VI. SUMMARY

In this study full length forisome gene is isolated from Phaseolus vulgaris and cloned in E. coli to aid further studies involving forisome protein. The spatial and temporal expression study of forisome gene is analysed in leaves, stem, roots and meristem at various stages by quantitative RT-PCR. This research paves a way for future research work involving forisome proteins in developing microfluidic lab on chip systems, artificial muscle, microgrippers, scaffolds in tissue engineering and vectors in drug delivery system.

Five plants of Fabaceae family viz. Vigna mungo (L.), Cicer arietinum (L.), Vigna unguiculata (L.), Phaseolus vulgaris (L.) and Vigna angularis (L.) were cultivated under natural conditions for sixteen days. The plants were watered at regular intervals. The growth parameters were viz. root length, shoot length, number of branches and number of leaves was monitored at regular intervals.

Total RNA was isolated from young leaves, stems, roots and meristems. First strand of cDNA was synthesised with oligo dT primer and screened for forisome gene. Gene specific degenerate primers were used for the amplification of the forisome gene. The gene specific degenerate primers were designed based on the existing sequences in National Centre for Biotechnology Information (NCBI).

The cDNA's having more than 1500 base pairs in size were sequenced by di-deoxy chain termination method. Among the five plants screened, the forisome gene sequence obtained from Phaseolus vulgaris was found to be the best based on gene coverage and gene sequence quality. The sequence was blasted using Basic Local Alignment Search Tool (BLAST) against the existing forisome gene
sequences in National Centre for Biotechnology Information (NCBI). There was 87-90% sequence homology with Glycine max sieve element occlusion protein (SEO-F4) with accession number of HM162864. This is the first report of forisome (tailed) gene sequence from Phaseolus vulgaris.

DNA barcoding is a method of scientific classification that uses a short genetic marker in an organism's DNA to identify particular species. Since Phaseolus vulgaris showed the maximum gene coverage, further work was carried out in Phaseolus vulgaris. The species of Phaseolus vulgaris was confirmed by three DNA marker genes psbA-trnH, rpoC1 and matK, using chloroplast genomic sequence as template. Forward and reverse primers were used to amplify the specific target regions. The amplified product was sequenced and was blasted against the existing nucleotide sequence in the database of NCBI.

Forisome proteins from Phaseolus vulgaris were isolated from six weeks old Phaseolus vulgaris plants were used to isolated protein. The separation was performed using sucrose density gradient centrifugation (20 to 80%). The isolated forisome protein was confirmed by polyacrylamide gel (SDS-PAGE). Protein bands were revealed by staining with Coomassie brilliant blue. After comparing the molecular weight of the isolated forisome protein with the known protein marker, the molecular weight of separated forisome protein was determined to be 75 kDa.

Quantitative real-time PCR (RT-qPCR) is an accurate and widely used technique to analyze expression levels of selected genes. Forisome gene expression studies were carried out during the developmental stages of the plant. Gene expression studies were carried out using real time PCR and four traditional internal controls were analysed and screened viz. (GADPH - Glyceraldehyde -3- Phosphate
Dehydrogenase, ACT 2 - Actin, EF1A - Elongation Factor-1 Alpha and EIF4 - Eukaryotic Initiation Factor-4). Among the four internal controls EF1A showed the most stable expression in all the parts of the plants. Hence EF1A was used as a reference gene to study the expression of forisome gene Spatial and temporal expression of forisome gene was carried out at regular time interval (4th, 7th, 10th, 13th and 16th day) of Phaseolus vulgaris growth.

The full length cDNA of the forisome gene from Phaseolus vulgaris was generated using 3’ and 5’ Rapid amplification of cDNA ends (RACE). RACE was done using a double-stranded (ds) adaptor which was ligated to both ends of ds cDNA by T4 DNA ligase. These adaptor - ligated ds 5’ - and 3 ’- cDNA fragments got selectively amplified by the PCR with a combination of gene-specific primer (GSP) and adaptor primer (AP). Thus full length forisome gene was obtained.

Using the forisome gene, amino acid sequence was deduced using ExPASy tool. The secondary structure of the forisome protein was depicted using predict protein software and was found to have a rod like coiled α sheets, β plates and conserved sequences of thioredoxin-fold. Conserved domains were also analysed using InterProScan software. Coiled coil structure was analysed using ExPASy software. Ramachandran Plot was also drawn to understand the stability of the structure.

TA vector is a unique vector that avoids the use of restriction enzymes and relies on the ability of adenine (A) and thymine (T) (complementary base pairs) on different DNA fragments to hybridize. Taq DNA polymerase preferentially adds an adenine to the 3’ end of the PCR product. Such PCR amplified inserts are cloned into linearized TA vectors that have complementary 3’ thymine overhangs.
The full length amplified forisome gene was ligated to the linear vector DNA and transformed into competent cells of E.coli DH5α.

The transformed bacteria with the recombinant vector were initially screened based on blue white colonies. Cells transformed with vectors containing recombinant DNA produces white colonies and cells transformed with non-recombinant plasmids (i.e. only the vector) grow into blue colonies. The white colonies contain plasmids with the gene of interest. The target gene disrupts the β-galactosidase gene and therefore the colonies appear white. However, plasmids contain ampicillin resistance gene and that was used for selection of the bacteria.

Colony PCR was done to confirm the size and the presence of the forisome gene. Amplification of the plasmid insert was achieved by using gene specific forward and reverse primers. It was then sequenced and confirmed for the presence of full length forisome gene in the TA vector.