Fungal diseases have been one of the principle causes of crop losses from the very early days of organised agriculture. Chemical control of the disease is costly and deleterious to the environment, and eventually becomes less efficient due to the evolution of the pathogen. Breeding for disease resistance is a time consuming process that often took decades to complete in the case of perennial tree crops. Significant new advances at the molecular level in the field of plant-pathogen interactions form the basis for novel transgenic approaches to crop protection. The cloning of disease resistance and defense-related genes and the dissection of the signal transduction components of the hypersensitive response and systemic acquired resistance pathways has greatly increased the diversity of options available for transgenic disease resistance. Establishment of the role of the candidate gene in defense responses would be essential for engineering effective and durable resistance to pathogens in the field.

In the present study, the pathogenesis related β-1,3-glucanase gene in Hevea was investigated for its role in combating the abnormal leaf fall disease caused by Phytophthora spp. Induction of β-glu has been well documented as a part of plant’s broad generalized defense mechanism against a variety of pathogenic fungi in many plant species. Accumulation of this enzyme has always been associated with the advent of hypersensitive response at the infection site and development of systemic acquired resistance throughout the plant. They are hydrolytic enzymes, able to catalyze the endo-type hydrolytic cleavage of β-1,3-linked glucans, which are the principal cell wall component in many phytopathogenic fungi. Thus they can act directly by degrading the cell wall of the pathogen. They can also perform in an indirect way, by releasing the fungal cell wall components that can act as elicitors of other forms of active host defense.
responses. For these reasons, considerable effort has been aimed at isolating and characterising these plant hydrolases to evaluate their potential for improving disease resistance of plants against fungi.

Gene specific primers were used to amplify the β-glu gene from Hevea genomic and cDNA. For this purpose DNA and RNA were isolated with good concentration and purity from Hevea (clone RRI 105) leaf tissues. RNA was isolated from latex also. First strand cDNA was synthesised from the isolated RNA through reverse transcription. PCR conditions were optimised to amplify the bands from genomic DNA and cDNA. A 1.25 kb and a 1.12 kb fragments were amplified from genomic and cDNA respectively. These PCR fragments were cloned in pGEM vectors and sequenced. Both were found to be identical except for the presence of a 132 bp intron in the genomic sequence. The Hevea β-glu gene shows similarities with the other reported β-glu genes from different plant species, but the intron size varies. The position of the intron was found to be same in all reported plant glucanases. The genomic DNA sequence of the β-glu gene in Hevea was reported for the first time in this study. Deduced amino acid sequence shows that it encodes a protein of 374 amino acids with 36 amino acid N-terminal signal peptide and 22 amino acid C-terminal for vacuolar targeting, which are removed during final processing. It is basic class I glucanase with a theoretical pI value of 8.96 with a predicted size of 41.2 kDa. Differences were observed at certain positions when compared with three earlier reported sequences of mature β-glu from Hevea. A protein glycosylation site reported in β-glu from clone RRIM 600 was absent in the present protein from clone RRI 105. Southern hybridisation confirms the presence of a low copy number gene in 14 different genotypes of rubber tree, which include both tolerant and susceptible varieties.

Most of the high yielding clones of rubber tree are susceptible to abnormal leaf fall, which is the most destructive disease of the rubber plantations in India. However, some clones show certain degree of tolerance to Phytophthora infection. To determine the effectiveness of β-glu to resist Phytophthora infection in Hevea, a susceptible clone (RRIM 600) and a highly tolerant clone (RRI 105) were analysed for induction of glucanase upon pathogen infection. Two experimental approaches, northern hybridisation and relative quantitative RT-PCR, were
performed to study the gene expression. β-Actin and 18S RNA genes were used as internal controls in RT-PCR and northern analysis respectively. Expression levels were calculated by measuring the band intensity. The level and onset of β-glucanase expression were found to be positively correlated to the level of resistance to the pathogen. Present study reports the induction of β-glucanase in both the tolerant and susceptible clones of Hevea, upon pathogen infection. However, the timing and magnitude of induction varies between the clones. In northern no signals were generated in the uninfected control samples. After inoculation, an exponential increase in β-glucanase transcript levels observed in both clones and it reached a peak at 48 h after inoculation. But a faster rate of increase was observed in tolerant clone and this was more evident in due course. Four days after inoculation, the transcript levels remains 15-fold higher than its basal levels in tolerant clones, while the β-glucanase mRNA levels were depleted to drastically low levels at this stage in susceptible clone. Relative RT-PCR analysis with house keeping actin gene as internal control also indicate similar trends, validating its usefulness in studying the differential gene expression. As both northern and RT-PCR give a measure of only the β-glucanase mRNA transcript levels, to confirm the presence of the end product glucanase enzyme assay was also carried out using laminarin as the substrate. The enzyme activity was found to vary between clones and treatments in accordance with the northern and RT-PCR results. The faster, higher and more prolonged induction of β-glucanase observed in the case of tolerant clone as reported in the case of many other plant species, was shown to have important implications in combating the Phytophthora challenge in Hevea. Although lesions were formed on tolerant clones, this enzyme may play a significant role in disease resistance by limiting the extension of fungal hyphae within the necrotic tissue.

As reported earlier, the defense-related β-glucanase was found to be expressed constitutively in the latex of rubber trees. Tapping to collect latex is a process of controlled wounding and hence the defense-related genes may be expressing in order to sanitize and seal the wounded sites of the plant. In the present study it was observed that the level of this constitutive expression was almost similar in tolerant and susceptible clones. However, the more intense signals obtained with the latex of a wild accession, suggests a role for latex glucanase in fungal resistance.
Induction of β-glucan in *Hevea* during pathogen infection indirectly suggests their role in plant defense. In order to find out a more direct evidence for the inhibition of pathogenic fungal growth by host β-glucan, a functional cDNA clone was constructed that could express the rubber β-glucan in *E.coli*. There are many reports on the *in vitro* anti-fungal properties of plant glucanases. Class I basic vacuolar isoforms are tested positively in most of the anti-fungal assays. The effect seems to be enhanced greatly when combinations of glucanase and chitinase were used. While most of the studies utilize native enzymes purified from infected plant tissues, in the present study a recombinant *Hevea* β-glucan expressed in *E.coli* was tried. The sequence coding for mature functional protein, excluding the N and C-terminal extensions of pre-proprotein, was cloned unidirectionally in pET vector and transformed to an expression host of *E.coli*. Sequencing of the vector ensured correct orientation and reading frame of the cloned insert. Conditions were optimised for the induction of the recombinant protein in soluble form. The target gene was under the control of a T7 promoter, which could be activated in host cells that contain a chromosomal copy of the T7 RNA polymerase gene. This T7 RNA polymerase gene is under the control of a lac promoter and hence, could be induced with IPTG. Thus, the addition of IPTG to a growing culture induces T7 RNA polymerase, which in turn, transcribes the target DNA in plasmid. Under optimum conditions, a band of expected size (55.6 kDa) was detected in SDS-PAGE of the soluble protein fraction, isolated from the induced colonies. The target protein, formed in fusion with an N-terminal affinity tag, was purified by passing through an affinity column. Western blotting and thrombin cleavage of the N-terminal fusion tag confirmed its identity. Hydrolysis of its substrate, laminarin, ensured that the purified recombinant protein is in an active form.

The purified *Hevea* β-glucan was assayed for its anti-fungal activity against the ALF causing *P.meadii* using filter paper disc method. A single mycelial plug was inoculated in the center of a PDA plate and were incubated at 25°C. Sterile filter paper discs of 5 and 9 mm diameter were laid on the agar surface 1 cm from the periphery of the petridish and was soaked with 10 μg of the purified enzyme solution. Clear inhibition zones, observed around the filter discs treated with the enzyme, indicate the anti-fungal property of the purified protein. These inhibitory
zones remained visible for at least one week and then the fungus starts to overgrow the initial inhibition zones. β-Glu can destroy the fungi by thinning their cell wall at the hyphal tips by degrading the β-glucan that results in swelling and ultimate burst of the hyphal tip. The possible role of host β-glu in resisting the Phytophthora attack in Hevea that has been evident by its over-expression during fungal infection is being confirmed through the direct fungicidal action of the purified glucanase reported herein.

Last 15 years have shown considerable progress in understanding the structure and regulation of plant β-1,3-glucanases. It is now recognized that higher plant species produce a broad range of β-glu differing in primary structure, cellular localization and catalytic activity. Their involvement in disease resistance has been confirmed through their induction during pathogenesis, in vitro anti-fungal properties, and finally through the development of transgenic plants with enhanced resistance. Host β-glu will be predominantly important in combating Oomycete pathogens like Phytophthora because their cell wall is made principally of β-1,3 linked glucans. The most destructive disease of rubber tree in India is ALF caused by different species of Phytophthora. Although many promising clones were evolved in the last few decades, the narrow genetic base of rubber trees along with its long breeding cycle, high juvenility period and highly heterozygous nature remains as the major limitations for evolving fungal resistant plants through conventional breeding programmes. Further, a single genotype, RRII 105, occupies more than 70% of our rubber plantations. The intensive use of a monoculture crop with little genetic diversity may significantly enhance their susceptibility to increasingly aggressive pathogens. Considering the obstacles involved and time taken for conventional techniques, alternative molecular strategies involving modern tools of molecular biology hold great potential for crop protection programmes in Hevea. Since the present study also supports the importance of β-glu in plant defense, it can be presumed that the resistance of rubber plants to fungal attack can be greatly enhanced through development of transgenic plants, which over-express this anti-fungal protein. Prospects seems to be promising, as efficient protocols for genetic transformation and plant regeneration, which often are the major constrains in the development of transgenics in many crops, is already available in the case of rubber.