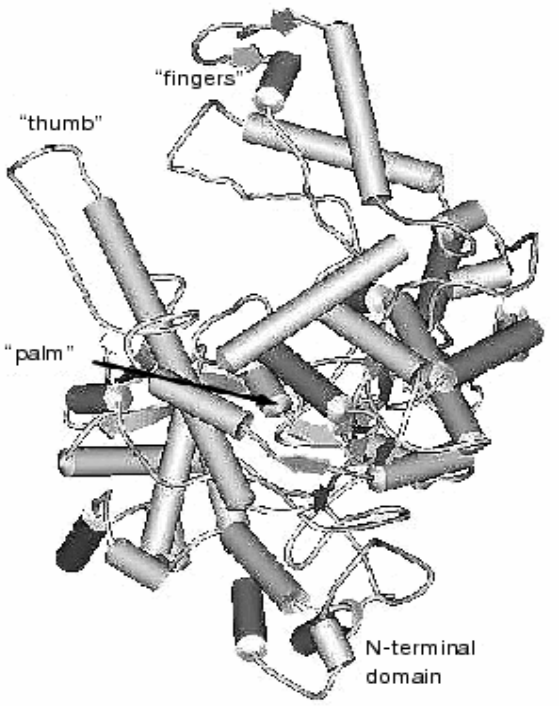
# I. BACTERIOPHAGE T7 RNA POLYMERASE

## Basic properties of bacteriophage T7 RNA polymerase

The transcription in prokaryotic and eukaryotic cells is carried out by the complex multi-subunit RNA polymerases. On the other hand RNA polymerases of certain phages such as T7, T3, SP6, and K11, and also mitochondrial RNA polymerases (McAllister and Raskin, 1993; Sousa, 1996) are single subunit enzymes. All these enzymes are able to perform the complete transcriptional cycle in the absence of any additional protein factors. This family of RNA polymerases is also characterized by higher (compared to bacterial RNA polymerases) rate of RNA synthesis and specificity towards their promoters. All these properties make the phage polymerases a rather convenient model for the investigation of the physicochemical aspects of transcription. The most extensively studied enzyme in this group is bacteriophage T7 RNA polymerase (T7 RNAP).

T7 RNAP was first isolated from bacteriophage T7-infected *Escherichia coli* cells in 1968 (Chamberlin *et al.*, 1970). The primary structure of T7 RNAP was determined during the 1980s (Moffatt *et al.*, 1984). Now, based on the X-ray analysis of four T7 RNAP crystal structures (Sousa *et al.*, 1993; Cheetham and Steitz, 1999), a model of the initiation complex has been published (Cheetham and Steitz, 2000). T7 RNAP transcribes late genes of bacteriophage T7. Owing to its molecular properties, the enzyme is widely used as a tool for synthesis of specific transcripts, as well as being a suitable model for studying the mechanisms of transcription. The polypeptide chain of the enzyme consists of 883 amino acid (aa) residues (MW 98092 Da) (Mofatt *et al.*, 1984). T7 RNAP is structurally related to the members of a super family of nucleotide polymerases that includes single subunit DNAPs and RNAPs such as *E. coli* DNAP I and reverse transcriptases. X-ray studies have demonstrated a marked resemblance between the three-dimensional structures of T7 RNAP.




Three-dimensional structure of T7 RNAP (Cheetham and Steitz, 1999)

Thus, despite the almost complete lack of sequence homology, T7 RNAP and Klenow fragment of *E. coli* DNAP I demonstrate a very high structural similarity: when polymerization domains of these enzymes are superimposed, all K-helices and L-strands (except one) in the two structures correspond to each other. The shapes of these domains resemble the right arm of a man and consist of the subdomains `palm', `thumb' and `fingers' (Cheetham and Steitz, 1999).

## T7 promoter and terminator

BglII — **T7-Promoter** →

***AGA TCT*** **CGA TCC CGC GAA ATT AAT ACG ACT CAC TAT AG**G GAG ACC

rbs

ACA ACG GTT TCC CTC TAG AAA TAA TTT TGT TTA ACT TTA AGA AGG AGA

NcoI

TAT A*CC ATG G*CA GCA **GCC ATC ATC ATC ATC ATC ACA GCA GCG GCC**

NdeI XhoI BamHI

**TGG TGC CGC GCG GCA GC*C ATA TGC TCG AGG ATC C*GG CTG CTA ACA**

**AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCG CTG AGC AAT**

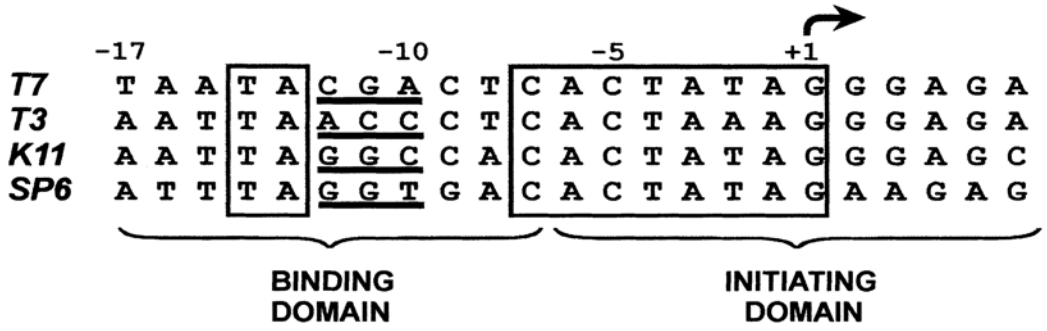
**T7-terminator**

**AACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG**

The natural T7 promoter shares a highly conserved sequence covering bp -17 to +6 relative to the start of the RNA chain (Dunn and studier, 1983). Efficient termination signals for T7 RNA polymerase also appear to be rare, so that the enzyme should be capable of making complete transcripts from almost any DNA. Transcription does terminate efficiently at T¢, the natural terminator from T7 DNA (Carter *et al*., 1981; Studier and Moffatt, 1986).

**T7 RNAP-promoter interactions**

T7 RNAP is a common paradigm for studies of transcription initiation, as it is a single-subunit enzyme sharing many of the biochemical characteristics of the more complex multi-subunit RNAPs from prokaryotes and eukaryotes (Chamberline *et al*., 1970). T7 RNAP catalyzes the transcription from late promoters of bacteriophage T7 recognizing the 23 bp consensus sequence.




Consensus sequences of class III promoters of bacteriophages T7, T3, K11, and SP6. The identical sites are boxed. The `specificity triplets' are underlined.

In spite of the fact that its binding affinity is 2-3 orders lower than for E. coli RNAP, T7 RNAP is absolutely specific to T7 promoters, and exhibits no affinity even to T3 promoters (Chaberline *et al*., 1970). Their consensus sequences differ only in the triplet (310 to 312), so the latter is believed to play the major role in the recognition by the respective RNAPs (Klement *et al.*, 1990). Mutagenesis studies indicate that T7 promoters apparently consist of two functional sites: binding (317 to 36) and initiating (36 to +6). T7 RNAP interacts with promoter asymmetrically, contacting the sequences 317 to 313, 37 to 31 and 314 to 39, and 33 to +2 of the coding and non-coding chains, respectively (Muller *et al*., 1989).

**Bacteriophage T7 RNAP and nuclear localization signal peptide**

A potential barrier to use T7 RNA polymerase for selective transcription in eukaryotic cell is entrance of the enzyme into the cell nucleus. As a prokaryotic protein, T7RNA polymerase would not be expected to contain signals for nuclear localization, and the protein seems rather large to freely diffuse through nuclear pores (Paine *et al*., 1975). However, ability to penetrate the nucleus may be needed for realizing the full potential of T7 RNA polymerase for selective transcription in eukaryotic cells or whole animals (Dunn *et al*., 1988).

Nuclear localization appears to be specified by relatively small signals in nuclear proteins (Dingwall *et al*., 1982; Robert *et al*., 1987). In the case of large-T antigen of SV40, the nuclear location signal has been shown to be short, predominantly basis amino acid sequence that is both necessary and sufficient for transport into the nucleus (Kalderon *et al*.,1984; Colledge *et al.*, 1986). This signal can also direct a normally cytoplasmic protein to the nucleus (Robert *et al*., 1987).

Insertion of the nuclear location signal between codons 10 and 11 of T7 RNA polymerase has only minimal effect on transcription activity in *E.coli*, but its insertion four codons from the C terminus abolishes activity. Fusion protein having only foreign codons ahead of codon 11 also has transcription activity in *E.coli*. Such fusion protein can be expressed transiently from plasmids microinjected into monkey cells, using SV40 expression signals, and detected by immunofluorescence. A fusion protein containing a nuclear location signal localizes predominantly in the nucleus whereas those which lack the signal localize predominantly in the cytoplasm. Ability to direct T7 RNA polymerase to the nucleus may be an advantage in attempting to make enzyme useful for selective transcription in eukaryotic cells.

## II. TRANSGENE EXPRESSION UNDER VARIOUS TISSUES SPECIFIC PLANT PROMOTERS USED IN THE PRESENT STUDY

The success of foreign gene expression in transgenic plants depends very much on promoter sequences (Vasil, 1994). To express the transgenes in plant cells, appropriate promoter sequences have to be introduced alongside the gene to ensure efficient transcription of mRNA (Shimamoto *et al*., 1989). A large number of promoters have been used in plant transformation and several promoter sequences have been isolated from monocots for tissue specific use in cereal species for the efficient expression of the transgenes. Some of plant promoters analysed for tissue specific expression is shown below:

| **Gene/ Promoter** | **Plant** | **Transgenic host** | **Organ/ Tissue** | **Reference** |
|---|---|---|---|---|
| *rbc*S | Pea | Tobacco | Green | Fluhr *et al*., 1985 |
| *rbc*S | Pea | Petunia | Green | Fluhr *et al.*, 1986 |
| *rbc*S | N.*Plumba* | Petunia, tobacco | Green | Poulsen *et al*., 1986 |
| *Cab* | Pea | Tobacco | Green | Lamppa *et al*., 1985 |
| *Cab* | Wheat | Tobacco | Green | Lamppa *et al.*, 1985 |
| *ST-LS1* | Potato | Tobacco | Green | Stockhaus *et al.*, 1987 |
| Patatin | Potato | Potato | Tuber | Twell and Ooms, 1987 |
| Lehaemoglo-bin | Soybean | Lotus | Nodule | Stougaard *et al*., 1987 |
| Nodulin N23 | Soybean | Lotus, *Trifolium* | Nodule | Jorgensen *et al.*, 1988 |
| β-Phaseolin | French bean | Tobacco | Seed | Sengupta-Gopalan *et al.*, 1985 |
| β-Conglycin | French | Tobacco | Seed | Sengupta-Gopalan *et al.*, 1985 |
| α'-Subunit | Soybean | Petunia, Tobacco | Seed | Chen *et al.*, 1986 |
| β-subunit | Soybean | Tobacco | Seed | Bray *et al*., 1987 |
| Lectin | Soybean | Tobacco | Seed, Root | Okamuro *et al.*, 1986 |

| | | | | |
|---|---|---|---|---|
| Glutenins | Wheat | Tobacco | Seed | Colot *et al*., 1987 |
| Zein | Maize | Tobacco | Seed | Matzke *et al*., 1990 |
| Legumin | Pea | *N. plumba* | Seed | Ellis *et al*., 1988 |
| EPSP Synthase | Petunia | Petunia, Tobacco | Flower Pollen | Benfey and Chua , 1989 |
| *Kin1/Cor6.6* | *Arabidopsis* | Tobacco | Guard cell | Wang *et al.,* 1995 |
| *GluB1* | Pea | Rice | Seed | Goto *et al*., 1999 |
| *Pal 1* | *Arabidopsis* | *Arabidopsis*, Tobacco | Vascular cell | Ohl *et al.*, 1990 |
| *Pds* | Tomato | *Arabidopsis* | Fruit | Rosati *et al*., 2000 |

## 1. Promoters analysed for foreign genes under T7-expression system

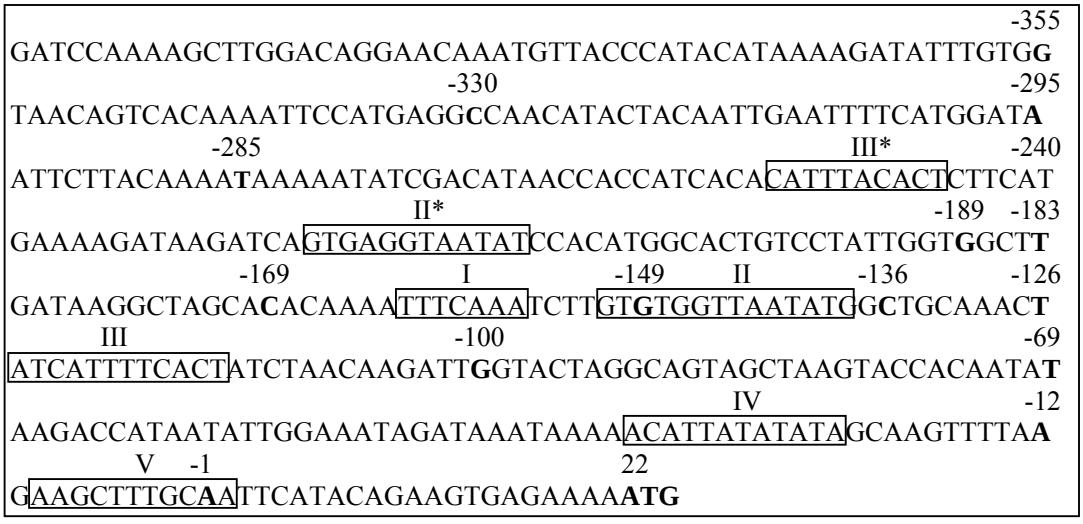
### Cauliflower mosaic virus 35S promoter

The cauliflower mosaic virus (CaMV) 35S promoter is a strong, constitutive promoter used in many transformation studies (Benfey and Chua, 1990), including those for the monocotyledons rice (Christou *et al*., 1991, Datta *et al*., 1990), wheat (Vasil *et al*., 1992), and maize (Gordon-Kamm *et al*., 1990). However, although this promoter was active in rice, transcript accumulation was relatively low compared to tobacco (McEloy *et al.,* 1991). The level of transgene expression under this promoter has been shown to vary between different species of plants and different tissues with the same plant (Nillson *et al*., 1996).

The expression pattern of a CaMV 35S-promoter-GUS fusion in primarily at or around vascular tissue in leaves, roots and flowers (Terada and Shimamoto, 1990). The embryo and endosperm of both dormant and germinating seed also showed GUS expression. Battraw and Hall (1990) found wider expression of a similar construct; expression was detected in most cell types of the leaves and roots of transgenic Taipei 309 rice plants. Unfortunately the levels of gene expression produced by this promoter in cereals are less than in dicots (Fromm *et al*., 1985).

**Ribulose-bisphosphate carboxylase (*rbc*S) promoter**

The pea *rbc*S-3A gene is a member of the gene family that codes for small subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*S), the key enzyme in photosynthetic carbon assimilation. The expression of *rbc*S genes is regulated by at least three parameters: Tissue type, light condition, and stage of development (Tobin and Silverthorne, 1985; Kuhlemeier *et al*., 1987). The *rbc*S-3A transcript levels are highest in leaves, lower in stem, and below the limits of detection in root (Coruzzi *et al*., 1986; Fluhr *et al*., 1986). Gene transfer experiments demonstrated that a 280 base pair (bp) enhancer-like element in the upstream region (at positions -330 to -50 relative to the transcription start site can confer both light induction and organ specificity upon a reporter gene (Fluhr *et al.*, 1986).




*In vitro* protein binding experiments identified a protein factor, GT1 that binds to two conserved sequences, box II and III, in the -150 region. Two additional binding sites, boxes II* and III*, with sequence homology to the box II and III were detected further upstream, around position –220 (Green *et al.*, 1987). These boxes have strong sequence homology to the constitutive enhancer elements from simian virus 40 (SV40) and adenovirus, but detail *in vivo* experiments showed that they can function as negative light-regulatory elements (LREs) (Kuhlemeier *et al.*, 1987).

### *Kin1* and *Cor6.6* promoters

The gene *kin1*, identified in *Arabidopsis thaliana* by Kurkela and Franck (1990), codes for 6.5 kDa protein those compositional similarity to the fish alanine-rich antifreeze protein (Davies and Hew, 1990) and its induction by low temperature has led to the suggestion that the gene product may function as an antifreeze protein (Kurkela and Franck, 1990). The gene *kin1* belongs to a family of two in *Arabidosis thaliana* and second gene was reported as *cor6.6* (Gilmour *et al*., 1991) and as *kin2* (Kurkela and Borg Franck, 1992). The mRNA levels of both genes increased dramatically upon low temperature treatment and was also inducible by ABA (Hejela *et al*.,1990). The *Arabidopsis thaliana* genes *kin1* and *cor6.6* belong to the same family and expression at higher levels following low temperature and ABA treatments. The putative promoters of both genes were fused to the GUS gene and GUS expression was analysed in transgenic tobacco and *Arabidopsis* plant showed that GUS activity directed by both *kin1* and *cor6.6* promoters were significantly induced by ABA, hydration and osmoticum (Wang *et al*., 1995). Transgenic plant with either the *kin1* or *cor6.6* promoters showed strong GUS expression in pollen, developing seeds, trichomes and most interestingly, in guard cells. During pollen development, maximum GUS activity was found in mature pollen. In contrast, the maximum GUS activity during seed development was during early embryogenesis. There was no maijor qualitative difference in pattern of GUS expression between *kin1* and *cor6.6* promoters and the results were similar for transgenic tobacco and *Arabidopsis* (Wang and Cutler, 1995).

### Phenylalanine ammonia-lyase (PAL) promoter

Phenylalanine ammonia-lyase catalyzes the deamination of L-phenylalanine to trans-cinnamic acid, which is the first step in the biosynthesis of large class of plant natural products based on the phenylpropane skeleton, including lignin monomers as well as certain classes of phytoalexins (Dixon *et al.*, 1983; Jones, 1984). In dicots, induction of lignin deposition in peripheral tissues and accumulation of furanocoumarin and isoflavonoid-derived phytoalexins help protect plants against

mechanical damage and potential pathogens (Dixon *et al*., 1983; Lamb *et al.*, 1989), and in monocots, enhancer lignin synthesis is a major component in a battery of induced defense response (Beardmore *et al*., 1983; Ride, 1975; Vance *et al*., 1980). Transgenic tobacco plants with suppressed levels of PAL activity deposit less lignin and are more sensitive to pathogen attack than wild type tobacco plants (Minami *et al*., 1989).

PAL is encoded by small gene family in bean (Cramer *et al*., 1989), pea (Kawamata *et al*., 1992), parsley (Maher et al., 1994), *Arabidopsis* (Ohl *et al.*, 1990), alfalfa (Gowri *et al*., 1991), poplar (Subramaniam *et al*., 1993) and tobacco (Pellegrini *et al.*, 1994). Transcripts of individual PAL genes show relatively different patterns of accumulation (Liang *et al.*, 1989). The expression of PAL genes is induced in suspension-cultured cells by elicitors from fungal cell wall and in plants by pathogen attack (Lois and Dietrich, 1989). PAL expression is strongly activated during the hypersensitive response in tobacco, potato and parsley plants infected by tobacco mosaic virus (TMV), *Phytophthora infestans* and *Phytophthora mesgasperma* sp. glycinea respectively (Cuypers *et al.*, 1988; Pellegrini *et al*., 1994). Nuclear run-off transcription experiments have shown that elicitor and UV-light induction of PAL mRNA accumulation in cell suspension cultures of bean and parsley reflect transient stimulation of PAL gene transcription (Lawton and Lamb, 1987; Lois *et al*., 1989). Likewise, analysis of the bean PAL2 promoter in transgenic tobacco and potato plants containing PAL2-β-glucuronidase (GUS) gene fusions has demonstrated tissue and cell type-specific PAL transcription during development and in response to wounding and light (Bevan *et al*., 1989; Liang *et al.*, 1989). In all cases so far investigated in higher plants, the changes of PAL activity are regulated at the transcription level (Zhu *et al.*, 1995).

The PAL1 promoter (from *Arabidopsis*) was activated early in seedling development and in adult plants was strongly expressed in the vascular tissues of roots and leaves, but was not active in the root tip or the shoot apical meristem. In

flowers, expression was observed in sepals, anthers, and carpels, but not in petals. Transcripts encoded by the endogenous PAL genes and GUS transcripts from the PAL1-GUS gene fusion were induced by wounding, $HgCl_2$ stress and light (Ohl *et al*., 1990).

In rice, PAL transcripts accumulated to a high level in stems, with lower levels in roots and leaves. The transcription of the ZB8 gene was investigated by fusing its promoter to the reporter gene GUS shown that high levels of GUS activity were observed in stem, moderate levels in roots and low levels in leaves of transgenic rice and tobacco plants. In transgenic rice the promoter was active in root apical tips, lateral root initiation sites, and vascular and epidermal tissues of stems and roots. In rice flower, high GUS activity was observed in floral shoots, receptacles, anthers and filaments, occasionally GUS activity was also detected in lemma and awn tissues. In tobacco flower, high GUS activity was detected in the pink part of petals. The infection of tobacco mosaic virus in the tobacco leaves induced GUS activity to a high level. Fungal cell wall elicitors strongly induced GUS activity and GUS transcripts to high levels in transgenic rice suspension-cultured cells (Zhu *et al*., 1995).

### ***GluB-1*** **promoter**

Glutelins are the predominant storage proteins in rice seeds, accounting for 80% of the total endosperm protein. They begin to accumulate six days after the anthesis and continue to accumulate until about 20 days after flowering (Takaiwa *et al*., 1991). The glutelins consist of heterogeneous acidic (37-39 kDa) and basic subunits (22-23 kDa) linked by a disulfide bond. Both subunits are derived from a common large 57 kDa precursor which is post-translationally cleaved (Szabados *et al.*, 1990).

Rice glutelins are coded for by small multigene family (Takaiwa *et al.*, 1989; Takaiwa and Oono, 1991), which can be subdivided into two subfamilies,

subfamily A containing types I, II and III with a total of at most 5 members and subfamily B. The degree of homology among all subfamily A members is above 80% in their coding regions, and their 5'-flanking region are relatively well conserved to a position 170 bp upstream from the transcription start site (Okita *et al*., 1989).

The analysis of *cis*-acting elements involved in tissue-specific expression has been carried out for soybean conglycinin (Chen *et al.*, 1986; Fujiwara and Beachy, 1994), pea legumin (Shirsat *et al*., 1990), bean phaseolin (Leisy *et al*., 1990; Yoshihara and Takaiwa, 1996; Zhao *et al*., 1994). Furthermore, sites for binding of nuclear proteins found in maturing seeds have been reported in upstream region required for such expression (Thomas, 1993).

Studies aimed at understanding the regulatory mechanisms responsible for the endosperm-specific expression revealed the presence of various *cis*-regulatory elements in the 5'- flanking region of the glutelin *GluB-1* gene The essential *cis*-regulatory elements governing the spatially and temporally specific expression of the glutelin gene expression were located within the first 245 bp of the promoter region of the *GluB-1* gene from the site of initiation of transcription (Takaiwa *et al.*, 1996).

### *Pds* promoter

Carotenoids are terpenoid pigments which are accumulated in the chloroplasts of leaves and in the chromoplasts of many flowers and fruits. Phytoene desaturase (Pds), the second dedicated enzyme in carotenoid biosynthesis, is encoded in tomato by a single copy gene. A 2 kb fragment from the tomato *Pds* gene, comprising 1.5 kb from the promoter and 0.5 kb from the 5' non-translated region, is able to drive developmentally regulated expression of the GUS reporter gene in transgenic tomato and tobacco plants (Giuliano *et al.*, 1993). In tomato, high levels of *Pds*/GUS expression are found in organs and at stages of development where

chromoplasts are formed: petals, anthers and ripening fruits. Tobacco petals and fruits, which do not contain chromoplasts, show instead low levels of *Pds*/GUS expression. Transgenic tobacco seedlings were subjected to treatment with a range of inhibitors of carotenoid and chlorophyll biosynthesis. In green tissues, carotenoid and chlorophyll levels are tightly co-regulated and that a chemically induced arrest in pigment biosynthesis results in activation of the *Pds* promoter.

High levels of GUS activity under Pds promoter were found only in plant containing the -1530/+564 promoter region, suggesting that the 5' non translated leader contain sequences necessary for high level of expression. The tomato flowers contain high level of carotenoids, mainly xanthophylls, located in chromoplast. *Pds* mRNA level are induced approximately 10-fold during anthesis, when carotenoid accumulation is maximal (Giuliano *et al.*, 1993). The mRNA is localized in chromoplast-containing organs, that is, petals and anthers. In tomato transgene, GUS activity reaches maximal levels during anthesis. High levels of expression are found in petals and anther, and in the pistil. The *Pds*/GUS transgene show low level of expression in pericarp from unripe fruit (Immature Green and Mature Green). A seven-to eight fold induction is observed between the mature green and the turning stage of fruit ripening (Corona *et al.*, 1996).

## 2. Expression of foreign genes investigated under T7-expression system

### GUS - Reporter gene

Reporter genes are used in plant transformation for the analysis of promoter activities, for monitoring the efficiency of selection and for following the inheritance of foreign genes in subsequent generations. The *gus*A (*uid*A) locus of *E.coli*, which encodes the β-glucuronidase enzyme, is the most popular reporter gene used in plant transformation (Jefferson *et al*., 1987, 1991).The β - glucuronidase catalyses the hydrolysis and cleavage of a wide range of fluoromatric and histochemical β - glucuronidase substrates. The activity of the GUS enzyme

can be easily and sensitively assayed in plants as there is no background activity, the expression of *gus*A gene fusions can be quantified by fluorometric assay, and histochemical analysis can be used to localize gene activity in transgenic tissues (Jefferson, 1987; Jefferson *et al.*, 1991).

**Iron storing Ferritin gene**

Ferritin is an iron-storage protein found in animals, plants and bacteria (Yablonski and Theil, 1992; Dix *et al.*, 1992). Comprising 24 homologous or heterologous subunits, it assembles to form a large complex (45-kDa) that stores up to 4500 iron atoms in its central cavity (Theil, 1987; Andrews *et al.*, 1992). The ferritin gene has isolated and sequenced in plants, including soybean (Ragland *et al.,* 1990, 1993), French ban (Spence *et al*., 1991), pea (Lobreaux *et al.*, 1992) and maize (Lobreaux *et al*., 1992). Although the amino acid sequence homology between plant and mammalian ferritin is low [39-49% within the transit and extension peptide (Andrews *et al*., 1992); 56-66% within the additional peptide (Ragland *et al.,* 1990)], its molecular conformation and iron-storage capacity is conserved.

Ferritin is thought to play two main roles in living cells, both through modulation of iron content (Theil, 1990). One is to provide iron for the synthesis of iron protein such as ferredoxin and cytochromes. The other is to prevent damage from free radicals produced by iron/dioxygene interactions. In animals, ferritin synthesis is regulated at the translational, not the transcriptional levels. Translation of the ferritin mRNA increases in response to high levels of iron, thus producing more ferritin for storage, and conversely, translation is decreased by low levels of iron. In plants, however, iron-dependent regulation of phytoferritin synthesis occurs at the transcriptional level (Van der Mark *et al*., 1983; Lescure *et al.*,1991) or post-transcriptionally in developing nodules and is also responsive to developmental cues (Lobreaux and Briat, 1991; Ragland and Theil, 1993).

The maximum iron content in leaves of transgenic tobacco plants was approximately 30% higher than that of non-transformants (Goto *et al*., 1999). The seeds from the transgenic rice accumulated up to threefold more iron than normal seeds (Goto *et al.,* 1999). Beard *et al.* (1996) re-valued the dietary iron sources and showed that iron stored in ferritin is bioavalable in animals made anemic by dietary iron deficiency, the bioavalability of extrinsic ferritin as an iron supplement is high as that of FeSO4. Thus, from a nutritive point of view, edible vegetables accumulating ferritin can be beneficial due to increased iron absorption (Goto *et al*., 1999).

**Hepatitis B surface antigen (HBsAg) – Edible vaccine candidate antigen**

Over two billion people worldwide are infected with hepatitis B, a serious liver infection that can result in jaundice, cirrhosis, and liver cancer. Although an injectable vaccine now exists, its expense and requirement for refrigeration makes it unavailable to more than one-third of the world's population, especially in poor countries where the vaccine is in urgent need (Walmsley and Arntzen, 2000; Richter *et al*., 2000).

The current hepatitis vaccine extracted from yeast requires chemical modification to become active, increasing the cost of the vaccine, which also must be stored under refrigeration. This has severely limited its utility in third world regions where the disease in rampant. Edible vaccines have the potential to address many of the problems facing synthesis and distribution of vaccines. Plant-based vaccines can be grown locally, reducing the cost and complications of transportation, while the stability of proteins in intact plants removes the need for refrigeration. Furthermore, the edible nature of the vaccines eliminates the need for syringe-based delivery, saving money and reducing the risk of infections (Langridge, 2000).

To overcome this limitation, studies various ways to increase plant production of the hepatitis B antigen, HBsAg, in potato, the results of their efforts, along with

further proof of the effectiveness of this form of the vaccine (Richter, *et al*., 2000). One of the main concerns about using a plant-based method of administering a hepatitis vaccine has been that transmission of hepatitis is believed to be non-enteric (not through the digestive tract). Although the plant-produced form of HBsAg was capable of initiating an immune response when injected into mice, they still needed to establish that the protein contained in potato tissue could stimulate the same response when fed to mice. The ability of plant-produced HBsAg to trigger an immune response when administered orally had been previously established (Kapusta *et al.,* 1999; Walmsley and Arntzen, 2000).

**Provitamine A**

Lycopene is the main carotene accumulated in ripe tomato fruits. This linear carotenoid is synthesised in plants through a pathway starting from geranylgeranyl diphosphate and is the biosynthetic precursor of most cyclic carotenoids, including beta-carotene. Lycopene is converted into beta-carotene by the action of lycopene beta-cyclase (β-Lcy), an enzyme introducing beta-ionone rings at both ends of the molecule (Hugueney *et al*., 1995; Cunningham *et al*., 1998). Beta-carotene is the major dietary precursor of vitamin A (Lakshman. and Okoh, 1993). Vitamin A deficiency (VAD) is a major public health problem in several countries, most of them localised in the developing world. Beta-carotene supplementation of the diet in areas at risk of VAD decreases morbidity and mortality related to several pathological conditions (Fawzi *et al*., 1993; West *et al*., 1999). The US Recommended Dietary Allowance (RDA) is 1 mg/day retinol equivalents (approximately 6 mg beta-carotene equivalents). Lycopene does not have pro-vitamin A activity, but it is a good dietary antioxidant. High plasma lycopene levels have been associated with a decreased incidence of prostate cancer (Gann *et al*., 1999). Metabolic engineering is one of the possible approaches to improve the levels of vitamins/antioxidant compounds in crop plants. The engineering of high vitamin E (alpha-tocopherol) levels has been obtained in *Arabidopsis* seeds (Shintani and DellaPenna, 1998). A major recent breakthrough has been the

engineering of beta-carotene in the endosperm of rice, a major staple food in areas at high risk of VAD (Ye *et al*., 2000).

The regulation of genes mediating plant carotenoid biosynthesis has made great progress in the past few years (Bartley *et al*., 1994; Cunningham and Gantt, 1998). The genes controlling lycopene synthesis in tomato, such as *Psy1* and *Pds* (encoding, respectively, the fruit-specific phytoene synthase and the phytoene desaturase) are up-regulated during fruit ripening (Corona *et al*., 1996), while those controlling lycopene cyclisation, like *β-Lcy* or *ε-Lcy* are down-regulated (Pecker *et al*., 1996; Ronen *et al*., 1999). As a result, beta-carotene in ripe tomato fruits does not exceed 15% of the total carotenoids. Several attempts have been made to engineer higher lycopene levels in tomato fruit, but none of them has met success. In one of these attempts, a bacterial phytoene desaturase (crtI, able to transform phytoene into lycopene) was fused to a plastidic transit peptide and introduced in tomato plants under the control of the CaMV *35S* promoter (*35S/tp/crtI*). This experiment, aimed at increasing lycopene levels, has unexpectedly resulted in a threefold increase in beta-carotene, but not in lycopene. The total carotenoid levels are in fact decreased in the transformants, and the reasons for this result are still poorly understood (Giuliano *et al.*, 2000). A slight enrichment of beta-cyclic carotenoids in leaves is observed in the transformants due to the constitutive nature of the promoter used.

## III. REGULATED FOREIGN GENE EXPRESSION IN TRANSGENIC PLANTS

The ability to analyze the expression of modified gene in a multicellular organism has contributed greatly to the understanding of gene regulation. Transgenic organisms are of particular use in the study of development, since gene expression can be analyzed at different stages and in various tissues. In higher plants, DNA can be introduced into somatic cells by *Agrobacterium*-mediated (Horsch *et al.,* 1985) or direct gene transfer (Paszkowski *et al.*, 1992). Stably integrated transgenes inherit to their offspring in a Mendelian fashion. The environmental stimuli

influencing gene expression that have been investigated with transgenic plants include light, heat, anaerobic stress, wounding (Broglie *et al.*, 1984; Timko *et al.*, 1985; Walker *et al.*, 1987) and chemical (Gatz *et al.,* 1992, 1995; Weinmann *et al*., 1994).

The ability to control expression of a gene via highly specific mechanism offers unique opportunities to study the physiological functions of certain gene products at different stages of development. The correlation of the phenotype with the kinetics of induction allows differentiation between primary and secondary consequences, which generates another advantage of a regulated expression system. Moreover, a stringently regulated promoter is absolutely required, if the expression of gene product interferes with the regeneration process. Some of the promoters and inducers characterized for controlled expression of foreign genes are listed below:

| **Gene** | **Plant** | **Transgenic host** | **Inducer** | **Reference** |
|---|---|---|---|---|
| *rbc*S | Pea | Tobacco | Light | Fluhr *et al*., 1985. |
| *rbc*S | Pea | Tobacco | Light | Sengupta-Gopalan *et al.*, 1985. |
| *rbc*S | *N.plumba* | Petunia | Light | Poulsen *et al*., 1986 |
| *rbc*S | Soybean | Petunia | Light | Shirley *et al*., 1987 |
| *Cab* | Pea | Tobacco | Light | Simpson *et al*., 1986 |
| *Cab* | Wheat | Tobacco | Light | Nagy *et al*., 1986, 1987 |
| *Cab* | *N.plumba* | Tobacco | Light | Castresana *et al.*, 1988 |
| Chalcone Synthase | *A. majus* | Tobacco | Light | Kaulen *et al*., 1986 |
| *ST-LS1* | Potato | Tobacco | Light | Stockhaus *et al.*, 1987 |
| *Hse* | Maize | Tobacco | Heat | Rochester *et al*., 1986 |
| *Hse* | Soybean | Tobacco | Heat | Baumann *et al*., 1987 |
| *ADH* | Maize | Tobacco | Anaerobic stress | Ellis *et al*., 1987 |

| | | | | |
|---|---|---|---|---|
| Protease inhibitor | Potato | Tobacco | Wounding | Sanchez-Serrano *et al*., 1987. |
| *Cab* | Wheat | Tobacco | Circadian clock | Nagy *et al*., 1988. |
| *Kin1/cor6.6* | *Arabidopsis* | Tobacco | Cold, ABA | Wang *et al.,* 1995. |
| *Ttriplex 35S* | Tobacco | Tobacco | Tetracycline | Gatz *et al*., 1992. |
| *Pal* 1 | *Arabidopsis* | Tobacco | Wounding, $HgCl_2$ | Ohl *et al.*, 1990. |

**Tetracycline inducible transgene expression in plants**

The Tn10 encoded tet repressor (TetR) regulates the expression of the tetracycline (Tc) resistance operon by binding to nearly identical operator sequences that overlap with three divergent promoters (Bertrand *et al.*, 1993; Hillen *et al*., 1984). The genes of the *tet* operon are only transcribed in the presence of the inducer Tc, which prevents the repressor from binding to its operator sequences. Using the Tn10 encoded Tet repressor (TetR) in combination with a suitably engineered cauliflower Mosaic Virus (CaMV) 35S promoter with three integrated tet operator sites, (Gatz *et al*., 1992) have shown the Tc inducible expression of a reporter gene tobacco.