CHAPTER I
INTRODUCTION

Thailand is one of the world rice producers among others big sources in the Southeast Asia such as China, India, Indonesia, Bangladesh and Vietnam. It has been estimated that Thailand share approximately 4% of the world rice production, With substantial export share of 30% in the world market. Thailand has been one of the rice exporters to all parts of the world for almost 20 years. It was reported that in 1999/2000 crop Thailand planted about 59.1 million rais (9.0 million hectares) of rice to produce 22.5 million tons of paddy, yielding roughly 15 millions Tons of milled rice. Of this, approximately 9 million tons was used for local consumption. The balance is about 6 million tons left available for export (Thailand, Office of Agricultural Economics., 2003)

Abiotic stress are prevalent in nature and can substantially diminish play yields. Based on the FAO/Unesco Soil Map of the world, the total area of saline soils is 397 million ha and that of sodic soils is 434 million ha, which are not necessarily arable but cover all salt –affected lands at global level. Of the current 230 million ha of irrigated land, 45 million ha are salt-affect soils (19.5 percent) and of the almost 1,500 million ha of dryland agriculture, 32 million are salt-affected soils (2.1 percent) to vary degrees by human-induced processes (FAO, 2000)

Plant responses to stressful environmental factors can be part of the mechanisms that permit the plant to withstand the stress. Some of the most universal features of plant form, such as waxy coatings on shoots, reduction of leaf growth or production of specialized leaf surfaces to avoid transpiration, and pores that can open and close on leaves, seem largely designed to cope with the challenge. However, this ability may not always increase the ability to plants to survive in natural system. Conventional breeding has been used regularly for crop improvement and production of a new variety. This process is simple in outline but complex in practice. Not only plant-breeding programmes are long-term and expensive, They also require the cultivation and analysis of large numbers of plants which takes both time and space.

Additionally, in some cases, the breeder has limited genetic variation for incorporation into new varieties (Connett and Barfoot, 1992). In addition to classical breeding approaches, recent work has used genetic transformation to introduce various genes into plants for better tolerance to abiotic stress.

Most recent research on desiccation tolerance has focused on discovering the mechanisms of desiccation tolerance, partly in the hopes of some day engineering tolerance in economically important species and banishing the specter of famine from drought (Alpert and Oliver., 2002). One approach of physiological research in dehydration tolerance has been used specific structures or species that can withstand severe desiccation. Most prominent in this category are seeds.

Seeds develop in 3 stages: 1) Embryogenesis, the formation of the embryo, during which all the cells are formed. 2) Seed formation, when the cells swell and accumulate storage proteins and lipids. 3) Maturation, a period of drying out when the seed is dormant and as much as 90% of the original water is removed in attaining a state of dormancy with unmeasurable metabolism. This desiccation state allows survival under extreme environmental conditions and favors wide dispersal. The embryo cannot withstand desiccation at all developmental stages; tolerance is usually acquired well before maturation drying but is lost as germination progresses (Ingram and Bartels., 1996).

1.1 LEA protein

LEA (Late Embryogenic Abundant Protein) proteins represent one major group of proteins that are reported to be expressed in response to dehydration in desiccation tolerant plants (Close., 1997). LEA proteins comprise a large family of plant proteins that accumulate to high levels during late stages of embryo development (Galau et al., 1986). Expression studies show that LEA proteins are generally associated with cellular dehydration in seeds and in response to water deficit in vegetative tissues. Treating plant tissues with the plant hormone ABA can also induce the expression of *Lea* genes. A common feature of most LEA proteins is their high hydrophillicity, which remains solubility after boiling. LEA proteins are highly

hydrophillic, it appears unlikely that they occur in specific cellular structure. Also, their high concentrations in the cell and biased amino acid compositions suggest that they do not function as enzymes. The randomly coiled moieties of some LEA proteins are consistent with a role in binding water. Total desiccation is probably lethal, and therefore such proteins could help maintain the minimum cellular water requirement (Ingram and Bartel., 1996)

LEA protein from different plant species have been divided into groups based on predicted biochemical properties and sequence similarities (Dure et al., 1989). The strong conservation of motifs in LEA proteins evolution points to domains with functional constraint has observed. One such motif, which was characteristic for group 1 LEA proteins, was internal 20 amino acids motif that could be repeated up to four times depending on the species and was first found in the wheat EM protein (Burns et al., 1997). Group 2 LEA proteins, also referred as dehydrins, are the most widely studied LEA proteins (Close., 1997). Many homologues have been isolated from species ranging from gymnosperms to dicotyledonous and monocotyledonous angiosperms. Dehydrins are characterized by a lysine rich 15 amino acids motif (termed the K-segment) that was predicted to form an amphipathic α-helix, a tract of continous serine residues and a conserved motif containing the consensus sequence DEYGNP that was found close to the N-terminus of the protein. Group 3 LEA proteins were characterized by having various number of a tandemly repeated 11-amino acids motif. This motif was predicted to form an alpha helix which was amphiphilic, having a hydrophobic surface (Chen and chen., 1996). In relation to the others, LEA proteins belonging to groups 4 and 5 were less frequently represented in the literature. Group 4 was characterized by a conserved N-terminus predicted to form α-helics and a diverse C-terminal part with a random coil structure. Group 5 LEA proteins contained more hydrophobic residues than groups 1 to 4 and consequently were not soluble after boiling, leading to the suggestion that they probably adopted a globular conformation (Phillips et al., 2002)

1.2 The *HVA*1 gene

The *hva*1 gene was first isolated from the aleurone layers of barley seeds as an ABA-inducible gene. The deduced amino acid sequence from the corresponding cDNA indicates that *hva*1 encodes a 22 kDa protein which contains nine imperfects of 11 amino acids motif, a characteristic feature of group 3 LEA proteins. *HVA*1 mRNA was rapidly induced in young seedlings (3 days after imbibition) by ABA and a series of stress conditions including partial dehydration, cold, NaCl, and heat (Sivamani et al., 2000)

Xu et al. (1996) produced transgenic rice plants expressing the barley *hva*1 gene, driven by a constitutive promoter. This lead to the constitutive accumulation of HVA1 protein in both leaves and roots of transgenic rice plants. The second generation transgenic rice plants showed increased tolerance to water deficit and salinity. In a second study, *HVA*1 was introduced into spring wheat (Sivamani et al., 2000). High levels of expression of the *hva*1 gene, regulated by a maize ubiquitin promoter, were observed in leaves and roots of independent transgenic wheat plants. Progenies of four selected transgenic wheat lines were tested under greenhouse conditions for tolerance of soil water deficit. Potted plants were grown under moderate water deficit and well-watered conditions, respectively. Two homozygous and one heterozygous transgenic lines expressing the *hva*1 gene had significantly higher use efficiency values as compared to the non-expressing transgenic and non-transgenic controls under moderate water deficit conditions. The two homozygous transgenic plant lines also had significantly greater total dry mass, root fresh and dry weights, and shoots dry weight compared to the two controls under soil water deficit conditions. As in the case for all LEAs, the precise mode of action of HVA1 under drought conditions remains unclear.

1.3 Rice transformation

Early success in the production of transgenic rice involved electroporation and the polyethylene glycol-mediated (PEG-mediated) transfer of genes. First fertile transgenic *japonica* (Shimamoto et al., 1989) and *indica* (Datta et al., 1990) rice were also obtained from protoplasts by using electroporation and PEG, respectively. The limitation of these methods was the requirement of regeneration system from protoplasts. Although some successful results were reported. Plant regenerated from protoplasts were often sterile and phenotypically abnormal. Some of these problems were probably related to the use of cells that have been cultured for long periods. (Hiei et al., 1997) In 1991, Christou et al. reported the recovery of first transgenic rice plant using particle bombardment method and since then this method has been extensively used for rice transformation. This technique is based on high velocity bombardment of plant cells with DNA-coated microprojectiles (tungsten or gold) accelerated by gun powder discharge or pressurized helium or electric current (Sanford, 1990). Like other methods, the particle acceleration too has its drawbacks. Some transgenic plants produced from bombarded explants have been reported to have very high transgene copy numbers and complex integration patterns. Since clustered inserts may increase the risk of gene silencing and DNA rearrangements (Repellin et al., 2001). The other major drawback was the restricted availability of the equipment because of its high cost (Christou and Ford, 1995). Hiei et al., (1994) reported a method for efficient production of transgenic rice plants from calli of *japonica* cultivars that had been cocultivated with *A. tumefaciens*. Their evidence was based on molecular analysis and genetic studies of a large number of transgenic plants and the analysis of sequence of T-DNA junctions in rice. Rashid et al. (1996) reported the successful application of such a method to *Basmati* cultivars of *indica* rice after only minor modifications. Park et al. (1996) reported transformation of rice with isolated shoot apices as explant used for co-cultivation and regenerated transgenic plants. Because of low copy integration, precise mode of T-DNA transfer and inexpensive nature, *Agrobacterium*-mediated transformation has a potential to develop into a method of choice.

1.4 *Agrobacterium*-mediated transfer of foreign genes into target plants

Agrobacterium tumefaciens is a soil bacterium that can genetically transform plant cells with a segment of DNA (transfer DNA, abbreviated as T-DNA) from a tumor-inducing plasmid (Ti plasmid) with the resultant production of a crown gall, which is a plant tumor (Hiei et al., 1997).

Virulent *Agrobacterium* strains contain a very large plasmid, known as the tumor-inducing or Ti plasmid. These plasmids vary in size between 130 and 230 kb in individual strains of bacteria. Non-virulent *Agrobacterium* strains, which are unable to infect plants, contain no Ti plasmid (Hughes ., 1996). The T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins; and the genes encoding for the synthesis of opines. These compounds resulted from condensation between amino acids and sugars, which are produced and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and for the bacterium-bacterium plasmid conjugative transfer genes (Riva et al., 1998).

The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the co-operative action of proteins encoding by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The 30 kb virulence (*vir*) region is a regulon organised in six operons that are essential for the T-DNA transfer (*vir*A, *vir*B, *vir*D, and *vir*G) or for the increasing of transfer efficiency (*vir*C and *vir*E) (Riva et al., 1998). Regulation of the *vir*-genes is mediated by the proteins of the *vir*A and *vir*G genes, which together encode a two component regulatory system with the *vir*A protein acting as the chemoreceptor (of the phenolic compounds) and VirG functioning as a transcriptional activator of the *vir*-genes after having been phosphorylated by VirA on a critical aspartate residue (Hooykaas., 2000) (Fig 1.1).

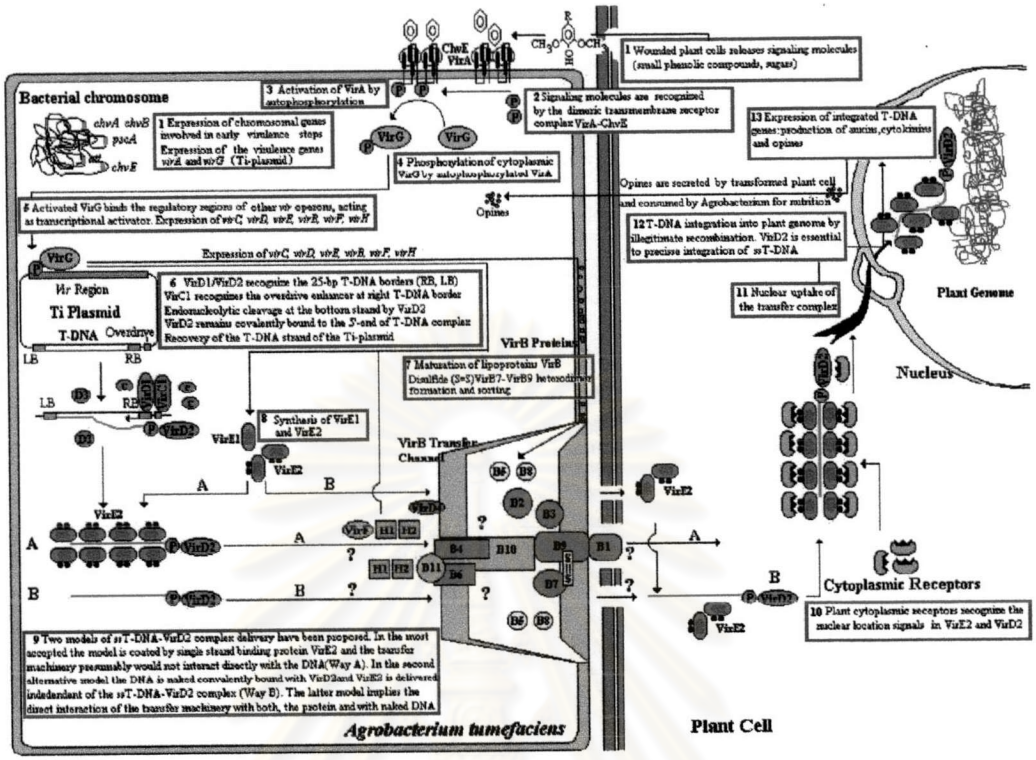

Figure 1.1 Basic steps in the transformation of plant cells by *A. tumefaciens* (Zupan and Zambryski, 1995)

Following *vir* gene induction, the production of a transfer intermediate begins with the generation of the T-strand, an single strand copy of the T-DNA (Stachel et al., 1986). VirD1 and VirD2 are essential for this process (Filichkin and Gelvin, 1993). Together, VirD1/VirD2 recognize the 25-bp border sequence and produce an single strand endonucleolytic cleavage in the bottom strand of each border . These nicks are used as the initiation and termination sites for T-strand production. After nicking, VirD2 remains tightly associated with the 5′ end of the T-strand. The lone VirD2 at the 5′ end gives the T-complex a polar character that may ensure that, in subsequent steps, the 5′ end is the leading end. T-strand production is thought to result from the displacement of the bottom strand of the T-DNA between the nicks (Zupan and Zambryski., 1995).

The T-strand must travel through numerous membranes and cellular spaces before arrival in the plant nucleus. Thus, to preserve its integrity, it was hypothesized that the T-strand likely travels as an single strand DNA-protein complex. VirE2 is an inducible single strand nucleic acid-binding protein encoded by the *vir*E locus that binds without sequence specificity. VirE2 binds tightly and cooperatively, which means that a T-strand would be completely coated. Consequently, degradation by nucleases will be prevented and, indeed, *in vitro* binding of VirE2 renders single strand DNA resistant to nucleolytic degradation. Finally, binding of VirE2 unfolds and extends single strand DNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. The T-strand along with VirD2 and VirE2 are termed the T-complex. Subsequently, the T-complex must exit the bacterial cell passing through the inner and outer membranes as well as the bacterial cell wall. It must then cross the plant cell wall and membrane. Once inside the plant cell, The T-complex targets to the plant cell nucleus and crosses the nuclear membrane, after which the T-strand becomes integrated into a plant chromosome (Zupan and Zambryski., 1995).

A number of sophisticated plant-transformation vectors, designed on the basic of this naturally occuring gene-transfer mechanism, have been developed and such vectors are widely employed in plant molecular biology and in the genetic engineering of plants. In the Ti plasmid, the gene responsible for the transfer of T-DNA are clustered together in a region known as the virulence region (Klee et al., 1983). The transfer process is active even when the virulence genes and the T-DNA are located on separate replicons in an *A. tumefaciens* cell. In a widely used binary system, a Ti plasmid serves as a helper, providing the virulence functions, and an artificial T-DNA, which contains a selectable marker gene and other genes of interesting, is placed on a second, small plasmid, which is often referred to as a binary vector (Hiei et al., 1997). The use of such vectors for gene delivery to higher plants has several advantages in addition to its convenience and efficiency; it allow the transfer of large segments of DNA with minimal rearrangement and the integration of small numbers of copies of genes into plant chromosome (Klee et al., 1987). Foreign genes delivered by this method are usually transmitted to progeny plants in a Mendelian manner (Budar et al., 1986)

1.5 Rice transformation using *Agrobacterium*

Tingay et al., (1997) transformaed immature barley embryo explants with *Agrobacterium tumefaciens* carrying a binary vector coding for chimaeric bacterial genes, *bar* and *gus*. From 1282 embryos, plants were recovered for 54 independently transformed lines, giving a transformation efficiency of 4.2%. Integration of both genes was confirmed by gel blot hybridization analysis of DNA from the transformed plants. Both marker genes, *bar* and *gus*, were expressed and co-segregated in the T1 progeny plants.

Rashid et al., (1996) developed the reproducible system production of transgenic plants in *indica* rice using *Agrobacterium* – mediated gene transfer. Three-week-old scutella calli served as an excellent starting material. These were infected with an *Agrobacterium tumefaciens* strain EHA101 carrying a plasmid pIG121Hm containing genes for β-glucuronidase (GUS) and hygromycin resistance (HygR). Hygromycin (50 mg/l) was used as a selectable agent. Inclusion of acetosyringone (50 μM) in the *Agrobacterium* suspension and co-culture media proved to be indispensable for successful transformation. Transformation efficiency of *Basmati* 370 was 22% which was as high as reported in *japonica* rice and dicots. A large number of morphologically normal, fertile transgenic plants were obtained. Integration of foreign genes into the genome of transgenic plants was confirmed by Southern blot analysis. GUS and HygR genes were inherited and expressed in R1 progeny. Mendelian segregation was observed in some R1 progeny.

1.6 Selectable Marker Genes

A selectable marker gene is used to recover transformants after a gene transfer experiment. It encodes a protein that confers on transformed cells and the ability to grow on media containing a toxic compound for untransformed cells. Transformant will emerge from the mass of untransformed tissue because of the advantage given by the expression of the resistance gene. The gene product of a selectable marker gene can be a detoxifying enzyme able to degrade the selective agent (Guerineau., 1995).

1.7 Hygromycin phosphotransferase gene (*hpt* gene)

Hygromycin B is an aminocyclitol produced by *Streptomyces hygroscopicus*. It inhibits protein synthesis in both prokaryotes and eukaryotes, interfering with ribosomal translocation and aminoacyl-tRNA recognition and causing misreading of mRNA (Zheng et al., 1991). The *hpt* combination was successfully employed in the genetic transformation of tobacco , *Arabidopsis*, maize and rice (Schrott et al., 1995). Hygromycin is a more potent phytotoxic compound than kanamycin; especially in cereal crops (Galum and Breiman., 1997). Hygromycin allows clear discrimination between transformed and non-transformed tissues and problems with albinos or the fertility of regenerants have not been reported (Ayres et al., 1994)

1.8 Bialaphos Resistance Gene (*bar*)

Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* It consists of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of these residues by peptidases, PPT is a potent inhibitor of glutamine synthetase (GS). This enzyme plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants (Block et al. 1987). It is the only enzyme that detoxifies ammonia produced during nitrate reduction, photorespiration, and amino acid degradation in plant cells. Inhibition of GS by PPT causes rapid accumulation of ammonia which leads to death of the plant cell. The bialaphos resistance gene (*bar*) from *Streptomyces hygroscopicus* which is involved in the bialaphos biosynthesis pathway. It encodes a phosphinothricin acetyltransferase, which acetylates the free NH_2 group of PPT and prevents

autotoxicity in the producing organism. The *bar* gene has been a useful selectable marker for obtaining transgenic monocots (Irvine and Meagher., 1996) such as corn (Gordon-Kamm et al., 1990), oat (Somers et al., 1992), rice (Toki et al., 1992), wheat (Vasil et al., 1992; Weeks et al.., 1993), barley (Wan et al., 1994)

1.9 Reporter gene in plants

Reporter genes are coding sequences that, upon expression in the transgenic plant, provide a clear indication that genetic transformation did take place (Galun and Breiman, 1997). In general, reporter genes should have the following characteristics: 1) the genetic organization should be well described, 2) the gene products should not be present in the organism or tissue under study, 3) the gene products should be well characterized with regard to biochemical activity, 4) substrate dependence and stability, and 5) the product of the reaction catalyzed by the reporter gene product should be stable, easily detectable, and quantifiable (Crazzolara et al., 1995).

1.10 Green Fluorescent Protein (GFP)

The jellyfish (*Aequorea victoria*) green fluorescent protein (GFP) gene has recently been shown to possess a number of desirable traits as a reporter especially in living tissue (Sheen et al., 1995). The green-fluorescent protein (GFP) emits bright fluorescence (λ_{max}=509 nm., with minor peak at 540 nm) upon excitation with ultraviolet (λ_{max}=395 nm) or blue (λ_{max}=475 nm) light. The 29.3 kDa protein consists of 238 amino acids and deletion mapping has established that almost the complete sequence (from amino acid 2 to 232) is required to maintain fluorescence. (Elliott et al., 1999).

The structure of GFP has been solved using seleniomethionyl-substituted protein and multi-wavelength anomalous dispersion (MAD) phasing methods. The eletron density maps produced by the MAD phasing were very clear, revealing a dimer comprised of two quite regular-barrels with 11 strands on the out side of cylinders. The fluorophore is highly protected, located on the central helix within a couple of Angstroms of the geometric center of cylinder (Yang et al., 1996) (Fig 1.2). *Aequorea* GFP owes its visible absorbance and fluorescence to a *p*-hydroxybenzylideneimidazolinone chromophore (Prasher et al., 1992). The chromophore formation is now beleived to proceed in three steps: Cyclization and dehydration of the main chain atom of Ser-65-Typ-66-Gly-67 to an imidazolone ring after the protein is folded, and oxidation of the CA-CB bond to residue 66 to acquire the extended conjugated system necessary for long-wavelength absorption (Palm and Wlodawer., 1999) (Fig 1.3). The fluorescence is independent of cell type or location (Urwin et al., 1999). GFP formation and fluorescence have been established in number of species (Cubitt et al., 1995) with only molecular oxygen being identified as a possible cofactor for chromophore formation. Hence the formation of the fluorescent chromophore is either autocatalytic or requires only ubiquitous cellular components (Chalfie et al., 1994)

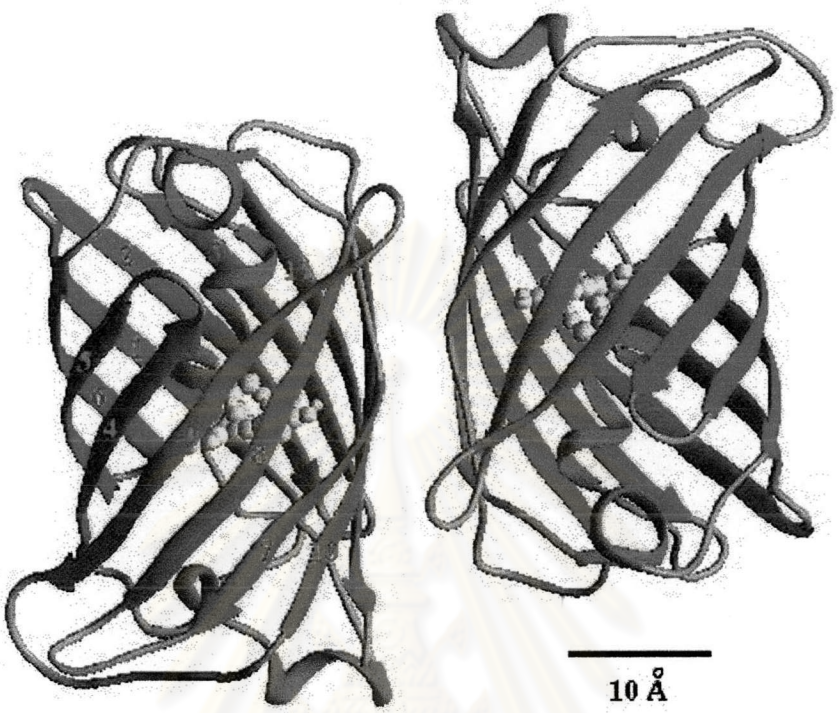

Figure 1.2 The overall shape of the green fluorescent protein and its association into dimer. Eleven strands of β-sheet (green) formed the wall of a cylinder. Short segments of α-helices (blue) capped the top and bottom of the 'β-can' and also provided a scaffold for the fluorophore which was near geometric center of the can. This folding motif, with β-sheet outside and helix inside, represented a new class of proteins. Two monomers were associated into a dimer in the crystal and in solution at low ionic strengths. This view was directly down the two-fold axis of the non-crystallographic symmetry (Yang et al. 1996).

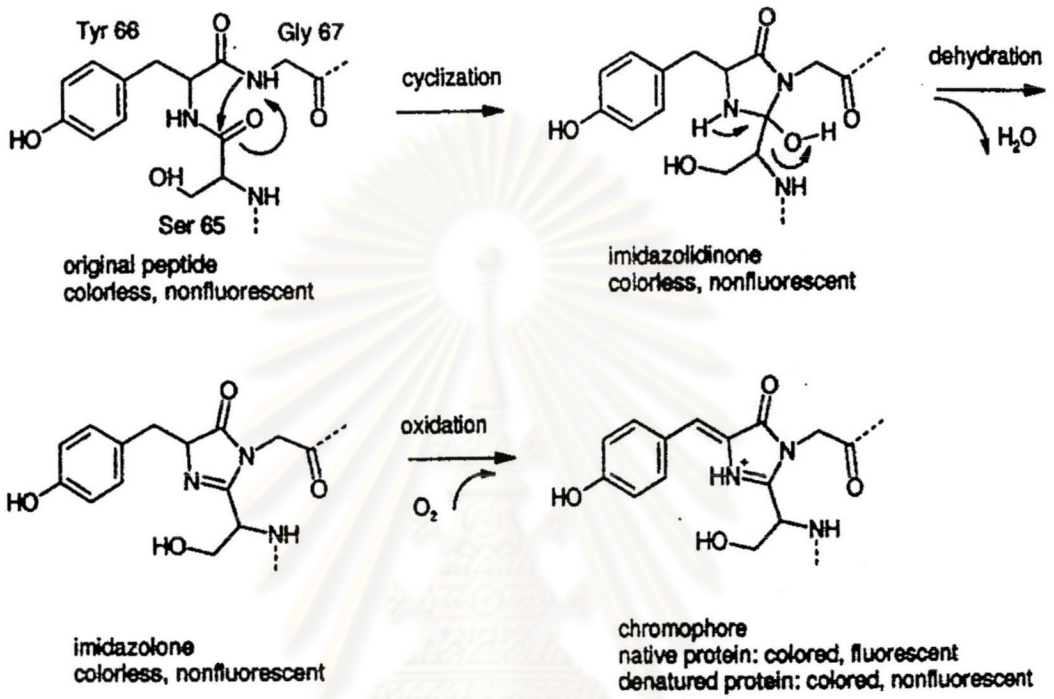

Figure 1.3 Steps in the formation of the chromophore

GFP has attracted widespread interest and is considered to have several advantages over other visual marker genes. The prime advantage of GFP as a reporter gene is that no exogenously supplied substrate or cofactors need to supply substrates and cofactors to produce positive signals. The detection of the commonly used reporters, such as β-glucuronidase (GUS) (Jefferson, 1987; Jefferson et al., 1987), β-galactosidase (LacZ) (Helmer et al., 1987; Terri et al., 1989), chloramphenicol acetyltransferase (CAT) (Seed and Sheen., 1988), and luciferase (LUC) (Gallie et al., 1989; Millar et al., 1992; Ow et al., 1986) requires either exogenous substrates/co-factors or antibodies. Their application are sometimes limited by problems in substrate uptake, leaky product, cell fixation, and cell permealization especially in multicellular organisms (Sheen et al., 1995).

Objectives of the thesis

1) To transform *indica* rice *O. sativa* cv. KDML 105 with green fluorescent protein by co-cultivation with *Agrobacterium* and confirm stable integration and transmission of the transgene in T_0 and T_1 regeneration

2) To transform *O. sativa* cv. KDML 105 with the barley *hva*1 gene by co-cultivation with *Agrobacterium* and to detect expression of this gene in T_0 plants by RT-PCR