Ginger is one of the most important horticultural crop globally and used for its various medicinal as well as culinary preparations. The production of ginger is largely affected by *Fusarium oxysporium* f. sp. *Zingiberi* in India and in the Northeasten region of India. As such, we need to explore the plant’s own defense mechanism for an alternative to improve the plant varieties, as it is an asexual plant. There are also reports on successful transfer and introgression of resistance gene. Formation of natural seed set is hardly found and rare in ginger because of its stylar and stigmatic incompatibility (Dhamayanthi et al., 2003). As such transgenic intervention seems to be the only way to improve the cultivars. In plants like potato, rice, etc., it has been successfully shown earlier that introgression of alien gene has resulted in pathogen resistance. Thus, RGCs holds much promise to develop molecular tools to assist in future R gene isolation and introgression into the plant. In this study RGCs are isolated from *Zingiber* spp. found in North East India

### 5.1 PCR amplification of RGCs from *Zingiber* spp.

In this study, a PCR based approach have been opted for isolating and characterizing *Zingiber* RGCs. PCR with degenerate oligonucleotide primers has led to isolation and cloning of R-genes in various plant species in earlier works (Kanazin et al., 1996). The amplification of fragments of predicted band size i.e., 600bp, 500bp and 550bp with the degenerate primers, signifies the presence and detection of RGCs in
Zingiber spp. Apart from these amplicons, a non-specific 350bp fragment was found to amplify in some samples. It has been reported that from earlier works that non-specific fragments usually co-amplify from degenerate primers, which is a common feature (Di Gaspero et al., 2002) and (Lopez et al., 2003). A total of 16 sequences were derived using the degenerate primers along conserved motifs of the NBS-LRR genes. Degenerate primers amplifying the R- gene conserved motifs specify that the 14 accessions could lead to the identification and isolation of Fusarium resistance gene from Zingiber and its further analysis.

5.2 Sequence characterization of Zingiber RGCs

The significant level of homology of the sequences with R gene databases in GenBank shows that the sequences amplified were identical to the R-genes reported. This signifies the probability of presence of motifs of NBS-LRR class related to resistance proteins. Therefore, the sequences derived from Zingiber could be considered as potential RGCs. The range of sequence identity in BLAST analysis shows a strong identity to the largest class of R-gene with all the sequences being clustered in the NBS-LRR class.

Such a wide prevalence of the NBS-LRR gene signifies their ancient origin (Dangl and Jones, 2001). As the Zingiber RGCs show a high sequence identity to known R-genes in database, consist of conserved NBS-LRR domain found in other RGCs and represents ORF of more than 100 amino acids uninterrupted, they seem to be a strong candidate for R-gene isolation (Deng et al., 2001; Noir et al., 2001). It is observed that the number of RGCs identified in a plant varies from each other. For
instance, 12 sequences were characterized as RGCs in *Kaempferia* (Joshi R. K. et al., 2012). In alfalfa (Cordero and Skinner, 2002) and Citrus (Deng et al., 2000) about 54% and 75% respectively, of the sequences characterized were found to be RGCs, while in coffee only 15% were RGCs (Noir et al., 2001). The level of sequence identity between *Zingiber* RGC sequences and known resistance genes in the top blast hits varied from 87% to 91% between Zne19p6 and Zne31p6 respectively to *Zingiber officinale* clone ZoP26 (e-value: 1e-106) and *Zingiber zerumbet* clone ZzP226 (e-value: 1e-81). The percent identity of *Zingiber* RGCs lies within 80-91%. A comparable range of identities is also found in RGCs from other plant when similar approach is undertaken. BLASTP analysis in GenBank database indicates the presence of NB-ARC domain which shows that the RGCs are analogous to plant R-gene products and to the human Apaf-1 proteins (Van der Biezen and Jones, 1998). Studies on the multiple sequence alignment of the amino acid sequences show the presence of specific resistance nucleotide binding site (RNBS) and kinase 2 motifs. The priming sites, P-loop and GLPL are also highlighted. It has been reported that the last residue of kinase-2 motif of identified RGCs can be used to predict with high accuracy whether they belong to the TIR or non-TIR subclass of NBS-LRR R-genes (Meyers et al., 1999). The *Zingiber* RGCs were observed to belong to the non-TIR NBS-LRR subclass as a tryptophan residue is found to exist at the end of kinase-2 motif and the RNBS-A and RNBS-B which are reported to be non-TIR specific motifs are found within the NBS domain (Meyers et al., 1999; Pan et al., 2000). Earlier studies have shown that the TIR domain has not been reported in the NBS-LRR R-genes of other monocots like wheat (Dilbirligi and Gill, 2003), rice (Monosi et al., 2004; Zhou et al., 2004) and maize (Xiao et al., 2006).
a. **Phylogenetic relationships of Zingiber RGCs**

The phylogenetic tree constructed based on CLUSTALW alignment of amino acid sequences of resistance gene candidate (RGC) of *Zingiber* spp. shows their relation with the RGCs or R-genes from other plant species. The RGCs are separated into two major branches and classified into four classes (A-D). The RGCs clustered together are found to have similar domain structure and functions. Group A comprising 9 RGCs are sequences with the highest level of similarity with other R-genes. Sequences with multiple stop codons were observed to correspond to pseudogenes which are non-functional genes. The RGCs detected as pseudogenes were grouped and form a unique class which lies within class B. Such non-coding RGCs have been found to be reported in the paralogs of R-genes Xa21, Cf9, Pto and Dm3 (Song et al., 1997; McDowell et al. 1998) and observed in PCR experiments (Aarts et al., 1998; Vicente and King, 2001). The detection of such pseudogenes is reported to suggest their role in evolution of new specificities by methods of recombination and gene conversion (Michelmore and Meyers, 1998). Pseudogenes seem to have the potential of becoming a new gene (Brosius and Gould, 1992).

b. **Expression analysis**

The RT-PCR products amplified with specific primers designed which were around 200pb and 300bp fragments, were related with putative disease related proteins and show similarity with the R-genes of other plant species. The expressed *Zingiber* RGCs consist of probable disease resistance proteins and certain uncharacterised proteins. While, the expression of the RGC sequences were not detected as predicted. There are reports from earlier studies that the majority of the NBS-LRR resistance
genes are generally expressed at a low levels, they are usually unaffected by pathogen infection and transcripts might be difficult to detect even by gel blot analysis (Hulbert et al., 2001). Expression of R-genes has been found in only highly resistant varieties, but not in partially resistant varieties (Swetha et al., 2007). The amplification of such unrelated sequences may be due to the amplification on basis of P-loop alone as reported in literature (Rigden et al., 2000). The lack of detection and its expression may be due to presence of some non-functional promoter preceding the sequences or the RGCs might correspond to pseudogenes.

Reports on the expression of RGCs or R genes are found to be very few and constitutive expression of R-gene is still a topic on discussion. This seems to be because of a very low transcript level in many of the plants preventing the RGCs to be detected. Graham has also stated that the R-genes have a low transcript level. Expression studies of many R-genes suggest that they express at a moderate to high level if induced following infection (Yoshimura et al., 1998). In this study the lack of signals in cDNA may be due to interfering sequences.