CHAPTER II

LITERATURE REVIEWS

Orchid export

Orchid is monocotyledonous plant which is classified into the family *Orchidaceae*, the largest family of plants with an estimated 17,000-35,000 species worldwide (Tsai, et al., 2006). Orchids are found throughout the world from the tropics to the subarctic zone but most species are distributed in tropical regions. Numbers of orchid hybrids have been naturally produced and horticulturally created. For the last 150 years, over 110,000 hybrids have been produced (Royal horticultural societies, 2011). To satisfy the customer demand, orchid breeders have continued to produce new varieties of hybrid orchid with new color, size and form.

Orchid is an important economic flower of Thailand. Although the world's largest orchid exporter is the Netherlands, Thailand is the world's largest tropical orchid exporter. Statistically, Thailand exports orchid around 30,447 and 19,424 tons in plants and fresh flowers with value of 586 and 2,300 million Baht, respectively (Data from Office of Agricultural Economics and Department of Agriculture Extension, Thailand, 2012). Most of the export orchids are *Dendrobium*, *Mokara*, *Aranda*, *Oncidium* and *Vanda*. Major trade partners of Thailand are Japan, USA, Italy, the Netherlands, China, Taiwan and Korea.

The Department of Agriculture Extension (Customs department, Thailand, online, 8 May 2013) reported that the export amount of fresh orchids flower increased from about 18,627 tons (approximate 2,136 million Baht) in 2004 to about 24,566 tons (approximate 2,545 million Baht) in 2007 (Table 1). However, the export value of orchids decreased in 2011-2012 as most orchid farms around Bangkok, particularly in Nakorn Pathom province where it is the largest area of export orchid production, have been devastated from Thailand flood disaster in the second half of 2011.

Table 1 Export values and quantity of Thai orchids during 2004 – 2012

Year	Plants		Fresh orchid flower	
	Quantity (Tons)	Value (Million Baht)	Quantity (Tons)	Value (Million Baht)
2004	26,376.03	344.62	18,627.00	2,136.06
2005	30,402.18	446.67	21,207.00	2,538.55
2006	34,154.82	430.44	23,348.00	2,490.95
2007	35,731.75	400.42	24,566.00	2,545.40
2008	38,997.47	423.44	25,152.00	2,417.58
2009	30,899.08	372.15	24,601.00	2,381.28
2010	29,987.70	422.45	25,270.00	2,312.90
2011	30,344.96	553.20	24,644.00	2,246.67
2012	30,447.05	586.16	19,424.00	2,046.17

Source: Office of Agricultural Economics, 2013

***Dendrobium* Sonia cv. Earsakul**

D. Sonia cv. Earsakul is one of the most important orchids of Thailand. With the exotic form of this tropical orchid, it is a popular cut flower all over the world. *D.* Sonia cv. Earsakul is a hybrid cross between mutants of *D.* Sonia Jo Daeng. The flower has purple (Purple Group N 78 A) with white base (White Group 155 C) of sepals and petals. (Figure 1). It is an easy growing and tolerant to weather conditions (Office of Management Agricultural Products, Department of Agriculture Extension)

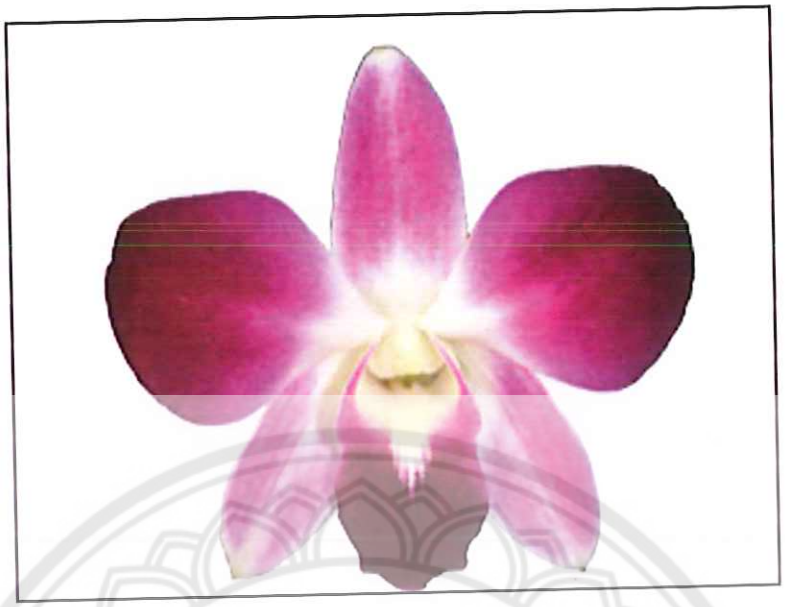

Figure 1 The flower of *Dendrobium* Sonia cv. Earsakul

Anthocyanin biosynthesis pathway

Anthocyanin is one of plant pigments and contributes to the wide range of flower colors. The common anthocyanins are derived from three anthocyanidins, including cyanidin, pelargonidin and delphinidin. Different modifications of these anthocyanidins such as methylation, glycosylation and acylation result in diverse anthocyanins, for example gentiodelphin and gentiocyanin (reviewed in Nakatsuka, et al., 2010). Anthocyanins are members of colored flavonoids which vary from pale red, purple and blue (reviewed by Tanaka, et al., 1998). The basic skeleton of anthocyanin biosynthetic pathway shown in Figure 2 demonstrates general pathway leading to cyanidin, pelargonidin and delphinidin-based anthocyanins which contribute to red-magenta, orange-brick red and purple-blue, respectively. The precursors for the synthesis of all flavonoids, including anthocyanins, are malonyl-COA and p-coumaroyl-COA. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-COA with p-coumaroyl-COA to yield tetrahydroxychalcone. Chalcone isomerase (CHI) then catalyzes the stereospecific isomerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin. Naringenin is converted to dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H). DHK can subsequently be hydroxylated by flavonoid 3'-hydroxylase (F3'H) to

produce dihydroquercetin (DHQ) or by flavonoid 3',5'-hydroxylase (F3'5'H) to produce dihydromyricetin (DHM). F3'5'H can also convert DHQ to DHM. At least three enzymes are required for converting the colorless dihydroflavonols (DHK, DHQ, and DHM) to anthocyanins. The first of these enzymatic conversions is the reduction of dihydroflavonols to flavan 9,4-cis-diols (leucoanthocyanidins) by dihydroflavonol 4-reductase (DFR). Further oxidation, dehydration, and glycosylation of the different leucoanthocyanidins produce the corresponding red cyanidin, brick-red pelargonidin and blue delphinidin pigments. Anthocyanidin 3-glucosides may be modified further in many species by glycosylation, methylation, and acylation. (Holton and Cornish, 1995).

***Dihydroflavonol 4-reductase* gene**

Dihydroflavonol 4-reductase (DFR) catalyzes a key step late in the biosynthesis of anthocyanins. DFR is an enzyme required to produce cyanidin, pelargonidin, and delphinidin based anthocyanins, which tend to yield red-magenta, orange-brick red and purple-blue, respectively (Forkmann, et al., 2001; To and Wang, 2006; Tanaka, et al., 2010). Therefore, lacking of DFR activity causes no anthocyanin synthesis leading to colorless or white flowers. The *DFR* gene was first isolated and cloned from Snapdragon and Maize (O'Reilly, et al., 1985). So far, a number of *DFR* genes have been isolated and characterized in many plants. *DFR* expression in most flowers, including *Torenia hybrida* (Ueyama, et al., 2002), *G. triflora* (Nakatsuka, et al., 2005), *D.* Jaquelyn Thomas 'Uniwai Prince' and *D.* Icy Pink 'Sakura' (Mudalige-Jayawickrama, et al., 2005), *P. hybrida* (Saito, et al., 2006), *Nierembergia* sp. (Ueyama, et al., 2006), *D.* Sonia cv. Earsakul (Pitakdantham, et al., 2011), and *Ascocenda* sp. (Kunu et al., 2012), is developmentally regulated. In some plants, such as petunia (*Petunia* hybrid) and cymbidium orchid (*Cymbidium* hybrid), DFR is substrate specific and unable to utilize dihydrokaempferol. These plants naturally lack or rarely contain pelargonidin-based anthocyanins and therefore do not produce flowers of orange/brick red colors (Meyer, et al., 1987). However, suppression of *DFR* leads to the production of flowers with pale or white colors (Aida, et al., 2000).

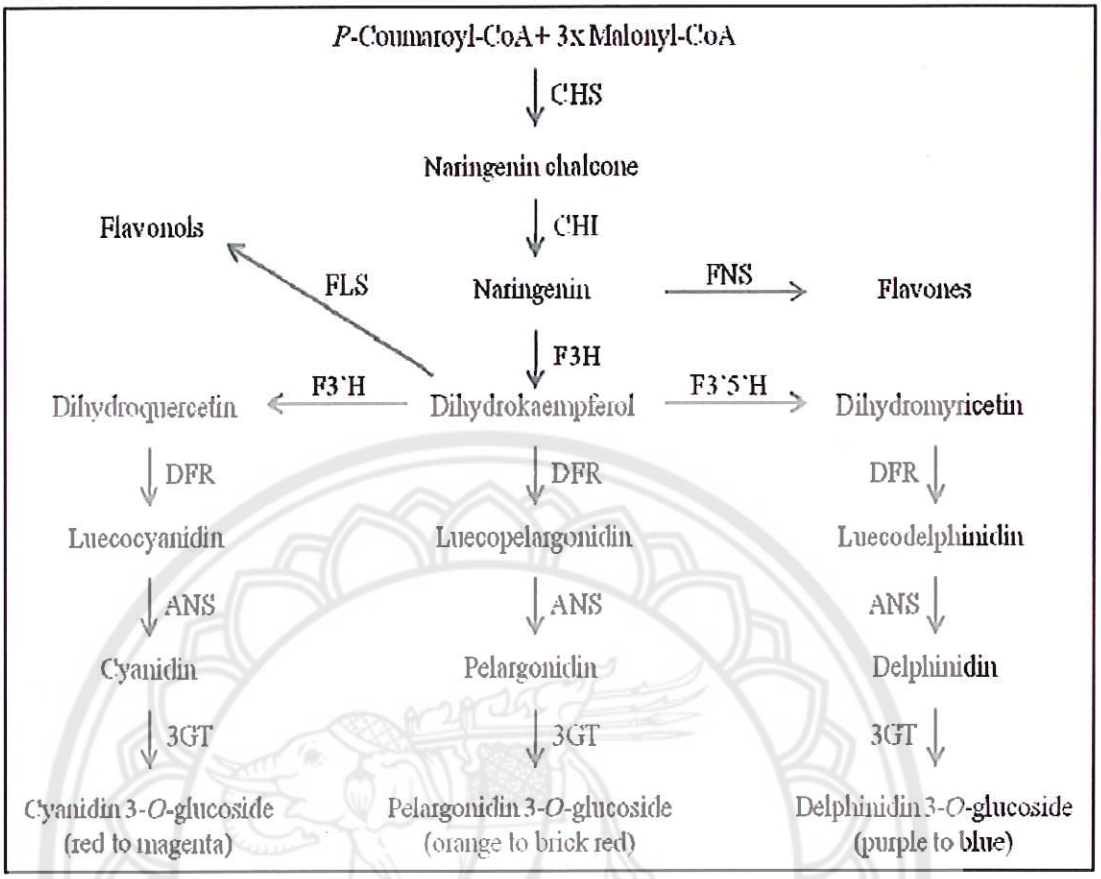

Figure 2 Schematic representation of the anthocyanin biosynthetic pathway

Note: CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase: F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, flavonoid 3-glucosyl transferase.

Source: Modified from Park, et al., 2007

Down-regulation of gene expression by RNA-induced gene silencing

Down-regulation of the target gene in the target plant has proposed to reduce gene expression. There are three major mechanisms to suppress the target gene;

1. Sense suppression (Co-suppression)

Co-suppression is a term that has been used to describe transcriptional silencing in plants (Jones, et al., 1998) and results in suppression of the transgenes as well as of the native homologue (Hamada and Spanu, 1998). There are several reports using this technique for modification of flower color. For example, Suzuki, et al. (2000) reported the modification of *Torenia hybrid* cv. Summerwave blue by cosuppressing expression of *CHS* and *DFR* resulting in white and blue/white varieties. A pink variety was also obtained by cosuppressing the *F3'5'H* gene. Co-suppression of the *FNSII* gene in the torenia successfully decreased the amount of flavones and increased the amount of flavanones, and yielded paler flower color (Ueyama, et al., 2002).

2. Anti-sense suppression

Anti-sense suppression is a technique used to silent expression of one or more genes by introduction of an antisense RNA molecule into a cell to inhibit translation of a complementary mRNA. This technique has been used to modify flower color of several ornamental plants. For example, Nielsen, et al. (2002) reported suppression of the lisianthus *FLS* gene by antisense technique resulting highly accumulation of dihydroflavonol and changing the flower color from the pink-purple to magenta. Nishihara, et al. (2003) transformed blue gentian (*Gentian triflora*) using an antisense gentian *CHS* gene to modify flower color to pale blue to white flowers.

3. RNA interference (RNAi)

RNA silencing is a gene regulatory mechanism that limits the transcript level either suppressing transcription or by activating a sequence-specific RNA degradation process (posttranscriptional gene silencing, PTGS, or RNA interference, RNAi) (Agrawal, et al., 2003). RNAi is a post-transcriptional process triggered by double-standed RNA (dsRNA). A simplified model for the RNAi pathway (Figure 3) is a two step mechanism. The first step, the dsRNA silencing trigger is recognized by an RNase termed Dicer, which cleaves the dsRNA into 21-23 nucleotides (nt) termed short interfering RNA (siRNA). The second step, siRNAs are incorporated into an RNA-induced silencing complex (RISC), which identifies substrates through their homology to siRNA and targets the equivalent mRNAs for destruction (Humanes, et al., 2008). RNAi is a new and effective technology used to analyze gene function. This

technique is also used to silence mRNAs of anthocyanin biosynthetic genes for flower color modification. Katsumoto, et al. (2007) successfully generated the blue-hued rose accumulating of delphinidin by down-regulation of the endogenous rose *DFR* gene using RNAi and overexpression of the iris *DFR* and the viola *F3'5'H* genes. Nakatsuka, et al. (2007) used a chimeric RNAi construct for suppression of *FLS* and *F3'H* genes and expression of the gerbera *DFR* gene into tobacco and successfully produced red-flowered tobacco plants. Nakatsuka, et al. (2008) used RNAi technology to suppress *CHS, ANS* and *F3'5'H* genes of blue gentian plant. The petals of transgenic gentian plants with suppressed *CHS, ANS* and *F3'5'H* genes exhibited pure white to pale-blue color, only pale-blue and magenta flower colors, respectively. Kamiishi, et al. (2012) reported that using RNAi targeting *CHS* of the liliaceous ornamental *Tricyrtis* sp. altered reddish-purple spotted tepals to completely white tepals.

Plant transformation

Plant transformation refers to the introduction and integration of exogenous DNA into plant cells and the consequent regeneration of transgenic plants. Transfer of DNA into plant cells can lead to transient or stable expression of the introduced DNA (Newell, 2000). Delivery of exogenous DNA to plant cells and gene transformation generally can be indirect and direct methods. For an indirect method, DNA of interest are introduced into plant cells via *Agrobacterium tumerfaciens* or *A. rhizogenes* whereas a direct method does not require bacterial cells as mediators to transfer DNA into plant cells, for example, microparticle bombardment (biolistics), microinjection, chemical (PEG) treatment of protoplasts and electroporation of protoplasts. Though all methods have advantages that are unique to each of them, at present, *Agrobacterium*-mediated and particle bombardment transformation are the most commonly used methods for plant transformation (Barampuram and Zhang, 2011)

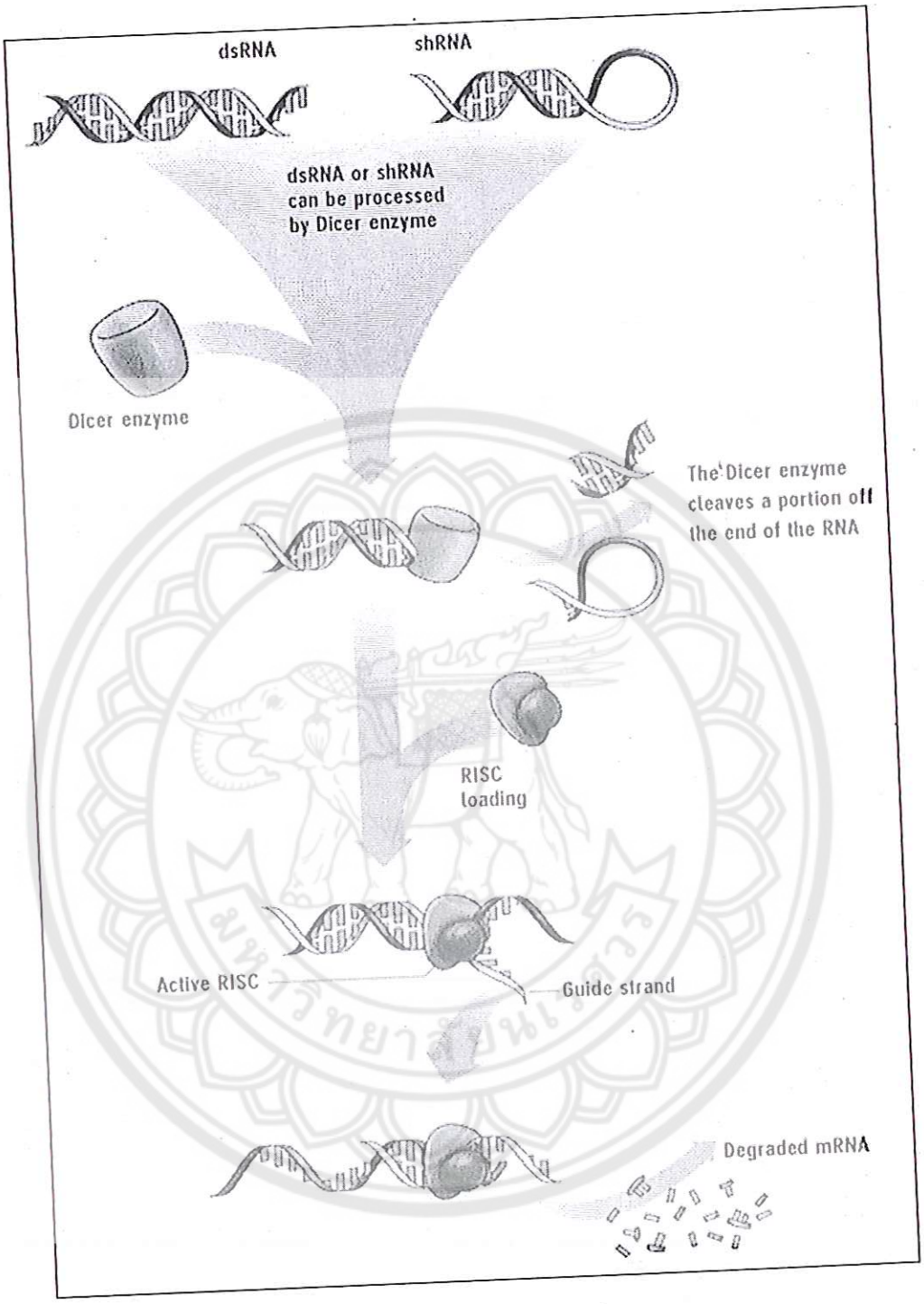

Figure 3 A simplified model for the RNAi pathway; dsRNA, double-stranded RNA; shRNA, small hairin RNA; RISC, RNA-induced silencing complex

Source: Marina biotech, 2013

Stable genetic transformation of plants involves the delivery of DNA into the nucleus of a competent cell and the recovery of fertile plants from that transformed cell. In most cases, plant transformation depends on the ability of the transformed plant cells and tissues to regenerate whole plants through the tissue culture process (Finer, 2010; Jones and Sparks, 2009). Therefore, stable transformation is often a time-consuming process involving tissue culture techniques that facilitate the growth of whole plants from tissue explants (Newell, 2000). Alternatively, transient gene expression in plants is a valuable tool for indication of successful gene introduction and function, and for promoter testing. It only takes one to several days to perform the assay. Most stable transformation procedures were developed based on optimization of DNA delivery using transient expression analyses (Finer, 2010; Jones, et al., 2009). In an aspect of functional genomics, transient expression can be used both to over-express and to silence genes of interest. The methods of inducing transient expression include particle bombardment, protoplast transfection, and *Agrobacterium*-mediated transformation, each with advantages and disadvantages.

Agroinfiltration or *Agrobacterium tumefaciens*-mediated infiltration is a rapid tool for functional gene assay via *Agrobacterium*-mediated transient gene expression and silencing. The agroinfiltration transient assay system has been effectively used in leaves of *Nicotiana benthamiana* (Goodin, et al., 2008) and has been optimized for other species, including *Lactuca sativa* (lettuce), *L. serriola* (wild lettuce), *Solanum lycopersicum* (tomato), some cultivars of *Arabidopsis thaliana* (Wroblewski, et al., 2005), tobacco (Yang, et al., 2000; Sparkes, et al., 2006) and grapevine (Santos, et al., 2008; Zottini, et al., 2008). Agroinjection for transient assays in tomato and strawberry fruits has also been developed (Orzaez, et al., 2006; Hoffmann, et al., 2006). Transient assays via agroinfiltration in floral tissues have been successfully utilized in *Antirrhinum majus* (Shang, et al., 2007) and Roses (Yasmin, et al., 2010). Agroinfiltration has also been used as a delivery system for transient RNAi to suppress anthocyanin biosynthetic genes such as *CHS* of *Antirrhinum majus* (Shang, et al., 2007) and *F3H* of strawberry fruit (Jiang, et al., 2013)

Flower color engineering by modification of *DFR* activity

DFR is a key enzyme of anthocyanidin synthesis as mentioned above. Modification of DFR activity via genetic transformation will lead to inhibition of anthocyanin synthesis or convert the direction of anthocyanin biosynthetic pathway. Brugliera, et al. (2000) modified the flower color of carnation from red to purple by introducing petunia *DFR* and *F3'5'H* into the carnation causing the flower richly accumulated delphinidin. Aida, et al. (2000) used the antisense *DFR* gene to suppress the *DFR* expression of *Torenia* hybrid resulting the flower turned pale to white. Polashock, et al. (2002) reported that overexpression of cranberry *DFR* in tobacco altered the flower color from pale pink to dark pink. Tsuda, et al. (2004) successfully modified the orange flower of petunia using RNAi technology to silence *F3'H* gene and overexpressed the *DFR* of rose. Katsumoto, et al. (2007) created the so called blue rose by suppression of its own *DFR* using RNAi technology and overexpression of *Iris DFR* and *Viola F3'5'H*.

Gateway technology for hairpinRNA construction

The Gateway cloning technology is a novel universal for cloning and subcloning DNA sequences, facilitation gene functional analysis. Once in this versatile operating system, DNA segments are transferred between vectors using site-specific recombination. The powerful system can easily transfer one or more DNA sequences into multiple vectors in parallel reactions, while maintaining orientation and reading frame. The Gateway site-specific recombinations are two proprietary enzyme mixes, called "LR Clonase", and "BP Clonase" reactions. The system requires the initial insertion of a DNA fragment into a plasmid with two flanking recombination sequences called "att L 1" and "att L 2", to develop a Gateway Entry clone (special Invitrogen nomenclature)

The gene cassette in the Gateway Entry clone can then be simply and efficiently transferred into any Gateway Destination using the proprietary enzyme mix, "LR Clonase". In this project, pSTARGATE (CSIRO, Australia) (Figure 4) and pWATERGATE (CSIRO, Australia) (Figure 5) were used as Gateway Destination vector to generate *DFR*-hairpinRNA (hpRNA) construct. The hpRNA construct

pSTARGATE is driven by the ubiquitin promoter whereas the hpRNA construct of pWATERGATE is driven by the ARbcS promoter.

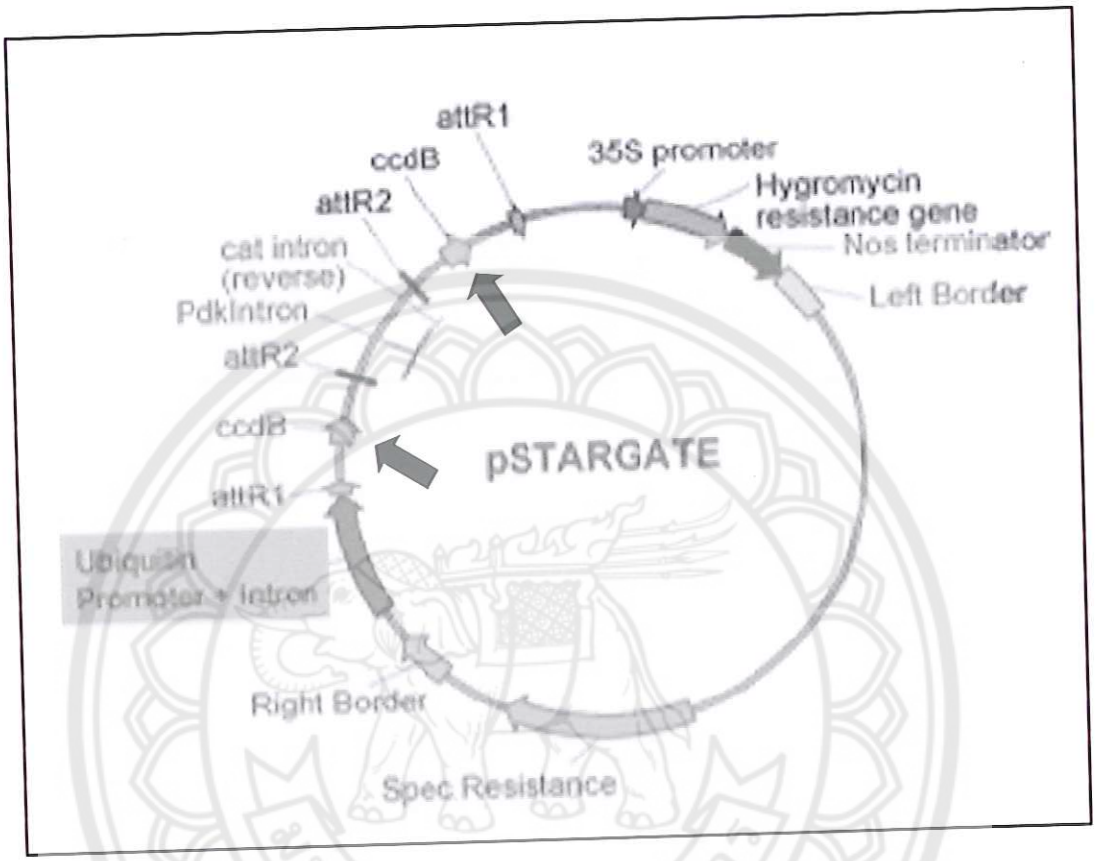

Figure 4 Vector map of pSTARGATE; (Black arrows show the exchanging sites between Entry clone and Destination vector)

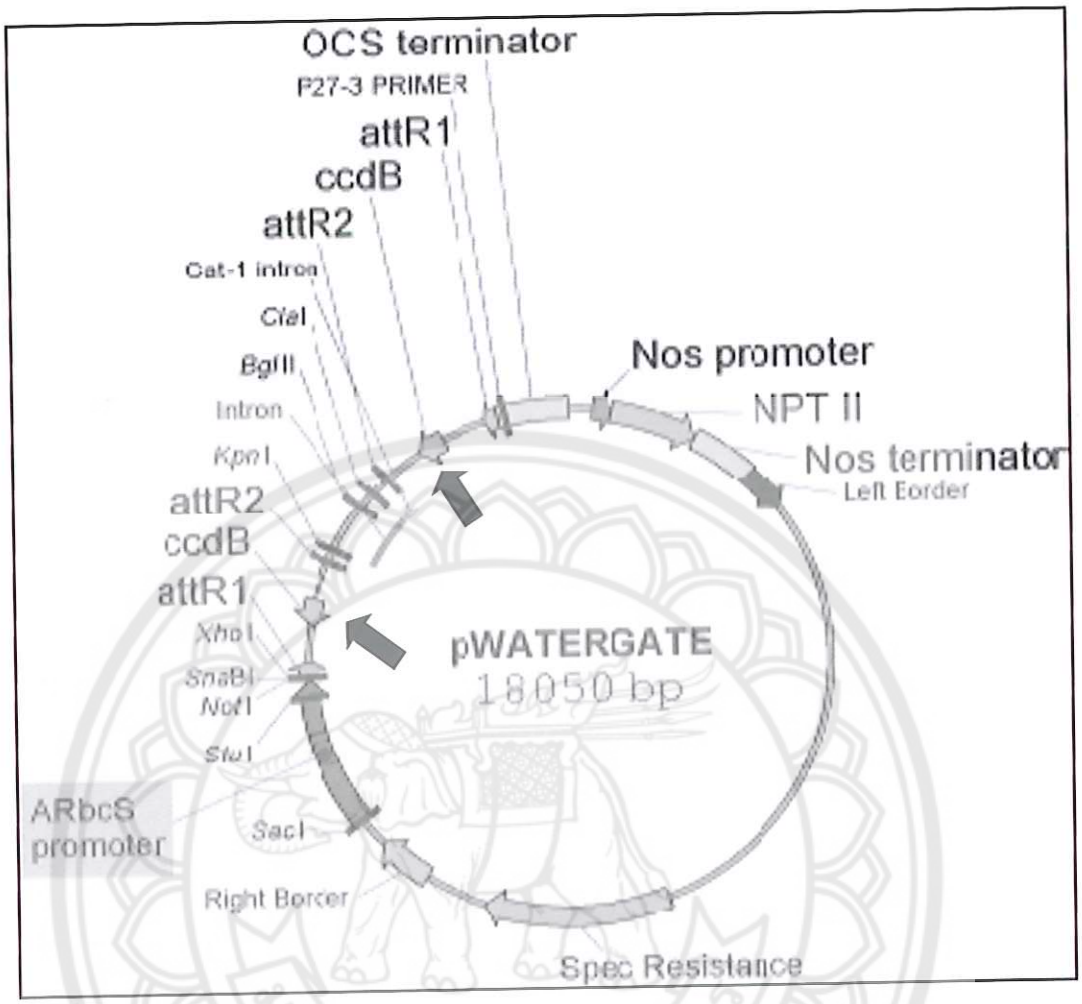

Figure 5 Vector map of pWATERGATE; (Black arrows show the exchanging sites between Entry clone and Destination vector)