SUMMARY AND CONCLUSIONS

Phenylalanine ammonia lyase the first enzyme in the phenylpropanoid pathway catalyzes the committed step of oxidative deamination of phenylalanine to trans-cinnamic acid and being inducible in response to various stress conditions thought to have a major regulatory role in the pathway. The PAL enzyme levels get enhanced in plant systems to a variety of biotic/abiotic factors such as pathogen infection, elicitors isolated from fungal cell-walls, mechanical wounding, UV, light, ethylene, HgCl₂, etc. The enhanced levels of the 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 under variety of conditions.

One of the pal genes, named Atpal1 from Arabidopsis thaliana has been isolated and sequenced in the laboratory and is a typical eukaryotic gene characterized by the presence of two exons and single intron including its upstream and downstream regions. The entire sequence is present on a 4.8 kb EcoRI fragment containing both the upstream and downstream regions. A single PstI site cleaves and generates two fragments, a 2.8 kb fragment containing the coding region and a 2.0 kb fragment containing the upstream region of the gene. The genomic Southern hybridization results indicate that the Atpal1 gene is a member of a small multigene family in Arabidopsis genome.
In this study, the expression pattern of the *Atpa/1* promoter with respect to tissue-specificity, developmental programmes and in response to various stress factors have been analyzed in transgenic tobacco. Since the *pal* gene is a member of a small multi-gene family other members of the gene family have been isolated in order to use them for studying the expression patterns directed by different members.

The *pal* gene isolated earlier in the laboratory was used as a probe to screen the genomic library of *Arabidopsis thaliana* (Columbia) to isolate the other members of the gene family. Extensive screening of the genomic library led to isolation of five clones. Restriction enzyme and Southern blot analyses led to the categorization of the clones into three groups, one clone was identical to *Atpa/1* isolated earlier in the lab, while three of the remaining four clones were similar and named *Atpal2*. The last clone whose restriction pattern does not match with any of the isolated genes of the *Arabidopsis pal* gene family could possibly represent a new member of the gene family.

Chimeric gene construct was generated by cloning the promoter region of the *pal* gene upstream to the GUS coding sequences. The promoter region of the *pal* gene was isolated as an EcoRI-BgIII fragment from the 4.8 kb complete gene fragment and cloned upstream to the GUS coding sequences in the binary vector pBI101 in a correct orientation. The *pal promoter:gus* chimeric gene construct was transferred to tobacco plants using *Agrobacterium* mediated transformation system. Tobacco plants were used as a model system
to study the expression pattern directed by the \textit{Atpa/1} promoter.

Fifty six transgenic plants were raised. Out of these majority of the independent transformants were obtained from leaf discs derived from a single tobacco plant which was initially transformed with the construct and the other category of transgenic plants were primary transformants, transformed only once. The transgenic nature of the regenerating initials and the plantlets were confirmed by assaying for the GUS activity.

The \textit{Atpa/1} promoter is active only in the vascular tissues and expression is confined to mid-ribs, veins, and veinlets of the leaf, xylem tissues of stem and petiole. The tissue specificity with respect to expression in xylem tissues of the \textit{Atpa/1} gene expression could be visualized in terms of lignification as \textit{pal} being the first enzyme of the pathway. The \textit{Atpa/1} promoter is most active in the roots as compared with shoots and leaves and such a picture is also reflected at the GUS transcript level from the these organs. Expression of \textit{Atpa/1} in the floral tissues was confined to the ovary and stigma and margins of the petals and sepals. The activation of the \textit{pal} promoter is consistent with the presence of anthocyanins and flavanoids in these tissues as they are end-products of the phenylpropanoid pathway.

The pattern of GUS expression conditioned by \textit{ATpal1} promoter was analyzed during various stages of seedling growth indicated it to be active in the vascular tissues. The \textit{Atpa/1} promoter is active only in the root tissues in the early stages of development. After the 10th day the pattern of expression
shows a dramatic shift. Shoot including leaves show very high expression and subsequently the pattern of activation becomes confined to the vascular tissues apparently indicating a gradual shift from developmental to tissues specific pattern of expression. As pal directed expression is confined to the vascular tissues in the developing seedlings it thus could serve as a very good marker for xylem differentiation during the vascular development.

In order to check the activation of pal1 promoter to mechanical wounding, the leaves or stems of the transgenic plants were mechanically wounded. The Atpal1 promoter shows localized activation in wounded leaves or stems. In the stems the cortex cells surrounding the wound zone also show some activation of the Atpal1 promoter, however, in the leaf tissues only vascular tissue show activation. The activation is consistent with the role of PAL in the synthesis of wound protectant, cinnamic acid esters- the end product of the phenylporpanoid pathway.

The Atpal1 promoter shows different patterns of activation in response to specific pathogen of tobacco Pythium and unspecific fungus usually a contaminant on the plates Aspergillus. In response to Pythium the initial contact points of the fungal hyphae show a very strong activation of the promoter whereas, a generalized defused activation to Aspergillus is observed. In response to Pythium infection, the pattern of pal1 activation as observed in the healthy and uninfected seedlings of equivalent age, appears to be suppressed. The fungal pathogen contains elicitors which lead to the activation of the pal
gene and the seedlings of that age would also have the signals for the programmed developmental activation of \textit{pal} gene in the seedlings. However, in the \textit{Pythium} infected seedlings the elicitor effect is seen only at the site of infection and no activation appears to be visible in response to developmental signals. It is therefore, conjectured that fungus contains some sort of suppressors that not only inhibit the effect of elicitors but also overcome the developmental signals and possibly inhibits the spread of the activation signals from the immediate vicinity of the site of infection leading only to the localized expression. This view of repression gets corroborated as the promoter though having the conserved consensus sequence elements box A and box B responsive to elicitors, are not able to bring the generalized activation upon infection with \textit{Pythium} as seen in the case of \textit{Aspergillus} infected seedlings.

In order to analyze the inducibility of \textit{At}pal1 promoter to UV and white light, seedlings were exposed to UV and white light for a period of 5 min. In response to UV the maximum transcriptional activation is seen 8 hr after the exposure. Flavanoids which are considered to be UV screens provide protection to the plant against the damaging UV rays and are end-products of phenylpropanoid pathway. Exposure to UV apparently would require more synthesis of flavanoids and thus it is conjectured that the \textit{At}pal1 gene will get activated in response to UV. In response to white light, the activation of \textit{At}pal1 promoter is very fast, the GUS expression is seen within 15 mins after the exposure. Three activation peaks 30 mins, 4 and 12 hr from the time exposure
were observed in white light treatment in comparison to the UV induction.

In order to visualize which of the components of white light i.e., blue or red light is responsible for this activation the effects of these two lights were studied separately. Differential activation of the Atpal1 promoter was observed in response to these two components of light. Higher levels of activation to blue light were observed in comparison to red light but faster activation of the promoter was seen with the red light treatment. The blue light induced Atpal1 activation is apparently several folds higher than white light activation and thus appears that in the presence of white light, the blue light component is not able to bring about the activation of the promoter. This may either be due to inhibitory effects of other light components on blue light or the inability of the blue light induced trans-acting proteins from binding to the blue light responsive elements in the presence of phytochrome-mediated signal transduction pathway. The second possibility appears likely since the phytochrome mediated activation is much faster than the blue light mediated activation.

To analyze the involvement of phytochrome mediated red light activation of the Atpal1 promoter the red-far red light reversibility was tested. The results show that the red light activation is as fast as white light but the levels of activation are much higher. The exposure of these seedlings to saturating far red light treatment leads to abolition of the two major peaks but does not abolish one of the peaks indicating the possibility of the promoter being under the control of both the light-stable type II as well as light-labile type I class of
phytochromes.

Salicylic acid (SA) has shown to play an important role in systemic acquired resistance (SAR) in plants and is considered to be a molecule involved in signal transduction in plant defense responses. In the presence of SA a maximum activation is seen after 10 hr and analysis of the promoter sequences indicated the presence of the conserved 10 bp consensus sequence which is present in all the SA activated genes which play a dominant role in the SAR.

Owing to the important role of Ca²⁺ as a secondary messenger, its ability to activate the \( Atpa/l \) promoter in the transgenic seedlings was analyzed. The activation brought about by Ca²⁺ on the \( Atpal1 \) promoter is higher than seen in any other treatment indicating a role of the divalent cation Ca²⁺ as a secondary messenger in the signal transduction pathway \( Atpal1 \) to various conditions. This activation could be either brought about by calmodulin dependent or independent pathways. MgCl₂ was used as control to neutralize the effect of chloride ions and surprisingly even Mg²⁺ ions seem to bring in the activation of the promoter.

Sucrose has been considered to be an activator of the \( chs \) promoter since it seems to mimic the effect of elicitors. Therefore it was expected that sucrose will also activate the \( Atpal1 \) promoter. However experiments indicate that sucrose is inhibitory to \( Atpal1 \) activation. Sucrose is used a carbon source in the MS medium and seedlings grown in presence or absence of sucrose showed differential expression. A seven fold higher expression is seen in the
absence of sucrose. This type of high level of activation of the promoter in the absence of sucrose could either be due to inhibitory effect of sucrose or the activation of the \textit{Atpal1} to the stress on the seedlings due to the non-availability of readily available carbon source.

Majority of the transgenic plants were independent secondary transformants regenerated from transformed leaf discs derived from a single non-GUS expressing primary transformant. An analysis of GUS expression under the control of \textit{Atpal1} promoter revealed the different GUS levels among various transgenic plants. On the basis of level of GUS expression, the transgenic plants could be classified into three distinct categories (i) low expressing plants, (ii) intermediate expressing plants and (iii) high expressing plants. This kind of differential expression was also reflected in the GUS transcript levels in these categories of plants. Ocasionaly under certain conditions in the primary transformants no expression was observed. The differential expression among the population of transgenic plants could be due to (i) copies of the transgene integrated (ii) the site of integration of the transgene and/or (iii) the degree of methylation of the promoter sequences. These possibilities were experimentally investigated.

The presence of a common band in plants of all the three categories in the Southern blots might be explained by the retransformation event. Southern hybridization experiments involving the DNA isolated from three categories of the plant using GUS, \textit{nptII} or \textit{Atpal1} promoter sequences indicate the following
(i) More than one copies of the transgene are integrated in all the plants except in some the primary transformants. Some of the copies were of the expected size on the basis of the restriction pattern of the gene construct. However, smaller bands were also observed. (ii) The intensity of the hybridization signals of all the bands was not similar. In all the categories of plant except the primary transformant, very high intensity band was observed with all the three probes (GUS, nptII or Atpa1). One of the intermediate expressing plant had another band of low molecular weight. This high intensity low molecular weight band appears to contain very high copy number of that sized fragment. (iii) The GUS expression does not show any correlation either to number of copies of transgene present or the site of integration.

In order to check the structural integrity of the Atpa1:gus construct in transgenic plants DNA digested with HindIII+EcoRI restriction enzymes was probed with GUS coding sequences. Since the EcoRI and HindIII are internal sites in the construct, a 4.1 kb fragment is expected to hybridize to the GUS sequences. The results indicate (i) This region is intact in majority of plants, (ii) besides the expected sized band, bands of high and low molecular weight were present, and (iii) in two of the intermediate plants, no band of the expected size was observed.

In the Southern blot experiments three basic observations were (i) the presence of small sized bands hybridizing to all the three probes (GUS, nptII or Atpa1 promoter), (ii) increase in the copy number of the smaller bands, and (iii)
the absence of the expected size band in two of the intermediate plants and the presence of larger and smaller sized bands in EcoRI-HindIII double digests. The increase in copy number could either be due integration of truncated copies of the construct in tandem. However, the hybridization of all the three probes to a high copy number off smaller fragment suggest the possibility of localized rearrangement followed by amplification of the sequences. The absence of expected size band in the double digests of two intermediate plants and presence of hybridizing larger or smaller bands support the possibility of rearrangements.

The role of methylation of the promoter sequences in the differential expression of the transgene was analyzed as hypermethylation is invariably correlated to silencing and lower expression, while hypomethylation to high expression. The upstream region of Atpal1 gene contains 56 possible methylation (CpG and CpNG islands). Presence of methyl groups in the promoter region of the genes are easily monitored by using sensitive/insensitive isoschizomeric restriction enzymes Mspl/Hpall (CCGG), in the Southern blot experiments. Since Atpal1 upstream region does not contain any sites for Mspl/Hpall, an indirect strategy was used. The effect of general demethylating agent, 5’-azacytidine on GUS expression in all the three categories of plants were monitored. Treatment of the low, intermediate and high expressing seedlings with azacytidine enhanced the level of expression in low and intermediate, while no discernible change was observed in the high expressing
seedlings. Surprisingly in the low and intermediate expressing plants treated with azacytidine, the enhanced levels of GUS activity did not attain the levels of high expressing plants. The following can be concluded from the above set of results (i) the major part of differential GUS expression observed among the transgenic tobacco plants could be accounted for by the degree of methylation. (ii) The part of the variability seen in the expression is due to factors other than methylation.

It was conjectured that if methylation of the promoter would take place than it would also spread over to the neighbouring regions of the transgene. Thus, analyzing the methylation pattern within the neighbouring regions may provide an index of the methylation in differential pattern of expression of the transgene. Using this strategy the isoschizomeric combination of enzymes SstI/SacI (GAGCTC) was used whose site is present in the nos terminator after the GUS coding sequences. SstI enzyme would not effect cleavage if cytosines are methylated while SacI will effect the cleavage even if the cytosines are methylated. Southern blots with GUS indicated the involvement of methylation in only two of the low expressing plants where a shift in bands pattern was seen. These results corroborate the earlier conclusion that methylation alone may not be responsible for this differential expression in the transgenic plants. It is possible that localized rearrangement creates the GUS coding sequences in the anti-sense orientation thereby resulting in the synthesis of anti-sense transcript of GUS. Anti-sense may also be produced by the integration of the
construct beginning from the left downstream to a strong endogenous promoter.

Among the transgenic plants one of the plant displayed different morphological features from that of the other transformed plants. The plant has following characters like - bushy appearance, shorter petioles, shorter internodes and was sterile due to shorter length of stamens. It also contains auxiliary buds at each of the axils of the leaf indicating a partial loss of apical dominance. GUS expression monitored histochemically showed the loss of tissue-specific ability of expression, showing expression in all the cells. The very high GUS expression was also confirmed spectrofluorimetrically. It is likely that during the process of second transformation, the construct was integrated into the same locus of the other homologue in the earlier integration event took place leading to the mutation. The mutant gene seem to be responsible for many phenotypic characters in the plants. As the plants were sterile they were clonally propagated by tissue-culture. Some of the regenerants showed an apparent reversibility in the characters of the mutant to normal plants. These set of regenerants could be (i) somaclonal variants or (ii) loss of the specific transgene insert consequently leading to plant with normal morphology. Further analysis is required to make any concrete conclusion about the possible reason for the production of the T-DNA insertion mutant.