CHAPTER II

LITERATURE REVIEWS

2.1. Traditional record

P. mirifica, a legume plant is a well-known Thai traditional plant in the name of "Kwao Krua Khao or White Kwao-Krua". The traditional consumption of the plant tuberous part is believe to be rejuvenation for menopausal women according to the description of a Thai translation of the Burmese-derived pamphlet (Suntara 1931). He did test the compound medicine from this plant for daily taken. The pamphlet described the improvement of sleep disorder, relieve of menopausal vasomotor symptoms (hot flashes, night sweats and hyperhidrosis) that associated with menopause. It is convincing that the tuber of *P. mirifica* in an appropriated dose has properties related with a rejuvenating agent for menopausal women. The plant was recognized as a reputed rejuvenator (Kerr 1932). The new scientific name was set to be *Pueraria mirifica* (mirifica in Latin mean "amazing" or "wonderful") with a synonym of *Pueraria candoli* var. *mirifica*, whereas, *Pueraria candollei var. candollei* have bigger corolla, calyx and has longer inflorescence than *Pueraria candollei var. mirifica* (Niyomdham 1992).

2.2. The taxonomy

The taxonomy of *P. mirifica* is present in Table 1; (Ridley 1922, Suvatti 1978). *P. mirifica* is distributed in deciduous or dry forest areas and in mountainous forests with sandy soils (80-800 meters level) (Kashemsanta et al 1952, Panriansaen 2000) which is almost found in at least 28 provinces in Thailand (Subtaeng & Cherdshewasart 2003). The plant is not found in the forest with high-density trees (Figure 1). The vine of *P. mirifica* elongated for climbing over the trees while spread on the ground in an open area (Panriansaen 2000, Panriansaen & Chershewasart 2003).

Table 1 The taxonomy of *P. mirifica*

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae or Leguminosae
Subfamily	Faboideae or Papilionoideae
Tribe	Phaseoleae
Subtribe	Glycininae
Genus	*Pueraria*
Species	*P. mirifica*

Figure 1 The plant cultivar of *Pueraria mirifica* with abundance leaves production

Figure 2 Leaves morphometry of *P. mirifica*

The trifoliolate leaves (Figure 2) are typical for the tribe Phaseoleae, as are the general build of the papilionoid flower (Figure 3) and the flat to cylindrical fruit, the pod. The length of the inflorescence of the flowers is approximately 15-40 cm. The flower contains five sepals and the petals are one standard with two keels. Stipules may or may not have basal lobes, which are single, twin-lobed or incised. The peduncles that carry the flowers bear short-shoots or thickened nodes called brachyblasts, which carry 3 (rarely 2) or 4–7 flowers per node. Flowers are mostly without a pedicel, have a bract that drops early, and the calyx densely pubescent has two lateral bracteoles. Flowers were appeared in February-March and fruiting is appeared in April. The calyx has five lobes, but the two uppermost are almost or entirely connate. The length of the calyx lobes varies among each other, the lowest usually the longest. The standard petal is clawed, and has two auricles near the base of the blade. A pair of bulges and callosities is often discernible at the ventral (abaxial) side of the standard. Petal color varies from pure white to blue and purple. The stamens are combined into a tube of nine, with the tenth (vexillar) stamen connate to the tube, at least in the middle; in several species the vexillar stamen becomes free when the flowering proceeds. The ovary is elongate, often hairy, sometimes glabrous, with athread-shaped style, ending in a globular stigma with short papillae, often penicillate below the knob. There are 5–20 ovules. The flowers are fragrant, attracting insects.

Figure 3 Flowers of *P. mirifica* in late winter

The pod is slender typically short or elongate, either glabrous or hairy, pale brown to black, and carry up to 10 single seeds when fully matured and dried which turn into various color (Cherdshewasart et al 2009, Smitasiri & Wungjai 1986). The brown or black seeds are bean-like, flattened-oblong or barrel-shaped, and the surface is minutely shagreened. The hilum is surrounded by a small strophiole (rim-aril). Germination is epigeal, bringing the cotyledons above ground. The first two leaves are simple and opposite, the following leaves are trifoliolate and alternate (Keung 2002). The root forms tuber in underground (Figure 4) with long tuberous or globular like Jimaca (*Pachyrhizus erosus*) root which has brown color. The size of tuberous root varies by age, cultivar and environmental factors.

Morphometric analysis revealed a low level of variation between cultivars in Thailand. Leaves were collected for 39 locations across Thailand with seed pods and flowers (Figure 5) (Suwanvijitr et al 2010).

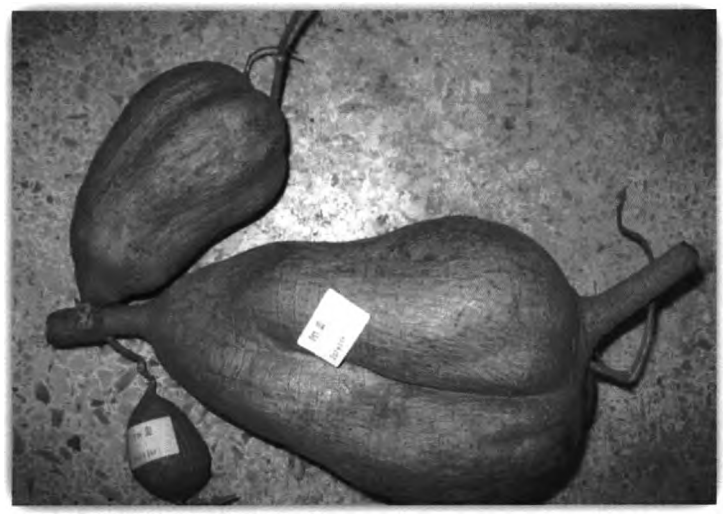


Figure 4 Tuberous roots of *P. mirifica*

2.3. The key chemical constituents

P. mirifica is found to contain at least 20 chemical compounds (Figure 5), some of these is phytoestrogen with similar biological effects to estrogen. Since 1986, the chemical constituents named puerarin 6"-O-β-apiofuranoside which is a C-glycosylisoflavone O-glycoside (Ingham et al 1986) has been discovered. The chemical structure was not classified as a steroid (Benson et al 1961). The other compounds, mainly found in *P. mirifica* were isoflavonoids, chromenes, coumarins, sterols, pterolcapans and acid.Isoflavonoids belong to the class of phytoestrogen (chemical compound produced by plant) which acts like estrogen in human and animal organisms. Phytoestrogen is known as hormone replacement for post-menopausal women by binding with estrogen receptor in the cell. The isoflavonoids also show biphasic effect (Cherdshewasart et al 2004). When the organism is in the lack of estrogen condition, the isoflavonoid will bind to estrogen receptors in the cell resulting in hormone-replacing activities. In contrast, when estrogen is present; isoflavonoid will compete with estrogen to bind estrogen receptors in cells. Isoflavonoids (like the other phenylpropanoids e.g. flavones, chalcones, lignans, stilbenes etc.) are secondary products of a particular pathway of phenylpropanoid metabolism in higher plants. The structure of isoflavonoids comprises of phenyl group and phenolic group (Figure 6 and Figure 7). They occur in plants either frees (aglycones) or glycosylated, depending on species and a physiological status.

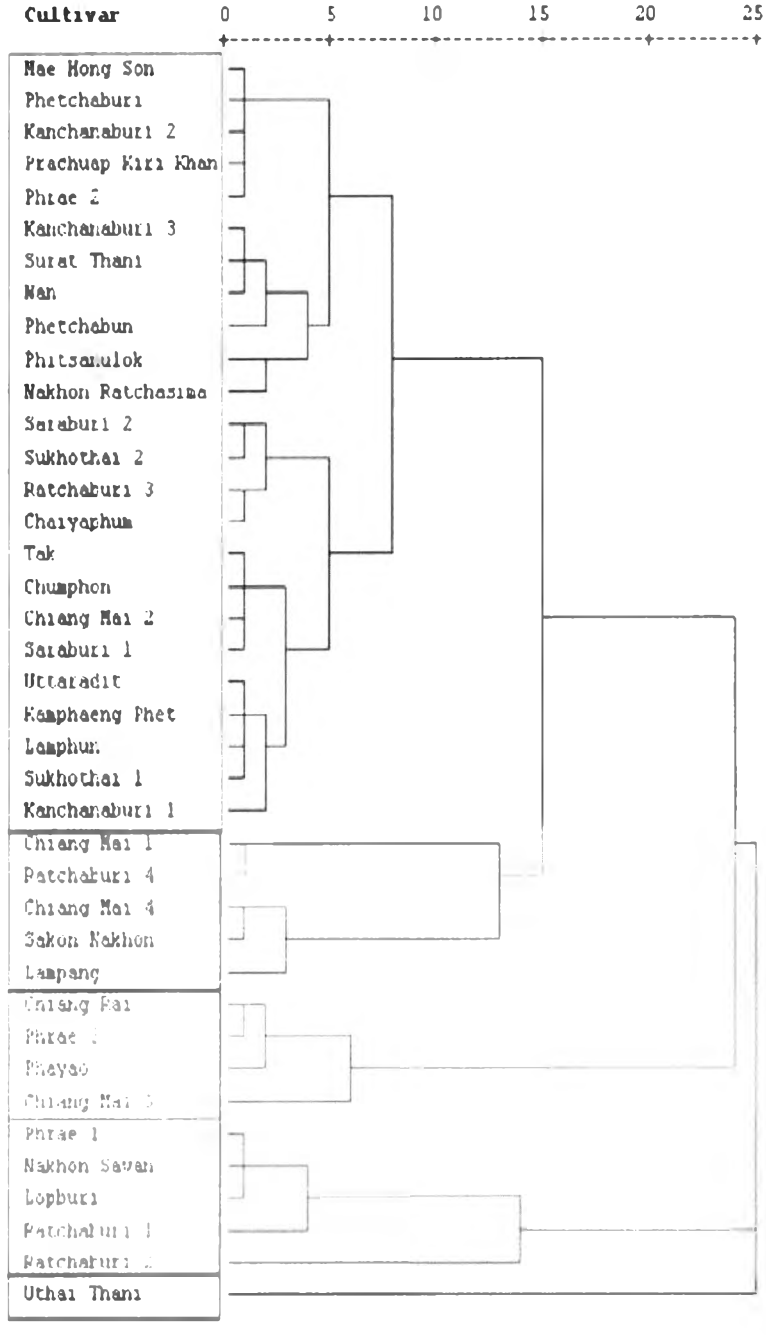


Figure 5 Leaf morphometric dendrogram created by the Between-groups linkage method of cluster analysis. *P. mirifica* is classified by the province of collection and sequential number modified from (Suwanvijitr et al 2010)

Aglycones are considered to be the biologically active substances; glycosides represent a deposit form of isoflavonoids. There was a study of the absorption of isoflavonoids; daidzein, genistein, daidzin and genistin in the rat stomach by feeding the same concentration. The result found only daidzein and genistein in blood plasma but no daidzin and genistin. It implied that in stomach, the absorption process may involve in transportation. Normally, in animal stomach have bacteria that can hydrolyze glucose and transform to aglycoside form (Piskula et al 1999). Chromene is benzene ring conjugated with pyran ring (Benzopyran) (Figure 6) which plant produces for photochemical properties (Thomas & Zachariah 2013). The chromene derivatives exhibit high potential biological activity such as antitumor, antivascular (Gourdeau et al 2004), antimicrobial (Sangani et al 2012), antioxidant (Mladenovic et al 2011) etc. Miroestrol, derived chromene compounds, has been firstly reported in 1958 (Pope et al 1958) in *P. mirifica*. The chromene derivatives were exhibited the estrogenic effect (Benson et al 1961, Chansakaow et al 2000a, Jones & Pope 1960, Pope et al 1958). Coumestans are oxidation products of pterocarpanes that are similar to coumarin and are rather broadly distributed in plants (Tuskaev 2013).

2.3.1. Daidzein

Daidzein (4',7-Dihydroxyisoflavone) is an isoflavonoid glycoside mainly found in the composition of secondary metabolite in Legume plant. It was first extracted from *P. mirifica* root (Ingham et al 1986). Kaufman and colleges from Michigan University, U.S.A. was interesting in plant isoflavonoid in legume plant and first discovered "Daidzein" in *P. lobata* (Kaufman et al 1997). Daidzein not only have estrogenic and antioxidant properties in mammal (Liu et al 2006b) but also decreased the risk of several chronic pathologies for example cancer (Messina 1999) coronary heart disease (Segasothy & Phillips 1999) and osteoporosis (Setchell & Cassidy 1999). Daidzein played important role in Malignant gliomas cell death program by inducing cell apoptosis (tumor necrosis factor-related apoptosis-inducing ligand, TRAIL) but did not effected normal cell (Siegelin et al 2009). Genistein also reduced the inflammation and used as an agent for antifibrotic effect in Wistar rats (Soumyakrishnan et al 2014). Daidzein inhibits the proliferation of cancer-derived cells (Lee et al 2011) anti-breast cancer (Liu et al 2012) by induce cell apoptosis associated with cell cycle arrest at the G0/G1 or G2 phase, depending on the cancer cell type. In some case, daidzein induced apoptosis directly without altering the cell

cycle distribution (Su et al 2000). Recently, daidzein was found to be a candidate for increase skin collagen synthesis and inhibit collagen degradation (Zhao et al 2014) Many researchers synthesized this substance for pharmaceutical and food industry because of its various benefit properties (Baraldi et al 1999, Meng et al 2006, Shimoda et al 2011, Soidinsalo & Wahala 2007, Wahala et al 1995, Wahala et al 1998, Whalley et al 1998). Daidzein is intake to the body by intestinal absorption (Foti et al 2006, Kishida et al 2008).

2.3.2. Genistein

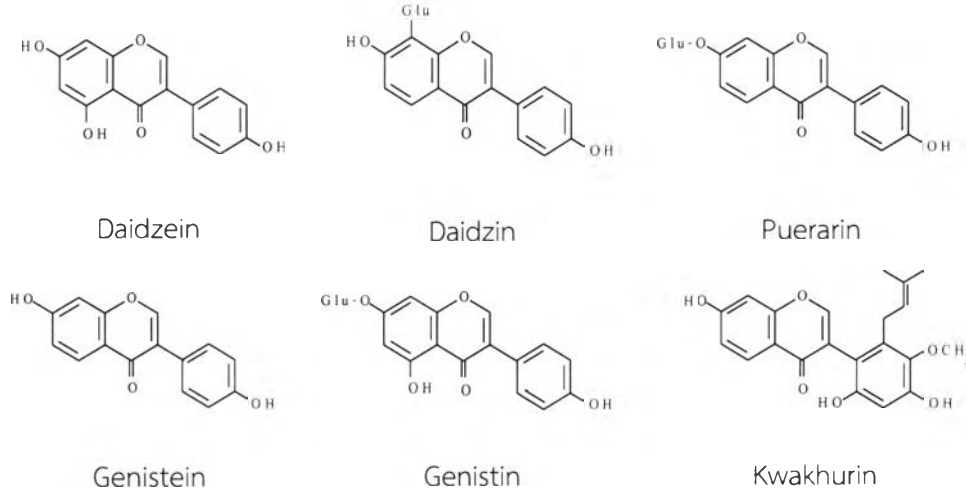
Genistein (4',5,7-trihydroxyisoflavfone) is aglycoside form of genistin, mostly found in Legume plant. There was an applied analytical method for extraction of daidzein and genisein in food by hydrolysed with *Aspergillus niger* (Liggins et al 1998). Genistein harbours various biological effects such as in plant, genistein act as an antimicrobial agent (Zhang et al 2008). In clinical trial, genistein played role in animal cells by inhibit tyrosine kinase enzyme which caused the growth of cancer (Dixon & Ferreira 2002). Genistein inhibit the growth of colon (MD-26) (Yu et al 2005), breast (MCF-7) (van Meeuwen et al., 2007) and prostate cancer cell (Nadal-Serrano et al 2013, Privat et al 2009, van Duursen et al 2011, Yang et al 2007b). Genistein prevents cardiovascular disease by reduced LDL cholesterol and inhibited pro-inflammatory cytokinase (Rimbach et al 2008). In term of diabetic therapy, genistein extracted from soybean can enhance insulin secretion in rats (Liu et al 2006a). Genistein extracted from Pueraria radix prevents bone loss and increased the bone mass (Fu & Liu 2009, Wang et al 2005). Kyuhou studied the effective of genistein on nervous cell and found that the effect of genistein is mediated through estrogen receptors. These findings suggested that genistein was benefit for the prevention of Parkinson's disease in post-menopausal women (Kyuhou 2008). Recently, genistein is becoming an alternative option for obesity and diabetes treatments (Behloul & Wu 2013).

Table 2 The summary of the chemical constituents in *P. mirifica*

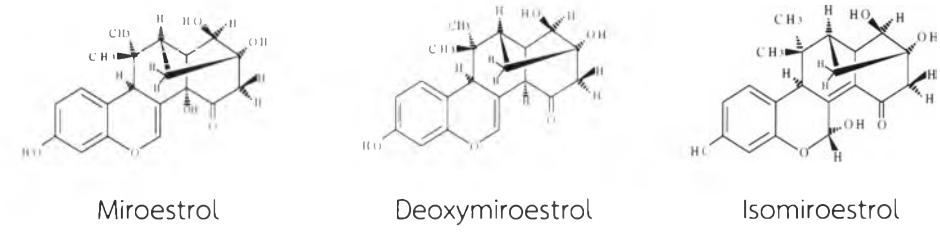
Categories	Chemical constituents	References
Isoflavonoids (aglycosidic form)	Daidzein	(Ingham et al 1986, Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2007c)
	Genistein	(Ingham et al 1986, Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2007c)
	Kwakhurin	(Ingham et al 1986)
	Kwakhurin hydrate	(Ingham et al 1986)
Isoflavonoid (glycosidic form)	Daidzin (daidzein-7-o-glucoside)	(Ingham et al 1986, (Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2007c)
	Genistin (genistein-7-o-glucoside)	(Ingham et al 1986, (Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2007c)
	Mirificin (puerarin6'-o-β-apiofuranoside)	(Ingham et al 1986)
	Puerarin (daidzein-8-glucoside)	(Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2007c, Cherdshewasart & Sutjit 2008, Subtaeng & Cherdshewasart 2003)
	Puerarin 6''- monoacetate	(Ingham et al 1989)
Chromenes	Miroestrol	(Pope et al 1958) (Jones & Pope 1960)
	Deoxymiroestrol	(Chansakaow et al 2000a)
	Isomiroestrol	(Chansakaow et al 2000a)
Coumestans	Coumestrol	(Ingham et al 1986, Ingham et al 1988)
	Mirificoumestan	(Ingham et al 1988)
	Miricoumestan glycol	(Ingham et al 1988)
	Miricoumestan hydrate	(Ingham et al 1988)
Sterols	β-sitosterol	(Hoyodom 1971)
	Stigmasterol	(Hoyodom 1971)
Pterolcapans	Pueriicapene	(Chansakaow et al 2000b)
	Tuberosin	(Chansakaow et al 2000b)
Acid	Tetracosanoic acid	(Chansakaow et al 2000b)

Verified from Cherdshewasart, 2009

Isoflavononoids



Chromenes



Coumestans

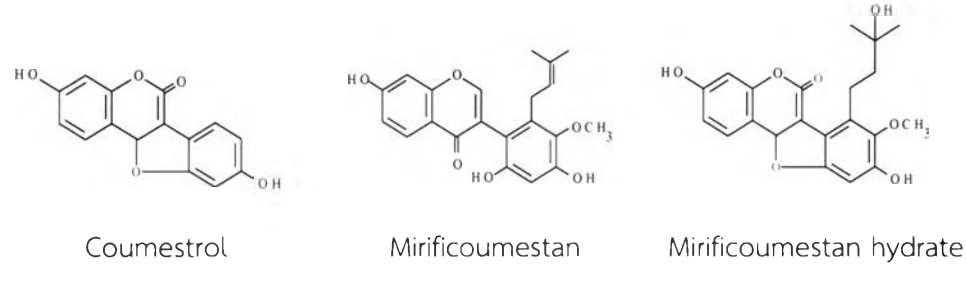
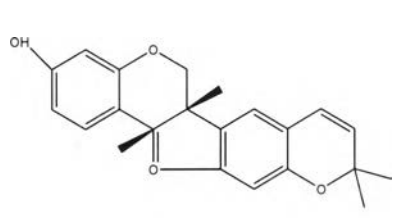
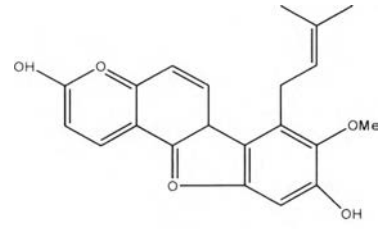


Figure 6 The structure of chemical compounds (Isoflavonoids, Chromenes and Coumestans) in *P. mirifica*

Pterocarpans

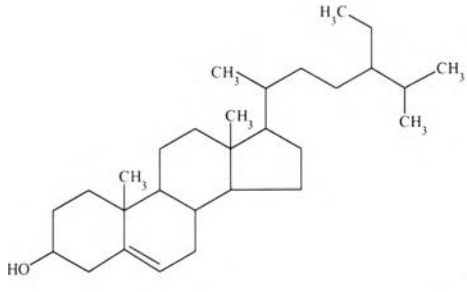


Tuberosin

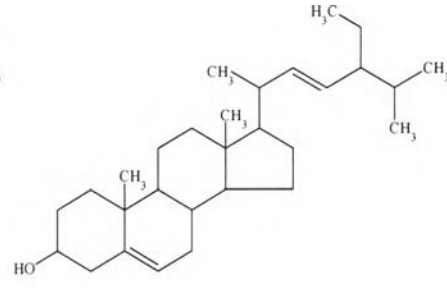


Puemirificarpene

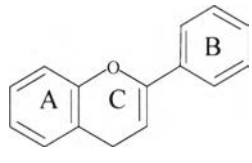
Sterols



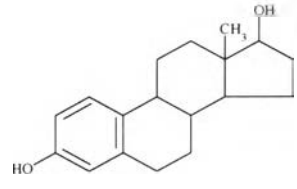
β-sitosterol



Stigmasterol



Isoflavonoid nucleus



17β-estradiol

Figure 7 The structure of chemical compounds (Pterocarpans Sterols and isoflavonoid nucleus) in *P. mirifica* and isoflavonoid nucleus compare with estrogen

2.3.3. Kwakhurin

Kwakhurin is a unique isoflavonoids found in *P. mirifica* that have a rejuvenating effect (Dweck 2009, Misako et al 2004). The synthesis of Kwakhurin had late done in the year later (Ito et al 2005). Although there are few reports in terms of

individual biological activity, Kwakhurin still interesting in terms of estrogenic activity comparing with the potential compounds such as miroestrol and deoxymiroestrol (Shimokawa et al 2013).

2.3.4. Kwakhurin hydrate

There has been report Kwakhurin hydrate is the isoflavonoid compound specifically found in *P. mirifica* as same as Kwakhurin (Dweck 2009). Kwakhurin hydrate is a hydroxyl conjugated Kwakhurin. There has no reported in term of biological activity.

2.3.5. Daidzin

Daidzin is the 7-O-glucoside of daidzein found in Kudzu root (*P. lobata*) (Van Hung & Morita 2007), Kudzu vine (Kirakosyan et al 2003) and other legume plants (Hati et al 2014). There has been reported of the potential for treatment of alcohol dependency that could be an antidipsotropic agent (Keung & Vallee 1998). So, the synthesis of daidzin for "alcohol addiction remedy" was studied (Gao et al 2003) Although, daidzin has less bioactive compound when compared with daidzein, human body can intake daidzin and convert to daidzein in human small intestine (Wu et al 2013)

2.3.6. Genistin

Genistin is a glycoside form of genistein which can hydrolyze to genistein and glucose. Genistin was first isolated from soy dietary food in 1931 (Walz 1931). The study of genistin in intestinal metabolism showed 14% of genistin can be absorbed. Genistein, not genistin, is found in intestine. The study can conclude that most of genistin has changed to genistein before absorbed to small intestine (Andlauer et al 2000). The growth inhibitory activity of genistin and daidzin on pBR322 DNA cleavage showed both daidzin and genistin can protect DNA damage and exhibited a superoxide dismutaselike effect, but only genistin reduce significantly the vitality of melanoma cell line (M14) cells (Russo et al 2006). Both of genistin and genistein suppressed LPS (lipopolysaccharide)-induced liver pro-inflammatory cytokine necrosis factor-alpha (TNF-alpha), interleukin (IL)-1beta, and IL-6 production in vivo and *in vitro* (Zhao et al 2006).

2.3.7. Mirificin

Mirificin or puerarin-6"-*O*-apiofuranoside is a natural compound that give yield puerarin and sugar apoise when heating with 2*N*-HCl (Ingham et al 1986) There has few report in term of biological activity. Puerarin and mirificin have estrogenic activity but puerarin has more estrogenic effect than mirificin (Nilanidhi et al 1957).

2.3.8. Puerarin (daidzein-8-glucoside)

Puerarin is an isoflavonoid class of phytoestrogen with (Ingham et al 1986) which mainly found in *P. mirifica* and *P. lobata* (Boue et al 2003, Chen et al 2001, Cherdshewasart et al 2007b, Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2007c, Guerra et al 2000, Kaufman et al 1997, Kirakosyan et al 2003, Liu et al 1998, Yu et al 1992). Puerarin treatment significantly increased the activation of casepase-3, a key executioner of apoptosis (Yu & Li 2006). puerarin and daidzein extracted from the tuber exhibited antioxidant activity as strong as α-tocopherol in DPPH assay (Cherdshewasart et al 2004). Puerarin showed cardioprotective effect (Gao et al 2006). This effect was attenuated by treatment with paxilline, an antagonist of the calcium-activated potassium channel. Furthermore, Puerarin also decreased the death of myocyte and restrained reactive oxygen species release in isolated cardiomyocytes subjected to simulated ischemia and reperfusion injury or oxidative stress induced by H_2O_2. puerarin restrained paxilline (calcium-activated potassium channel blocker) and chelerythrine (protein kinase C inhibitor), thus puerarin plays an important role in protective against cardiovascular disease by opening the calcium-activated potassium channel and activating protein kinase C, as well as directly *via* its antioxidant activity (Gao et al 2007). The behaviour and brain neuronal activity was activated by the cAMP/PKA signaling pathway *via* membrane-associative estrogen receptors. Furthermore, the membrane-permeant inhibitor of protein kinase A (PKA) H89 also completely inhibited this potentiation. These results suggested that puerarin should be the candidate compound for the preventive of dementia for example neurological degenerative disease (Alzheimer's disease) and vascular disorder disease (Lin et al 2007). In diabetic therapy, puerarin protect against high glucose-induced acute dysfunction of vasculature through its antioxidative properties (Meng et al 2009), glucose-induced injury through upregulation of HO-1 expression in blood vessels (Meng et al 2008) and reduced high-glucose vascular

smooth muscle cell proliferation by blocking PKCβ2/Rac1-dependent signaling and improved vascular function (Zhu et al 2010).

2.3.9. Puerarin 6"-monoacetate

Puerarin 6"-monoacetate is biotransformed of puerarin which catalyzed by bacteria (for example, *Bacillus cereus*) in soil (Yu et al 2012b).

2.3.10. Miroestrol

There were human and animal studies with miroestrol in England in the early 1960's. Miroestrol was the first isolated chemical compound extracted from *P. mirifica* found in the amount of approximately 1.5 mg/ 100 g dry tuber (Jones & Pope 1961) with estrogenic activity in rat vaginal cornification test (Jones et al 1961). The estrogenic activity was tested in mouse and overiectomized rat by subcutaneous injection of miroestrol in 70% propyleneglycol (0.01, 0.05 and 0.1 μg/day for 20 days). The result showed the mammary duct growth in the rat and is 2.2 times that was qualitatively similar to mice oestradiol and oestrogen (Benson et al 1961). Miroestrol exhibited 0.25 times that of 17β-estradiol (vaginal cornification test) approximately 3 times that of stilbesterol in the immature mouse uterine growth test and 2/3 that of stilbesterol (Jones et al 1961). The potential of miroestrol and deoxymiroestrol to modify the hepatic enzymes involved in bile salt transportation were examined in comparison with estradiol. The results showed that both miroestrol and deoxymiroestrol significantly risk of hepatotoxicity and intrahepatic cholestasis (Udomsuk et al 2012). Many studies both in mammal and MCF-7 human breast cancer cell test (Matsumura et al 2005) have confirmed the potential estrogenic effect of miroestrol extracted from *P. mirifica*, the miroestrol has been interested in industry level for chemical synthesis (Corey & Wu 1994).

2.3.11. Deoxymiroestrol and isomiroestrol

Deoxymiroestrol and isomiroestrol have been found to be even more potent than miroestrol in term of estrogenic potency. Deoxymiroestrol has an anti-breast cancer (MCF-7) approximately 10 times greater than miroestrol. However, it was easily oxidized by the air and converted to miroestrol and isomiroestrol (Chansakaow et al 2000b). The difference between miroestrol and deoxymiroestrol is an Alkyl

group (-OH group in deoxy- and –H group in miroestrol). Deoymiroestrol is easily oxidized with oxygen in an environment while *P. mirifica* was preparing for an experiment (Chansakaow et al 2000a). So, it can conclude that the most estrogenic effect of *P. mirifica* plant comes from miroestrol.

2.3.12. Coumestrol and coumestan derivatives

Coumestrol (3,9-dihydroxycoumestan) and coumestan derivatives (mirificoumestan, mirificoumestan glycol and Mirificoumestan hydrate) were found in many plants such as legumes (*P. mirifica*, *P. lobata*, soybeans), Brussels, sprouts, spinach etc. In molecular biology study, coumestrol activated farnesoid X receptor (FXR) and regulated the expression of genes involved in lipid and glucose metabolism. This result could benefit on metabolic disorders, including hypertriglyceridemia and diabetes (Takahashi et al 2008). Coumestrol has strongly inhibited benzidine (human bladder carcinogen) mediated lipid peroxidation in a time dependent manner on cytochrome P-450 and peroxidase human liver microsomes (Makena & Chung 2007). The neurobehavioral actions of coumestrol in ovariectomized (OVX) female rats increased ERβ mRNA expression in the paraventricular nucleus (PVN) that negative of estrogen effect. After 10-day-treatment with coumestrol, LH secretion was increased in OVX wild-type mice. These result shown the same direction as the daily feeding of *P. mirifica* powder in OVX rats (Whitten et al 2002).

2.3.13. β-sitosterol and stigmasterol

The two compounds belong to phytosterols which found in higher plants especially edible plant such as soybean (Barnes et al 2006, Kritchevsky & Chen 2005). β-sitosterol and Stigmasterol played anti-snake venom activity (Gomes et al 2007). Moreover, the two compounds also strong inhibited on the dRP lyase activity of DNA polymerase (Li et al 2004) and intestinal absorption of chloresterol (Batta et al 2006). β-sitosterol decreased secretion of apolipoprotein B48 from Caco2 human intestinal cells (Ho & Pal 2005). Stigmasterol exhibited anti-diabetic and antiperoxidative properties (Panda et al 2009). β-sitosterol and Stigmasterol were separated (Gomes et al 2007, Xu et al 2005) and synthesized (De-Eknamkul & Potduang 2003, Hang & Dussault 2010, Kongduang et al 2008) for pharmacological term.

2.3.14. Tuberosin and puerimicapene

There has been reported in legume plant (Chansakaow et al 2000b). In *P. lobata* also found when enhanced with $CuCl_2$ treatment (Hakamatsuka et al 1991). Tuberosin showed antioxidant activity by significantly scavenged all the species of free radicals (Pandey & Tripathi 2010) and anti-inflammatory by improved red blood cell actioxidant activity (Pandey et al 2013).

2.3.15. Tetracosanoic acid

Tertracosanoic acid is an fatty acid normally found to be a constituent in plants (Guo et al 2012, Tang et al 2013) and plays important role in many functions (Elias 1983). This compound also exhibited antibacterial activity (Teponno et al 2006).

2.4. The plant dried tuberous powder estrogenic activity tests

P. mirifica tuberous powder is extracted and admixed in industrial cosmetics such as a firming breast lotion or cream, eye gel, and skin moisturizer, the benefits of skin application are mostly used for anti-wrinkle and breast firming. Additionally, this plant powder was manufactured as a food supplement for anti-aging (Dweck 2009). There were also the tests of the crude extract of the tuberous powder in animals which confirmed the plant estrogenic properties (Chansakaow et al 2000b). Uterotrophic assay (Cherdshewasart et al 2007a) and YES assay (Boonchird et al 2010).

In term of in vivo assay, *P. mirifica* played preventive effects on bone loss in ovariectomized rats (Urasopon et al 2007, Urasopon et al 2008). *P. mirifica* crude extract played the estrogenic activity dose-dependent on both uterotrophic and vaginal cornification assays (Cherdshewasart et al 2008). Puerariae powder has an additive effect along with microgrooved tonographical stimulation, to promote changes in the STRO-1+proteome that effect cell phenotype which did not affect cell growth and cell viability (Kantawong et al 2010)T

2.5. The plant crude extracts estrogenic activity tests

P. mirifica crude extracts showed a biphasic response to MCF-7 cells with a strong binding with estrogen at low concentration and antiproliferative effect at high

concentration with ED_{50} value of 642.83 μg/mL in competition with estrogen receptors but no proliferative and anti-proliferative effect in HeLa/MCF7 cells at 100 and 1,000 μg/mL (Cherdshewasart et al 2004). Furthermore, *P. mirifica* crude extract was responded in dose-dependent manner on MCF-7 cells (Cherdshewasart et al 2007b) and HepG2 cells. The mechanism of action of the plant extract was evaluated. It was found that the chemicals needed a metabolic activation to promote their actions within human cells. Recombinant yeast exhibited no estrogenic activity because it lacked metabolic enzyme (Lee et al 2002). In term of *in vitro*, *P. mirifica* established strong estrogenic effects in the test MCF-7 proliferation/antiproliferation assay (Cherdshewasart et al 2007b). The major isoflavonoids in crude extraction of *P. mirifica* leaves has been separated and showed estrogenic activity (Jungsukcharoen et al 2014).

2.6. Plant proteomic study

Proteins are an important class of biological macromolecules present in all organisms. All proteins are polymers of amino acids classified by their physical size, proteins are nanoparticles (definition: 1-100 nm). The total complement of proteins present at a time in a cell or cell type is known as its proteome, and the study of such large-scale data sets defines the field of proteomics. In plant proteomic studies, a large-scale study on proteomics in soybean (legume family) began in 2002 (Herman et al 2003). Recently, *P. mirifica* plant proteomics was preliminary studied (Chershewasart et al 2011)

2.6.1. The protein extraction method

The beginning of proteomic study is to find the practical protein extraction method for specific plant not only the abundance proteins could be gained but also the clearly separated well performed for selecting the right extraction method. Due to plant There are many protein extraction methods have been developed for plant proteome analysis (Jiang et al 2004, Natarajan et al 2005a, Sheoran et al 2009, Wang et al 2006) including trichloroacetic acid (TCA)-acetone, phenol, direct iso-electric focusing (IEF) buffer, and Tris-HCl. The protein solubilization methods suitable for proteomic analysis in soybean seed proteins were established. TCA-acetone and phenol protein extraction methods were found to be superior to other two tested methods because they can remove a large proportion of non-protein materials which

can interfere with plant proteomics and separation proteins, (Natarajan et al 2005a). TCA-acetone method is precipitating technique for separate protein out of the contaminants such as salts, hydrophilics, detergents and lipids by using the solubility principle. The obtained proteins still contaminated by debris (nucleic acid, polysaccharides and phenolic compounds) and proteins was hardly redissolved, especially, when protein pellet was quite acidic. Whereas, Tris-HCl extraction method widely used to extract proteins from plant samples and protect protein from denaturation by using glycerol but the limitation of this technique is use for soluble protein only and not suitable for dinoflagellated protein. Phenol extraction was used for removing nucleic acid and carbohydrates. This technique also reduced the contaminant background of 2D gel by reduce molecular interaction between proteins and another materials (Chatterjee et al 2012)

2.6.2. Protein separation and identification

After the practical protein extraction method had obtained, separation technique was employed for proteome patterns. 2 Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) is well-known technique for protein separation. First Dimension focusing on their Isoelectric point (pI) and second dimension focusing on their molecular weight (MW) (Markham 1956). The proteomic reference map of soybean leaves was also established by using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as a separation technique (Natarajan et al 2006) and identified by mass spectrometry (MS). The result indicated that this 2D-PAGE, combined with MALDI-TOF-MS and LC-MS/MS, is a sensitive and powerful technique (Biemann 1963) for separation and identification of soybean leaf proteins.

2.6.3. Functional protein in plant

The proteomics and functional analysis of soybean were revealed (Komatsu & Ahsan 2009). Many studies of tuber proteomes in plants have been done, for example: Ginger root extract (Fasoli et al 2012), cassava (Owiti et al 2011), curcuma (Boonmee et al 2011, Chokchaichamnankit et al 2009), grape (Niu et al 2013), potato tubers (Yu et al 2012a) and carrot root were studied (Louarn et al 2012).

Protein expressions are different due to the biotic and abiotic factors such as climate change, plant pathogenesis, herbivores, insects etc. The climate changes have been one of the most important in growing plants and plant production. The

climatic factors include photosynthetic stress, air pollutants, thermal stress (heat and cold), osmotic stress (drought, salt, flooding stress) and metal stress (Hashiguchi et al 2010). Some evidence shows high temperature and humidity (HTH) stress play important role in differentially expressed proteins in soybean (Wang et al 2012). Protein alteration in different season in Legume plant has never been reported. Low temperature stress (less than 20°C) cause considerable agricultural yields loss in crops (maize, rice and chickpea) (Thakur et al 2010). Low temperature exposure coordinately induces the accumulation of PAL (Phenylalanine Ammonia Lyase) and CHS (Chalcone Synthase) mRNAs only in the light (Leyva et al 1995, Timperio et al 2008), beside; high temperature stress (heat stress) causes oxidative damage that manifests in lipid peroxidation.

Plant response to heat stress by produce detoxification enzymes such as glutathione S-transferase, catalases, superoxide dismutase (SOD) and ascorbate peroxidases (APX) for protection (Timperio et al 2008). The optimal temperature is 22°C and the maximum is about 40°C (Nieuwelink 2005). Plant pathogenesis also important for protein expressed. The stress response and defense mechanism were reacted to protect plant for surviving in difficult condition. In plant also found proteins related with this situation, for example, Pathogen- and wound-inducible antifungal protein CBP20 precursor (Ponstein et al 1994), Leaf rust resistance protein Lr10 (Heath 2000).

In tuberous proteomics record, the specific proteins involved in potato tuber development were including 6-fructokinase, phytoalexin-deficient 4-1, metallothionein II-like protein, and malate dehydrogenase, whereas, Novel stage-specific proteins identified during *in vitro* tuberization were ferredoxin–NADP reductase, 34 kDa porin, aquaporin, calmodulin, ripening-regulated protein, and starch synthase (Niu et al 2013). Methionine synthase, cysteine synthase (involved in amino acid biosynthesis), heat shock protein, protein disulfide-isomerase (involved in protein folding) and superoxide dismutase (detoxification) are commonly found in carrot root (Louarn et al 2012). Zingipain-2OS, zingipain-1OS and cystein protease (milk coagulating protease) were found in ginger root extract (Hashim et al 2011). Sporamin (storage protein) was mainly found in sweet potato *(Ipomoea batatas)* (Maeshima & Asahi 1978) and was highly expressed in the dormant period in curcuma rhizome (Boonmee et al 2011, Chokchaichamnankit et al 2009). UDP-glucose (flavonoid3-O-glucosyltransferase, UFGT), which is secondary metabolism, involved protein found in grapevine (*Vitis vinifera*) (Niu et al 2013).

There were reports set group of plant functional into 8 classes: (Chokchaichamnankit et al 2009)

1. Cell structure – Protein involved in construction of cell compartments.

2. Defense – For protecting itself from environment such as plant pathogenesis, herbivores, insects etc.

3. Metabolism – Involved in carbohydrate metabolism (glycolysis, gluconeogenesis).

4. Photosynthesis - Involved in photosynthesis reaction.

5. Protein synthesis – Focusing on the role in controlling cell growth division and development.

6. Storage protein – Plant use for reservation of metal ions and amino acids.

7. Stress response – Plant produces for response of stress condition such as Climate change (hot/cold temperature), salinity etc.

8. Transport – Due to plasma membrane in plant cell is selective permeable, protein is required to transport protons, organic/inorganic ions solutes through plasma membrane and tonoplast at rates sufficient of cell (Chrispeels et al 1999).

2.6.4. Factors resulting proteomic change

Plant proteomics pattern is varied by environmental condition under abiotic stress condition. Abiotic stress condition causes plants adapt themselves to survive the stress conditions in generally term of acclimation which lead to the development of transcriptome, proteome and metabolome (Bogeat-Triboulot et al 2007).

Plant stress response is a dynamic process which is dependent on stress intensity and stress duration. Several stages of plant stress response could be distinguished as in Figure 8 (Kosova et al 2011). The corresponding of each stage of plant stress is response to the composition of different proteome. Plant which has no stress exhibits active growth and development relied on cell division. These processes are also associated with a *de novo* biosynthesis of several cellular components. In stressed plants, an intense re-organization of cellular metabolism has been investigated. There is a shift from an active growth and developmental progress to stress acclimation. For stress condition, Firstly, the Early stages of plant stress

response (alarm phase) induce stress responsive signaling pathways and a strong oxidative stress. The following stages (acclimation phase) are associated with a *de novo* biosynthesis of several stress protective proteins (e.g., chaperones, COR/LEA, PCs, ROS scavenging enzymes) and other compounds (e.g., antioxidants - carotenoids, tocopherols; osmoprotectants - GB, proline). During recovery stage, processes leading to activate the degradation of stress-protective compounds and set up a new cellular homeostasis.

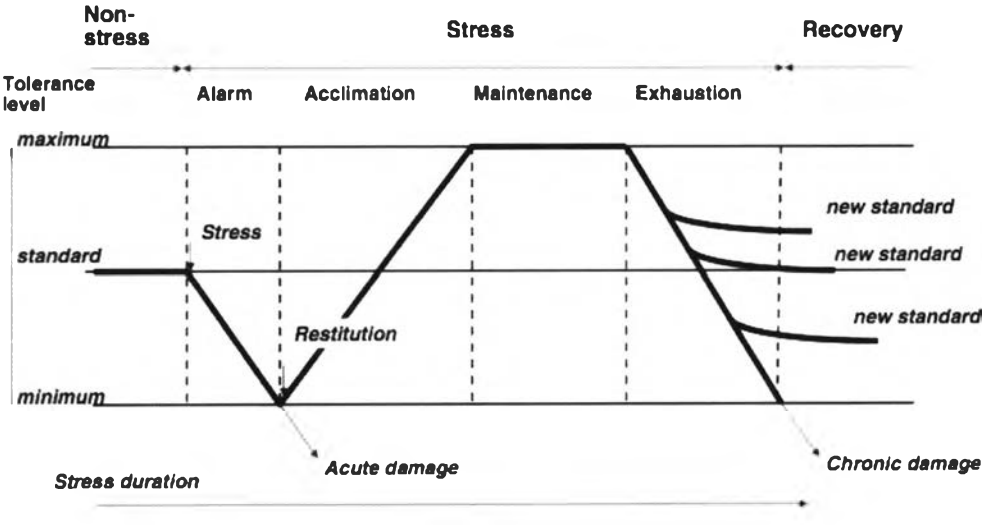


Figure 8 A generalized scheme of a dynamics of plant response in an abiotic stress condition.

2.6.4.1. Cold

Cold stress is a climatic factor in plants. The dropped temperature is associated with the alteration of energy metabolism (Sha Valli Khan et al 2014) by decrease the rate of enzyme-catalyzed reactions caused metabolic imbalance. Plants response to cold temperature by generate reactive oxygen species (ROS), which interact with several cellular components, including DNA, proteins, lipids, and pigments and have been shown to exhibit complementary protective responses to cope with the cold stress (Yamaguchi-Shinozaki & Shinozaki 2006). Some Study reported that proteins involved in energy metabolism and in protein destination, storage and assimilation were up-regulated in cold stress (treated with 10°C, 72 h) (Lee et al 2009). On the other hand, Heat shock protein 70 was detected in plant by

functioning as molecular chaperones and preventing aggregation of the denatured proteins and facilitating refolding under chilling or thermal stress (Renaut et al 2004). The increasing of Cu/Zn superoxide dismutase (Cu/Zn-SOD) in cold temperature was reported (Degand et al 2009). Moreover, cold temperature also involved in photosynthesis pathway by up-regulated the level of plastocyanin and Rubisco subunits (In stroma cell) (Gao et al 2009).

2.6.4.2. Heat

Heat stress caused folding and denaturation in several intracellular protein and membrane complexes. The existence proteins normally found in heat stress condition belong to chaperones (heat-shock proteins (HSPs) family) which assist the non-covalent protein unfolding (non-aggregation) in heat (Baniwal et al 2004). For energy metabolism protein, heat stress increased the accumulation of enzymes involved in biosynthesis of UDP-glucose (UDP- glucose pyrophosphorylase UGPase), thiamine and dehydro- genation of pyruvate (pyruvate dehydrogenase) and transketolase (Lee et al 2007). These results also exhibited the up-regulation of pyruvate activity (pyruvate dehydrogenase complex) that involved in carbon transferred from glycolysis to Calvin cycle and an increase in the activity of Rubisco activases and pentose phosphate pathway (PPP) involved in regeneration of ribulose-1,5-bisphosphate (RuBP) (substrate for Rubisco) (Lee et al 2007, Zhang et al 2010).

2.6.4.3. Drought

Drought stress directly impacts in the rehydration of cell. The cellular metabolism that associated with an osmotic adjustment could be observed. The results found "peroxidase" (ROS scavenging enzyme) which leaded to enhance cell wall loosening upon dehydration stress. Under dehydration stress condition, several β-D-glucosidases were up-regulated because these enzymes associated with the cleavage of abscisic acid (ABA) from saccharide conjugates which is a prerequisite for ABA activity in carbohydrate metabolism (Hajheidari et al 2005). Besides, the defensed proteins named polygalacturonase inhibitor proteins, nodulin precursors, chitinases and osmotin were found (Zhu et al 2007).

2.6.4.4. Waterlogging

Waterlogging is a saturation of soil with groundwater and water may remain on the ground when water is exceeding limit to absorb cause oxygen obstruction. The lack of oxygen leads plant to develop anaerobic metabolism (glycolysis followed by pyruvate fermentation instead of oxidative decarboxylation and subsequent decomposition *via* Krebs cycle) and also to program cell death, PCD of root cortex cells in order to form lysigenous paerenchyma and provide oxygen to roots from un-flooded shoot parts of the plant (Kosova et al 2011). Waterlogging was resulting in enhancement of H_2O_2 levels and lipid peroxidation indicating that this stress factor has an oxidative component. At proteome level, waterlogging induces changes in abundance of proteins were associated with several processes including defense mechanisms, disease resistance, energy metabolism, PCD, photosynthesis, protein biosynthesis, redox homeostasis, RNA processing, signal transduction and stress (Ahsan et al 2007).

2.6.4.5. Mineral nutrients

Mineral nutrients are also an essential factor for plant growth and development. They function as cofactors in many enzymes and other proteins with special operations. For aluminium concentration, there has been a reported the effects of elevated concentrations of aluminium on rice roots. An induction of several enzymes involved in ROS detoxification (Cu/Zn-SOD, Gluthatione S-transferase, GST) and glutathione metabolism (cysteine synthase CS) was found for acting as an ion chelating agent (Yang et al 2007a). Effect of nitrogen deficiency in two winter wheat cultivars has studied (Bahrman et al 2004). The differentially expressed proteins included mainly proteins involved in carbon metabolism, namely glycolytic enzymes aldolase (ALDO), enolase (ENO) and phosphoglycer- ate kinase (PGK), Rubisco activase, OEE1 protein and β subunit of ATP synthase. Whereas the deficiency of iron (Fe concentration), the differentially proteins included ROS scavenging enzymes (APX, CAT, other peroxidases), enzymes involved in SAM biosynthesis (SAMS), proteins involved in ion transport (vacuolar proton ATPase subunit), cell signalling and protein degradation (β5 proteasome subunit) were expressed (Brumbarova et al 2008). The effect of boron also investigated

2.6.4.6. Herbicides

Herbicides are chemicals which adversely affect plant growth and development and thus they are used for elimination of several weeds. In the frame of toxicity investigations and risk assessment integral endpoints such as growth and reproduction are traditionally monitored. The effects of flumioxazin on grapevine proteome were studied (Castro et al 2005). Herbicide treatment exhibited a degradation of several photosynthetic proteins including Rubisco. It caused the less efficiency of photosynthetic processes and led the accumulation of several antioxidative enzymes and enzymes involved in biosynthesis of osmotically active compounds implicating processes leading to osmotic adjustment. Herbicides also enhanced the accumulation of uridylyl-glucoso-pyrophosphorylase (UGPase) and GAPDH indicated oxygen deprivation since GAPDH belongs to anaerobic proteins, ANPs. The application of flumioxazin also induced expression of several PR proteins, namely PR-10 proteins.

2.6.4.7. Mechanical stress - wounding

The studied of wound healing-periderm formation process in potato (*Solanum tuberosum*) tubers according to changes in proteome composition, two main phases of the wound healing process could be distinguished. (Chaves et al 2009) Early wound-healing events (0 to 4 days after slicing) induced an increased expression of several protease inhibitors, pathogenesis-related protein PR10, peroxidases, proteins with chitin-binding domains, chitinases and high molecular weight patatins while later phases (4 to 8 days after slicing) which are characterised by suberisation led to enhanced expression of suberisation-associated anionic peroxidase, chitinases, metacaspase, annexin p34, elicitor-inducible pro- tein EIG-J7, Ran protein and 20S proteasome α-subunit B.

2.7. Plant metabolomic study

The metabolomics is the study of how the pool of metabolites that involved in metabolism of cells also called the entire chemicals changes under various physiological or developmental conditions (temperature, rainfall amount, moisture etc.) or in response to genetic modification. Plant metabolomics represents the thorough profiling of metabolites that containing in plant organism. Proteomics and transcriptomics are both considered to be a flow of media relating to genetic

information, on the other hand, metabolomics are subjected to relate with plant phenotype expression (Figure 9). The application of metabolomics techniques to plant science was pioneered at the Max Planck Institute obtained a series of metabolome data from potato tubers using GC/MS. A pair-wise comparison of metabolite levels revealed that many pairs of metabolites exist, and showed high levels of correlation coefficients despite alterations in biological replicates (Weckwerth et al 2004). The investigators also demonstrated that the structure of the correlation network could be changed in a mutant with a silent phenotype, in which metabolic changes were observed despite a lack of visible changes. Theoretical studies revealed that factors such as equilibrium between neighboring metabolites shared biosynthetic enzymes and coordinated regulation of gene expression are likely responsible for the correlation between metabolite levels. Most metabolites that participate in common reactions are not correlated in this way, while some non-neighboring metabolites are highly correlated (Weckwerth et al 2004).

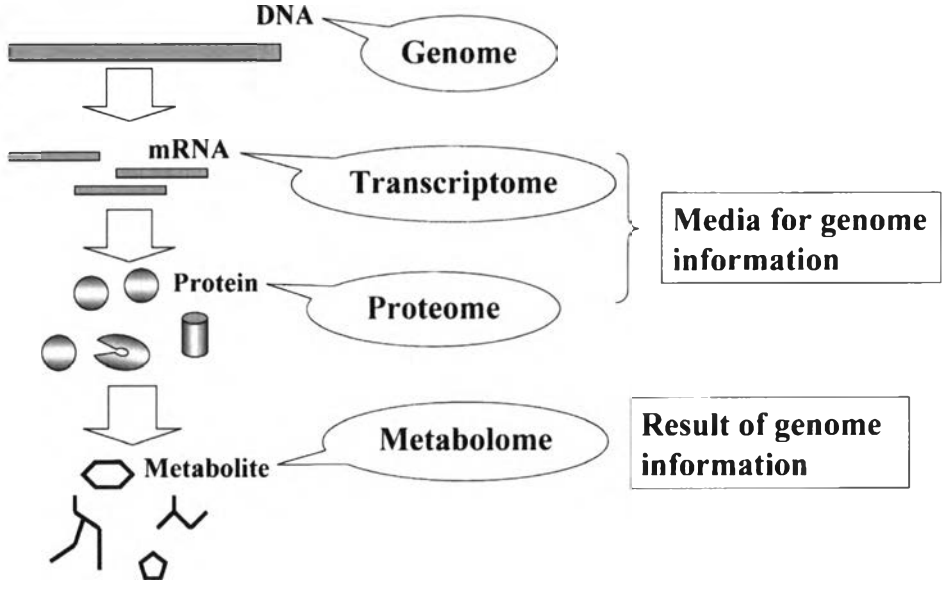


Figure 9 Metabolomics in functional genomomics (Derived from (Fukusaki & Kobayashi 2005)

2.7.1. The processes for metabolomics determination

The purpose of metabolomics determination is to gain the entire metabolites in target plant organism. For metabolomics, qualitative and quantitative analyses of all metabolites are employed. Metabolomics consists of several complicated technical elements described in Figure 10 with each step possibly giving rise to an experimental error. To establish a robust system, close collaboration among researchers in the fields concerned with analytical chemistry, organic chemistry, chemometrics, informatics, and bioscience is required. The conventional technique steps are described as follow.

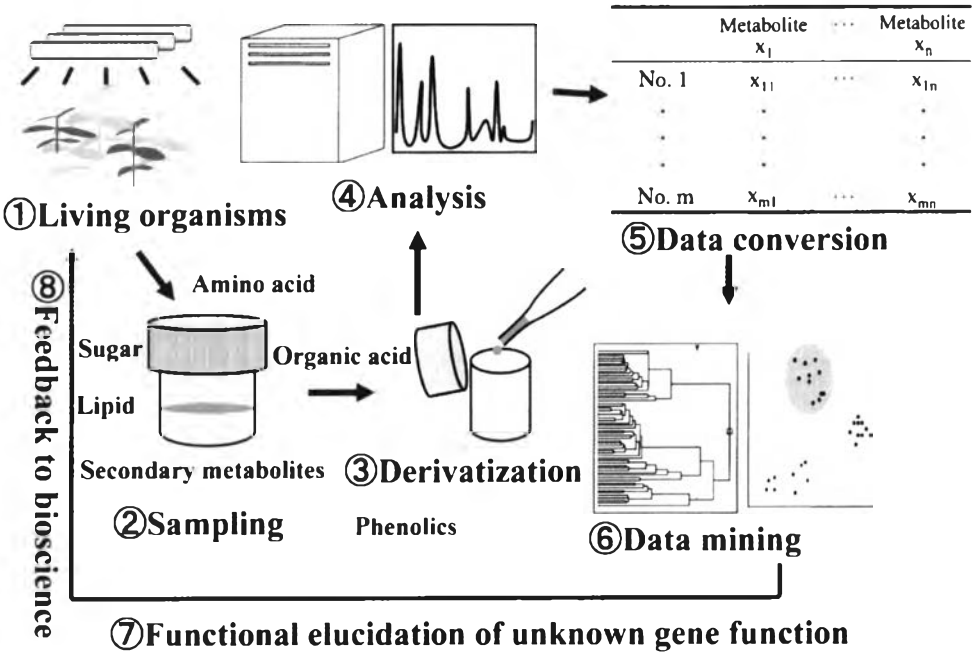


Figure 10 General scheme of metabolomics steps (Derived from (Fukusaki & Kobayashi 2005)

2.7.1.1. Plant cultivation

Plant metabolisms in the cell are varied by the environment, herbivore, insect and other stresses. The ability to produce various phytochemicals has evolved in plants for the purpose of self-defense, environmental adaptation and interaction with other organisms. Because humans utilize phytochemicals for numerous

purposes, including the production of pharmaceuticals, further understanding of the genetic background behind the diversity of secondary metabolites produced by plants will facilitate a more intensive application of these compounds. However, the lack in metabolome analysis of plant metabolites is the annotation of metabolite signals (Iijima et al 2008)

2.7.1.2. Sampling

Sampling is one of the most important steps. The sampling should be less variation to reduce significant error. To maintain variance at a minimum, not only the growth stage but also the exact time of sampling should be controlled. Furthermore, cultivation area and quantified sample should also be considered. The analytical method and instruments should be decided dependent on the characteristics of target metabolites in post-harvest period, including the number of metabolites being examined, and their respective quantities. The optimum preparation protocol should be developed depends on a case-by-case basis.

2.7.1.3. Derivatization and pretreatment

Derivatization of target metabolites is required subjected to the analytical techniques. For example, volatile compounds are applicable for GC-MS analysis. Most hydrophilic metabolites should be derivatized by sililation or other methods. High- performance liquid chromatography (HPLC) also requires derivatization in the case of UV or fluorescence detection. Specificity and efficiency are both important factors and it should be validated in their terms for the most effective. In addition, the stability of yielded derivatives should be evaluated (Blau 1977).

2.7.1.4. Analysis

Separation and identification technique are be employed for metabolomics analysis. Separation is the most important part for separate the contaminants out of the samples. Separation strongly affects both resolution and quantification. However, it might be costly and inconvenient to determine the best specification for both resolution and quantification. The Method for separation is normally includes chromatography or electrophoresis coupled to mass spectrometry. UV detection and electrochemical detection are also used for quantification. Although great effort has

been put into construction of the MS/MS spectral databases (Horai et al 2010), further enrichment is required for structural elucidation of a wider range of metabolites. By the way, the development of databases and methodology is still required to explore the diversity of plant secondary metabolites

2.7.1.5. Data conversion

Multivariate analyses are practically used in metabolomics research because rather complicated linear and nonlinear relationships must be elucidated. The target metabolite should be used as an independent variable and peak area should be used as a dependent variable. Data obtained by GC-MS or LC-MS should also be corrected by appropriate preprocessing. In fact, appropriate preprocessing is a prerequisite for data mining (Matsuda et al 2009).

2.7.1.6. Data mining

In metabolomics, multivariate analysis with an appropriate algorithm should be performed depending on data structure and mining intention. Multivariate analysis methodology used includes multiple regression, discriminant analysis, principal component analysis, hierarchical cluster analysis, factor analysis, canonical analysis. Among these methods, exploratory analysis tends to be used most often in plant metabolomics.

2.7.1.7. Functional elucidation of unknown gene function

The unknown gene function is being clarified and grouped.

2.7.1.8. Feedback to bioscience

When a certain working hypothesis has been established, a specific biosynthetic pathway can be proposed. For example, some metabolites upon which perturbations were imposed including physical stresses, biotic stress, mutation, transgenic stress should be evaluated in a small-scale experiment. Metabolomics will focus on the specific biosynthetic pathway or on the specific category of metabolites. Besides, when an exactly same result was obtained in the same experiment under the same specification with the same instrument by the same person, this is defined

as repeatability. The similar experiment is performed at a different place with different equipment by a different person and a similar result is obtained, this is termed reproducibility.

2.7.2. The metabolic engineering of isoflavonoid synthesis

The metabolic isoflavonoid biosynthesis pathway in soybean was revealed (Yu et al 2003). The three major types of isoflavones are daidzein, genistein, and glycitein which two of them are also found in *P. mirifica* (Cherdshewasart et al 2007c). These isoflavonoid compounds played defense mechanism in term of anti-microbial and/or anti-herbivore activities (Graham & Graham 1996). They are the main chemical compounds plants deploy to combat pathogens and disease (Dixon & Paiva 1995). Both flavonoids and isoflavonoid are chemotaxic to rhizobia and microbes (Barbour et al 1991).

Isoflavonoids are synthesized within the phenylpropanoid pathway (Figure 11) starting with phenylalanine. Phenylalanine is catalyzed by phenylalanine ammonia-lyase (PAL) to remove the amine group and change to cinnamic acid. Cinnamic acid 4-hydroxylase (C4H), belongs to group cytochrome P450 monooxygenase act as hydroxyl group addition, convert cinnamic acid to *p*-coumarate. Further, enzyme 4-coumarate coenzyme A ligase (4CL) activates the *p*-coumarate by conjugating a CoA at the three-carbon side chain. And then, chalcone synthase (CHS) carries out the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to form the C15 flavonoid skeleton. In most plant species, this compound is naringenin-chalcone (4,2,4,6′-tetrahydroxychalcone). The chalcone is synthesized by CHS and converts to flavanone naringenin (5,7,4′-trihydroxyflavanone) by the enzyme chalcone isomerase (CHI).

Naringenin is one of the shared substrates between flavonoid and isoflavonoid pathways. The modification of naringenin leads to produce various flavonoid compounds. The most common reaction using naringenin as a substrate is the addition of a hydroxyl group at the C3 position to form dihydrokaempferol as catalyzed by flavanone 3-hydroxylase (F3H) and a 2-oxoglutartate-dependent dioxygenase (Deboo et al 1995). The modification at the C3 position is essential for the production of anthocyanins and condensed tannins, which requires enzymes including dihydroflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), UDPG-flavonoid glucosyl transferase (UFGT), and others.

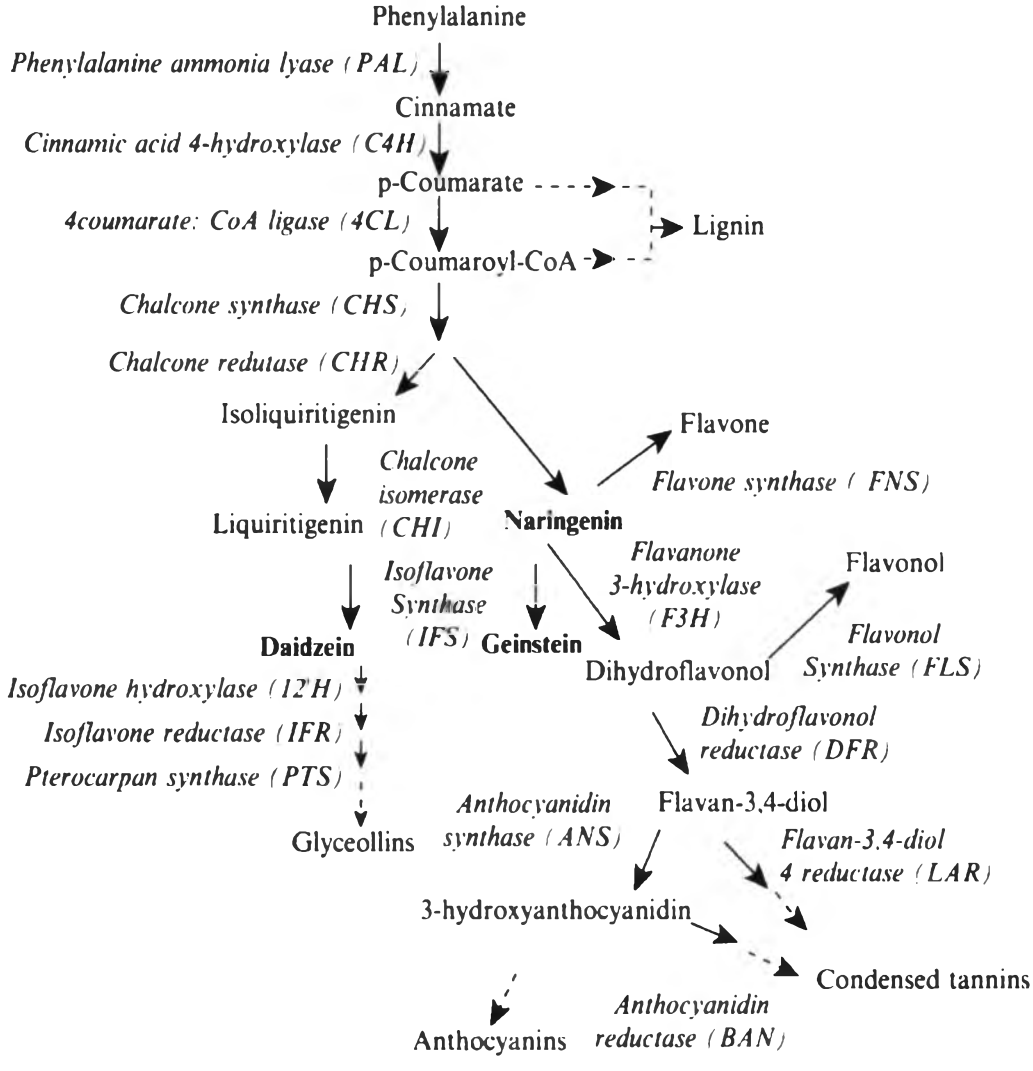


Figure 11 Biosynthetic pathway of phenylpropanoid pathway (including a list of enzymes and their abbreviations). (Yu et al 2003).

The enzyme isoflavone synthase (IFS) (cytochrome P450 monooxygenase) acts as the key metabolic entry point for the formation of all isoflavonoids. This enzyme plays two roles: it diverts naringenin formed by CHI into genistein production and, in conjunction with another legume-specific enzyme, chalcone reductase (CHR), forms daidzein. In this case, CHR, CHS, and CHI work in concert to produce isoliquiritigenin and then liquiritigenin, which is the precursor for daidzein. The IFS enzyme is discussed in detail later. Both daidzein and genistein can be conjugated sequentially with glucosyl transferase (UGT) to form daidzin and genistin. (Latunde-Dada et al 2001).