INTRODUCTION

Every living organism comes under various conditions of stress during its life span. Each of the organisms have evolved their own ingenious methods of overcoming the stress. In the case of plants the process of overcoming stress would have to be unique and efficient as they are immobile and static in nature, in contrast to the highly mobile animals. Plants in nature have to counter both the biotic and abiotic stresses at the same given place in contrast to animals. Biotic stress normally include the pathogen infection by the fungi, bacteria and viruses, infestation by nematodes etc. While, abiotic stresses include those such as high/low temperatures, UV irradiation, toxic contaminants in water and soil, drought and water logging and other associated environmental conditions.

To counter these adverse conditions plants have not only evolved morphological adaptions, but also system(s) of modulating their gene expression by transcriptional switching on and off a battery of genes. Two most studied examples of this modulation are the synthesis of (i) pathogenesis related proteins in response to viral infection and (ii) heat shock proteins in response to higher temperatures. This kind of modulation in the gene expression is also observed in the plant defense response to pathogens which often lead to systemic acquired resistance (SAR).

Infection of a plant by pathogen (biotic stress) takes place one time or the other in its life span and no plant is an exception to it. The establishment of the
pathogen on the plant leading to the infection or inability for the pathogen to cause infection depends upon the plant-pathogen interaction/perception. Specificity underlying the above mechanism is thought to be decided over by the compatible or incompatible interaction the pathogen has with the host plant. In an incompatible interaction the pathogen is unable to establish the infection as early molecular recognition, followed by rapid expression of defense responses leads to the hypersensitive reaction. Hypersensitive reaction basically encompasses the localised activation of plant defense responses and cellular necrosis around the infected region leading to the initial stop of the spread of infection (Lamb et al., 1989; Schmelzer et al., 1989). While, in the case of compatible interaction the pathogen is able to elude the host defense response mechanism (Lamb et al., 1989). This phenomena is based on the ‘gene for gene hypothesis’ proposed by Keen (1992) which has received considerable experimental support.

Plants on being infected with pathogen induces an elaborate set of defense responses similar to that of the immune system as represented in the vertebrate animals. The following are some of the major inducible biochemical responses which would play a active role in the defense of the plant; (i) activation of the phenylpropanoid pathway enzymes consequently leading to the accumulation of antimicrobial phytoalexins (Hahlbrock and Scheel, 1989; Dixon and Lamb, 19904), deposition of wall bound phenolics, lignin and callose leading to the reinforcement of the cell-wall (Brisson et al., 1994), (ii) accumulation of
hydroxy-proline rich glycoproteins in the cells (Keen, 1992), (iii) inducible synthesis of pathogenesis related (PR) proteins (Somssich et al., 1986) and (iv) production of proteinase inhibitors and lytic enzymes such as chitinases and glucanases which in turn could act on the cell-wall of the microbes (Bowles, 1990; Collinge et al., 1993). Many of the induced PR proteins are identified to be either a class of chitinases or glucanases, which in turn indicate the underlying mechanism of their role in plant defense (Collinge et al., 1993).

Plants depending upon the complexity of interaction/perception would activate defense responses by any of the above processes independently or synergistically activating all of them to contain the pathogen infection. This kind of activation of a panoply of genes by the plant to a pathogen attack would indicate a very efficient interplay of receptors, signals, signal transduction pathways and finally the gene activation, in some cases within few minutes (Cramer et al., 1985). Genes of phenylpropanoid pathway are one of the sets of genes activated in the plants during the stress conditions faced by it. All the various genes isolated of the phenylpropanoid pathway get activated to various stresses and their activation leads to the production of antimicrobial isoflavanoid phytoalexins, lignin deposition and UV protectant flavanoids.

PAL being the first enzyme in the phenylpropanoid pathway catalyzes the committed step of oxidative-deamination of phenylalanine to trans-cinnamic acid and being inducible by stress conditions shows to have major regulatory role in the pathway. The PAL enzyme levels gets enhanced in plant systems to a
variety of biotic/abiotic factors such as fungal cell-wall elicitors, pathogen infection, mechanical wounding, UV, light, ethylene, HgCl₂, etc. The enhanced levels of PAL enzyme in response to various stimuli can directly be correlated with the transcriptional activation of the pal genes. Thus, pal gene system appears to be a novel one in terms of being transcriptionally activated by a variety of conditions. A simplest model for transcription of an inducible eukaryotic gene is that in response to a signal, a trans-acting protein binds to its cognate cis-sequence element located in the upstream region of the gene, making the gene transcriptionally activated. The activation of pal in response to a diverse array of factors predicts the presence of multitude of cis elements, each one possibly responsive to a different factor or signal. It may alternatively be assigned that only a limited repertoire of responsive elements are present in the promoter and transcriptional response is mediated by a common set of trans-acting proteins, differentially modified post-translationally in response to a particular signal. It may be argued that a single gene may not respond to all the factors but various members of the gene family may get transcriptionally activated in response to different factors.

The pal genes serve as a good model system to investigate these alternatives. However, it would be desirable to have a single or a very limited number of members of the gene family. For this purpose Arabidopsis thaliana a small plant of the curciferae family appears to be an ideal system. The plant Arabidopsis has already become an ideal experimental system for plant
molecular genetic studies. Small genome size (7 X 10^7 bp), short life cycle, near absence of dispersed middle repetitive DNA and well-established classical genetics are some of its desirable features.

One of the pal genes, named *Atpal1* was already isolated and sequenced in the laboratory (Thungapathra, 1990). A single 4.8 kb EcoRI fragment contains the complete gene including its upstream and downstream regions. The fragment contains over 1.8 kb of the upstream region. In order to study the transcriptonal regulation of the gene, its upstream regions has to be analyzed for the presence of the sequence elements actually conferring the inducibility on the gene. Such a study could be done two different ways:- (i) Assay based on the expression system: The expression of a reporter gene under the control of the regulatory region of the gene is monitored either in a transient assay system or in transgenic plants after stable inrtegration of the gene construct following transformation and (ii) Assay based on the DNA-protein interaction: By using gel retardation or *in vitro* foot-printing, putative *cis* elements could be delineated. In this study the first assay described was used by making the *Atpal1* promoter:*gusA* gene construct, transferring it to tobacco plants via *Agrobacterium*-mediated transformation system and analyzing the GUS expression under various conditions. Besides this it is imperative to isolate the other members of the gene family.

The main objectives of the work presented in this dissertation are (i) Isolation and characterization of the others members of the *pal* gene family; (ii)
Generation of \textit{Atpal1:gusA} gene construct suitable for expression; (iii) Production of transgenic tobacco plants following \textit{Agrobacterium}-mediated gene transfer; (iv) Analysis of transgenic tobacco plants carrying the \textit{Atpal1:gusA} construct by monitoring the GUS expression in response to (a) developmental and tissue-specific pattern and (b) various biotic and abiotic stress factors; (v) Analyzing various transgenic plants with a view to monitor any variability in the pattern of GUS expression.
Fig. 1: A Schematic representation of the Phenylpropanoid pathway showing some of the important enzymes

PAL - Phenylalanine ammonia lyase
C4H - *trans*-Cinnamate-4 hydroxylase
4CL - 4-Cinnamate CoA ligase
SS - Stilbene synthase
CHS - Chalcone synthase
CCR - 4-Coumaryl-CoA ligase
CHI - Chalcone isomerase
IFR - Isoflavone reductase
COMT - Caffeic acid-o-methyl transferase
CAD - 4-Coumaraldehyde dehydrogenase
PHENYLPROPANOID PATHWAY

Phenylalanine → trans-cinnamic acid → 4-Hydroxy cinnamic acid → 4-Coumaraldehyde → 4-Coumaryl alcohol → Lignin precursors

Wall-bound Phenolics
Stilbene (resveratrol)

4CL
4-Hydroxy cinnamyl Co.A

Naringenin chalcone

CHS
Naringenin flavonone

IFR
Isoflavonoid Phytoalexins (glyceollin, Coumestrol, Kievitone)

CCR
Flavanoid pigments and UV protectants (anthocyanins, anthocyanidins, Keampferol)

COMT
Sinapyl esters
Ferulic esters

Furanocoumarin Phytoalexins (psoralens)

Chlorogenic acid and its esters

Caffeic acid

Lignin, suberin Structural polymers in xylem/cell-wall
LITERATURE REVIEW

STRUCTURE AND EXPRESSION OF PHENYLALANINE AMMONIA LYASE GENE

A diverse array of phenylpropanoid secondary metabolites are synthesized in higher plants by general phenylpropanoid pathway which may include its ancillary branch pathways. Each of the ancillary pathways derive their basic phenylpropanoid building unit from the core reactions of general phenylpropanoid metabolism (Fig 1). The ancillary branch pathways lead to the synthesis of lignins, suberins, wall-bound and soluble esters of cinnamate (Lewis and Yamamoto, 1990), stilbenes, flavanoids/coumarins (Hahlbrock and Scheel, 1989).

Phenylpropanoids play a key role in plant development and in protection against environmental stresses. They serve as UV absorbing flavanoids, low-molecular weight flower pigments, anti-microbial phytoalexins, wound protectant hydroxy-cinnamic acid esters and polymeric constituents of the surface/support structures such as lignin, suberin and other cell-wall components (Hahlbrock and Scheel, 1989; Rhodes, 1994). Phenylpropanoids or their derivatives have also been implicated in the plant-microbial recognition process involving Agrobacterium and Rhizobium. Acetosyringone derived from the phenylpropanoid wound metabolites and luteolin, a simple flavanoid are thought to play a role in virulence of Agrobacterium (Stachel and Zambryski,
1986) and induction of nodulation genes in *Rhizobium* (Downie and Johnston, 1986). Several genes of the pathway are also transcriptionally activated in plant-mycorrhizal symbiotic interaction (Harrison and Dixon, 1994).

**Role of Phenylpropanoids in Plant Defense**

Involvement of phenylpropanoids in plant defense had been postulated on the basis of synthesis and accumulation of phytoalexins in the host plants in response to pathogen infection. Infection of pea plants by the fungus *Nectaria haematococca* results in accumulation of phytoalexin, pisatin in the host plant with concomitant induction of phytoalexin detoxifying enzyme, pisatin demethylase (PDA) in the pathogen (Kistler and van Etten, 1984; Weltring et al., 1988). The results suggest (i) the involvement of phytoalexin in plant defense response and (ii) a direct relationship between the degree of pathogenicity of the fungus and detoxification of phytoalexins by specific fungal enzymes. Since phytoalexins are metabolic end products of the phenylpropanoid pathway, all the enzymatic steps (Fig. 1) leading to their synthesis should be inducible in response to fungal infection. In fact, it has been found to be the case (Lamb et al., 1989). Owing to the inducibility of the genes encoding these enzymes in response to pathogen infection, has led to the designation of these genes as plant defense genes. Phenylalanine ammonia lyase (PAL) is the first enzyme of the pathway (Fig. 1) and its inhibition by a specific inhibitor, α-amino-oxy-3-phenyl propionic acid renders soybean susceptible to normally
avirulent races of *Phytophthora megasperma* (Lamb et al., 1989). Induction of defense response by either fungal infection, elicitors isolated from fungal cell-walls or wounding leads to the transcriptional activation of defense genes as a part of massive shift in pattern of host gene expression (Chappell and Hahlbrock, 1984; Cramer et al., 1985; Lawton and Lamb, 1987). In elicitor treated bean suspension cultured cells, the defense genes encoding chitinases and enzymes of phenylpropanoid biosynthesis involved in phytoalexin and lignin production, including PAL, chalcone synthase (CHS), chalcone isomerase (CHI) and cinnamyl-alcohol dehydrogenase (CAD) are activated within 2 to 3 min of elicitor treatment (Lawton and Lamb, 1987; Walter et al., 1988; Hedrick et al., 1988). The rapid activation of these sets of genes indicates an active involvement of inducible defense system in plants in response to the stress conditions created by pathogen invasion, wounding etc. This inference gets corroborated by the timing of induction of PAL, CHS and CAD in a coordinated fashion and a different pattern of induction between compatible and incompatible interactions involving bean hypocotyls and its fungal pathogen, *Colletotrichum lindemuthianum*. The activation of these and other non phenolic defense responses is much more rapid in incompatible interactions than compatible ones (Hahlbrock and Scheel, 1989; Keen, 1992). On the other hand, in the case compatible interactions, there appears to be suppression of the phenylpropanoid pathway genes by the pathogen as has been observed in the case of pea (Yoshioka et al., 1992; Yamada et al., 1992). Similar observations
were made in the case of tomato plants infected with wilt fungus, *Verticillium alboatrum*. The resistant tomato lines show reduced PAL suppression as compared with the susceptible lines, wherein a close correlation is seen between rapid suberization of the xylem in the resistant plants and a rapid increase in the activity of PAL (Lee et al., 1992b).

**Role of Phenylpropanoids in General Plant Metabolism**

Phenylpropanoids besides being important in plant defense also play a role in development and structural organisation of the cells. Phenylpropanoids such as lignins, suberins and wall bound phenolic cinnamate esters are major structural constituents of the cell-wall and these components provide basic structural support to the plant (Lewis and Yamamoto, 1990; Bolwell, 1988; Carpita et al., 1993).

Phenylpropanoids also appear during seed germination and are distributed all over the seedling in a tissue-specific manner (Jahnen and Hahlbrock, 1988). High levels of PAL, CHS and CHI transcripts are found in the young roots and root-tips of the alfalfa (*Medicago sativa* L.) plants (Mckhann and Hirsh, 1994). Flavones and flavanones, the end-products of phenylpropanoid pathway play a major role in nodulation and evidence for their involvement comes from the activation of nodulation genes in the presence of flavones (Redmond et al., 1986). Role of phenylpropanoids has also been postulated in cheamo-atraction of rhizobia to roots (Ceatono-Annolles et al., 1988; Lynn and Chang, 1990) and
growth enhancement of rhizobia on the minimal media (Hartwig et al., 1991). The phenylpropanoids are also implicated in other interactive processes i.e., Agrobacterium infection (Stachel and Zambryski, 1986) and plant-mycorrhizal symbiosis (Harison and Dixon, 1994).

Recent evidences have indicated a role of phenylpropanoids in the process of fruit ripening. Transcript levels of PAL, CHS and CHI appear to be co-regulated along with 1-amino-cyclopropane-1-carboxylicacid (ACC) synthase transcripts. They all seem to be activated in synchrony to signals of fruit ripening in melon-fruit in contrast to ACC oxidase whose activation precedes that of the ACC synthase (Diallinas et al., 1994). Inhibition of the CHS activity by anti-sense chs gene construct produces self-sterile plants possibly implicating flavanoids in fertilization (Ylstra et al., 1994). This loss of fertility is attributed to inability of maintaining the structural integrity of pollen-tube apex, which leads to rupturing and loss of contents of the apex during the growth of the pollen tube in the style after pollination. In the normal wild-type plants massive accumulation of flavanols is seen in the pollen tube apex (Ylstra et al., 1994).

Catechin, a key component in determining the tea-quality is a phenylpropanoid. The various cultivars of tea contain different amounts of catechin. It is postulated that the regulated biosynthetic activites of the enzymes of the phenylpropanoid pathway determine the varying catechin contents in the tea plants. The PAL activity in different cultivars varies and shows a correlation with the catechin content (Matasumoto et al., 1994).
Phenylalanine ammonia-lyase (PAL)- first enzyme in the Phenylpropanoid pathway

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is the first and key enzyme of the phenylpropanoid pathway. PAL catalyzes the deamination of phenylalanine to trans-cinnamic acid and this reaction in turn regulates the metabolism of phenylalanine and the level of phenylpropanoids in plants. PAL is inhibited by its product trans-cinnamic acid (Jones, 1984) which suggests it being under strict regulatory control. PAL has been purified from a variety of sources including parsley, pea, potato and Phaseolus. Subcellular localization indicates that PAL is mainly cytoplasmic but some forms could also be associated with membranous organelles. Various isoforms have been isolated from different plants which suggested a possible association of different isoforms to various subcellular localizations (Jones, 1984).

PAL is a tetrameric enzyme with two active sites per tetramer containing dehydro-alanyl residues (Jones, 1984). Though the enzyme is a tetramer, each of the subunits are easily separable, their molecular weight range from 77 KDa in case of parsley (Bolwell, 1988) to 85 KDa in the case of Phaseolus vulgaris (Bolwell et al., 1985). Purification from the bean culture cells of the enzyme led to the resolution of at least four isoforms of the enzyme each differing in its pi and K_m values. The treatment of bean suspension culture cells with elicitors produces isoforms with had high pi and low K_m values as compared to the earlier isoforms (Bolwell, 1985). This kind of de novo production of isoforms of
Fig. 2: Schematic representation showing structure of various pal genes

Phaseolus pal2

Pea pal1

Arabidopsis pal 1

Parsley pal1

Rice pal1

Tomato pal5

Trifolium pal1

Populus pal g1

pal g2a

pal g2b

↓: Transcription start site
the enzyme could be due to transcriptional activation of the gene under the given set of conditions leading to the synthesis of new protein or post-translational modification resulting in various isoforms. Evidences in the case of chalcone synthase, indicates transcriptional activation of a separate sub-sets of the chs multi-gene family under the conditions of stress in alfalfa (Junghans et al., 1993).

**Structure of pal genes**

The process of isolation of pal gene began with the isolation of cDNA clones of parsely (Kuhn et al., 1984) and Phaseolus (Edwards et al., 1985). Since then pal specific cDNA clones have been isolated and characterized from a number of plant species such as sweet potato (Tanaka et al., 1989), alfalfa (Gowri et al., 1991), pine (Whetten et al., 1992), pea (Kawamata et al., 1992), melon fruit (Diallinas et al., 1994), tobacco (Pellegrini et al. 1994) and tea (Matsumoto et al., 1994). The deduced amino-acid sequence from the isolated clones shows that the enzyme contains 690 to 725 amino acids with an approximate molecular weight ranging from 77 to 83 KDa. Studies using cDNA’s implicate PAL in a variety of functions eg. injury/wound response in sweet potato (Tanaka et al., 1989), possible role in ripening of melon fruit (Diallinas et al., 1994), catechin content in tea plants (Matsumoto et al., 1994), and hypersensitive reaction in response to tobacco mosaic virus and fungal elicitor in tobacco (Pelligrini et al., 1994).
The isolation of the *pal* cDNAs established the basic similarity of the PAL enzyme and that of the *pal* genes showing transcriptional activation under a variety of conditions. However, in order to analyze the regulatory pattern of various *pal* genes, it is necessary to isolate the regulatory domains of these gene(s). Using cDNA clones as probes, *pal* genes were first isolated from parsley (Lois et al., 1989) and bean (Cramer et al., 1989) genomic libraries. Subsequently, *pal* genes have been isolated and characterized from a variety of plant species such as rice (Minami et al., 1989), *Arabidopsis* (Thungapathra, 1990; Ohl et al., 1990; Wanner et al., 1995), pea (Yamada et al., 1992), tomato (Lee et al., 1992) and *Trifolium* (Howles et al., 1994).

The characterization and DNA sequencing of different *pal* genes from various plant species reveals the basic similarities in its structure (Fig. 2). The structure of *pal* genes as deduced from sequence data and a comparison with cDNA sequences reveals that gene contains two exons and single intron. Only exception known so far from this general pattern is the *pal*3 gene of *Arabidopsis* which contains an additional intron in the second exonic region of the other genes. The position of first exon by and large is conserved. The gene is of a typical eukaryotic type and the nucleotides at the exon-intron junctions follow characteristic GT/AG rule (Breathnach and Chambon, 1981). The size of first exon varies from 308 to 431 bp. The second exon is large ranging from 1648 to 1748 bp. The second and third exons of the *Atpal*3 add up to 1753 bp which is almost of the size of second exon of the other *pal* genes. The first
exon of the Atpal3 is of 361 bp which falls within the size range of the first exon of other pal genes. The size of the intron is highly variable ranging from 90 bp in the case of pea pal2 to 1720 bp in the case of the bean pal2.

A comparison of nucleotide sequence of various genes reveals that exonic regions are highly conserved at nucleotide level to ~60-80% while intronic as well as 5′-upstream and 3′-downstream sequences are diverged. The intronic position in terms of amino acid also appears to be conserved. Splicing in the gene occurs at the amino-acid arginine and it appears conserved in all the pal genes analysed so far. The conservation of the amino-acid sequence around the splice site leads to a consensus "ELIRFL" (Glu-Leu-Ile-Arg-Phe-Leu). ELIR is encoded by the first exon and RFL is encoded by second exon. Slight divergence in this consensus is seen in the case of Atpal2 and the second splice site in Atpal3. Similar conservation at the nucleotide level is seen in majority of the isolated genes and has a consensus of CAG/gt......ag/GTT.

A deduced amino-acid sequence of PAL between the pal genes from the various plant species indicates homology of 65 to 90% with ATpal1. Atpal1 shows maximum homology of 95% with Atpal2, while shows only 65% homology with pine PAL and with the remaining pal genes from various plants it has a homology ranging from a maximum of 90% to a minimum of 65%. A phylogenetic tree constructed from the deduced amino acid sequences show a modular mode of evolution of the gene over different species (Wanner et al., 1995). The analysis shows a specified evolutionary pattern for moncots and
dicots. It further indicates the maximum conservation of the sequences among the members of the Solanaceae family showing a common lineage during the process of evolution. The unique Atpal3 is grouped away from all the identified dicot PAL proteins indicating that it is distantly related to them, possibly remnant of an ancestral rudimentary gene before possible divergence into monocots and dicots during the process of evolution.

Presence of isozymes of PAL in Parsley (Bolwell et al., 1985) and in bean (Liang et al., 1989) indicates two possibilities of its origin (i) all these isozymes could be coded by different genes in the genome or (ii) they are all coded by a single gene and the products are a consequence of post-transcriptional and post-translational modification. Isolation of different cDNA clones from the cDNA libraries like in parsley (Lois et al., 1989), bean (Cramer et al., 1989), alfalfa (Gowri et al., 1991) and pea (Kawamata et al., 1992) supports the first possibility. Thus, the isolation of different cDNA clones is a first step in characterization of various members of the multi-gene families of PAL in different species. On genomic Southern hybridization with the cDNA or genomic clones, multiple bands are visible supporting the presence of multi-gene family. This lays the foundation for the possibility of different genes coding for different isozymes. All the pal genes isolated from various plant species such as bean (Cramer et al., 1989), pea (Yamada et al., 1992), Arabidopsis (Wanner et al., 1995) etc. are all members of multi-gene family having two to six members. Potato and loblolly pine are the two present exceptions to the small multi-gene
family of *pal*. Loblolly pine is reported to contain a single *pal* gene (Whetten and Sedroff, 1992) while potato seem to contain over forty *pal* genes (Joos et al., 1992). The presence of multi-gene family led to the idea of each member playing a specified role in the plant which could be developmentally and environmentally regulated as has been observed in the case of bean (Shufflebottom et al., 1993) and hybrid aspen (Osakabe et al., 1995).

Even though PAL is represented by a small multi-gene family clustering of genes on a chromosomal segment is reported only in *Trifolium* (Howles et al., 1994) and hybrid aspen (Osakabe et al., 1995) while in all the other cases the genes seem to be located on various chromosomes.

**Expression Pattern of *pal* genes**

The *pal* genes, as already been discussed in the earlier sections are members of a multi-gene family in almost all the plant species studied so far. Presence of multiple isoforms of the enzyme and multiple genes differing in the regulatory domains of the genes suggest that each member of the *pal* gene family may play a specific role in plant development and defense. In order to monitor the expression of *pal* genes, the studies have concentrated on monitoring the transcript pattern of various genes. This has been accomplished initially by northern blot hybridization using *pal* sequences as a probe. The initial studies were designed to measure the *pal* transcript levels irrespective of which members of the gene family is transcriptionally active. Subsequently, gene
specific probes were used to monitor the differential activation of various members of the gene family with respect to the developmental or tissue-specific pattern of expression.

Transcriptional activation of *pal* to fungal elicitors

Studies demonstrated that in response to fungal infection or elicitors isolated from fungal cell walls, the level of *pal* specific transcript increases (Kuhn et al., 1984; Edwards et al., 1985) confirming the notion of *pal* being one of the defense genes. This enhanced levels of transcript could directly be correlated with the higher levels of the enzyme, PAL. Such a pattern of inducibility is also observed in the case of CHS (Ellis et al., 1988), another enzyme of the phenylpropanoid pathway. Levels of both *pal* and *chs* transcripts increase in the bean suspension cultures treated with elicitors isolated from the fungus, *Colletotrichum lindemuthianum*.

Correlating the defense activity the PAL enzyme, *pal* transcript accumulation in relation to fungal elicitors have been studied in various systems. Parsley cells treated with elicitors from *Phytophthora megasperma* showed massive accumulation of the *pal* transcript within 2 hr following the treatment (Lois et al., 1989). Using gene specific oligo-nucleotides they also showed that all the three cDNA’s isolated *pal1*, *pal2* and *pal3* were transcriptionally activated. Similar kind of studies using pea epicotyls treated with elicitors from *Mycosphaerella pinodes* showed transcriptional activation not
only of *pal* but also of *chs* with maximum accumulation around 5 hrs after the treatment (Yoshioka et al., 1992). In the case of tobacco leaves treated with megaspermin (elicitor) the transcriptional activation was seen with a maximum accumulation around 8 hrs after the treatment (Pellegrini et al., 1995). Studies in bean hypocotyls showed marked increase in expression of *pal1* and *pal3* over *pal2* in response to fungal infection (Liang et al., 1989a).

Recent studies in tomato have led to the identification of two transcripts for the same gene being transcribed by utilization of alternate initiation sites. Study with the various stimuli like wounding, light or *Verticillium* infection led to transient increase in the transcript. These modulatory, transient and instantaneous changes in the transcript levels was seen with the smaller transcript, while steady state was maintained in the case of longer transcript (Lee et al., 1994). These studies, indicating the preferential use of the certain initiation sites in response to stress, opens up plethora of questions on the complexity of signal transduction mechanism and its consequent role in activation of the gene.

*PAL* gene(s) get transcriptionally activated in response to wounding

The level of *pal* specific transcript increases in response to wounding in bean (Liang et al., 1989a), sweet potato (Tanaka et al., 1989) and melon-fruits (Diaillinas et al., 1994). The transcript level in response to wounding increased by 10 to 12 folds higher over the control plants and reached the maximum at
6 hrs after wounding and a similar kind of profile was also observed at the level of protein activity of PAL in the case of bean (Liang et al., 1989a). Two dimensional gel electrophoresis of total translatable products or immuno-precipitable PAL subunits with a molecular weight 77 KDa from wounded hypocotyls showed an increase in the forms with higher pi and lower K_m. The *in vitro* translation studies using sense and anti-sense transcripts, Liang et al., (1989) showed that *pal3* encodes a basic subunit isoform, the *pal2*, an acidic subunit isoform and the *pal1* encodes a subunit with the pi of intermediate range. These *in vitro* translation experiments confirm the earlier work of Bolwell et al., (1985) where in lower K_m and higher pi isoforms i.e. basic isoforms accumulate in response to wounding. Though detectable levels of transcript were not seen in the case of wounded leaves of tomato but, RT-PCR led to identification of the transcription of tomato *pal1*-truncated mRNA (Lee et al., 1992a).

**Regulated tissue-specific pal transcript accumulation**

Northern blot experiments in the various plant species like bean (Liang et al., 1989a), pea (Yamada et al., 1992) and *Arabidopsis thaliana* (Wanner et al., 1995) show the maximum *pal* transcript levels in roots of the seedlings with the corresponding PAL activity (Jahnen and Hahlbrock, 1988); Liang et al., 1989a). Studies in *Arabidopsis* have led to the identification of gene-specific transcripts in various organs. The *Atpal1* specific transcript shows higher levels than those
of the *Atpal2* in roots while, the *Atpal1* gene shows higher expression in all tissues as compared with that of *Atpal2* (Wanner et al., 1995). Recent studies in hybrid aspen (*Populus Kitakamiensis*) showed the organ-specific expression pattern of both the genes isolated (Osakabe et al., 1995). Gene-specific probes were used to show developmental control of transcript accumulation of both the *pal* gene members. *Populus palg1* transcript was detected at the highest level in young tissues like buds and young leaves while, *palg2a* accumulated specifically in mature stems (Osakabe et al., 1995).

**Light and UV induced pal transcription**

Etiolated rice leaves after exposure to light show accumulation of *pal* transcripts 3 to 4 hr after the illumination (Minami et al., 1989). Similar effect on activation of *pal* and 4cl is seen in etiolated parsley seedlings in response to UV irradiation in a co-ordinated fashion (Lois et al., 1989). In the case of etiolated bean hypocotyls (Liang et al., 1989a), a maximum accumulation of *pal* transcripts is seen within a period of 1 hr. The *pal1* and *pal2* transcripts accumulate more than the *pal3* transcripts in bean hypocotyls. Similarly, an enhancement of *pal* transcripts of both the *Populus palg1* and *palg2a* is observed when exposed to UV with peak at 6 hr (Osakabe et al., 1995). The different members of *pal* gene family in various plants species show different inducibilities in response to light or UV irradiation. This implies that, though there is a basic mechanism for activation of the *pal* genes, subtle differences
exist possibly in the signal transduction cascade among the various plant species.

**Expression in response to Ethylene**

Studies in carrot root show activation of all the genes of the phenylpropanoid pathway like *pal*, *chs 4-cl* etc. in response to ethylene (Ecker and Davis, 1987). There appears to be a correlation between the defense response of the plant and synthesis of ethylene. The fungal elicitors are known to induce the synthesis of ethylene concomitantly with activation of the *pal*, *chs* and *4-cl*. Ethylene is also involved in the process of fruit ripening and the signals of fruit ripening lead to co-ordinated regulation and increase in the levels of various transcripts like ACC synthase, ACC oxidase, PAL, CHS and CHI in the ripening of melon-fruit (Diallinas et al., 1994). These studies indicate that the transcriptional activation of the phenylpropanoid pathway genes follow a pattern similar to the genes of the ethylene biosynthetic pathway during the process of fruit ripening.

**Characterization of Regulatory domains**

The northern blot studies discussed in the earlier section indicate that *pal* genes(s) are transcriptional activated in response to a variety of biotic and abiotic factors such as elicitors isolated from fungal cell-walls, UV and visible light, and ethylene besides, tissue-specific and developmental cues. Transcriptional
activation of class II eukaryotic gene is thought to be brought about by a complex interaction of trans-acting proteins and specific DNA sequences. The level of transcripts made is thus dependent on an interplay of various protein-protein and DNA-protein interactions involving a fine modulation of different down and up regulatory events. There are two basic techniques used to define and delineate the sequence elements responsible for transcriptional activation. One method is based on the assays determining DNA-protein interaction (gel retardation and foot-printing) and the other on the expression of a reporter gene directed by the regulatory region following transfer to protoplasts (transient expression) or to plants (expression in transgenic plants).

Identification of regulatory domains

(a) Assays based on DNA-protein interaction:

Transcriptional activation is considered to be brought about by the binding of the cis-regulatory promoter sequence elements of the gene by transcriptional factors. Binding of the transcriptional factors to the promoter elements would lead to opening up of the chromatin leading to transcription. The regions of DNA-protein interactions in upstream region of parsley pal gene have been delineated by using in vivo foot-printing (Lois et al., 1989). Similar elements have also been identified of bean chs promoter another important enzyme in the phenylpropanoid pathway by using the similar technique (Lawton et al., 1990).
DNA-protein interaction of parsley pal promoter was detected as changes in the DMS (dimethyl sulfoxide) reactivity to residues and led to identification of two types of foot-prints. One being the constitutive foot-print within the range of -260 to -200 bp which indicates the possible role in constitutive expression and the Second being the inducible foot-print identified with stress (UV irradiation) in two regions i.e. -199 to -150 bp and -149 to -100 bp. The consensus sequence identified within this region is termed box A:

\[
A \ A \ C \ C C \\
C C A A C C C
\]

And box B:

\[
C T \ A C C T A C
\]

This kind of putative consensus sequence elements are seen in many of the genes encoding enzymes of the phenylpropanoid pathway (Sommer and Saedler, 1986; Lippardt et al., 1988; Feinbaum and Ausubel, 1988; Schulze-lefert et al., 1989) including the gene of this study (Thungapathra, 1990). These elements identified are seen in the case of many of the light regulated genes (Dean et al., 1988) suggesting their role in general as well as stress responses.

Studies in pea have led to the identification of putative domains/sequence elements in the promoter of pal gene to which nuclear transcriptional factors bind in response to both elicitors and suppressors for the pea pathogen, Mycosphaerella pinodes (Wada et al., 1995). Their initial studies indicate, after addition of elicitor, the transcript accumulation peaks within 20 min and could be very effectively blocked by the addition of supprescin (suppressor named supprescin). In vitro foot-print analysis was done for the promoter of pal gene
in the presence of elicitor, elicitor + supprescin and supprescin alone. This consequently led to the identification of certain putative elements to which transcriptional factors interact. Studies have delineated two binding domains spanning -110 to -41 and -257 to -187 bp region (Wada et al., 1995). These studies have led to a view that supprescin not only blocks the signal transduction pathway of elicitor mediated activation but also simultaneously initiates an independent signal transduction leading to the active role in deactivation of the genes.

Recent studies on the bean *pal* promoter using gel shift assays and methylation interference assays have identified four sequence segments within this region ranging from -193 to -182 bp representing G-box (Sablowski et al., 1994), -246 to -238 bp representing AC-I box, -131 to -120 bp comprising AC-II box and also an AC-III box by Hatton et al., (1995). Various transcription factors bind to these identified boxes and in turn interact to bring about complex tissue specific pattern of expression in plants. Thus, the combinatorial interactions involving cis-elements indicate flexible mechanism of regulating tissue-specific expression pattern in the plants.

(b) Promoter directed reporter gene expression

The *in vitro* and *in vivo* foot printing studies though are elegant way of identifying the various cis-regulatory elements, only determine the DNA-protein interactions. A basic question whether the interactions observed are in
fact responsible for expression needs to be addressed. Studies using the promoter regions cloned upstream to the reporter gene and studying its expression following transfer to a plant gives a better picture of the actual pattern of expression in a cell or tissue. The effect of progressive deletions of the promoter on the expression of the reporter gene is monitored. Two reporter genes have been used (i) chloramphenicol acetyl transferase (CAT) or (ii) β-glucuronidase (GUS).

Studies using the CAT reporter gene have been done in protoplasts to analyze the transient expression in bean using the 5’ upstream regions of the bean chs gene (Dron et al., 1988). Following this CAT activity has been studied in the pea protoplasts using the pea pal promoter (Hashimoto et al., 1992). These studies indicate the responsiveness of the pal promoter to the elicitor from Mycosphaerella pinodes which leads to massive transient CAT activity in electroporated protoplasts. Studies by Yamada et al. (1992) using the chimeric gene construct and nested 5’-deletion of the promoter identified the elicitor-responsive sequences within the promoter fragment of pea pal1 and pal2 using the transient expression system. Further studies indicate that the CAT activity is induced by the treatment with fungal elicitors and UV, suppressed by fungal suppressor or orthovanadate (an inhibitor of plasma membrane ATPase).

The transient expression system has been useful in studying the inducibility of a promoter, however it could not be utilized for localization of sequence elements concerned with developmental or tissue-specific expression. However,
in order to characterize the region containing the sequence elements conferring inducibility in response to developmental cues or tissue-specific signals, the gene constructs have to be stably integrated and transgenic plants have to be raised. The expression of GUS or other reporter gene in such a transgenic system is monitored. In this way the promoters of two pal genes from bean and one from Arabidopsis have been analyzed.

The bean pal2 promoter has been analyzed by two groups (Liang et al., 1989b; Bevan et al., 1989) using bean pal2:GUS constructs in transgenic tobacco or potato plants. The transgenic plants show a highly regulated tissue-specific pattern of expression. The expression is localized in the xylem vessels of the vascular bundles, epidermal cells and trichomes, growing meristematic shoot apex and in the axenically growing roots and root hairs. The expression is also associated with petals of the flowers and to a certain extent in the sepals. Similar kind of tissue specific pattern of expression was observed using the Arabidopsis pal1:GUS constructs. The precise spatial pattern of activity of bean pal2 and Arabidopsis pal1 promoters indicates many facets which can directly be correlated to the accumulation of specific phenylpropanoid products. This was inturn related to vascular expression associated with lignin deposition, light induction in epidermal cells, developmental expression in the growing apex of shoots and roots and anthocyanin pigmentation in the floral tissues. The promoter is also responsive to the environmental cues like wounding and a correlation was also seen at the transcript level for both GUS and endogenous
*pal* (Liang et al., 1989b). Though histochemical localization of the GUS expression was possible no extractable GUS activity in response to wounding was seen in the transgenic *Arabidopsis* plants (Ohl et al., 1990). Studies using the deletion analysis for delineating a possible presence of upstream elements led to the identification of putative *cis* elements responsible for tissue-specific expression and a putative cryptic phloem element (Levya et al., 1992; Hatton et al., 1995). Studies by Hatton et al. (1995) further indicated the presence of multiple *cis* elements which are considered responsible for this tissue-specific expression. Foot-printing and methylation interference studies along with the deletion constructs of the *pal2*:GUS in transgenic tobacco indicated the presence of redundant *cis* elements responsible for tissue-specific expression (Hatton et al., 1995).

In order to analyze the differential expression of the members of a gene family, expression of both the members of the gene family was monitored in transgenic tobacco using the bean *pal2* and *pal3* promoters. No qualitative difference in expression was seen indicating a possible overlapping functions of the members of the gene family.