in sunflower sprouts, the results demonstrated that it can slightly increase in the amount of DPPH radical scavenging activity, total phenolic, and melatonin contents (Cho et al., 2008).

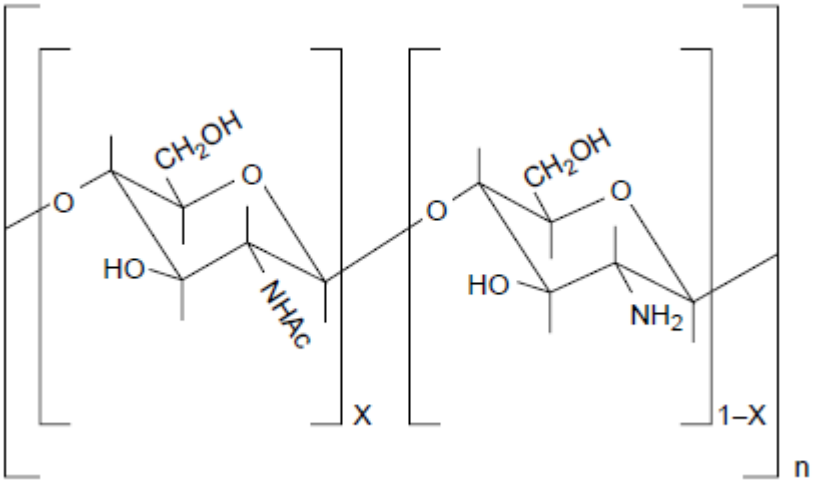


Figure 13. The chemical structure of chitosan with X 5 degree of acetylation and n 5 number of sugar units per polymer (Taken from Dodane & Vilivalam, 1998).

1.4.2 Methyl jasmonate

Methyl jasmonate is a fragrant volatile compound which firstly identified in *Jasminum grandiflorum* flowers. It also has proven that it can distribute widely in the plant kingdom (Cheong & Choi, 2003). Methyl jasmonate and its free-acid jasmonic acid are important in cellular regulators which involve in developmental processes of plants, for instance, seed germination, fertility, root growth, fruit ripening, and senescence (Wasternack & Hause, 2002) (Figure 14). Furthermore, they can trigger plant defense mechanism responding to low temperature, salinity, drought, insect-driven wounding, and various pathogen (Wasternack & Parthier, 1997). Methyl jasmonate elicitation can upregulate various genes such as genes involving in jasmonate biosynthesis, secondary metabolism, cell wall formation, and genes encoding defense proteins and stress protective. On the other hand, there is downregulation of several genes which involved in photosynthesis (Cheong &

Choi, 2003). These compounds are isolated and characterized as growth inhibitors (Ueda & Kato, 1980). There was a study in radish sprouts (*Raphanus sativus* L.) which showed that the total phenolic contents and DPPH free radical scavenging capacity were significantly increased by the methyl jasmonate treatment (Kim et al., 2006).

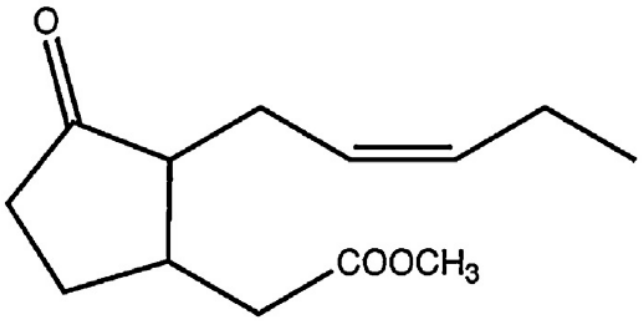


Figure 14. The structure of methyl jasmonate (Taken from Dar et al., 2015).

CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

10X reaction buffer with $MgCl_2$ (Thermo Scientific, USA)

2, 2-Diphenyl-1-picryl hydrazyl (≥95%) (Sigma Chemical Co., USA)

2, 4, 6-Tripyridyl-S-Trizine (≥98%) (Sigma Chemical Co., USA)

6-Hydroxy-2, 5, 7, 8-Tetranethylchroman-2-C (≥98%) (Sigma Chemical Co., USA)

Absolute ethanol (AnalaR NORMAPUR, Ireland)

Acetic acid (≥99.85%) (Sigma Chemical Co., USA)

Acetonitrile (Sigma Chemical Co., USA)

Agarose (Bioline, UK)

Bovine serum albumin (Sigma Chemical Co., USA)

Bromophenol blue (Carlo Erba Reagenti, Italy)

Chlorogenic acid (Sigma Chemical Co., USA)

Cynarin (Sigma Chemical Co., USA)

Folin-Ciocalteu's phenol reagent (Sigma Chemical Co., USA)

Gallic acid (≥97.5%) (Sigma Chemical Co., USA)

Iron (III) chloride hexahydrate (≥97%) (Sigma Chemical Co., USA)

Methanol (Sigma Chemical Co., USA)

Puerarin (Sigma Chemical Co., USA)

RiboLock RNase Inhibitor (Thermo Scientific, USA)

Sepasol-RNA I super G (Nacalai Tesque, Japan)

Sodium acetate trihydrate (≥99%) (Sigma Chemical Co., USA)

Sodium carbonate (≥99%) (Sigma Chemical Co., USA)

2.1.2 Enzymes

DNase I (Thermo Scientific, USA)

Taq DNA polymerase (New England Biolabs, UK)

2.1.3 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd., Japan)

Balance: PB303-L (Mettler Toledo, USA)

CentriVap Benchtop Vacuum Concentrators (Labconco, USA)

CFX96™ Real-Time PCR Detection System (BIO-RAD, USA)

Gel documentation apparatus: Gel Doc™ (Syngene, England)

Gel electrophoresis apparatus: Mupid® -exU (Advance Co., LTD, Japan)

HPLC Shimadzu: LC-20A (Shimadzu, Japan)

Laminar flow: Bio Clean Bench (SANYO, Japan)

Lyophilizer: Freezone 2.5 (Labconco, USA)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

Microwave oven: R-362 (SHARP, Thailand)

Mixer mill: MM400 (Retsch®, Germany)

PCR: T100™ Thermal Cycle (BIO-RAD, USA)

pH meter: S220 Seven compact™ pH/ion (Mettler Toledo, USA)

Refrigerator: Ultra low temperature freezer (New Brunswick Scientific, UK)

Refrigerated centrifuge: Legend XTR (Thermo Scientific, USA)

Spectrophotometer: DU® 530 (Beckman Coulter™, USA)

Synergy H1 Multi-Mode Reader (Biotek, USA)

2.1.4 Glasswares and plasticwares

1.5-ml microcentrifuge tube (Axygen Hayward, USA)

0.2 ml PCR thin wall microcentrifuge tube (Axygen Hayward, USA)

10-, 100-, 1000-µl pipette tips (Axygen Hayward, USA)

Glass bottles

NIPRO disposable syringe (Nissho, Japan)

Nylon syringe filter, 0.2 micron, 13 mm (Filtrex Technologies, USA)

12x32mm clear glass screw thread standard mouth vials (Vertical Chromatography, Thailand)

Black open-top cap with PTFE/white silicone septa (Vertical Chromatography, Thailand)

Glass, 100µL with bottom spring (Vertical Chromatography, Thailand)

2.1.5 Kits

iScript™ reverse transcription supermix for RT-qPCR (BIORAD, USA)

SsoFast™ Evagreen® Supermix (BIO-RAD, USA)

2.1.6 Oligonucleotide primers

All oligonucleotide primers used were synthesized by BIO BASIC Int, Canada

2.1.7 Plant materials

Thai sunflower seeds including 5A, 10A, S473, and S475 were kindly provided by Assistant Professor Thitiporn Machikowa, Ph.D. from Suranaree University of Technology. The seeds from Chiatai company (CH) was purchased from the market and UNK variety was purchased from the Internet (https://www.facebook.com/lungtoporganic).

2.1.8 Software and database

CFX manager software (BIO-RAD, USA)

ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

Gen5 Microplate Reader and Imager Software (BioTek, USA)

LCsolution (Shimadzu, Japan)

MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0 (Tamura, et al. 2013)

NCBI (http://www.ncbi.nlm.nih.gov/)

Sunflower genome database (www.sunflowergenome.org)

Translate tool (http://web.expasy.org/translate/)

2.2 Methods

2.2.1 Sunflower sprouts cultivation and elicitors treatment

The seeds were cultivated by soaking seeds in water for 10 minutes, wrapping with wet tissue until the roots emerge, and sowing in vermiculite for 5 days for comparing among six varieties and 3, 5, 7, 9 days for comparing among each

developmental stage at 12 light hours and 12 dark hours with 30°C air temperature and 70% relative humidity. There are five biological replicates in each variety.

For treating the sprouts with biotic elicitors including chitosan and methyl jasmonate (MeJA), Chitosan O-80 solution was prepared in 1% acetic acid at 1% (w/v) concentration and diluted with water to give a final chitosan concentration of 10, 50, 100 and 200 ppm and methyl jasmonate, was dissolved in 0.2% ethanol and was applied at 10, 50, 100 and 200 ppm. Treatments were applied by exogenous spraying (not as soaking solution treatment) once per day. Sprout samples were collected at 3, 5 and 7 days after sowing.

The samples were gently collected excluding roots, weighed (fresh mass), flash frozen in liquid nitrogen and stored at -80 °C.

2.2.2 Sunflower sprouts extraction

The sprouts were grounded by using Mixer mill MM400 (Retsch, Germany) at 30 Hz for 1 minute. Some of the samples were partly separated for lyophilizing for HPLC analysis. Sixty milligram fresh weight were measured for FRAP, DPPH, and total phenolic compounds assay. The samples were extracted with 1 ml 95% methanol, shaked vigorously at 1500 rpm for 5 min, 10°C before being centrifuged at 10,000 rpm for 5 min. Supernatant was placed in 1.5 ml tube. For HPLC analysis, 15 mg dry weight were measured. The samples were extracted with 1 ml 80% methanol, shaked vigorously at 2000 rpm for 30 min, 10°C before being centrifuged at 12,000 rpm for 3 min. Supernatant was placed in 1.5 ml tube and vacuum evaporation was conducted. The samples were resuspended with 100 μl 80% methanol. The supernatant was filtered through 0.2 micron (13 millimeter diameter) nylon filter membrane.

2.2.3 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay)

The DPPH assay was carried out as previously described (Brand-Williams, Cuvelier, & Berset, 1995). Briefly, 190 μl of freshly prepared DPPH (0.6 M) was mixed with 10 μl of tested compounds in 95% ethanol. After the mixtures were incubated at room temperature for 30 min, the absorbance at 515 nm was measured. Trolox (0, 25, 50, 100, and 200 μM) was used for the standard calibration curve. The results were expressed as mM trolox equivalent (TE)/g fresh weight sunflower sprouts.

2.2.4 Ferric reducing antioxidant power (FRAP assay)

The FRAP assay was carried out as previously described (Benzie & Strain, 1996). Briefly, 190 μl of 10:1:1 300 mM acetate buffer : 20 mM $FeCl_3 \bullet 6H_2O$: 10 mM 2, 4, 6-Tripyridyl-S-Trizine in 40 mM HCl was mixed with 10 μl of the tested compounds in 95% methanol. After the mixtures were incubated at room temperature for 30 min, the absorbance at 593 nm was measured. Trolox (0, 25, 50, 100, and 200 μM) was used for the standard calibration curve. The results were expressed as mM trolox equivalent (TE)/g fresh weight sunflower sprouts.

2.2.5 Folin–Ciocalteu – Total phenolic compound assay

Total phenolic content was estimated by the Folin–Ciocalteu method (Singleton & Rossi, 1965). Briefly, 36 μl of 1:10 diluted Folin–Ciocalteu reagent and 146 μl of 350 mM Na_2CO_3 were mixed with 18 μl of diluted sample in a 96-well-plate. After the mixtures were incubated at room temperature for 30 min, the absorbance at 765 nm was

measured. Gallic acid (0, 50, 100, 500, and 1000 μM) was used for the standard calibration curve. The results were expressed as mM gallic acid equivalent (GAE)/g fresh weight sunflower sprouts.

2.2.6 Bovine Serum Albumin (BSA)−antiglycation assay

The BSA−antiglycation assay was carried out as previously described (Sun et al., 2012). BSA (50 mg/mL) was dissolved in phosphate buffer (0.2 M, pH 7.4) with glucose (0.8 M). NaN_3 (0.2 g/L) was added to the test solution to ensure an aseptic condition. Aminoguanidine (AG) solution (1 mM) was used as the positive control. All incubations were carried out at 37 °C for 7 days in the absence or presence of inhibitors. The measurement of the fluorescent intensity of AGEs was performed using a Synergy H1 Multi-Mode Reader (BioTek, USA). The generation of AGEs was characterized by a typical fluorescence with excitation and emission maxima at 330 and 410 nm, respectively.

2.2.7 HPLC analysis

The equipment was a Shimadzu LC-20A instrument with a 320 nm uv detector. Separation was carried out with a Phenomenex Gemini C18 (50 × 4.6 mm) column. The mobile phase was composed of 1% acetic acid water (solvent A) and acetonitrile (solvent B) of the following gradients: 0 min, 100% A/0% B; 25 min, 55% A/45% B. The flow rate was set at 1.0 mL/min.

2.2.8 Database searching for chlorogenic acid and cynarin biosynthetic genes

Nucleotide blast in sunflower genome database (www.sunflowergenome.org) was conducted for finding the gene Hydroxycinnamoyl CoA quinate transferase (HQT) in sunflower sprouts which involves in chlorogenic acid and cynarin biosynthesis. This gene is similar to HQT gene from tomato (*Solanum lycopersicum*) (NM_001247921.2) (Moglia et al., 2014) (Figure 15). Open Reading Frame (ORF) by http://web.expasy.org/translate was performed and phylogenetic tree was constructed by using MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0 (Tamura et al., 2013). The tree was constructed together with the *Helianthus annuus* hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase 1, 2, 3, and 4 (HaHCT1, HaHCT2, HaHCT3, and HaHCT4) (no report), *Nicotiana tabacum* hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (NtHCT) (CAD47830) (Hoffmann et al., 2003), *Arabidopsis thaliana* hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase (AtHCT) (NP_199704) (Hoffmann et al., 2005), *Trifolium pratense* hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase 1 A, 1 B, and 2 (TpHCT1A, TpHCT1B, and TpHCT2) (ACI16630, ACI28534, and ACI16631) (Sullivan, 2009), *Pinus radiata* hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase (PrHCT) (ABO52899) (Wagner et al., 2007), *Avena sativa* hydroxycinnamoyl-CoA:hydroxyanthranilate N-hydroxycinnamoyltransferase (AsHHT) (BAC78633) (Yang et al., 2004), *Nicotiana tabacum* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (NtHQT) (CAE46932) (Niggeweg et al., 2004), *Solanum lycopersicum* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (SlHQT) (NP_001234850) (Moglia et al., 2014), *Cynara cardunculus* var. scolymus

hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (CcsHQT) (ABK79689) (Comino et al., 2007), *Cynara cardunculus* var. scolymus hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase 1 and 2 (HQT1 and HQT2) (CAM84302 and ACJ23164) (Sonnante et al., 2010). *Dianthus caryophyllus* anthranilate N-hydroxycinnamoyl/benzoyltransferase (HCBT) (CAB06430) (Yang et al., 1997), *Arabidopsis thaliana* spermidine hydroxycinnamoyl transferase (SHT) (NP_179497) (Grienenberger et al., 2009), *Clarkia breweri* benzoyl-coenzyme A (CoA):benzyl alcohol benzoyl transferase (CbBEBT) (AAN09796) (Auria et al., 2002), *Petunia* x *hybrida* benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase (PhBPBT) (AAU06226) (Boatright et al., 2004), *Arabidopsis thaliana* hydroxycinnamoyl-CoA:x-hydroxyacid O-hydroxycinnamoyltransferase (AtHHT) (ACY78659) (Gou et al., 2009), *Arabidopsis thaliana* fatty alcohol:caffeoyl-CoA caffeoyl transferase (FACT) (At5g63560) (Kosma et al., 2012), *Arabidopsis thaliana* spermidine disinapoyl transferase (AtSDT) (NP_179932) (Luo et al., 2009), *Glycine max* malonyl-CoA:isoflavone 7-O-glucoside-6-O-malonyltransferase (GmIF7MaT) (BAF73620) (Suzuki et al., 2007), *Dahlia variabilis* malonyl-CoA:anthocyanidin 3-O-glucoside-6-O-malonyltransferase (Dv3MAT) (AAO12206) (Suzuki et al., 2002), *Gentiana triflora* anthocyanin 5-aromatic acyltransferase (Gt5AT) (BAA74428) (Fujiwara et al., 1998), *Nicotiana tabacum* malonyl-CoA flavonoid/naphthol glucoside acyltransferase (NtMAT1) (BAD93691) (Taguchi et al., 2005), *Perilla frutescens* malonyl-CoA:anthocyanin 5-O-glucoside-6-O-malonyltransferase (Pf5MaT) (AAL50565) (Suzuki et al., 2001).

2.2.9 RNA extraction and cDNA synthesis

Total RNAs were extracted from sunflower sprouts in different developmental stages varying 3, 5, 7, and 9 day of cultivation by using the Sepasol extraction method (Sepasol-RNA I Super G; Nacalai tesque, Japan). The RNAs were incubated with DNase (Fermentas, USA) for 1 hr at 37ºC followed by heat-inactivation for 10 min at 65ºC. First-strand complementary DNA (cDNA) was synthesized with the iScript™ reverse transcription supermix for RT-qPCR (BIORAD, USA) using 20 µl of reaction mixture containing 1 µg of total RNA, 4 µl of iScript RT Supermix consisting of RT supermix with RNase H+ Moloney murine leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, dNTPs, oligo(dT), random primers, buffer, $MgCl_2$, and stabilizers. The complete reaction was then cycled for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C using a PCR: T100™ Thermal Cycle (BIO-RAD, USA). The reverse transcription reaction mixture was then used for polymerase chain reaction (PCR) amplification in the presence of the primers. The Primer3 plus software was used for primer designing. All primers used were listed in Table 2.

2.2.10 Gene expression analysis by real-time qRT-PCR

Real-time qRT-PCR was carried out in triplicates by using of 10 µl of reaction mixture that contained

component	volumn	
Ssofast Evagreen Supermix	5	µl
nuclease-free water	3	µl
Forward primer (10 µM)	0.5	µl
Reverse primer (10 µM)	0.5	µl
cDNA template (1 µg RNA-equivalent/µl)	1	µl
total volumn	10	µl

The real-time PCR reactions were performed using a CFX96™ Real-Time PCR Detection System (BIO-RAD, USA). The reactions were activated from denaturation (95°C for 10 min), cDNA was then amplified for 40 cycles consisting of denaturation (95°C for 20 sec) and annealing/extension (52°C for 10 sec). Denaturation at 95°C for 15 sec, then, a melt-curve was constructed (65°C for 15 sec, and then temperature was increased by 0.5°C until 90°C). At the end of annealing and extension step, and after each increment for melting curve, plate read was conducted every cycle.

Real-time PCR was performed with a series of template concentration which prepared by serial dilution of a template for determining amplification efficiency of each primer pair. C_T values of at least five cDNA concentrations were plotted on x-axis against log cDNA dilution on y-axis to create standard curve. PCR efficiency was then calculated from: $E = 10^{-1/slope}$, where E is the efficiency. Percentage of PCR efficiency or (E-1)*100% should be within 90-110%.

Relative gene expression levels were calculated by the ΔC_T method using a reference gene (Schmittgen & Livak, 2008) which tubulin was used as a reference gene. Fold change of gene expression between two samples is calculated by ratio (reference/target) = $2^{CT(reference) - CT(target)}$.

2.2.11 Statistical Analysis

Experimental results were obtained as the mean value ± standard deviation (SD) (n = 3-5). Statistical analyses were performed using the SPSS statistical package (SPSS, Inc., Chicago, IL). Mean values were compared using Duncan's multiple-range test. The statistical significances were achieved when $p < 0.05$.

Solanum lycopersicum hydroxycinnamoyl CoA quinate transferase (hqt), mRNA

NCBI Reference Sequence: NM_001247921.2

GenBank Graphics

>gi|924183451|ref|NM_001247921.2| Solanum lycopersicum hydroxycinnamoyl CoA quinate transferase (hqt), mRNA

ATTTCACAAATAATAATATTCCAAAAATACATATTTTTATTATTTTTAAAAACACCCTTCCCTCTCCTTG
ACCTAATTTTTTTTTTTACCTCTTTTGGAAATAAATGGGAAGTGAAAAAATGATGAAAATTAATATCAAA
GAATCAACACTAGTGAAACCATCAAAACCAACACCAACAAAGAGAATTTGGAGTTCTAATTTGGATTTAA
TTGTTGGAAGAATTCATCTTTTGACTGTTTATTTTTATAAACCAAATGGATCTTCAAATTTTTTTGATAA
TAAAGTTATTAAAGAAGCATTAAGTAATGTTTTAGTTTCATTTTATCCAATGGCTGGAAGATTAGGTAGG
GATGAACAAGGTAGAATTGAAGTTAATTGTAATGGTGAAGGTGTTTTGTTTGTTGAGGCTGAAAGTGATT
CATGTGTTGATGATTTTGGTGATTTTACACCATCTTTGGAACTTAGAAAACTCATTCCAAGTGTTGAAAC
CTCTGGAGATATCTCAACTTTCCCACTAGTTATATTTCAGATTACTCGTTTCAAGTGTGGCGGAGTCGCT
CTTGGTGGTGGAGTATTCCACACGTTATCCGATGGTCTCTCATCCATCCACTTCATCAACACGTGGTCGG
ACATCGCCCGTGGCCTCTCCGTCGCAGTCCCGCCGTTCATCGATCGGACGCTCCTCCGTGCAAGGGACCC
ACCGACATCTTCTTTCGAGCACGTTGAGTACCATCCTCCACCTACCCTAAACTCATCGAAAAATCGCGAG
TCCAGTACCACGACCATGTTGAAATTCTCGAGTGAACAACTCGGGCTTCTTAAGTCCAAGTCCAAAAATG
AGGGTAGCACCTATGAAATCCTCGCAGCCCATATTTGGCGATGCACGTGCAAGGCACGTGGATTGCCAGA
GGATCAATTGACCAAATTACACGTGGCCACCGACGGAAGGTCAAGGCTTTGCCCTCCCTTGCCACCGGGT
TACCTAGGAAACGTCGTGTTCACGGCAACCCCAATAGCTAAATCATGCGAACTTCAATCAGAGCCGTTGA
CAAATTCCGTCAAGAGAATTCACAACGAGTTGATCAAAATGGACGACAATTACCTAAGATCAGCACTGGA
TTACCTCGAATTACAACCTGATTTATCAACCCTAATTCGGGGCCCGGCTTACTTTGCTAGCCCTAACCTC
AATATTAATAGTTGGACTAGGTTGCCTGTCCATGAGTGTGATTTTGGATGGGGTAGGCCAATTCATATGG
GACCAGCTTGCATTTTATATGAAGGGACAATTTATATTATACCAAGTCCAAATTCTAAAGATAGGAACTT
GCGTTTGGCTGTTTGTCTAGATGCTGGTCACATGTCACTATTTGAAAAATATTTATATGAATTATGAGAT
ATTTTTCATATGAAAAAAAAAATCAAGAATGTACAACACAAATTGTGGTGTGATTT

Figure 15. Nucleotide sequences of HQT gene in tomato (*Solanum lycopersicum*) (NM_001247921.2).

Table 2. Oliginucleotide primers used in real-time qRT-PCR

Name	Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Annealing temp. (°C)
HaTubulin	tubulin	Forward	AGCGGAGAATTGTGATTGCT	222	52
		Reverse	TCTGCATTTTCCACAAGCTG		
HaHQT1	hydroxycinnamoyl-Coenzyme A: quinate hydroxyl cinnamoyl transferase 1 (HQT1)	Forward	GATGGCTGGACGGTTAGGTA	198	52
		Reverse	GCGAAAAACAGAGGAAACGA		
HaHQT2	hydroxycinnamoyl-Coenzyme A: quinate hydroxyl cinnamoyl transferase 2 (HQT2)	Forward	TGCTCCAACTTCTTTGATCCT	250	54
		Reverse	AAGAAGAAATGTCACCGGAAT		
HaHQT3	hydroxycinnamoyl-Coenzyme A: quinate hydroxyl cinnamoyl transferase 3 (HQT3)	Forward	CGGTTGGGAAGAGATGAGAG	164	52
		Reverse	ATGTCGCCGGAGTAATCAAC		

CHAPTER III
RESULTS

3.1 Growth, antioxidant activities, total phenolic contents, and major bioactive compound contents in six varieties of sunflower sprouts

3.1.1 Growth determination

Six varieties of five days old sprouts including 5A, 10A, S473, S475, UNK, and CH was weighted. The results demonstrated that the varieties from Suranaree University of Technology except S473 were significantly different comparing with those which were purchased from the market (UNK and CH).

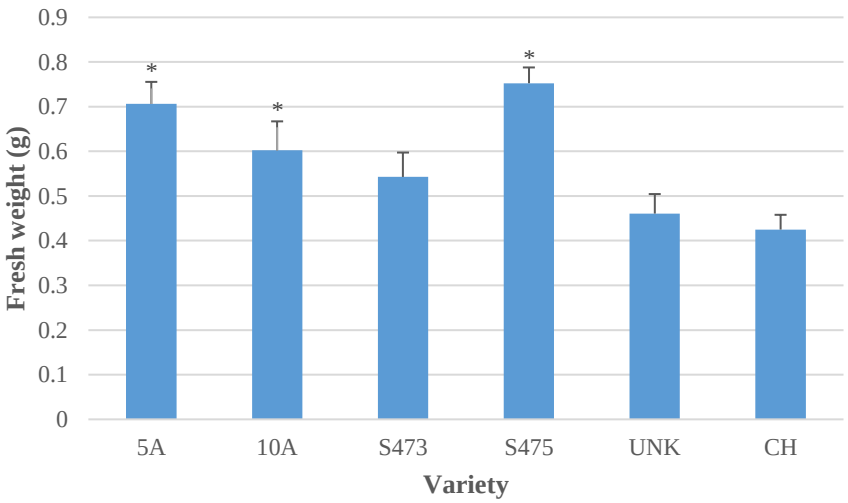
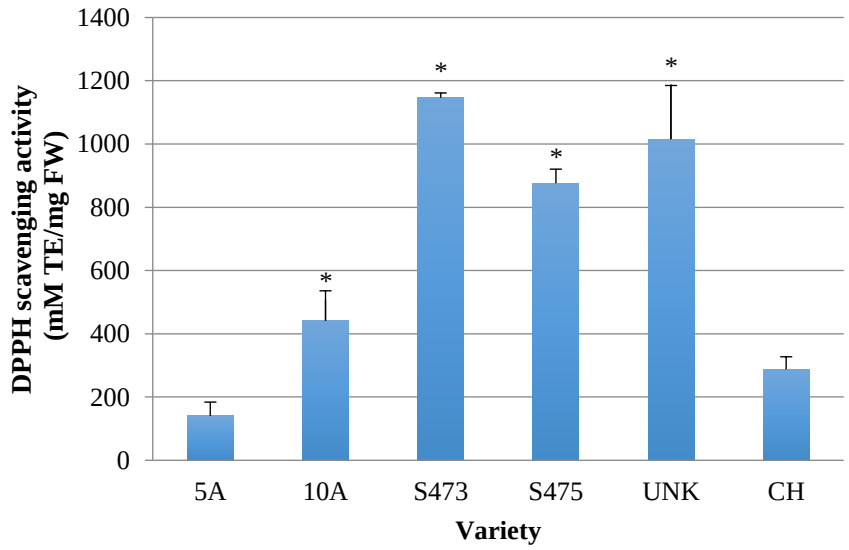


Figure 16. Fresh weight of five days old sunflower sprouts from six varieties. Each value represents the mean ± SE (n = 5) with * indicate significant differences compared to the CH variety ($P < 0.05$).

3.1.2 Antioxidant activity and total phenolic content determination

The results showed that the antioxidant activities of 10A, S473, S475 and UNK varieties were significantly different in DPPH assay (Figure 17A). Using FRAP and Folin-Ciocalteu assays, it was found that only UNK variety was significantly different from the other varieties which contained the highest antioxidant activity and total phenolic contents (Figure 17B and 17C). The antioxidant activity from DPPH assay was different from the other two assays which S473 and S475 varieties contained the highest level as same as UNK variety. While the results obtained from FRAP assay mostly was correlated to that of Folin-Ciocalteu assay. These results did not correlate to fresh weight which 5A, 10A, and S475 variety contained the highest fresh weight. Although, UNK variety gave almost the lowest fresh weight, it consisted of the highest antioxidant activity and total phenolic contents.

(A)



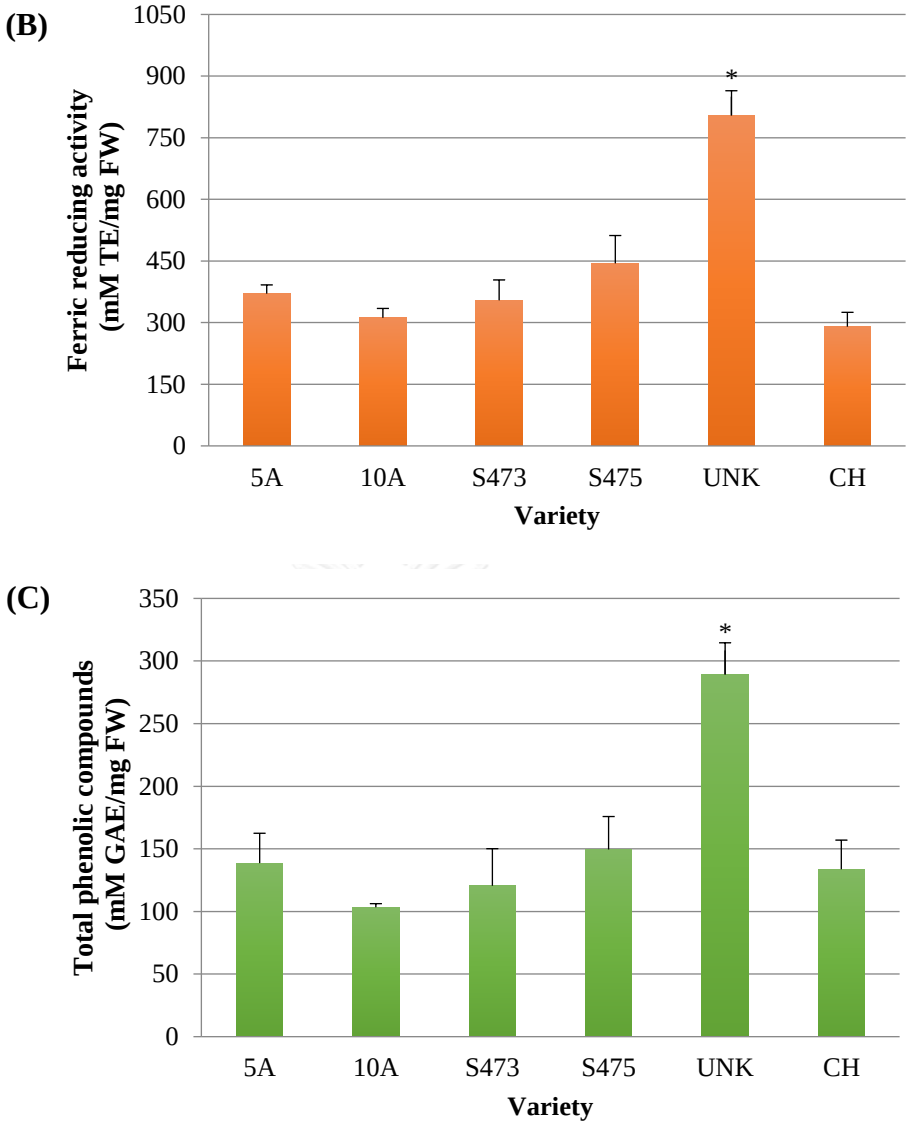
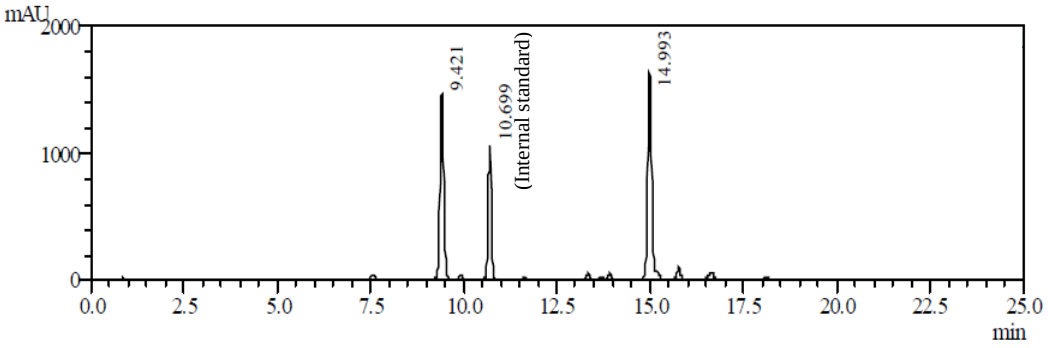


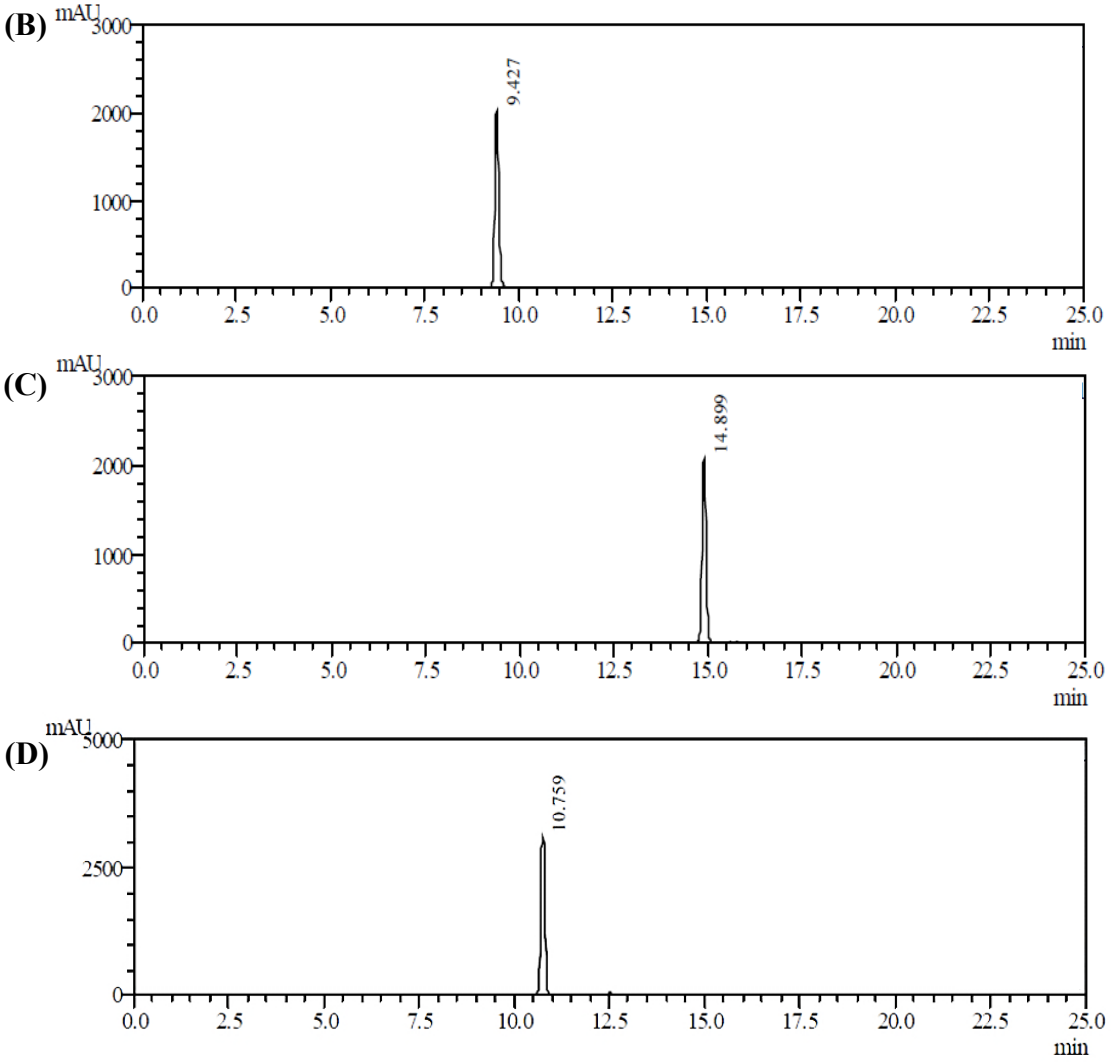
Figure 17. Antioxidant activity and total phenolic contents of five days old sunflower sprouts comparing among six different varieties. (A) Antioxidant activity measuring by 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay), (B) Antioxidant activity measuring by Ferric reducing antioxidant power (FRAP assay), and (C) total phenolic contents measuring by Folin-Ciocalteu assay. Each value represents the mean ± SE (n = 5) with * indicate significant differences compared to 5A variety ($P < 0.05$).

3.1.3 Chlorogenic acid & cynarin amounts by HPLC analysis

The contents of two major phenolic compounds in sunflower sprouts by HPLC analysis were analyzed. The results showed that two main peaks were detected with the retention times of 9.421 and 14.993, respectively (Figure 18A). These results were confirmed by using LC-MS analysis demonstrating that these two main peaks were the major phenolic compounds in sunflower sprouts including chlorogenic acid and cynarin according to Moglia et al., 2014 (Appendix), therefore, the standards of these two compounds were used to determine the amounts of chlorogenic acid (Figure 18B) and cynarin (Figure 18C). Puerarin was added in 80% methanol which was used in metabolite extraction to make an internal standard which provided the retention time of 10.759 (Figure 18D). HPLC results showed that the amounts of chlorogenic acid in all six varieties were not significantly different (Figure 18E). While, 10A and UNK variety contained the highest level of cynarin (Figure 18E). These results almost agree with the antioxidant activities and total phenolic contents especially UNK variety which contained the highest amount of antioxidant activities, total phenolic contents, and cynarin.

(A)




(B)
mAU
3000
2000
1000
0
9.427
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
min
(C)
mAU
3000
2000
1000
0
14.899
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
min
(D)
mAU
5000
2500
0
10.759
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
min

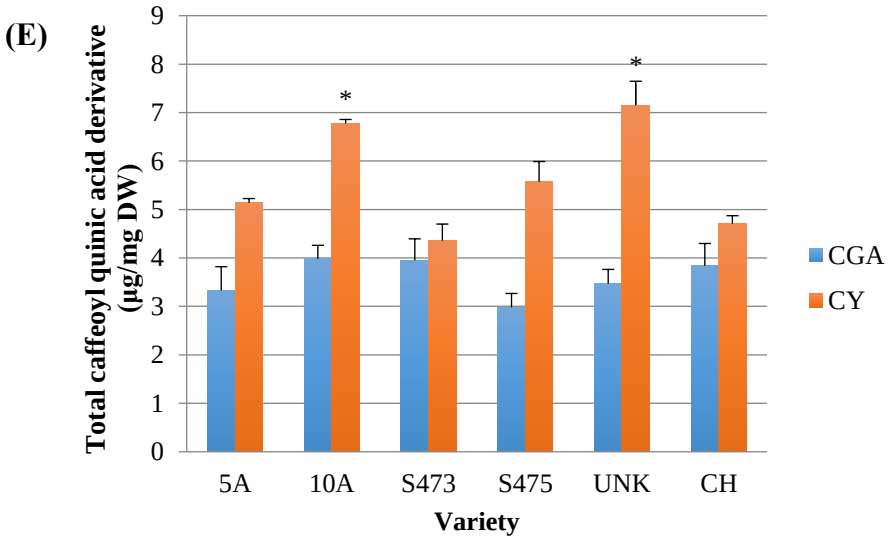

(E)
Total caffeoyl quinic acid derivative (µg/mg DW)
9
8
7
6
5
4
3
2
1
0
*
*
5A
10A
S473
S475
UNK
CH
Variety
CGA
CY

Figure 18. HPLC analysis of of five days old sunflower sprouts comparing among six different varieties (A) HPLC chromatogram of the *H. annuus* extract at 320 nm, (B) HPLC chromatogram of chlorogenic acid at 320 nm, (C) HPLC chromatogram of cynarin at 320 nm, (D) HPLC chromatogram of internal standard (puerarin) at 320 nm, (E) Chlorogenic acid (CGA) and cynarin (CY) compounds contents in different varieties of sunflower sprouts extracts by HPLC analysis. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 5A variety ($P < 0.05$).

3.2 Growth, antioxidant activities, total phenolic contents, and major bioactive compound contents comparing among different germination time

3.2.1 Growth determination

The 5A and CH varieties were selected to be the candidates from sunflower breeding program of Suranaree University of Technology and commonly purchasing from the market, respectively. Four germination time of 5A and CH varieties including day 3, 5, 7, and 9 were weighted and heighted. The results demonstrated that 5A variety contained more fresh weight than CH variety and the weight of CH slightly increased when the sprouts was growing (Figure 19). The height of each variety also corresponded to the fresh weight. In addition, the true leave obviously emerged when the sprouts were 9 days old (Figure 20).

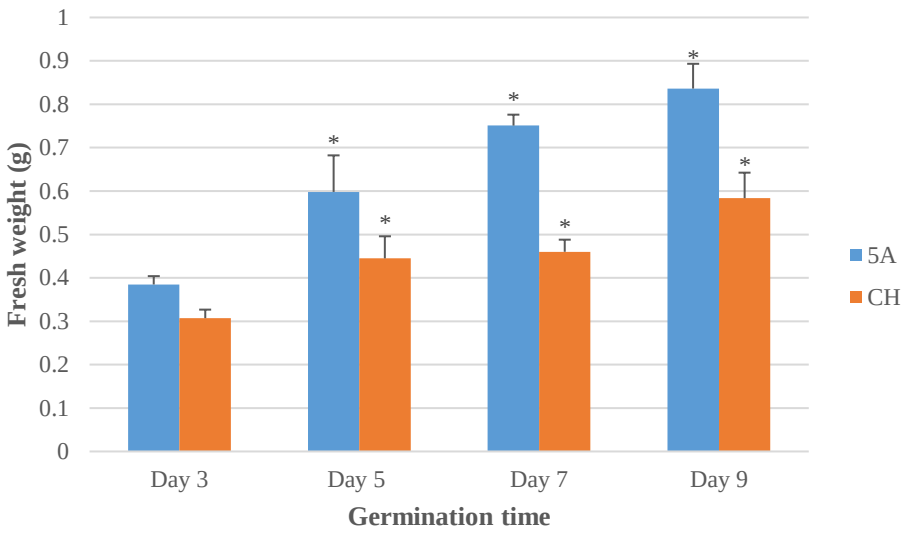


Figure 19. Fresh weight at different developmental stage of sunflower sprouts comparing among 5A and CH varieties. Each value represents the mean ± SE (n = 5) with * indicate significant differences compared to Day 3 of each variety ($P < 0.05$).

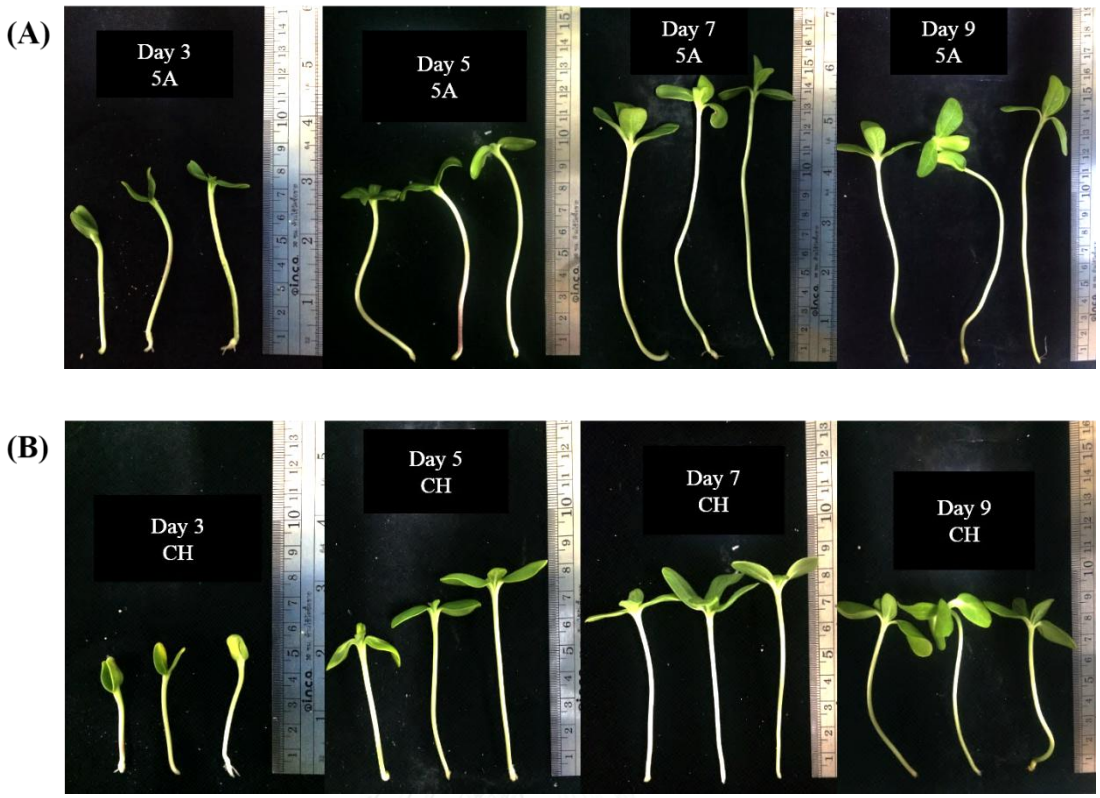
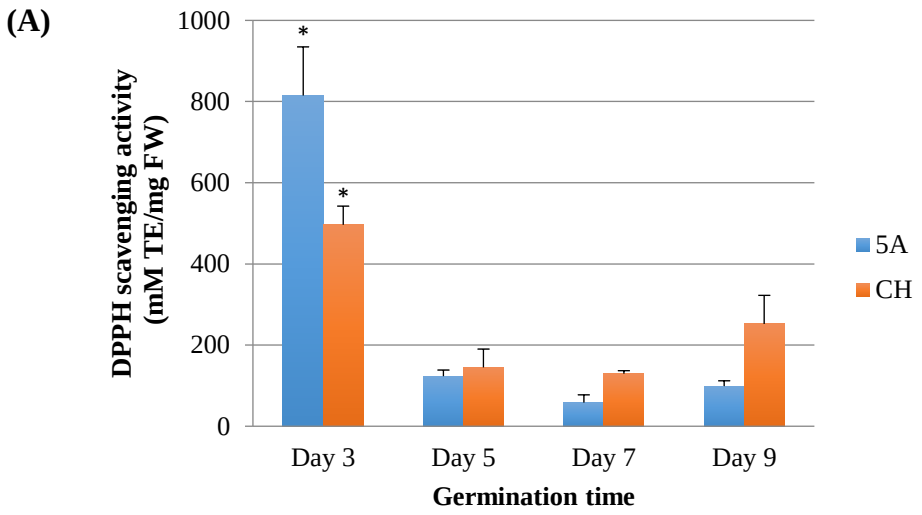


Figure 20. The height at different germination time of sunflower sprouts. Each germination time consisted of five biological replicates. (A) Germination time at day 3, 5, 7, and 9 of 5A variety, and (B) Germination time at day 3, 5, 7, and 9 of CH variety.

3.2.2 Antioxidant activity and total phenolic contents determination

The results at day 3 of germination time demonstrated that both 5A and CH varieties contained the highest antioxidant activity and total phenolic contents in all assays. The results of day 5, 7, and 9 were not significantly different. Comparing the results among three assays, it can be found that antioxidant activities and total phenolic contents had the similar trend (Figure 21). These results did not totally depend on fresh weight and height.



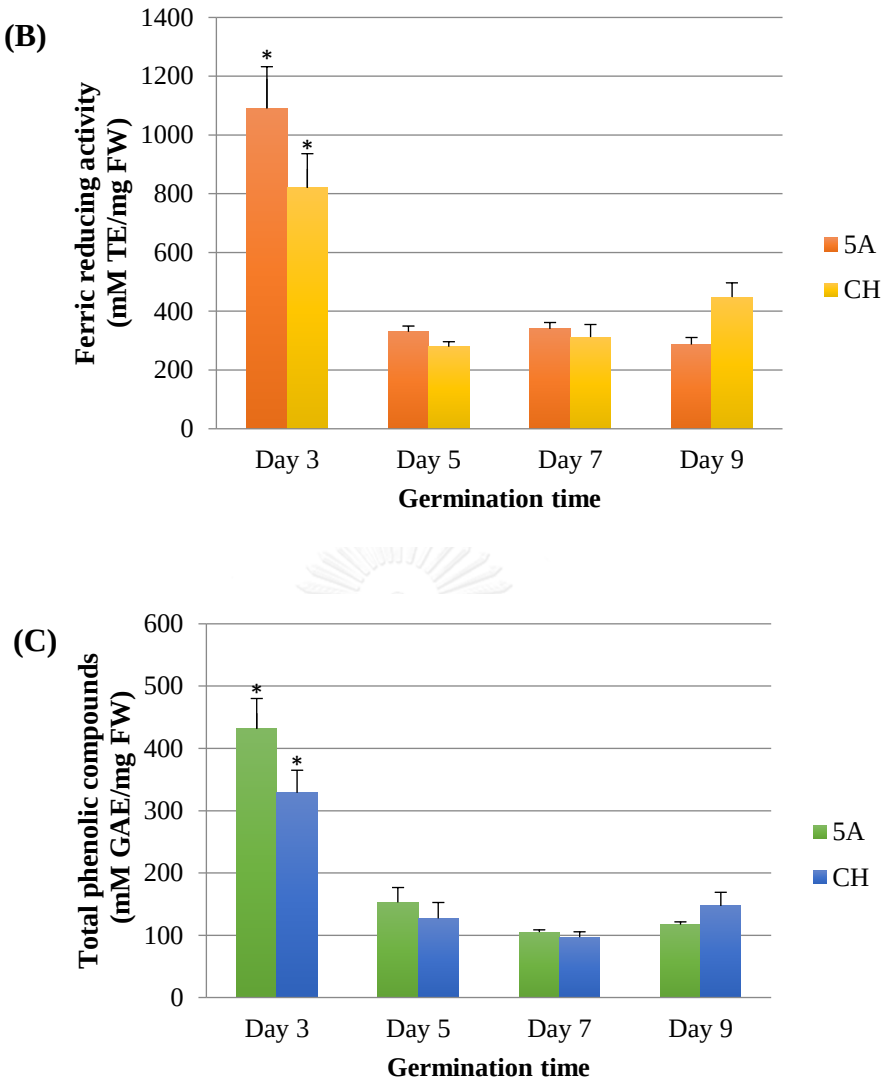
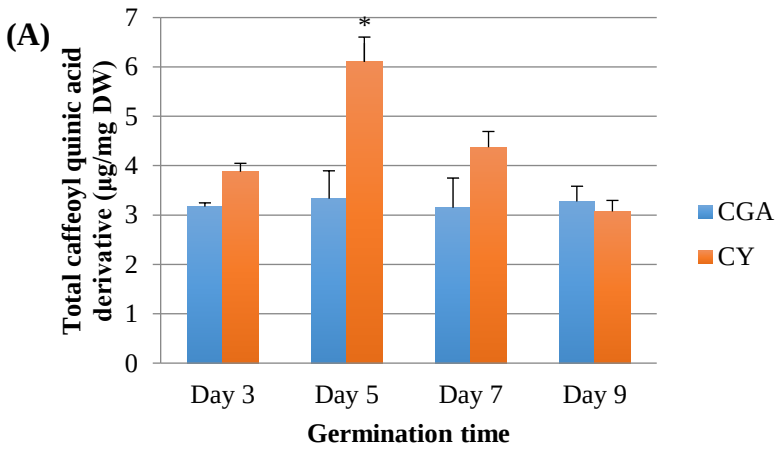


Figure 21. Antioxidant activity and total phenolic contents of 5A and CH varieties of sunflower sprouts comparing among different germination time. (A) Antioxidant activity measuring by 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay), (B) Antioxidant activity measuring by Ferric reducing antioxidant power (FRAP assay), and (C) Total phenolic contents measuring by Folin-Ciocalteu assay. Each value represents the mean ± SE (n = 5) with * indicate significant differences compared to day 5 of each variety ($P < 0.05$).

3.2.3 Chlorogenic acid & cynarin amounts by HPLC analysis

HPLC results showed that the amounts of chlorogenic acid in 5A variety at four germination time were not significantly different, whereas, the amount of cynarin was highest at day 5 of germination time (Figure 22A). The CH variety contained the highest level of chlorogenic acid and cynarin at day 3 and 5 of developmental stages (Figure 22B). These results do not completely agree with the antioxidant activities and total phenolic contents because both of them significantly increased at day 3 of germination time, however, the amounts of chlorogenic acid and cynarin at day 3 of CH variety still corresponded with antioxidant activities and total phenolic contents.



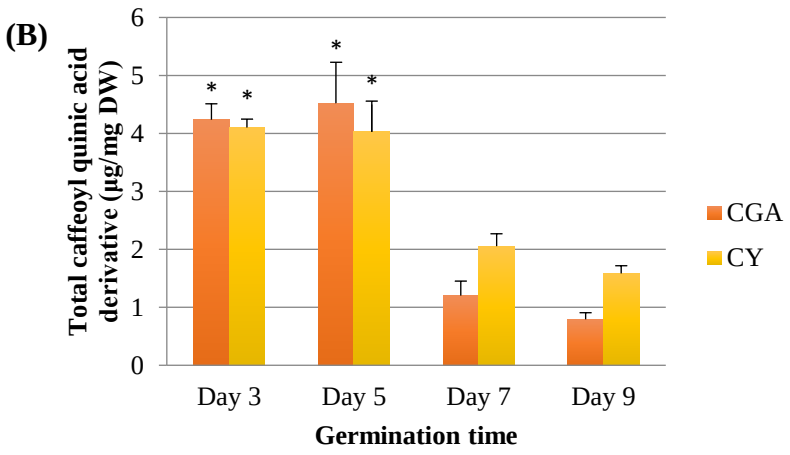


Figure 22. HPLC analysis of 5A and CH varieties of sunflower sprouts comparing among different germination time. (A) Chlorogenic acid (CGA) and cynarin (CY) compounds contents in different germination time of 5A variety of sunflower sprout extracted by HPLC analysis, and (B) Chlorogenic acid (CGA) and cynarin (CY) compounds contents in different germination time of CH variety of sunflower sprout extracted by HPLC analysis. Each value represents the mean ± SE (n = 5) with different * indicate significant differences compared to day 9 of each variety ($P < 0.05$).

3.3 Growth, antioxidant activities, total phenolic contents, and major bioactive compound contents comparing among different chitosan concentration

3.3.1 Growth determination

The 5A and CH varieties were selected to be the candidates from sunflower breeding program of Suranaree University of Technology and commonly purchasing from the market, respectively. Four concentration of chitosan were used including 10, 50, 100, and 200 ppm. The period of cultivation was divided into day 3, 5, and 7. Then, fresh weight was measured. The results demonstrated that there was a significant difference in 5A variety at

day 3 (10 and 50 ppm chitosan concentration) and day 7 (100 and 200 ppm chitosan concentration). Mostly different chitosan concentration did not affect to change the fresh weight in both varieties (Figure 23). The height in each developmental stage was similar and did not depend on varying chitosan concentration (data not shown).

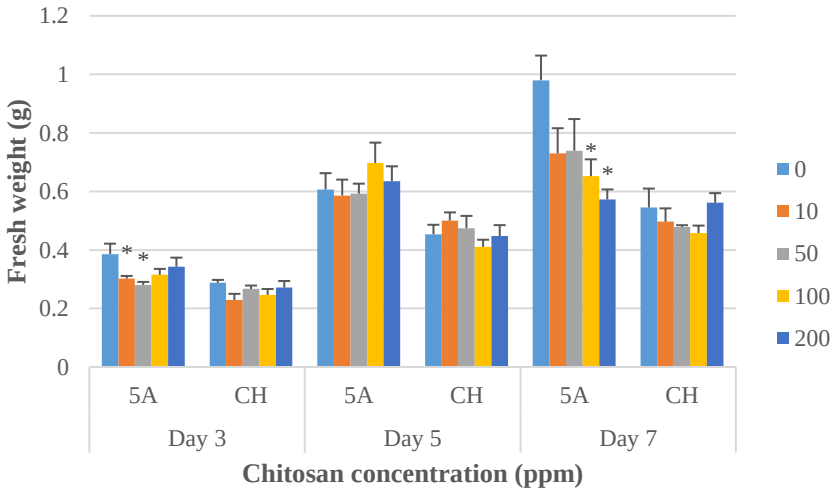
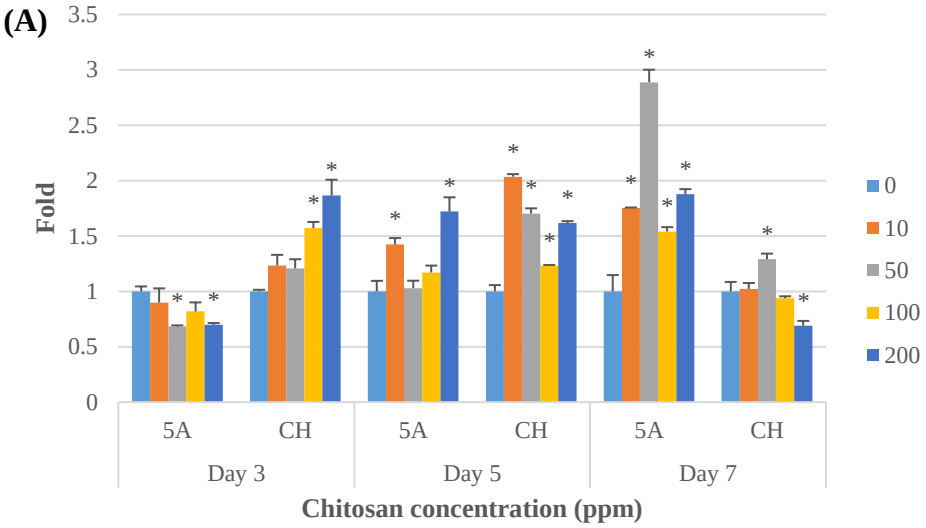
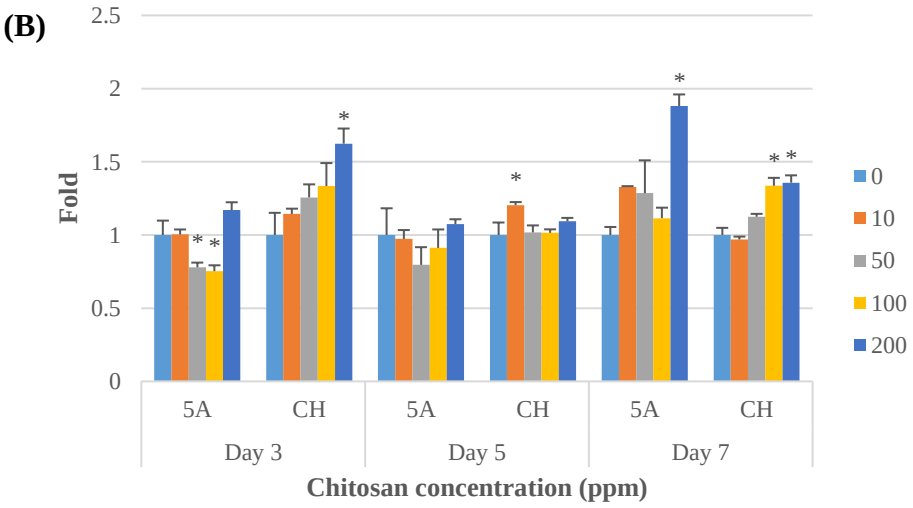


Figure 23. Fresh weight of different chitosan concentration and germination time of sunflower sprouts comparing between 5A and CH varieties. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 0 ppm concentration of each variety and germination time ($P < 0.05$).

3.3.2 Antioxidant activities and total phenolic contents determination

The 5A and CH varieties of chitosan elicited sunflower sprouts at different chitosan concentrations and germination time were measured for antioxidant activities by DPPH and FRAP assay and total phenolic contents by Folin-Ciocalteu assay (Figure 24). In 5A variety, the results from DPPH assay demonstrated that chitosan-treated sunflower sprouts showed the decrease in antioxidant activity at day 3 of germination time, on the other hand, the antioxidant activity increased at day 5 and 7 (Figure 24A). For FRAP and Folin-Ciocalteu assay of 5A variety, there was a decreasing trend at day 3 of germination time and the highest rise of antioxidant activity and total phenolic contents using 200 ppm chitosan concentration at day 7 (Figure 24B and 24C). For CH variety, there was a significant difference at day 3, 5, and 7 of DPPH assay but not with the same chitosan concentration in each day (Figure 24A). There was almost the same trend in FRAP and Folin-Ciocalteu assay showed that the significant difference was at day 3 with 200 ppm of chitosan concentration and day 5 at 10 ppm. Despite the antioxidant activity measuring by FRAP assay showed the significant difference in day 7, no significant difference was found in day 7 of Folin-Ciocalteu assay (Figure 24B and 24C).


(A)
Fold
3.5
3
2.5
2
1.5
1
0.5
0
5A
CH
Day 3
Day 5
Day 7
Chitosan concentration (ppm)
0
10
50
100
200


(B)
Fold
2.5
2
1.5
1
0.5
0
5A
CH
Day 3
Day 5
Day 7
Chitosan concentration (ppm)
0
10
50
100
200

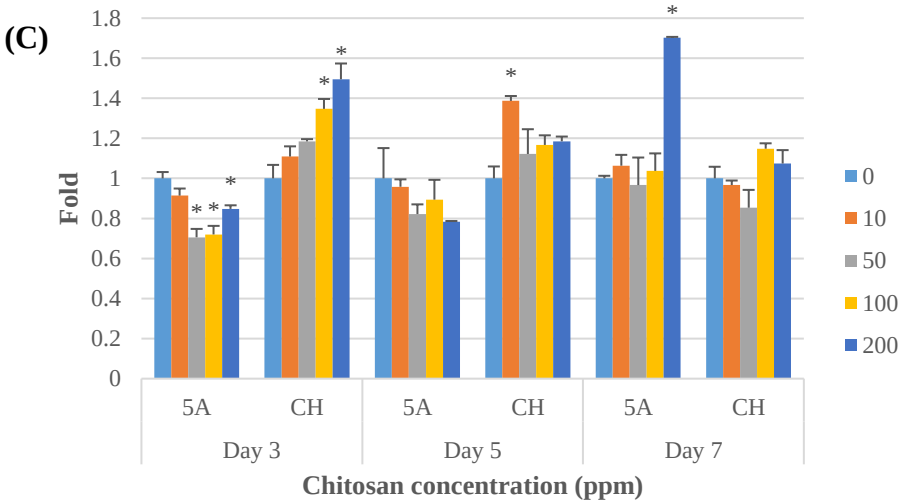
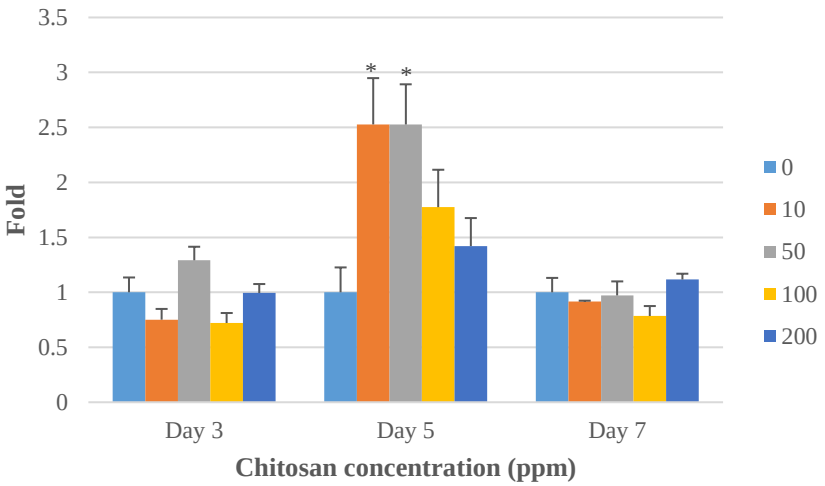

(C)
Fold
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5A
CH
Day 3
Day 5
Day 7
Chitosan concentration (ppm)
0
10
50
100
200

Figure 24. Antioxidant activity and total phenolic contents of different chitosan concentration and germination time of sunflower sprouts comparing between 5A and CH varieties. (A) Antioxidant activity measuring by 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay), (B) Antioxidant activity measuring by Ferric reducing antioxidant power (FRAP assay), and (C) total phenolic contents measuring by Folin-Ciocalteu assay. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 0 ppm concentration of each variety and germination time ($P < 0.05$).

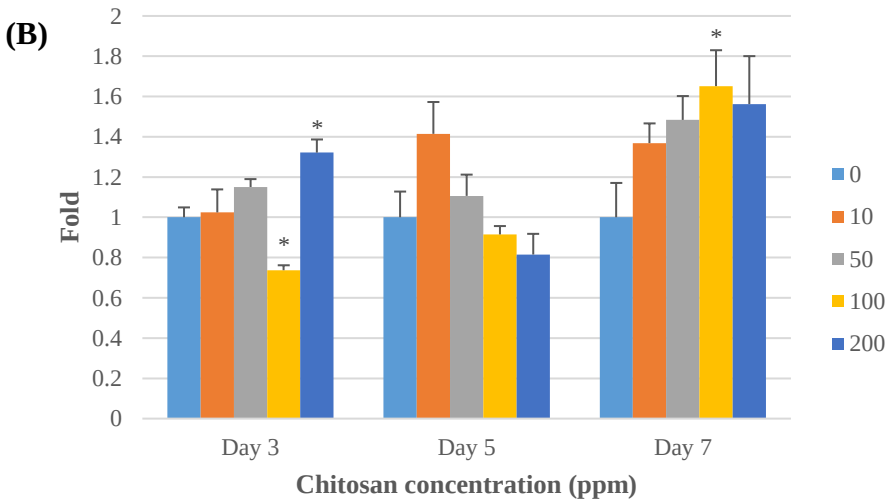
3.3.4 Chlorogenic acid & cynarin amounts by HPLC analysis

HPLC results showed that the amounts of chlorogenic acid in 5A variety at different chitosan concentration in day 3 and day 7 were not significantly different, whereas, the amount in day 5 at 10 and 50 ppm treatment were significantly different comparing to the control line (Figure 25A). There was also a significant difference in cynarin amouts of 5A variety in day 3 and day 7 at 200 and 100 ppm, respectively (Figure 25B). The level of both chlorogenic acid and cynarin fluctuated in almost chitosan concentration and germination time in CH variety (Figure 25C and 25D). However, the significant increase in cynarin amount was found in day 3 at 50 and 200 ppm. These results do not completely agree with the antioxidant activities and total phenolic contents.

(A)


3.5
3
2.5
2
1.5
1
0.5
0
Fold
*
*
Day 3
Day 5
Day 7
Chitosan concentration (ppm)
0
10
50
100
200

(B)


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold
*
*
*
Day 3
Day 5
Day 7
Chitosan concentration (ppm)
0
10
50
100
200

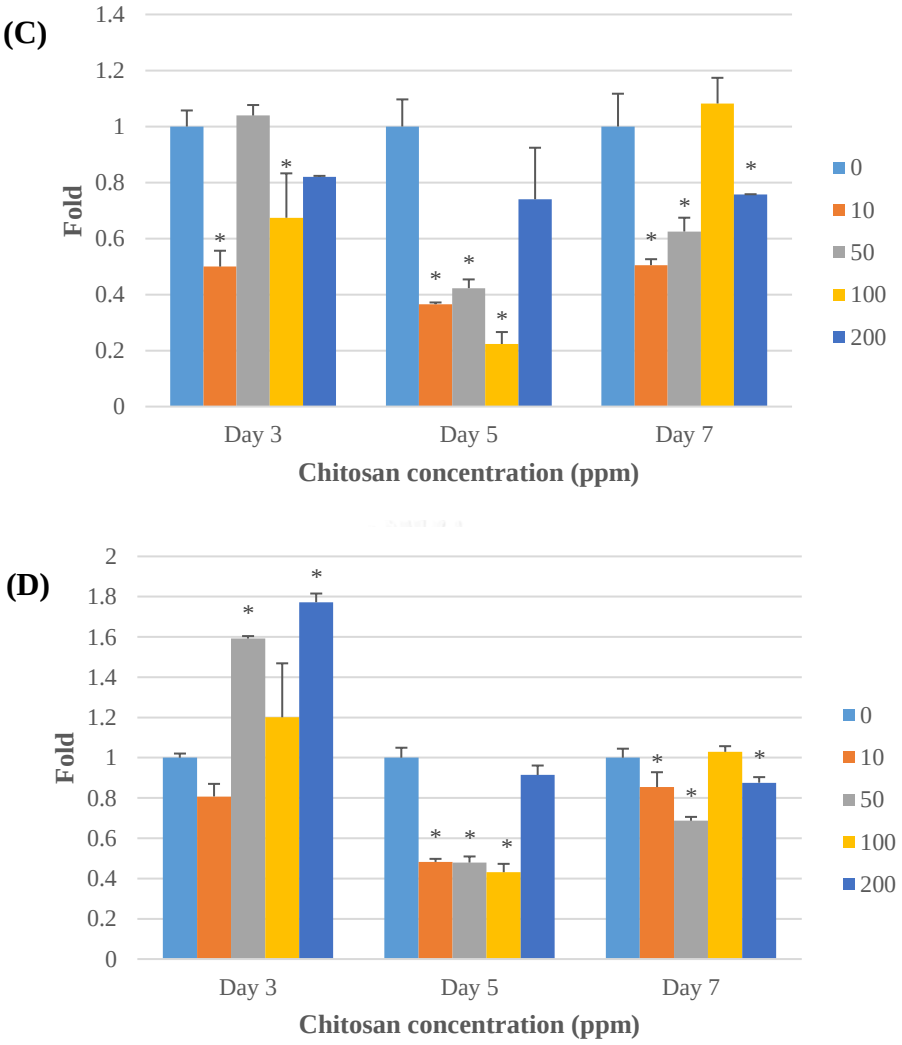


Figure 25. HPLC analysis of 5A and CH varieties of different chitosan concentration and germination time of sunflower sprouts comparing between 5A and CH varieties. (A) Chlorogenic acid (CGA) and (B) cynarin (CY) compounds contents in different germination time of 5A variety of sunflower sprout extracted by HPLC analysis, (C) Chlorogenic acid (CGA) and (D) cynarin (CY) compounds contents in different germination time of CH variety of sunflower sprout extracted by HPLC analysis. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 0 ppm concentration of each variety and germination time ($P < 0.05$).

3.4 Growth, antioxidant activities, total phenolic contents, and major bioactive compound contents comparing among different methyl jasmonate concentration

3.4.1 Growth determination

The 5A and CH varieties were selected to be the candidates from sunflower breeding program of Suranaree University of Technology and commonly purchasing from the market, respectively. Five concentration of methyl jasmonate were used including 0, 10, 50, 100, and 200 ppm. The period of cultivation was divided into 3, 5, and 7 days. Then, fresh weight and height were measured. The results demonstrated that different methyl jasmonate concentration strongly affect the fresh weight of both varieties (Figure 26). There was gradual decline in 5A variety from 0 to 200 ppm. The pattern of CH variety was different from 5A that the highest value of fresh weight was 10 ppm concentration of methyl jasmonate at every germination time, however, Significant difference was found only in day 5 of germination time. The height at each developmental stage was lowest in 200 ppm concentration. This concentration cannot be used to continuously analyze the antioxidant activities, total phenolic contents, chlorogenic acid amounts, and cynarin amounts because they were not in a suitable condition to consume and the height were too low (Figure 27).

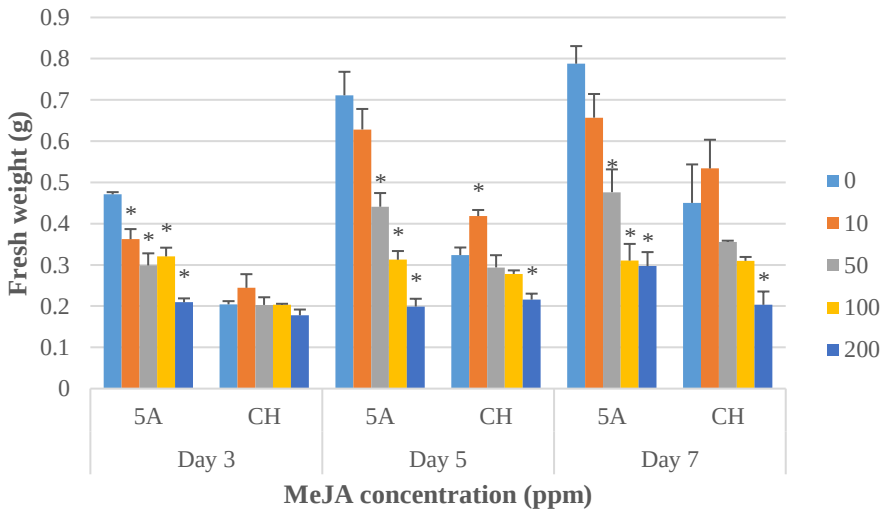
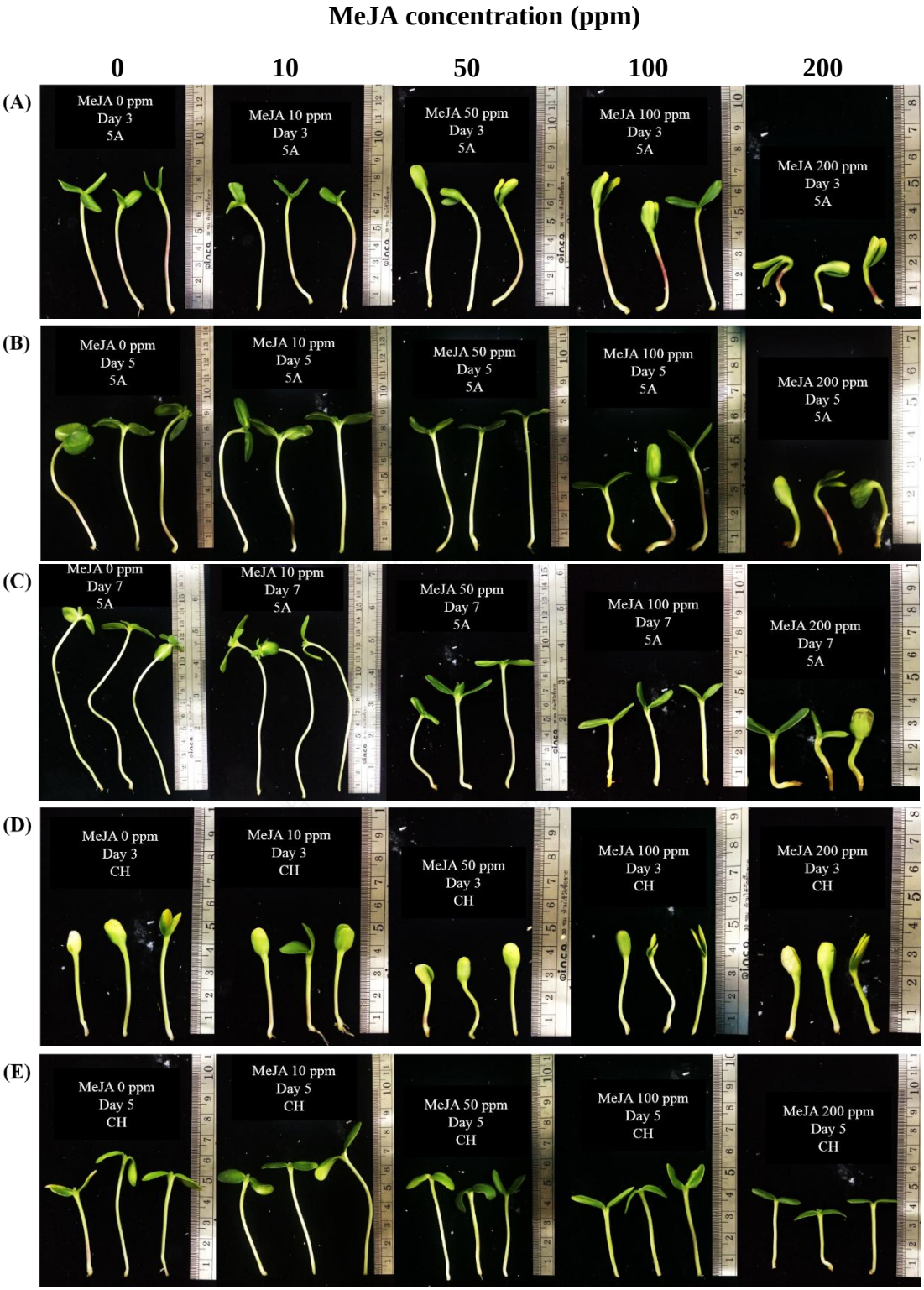


Figure 26. Fresh weight of different methyl jasmonate concentration and germination time of sunflower sprouts comparing between 5A and CH varieties. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 0 ppm concentration of each variety and germination time ($P < 0.05$).


MeJA concentration (ppm)
0
10
50
100
200
(A)
MeJA 0 ppm Day 3 5A
MeJA 10 ppm Day 3 5A
MeJA 50 ppm Day 3 5A
MeJA 100 ppm Day 3 5A
MeJA 200 ppm Day 3 5A
(B)
MeJA 0 ppm Day 5 5A
MeJA 10 ppm Day 5 5A
MeJA 50 ppm Day 5 5A
MeJA 100 ppm Day 5 5A
MeJA 200 ppm Day 5 5A
(C)
MeJA 0 ppm Day 7 5A
MeJA 10 ppm Day 7 5A
MeJA 50 ppm Day 7 5A
MeJA 100 ppm Day 7 5A
MeJA 200 ppm Day 7 5A
(D)
MeJA 0 ppm Day 3 CH
MeJA 10 ppm Day 3 CH
MeJA 50 ppm Day 3 CH
MeJA 100 ppm Day 3 CH
MeJA 200 ppm Day 3 CH
(E)
MeJA 0 ppm Day 5 CH
MeJA 10 ppm Day 5 CH
MeJA 50 ppm Day 5 CH
MeJA 100 ppm Day 5 CH
MeJA 200 ppm Day 5 CH

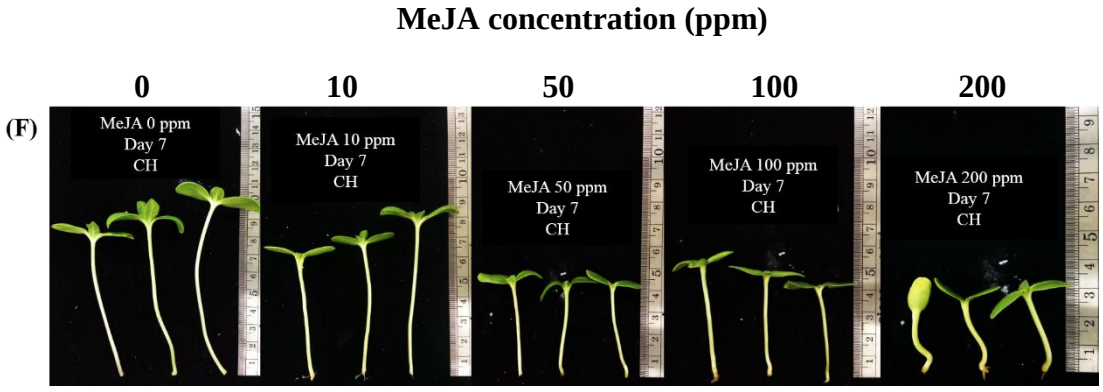
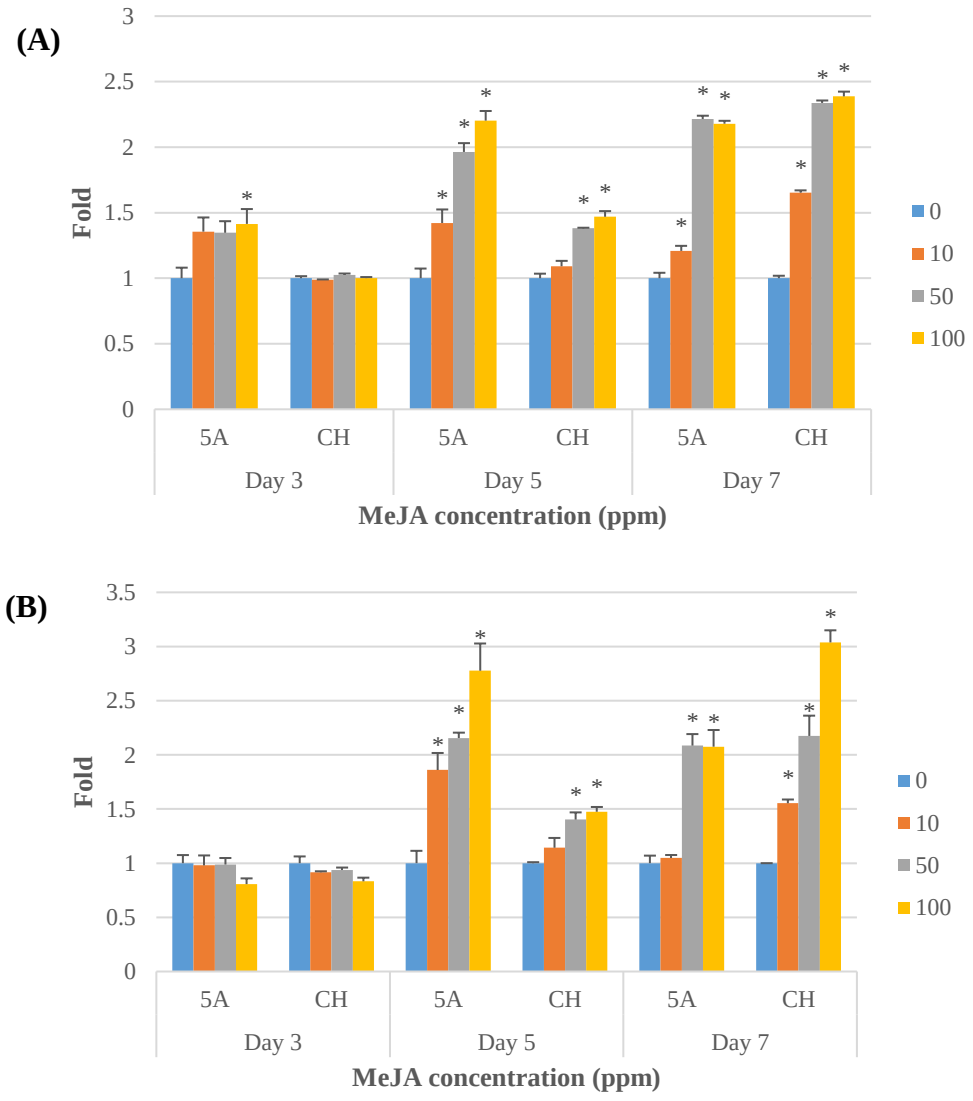


Figure 27. The height of the 5A and CH variety sunflower sprouts treated with methyl jasmonate at different germination time of. Each germination time consisted of three biological replicates. (A) Day 3 of 5A variety, (B) Day 5 of 5A variety, (C) Day 7 of 5A variety, (D) Day 3 of CH variety, (E) Day 5 of CH variety, and (F) Day 7 of CH variety.

3.4.2 Antioxidant activities and total phenolic contents determination

The 5A and CH varieties of chitosan elicited sunflower sprouts at different chitosan concentrations and germination time were measured for antioxidant activities by DPPH and FRAP assay and total phenolic contents by Folin-Ciocalteu assay (Figure 28). For 5A variety, there were a slightly significant difference in day 3 at 200 ppm measuring from DPPH and Folin-Ciocalteu assay. It obviously increased in day 5 and day 7 especially antioxidant activities from both DPPH and FRAP in day 5 were reached from 10 ppm to 100 ppm, while others started from 50 ppm to 100 ppm. The results also showed that there was no significant difference in day 3 of CH variety measuring by DPPH and FRAP assay, but that was occurred in day 3 of that variety measuring

by Folin-Ciocalteu at 50 and 100 ppm concentration. At day 5 and 7, there was a considerable rise in sunflower sprouts treated with 10, 50, and 100 ppm of methyl jasmonate.



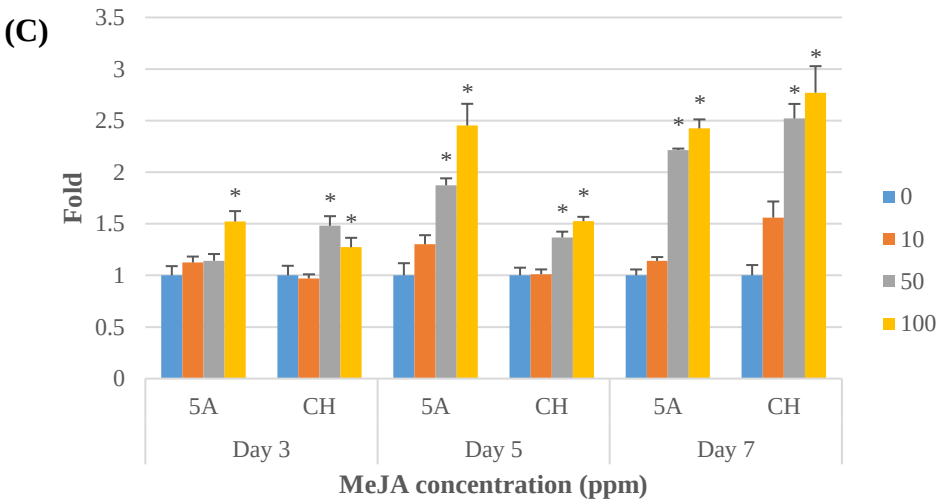
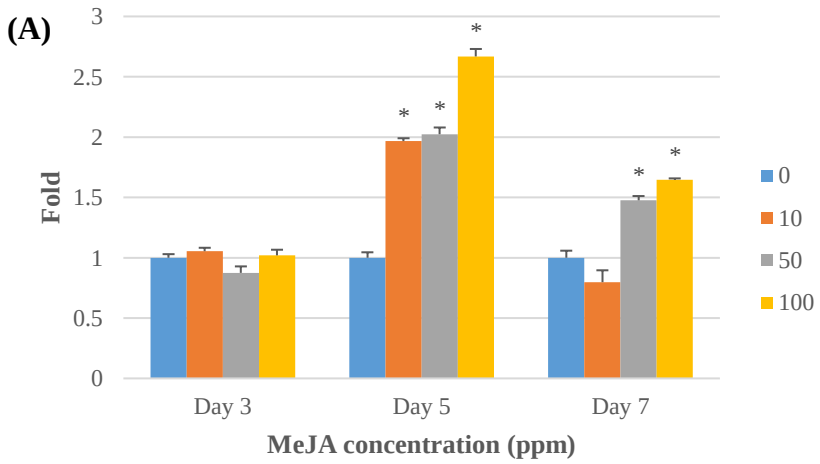
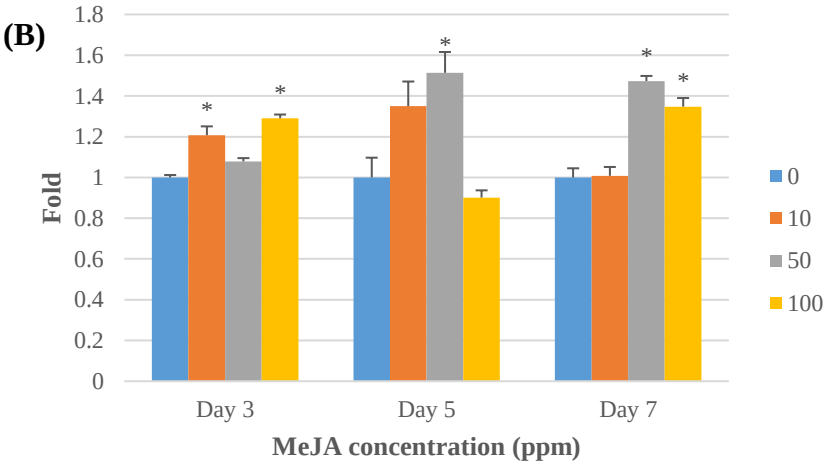


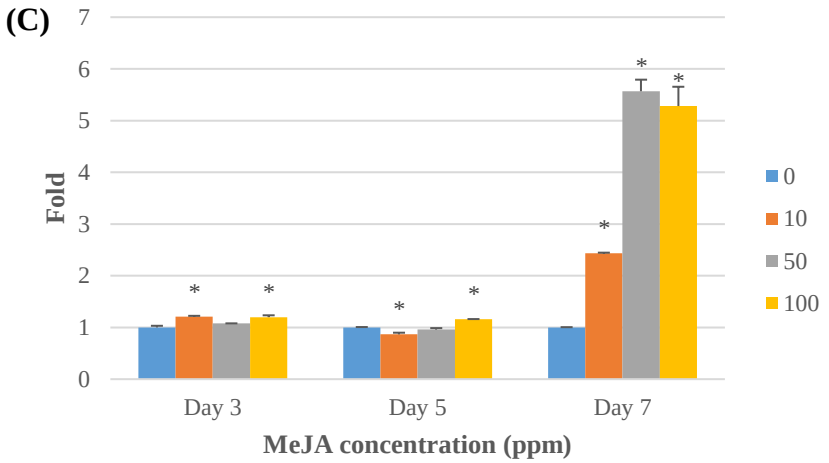
Figure 28. Antioxidant activity and total phenolic contents of different methyl jasmonate concentration and germination time of sunflower sprouts comparing between 5A and CH varieties. (A) Antioxidant activity measuring by 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH assay), (B) Antioxidant activity measuring by Ferric reducing antioxidant power (FRAP assay), and (C) total phenolic contents measuring by Folin-Ciocalteu assay. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 0 ppm concentration of each variety and germination time ($P < 0.05$).

3.4.4 Chlorogenic acid & cynarin amounts by HPLC analysis

HPLC results showed that the amounts of chlorogenic acid in 5A variety in different concentration of methyl jasmonate at three germination time were significantly different at day 5 and 7 with 50 and 100 ppm concentration of methyl jasmonate elicitation which day 5 was started to rise significantly from 10 ppm (Figure 29A). The amount of cynarin was increased rapidly at day 5 in 50 ppm before dramatically decreasing in 100 ppm and day 7 in 50 and 100 ppm which were significantly different (Figure 29B). The level of chlorogenic acid and cynarin in CH variety dramatically rose from 10 to 100 ppm of methyl jasmonate at day 7 of germination time (Figure 29C and 29D). The other results which I did not mention meaning for a slightly increase in these two bioactive compound amounts. These results significantly agree with the antioxidant activities and total phenolic contents.







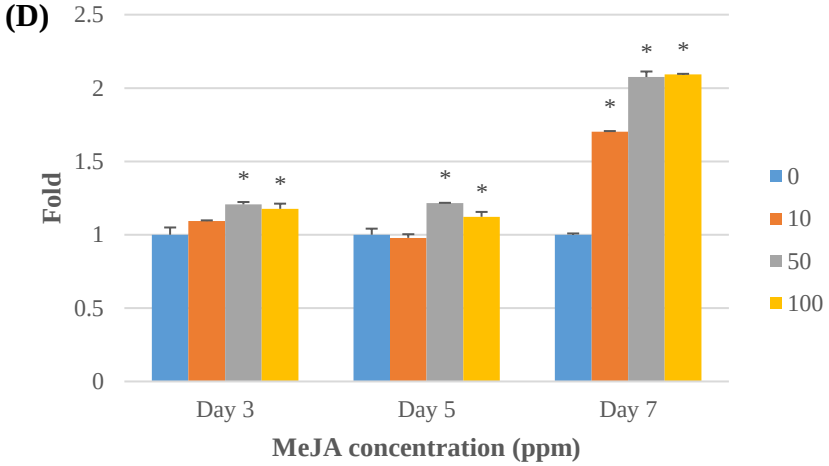


Figure 29. HPLC analysis of 5A and CH varieties of different methyl jasmonate concentration and germination time of sunflower sprouts comparing between 5A and CH varieties. (A) Chlorogenic acid (CGA) and (B) cynarin (CY) compounds contents in different developmental stages of 5A variety of sunflower sprout extracted by HPLC analysis, (C) Chlorogenic acid (CGA) and (D) cynarin (CY) compounds contents in different developmental stages of CH variety of sunflower sprout extracted by HPLC analysis. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to 0 ppm concentration of each variety and germination time ($P < 0.05$).

3.5 Antiglycative activity of chlorogenic acid and cynarin

Only HPLC standards including chlorogenic acid and cynarin were used in this experiment comparing with aminoguanidine which was a well-known synthetic antiglycative agent. The results showed that % inhibition of AGEs of both chlorogenic acid and cynarin was higher than the standard from 12.5 µg/ml concentration. Comparing at the same concentration (2.5 µg/ml), it can be found that aminoguanidine had higher % inhibition of AGEs than chlorogenic and cynarin around 20%. At all concentration tested, chlorogenic acid and cynarin have almost equal % inhibition of AGEs (Figure 30).

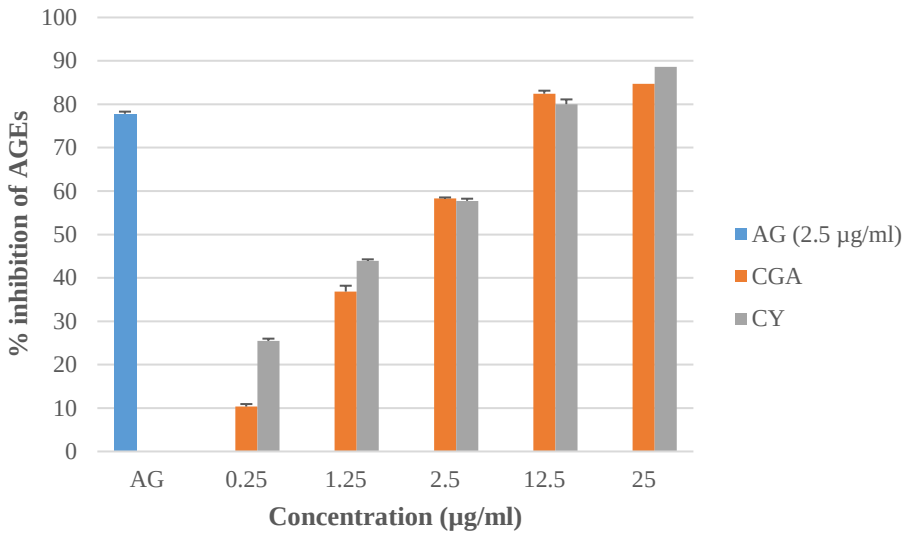


Figure 30. Inhibitory effects of chlorogenic acid (CGA) and cynarin (CY) against the formation of AGEs compared to 2.5 µg/ml of aminoguanidine (AG). Each value represents the mean ± SE (n = 3).

3.6 Biosynthetic genes database searching

The results from nucleotide blast in sunflower genome database (www.sunflowergenome.org) which was conducted for finding the genes that are similar to HQT gene from tomato suggested that three candidate genes, namely HaHQT1, HaHQT2, and HaHQT3. From the sequence alignment of these three genes (Figure 31), it can be found that HaHQT2 and HaHQT3 showed 90.2% similarity (appendix). This result can be confirmed by constructing phylogenetic tree comparing with other reported genes from Bontpart, et al. 2015. In addition, they all grouped together with the characterized HQT fron tomato (SlHQT) (Moglia et al., 2014). The result showed that HaHQT1 was related to HQT2 from *Cynara cardunculus* var. scolymus. HaHQT2 and HaHQT3 and this group was closely related to HQT1 from *Cynara cardunculus* var. scolymus (Figure 32).

```
CLUSTAL O(1.2.1) multiple sequence alignment

HaHQT1      atgaaaaccgatcaaaccaaaccaatgaacatcacaataaccaaatcatcaatcgttcca
HaHQT2      atggggac---tgttcaaaagacattgaacatcaacatcaaacaaacaacgtttgtacaa
HaHQT3      atgaaaactgatcacaacatcaagatgaacatcacaataaaaaactcaacaatcattcct
            ***   **   *      *      *********  ** *   *  ** *  *  * *

HaHQT1      ccatcagagaccattaatggatcctctaaccacatctggacctccaatttggatctagtt
HaHQT2      ccttcgaagcccacacaagcttctacaaagcagttatggacttccaacttggatttggtc
HaHQT3      ccatcagagcccatagcagacgctccaaagcagctatggacctctaacttggatcttgtt
            ** **  ** ***     *   *  * ** **  * ***** ** ** ****** * **

HaHQT1      gttggaagaattcatattctaacggtttatttctacaggcctaacggatctaacaactat
HaHQT2      gttggcagaatccatattttaaccgtttatttctaccgtccgatcgtttgctccaacttc
HaHQT3      gttggaagaattcatattttaaccgtttacttctacaggccgaacgggtcgtccaacttt
            ***** ***** ****** **** ***** ****** * ** * **  *    *****

HaHQT1      tttgatccgaatgtgatgaaacaggcgcttgctgatgtgcttgtgccgttttatccgatg
HaHQT2      tttgatcctcttgtcatgaagaaggcgcttgcagatgttcttgttacctttttatccaatg
HaHQT3      ttcgactcgaatgttatgaaaaaggcgcttgcggatgtgctcgtgccgttttatccgatg
            ** **  *   *** *****  ********** ***** ** **  * ******** ***

HaHQT1      gctggacggttaggtagagatgagagtggcagaattgtgattaattgtaatggtgagggt
HaHQT2      gctggtcgtatgggtaaagatcagaatggcaaagttgttattaattgtaatgatgagggt
HaHQT3      gctgggcggttgggaagagatgagagtggtaggattgttattaattgtaatggtgaagga
            ***** **  * ** * **** *** *** *   **** ************* *** **

HaHQT1      gctttgtttgttgaggctgaatcggattcgtgtttggatgattttggtgagtttactccg
HaHQT2      gttttgtttgtcgaagccgagtccgattctactttggatgattttggtgagtttacgccg
HaHQT3      gttttgttcgttgaggccgaaacggatgcgtgtttggatgatttcggagagtttactccg
            * ****** ** ** ** **  * *** *   ************ ** ******** ***

HaHQT1      tcgccggagtttcggagtcttactccgactgttgattattccggtgatattagttcgttt
HaHQT2      tcgccggagctccggcgacttacgcccactgtggattattccggtgacatttcttcttat
HaHQT3      tcgccggagtttcagatgctgacgcccagcgttgattactccggcgacatttcttcgtac
            ********* * * *   ** ** ** * ** ***** ***** ** ***  *** *

HaHQT1      cctctgtttttcgc----------------acaggtaactcattttaaatgtggagga
HaHQT2      ccgctgtttttttgctcaggtatattggtttgaccaggtaacccatttcaagtgtggagga
HaHQT3      ccgttgtttttcgc----------------acaggtactgcatttcaagtgtggagga
            **  ******* **                ******   ***** ** *********

HaHQT1      gtcgcacttggttgtggtgtattccatacattatctgacggtttatcatctctccatttc
HaHQT2      gttggtcttggttgtggtgtctttcatactttggctgatgggctatcctctctgcatttc
HaHQT3      gttggtcttggttgtggtgtctttcatactttggctgatgggctatcctctctgcatttc
            ** *  ************** ** ***** **  **** **  **** ***** ******

HaHQT1      ataaacacatggtccgatgtggctcgcgggctctctgtagccatcccaccgttcattgac
HaHQT2      atcaacacatggtccgacatggctcgcggcctctccattgccatcccaccatttatcgac
HaHQT3      atcaacacatggtccgacatggctcgcggcctctccattgccatcccaccatttatcgac
            ** **************  ********** *****  * *********** ** ** ***

HaHQT1      cgtaccctgctacgtgcacgcgtaccacccaccccaacacacgaccatgtcgaataccac
HaHQT2      cgcaccttgcttcgtgcacgtgaaccccccactcccacttttgaccatgtcgagtaccac
HaHQT3      cgcaccttgcttcgtgcacgtgaaccccccactcccacttttgaccatgtcgagtaccac
            ** *** **** ******** * *** ***** ** **    *********** ******

HaHQT1      ccacctccaaccatgatcaccacccaaaaa---accggttcactttctaaatcatccacc
HaHQT2      ccccctccgtcgatgaaacctgactcacagaagcctgaatccagccgaaagccctccacc
HaHQT3      ccccctccgtcgatgaaacctgactcacagaagcctgaatccagccgaaagccctccacc
            ** *****  * ****   *   *  * *     * *  **       **  * ******
```

```
HaHQT1      accatgctaaaactcacactagaccaactcaacaccctcaaagctaaagccaagagcgaa
HaHQT2      actattcttaagcttacacttgaccaactcaatgctctcaaagccgcagccaagaatgat
HaHQT3      actattcttaagcttacacttgaccaactcaatgctctcaaagccgcagccaagaatgat
            ** ** ** ** ** ***** **********  * ********   ********  **

HaHQT1      g---gcggggcaagttatagcacatacgagaccctcgcggctcacatatggcgatgcgcg
HaHQT2      ggtggaaacaccaactatagcacgtacgagatcctagcggctcacttatggcgttgcgcg
HaHQT3      ggtggaaacaccaactatagcacgtacgagatcctagcggctcacttatggcgttgcgcg
            *   *     * *  ******** ******* *** ********* ******* ******

HaHQT1      tgcaaggctagagggctcccagatgaccaactaaccaagctatacgtagccacagatgga
HaHQT2      tgcaaggctcgtggactccccgatgaccaactaaccaaactgtacgtggctacagacgga
HaHQT3      tgcaaggctcgtggactccccgatgaccaactaaccaaactgtacgtggctacagacgga
            ********* * ** ***** ***************** ** ***** ** ***** ***

HaHQT1      cggtcaagattgagtcctcacctcccacgaggctaccttgggaacgtggtctttacagcc
HaHQT2      cgatccagattgagcccccaactcccaccgggctacctagggaatgttgtgttcactgcc
HaHQT3      cgatccagattgagcccccaactcccaccgggctacctagggaatgttgtgttcactgcc
            ** ** ******** ** ** *******  ******** ***** ** ** ** ** ***

HaHQT1      accccggttgccaaatcgggtactcttacatccgactcattggtaaacgctgcgaaactg
HaHQT2      accccgattgctaaatcggctgacctcacgactcaaccgttggccaatgcagcatcgttg
HaHQT3      accccgattgctaaatcggctgacctcacgactcaaccgttggccaatgcagcatcgttg
            ****** **** ******* *   ** **  *  *  * ****  ** ** **     **

HaHQT1      attcataccacgttgaaaaaaatggacgatgactatttgagatcggctattgattacctc
HaHQT2      atccggacaactttgtcaaaaatggataacgactatttgcgatccgccatcgactacctt
HaHQT3      atccggacaactttgtcaaaaatggataacgactatttgcgatccgccatcgactacctt
            ** *  ** ** ***  *********  * ********* **** ** ** ** *****

HaHQT1      gagacacaacctgatatatccgctctaatccgtggacctagttactttgctagcccaaac
HaHQT2      gaggtgcagcccgatctatcagccttaattcgtggtccaagttactttgcgagcccgaac
HaHQT3      gaggtgcagcccgatctatcagccttaattcgtggtccaagttactttgcgagcccgaac
            ***   ** ** *** **** **  **** ***** ** ********** ***** ***

HaHQT1      cttaacataaacgcttggactagactacctgtacacgatgcggattttgggtggggtcgg
HaHQT2      ctaaacattaacacgtggactcggttgccagtgcatgatgcggatttcgggtggggtaga
HaHQT3      ctaaacattaacacgtggactcggttgccagtgcatgatgcggatttcgggtggggtaga
            ** ***** *** * ****** *  * ** ** ** ********** ********* *

HaHQT1      cccatttttatgggacccgcgaccatattgtacgagggaaccgtatatgttctacctagc
HaHQT2      cccgtattcatgggacccgcgtgtatattatacgaggggaccatttatgttctaccaagc
HaHQT3      cccgtattcatgggacccgcgtgtatattatacgaggggaccatttatgttctaccaagc
            *** * ** ************   ***** ******** *** * *********** ***

HaHQT1      ccgaacaacgatagaagtgtgtctttggcagtgtgcttagatgcaaaggaacaaccactt
HaHQT2      ccgaacaatgataggagtatgtcactagcagtgtgtttggatgcagatgaacagccattg
HaHQT3      ccgaacaatgataggagtatgtcactagcagtgtgtttggatgcagatgaacagccattg
            ******** ***** *** ****  * ******** ** ****** * ***** *** *

HaHQT1      tttgagaagtacttgtatgaattgtaa
HaHQT2      tttgagaagttcttatacgacttctaa
HaHQT3      tttgagaagttcttatacgacttctaa
            ********** *** ** ** ** ***
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Figure 31. Alignment of nucleotide sequences among HaHQT1, HaHQT2, and HaHQT3

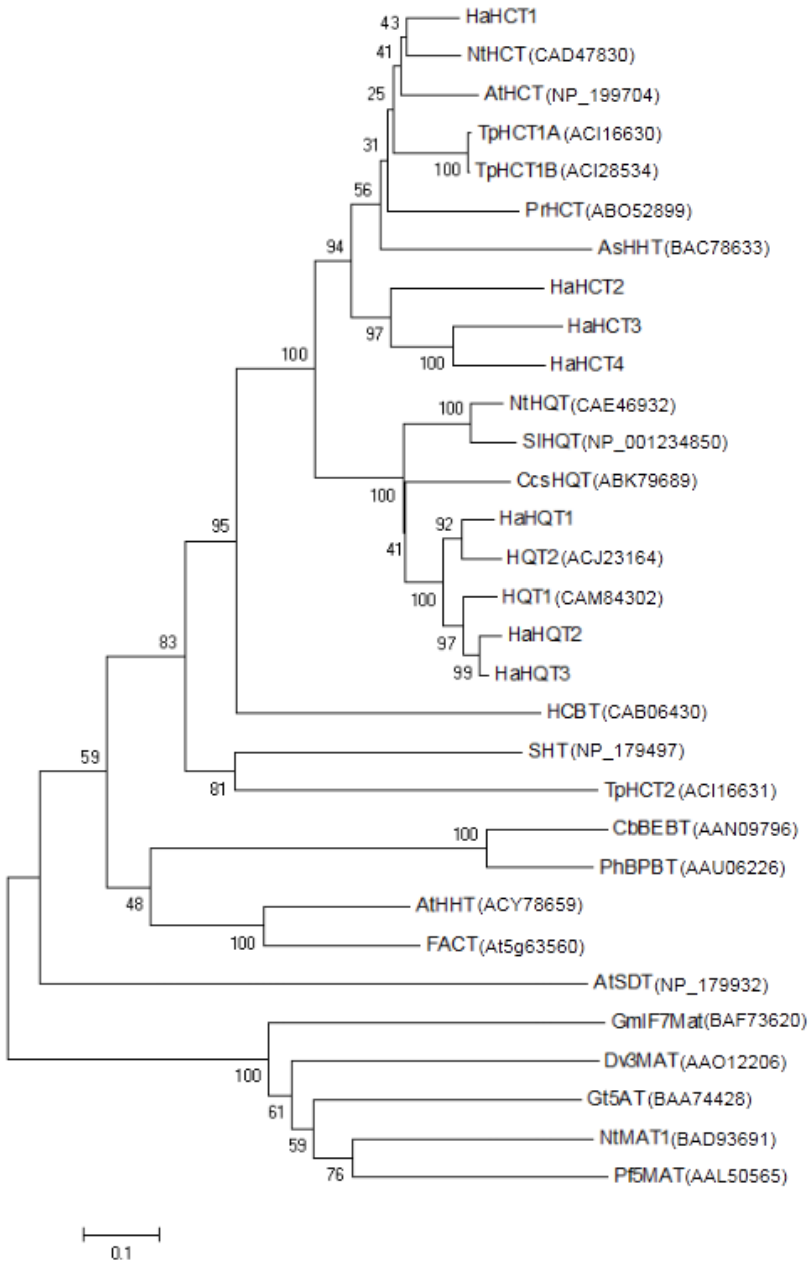
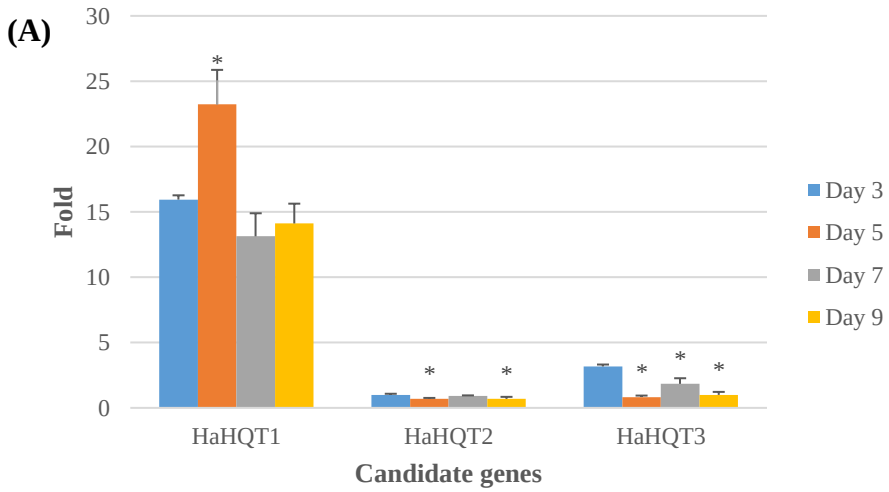


Figure 32. Phylogenetic tree of *Helianthus annuus* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (HaHQT) candidate genes. The tree was constructed together with those which were described in 2.2.8. Evolutional distances were estimated as the number of amino acids substitutions per site, considering Poisson correction. The bootstrap values were evaluated on the base of 1000 pseudoreplicates.

3.7 HQT gene expression level determination by real-time qRT-PCR

The relative HQT gene expression level of each developmental stage of sunflower sprouts was represented (Figure 33). The results demonstrated that candidate gene named HaHQT1 obviously had the highest expression level compared to HaHQT2 and HaHQT3. In 5A variety, the significant difference of HaHQT1 gene expression was occured in day 5 of developmental stage which correlated to cynarin amounts determined by HPLC (Figure 33A). The HaHQT1 of CH variety was slight difference from 5A variety which CH variety contained the highest gene expression level significantly at day 3 and 5. This result also concurred with chlorogenic acid and cynarin amounts from the HPLC results of CH variety (Figure 33B). In addition, from protein subcellular localization prediction, HaHQT1 and HaHQT2 were predicted as having a chloroplast transit peptide while HaHQT3 was predicted as having no targeting peptide that could be localized in the cytosol (Figure 34).



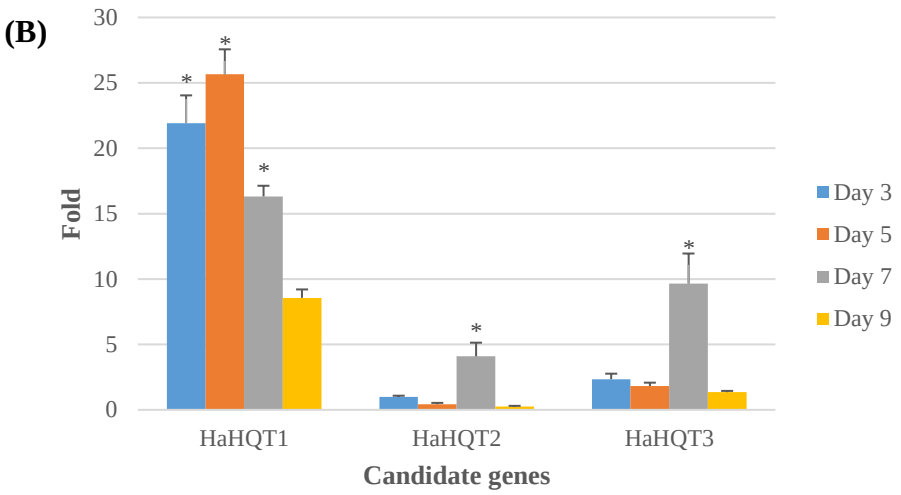


Figure 33. Relative gene expression level of candidate HaHQT genes (A) HaHQT genes in 5A variety at day 3, 5, 7, and 9 of germination time, and (B) HaHQT genes in CH variety at day 3, 5, 7, and 9 of germination time. Each value represents the mean ± SE (n = 3) with * indicate significant differences compared to day 3 of each candidate genes in 5A variety and day 9 of each candidate genes in CH variety ($P < 0.05$).

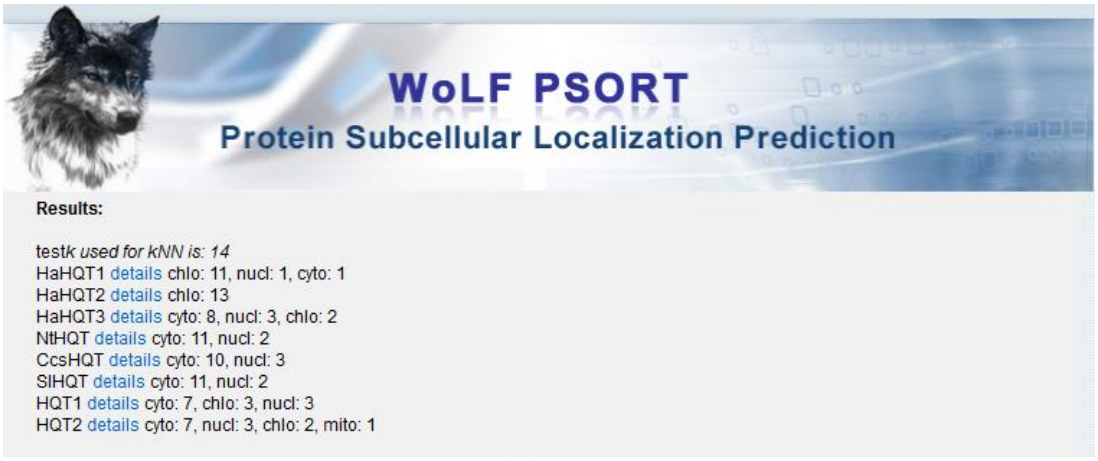


Figure 34. Protein Subcellular Localization Prediction of *Helianthus annuus* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyltransferase (HaHQT) candidate genes by WoLF PSORT. The prediction was performed together with those which were described in 2.2.8. The results were classified as follows: chlo, predicted as having a chloroplast transit peptide; nucl, predicted as having a nuclear targeting peptide; cyto, predicted as having no targeting peptide or localized in cytosol; mito, predicted as having a mitochondria targeting peptide.

CHAPTER IV

DISCUSSION

4.1 The correlation among antioxidant activities, total phenolic contents, and major bioactive compound contents in six varieties of sunflower sprouts

The UNK variety contained the highest antioxidant activities, total phenolic contents, and the amount of cynarin. Since it is an unknown variety, the origin, seed yields, and oil contents have not been displayed. However, it can be compared between inbred line (5A and 10A) and synthetic varieties (S473 and S475) in DPPH assay. Inbred lines were reported as drought sensitive varieties, while, the synthetic varieties were reported as drought tolerant (Saensee et al., 2012). The synthetic varieties and inbred lines contained high level and low level of antioxidant activity, respectively. This result correlated to the previous report that drought stress limited sunflower yield components and seed oil contents. Inbreeding plant will sometimes lead to unwanted genetic drift. The continuous overlaying of like genetics exposes recessive gene patterns that often lead to changes in reproduction performance, fitness, and ability to survive. However, the antioxidant activity measuring by DPPH assay seems to have less correlation between antioxidant activity and total phenolic contents comparing with FRAP assay. For DPPH assay, the presence of colored compounds such as carotenoids and anthocyanins in samples results in increasing the interferences. The more color in the samples, the smaller the absorbance decrease and the less antioxidant activity is measured. Another reason is the secondary reaction products between the samples and the chromogen. Therefore, the antioxidant activities were underestimated because of sample interferences which might be a problem for the quantitative analysis (Arnao,

2000). Moreover, DPPH radical can interact with other radicals. The time response curve to reach the steady state also acts as non-linear with different ratios of antioxidant/ DPPH (Brand-Williams et al., 1995). On the other hand, FRAP assay was reported to be a reproducible and linearly related to antioxidants' molar concentration (Hodzic et al., 2009). Huang et al., 2005 also demonstrated a good correlation ($R^2 > 0.99$) between the total phenols and antioxidant activity. It is may be redundant because these assays are based on similar redox reactions (Huang et al., 2005). However, I would like to measure antioxidant activity and total phenolic compounds by using many methods together to confirm the results. Although the total phenolic compounds assay seems to have the clear advantages rather than DPPH and FRAP assays, they are some disadvantages which simple phenols such as the phenolic group in tyrosine react with Folin-Ciocalteu reagent but they are not effective to radical scavenging antioxidants (Huang et al., 2005). The antioxidant activity of phenolic compounds depends on many factors such as the number of hydroxyl groups bonded to the aromatic ring, the site of bonding and mutual position of hydroxyls in the aromatic ring. For example, the increase in antioxidant activity also depends on the high number of hydroxyl groups and the presence of a second hydroxyl group on the ortho or para position of aromatic ring (Wang et al., 2003). The previous report in sunflower seeds found that chlorogenic acid, caffeic acid, and two caffeoylquinic derivative which the derivative 1 may be cynarin because of a very similar mass spectrum are the main acids presented in sunflower seeds (Pedrosa et al., 2000). Chlorogenic acid and cynarin were found in artichoke sprouts which is in the same family with sunflower (asteraceae). Chlorogenic acid which is derivatives of caffeic and quinic acid showed strong antioxidant activity, since the activity increased when quinic acid bind caffeic acid and decrease hydrogen

peroxide and DPPH radical scavenging activities of caffeic acid (Sroka & Cisowski, 2003). In addition, cynarin also showed high antioxidant activity because it contained two adjacent hydroxyl groups on each of its phenolic ring (Wang et al., 2003). Cynarin in sunflower sprouts was firstly reported and confirmed by HPLC and LC-MS analysis. Moreover, the contents of it was over 8% (w/w) which was higher than total amounts of caffeoylquinic acid derivatives in artichoke leaves (3-6.5% (w/w))(Sun et al., 2012). Therefore, we can confirm the presence of chlorogenic acid and cynarin in sunflower sprouts by using LC-MS and HPLC. From the results of comparing among six varieties at day 5 of germination time, It seems that cynarin is the most involved in antioxidant activities and total phenolic contents. The contents of cynarin were different in the varieties, while, those of chlorogenic acid were almost in the same level. This results corresponded to the results of chlorogenic acid and cynarin compound contents in artichoke which compared among 3 varieties (Wang et al., 2003).

4.2 The effect of germination time to antioxidant activities, total phenolic contents, and major bioactive compound contents

Germination time is another factor that can affect the antioxidant activities, total phenolic contents, and major bioactive compound contents. The results showed a decline of antioxidant activities and total phenolic compound contents after day 3 of germination time that similar to those of vegetables from Brassicaceae family. They compared the decrease in total contents from seeds to days 8 and 12 of germination, by approximately 50 and 65%, respectively, in broccoli; by 30% in kohlrabi; by 35 and 55%, respectively, in red cabbage and turnip; and 70, 75, and 75% in rutabaga, turnip greens, and radish, respectively (Baenas et al., 2012). The glucosinolates amounts

which is the major phenolic compounds in Brassicaceae also correlated to total phenolic contents. The general trend for the glucosinolates amounts also decrease over germinated time. Whereas, in sunflower sprouts the antioxidant activities and total phenolic contents in day 5, 7, and 9 of germination time were lower than day 3 of germination time. There was also a different pattern that amount in amounts of chlorogenic acid and cynarin between 5A and CH which was an inbred and unknown variety, respectively. The amounts of antioxidant activities, total phenolic contents, and amount of cynarin were corresponded in CH variety but chlorogenic acid was also synthesized at the nearly same amounts of cynarin. This may be occurred because CH variety was different from other varieties that chlorogenic acid usually lower than cynarin about 35-52% excluding S473. The amounts of chlorogenic acid and cynarin started to decrease after day 5 of germination time. The previous report explained that chlorogenic acid was protected from the oxidation and the level of protection rose exponentially when the amounts of cynarin increased (Sato et al., 1993). Therefore, the significant decreased in chlorogenic acid and cynarin amounts after day 5 leaded to lower antioxidant activities and total phenolic contents. Other reasons may be the activation of secondary metabolism during germination (Grubb & Abel, 2006) or the interference between phenolic compounds and fatty nutrients in the sprouts during sample extraction (Ciska et al., 2008). In contrast, there were many previous studies showed that germination promoted a dramatically increase in the amount of phytosterols and tocopherols (Shi et al., 2010), vitamins such as thiamine, ascorbic acid, and riboflavin (Ahmad & Pathak, 2000), isoflavones (Lee et al., 2007), and isoflavone aglycones (Kim et al., 2005).

4.3 The effect of biotic elicitors to antioxidant activities, total phenolic contents, and major bioactive compound contents

Elicitors can modulate biosynthetic rates, accumulation and/or vacuolar transit, turnover and degradation (Barz et al., 1990). Methyl jasmonate can highly trigger secondary metabolite production including chlorogenic acid and cynarin resulting in higher antioxidant activities and total phenolic compound contents compared to control line (0 ppm concentration). Chitosan can slightly induce antioxidant activities and total phenolic contents. This results were correlated to antioxidant activity measured by DPPH assay and total phenolic contents in chitosan treated sunflower sprouts reported by Cho et al., 2008. However, there is no previous report in using chitosan to induce secondary metabolite production in sunflower sprouts before. The results in this experiment showed that the amount of chlorogenic acid and cynarin were slightly increased in 5A variety. On the other hand, there was a problem in CH variety that the amounts of chlorogenic acid and cynarin fluctuated but in the same trend. It might be possible that the metabolic flux increased in the flavonoids biosynthesis pathway more than caffeoyl quinic acid derivative biosynthesis pathway (Figure 8.). Therefore, chlorogenic acid and cynarin were observed in the decreased amounts. There are many types of chitosan molecule and the oligomeric (O-80) type of chitosan which is a low molecular weight type (45,000 D) was chosen in this experiment. Indeed, it was claimed to be the most suitable type because it could increase the accumulative inflorescence number and trigger early flowering in *Dendrobium* orchid (Limpanavech et al., 2008). Although, methyl jasmonate was used as exogenous application to induce secondary metabolites biosynthesis, jasmonic acid signaling pathway included in various types of secondary metabolites in most plants. it was considered as intermediate signals instead

because many elicitors stimulate endogenous jasmonic acid biosynthesis in plants, the jasmonic acid signaling pathway is regarded as a transducer or mediator for elicitor signaling, leading to accumulation of plant secondary metabolites (Gundlach et al., 1992). Moreover, methyl jasmonate was reported to increase the phenylalanine ammonia lyase (PAL) activity which is the initial enzyme in the phenylpropanoid pathway (Kim et al., 2006). Therefore, the amounts of chlorogenic acid and cynarin which are the downstream products in phenylpropanoid pathway might be increased. The higher concentration of methyl jasmonate used, the higher chlorogenic acid and cynarin accumulation. However, the increase in methyl jasmonate concentration about 200 ppm resulted in lower growth sprouts. Jasmonic acid was identified to be a plant growth inhibitor and a senescence-promoting substance in many plants such as rice (Yamane et al., 1980) and wheat seedling (Dathe et al., 1981). The structure of jasmonic acid is closely similar to cucurbic acid which is a plant growth inhibitor isolated from seeds of *Cucurbita pepo* L. (Koshimizu et al., 1974). Therefore, the most appropriate concentration in methyl jasmonate elicited sprouts is 100 ppm.

4.4 Antiglycative activity of chlorogenic acid and cynarin

Chlorogenic acid and cynarin from sunflower sprouts can provide both antioxidant activities and antiglycative activity. Since the formation of AGEs correlated with oxidation, amadori products were modified to create protein dicarbonyl compound such as methylglyoxal, then this compounds participate in AGE formation (Hunt et al., 1993). The antioxidant activities of the pure compounds of chlorogenic acid and cynarin were reported. Chlorogenic acid was usually compared with caffeic acid, the results of

antioxidant activity (Chen & Ho, 1997) and antiglycative activity by using methylglyoxal instead of glucose (Gugliucci et al., 2009) associated in the same way that caffeic acid contained more of both activities than chlorogenic acid which is the caffeic acid derivatives. In sunflower sprouts, both antioxidant activity and antiglycative activity showed the highest among many other edible vegetables or even Chinese medicines and the trend of both activities were demonstrated in the same way (Sun et al., 2012). It can be explained that the high amount of chlorogenic acid and cynarin presented in sunflower sprouts leaded to high antioxidant activity and antiglycative activity more than other sprouts. For both chlorogenic acid and cynarin in my results displayed an AGE generation concentration-dependent inhibition. Moreover, chlorogenic acid and cynarin also have closely similar % inhibition of AGEs formation from 2.5 to 25 μg/ml concentration of the compounds. Their hydrophobicity, overall size, and polarizability can affect to antiglycative activity (Verma & Hansch, 2004). The inhibitory effect of cynarin against the formation of AGEs can comparable with the result from Sun, et al. 2012 which these two results were closely similar in high concentration including 25 and 12.5 μg/ml concentration of cynarin contained % inhibition of AGEs formation about 90 and 80 %, respectively. These two concentrations were higher than aminoguanidine (2.5 μg/ml) which was a positive control (about 78% inhibition). However, The antiglycative activity in chlorogenic acid cannot be compared with previous work because they used methylglyoxal which was a dicarbonyl compound produced from glycation of proteins by glucose instead of glucose in my experiment (Gugliucci et al., 2009). It can be suggested that the experiment should be also conducted *in vivo* to confirm that these two compounds can affect to antiglycation process in animal body.

4.5 Genes that might be involved in chlorogenic acid and cynarin biosynthesis

Since the sunflower genome has already been published (Kane et al., 2011) and transcriptome data in reproductive stage is available (Livaja et al., 2013). The 3 isoforms of genes involved in the biosynthesis of these two compounds including HaHQT1, HaHQT2, and HaHQT3 were identified. From the cDNA extracted from 5A and CH variety of sunflower sprouts at different germination time using the same samples as 3.2 demonstrated that the major isozymes involved in chlorogenic acid and cynarin productions in sunflower sprouts is HaHQT1 showing high expression level compared to HaHQT2 and HaHQT3. This enzyme involved in cynarin biosynthesis in 5A variety and involved in both chlorogenic acid and cynarin in CH variety. From the phylogenetic tree, HaHQT1 is closely related to HQT2 from artichoke which they indicated HQT1 more associate with chlorogenic acid contents (Sonnante et al., 2010). The HQT1 in artichoke is more correlate to HaHQT2 and HaHQT3 with low expression and no correlation between the amount of these two compounds and gene expression. However, both HQT1 and HQT2 from artichoke have ability to form chlorogenic acid, *p*-coumaroylquinate, and caffeoyl shikimate in vitro which only HQT2 can catalyze the reaction to produce *p*-coumaroylshikimate. These enzymes have the strong preference for quinate over shikimate (Niggeweg et al., 2004). The HQT2 in artichoke is involved in the reaction of coumaroyl-coA with quinic acid to synthesize coumaroyl quinic acid then it was hydroxylated by CYP98A type of C3′H to produce chlorogenic acid. Whereas, HQT1 involved in direct step to produce chlorogenic acid by catalyzing caffeoyl-CoA and quinic acid. The route of chlorogenic acid synthesis depended on the availability of cytoplasmic pools of the alternative acyl donors and acceptors and the

acyltransferases and C3′H specificity (Sonnante et al., 2010). The previous report in coffee bean showed that the different C3′H enzymes specificity operating in different cells may also influence the main route that depended on HQT1 enzyme for chlorogenic acid synthesis (Mahesh et al., 2007). The cynarin synthesis was recently reported in tomato that it come from CCT (chlorogenate:chlorogenate transferase) activity of HQT requiring low pH optimum, high concentration, and absence of acyl-CoA donor for its substrate (chlorogenic acid) in vacuole of plant cells. Moglia, et al. 2014 have already confirmed that HQT enzyme can localize both to vacuole as well as to cytoplasm of plant cell. When chlorogenic acid was accumulated in cytoplasm in the high amount, then it was transported to vacuole for storage. The CCT activity localization in vacuole results in cynarin biosynthesis. Although, low affinity of CCT for chlorogenic acid occurred, it can be synthesized by high vacuolar concentration of chlorogenic acid (Moglia et al., 2014).

From protein subcellular localization prediction of candidate HaHQT genes, when compared the HaHQT1 and HaHQT2 with other HQT genes from different plants, it can be found that HQT genes were commonly localized in cytosol. Moreover, there were a similarity (90.2%) between HaHQT2 and HaHQT3. Therefore, HaHQT2 and HaHQT3 should not be localized in different organisms and all of candidate genes possibly should be localized in cytosol. The experimental localization of these candidate genes should be performed to confirm this.

CHAPTER V
CONCLUSIONS

The differences in varieties, germination times, and responses to biotic elicitors affect the antioxidant activities, total phenolic contents, and bioactive compound contents including chlorogenic acid and cynarin in sunflower sprouts. The pure chlorogenic acid and cynarin also possess the antiglycative activity. The genes involved in the main bioactive compound biosynthesis was also clarify. Conclusions in each topic are as follows:

1. The UNK variety that was purchased from the internet showed the highest antioxidant activities, total phenolic, and cynarin contents compared with the inbred line (5A and 10A), the synthetic varieties (S473 and S475), and the CH variety which is also another unknown variety purchased from Chiatai company.
2. The germination time at day 3 demonstrated the highest amount in antioxidant activities, total phenolic contents, and major bioactive compound contents including chlorogenic acid and cynarin. The highest amount of cynarin was detected at day 3 for CH and day 5 for 5A.
3. Chitosan can slightly induce antioxidant activities and total phenolic contents in sunflower sprouts. However, the amounts of chlorogenic acid and cynarin mostly remained stable in 5A and decreased in CH.
4. Methyl jasmonate has an ability to increase chlorogenic acid and cynarin biosynthesis. The results also correlated with the antioxidant activities and total phenolic contents.

5. The pure chlorogenic acid and cynarin have a similar trend of % inhibition of AGEs in a dose-dependent manner.
6. The HaHQT1 gene was identified as a biosynthetic gene that might be involved in chlorogenic acid and cynarin biosynthesis in sunflower sprouts.

In the future work, I suggest that HaHQT1 gene should be isolated, characterized, and localized to confirm the biosynthetic route of chlorogenic acid and cynarin in sunflower sprouts.

REFERENCES

Ahmad, S., and Pathak, D. (2000). Nutritional changes in soybean during germination. *Journal of Food Science and Technology, 37,* 665-666.

Ahmed, N. (2005). Advanced glycation endproducts—role in pathology of diabetic complications. *Diabetes Research and Clinical Practice, 67,* 3-21.

Alda, S., Camelia, M., Diana, D., Liana, A., and Nita, L. (2011). The dynamic of pigments level in sunflower sprouts after zinc compounds supplementing in growth. *Journal of Horticulture, Forestry and Biotechnology, 15*, 212-216.

Asgary, S., Naderi, G., Sarrafzadegan, N., Ghassemi, N., Boshtam, M., Rafie, M., and Arefian, A. (1999). Anti-oxidant effect of flavonoids on hemoglobin glycosylation. *Pharmaceutica Acta Helvetiae, 73*, 223-226.

Baenas, N., Moreno, D. A., and García-Viguera, C. (2012). Selecting sprouts of Brassicaceae for optimum phytochemical composition. *Journal of Agricultural and Food Chemistry, 60*, 11409-11420.

Bailly, C., Bogatek-Leszczynska, R., Come, D., and Corbineau, F. (2002). Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Science Research, 12,* 47-55.

Balasaraswathi, R., and Sadasivam, S. (1997). Changes in oil, sugars and nitrogenous components during germination of sunflower seeds, *Helianthus annuus*. *Plant foods for human nutrition, 51,* 71-77.

Barz, W., Beimen, A., Drager, B., Jaques, U., Otto, C., Super, E., and Upmeier, B. (1990). Turnover and storage of secondary products in cell cultures. *Secondary Products from Plant Tissue Culture*, 79-102

Bau, H. M., Villaume, C., Nicolas, J. P., and Mejean, L. (1997). Effect of germination on chemical composition, biochemical constituents and antinutritional factors of soya bean (*Glycine max*) seeds. *Journal of the Science of Food and Agriculture, 73*, 1-9.

Benzie, I. F. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*, 70-76.

Bhatty, R. (1988). Composition and quality of lentil (*Lens culinaris* Medik): a review. *Canadian Institute of Food Science and Technology Journal, 21*, 144-160.

Boatright, J., Negre, F., Chen, X., Kish, C. M., Wood, B., Peel, G., Orlova, I., Gang, D., Rhodes, D., and Dudareva, N. (2004). Understanding in vivo benzenoid metabolism in petunia petal tissue. *Plant Physiology, 135*, 1993-2011.

Bontpart, T., Cheynier, V., Ageorges, A., and Terrier, N., (2015). BAHD or SCPL acyltransferase? What a dilemma for acylation in the world of plant phenolic compounds. *New Phytologist*, *208*, 695-707.

Brand-Williams, W., Cuvelier, M. E., and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology, 28*, 25-30.

Centeno, C., Viveros, A., Brenes, A., Canales, R., Lozano, A., and de la Cuadra, C. (2001). Effect of several germination conditions on total P, phytate P, phytase, and acid phosphatase activities and inositol phosphate esters in rye and barley. *Journal of Agricultural and Food Chemistry, 49*, 3208-3215.

Chen, J. H., and Ho, C.-T. (1997). Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *Journal of Agricultural and Food Chemistry, 45*, 2374-2378.

Cheong, J.-J., and Choi, Y. D. (2003). Methyl jasmonate as a vital substance in plants. *Trends in Genetics, 19*, 409-413.

Chirkov, S. (2002). The antiviral activity of chitosan (review). *Applied Biochemistry and Microbiology, 38*, 1-8.

Cho, M. H., No, H. K., and Prinyawiwatkul, W. (2008). Chitosan treatments affect growth and selected quality of sunflower sprouts. *Journal of Food Science, 73*, S70-S77.

Choi, J., Park, J. K., Lee, K. T., Park, K. K., Kim, W. B., Lee, J. H., Jung, H. J. and Park, H. J. (2005). In vivo antihepatotoxic effects of Ligularia fischeri var. spiciformis and the identification of the active component, 3, 4-dicaffeoylquinic acid. *Journal of Medicinal Food, 8*, 348-352.

Ciska, E., Honke, J., and Kozlowska, H. (2008). Effect of light conditions on the contents of glucosinolates in germinating seeds of white mustard, red radish, white radish, and rapeseed. *Journal of Agricultural and Food Chemistry, 56*, 9087-9093.

Clifford, M. N. (1999). Chlorogenic acids and other cinnamates–nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture, 79*, 362-372.

Combs Jr, G. F. (1991). The vitamins: fundamental aspects in nutrition and health. *American Journal of Clinical Nutrition, 53*, 755-763.

Comino, C., Lanteri, S., Portis, E., Acquadro, A., Romani, A., Hehn, A., Larbat, R., and Bourgaud, F. (2007). Isolation and functional characterization of a cDNA

coding a hydroxycinnamoyltransferase involved in phenylpropanoid biosynthesis in *Cynara cardunculus* L. *BMC Plant Biology, 7*, 14.

D'Auria, J. C., Chen, F., and Pichersky, E. (2002). Characterization of an acyltransferase capable of synthesizing benzylbenzoate and other volatile esters in flowers and damaged leaves of Clarkia breweri. *Plant Physiology, 130*, 466-476.

D'Auria, J. C. (2006). Acyltransferases in plants: a good time to be BAHD. *Current Opinion in Plant Biology, 9*, 331-340.

Dar, T. A., Uddin, M., Khan, M. M. A., Hakeem, K., and Jaleel, H. (2015). Jasmonates counter plant stress: a review. *Environmental and Experimental Botany, 115*, 49-57.

Dathe, W., Ronsch, H., Preiss, A., Schade, W., Sembdner, G., and Schreiber, K. (1981). Endogenous plant hormones of the broad bean, *Vicia faba* L.(-)-jasmonic acid, a plant growth inhibitor in pericarp. *Planta, 153*, 530-535.

Dodane, V., and Vilivalam, V. D. (1998). Pharmaceutical applications of chitosan. *Pharmaceutical Science & Technology Today, 1*, 246-253.

Fujiwara, H., Tanaka, Y., Yonekura-Sakakibara, K., Fukuchi-Mizutani, M., Nakao, M., Fukui, Y., Yamaguchi, M., Ashikari, T., and Kusumi, T. (1998). cDNA cloning, gene expression and subcellular localization of anthocyanin 5-aromatic acyltransferase fromGentiana triflora. *The Plant Journal, 16*, 421-431.

Gebhardt, R. (1998). Inhibition of cholesterol biosynthesis in primary cultured rat hepatocytes by artichoke (*Cynara scolymus* L.) extracts. *Journal of Pharmacology and Experimental Therapeutics, 286*, 1122-1128.

Ghaouth, A., Arul, J., Ponnampalam, R., and Boulet, M. (1991). Chitosan coating effect on storability and quality of fresh strawberries. *Journal of Food Science, 56*, 1618-1620.

Gou, J. Y., Yu, X. H., and Liu, C. J. (2009). A hydroxycinnamoyltransferase responsible for synthesizing suberin aromatics in Arabidopsis. *Proceedings of the National Academy of Sciences, 106*, 18855-18860.

Granato, D., Santos, J. S., Maciel, L. G., and Nunes, D. S. (2016). Chemical perspective and criticism on selected analytical methods used to estimate the total content of phenolic compounds in food matrices. *TrAC Trends in Analytical Chemistry, 80*, 266-279.

Grienenberger, E., Besseau, S., Geoffroy, P., Debayle, D., Heintz, D., Lapierre, C., Pollet, B., Heitz, T., and Legrand, M. (2009). A BAHD acyltransferase is expressed in the tapetum of Arabidopsis anthers and is involved in the synthesis of hydroxycinnamoyl spermidines. *The Plant Journal, 58*, 246-259.

Grubb, C. D., and Abel, S. (2006). Glucosinolate metabolism and its control. *Trends in Plant Science, 11*, 89-100.

Gugliucci, A., Bastos, D. H. M., Schulze, J., and Souza, M. F. F. (2009). Caffeic and chlorogenic acids in Ilex paraguariensis extracts are the main inhibitors of AGE generation by methylglyoxal in model proteins. *Fitoterapia, 80*, 339-344.

Gundlach, H., Muller, M. J., Kutchan, T. M., & Zenk, M. H. (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proceedings of the National Academy of Sciences, 89*, 2389-2393.

Halliwell, B. (1990). How to characterize a biological antioxidant. *Free Radical Research Communications, 9*, 1-32.

Harmuth-Hoene, A. E., Bognar, A., Kornemann, U., and Diehl, J. (1987). The influence of germination on the nutritional value of wheat, mung beans and chickpeas. *Zeitschrift fur Lebensmittel-Untersuchung und-Forschung, 185*, 386-393.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C., Pollet, B., and Legrand, M. (2005). Acyltransferase-catalysed p-coumarate ester formation is a committed step of lignin biosynthesis. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology, 139*, 50-53.

Hoffmann, L., Maury, S., Martz, F., Geoffroy, P., & Legrand, M. (2003). Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. *Journal of Biological Chemistry, 278*, 95-103.

Huang, D., Ou, B., and Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841-1856.

Hunt, J. V., Bottoms, M. A., and Mitchinson, M. J. (1993). Oxidative alterations in the experimental glycation model of diabetes mellitus are due to protein-glucose adduct oxidation. Some fundamental differences in proposed mechanisms of glucose oxidation and oxidant production. *Biochemical Journal, 291*, 529-535.

Hutcheson, S. W. (1998). Current concepts of active defense in plants. *Annual Review of Phytopathology, 36*, 59-90.

Jimenez-Escrig, A., Jimenez-Jimenez, I., Sanchez-Moreno, C., and Saura-Calixto, F. (2000). Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2, 2-diphenyl-1-picrylhydrazyl. *Journal of the Science of Food and Agriculture, 80*, 1686-1690.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences, 103*, 11086-11091.

Kane, N.C., Gill, N., King, M.G., Bowers, J.E., Berges, H., Gouzy, J., Bachlava, E., Langlade, N.B., Lai, Z., Stewart, M., and Burke, J.M. (2011). Progress Towards a reference genome for sunflower. *Botany, 89*, 429-437.

Kaul, A., and Khanduja, L. (1998). Polyphenols inhibit promotional phase of tumorigenesis: relevance of superoxide radicals. *Nutrition and Cancer, 32* , 81-85.

Kendra, D. F., and Hadwiger, L. A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal toFusarium solani and elicits pisatin formation inPisum sativum. *Experimental Mycology, 8*, 276-281.

Kim, H. J., Chen, F., Wang, X., and Choi, J. H. (2006). Effect of methyl jasmonate on phenolics, isothiocyanate, and metabolic enzymes in radish sprout (*Raphanus sativus* L.). *Journal of Agricultural and Food Chemistry, 54*, 7263-7269.

Kim, S. (1998). Applications of chitin and chitosan in agriculture. *Korean J Chitin Chitosan, 3*, 327-342.

Kim, W. J., Lee, H. Y., Wonl, M. H., & Yoo, S. H. (2005). Germination Effect of Soybean on Its Contents of Isoflavones and Oligosaccharides. *Seed, 37*, 23-36.

Koshimizu, K., Fukui, H., Usuda, S., and Mitsui, T. (1974). *Plant growth inhibitors in seeds of pumpkin.* Paper presented at the Plant Growth Substances; Proceedings of the International Conference.

Kosma, D. K., Molina, I., Ohlrogge, J. B., and Pollard, M. (2012). Identification of an Arabidopsis fatty alcohol: caffeoyl-coenzyme A acyltransferase required for the synthesis of alkyl hydroxycinnamates in root waxes. *Plant Physiology, 160*, 237-248.

Lallemand, L. A., Zubieta, C., Lee, S. G., Wang, Y., Acajjaoui, S., Timmins, J., McSweeney, S., Jez, J. M., McCarthy, J. G. and McCarthy, A. A. (2012). A structural basis for the biosynthesis of the major chlorogenic acids found in coffee. *Plant Physiology, 160*, 249-260.

Larimore, B. B. (1975). Sprouting for All Seasons: How and what to Sprout, Including Delicious, Easy-to-prepare Recipes. Horizon Publishers.

Lee, S. J., Ahn, J. K., Khanh, T. D., Chun, S. C., Kim, S. L., Ro, H. M., Song, H.K., Chung, I. M. (2007). Comparison of isoflavone concentrations in soybean (*Glycine max* (L.) Merrill) sprouts grown under two different light conditions. *Journal of Agricultural and Food Chemistry, 55*, 9415-9421.

Lee, Y. S., Kang, C. S., and Lee, Y. S. (1999). Effects of chitosan on production and rot control of soybean sprouts. *Korean Journal of Crop Science, 44*, 368-372.

Lee, Y. S., Kim, Y. H., and Kim, S. B. (2005). Changes in the respiration, growth, and vitamin C content of soybean sprouts in response to chitosan of different molecular weights. *HortScience, 40*, 1333-1335.

Liang, N., and Kitts, D. D. (2014). Antioxidant property of coffee components: assessment of methods that define mechanisms of action. *Molecules, 19*, 19180-19208.

Lintschinger, J., Fuchs, N., Moser, H., Jager, R., Hlebeina, T., Markolin, G., and Gossler, W. (1997). Uptake of various trace elements during germination of wheat, buckwheat and quinoa. *Plant Foods for Human Nutrition, 50*, 223-237.

Livaja, M., Wang, Y., Wieckhorst, S., Haseneyer, G., Seidel, M., Hahn, V., Knapp, S.J., Taudien, S., Schon, C.C., and Bauer, E. 2013. BSTA: a targeted approach combines bulked segregant analysis with next-generation sequencing and de novo transcriptome assembly for SNP discovery in sunflower. *BMC Genomics, 14*, 1.

Lizama-Uc, G., Estrada-Mota, I. A., Caamal-Chan, M. G., Souza-Perera, R., Oropeza-Salín, C., Islas-Flores, I., and Zuniga-Aguilar, J. J. (2007). Chitosan activates a MAP-kinase pathway and modifies abundance of defense-related transcripts in calli of *Cocos nucifera* L. *Physiological and Molecular Plant Pathology, 70*, 130-141.

Luo, J., Fuell, C., Parr, A., Hill, L., Bailey, P., Elliott, K., Fairhurst, S.A., Martin, C., and Michael, A. J. (2009). A novel polyamine acyltransferase responsible for the accumulation of spermidine conjugates in Arabidopsis seed. *The Plant Cell, 21*, 318-333.

Mahesh, V., Million-Rousseau, R., Ullmann, P., Chabrillange, N., Bustamante, J., Mondolot, L., Morant, M., Noirot, M., Hamon, S., de Kochko, A., and Werck-Reichhart, D. (2007). Functional characterization of two p-coumaroyl ester 3′-hydroxylase genes from coffee tree: evidence of a candidate for chlorogenic acid biosynthesis. *Plant Molecular Biology, 64*, 145-159.

Martinez-Villaluenga, C., Penas, E., Ciska, E., Piskula, M. K., Kozlowska, H., Vidal-Valverde, C., and Frias, J. (2010). Time dependence of bioactive compounds

and antioxidant capacity during germination of different cultivars of broccoli and radish seeds. *Food Chemistry, 120*, 710-716.

Marton, M., Mandoki, Z., and Csapo, J. (2010). Evaluation of biological value of sprouts-I. Fat content, fatty acid composition. *Acta Universitatis Sapientiae Alimentaria, 3*, 53-65.

McDougall, B., King, P. J., Wu, B. W., Hostomsky, Z., Reinecke, M. G., and Robinson, W. E. (1998). Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrobial Agents and Chemotherapy, 42*, 140-146.

Merriam-Webster. (2002). "antioxidant". *Webster's Third New International Dictionary, Unabridged*. http://unabridged.merriam-webster.com (June 9, 2016).

Moglia, A., Lanteri, S., Comino, C., Hill, L., Knevitt, D., Cagliero, C., Rubiolo, P., Bornemann, S., and Martin, C. (2014). Dual Catalytic Activity of Hydroxycinnamoyl-Coenzyme A Quinate Transferase from Tomato Allows It to Moonlight in the Synthesis of Both Mono-and Dicaffeoylquinic Acids. *Plant Physiology, 166*, 1777-1787.

Monsen, E. R. (2000). Dietary reference intakes for the antioxidant nutrients: vitamin C, vitamin E, selenium, and carotenoids. *Journal of the American Dietetic Association, 100*, 637-640.

Montesano, M., Brader, G., and Palva, E. T. (2003). Pathogen derived elicitors: searching for receptors in plants. *Molecular Plant Pathology, 4*, 73-79.

Mostafa, M., Rahma, E., and Rady, A. (1987). Chemical and nutritional changes in soybean during germination. *Food Chemistry, 23*, 257-275.

Nathan, D. M. (1993). Long-term complications of diabetes mellitus. *New England Journal of Medicine, 328,* 1676-1685.

Niggeweg, R., Michael, A. J., and Martin, C. (2004). Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nature Biotechnology, 22*, 746-754.

Obizoba, I. C. (1991). Effect of sprouting on the nitrogenous constituents and mineral composition of pigeon pea (*Cajanus cajan*) seeds. *Plant Foods for Human Nutrition, 41,* 21-26.

Pająk, P., Socha, R., Gałkowska, D., Rożnowski, J., and Fortuna, T. (2014). Phenolic profile and antioxidant activity in selected seeds and sprouts. *Food Chemistry, 143,* 300-306.

Pedrosa, M. M., Muzquiz, M., García-Vallejo, C., Burbano, C., Cuadrado, C., Ayet, G., and Robredo, L. M. (2000). Determination of caffeic and chlorogenic acids and their derivatives in different sunflower seeds. *Journal of the Science of Food and Agriculture, 80,* 459-464.

Peer, D., and Leeson, S. (1985). Nutrient content of hydroponically sprouted barley. *Animal Feed Science and Technology, 13,* 191-202.

Peluso, G., De Feo, V., De Simone, F., Bresciano, E., and Vuotto, M. L. (1995). Studies on the inhibitory effects of caffeoylquinic acids on monocyte migration and superoxide ion production. *Journal of Natural Products, 58,* 639-646.

Plaza, L., de Ancos, B., and Cano, P. M. (2003). Nutritional and health-related compounds in sprouts and seeds of soybean (*Glycine max*), wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa*) treated by a new drying method. *European Food Research and Technology, 216,* 138-144.

Pulido, R., Bravo, L., and Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry, 48*, 3396-3402.

Quinhone, A., and Ida, E. I. (2015). Profile of the contents of different forms of soybean isoflavones and the effect of germination time on these compounds and the physical parameters in soybean sprouts. *Food Chemistry, 166*, 173-178.

Saensee, K., Machikowa, T., and Muangsan, N. (2012). Comparative performance of sunflower synthetic varieties under drought stress. *International Journal of Agriculture and Biology, 14*, 929-934.

Sangronis, E., and Machado, C. (2007). Influence of germination on the nutritional quality of *Phaseolus vulgaris* and *Cajanus cajan*. *LWT-Food Science and Technology, 40*, 116-120.

Satô, M., Hiraoka, A., and Watanabe, M. (1993). Inhibition of γ-irradiation-induced oxidation of chlorogenate by 1, 3-dicaffeoylquinate in *Xanthium occidentale*. *Phytochemistry, 33*, 1357-1360.

Savage, G. (1990). Nutritional Value of Sprouted Mung Beans. *Nutrition Today, 25*, 21-24.

Sawa, T., Nakao, M., Akaike, T., Ono, K., and Maeda, H. (1999). Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: implications for the anti-tumor-promoter effect of vegetables. *Journal of Agricultural and Food Chemistry, 47*, 397-402.

Scandalios, J. G., Guan, L., and Polidoros, A. N. (1997). Catalases in plants: gene structure, properties, regulation, and expression. *Cold Spring Harbor Monograph Series, 34*, 343-406.

Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols, 3*, 1101-1108.

Schoch, G., Goepfert, S., Morant, M., Hehn, A., Meyer, D., Ullmann, P., and Werck-Reichhart, D. (2001). CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. *Journal of Biological Chemistry, 276*, 36566-36574.

Shi, H., Nam, P. K., and Ma, Y. (2010). Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination. *Journal of Agricultural and Food Chemistry, 58*, 4970-4976.

Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144-158.

Sonnante, G., D'Amore, R., Blanco, E., Pierri, C. L., De Palma, M., Luo, J., Tucci, M., and Martin, C. (2010). Novel hydroxycinnamoyl-coenzyme A quinate transferase genes from artichoke are involved in the synthesis of chlorogenic acid. *Plant Physiology, 153*, 1224-1238.

Sroka, Z., and Cisowski, W. (2003). Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food and Chemical Toxicology, 41*, 753-758.

Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. (1995). Molecular genetics of plant disease resistance. *Science, 268*, 661.

Stern, D. M., Du Yan, S., Yan, S. F., and Schmidt, A. M. (2002). Receptor for advanced glycation endproducts (RAGE) and the complications of diabetes. *Ageing Research Reviews, 1*, 1-15.

Strack, D., and Gross, W. (1990). Properties and activity changes of chlorogenic acid: glucaric acid caffeoyltransferase from tomato (*Lycopersicon esculentum*). *Plant Physiology, 92*, 41-47.

Sugano, M., Yoshida, K., Hashimoto, M., Enomoto, K., and Hirano, S. (1992). Hypocholesterolemic activity of partially hydrolyzed chitosans in rats. *Advances in Chitin and Chitosan, 2*, 472-478.

Sullivan, M. (2009). A novel red clover hydroxycinnamoyl transferase has enzymatic activities consistent with a role in phaselic acid biosynthesis. *Plant Physiology, 150*, 1866-1879.

Sun, Z., Chen, J., Ma, J., Jiang, Y., Wang, M., Ren, G., and Chen, F. (2012). Cynarin-rich sunflower (*Helianthus annuus*) sprouts possess both antiglycative and antioxidant activities. *Journal of Agricultural and Food Chemistry, 60*, 3260-3265.

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Nakao, M., Tanaka, Y., Yamaguchi, M.A., Kusumi, T., and Nishino, T. (2001). Malonyl-CoA: Anthocyanin 5-O-Glucoside-6‴-O-Malonyltransferase from Scarlet Sage (*Salvia splendens*) Flowers. Enzyme purification, gene cloning, expression, and characterization. *Journal of Biological Chemistry, 276*, 49013-49019.

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Yamaguchi, M. A., Tanaka, Y., Kusumi, T. and Nishino, T. (2002). cDNA

cloning, heterologous expressions, and functional characterization of malonyl-coenzyme A: anthocyanidin 3-O-glucoside-6"-O-malonyltransferase from dahlia flowers. *Plant Physiology, 130*, 2142-2151.

Suzuki, H., Nishino, T., and Nakayama, T. (2007). cDNA cloning of a BAHD acyltransferase from soybean (*Glycine max*): isoflavone 7-O-glucoside-6 ″-O-malonyltransferase. *Phytochemistry, 68*, 2035-2042.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., and Suzuki, M. (1986). Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. *Carbohydrate Research, 151*, 403-408.

Taguchi, G., Shitchi, Y., Shirasawa, S., Yamamoto, H., and Hayashida, N. (2005). Molecular cloning, characterization, and downregulation of an acyltransferase that catalyzes the malonylation of flavonoid and naphthol glucosides in tobacco cells. *The Plant Journal, 42*, 481-491.

Tamagnone, L., Merida, A., Stacey, N., Plaskitt, K., Parr, A., Chang, C.-F., Lynn, D., Dow, J. M., Roberts, K., and Martin, C. (1998). Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. *The Plant Cell*, *10*, 1801-1816.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*, 2725-2729.

Thornalley, P. J. (2003). Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Archives of Biochemistry and Biophysics, 419*, 31-40.

Tian, B., Xie, B., Shi, J., Wu, J., Cai, Y., Xu, T., Xue, S., and Deng, Q. (2010). Physicochemical changes of oat seeds during germination. *Food Chemistry, 119*, 1195-1200.

Ueda, J., and Kato, J. (1980). Isolation and identification of a senescence-promoting substance from wormwood (*Artemisia absinthium* L.). *Plant Physiology, 66*, 246-249.

Ulbrich, B., and Zenk, M. H. (1979). Partial purification and properties of hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase from higher plants. *Phytochemistry, 18*, 929-933.

Vasconsuelo, A., and Boland, R. (2007). Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Science, 172*, 861-875.

Verma, R. P., and Hansch, C. (2004). An approach towards the quantitative structure–activity relationships of caffeic acid and its derivatives. *ChemBioChem, 5*, 1188-1195.

Vignoli, J., Bassoli, D., and Benassi, M. (2011). Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: The influence of processing conditions and raw material. *Food Chemistry*, *124*, 863-868.

Villegas, R., and Kojima, M. (1986). Purification and characterization of hydroxycinnamoyl D-glucose. Quinate hydroxycinnamoyl transferase in the root of sweet potato, *Ipomoea batatas* Lam. *Journal of Biological Chemistry, 261*, 8729-8733.

Vlassara, H., and Palace, M. (2002). Diabetes and advanced glycation endproducts. *Journal of Internal Medicine, 251*, 87-101.

Waginger, H. (1984). *Production and use of sprouted grains [barley, oats]*. Paper presented at the 6. International Congress on Soilless Culture. Lunteren (Netherlands). 29 Apr-5 May 1984.

Wagner, A., Ralph, J., Akiyama, T., Flint, H., Phillips, L., Torr, K., Nanayakkara, B., and Te Kiri, L. (2007). Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase in Pinus radiata. *Proceedings of the National Academy of Sciences, 104*, 11856-11861.

Wang, M., Simon, J. E., Aviles, I. F., He, K., Zheng, Q.-Y., and Tadmor, Y. (2003). Analysis of antioxidative phenolic compounds in artichoke (*Cynara scolymus* L.). *Journal of Agricultural and Food Chemistry, 51*, 601-608.

Wasternack, C., and Hause, B. (2002). Jasmonates and octadecanoids: signals in plant stress responses and development. *Progress in Nucleic Acid Research and Molecular Biology, 72*, 165-221.

Wasternack, C., and Parthier, B. (1997). Jasmonate-signalled plant gene expression. *Trends in Plant Science, 2*, 302-307.

Wolff, S. P., and Dean, R. (1987). Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation'in diabetes. *Biochemical Journal, 245*, 243-250.

Yamane, H., Sugawara, J., Suzuki, Y., Shimamura, E., and Takahashi, N. (1980). Syntheses of Jasmonic Acid Related Compounds and Their Structure-Activity Relationships on the Growth of Rice Seedings. *Agricultural and Biological Chemistry, 44*, 2857-2864.

Yan, S. D., Schmidt, A. M., Anderson, G. M., Zhang, J., Brett, J., Zou, Y. S., Pinsky, D., and Stern, D. (1994). Enhanced cellular oxidant stress by the interaction of

advanced glycation end products with their receptors/binding proteins. *Journal of Biological Chemistry, 269*, 9889-9897.

Yang, Q., Reinhard, K., Schiltz, E., and Matern, U. (1997). Characterization and heterologous expression of hydroxycinnamoyl/benzoyl-CoA: anthranilate N-hydroxycinnamoyl/benzoyltransferase from elicited cell cultures of carnation, Dianthus caryophyllus L. *Plant Molecular Biology, 35*, 777-789.

Yang, Q., Xuan Trinh, H., Imai, S., Ishihara, A., Zhang, L., Nakayashiki, H., Tosa, Y., and Mayama, S. (2004). Analysis of the involvement of hydroxyanthranilate hydroxycinnamoyltransferase and caffeoyl-CoA 3-O-methyltransferase in phytoalexin biosynthesis in oat. *Molecular Plant-Microbe Interactions, 17*, 81-89.

Youn, S. K., Kim, Y. J., and Ahn, D. H. (2001). Antioxidative effects of chitosan in meat sausage. *Journal-Korean Society of Food Science and Nutrition*, *30*, 477-481.

Young, D. H., Kohle, H., and Kauss, H. (1982). Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells. *Plant Physiology, 70*, 1449-1454.

APPENDIX

APPENDIX A

Protocols

1. Chemical reagents preparation for DPPH assay

1.1 Stock DPPH 0.6 M 50 ml: 12 mg DPPH + 50 ml absolute methanol

1.2 DPPH working solution: 10 ml of 0.6 M DPPH + 45 ml 95% methanol

1.3 10 mM Trolox (Standard solution) 1 ml: 2.5 mg Trolox + 1 ml 95% methanol

Dilution

- 0 µM 1 ml: 1000 µl of 95% methanol
- 25 µM 1 ml: 25 µl of 1000 µM Trolox + 975 µl of 95% methanol
- 50 µM 1 ml: 50 µl of 1000 µM Trolox + 950 µl of 95% methanol
- 100 µM 100 µl: 10 µl of 1000 µM Trolox + 90 µl of 95% methanol
- 200 µM 100 µl: 20 µl of 1000 µM Trolox + 80 µl of 95% methanol
- 400 µM 100 µl: 40 µl of 1000 µM Trolox + 60 µl of 95% methanol
- 600 µM 100 µl: 60 µl of 1000 µM Trolox + 40 µl of 95% methanol
- 800 µM 100 µl: 80 µl of 1000 µM Trolox + 20 µl of 95% methanol
- 1000 µM 1 ml: 100 µl of 10 mM Trolox + 900 µl of 95% methanol

2. Chemical reagents preparation for FRAP assay

2.1 300 mM Acetate buffer pH 3.6

2.2 20 mM $FeCl_3 \cdot 6H_2O$ 10 ml: 0.0541 g $FeCl_3 \cdot 6H_2O$ + 10 ml DI water

2.3 10 mM TPTZ in 40 mM HCl 10 ml: 0.031 g TPTZ + 10 ml of 40 mM HCl (dissolve at 50 °C)

2.4 FRAP working solution: Mix ratio

300 mM Acetate buffer : 20 mM $FeCl_3 \cdot 6H_2O$: 10 mM TPTZ in 40 mM HCl = 10 : 1 : 1

2.5 Standard solution (same DPPH assay)

3. Chemical reagents preparation for Folin-Ciocalteau assay

1. 95% Methanol 100 ml: 95 ml absolute methanol + 5 ml DI water

2. 350 mM Na_2CO_3 100 ml: 3.70965 g Na_2CO_3 + 100 ml DI water

3. 10% Folin reagent from 2M Folin reagent 100 ml:

10 ml Folin reagent + 90 ml DI water

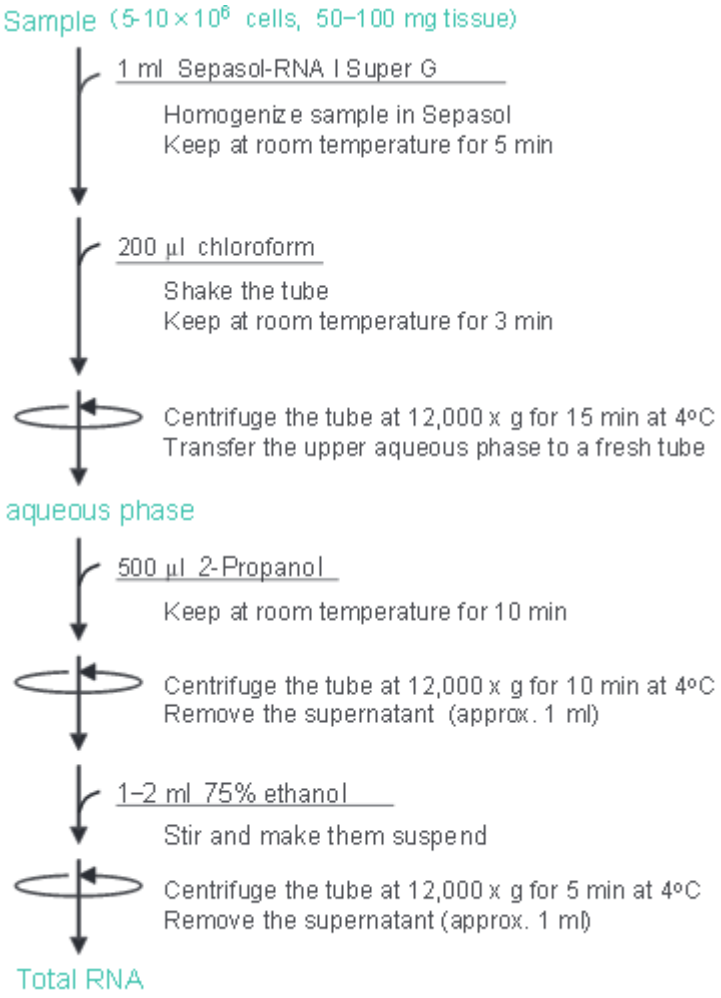
4. 10 mM Gallic acid 50 ml (standard stock solution):

0.08506 g Gallic acid (anhydras) + 50 ml 95% methanol

Dilution:

- 50 µM 1 ml: 50 µl of 1000 µM Gallic acid + 950 µl of 95% methanol
- 100 µM 1 ml: 100 µl of 1000 µM Gallic acid + 900 µl of 95% methanol
- 500 µM 1 ml: 500 µl of 1000 µM Gallic acid + 500 µl of 95% methanol
- 1000 µM 10 ml: 1 ml of 10 mM Gallic acid + 9 ml of 95% methanol
- 1500 µM 1 ml: 150 µl of 10 mM Gallic acid + 850 µl of 95% methanol
- 2000 µM 1 ml: 200 µl of 10 mM Gallic acid + 800 µl of 95% methanol

4. **RNA extraction by Sepasol-RNA I super G (Nacalai Tesque, Japan)**



5. Removal of genomic DNA from RNA preparations (Thermo Scientific, USA)

1. Add to an RNase-free tube:

RNA	1 µg
10X reaction buffer with $MgCl_2$	1 µL
DNase I, RNase-free (#EN0521)	1 µL (1 U)
DEPC-treated Water (#R0601)	to 10 µL

2. Incubate at 37 °C for 30 min.
3. Add 1 µL 50 mM EDTA and incubate at 65 °C for 10 min. RNA hydrolyzes during heating with divalent cations in the absence of a chelating agent (5). Alternatively, use phenol/chloroform extraction.
4. Use the prepared RNA as a template for reverse transcriptase.

Note

- Do not use more than 1 U of DNase I, RNase-free per 1 µg of RNA.
- If using DNase I, HC, enzyme can be diluted in 1X DNase reaction buffer just prior to use, or in storage buffer (not supplied *see composition on reverse page*) for longer storage.
- Volumes of the reaction mixture and 50 mM EDTA solution can be scaled up for larger amounts of RNA. The recommended final concentration of RNA is 0.1 µg/µL.
- Thermo Scientific RiboLock RNase Inhibitor (#EO0381), typically at 1 U/µL, can also be included in the reaction mixture to prevent RNA degradation.

6. cDNA synthesis by iScript™ reverse transcription supermix for RT-qPCR (BIORAD, USA)

1. Prepare the following reaction mixture in 0.2 ml tube, * do not add iScript RT supermix until step 3

Component	Volume per Reaction, µl
iScript RT Supermix	4
RNA template (1 µg–1 pg total RNA)	Variable
Nuclease-free water	Variable
Total volume	**20**

2. Add iScript RT supermix and incubate at the following temperature program in thermal cycler:

Priming	25°C	5 minutes
Reverse transcription	42°C	30 minutes
RT inactivation	85°C	5 minutes
Hold at 4°C		

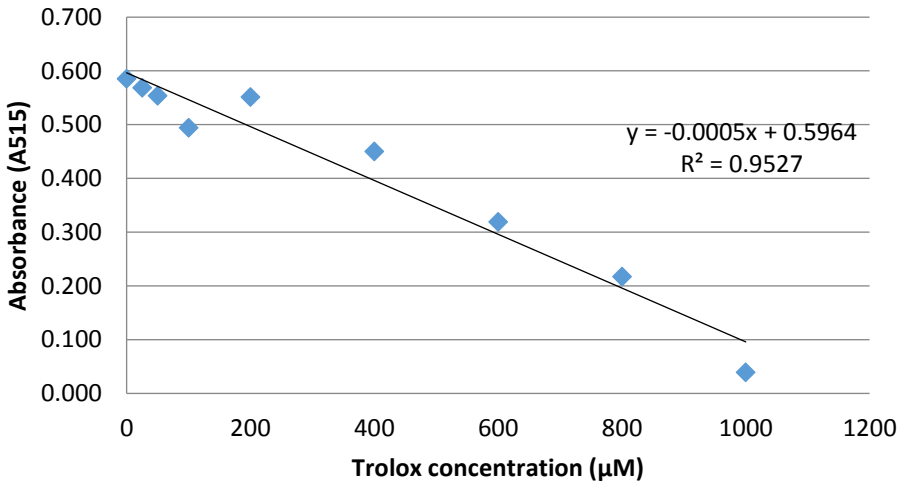
3. Place cDNA at -20°C for long-term storage

APPENDIX B

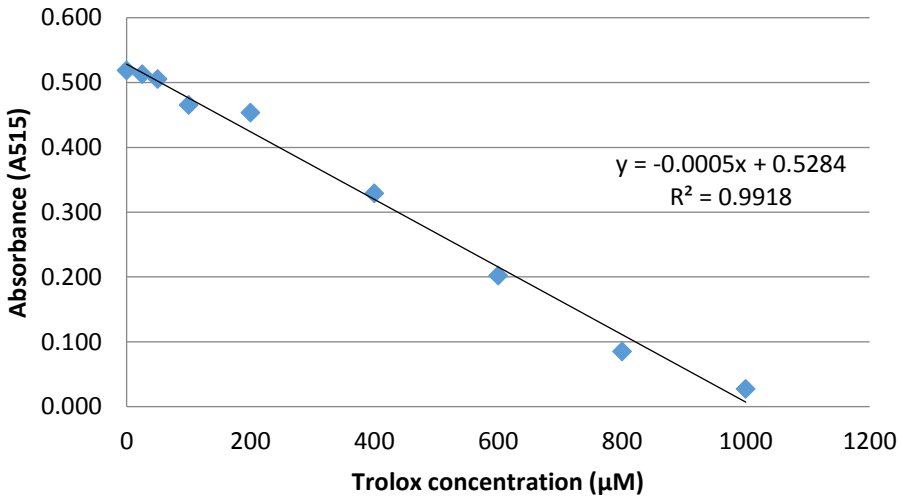
Standard curves

1. DPPH assay

1.1 Comparing among six varieties and different germination time

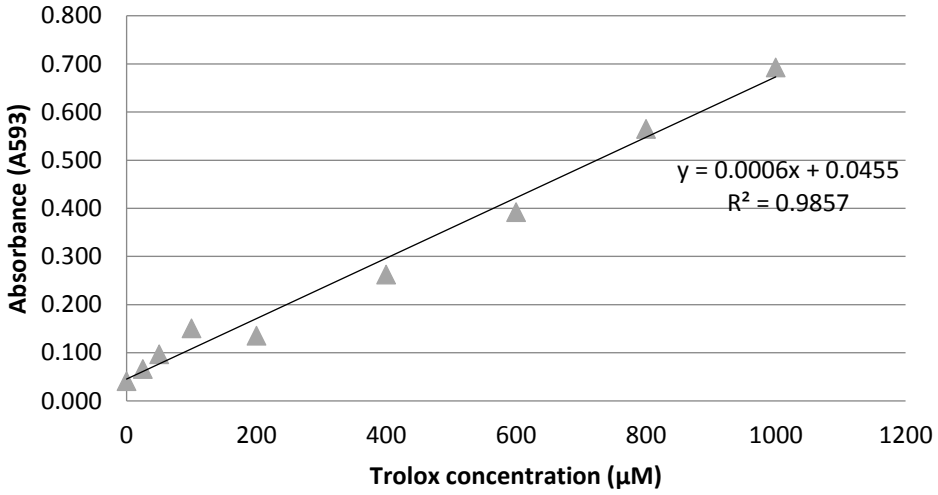


1.2 Comparing among different elicitors concentration

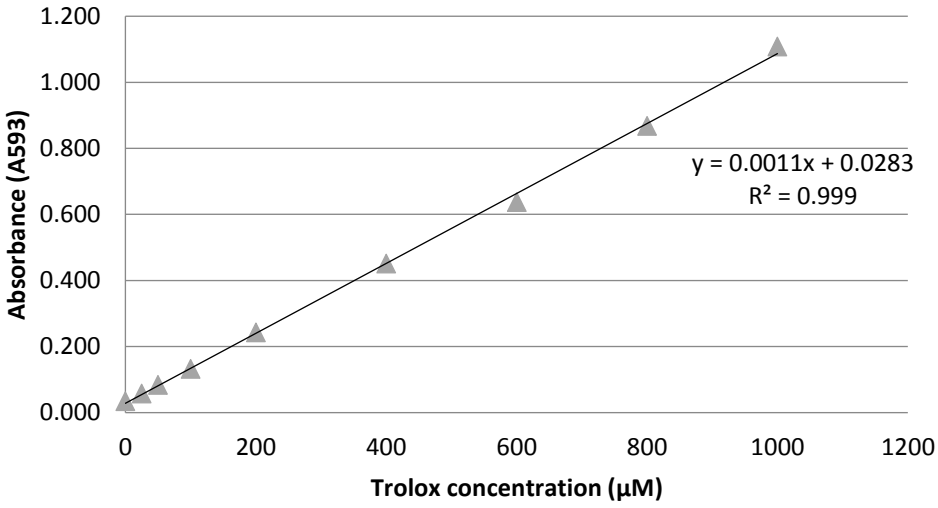


2. FRAP assay

2.1 Comparing among six varieties and different germination time

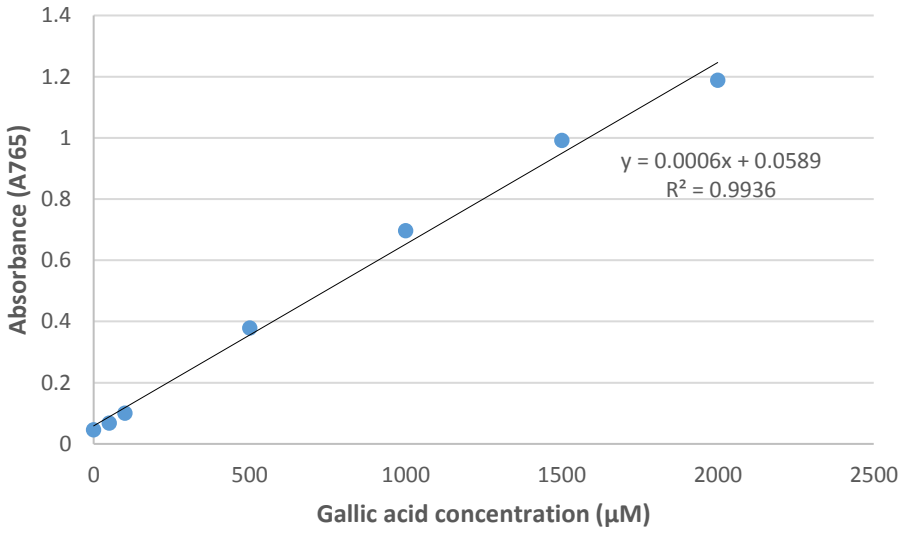


2.2 Comparing among different elicitors concentration

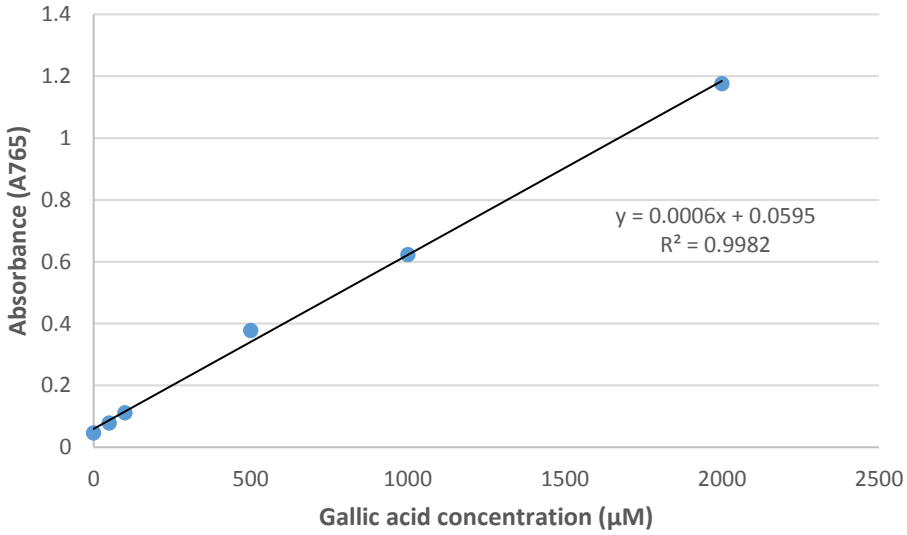


3. Folin-Ciocalteau assay

3.1 Comparing among six varieties and different germination time

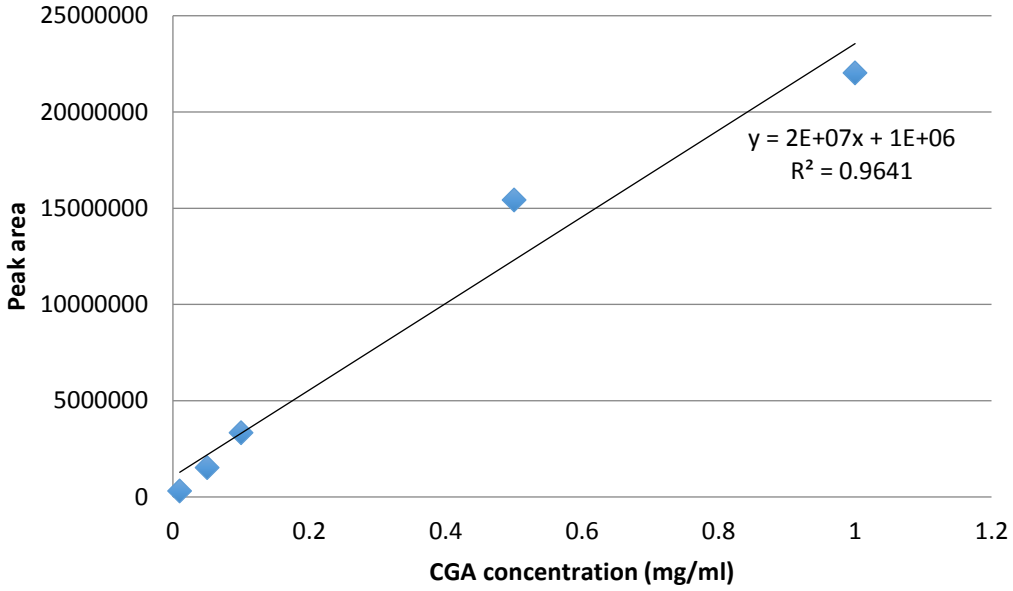


3.2 Comparing among different elicitors concentration

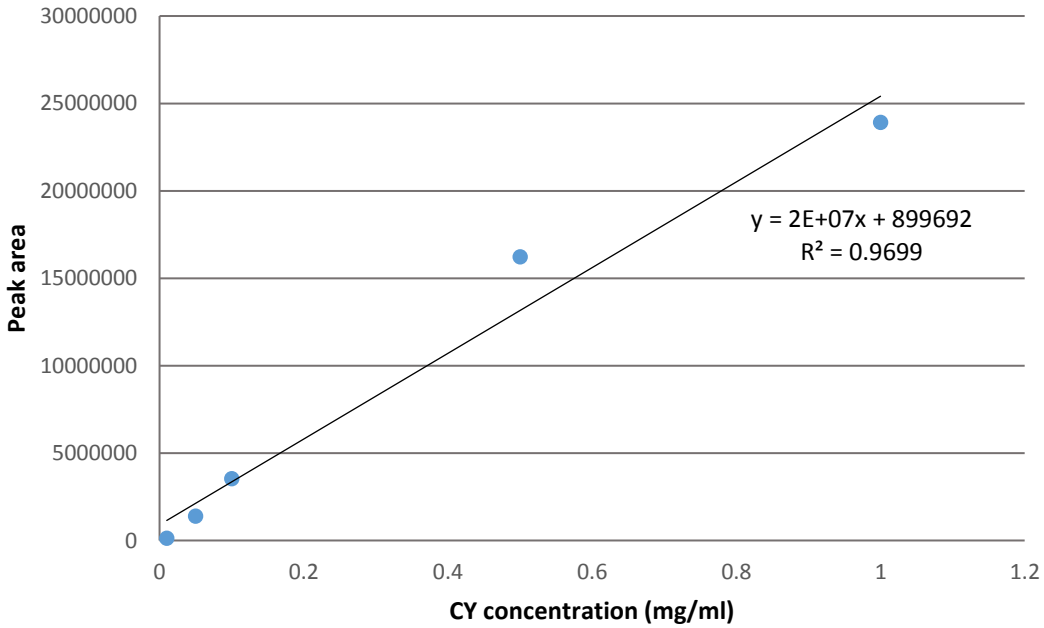


4. HPLC analysis

4.1 Chlorogenic acid

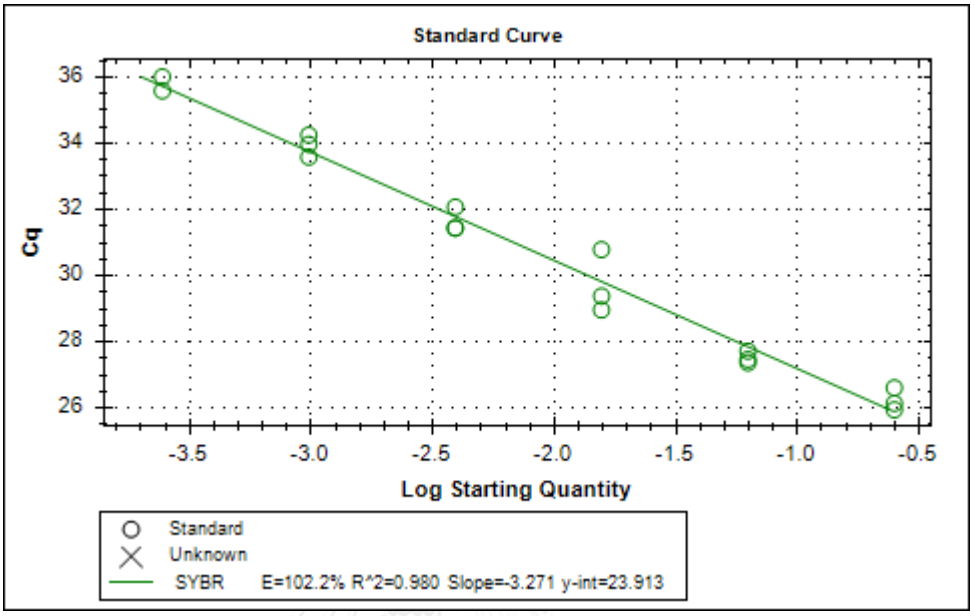


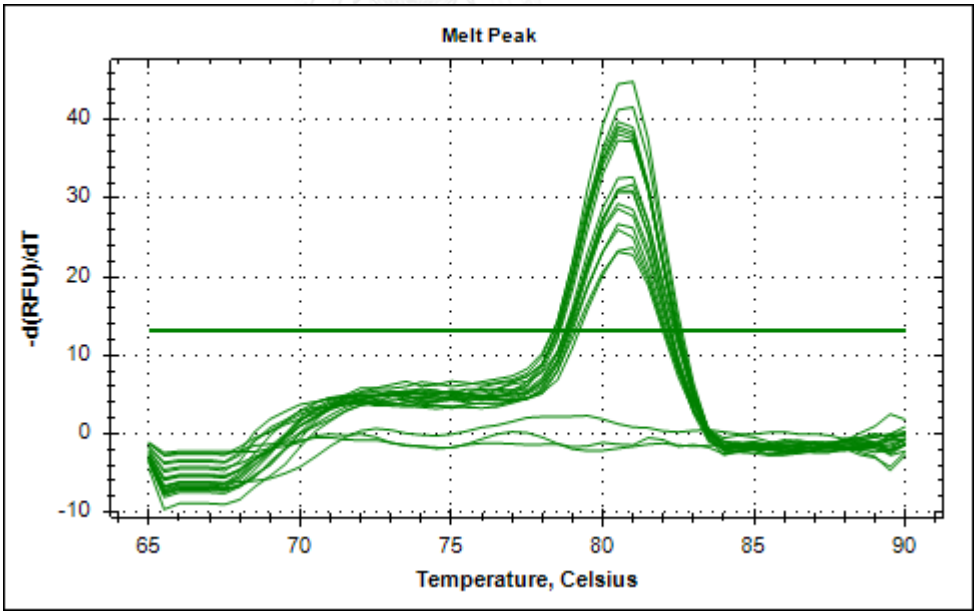
4.2 Cynarin



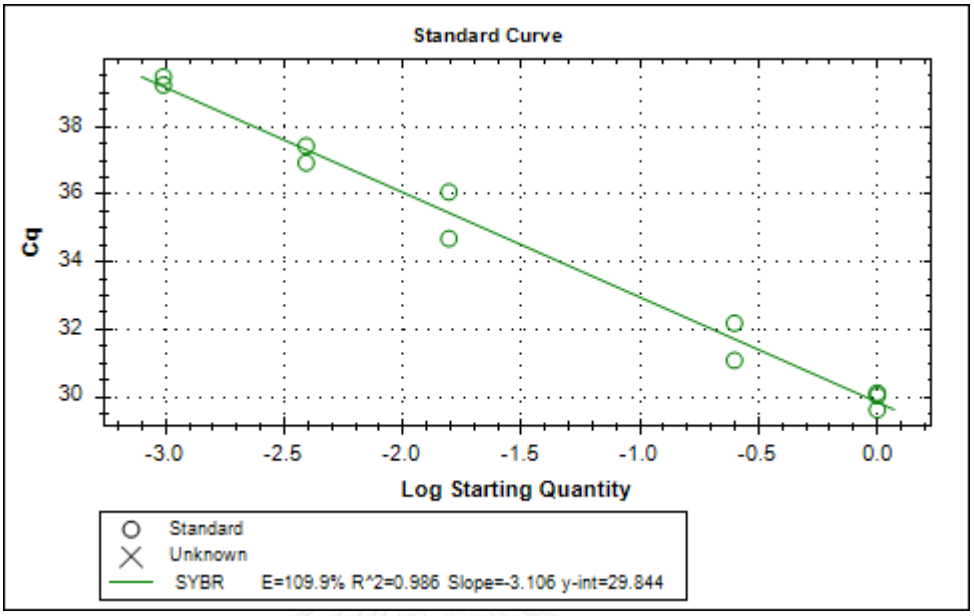
5. Gene expression analysis by real-time qRT-PCR

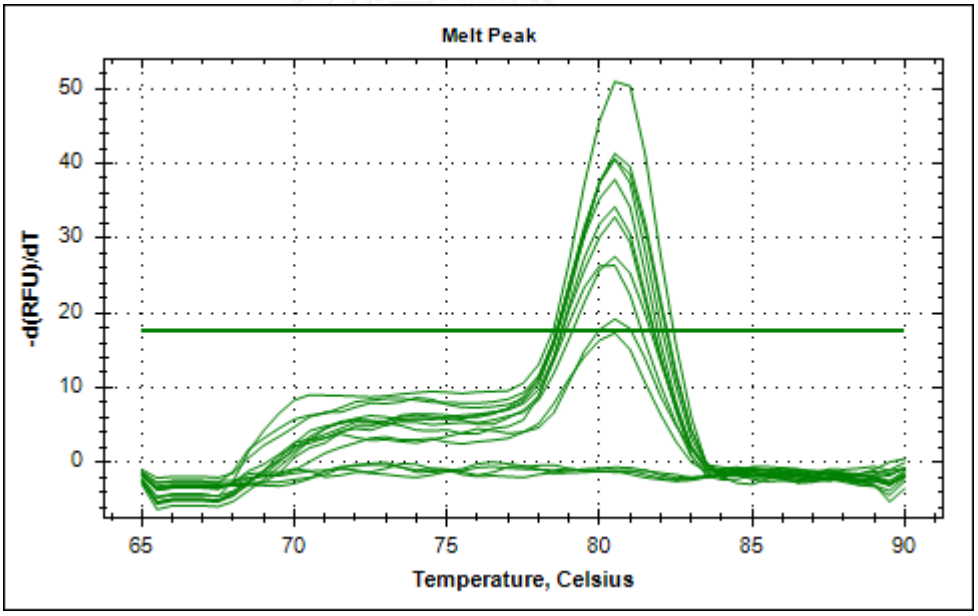
5.1 HaTubulin



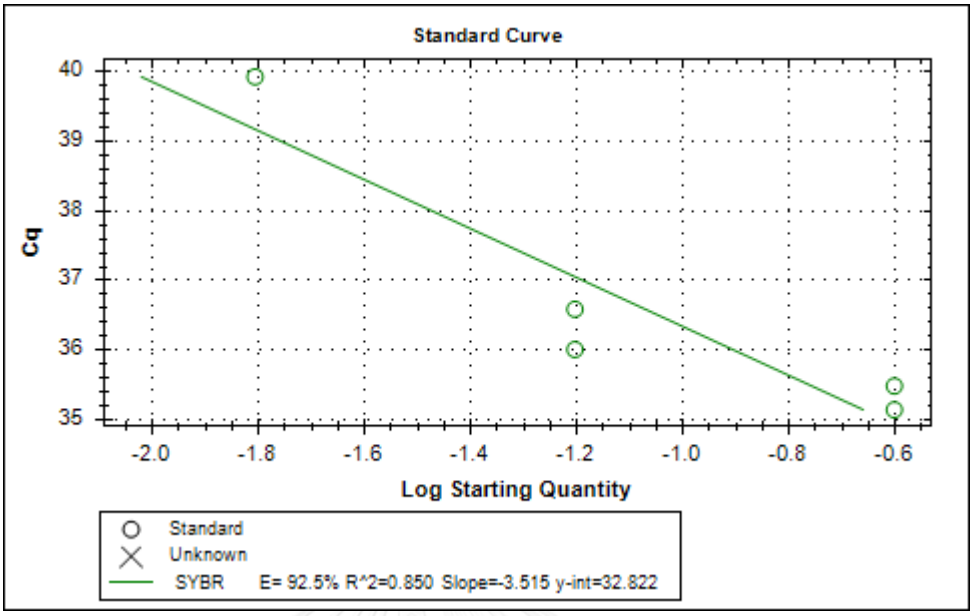


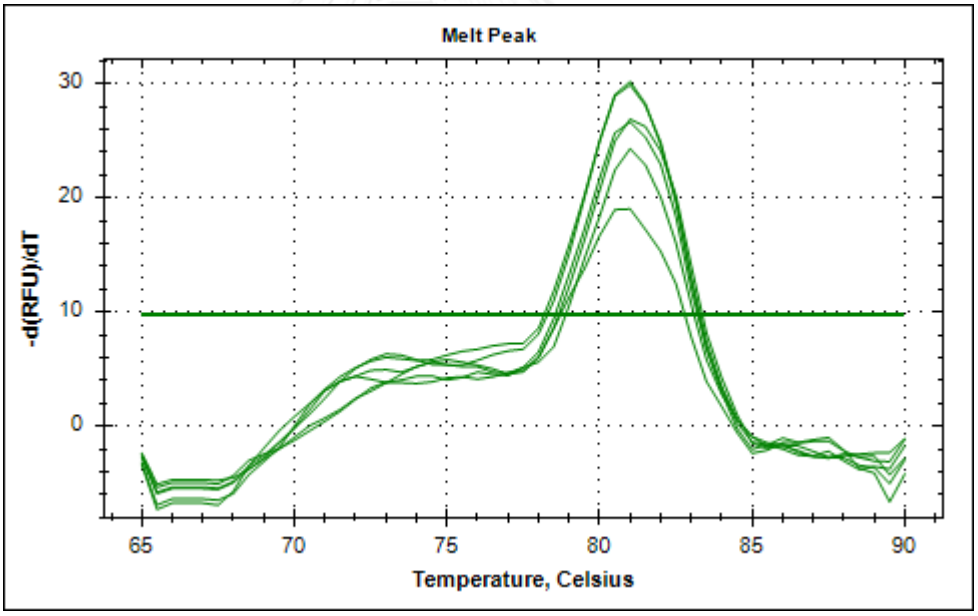
5.2 HaHQT1


Standard Curve
Cq
38
36
34
32
30
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
Log Starting Quantity
Standard
Unknown
SYBR E=109.9% R^2=0.986 Slope=-3.106 y-int=29.844

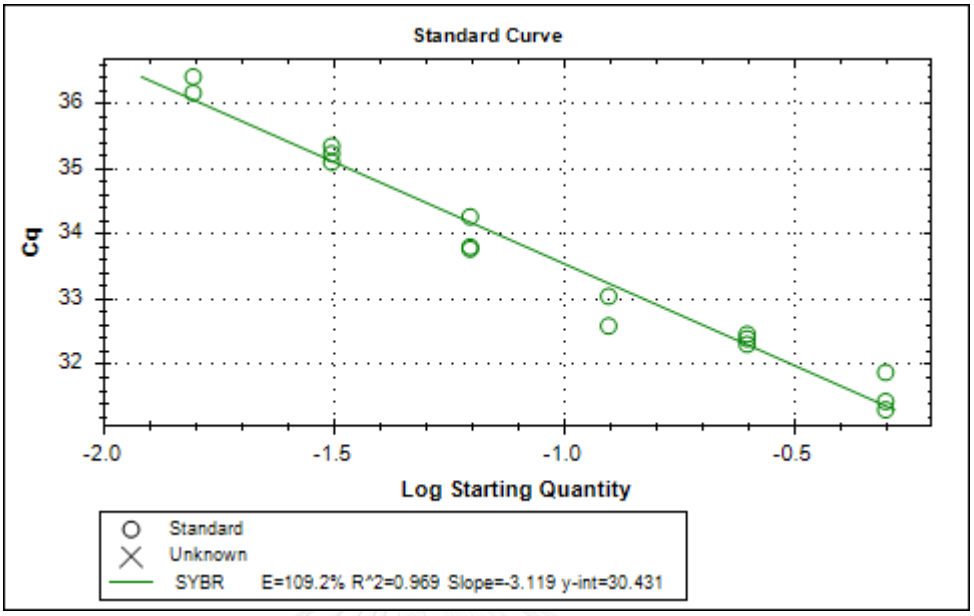

Melt Peak
-d(RFU)/dT
50
40
30
20
10
0
65
70
75
80
85
90
Temperature, Celsius

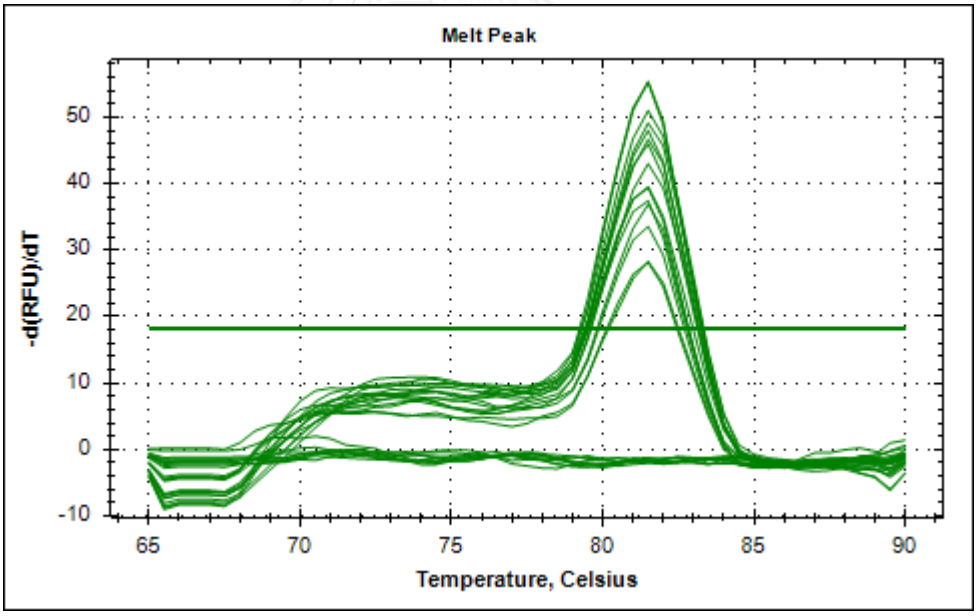
5.3 HaHQT2


Standard Curve
Cq
40
39
38
37
36
35
-2.0
-1.8
-1.6
-1.4
-1.2
-1.0
-0.8
-0.6
Log Starting Quantity
Standard
Unknown
SYBR E= 92.5% R^2=0.850 Slope=-3.515 y-int=32.822


Melt Peak
-d(RFU)/dT
30
20
10
0
65
70
75
80
85
90
Temperature, Celsius

5.4 HaHQT3

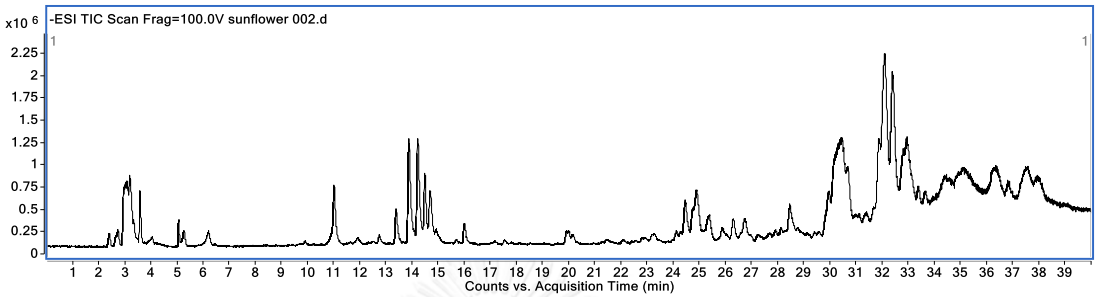

Standard Curve
Cq
36
35
34
33
32
-2.0
-1.5
-1.0
-0.5
Log Starting Quantity
Standard
Unknown
SYBR E=109.2% R^2=0.969 Slope=-3.119 y-int=30.431


Melt Peak
-d(RFU)/dT
50
40
30
20
10
0
-10
65
70
75
80
85
90
Temperature, Celsius

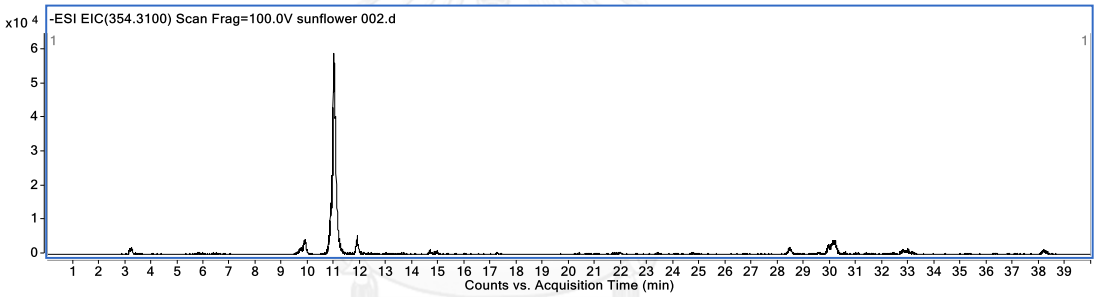
APPENDIX C

LC-MS/MS results

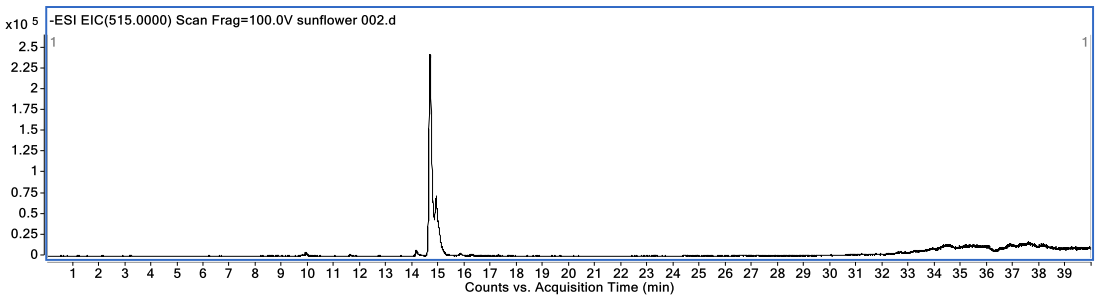
1. LC analysis



2. EIC; 354.31 m/z>> Chlorogenic acid

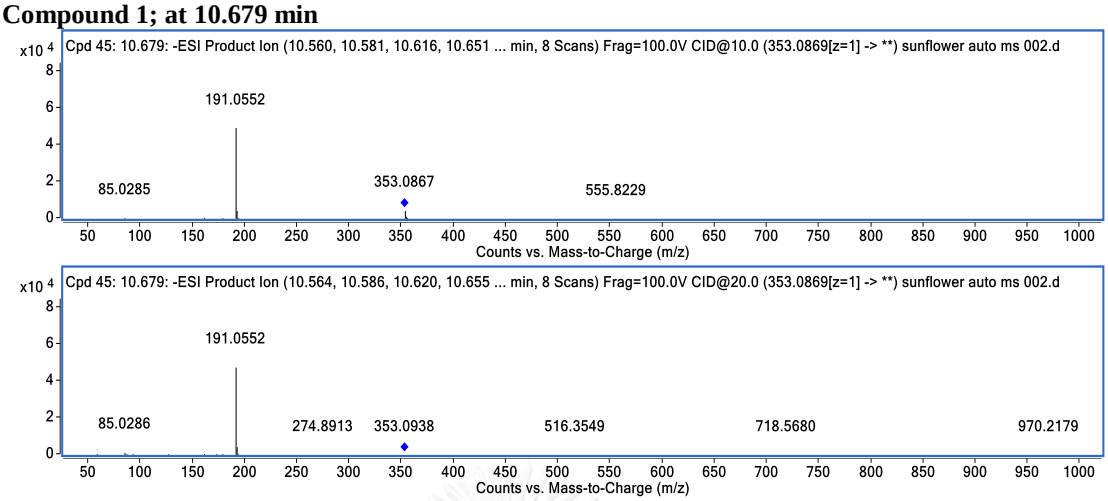


3. EIC; 515 m/z>>1,5-diCQA or 3,5-diCQA

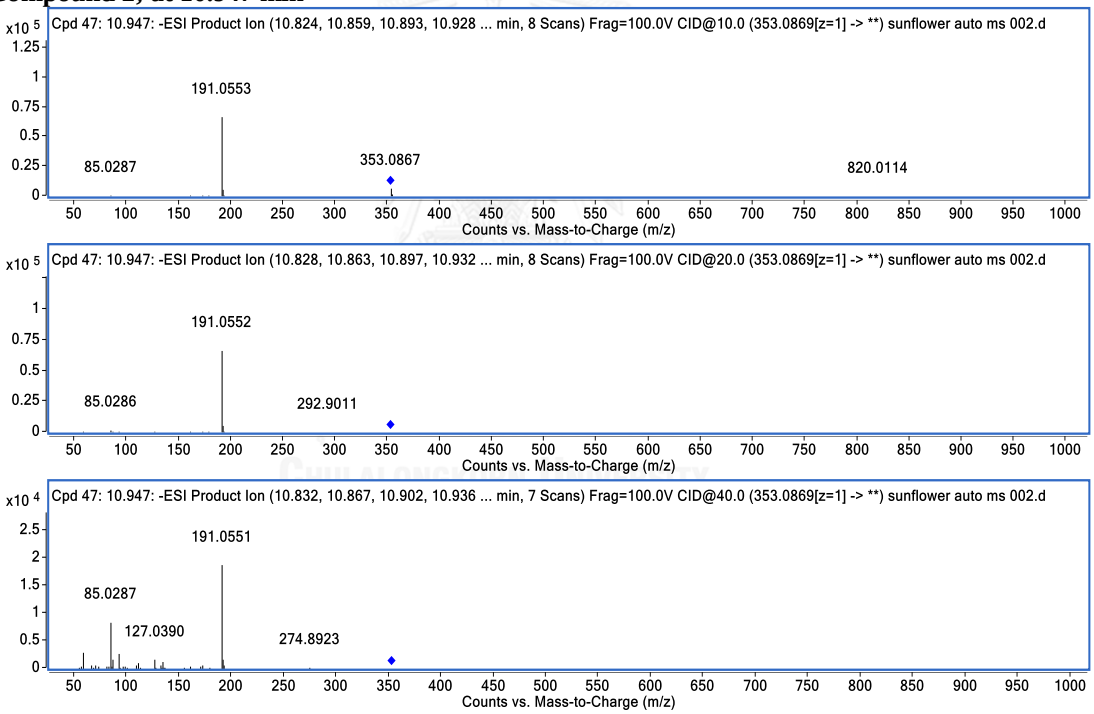


MS/MS 354.31 m/z>> Chlorogenic acid

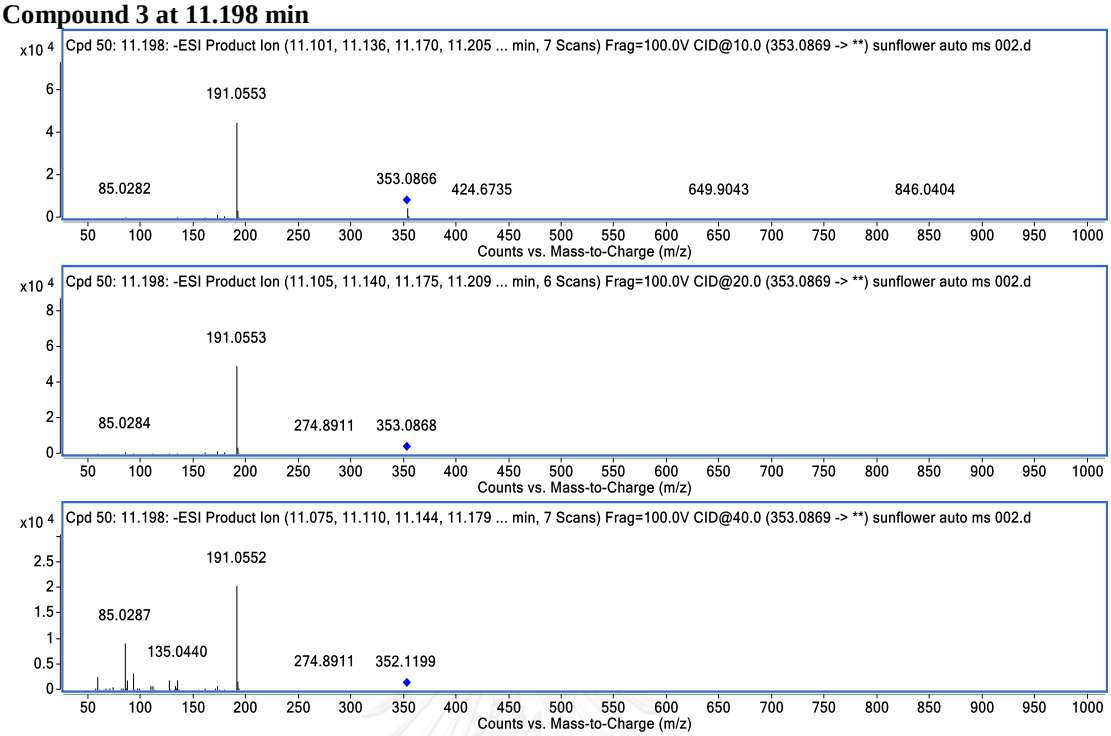
Compound 1; at 10.679 min



Compound 2; at 10.947 min



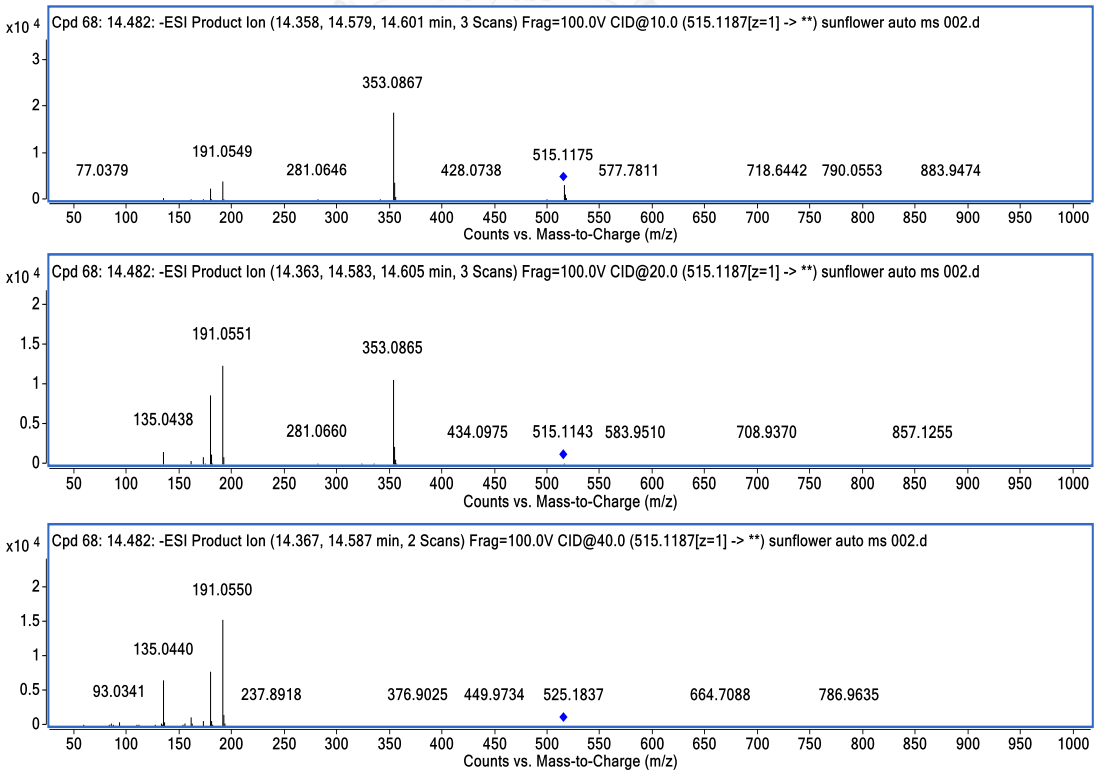
Compound 3 at 11.198 min



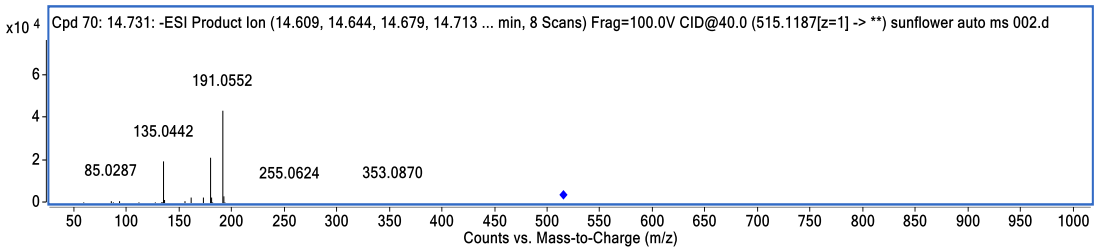
***To sum up, compound 1, 2 and 3 might be the same compound.**

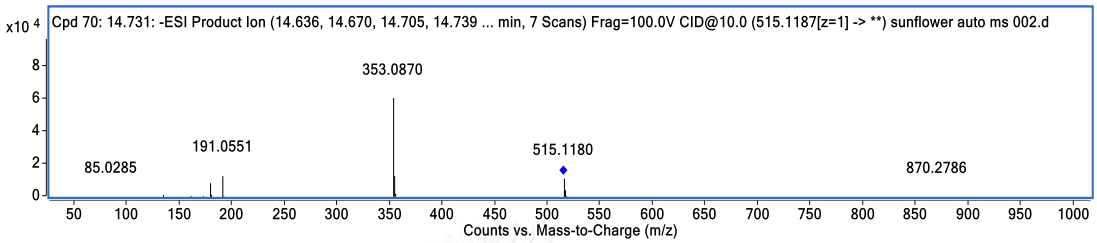
MS/MS 515 m/z>>1,5-diCQA or 3,5-diCQA

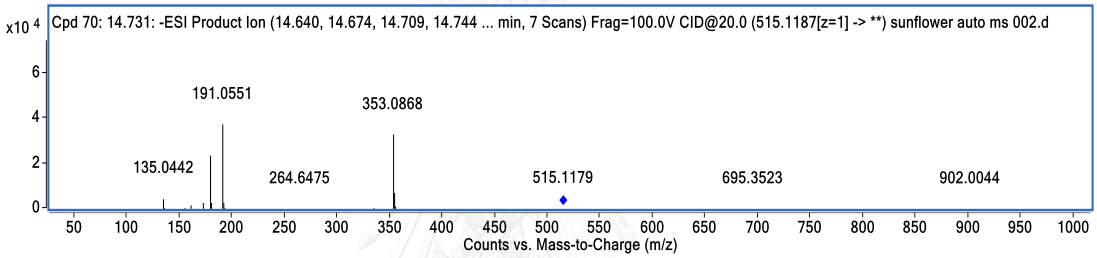
Compound 1; at 14.482 min



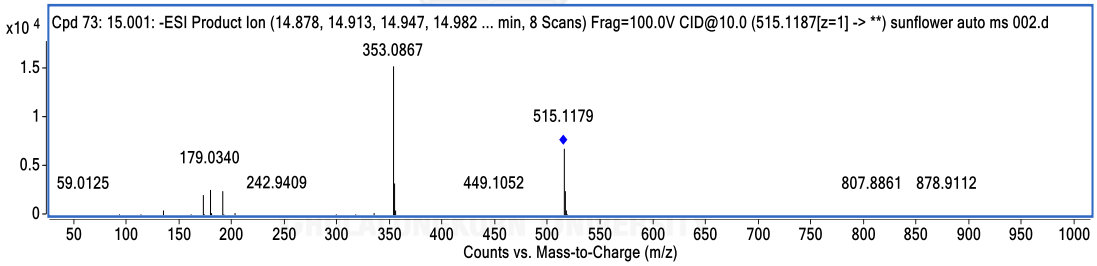
Compound 2; at 14.731 min

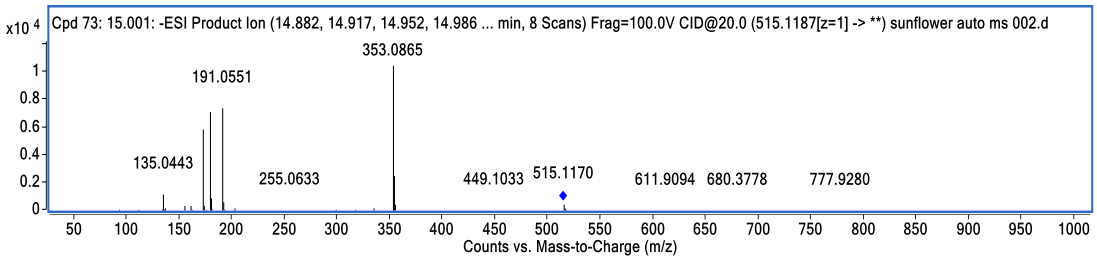


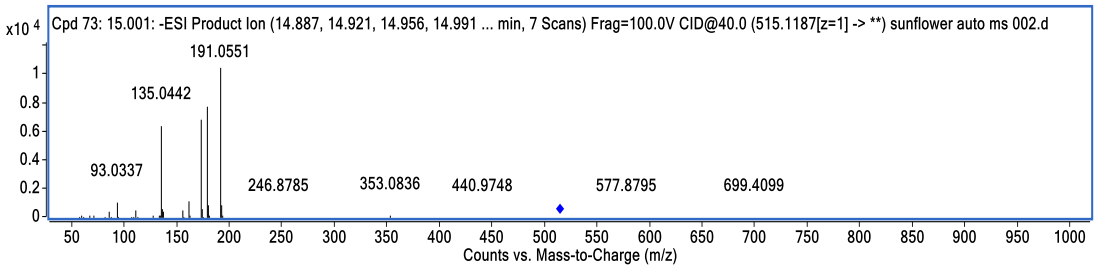




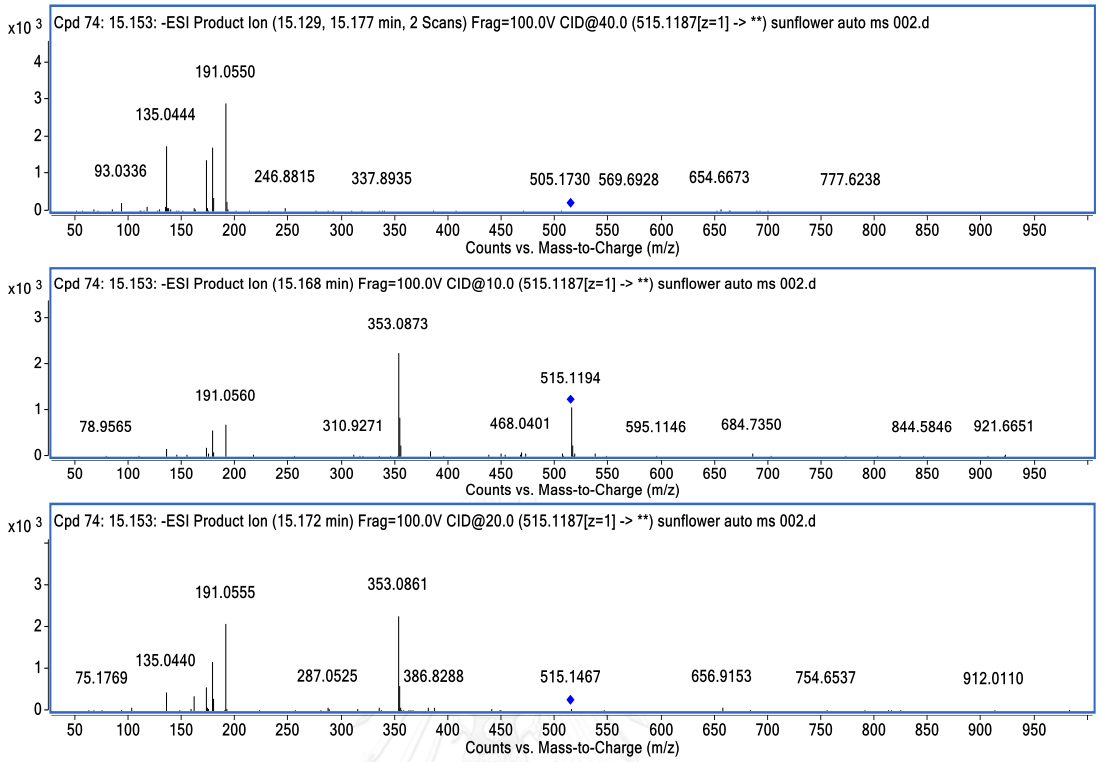
Compound 3; at 15.001 min







Compound 4; at 15.153 min



***The fragmentation is 191, 179, 135 which is similar to standard 3,5-diCQA. Therefore, the compound 1, 2, 3 and 4 is 3,5-diCQA.**

APPENDIX D

Sequence alignments

1. Sequence alignment among all genes used in this experiment

```
CLUSTAL O(1.2.1) multiple sequence alignment


GmIF7Mat        -------------------------MAETPTLRIHEVCPISPPQET--PSTTIPFTFFD
Gt5AT           -------------------------MEQIQMVKVLEKCQVTPPSDTTDVELSLPVTFFD
Dv3MAT          -------------------------MDNIPNLTILEHSRISPPPST-IGHRSLPLTFFD
NtMAT1          -----------------------------MASVIEQCQVVPSPGS-ATELTLPLTYFD
Pf5MAT          -----------------------------MTTTLLETCRILPPPT---DEVSIPLSFFD
AtSDT           --------------------MPIHIGSSIPLMVEKMLTEMVKPSKHIPQ--QTLNLSTLD
CbBEBT          ---------------------MAHDQSLSFEVCRRKPELIRPAKQTPH--EFKKLSDVE
PhBPBT          --------------------MDSKQSSELVFTVRRQEPELIAPAKPTPR--ETKFLSDID
AtHHT           MVAENNKNKDVTLSASMDNNNNNIKGTNIHLEVHQKEPALVKPESETRK--GLYFLSNLD
FACT            ------------------------MADSFELIVTRKEPVLVSPASETPK--GLHYLSNLD
TpHCT2          -----------------------------MVTIKNSYTVIPEEPTPQ--GRLWLSDKD
SHT             -----------------------------MAPITFRKSYTIVPAEPTWS--GRFPLAEWD
HCBT            -----------------------------MSIQIKQSTMVRPAEETPN--KSLWLSKID
HQT1            ------------MGSDQATIAVPNEHHKLMNINIKHSSFVQPSQPTPS--STIWTSNLD
HaHQT2          ------------------------MGTVQKTLNINIKQTTFVQPSKPTQASTKQLWTSNLD
HaHQT3          ----------------------MKTDHNIKMNITIKNSTIIPPSEPIADAPKQLWTSNLD
HaHQT1          ----------------------MKTDQTKPMNITITKSSIVPPSETINGSSNHIWTSNLD
HQT2            ----------------------MGSDQKMMMNIDIMKSSIVPPSELIADCPKQLWTSNLD
CcsHQT          -----------------------------MELTVKESLMVKPSKPTPN--QRLWNSNLD
NtHQT           ------------------------MGSEKMMKINIKESTLVKPSKPTPT--KRLWSSNLD
SlHQT           ------------------------MGSEKMMKINIKESTLVKPSKPTPT--KRIWSSNLD
HaHCT3          --------------------------MKVVVRESTMVRPAEGTPT--IKLWNSCLD
HaHCT4          ------------------------------------------------------------
AsHHT           ----------------------------MKITVRSSTVVVPAAETPR--VRLWNANPD
HaHCT2          ----------------------------MKIVVRESTMVSPKEETPK--INLWCSNLD
PrHCT           ----------------------------MLITVKNSQMVRPAAPTPQ--RDLWNSNVD
AtHCT           ----------------------------MKINIRDSTMVRPATETPI--TNLWNSNVD
TpHCT1A         ----------------------------MIINVRDSTMVRPSEEVTR--RTLWNSNVD
TpHCT1B         ----------------------------MIINVRDSTMVRPSEEVTR--RTLWNSNVD
HaHCT1          ----------------------------MKIEVRESTMVKPAEETPK--INLWNSNVD
NtHCT           ----------------------------MKIEVKESTMVKPAAETPQ--QRLWNSNVD


GmIF7Mat        VLW--LRLPPVERLFFYSFPNPTT-----TSSFFDTTILPNLKHSLSLTLHHFPPLAGTI
Gt5AT           IPW--LHLNKMQSLLFYDFPYPR--------THFLDTVIPNLKASLSLTLKHYVPLSGNL
Dv3MAT          IAW--LLFPPVHHLYFYHFPYSK--------SHFTETVIPNLKHSLSITLQHYFPFVGKL
NtMAT1          HVW--LAFHRMRRILFYKLPISR--------PDFVQTIIPTLKDSLSLTLKYYLPLAGNV
Pf5MAT          MKW--LHFHPLRRLLFYDHPCSK--------PQFLDAIVPHLKQSLSLTLKHYLPVAGNL
AtSDT           NDPY-NEV-IYKACYVFKAKNVAD-------DDNR--PEALLREALSDLLGYYYPLSGSL
CbBEBT          DQE-GLRF-QIPVIQFYKHNNESM-------QERD--PVQVIREGIARALVYYYPFAGRL
PhBPBT          DQE-GLRF-QIPVINFYRKD-SSM-------GGKD--PVEVIKKAIAETLVFYYPFAGRL
AtHHT           QNI---AV-IVRTIYCFKS---------EERGNEE--AVQVIKKALSQVLVHYYPLAGRL
FACT            QNI---AI-IVKTFYYFKS---------NSRSNEE--SYEVIKKSLSEVLVHYYPAAGRL
TpHCT2          QVA--TQH-HTPTIYIYKPNQNQE----------N--VIETLKNSLSKILVHYYPIAGRL
SHT             QVG--TIT-HIPTLYFYDKPSESF--------QGN--VVEILKTSLSRVLVHFYPMAGRL
HCBT            MILRTPYS-HTGAVLIYKQPDNNEDNIHPSSSMYF--DANILIEALSKALVPYYPMAGRL
HQT1            LVV--GRI-HILTVYFYRP---------NGASNFF--DADVMKKALADVLVSFYPMAGRI
HaHQT2          LVV--GRI-HILTVYFYRP---------IVCSNFF--DPLVMKKALADVLVTFYPMAGRM
HaHQT3          LVV--GRI-HILTVYFYRP---------NGSSNFF--DSNVMKKALADVLVPFYPMAGRL
HaHQT1          LVV--GRI-HILTVYFYRP---------NGSNNYF--DPNVMKQALADVLVPFYPMAGRL
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HQT2          LVV--GRI-HILTVYFYRP---------NGSSKFF--DPNVMKKALADVLVSFYPMAGRL
CcsHQT        LVV--GRI-HILTVYFYRP---------NGSSNFF--DSGVLKKALADVLVSFFPMAGRL
NtHQT         LIV--GRI-HLLTVYFYKP---------NGSSNFF--DSKIMKEALSNVLVSFYPMAGRL
SlHQT         LIV--GRI-HLLTVYFYKP---------NGSSNFF--DNKVIKEALSNVLVSFYPMAGRL
HaHCT3        LTA--PNF-HIQLVYFYRP---------NGAANFF--DTKVMKDALSRALVAFYPMAGRF
HaHCT4        -MA--PNY-HTPVVYFYRP---------NGVVNFF--DTKVMKDALSRALVAFYPMAGRF
AsHHT         LVV--PRF-HTPSVYFYRRGGE-----DGGDACYF--DAGRMRRALAEALVPFYPMAGRL
HaHCT2        LMV--PNV-HTQTVYIYRP---------NGAANFF--DTKPMKDALSKALVPFYPVGGRL
PrHCT         LVV--PRI-HTASVYFYRP---------TGSPDFF--SMNILRDALSKLLVPFYPMAGRL
AtHCT         LVI--PRF-HTPSVYFYRP---------TGASNFF--DPQVMKEALSKALVPFYPMAGRL
TpHCT1A       LVV--PNF-HTPSVYFYRSN--------NGTSNFF--DAKIMKEALSKVLVPFYPMAGRL
TpHCT1B       LVV--PNF-HTPSVYFYRSN--------NGTSNFF--DAKIMKEALSKVLVPFYPMAGRL
HaHCT1        LVV--PNF-HTPSVYFYRP---------NGAPDFF--DPKVMKDALSRALVPFYPMGGRL
NtHCT         LVV--PNF-HTPSVYFYRP---------TGSPNFF--DGKVLKEALSKALVPFYPMAGRL
                           :                        :  .::  *  : *  * .

GmIF7Mat      TW-PL---HTPLPLITYT-PGNSIPFRIAES-NADFNTLSSNLS-EVNNHRRN-LIPHLP
Gt5AT         LM-PIKSGEMPKFQYSRD-EGDSITLIVAES-DQDFDYLKGHQL-VDSNDLHG-LFYVMP
Dv3MAT        IVYPNPHDSTRKPEIRHV-EGDSVALTFAET-TLDFNDLSANHP-RKCENFYP-LVPPLG
NtMAT1        AC-PQ-DW-SGYPELRYV-TGNSVSVIFSES-DMDFNYLIGYHP-RNTKDFYH-FVPQLA
Pf5MAT        LY-PS-SNTDQKPRLRCV-AGDSVPLTIAES-TTDFDMLTGNHA-RDADQFYD-FVAPMP
AtSDT         KRQE----SDRKLQLSCGGDGGGVPFTVATA-NVELSSLKNLEN-IDSDTA---LNFLP-
CbBEBT        REVD-----GRKLVVECTGEG--VMFIEADA-DVTLEQFGDAL-QPPFPCFDQ-LLFDV-
PhBPBT        REGN-----DRKLMVDCTGEG--VMFVEANA-DVTLEEFGDEL-QPPFPCLEE-LLYDV-
AtHHT         TISP-----EGKLTVDCTEEG--VVFVEAEA-NCKMDEIGDIT--KPDPETLGKLVYDV-
FACT          TISP-----EGKIAVDCTGEG--VVVVEAEA-NCGIEKIKKAISEIDQPETLEKLVYDV-
TpHCT2        CYSDE-EVDSCRVELNLNAKG--AILLEAET-TKTIHDYGDFS---PSDLTKE-LIPII-
SHT           RWLP-----RGRFELNCNAEG--VEFIEAES-EGKLSDFKDFS---PTPEFEN-LMPQV-
HCBT          KING------DRYEIDCNAEG--ALFVEAES-SHVLEDFGDFR---PNDELHRVMVPTC-
HQT1          SRDQ-----NGRLEINCNGEG--VLFVEAES-DSTLDDFGEFT---PSPELRR-LTPTV-
HaHQT2        GKDQ-----NGKVVINCNDEG--VLFVEAES-DSTLDDFGEFT---PSPELRR-LTPTV-
HaHQT3        GRDE-----SGRIVINCNGEG--VLFVEAET-DACLDDFGEFT---PSPEFQM-LTPSV-
HaHQT1        GRDE-----SGRIVINCNGEG--ALFVEAES-DSCLDDFGEFT---PSPEFRS-LTPTV-
HQT2          GRDE-----TDRIVINCNNEG--VLFVEAES-DSTLDDFGELK---PSPEFRQ-LTPSV-
CcsHQT        GNDG-----DGRVEINCNGEG--VLFVEAEA-DCSIDDFGEIT---PSPELRK-LAPTV-
NtHQT         ARDE-----QGRIEINCNGEG--VLFVEAES-DAFVDDFGDFT---PSLELRK-LIPTV-
SlHQT         GRDE-----QGRIEVNCNGEG--VLFVEAES-DSCVDDFGDFT---PSLELRK-LIPSV-
HaHCT3        KRGE-----DGRVEVDCQGQG--GLFLEAES-DGEINDFSDFA---PKFKFLK-LVPVV-
HaHCT4        VQDQ-----DGRVEIDCQGQG--ALFFDAES-DGVVDDFDDFA---PQIKFLK-LVPMV-
AsHHT         AHDE-----DGRVEIDCNAEG--VLFVEADAPDGAVDDFGDFV---PTMGLKR-LIPTV-
HaHCT2        KEDK-----NGRIEIDCQGQG--VLFVEAES-VGXXXXXX--------------------
PrHCT         KRDP-----DGRIEINCNGEG--VLLVEAIT-DSVIDDFGDFA---PTMELKQ-LIPKV-
AtHCT         KRDD-----DGRIEIDCNGAG--VLFVVADT-PSVIDDFGDFA---PTLNLRQ-LIPEV-
TpHCT1A       RRDE-----DGRVEIDCDGQG--VLFVEADT-GAVIDDFGDFA---PTLELRQ-LIPAV-
TpHCT1B       RRDE-----DGRVEIDCDGQG--VLFVEADT-GAVIDDFGDFA---PTLELRQ-LIPAV-
HaHCT1        KRDE-----DGRIEIDCRGQG--VLFVEAES-DGVVDDFGDFA---PTLELRK-LIPAV-
NtHCT         CRDE-----DGRIEIDCKGQG--VLFVEAES-DGVVDDFGDFA---PTLELRQ-LIPAV-
                              *    .  : :

GmIF7Mat      TS------HEEASVLALQ------LTHFP-NQGYSIGITSHHAALDGKSSTLFMKSWAHI
Gt5AT         RVIRTMQDYKVIPLVAVQ------VTVFP-NRGIAVALTAHHSIADAKSFVMFINAWAYI
Dv3MAT        NA-VKESDYVTLPVFSVQ------VTYFP-NSGISIGLTNHHSLSDANTRFGFLKAWASV
NtMAT1        EP-KDAPGVQLAPVLAIQ------VTLFP-NHGISIGFTNHHVAGDGATIVKFVRAWALL
Pf5MAT        PI-AEEFECKIVPVFSLQ------VTLFP-GRGICIGLSNHHCLGDARSVVGFVLAWASI
AtSDT         ---VLHVDIDGYRPFALQ------VTKFE-CGGFILGMAMSHAMCDGYGEGHIMCALTDL
CbBEBT        ---PGSGGILDSPLLLIQ------VTRLK-CGSFIFALRLNHTMADAAGIVLFMKAVGEM
PhBPBT        ---PGSAGVLHCPLLLIQ------VTRLR-CGGFIFALRLNHTMSDAPGLVQFMTAVGEM
AtHHT         ---VDAKNILEIPPVTAQ------VTKFK-CGGFVLGLCMNHCMFDGIGAMEFVNSWGQV
FACT          ---PGARNILEIPPVVVQ------VTNFK-CGGFVLGLGMNHNMFDGIAAMEFLNSWAET
TpHCT2        ---DYNQPFQEMPLLVVQ------LTSFKNNQGFALGVAFSHSLSDGTGAVKFINSWAKI
SHT           ---NYKNPIETIPLFLAQ------VTKFK-CGGISLSVNVSHAIVDGQSALHLISEWGRL
HCBT          ---DYSKGISSFPLLMVQ------LTRFR-CGGVSIGFAQHHHACDGMSHFEFNNSWARI
HQT1          ---DYSGDISSYPLFFAQ------VTHFK-CGGVALGCGVFHTLADGLSSIHFINTWSDM
HaHQT2        ---DYSGDISSYPLFFAQVYWFDQVTHFK-CGGVGLGCGVFHTLADGLSSLHFINTWSDM
```

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HaHQT3          ---DYSGDISSYPLFFAQ------VLHFK-CGGVGLGCGVFHTLADGLSSLHFINTWSDM
HaHQT1          ---DYSGDISSFPLFFAQ------VTHFK-CGGVALGCGVFHTLSDGLSSLHFINTWSDV
HQT2            ---DYSGDISSYPLFFAQ------VTHFK-CGGVALGCGVHHTLSDGLSSLHFINTWSDM
CcsHQT          ---DYSDQVSSYPLCITQ------VTRFN-CGGVSLGCGLHHTLSDGLSSLHFINTWSDK
NtHQT           ---DTSGDISTFPLIIFQ------VTRFK-CGGVSLGGGVFHTLSDGLSSIHFINTWSDI
SlHQT           ---ETSGDISTFPLVIFQ------ITRFK-CGGVALGGGVFHTLSDGLSSIHFINTWSDI
HaHCT3          ---DYSLGIDSYPLLVVQ------VTYFK-CGGVSLGVGLHHRVVDGSSAMHFINTWSDI
HaHCT4          ---DYSLGIESYPLLVLQ------VTYFK-CGGVSLTVSMHHYLADGPSAMHFINTWADM
AsHHT           ---DFTGGISSYPLLVVQ------VTHFK-CGGVALGIAMQHHVADGFSGLHFINSWSDL
HaHCT2          ---------XXXXXXXXQ------VTFFK-CGGVSLGVGMHHYVCDGTSALHFMNTWADM
PrHCT           ---NYSEDISSYPLLVLQ------VTFFK-CGGVSLGVGMQHHVADGYAGIHFINTWSDV
AtHCT           ---DHSAGIHSFPLLVLQ------VTFFK-CGGASLGVGMQHHAADGFSGLHFINTWSDM
TpHCT1A         ---DYSRGIESYPLLVLQ------VTYFK-CGGVSLGVGMQHHVADGASGLHFINTWSDV
TpHCT1B         ---DYSRGIESYPLLVLQ------VTYFK-CGGVSLGVGMQHHVADGASGLHFINTWSDV
HaHCT1          ---DYSQGIESYPLLVLQ------VFNKF-CGGVSLGVGMQHHAADGASGLHFINTWSDM
NtHCT           ---DYSQGIQSYALLVLQ------ITHFK-CGGVSLGVGMQHHAADGASGLHFINTWSDM
                                *     :      .  .    *   *.     :

GmIF7Mat        CSYLNTSPEEPLLFSLPKHLTPSFDRSV-IRDPLGI------GEIYAKSWTSFGGATNDR
Gt5AT           NKFGKDA----DLLSA--NLLPSFDRSI-IKDLYGL------EETFWNEMQDVLEMF---
Dv3MAT          CETGEDQ----PFLKN--GSPPVFDRVV-VNPQLYE------NRLNQTRL--GTFYQ---
NtMAT1          NKFGGDE----QFLAN--EFIPFYDRSV-IKDPNGV------GMSIWNEMKKYKHMM---
Pf5MAT          NKFGGDE----EFLSENGESLPIFDRSL-IKDPLEI------DTIFWKVLRNIP--L---
AtSDT           AGGKKK-P----------MVTPIWERER-LVGKPED----DQPPF-----VPGDDTA-AS
CbBEBT          ARGAAT-P----------STLPVWDRHI-LNARVPP-----QVTFNHREYEEVKGT---I
PhBPBT          ARGATA-P----------STLPVWCREL-LNARNPP-----QVTCTHHEYEEVPDTK-GT
AtHHT           ARGLPL-----------TTPPFSDRTI-LNARNPP-----KIENLHQEFEEIEDKS-NI
FACT            ARGLPL-----------SVPPFLDRTL-LRPRTPP-----KIEFPHNEFEDLEDIS-GT
TpHCT2          ARGETLEP----------NELPFLDRTL-IKFSHTP---SKVPRFEHIELKPLPLII-GR
SHT             ARGEPL-----------ETVPFLDRKI-LWAGEPLPPFVSPPKFDHKEFDQPPFLI-GE
HCBT            AKGLLP-----------ALEPVHDRYLHLRLRNPP-----QIKYTHSQFEPFVPSL-PN
HQT1            ARGLSI-----------AIPPFIDRTL-LRAREPP-----TPTFDHIEYHAPPSMK-TI
HaHQT2          ARGLSI-----------AIPPFIDRTL-LRAREPP-----TPTFDHVEYHPPPSMK-PD
HaHQT3          ARGLSI-----------AIPPFIDRTL-LRAREPP-----TPTFDHVEYHPPPSMK-PD
HaHQT1          ARGLSV-----------AIPPFIDRTL-LRARVPP-----TPTHDHVEYHPPPTMI-TT
HQT2            ARGLSV-----------AIPPFIERTL-LRAREPP-----TPTYDHVEYHSPPSMN-TT
CcsHQT          ARGLSV-----------AIPPFIDRSL-LRARDPP-----TAMFEHLEYHSPPSLI-AP
NtHQT           ARGLSV-----------AIPPFIDRTL-LRARDPP-----TSSFEHVEYHPPPSLI-SS
SlHQT           ARGLSV-----------AVPPFIDRTL-LRARDPP-----TSSFEHVEYHPPPTLN-SS
HaHCT3          ARGLDI-----------TLPPFIDRTL-LRAQDPP-----RPTFDHVEFHSDPVVN-AP
HaHCT4          SRGLDI-----------NLPPFIDRTL-LRARDPP-----QPTFEHIEYHPDPT---MP
AsHHT           CRGVPI-----------AVMPFIDRTL-LRARDPP-----VPTHPHIEYQPAPAML-GS
HaHCT2          ARGLDL-----------TIPPFIDRTL-LRARDPP-----RPVYDHIEYHVGRPMK-LP
PrHCT           ARGLDI-----------TLPPFIDRTL-LRARNPP-----TPKFQHIEYQQPPPLK-DT
AtHCT           ARGLDL-----------TIPPFIDRTL-LRARDPP-----QPAFHHVEYQPAPSMK-IP
TpHCT1A         ARGLDV-----------SIPPFIDRTL-LRARDPP-----RPVFDHIEYKPPPSMK-TT
TpHCT1B         ARGLDV-----------SIPPFIDRTL-LRARDPP-----RPVFDHIEYKPPPSMK-TT
HaHCT1          ARGLDI-----------TIPPFIDRTL-LRARDPP-----QPVFDHIEYQPAPPMK-NA
NtHCT           ARGLDL-----------TIPPFIDRTL-LRARDPP-----QPQFPHVEYQPPPTLK-VT
                                   *   *   :

GmIF7Mat        SLNVWDTLGGNQTDLVKGLFELTPLDIKKLKKLAESKFVVGDNKKKVRVTSFTVTCAYLL
Gt5AT           ---SRFGSKPPRFNKVRATYVLSLAEIQKLKNKVLNL---RGSEPTIRVTTFTMTCGYVW
Dv3MAT          ----APSLVGSSSDRVRATFVLARTHISGLKKQVLTQ---LP--MLEYTSSFTVTCGYIW
NtMAT1          ----KMSDVVTPPDKVRGTFIITRHDIGKLKNLVLTR---RP--KLTHVTSFTVTCAYVW
Pf5MAT          ----KPSSFPLPTNRVRATFVLSQSDIKRLKHLAN------N--NLVQPSSFVVAAAYIW
AtSDT           PYL-------PTDDWVTEKITIRADSIRRLKEATLKE----YDFSNETITTFEVIGAYLW
CbBEBT          FTPF--------DDLAHRSFFFGSTEISAMRKQIPPHL-------RSCSTTIEVLTACLW
PhBPBT          LIPL--------DDMVHRSFFFGPTEVSALRRFVPPHL-------HNCST-FEVLTAALW
AtHHT           NSL------YTKEPTLYRSFCFDPEKIKKLKLQATENS---ESLLGNSCTSFEALSAFVW
FACT            GKL------YSDEKLVYKSFLFGPEKLERLKIMAET-----------RSTTFQTLTGFLW
TpHCT2          KDTSE----ERKKKTTATLLKLSYDQVEKLKKKANDFLS-MKKKGSRPFSKFEAIGAHLW
SHT             TDNVE----ERKKKTIVVMLPLSTSQLQKLRSKANGS---KHSDPAKGFTRYETVTGHVW
HCBT            ELL------DGKTNKSQTLFKLSREQINTLKQKLDLS-----SNTTTRLSTYEVVAGHVW
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```
HQT1          SQNPES----SRK-PSTTVLKLTLDQLNVLKASAKND------GSNTTYSTYEILAAHLW
HaHQT2        SQKPES----SRK-PSTTILKLTLDQLNALKAAAKND------GGNTNYSTYEILAAHLW
HaHQT3        SQKPES----SRK-PSTTILKLTLDQLNALKAAAKND------GGNTNYSTYEILAAHLW
HaHQT1        Q---KTG--SLSK-SSTTMLKLTLDQLNTLKAKAKSE------GG-ASYSTYETLAAHIW
HQT2          AQKPGSG--SLSK-SSTTMLKLTLDQLNSLKAKAKSE------SG-STHSTYEILAAHIW
CcsHQT        SQNQNFT--SHPKLASTAMLRLTLDQINGLKSKAKGD------GS-VYHSTYEILAAHLW
NtHQT         SKSLES---TSPKPSTTTMLKFSSDQLGLLKSKSKHD------GS-----TYEILAAHIW
SlHQT         KN---------RESSTTTMLKFSSEQLGLLKSKSKNE------GS-----TYEILAAHIW
HaHCT3        LHVP-----SHETKTVCSVFKLTRDQLDTLKTKSKEA---DGNTNTISYSSFEILSGHVW
HaHCT4        LQVS-----SDETETIFSIFKLTRDQLNLLKAQSKED------ENTINYSSFEILSGHVW
AsHHT         EEPQALAGKPESPPTAVDIFKLSRSDLGRLRAQLPTG------EGAPRFSTYAVLGAHVW
HaHCT2        LE-------AQSQEKAVSIFKLTRDQLNTLKTKLRED------GSPINYSSFETLSGHVW
PrHCT         SGIMN----GEKTDISVAIFKLTKEQLEILKGKAREN------GNNIAYSSYEMLSGHIW
AtHCT         LDPSK----SGPENTTVSIFKLTRDQLVALKAKSKED------GNTVSYSSYEMLAGHVW
TpHCT1A       QQSTK----PGSDGAAVSIFKLTREQLSTLKAKSKEA------GNTIHYSSYEMLAGHVW
TpHCT1B       QQSTK----PGSDGAAVSIFKLTREQLSTLKAKSKEA------GNTIHYSSYEMLAGHVW
HaHCT1        PTTT---NDQPVPETAVSIFKLTRDQLNALKAKSKEA------GNTISYSSYEMLSGHVW
NtHCT         PENTP--ISEAVPETSVSIFKLTRDQINTLKAKSKED------GNTVNYSSYEMLAGHVW
                                :    :  ::                         . :

GmIF7Mat      SCAVKAEQPN---------CERVPFVFNVDCRARLD-PPIPETYFGNCVVALLASAKREE
Gt5AT         TCMVKSKDDVVSEESSNDENELEYFSFTADCRGLLT-PPCPPNYFGNCLASCVAKATHKE
Dv3MAT        SCIVKSLVNMG---EKKGEDELEQFIVSVGCRSRLD-PPLPENYFGNCSAPCIVTIKNGV
NtMAT1        TCIIKSEAATG---EEIDENGMEFFGCAADCRAQFN-PPLPPSYFGNALVGYVARTRQVD
Pf5MAT        SCMVKSGD-------GGEANAPELFVIPADARGRTN-PPVPANYFGNCIVGGVVKVEHEK
AtSDT         KSRVKALNLD--------RDGVTVLGLSVGIRNVVD-PPLPDGYYGNAYIDMYVPLTARE
CbBEBT        RCRTLAIKPN--------PDEEVRMICIVNARSKFN-PPLPDGYYGNAFAIPAAVTTAGK
PhBPBT        RCRTISIKPD--------PEEEVRVLCIVNARSRFN-PQLPSGYYGNAFAFPVAVTTAEK
AtHHT         RARTKSLKML--------SDQKTKLLFAVDGRAKFE-PQLPKGYFGNGIVLTNSICEAGE
FACT          RARCQALGLK--------PDQRIKLLFAADGRSRFV-PELPKGYSGNGIVFTYCVTTAGE
TpHCT2        RCASKARGLE--------DDQESVVRFHADIRRRIN-PPLPQNFFANALALTATKGCVGE
SHT           RCACKARGHS--------PEQPTALGICIDTRSRME-PPLPRGYFGNATLDVVAASTSGE
HCBT          RSVSKARGLS--------DHEEIKLIMPVDGRSRINNPSLPKGYCGNVVFLAVCTATVGD
HQT1          RCACKARGLP--------DDQLTKLYVATDGRSRLS-PQLPPGYLGNVVFTTTPVAKSGD
HaHQT2        RCACKARGLP--------DDQLTKLYVATDGRSRLS-PQLPPGYLGNVVFTATPIAKSAD
HaHQT3        RCACKARGLP--------DDQLTKLYVATDGRSRLS-PQLPPGYLGNVVFTATPIAKSAD
HaHQT1        RCACKARGLP--------DDQLTKLYVATDGRSRLS-PHLPRGYLGNVVFTATPVAKSGT
HQT2          RCACKARGLP--------DDQLSKLYVATDGRSRLS-PRLPPGYLGNVVFTATPVAKSGD
CcsHQT        RCACEARGLS--------DDQPTKLYVATDGRSRLN-PPLPPGYLGNVVFTATPIAKSGE
NtHQT         RCTCKARALS--------DDQLTKLHVATDGRSRLC-PPLPPGYLGNVVFTGTPMAKSSE
SlHQT         RCTCKARGLP--------EDQLTKLHVATDGRSRLC-PPLPPGYLGNVVFTATPIAKSCE
HaHCT3        KCVCKARGLP--------NYLDTKLNMAVDGRARFQ-PPLPPGYFGNVVFTTTAIATAGE
HaHCT4        KCVCKARGLP--------DDQGTKLDIPTDGRARFQ-PHLPPGYFGNAIFTTAVIATAGE
AsHHT         RCASLARGLA--------PEQPTKLYCATDGRQRLT-PTHPDGYFGNVIFTATPLAEAGK
HaHCT2        KCVCRARGLP--------VDQETKLYFAADGRAHLE-PALPAGYFGNVIFSNNVTSTVGE
PrHCT         RCACKARNLA--------EDQETKLYIATDGRNRLR-PSIPPGYFGNVIFTTTPMAVTGD
AtHCT         RSVGKARGLP--------NDQETKLYIATDGRSRLR-PQLPPGYFGNVIFTATPLAVAGD
TpHCT1A       RSVCKARSLP--------DDQETKLYIATDGRARLQ-PPPPPGYFGNVIFTTTPIAIAGD
TpHCT1B       RSVCKARSLP--------DDQETKLYIATDGRARLQ-PPPPPGYFGNVIFTTTPIAIAGD
HaHCT1        RCVCKARGLP--------DDQDTKLYIATDGRARLR-PSLPPGYFGNVIFTTTPIAVAGD
NtHCT         RSTCMARGLA--------HDQETKLYIATDGRSRLR-PSLPPGYFGNVIFTTTPIAVAGD
               .   :                .      *     *  *  : .*

GmIF7Mat      LLGEEAFFKSVIGISEELNG---LEGDVLNGADKWIPKIQSVV----------------SE
Gt5AT         LVGDKGLLVAVAAIGEAIEKRLHNEKGVLADAKTWLSESNGIP-----------------
Dv3MAT        LKGENGFVMAAKLIGEGISKMVNKKGGILEYADRWYDGFKI-------------------
NtMAT1        LAGKEGFTIAVELIGEAIRKRMKDEEWILSG--SWFKEYDKVD-----------------
Pf5MAT        MAGNEGFVIAAEAIAGEIKNKMNDKEEILKGAENWLSEIWKCM-----------------
AtSDT         VEEF-TISDIVKLIKEAKRNAH-DKDYLQEEL-ANTEKIIKMNLT-------IKGK----
CbBEBT        LCNN-PLGFALELIRKAKREV--TEEYMHSVA-DLMV---------------ATGRPHFT
PhBPBT        LCKN-PLGYALELVKKTKSDV--TEEYMKSVA-DLMV---------------IKGRPHFT
AtHHT         LIEK-PLSFAVGLVREAIKMV--TDGYMRSAI-DYFEVTRARPSL----------------
FACT          VTLN-PLSHSVCLVKRAVEMV--NDGFMRSAI-DYFEVTRARPSL----------------
TpHCT2        ITSK-PLGYVAQKIREGTELV--KDDFIKSQI-DVIRSFRKMDDAMKLFLGDETEKAPYF
```

```
SHT             LISN-ELGFAASLISKAIKNV--TNEYVMIGI-EYLKNQKDLKKFQDL-HALGSTEGPFY
HCBT            LSCN-PLTDTAGKVQEALKGL--DDDYLRSAI-DHTESKPDLPVP-------YMGSPEKT
HQT1            LTTQ-SLSNAASLIRTTLTKM--DNNYLRSAI-DYLEVQPDLSAL-------IRG-PSYF
HaHQT2          LTTQ-PLANAASLIRTTLSKM--DNDYLRSAI-DYLEVQPDLSAL-------IRG-PSYF
HaHQT3          LTTQ-PLANAASLIRTTLSKM--DNDYLRSAI-DYLEVQPDLSAL-------IRG-PSYF
HaHQT1          LTSD-SLVNAAKLIHTTLKKM--DDDYLRSAI-DYLETQPDISAL-------IRG-PSYF
HQT2            LTSK-SLSNTAKLIHTTLTKM--DDDYLRSAI-DYLESQPDLSAL-------IRG-PSYF
CcsHQT          FKSE-SLADTARRIHSELAKM--DDQYLRSAI-DYLELQPDLTAL-------VRG-PTYF
NtHQT           LLQE-PLTNSAKRIHSALSKM--DDNYLRSAL-DYLELLPDLSAL-------IRG-PTYF
SlHQT           LQSE-PLTNSVKRIHNELIKM--DDNYLRSAL-DYLELQPDLSTL-------IRG-PAYF
HaHCT3          IQLK-PTWQAASKIHNALVTM--NNDYLRSAV-DYLEQHLDQKP------------MVSY
HaHCT4          IQSK-PTWYAASKIHNALVTM--NSDYLKSAL-DYLEQHLDQKP------------KVSY
AsHHT           VTGS-L-ADGATTIQDALEKM--DDEYCHSAL-DYLELQPDLSAL-------VRG-AHTF
HaHCT2          ILSN-PSWFAASKIREGVVRR--TNDYLRSII-DYLELQPNMLSL-------AGATMETY
PrHCT           IISK-PTYYAASVIHEALGRM--DDEYLRSAL-DYLELQPDLTAL-------VRG-AHTF
AtHCT           LLSK-PTWYAAGQIHDFLVRM--DDNYLRSAL-DYLEMQPDLSAL-------VRG-AHTY
TpHCT1A         LMSK-PTWYAASRIHNALSRM--DNEYLRSAL-DFLELQPDLKAL-------VRG-AHTF
TpHCT1B         LMSK-PTWYAASRIHNALSRM--DNDYLRSAL-DFLELQPDLKAL-------VRG-AHTF
HaHCT1          LQSK-PTWYAAGKIHDALARM--DNDYLRSAL-DYLELQPDLKAL-------VRG-AHTF
NtHCT           IQSK-PIWYAASKLHDALARM--DNDYLRSAL-DYLELQPDLKAL-------VRG-AHTF
                .           :           .

GmIF7Mat        TPRLFSVAGSPRFEV-YGIDFGWGRPEKVDVTS----VDKTGAF--SLSESRD---HSGG
Gt5AT           SKRFLGITGSPKFDS-YGVDFGWGKPAKFDITS----VDYAELI--YVIQSRD---FEKG
Dv3MAT          PARKMGISGTPKLNF-YDIDFGWGKAMKYEVVS----IDYSASV--SLSACKE---SAQD
NtMAT1          AKRSLSVAGSPKLDL-YAADFGWGRPEKLEFVS----IDNDDGISMSLSKSKD---SDGD
Pf5MAT          GMSVLGISGSPKFDL-SNADFGWGKARKLEVVS----IDGEKY-TMSL---CN---SDCG
AtSDT           KDGLFCLTDWRNIGIFGSMDFGWDEPVNIVPVVPSE-TARTVNM--FMRPSRLESDMVGG
CbBEBT          VVNTYLVSDVTRAGF-GEVDFGWGEAVYGGPAKGGVGVIPGVTS--FYIPLRNR-QGEKG
PhBPBT          VVRTYLVSDVTRAGF-GEVDFGWGKAVYGGPAKGGVGAIPGVAS--FYIPFRNK-KGENG
AtHHT           -SSTLLITTWSRLGF-HTTDFGWGEPILSGPVA----LPEKEVT--LFLSHGE---QRRS
FACT            -TATLLITSWAKLSF-HTKDFGWGEPVVSGPVG----LPEKEVI--LFLPCGS---DTKS
TpHCT2          GNPNFQVASWTGMPF-YEADFGYGKPIYFGYAG----VSPHDRA--YITLSPD---GDGS
SHT             GNPNLGVVSWLTLPM-YGLDFGWGKEFYTGPGT----HDFDGDS--LILPDQN---EDGS
HCBT            LYPNVLVNSWGRIPY-QAMDFGWGSPTFFGISN----IFYDGQC--FLIPSQN---GDGS
HQT1            ASPNLNINTWTRLPV-HDADFGWGRPVFMGPAC----ILYEGTI--YVLPSPN---NDRS
HaHQT2          ASPNLNINTWTRLPV-HDADFGWGRPVFMGPAC----ILYEGTI--YVLPSPN---NDRS
HaHQT3          ASPNLNINTWTRLPV-HDADFGWGRPVFMGPAC----ILYEGTI--YVLPSPN---NDRS
HaHQT1          ASPNLNINAWTRLPV-HDADFGWGRPIFMGPAT----ILYEGTV--YVLPSPN---NDRS
HQT2            ASPNLNINAWTRLPV-YDADFGWGRPIFMGPAC----ILYEGTI--YVLPSPN---NDRS
CcsHQT          ASPNLNINSWTRLPI-YESDFGWGRPIFMGPAS----ILYEGTI--YIIPSPS---GDRS
NtHQT           ASPNLNINSWTRLPV-HDSDFGWGRPIHMGPAC----ILYEGTV--YILPSPN--SKDRN
SlHQT           ASPNLNINSWTRLPV-HECDFGWGRPIHMGPAC----ILYEGTI--YIIPSPN--SKDRN
HaHCT3          KHTNLRITSWARLPI-NDADFGWGRPIYTGPTW----IPLEGYV--LCYQDPI---NDGS
HaHCT4          KYTNLRIISWARLLI-HEADFGWGRPIFMGPTS----IPLGSC---YFLPSPI---NDGS
AsHHT           RCPNLGLTSWVRLPI-HDADFGWGRPVFMGPGG----IAYEGLA--FVLPSAN---RDGS
HaHCT2          KSSNVAITTWAWLPI-HDADFGWGRPVFMGPGG----VGIEGIS--TMVPSPI---NDGS
PrHCT           RCPNIGITSWSRLPI-HDADFGWGRPIFMGPGG----IAYEGLA--FVLPSSV---NDGS
AtHCT           KCPNLGITSWVRLPI-YDADFGWGRPIFMGPGG----IPYEGLS--FVLPSPT---NDGS
TpHCT1A         KCPNLGITSWARLPI-HDADFGWGRPIFMGPGG----IAYEGLS--FIIPSST---NDGS
TpHCT1B         KCPNLGITSWARLPI-HDADFGWGRPIFMGPGG----IAYEGLS--FIIPSST---NDGS
HaHCT1          KCPNLGITSWARLPI-HDADFGWGRPIFMGPGG----IAYEGLS--FVLPSPD---NDGS
NtHCT           KCPNLGITSWSRLPI-HDADFGWGRPIFMGPGG----IAYEGLS--FILPSPT---NDGS
                      :           ***:

GmIF7Mat        IQIGLALTKNQMEAFSRVFAQGLESLES-------------
Gt5AT           VEIGVSLPKIHMDAFAKIFEEGFCSLS--------------
Dv3MAT          FEIGVCFPSMQMEAFGKIFNDGLESAIAS------------
NtMAT1          LEIGLSLSKTRMNAFAAMFTHGISFL---------------
Pf5MAT          LEVGLSLPGERMEAFAAIFADGLAKLDSS------------
AtSDT           VQIVVTLPRIAMVKFKEEMEALE------------------
CbBEBT          IVLPICLPSAAMEIFAEALNNTLNGKEIEIAKHFTQSS--L
PhBPBT          IVVPICLPGFAMEKFVKELDSMLKGDAQLDNKKYAFITPAL
AtHHT           INVLLGLPATAMDVFQEQFLQI--------------------
```

```
FACT          INVLLGLPGSAMKVFQGIMDI--------------------
TpHCT2        VIVSLHFQMAHLELFKKYFYEDI------------------
SHT           VILATCLQVAHMEAFKKHFYEDI------------------
HCBT          MTLAINLFSSHLSLFKKYFYDF-------------------
HQT1          MSLAVCLDADEQPLFEKFLYDF-------------------
HaHQT2        MSLAVCLDADEQPLFEKFLYDF-------------------
HaHQT3        MSLAVCLDADEQPLFEKFLYDF-------------------
HaHQT1        VSLAVCLDAKEQPLFEKYLYEL-------------------
HQT2          VSLAVCLDANEQPLFEKFLYEF-------------------
CcsHQT        VSLAVCLDPDHMSLFRKCLYDF-------------------
NtHQT         LRLAVCLDADHMPLFEKYLYEF-------------------
SlHQT         LRLAVCLDAGHMSLFEKYLYEL-------------------
HaHCT3        LSIIIGLEAEQMKLFSSFLYEI-------------------
HaHCT4        LSIIIGLEAEQMKLFSKLVYAI-------------------
AsHHT         LSVAISLQAEHMEKFRKMIFDF-------------------
HaHCT2        LSIVISLQAEQLKLFKKFLYDI-------------------
PrHCT         LSVALGLQPDHMVRFAKMLYEI-------------------
AtHCT         LSVAIALQSEHMKLFEKFLFEI-------------------
TpHCT1A       LSVAIALQHEHMKVFKEFLYDI-------------------
TpHCT1B       LSVAIALQHEHMKVFKEFLYDI-------------------
HaHCT1        LSIAISLQAEHMKLFSNLLYDI-------------------
NtHCT         QSVAISLQAEHMKLFEKFLYDF-------------------
                :   :       *    .
```

2. Sequence alignment between HaHQT2 and HaHQT3

```
# Aligned_sequences: 2
# 1: HaHQT2
# 2: HaHQT3
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1355
# Identity:    1222/1355 (90.2%)
# Similarity:  1222/1355 (90.2%)
# Gaps:          37/1355 (2.7%)
# Score: 5631.5
#
#
#=======================================

HaHQT2             1 atggggactgttcaaaagacattgaacatcaacatca-aacaaacaacgt     49
                     |||...||||.|||.||.|   |.||.||.|||||| ||.|||.|||
HaHQT3             1 atgaaaactgatcacaaca---tcaagatgaacatcacaataaaaaac--     45

HaHQT2            50 ttgtacaa--------ccttcgaagcccacacaagcttctacaaagcagt     91
                      |..||||        ||.||..||||||.|..||...||.||||||||.
HaHQT3            46 -tcaacaatcattcctccatcagagcccatagcagacgctccaaagcagc     94

HaHQT2            92 tatggacttccaacttggatttggtcgttggcagaatccatattttaacc    141
                     |||||||.||.|||||||||.|.||.|||||.|||||.||||||||||||
HaHQT3            95 tatggacctctaacttggatcttgttgttggaagaattcatattttaacc    144

HaHQT2           142 gtttatttctaccgtccgatcgtttgctccaacttctttgatcctc--tt    189
                     |||||.||||||.|.||||.||..|..||||||||.||.||  |||  .|
HaHQT3           145 gtttacttctacaggccgaacgggtcgtccaacttttttcga--ctcgaat    192

HaHQT2           190 gtcatgaagaaggcgcttgcagatgttcttgttacctttttatccaatggc    239
                     ||.|||||.||||||||||.|||||.||.||..|.||||||||.|||||
HaHQT3           193 gttatgaaaaaggcgcttgcggatgtgctcgtgccgttttatccgatggc    242


HaHQT2           240 tggtcgtatgggtaaagatcagaatggcaaagttgttattaattgtaatg    289
                     |||.||..||||.|.||||.|||.|||.|...||||||||||||||||||
HaHQT3           243 tgggcggttgggaagagatgagagtggtaggattgttattaattgtaatg    292

HaHQT2           290 atgagggtgttttgtttgtcgaagccgagtccgattctactttggatgat    339
                     .|||.||.||||||||.||.||.||||||..|.|||.|...||||||||||
HaHQT3           293 gtgaaggagttttgttcgttgaggccgaaacggatgcgtgtttggatgat    342

HaHQT2           340 tttggtgagtttacgccgtcgccggagctccggcgacttacgcccactgt    389
                     ||.||.||||||||.|||||||||||||.|.|.|.|...||.|||||||..||
HaHQT3           343 ttcggagagtttactccgtcgccggagtttcagatgctgacgcccagcgt    392

HaHQT2           390 ggattattccggtgacatttcttcttatccgctgtttttttgctcaggtat    439
                     .|||||.||||||.||||||||||||.||.|||.|||||||.||.||||  |
HaHQT3           393 tgattactccggcgacatttcttcgtacccgttgtttttcgcacagg--t    440

HaHQT2           440 attggtttgaccaggtaacccatttcaagtgtggaggagttggtcttggt    489
                     |.||                ||||||||||||||||||||||||||||||
HaHQT3           441 actg----------------catttcaagtgtggaggagttggtcttggt    474

HaHQT2           490 tgtggtgtctttcatactttggctgatgggctatcctctctgcatttcat    539
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3           475 tgtggtgtctttcatactttggctgatgggctatcctctctgcatttcat    524

HaHQT2           540 caacacatggtccgacatggctcgcggcctctccattgccatcccaccat    589
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3           525 caacacatggtccgacatggctcgcggcctctccattgccatcccaccat    574

HaHQT2           590 ttatcgaccgcaccttgcttcgtgcacgtgaacccccactcccacttttt    639
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
HaHQT3          575 ttatcgaccgcaccttgcttcgtgcacgtgaaccccccactcccacttttt    624

HaHQT2          640 gaccatgtcgagtaccacccccctccgtcgatgaaacctgactcacagaa    689
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          625 gaccatgtcgagtaccacccccctccgtcgatgaaacctgactcacagaa    674

HaHQT2          690 gcctgaatccagccgaaagccctccaccactattcttaagcttacacttg    739
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          675 gcctgaatccagccgaaagccctccaccactattcttaagcttacacttg    724

HaHQT2          740 accaactcaatgctctcaaagccgcagccaagaatgatggtggaaacacc    789
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          725 accaactcaatgctctcaaagccgcagccaagaatgatggtggaaacacc    774

HaHQT2          790 aactatagcacgtacgagatcctagcggctcacttatggcgttgcgcgtg    839
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          775 aactatagcacgtacgagatcctagcggctcacttatggcgttgcgcgtg    824

HaHQT2          840 caaggctcgtggactccccgatgaccaactaaccaaactgtacgtggcta    889
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          825 caaggctcgtggactccccgatgaccaactaaccaaactgtacgtggcta    874

HaHQT2          890 cagacggacgatccagattgagcccccaactcccaccgggctacctaggg    939
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          875 cagacggacgatccagattgagcccccaactcccaccgggctacctaggg    924

HaHQT2          940 aatgttgtgttcactgccaccccgattgctaaatcggctgacctcacgac    989
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          925 aatgttgtgttcactgccaccccgattgctaaatcggctgacctcacgac    974

HaHQT2          990 tcaaccgttggccaatgcagcatcgttgatccggacaactttgtcaaaaa   1039
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3          975 tcaaccgttggccaatgcagcatcgttgatccggacaactttgtcaaaaa   1024

HaHQT2         1040 tggataacgactatttgcgatccgccatcgactaccttgaggtgcagccc   1089
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3         1025 tggataacgactatttgcgatccgccatcgactaccttgaggtgcagccc   1074

HaHQT2         1090 gatctatcagccttaattcgtggtccaagttactttgcgagcccgaacct   1139
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3         1075 gatctatcagccttaattcgtggtccaagttactttgcgagcccgaacct   1124

HaHQT2         1140 aaacattaacacgtggactcggttgccagtgcatgatgcggatttcgggt   1189
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3         1125 aaacattaacacgtggactcggttgccagtgcatgatgcggatttcgggt   1174

HaHQT2         1190 ggggtagacccgtattcatgggacccgcgtgtatattatacgaggggacc   1239
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3         1175 ggggtagacccgtattcatgggacccgcgtgtatattatacgaggggacc   1224

HaHQT2         1240 atttatgttctaccaagcccgaacaatgataggagtatgtcactagcagt   1289
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3         1225 atttatgttctaccaagcccgaacaatgataggagtatgtcactagcagt   1274

HaHQT2         1290 gtgtttggatgcagatgaacagccattgtttgagaagttcttatacgact   1339
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
HaHQT3         1275 gtgtttggatgcagatgaacagccattgtttgagaagttcttatacgact   1324

HaHQT2         1340 tctaa   1344
                    |||||
HaHQT3         1325 tctaa   1329
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VITA

Miss Patwira Boonjing was born on July 9th, 1991. She is the oldest child and grew up in Bangkok with her family. She received her Bachelor's degree from Department of Plant Science, Faculty of Science, Mahidol University, with Second-Class Honors in 2013. After that, she continues studying in Program in Biotechnology, Faculty of Science, Chulalongkorn university, for her Master's degree. She participated in many scientific conferences during those years. She has been recognized for Good Poster Presentation Award in The 5th International Biochemistry and Molecular Biology Conference in 2016. She plans to pursue her Ph.D. study in the field of Biological Science.