CLONING AND CHARACTERIZATION OF A FULL-LENGTH *CAB* GENE ENCODING THE LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN IN *HEVEA BRASILIENSIS*

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A full-length genomic DNA encoding the light harvesting chlorophyll a/b binding protein (*CAB*), expressed mainly in green leaves, for which the expression is reported to be directly related to the juvenility of the plant tissues, was cloned and sequenced. Initially *CAB* gene was amplified through PCR from the genomic as well as cDNA of *Hevea brasiliensis* using primers designed from the conserved regions of the *CAB* gene reported earlier from related plant species. The PCR amplified fragment (0.5 kb) was eluted, cloned and sequenced. The sequence revealed the presence of 525 bps that showed 91 per cent homology with *CAB* mRNA from *Ricinus communis*. Further, full length *CAB* gene (0.8 kb) was amplified from genomic DNA using *Ricinus communis* specific primers. The sequence revealed the presence of a fragment of 802 bp which showed 90 per cent sequence homology with the reported cDNA sequence of *CAB* gene from *Ricinus communis*. The region amplified in the present study contains the full protein coding sequence with no introns. The phylogenetic tree showed the close relationship of the *CAB* gene of *Hevea* with *Ricinus communis* and *Populus trichocarpa* apart from the other eukaryotic plants. The amino acid sequence deduced spans an open reading frame of 265 amino acids with predicted molecular weight of 28 kDa and iso-electric point (pI) as 5.45. A protein model was also predicted for the amino acid sequence. This is the first report on isolation and characterization of a full length *CAB* gene from *Hevea brasiliensis*.

Key words*: CAB* gene, Cloning, Characterization, Differential expression

INTRODUCTION

Hevea being a cross pollinated, the seedlings are highly heterozygous. Through conventional breeding elite clones have been developed and are propagated commercially by bud grafting. Plant regeneration systems in *Hevea* need to be developed from clonal materials which are physiologically mature for use in genetic transformation experiments. The explants collected from mature clonal materials are highly recalcitrant to *in vitro* culture when compared with seedling derived materials in *Hevea*. Despite these limitations, plant regeneration systems have been developed in *Hevea* from different explants and these are being utilized in transgenic plant development (Thulaseedharan *et al*., 2014; 2017). Somatic embryogenesis and plant

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regeneration were also achieved from leaf explants of six month old, glass house grown bud grafted plants of *Hevea* (Kala *et al.,* 2006). It was observed that physiological age of the explant source determines its embryogenic capacity which decreased with maturity of the source plant. With leaf explants collected from plants of different physiological maturity, variation was observed in the rate and time taken for embryogenic callus initiation and embryo induction (Kala *et al.,* 2009). It is also reported that the frequency and time taken for embryogenic callus initiation was influenced by the explant source in *H. brasiliensis* (Lardet *et al*., 2009).

Juvenility of plant materials is a key factor in micro propagation because the regeneration ability of woody plants decreases with maturity (Huang *et al*., 2000). Juvenile - mature phase change is associated with changes in gene expression. In several crops, comparison of cDNA libraries made from RNA extracted from juvenile and mature shoots indicated differences in gene expression which in turn influence the *in vitro* culture response (Hutchison *et al.,* 1988). Among the several genes differentially expressed during phase change, genes encoding elements of the photosynthetic apparatus such as the chlorophyll a/b binding *CAB* protein have also been reported. The largest class of juvenile-induced genes was comprised of those involved in photosynthesis. It is also well established that photosynthetic rates and related physiological attributes differ between juvenile and reproductively mature individuals in many species examined (Bond, 2000). Higher expression of the *CAB* gene is an indication of the juvenile nature of the tissue which imparts more regeneration ability in tissue culture. The *CAB* proteins of the light-harvesting complex of PSI and II are encoded by a nuclear gene family, synthesized as precursor polypeptides in the cytoplasm, and transported into the chloroplast. *CAB* proteins are located in the chloroplast thylakoid membrane where they bind Chl a, Chl b, and carotenoid pigments to form pigment/protein complexes that transfer absorbed light energy to photo system reaction centers (Chang and Walling, 1991).

CAB protein gene was differentially expressed in juvenile and mature plants. Steady-state *CAB* mRNA levels are relatively higher in newly expanding shoot foliage from juvenile plants of eastern larch compared to mature plants. In both larch and English ivy, a comparison of cDNA libraries made from RNA extracted from juvenile and mature foliage indicate that there are differences in gene expression between juvenile and mature shoots (Hutchinson *et al*., 1988; Hackett *et al*., 1991). The expression of sequences for the *CAB* gene decreased with maturation (Hutchison *et al*., 1990; Woo *et al*., 1994). Experiments showed difference in *in vitro* culture response of leaf explants which is also a major site of *CAB* gene function. In the present work, an attempt has been made to isolate and characterize chlorophyll a/b binding *CAB* protein gene from *Hevea brasiliensis*.

MATERIALS AND METHODS

Cloning and partial sequence characterization of the *CAB* gene from genomic DNA

Genomic DNA was isolated from young, uninfected leaves collected from glasshouse grown bud grafted plants of *Hevea brasiliensis* (clone RRII 105) following the modified CTAB protocol of Doyle and Doyle (1990). Primers were designed from the conserved middle portion of *CAB* gene sequences previously reported in *Ricinus* (accession no. XM_002524570.1), tobacco (AY219853.1),

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tobacco         GTGCAAAGCCCATGAAACTCAAGCCTCAAATCAACTCTTTCTTTTTGTGCATTCAAGAGT 960
Pisum           ------------------------------------------------------------
Fagus           --------------------------------AATTCGGCACGAGATTAATTTCTCAATTT 29
Vigna           ----------------GACAACTCCCACACCTCCAACTACAAACCACAAAGAAAAAGGA 43
Brassi          ---GGCCGGGGACCCATCTCTTGGCTCATAAACAACAACCAATAAACGTTTTATTTCGCT 57
ara             -----------ACCCATTTCTTGGCTTACAA-CAACAAATCTTAAACGTTTTACTTTGTG 48
Zea             ---------------CTCTCACCAGTCACAAAAAAAAACCCATAAAATCAACAAAAAAGA 45

tobacco         TATCATTTTACTCCTACAATGGCTGCTTCTACCACAGCTCTTTCTTCTCC---TTTTGCC 1017
Pisum           ------------------ATGGCCGCTTCATCCATGGCTCTCTCTTCCCCAACCTTGACT 42
Fagus           TGTCATTAAATTCCAGCAATGGCAGCCTCAACCATGGCTCTCTCTTCCCCATCTCTTGCC 89
Vigna           AAGTTCTTTGCAACAATGGCTGCTGCATCCTCCATGGCACTCTCATCCCCCTCCTTCGCT 103
Brassi          CAGCACT---CAACCGCAATGGCCGCCTCAACAATGGCTCTCTCCTCCCCTGCCTTCGCC 114
ara             CTGCACTACTCAACCTCAATGGCCGCCTCAACAATGGCTCTCTCCTCCCCTGCCTTCGCC 108
Zea             AGAAAC------GTTCTAATGGCTGCCTCAACCATGGCTCTCTCCTCCCCTGCCTTCGCC 99
                            ** ** **  * *  ** ** ** ** **     *  *

tobacco         GGAAAGGCGGTAAAACTCTCACCATCTTCCTCTGAAGTTACTGGAAATGGGAAAGTTACT 1077
Pisum           GGCAAGCCAGTCAAGCTGAACCCATCAAGCCAAGAATT---GGGAGGTGCAAGGTTCACC 99
Fagus           GGCAAGGCAGTGAAGCTTGCCCCTTCAACCCCAGAGCT---CAATGTTGGACGTGTCAGC 146
Vigna           GGCAAGGCCGTCAAGCTTGCCCCATCAGCCCCCGAAGT---------CGGGAGGGCCAGC 154
Brassi          GGAAAGGCCGTCAAGCTTTCCCCAGCAGCATCAGAAGTCCTTGGAAGCGGCCGTGTGACA 174
ara             GGTAAGGCCGTCAAGCTTTCCCCCGCGGCATCTGAAGTCCTTGGAAGCGGCCGTGTGACA 168
Zea             GGAAAGGCCGTGAAGCTTTCTCCTTCAGCATCAGAAGTCCTTGGAAGCGGCCGTGTGACA 159
                ** *** * ** ** **    **  *       **  *          *       *

tobacco         ATGAGGAAGACTGCTAACAAGGCCAAGCCTGTCTCTTCTGGTAGCCCATGGTATGGTCCT 1137
Pisum           ATGAGGAAGTCTGCTACCACCAAGAAAGTAGCCTCCTCTGGAAGCCCATGGTACGGACCA 159
Fagus           ATGAGGAAATCAGCCAGCAGGAA------TGTTTCCTCTGGAAGCCCATGGTACGGCCCA 200
Vigna           ATGAGGAAGACCGTCACCAAGCA------GGTTTCCTCCGGAAGCCCATGGTACGGCCCA 208
Brassi          ATGAGGAAGACCGTAGCCAAGCCAAA---GGGCCCATCAGGCAGCCCATGGTACGGATCC 231
ara             ATGAGGAAGACTGTTGCCAAGCCAAA---GGGCCCATCAGGCAGCCCATGGTACGGATCT 225
Zea             ATGAGGAAGACCGTCGCCAAGCCAAA---GGGCCCATCGGGCAGCCCATGGTACGGTTCC 216
                ********  * *    **           *   * ** ** ********** **  *

tobacco         GACCGTGTCAA<b>GTACTTGGGCCCATTCTC</b>TGGTGAGTCTCCAAGCTACTTGACTGGTGAG 1197
Pisum           GACCGTGTTAA<b>GTACTTAGGCCCATTCTC</b>CGGTGAGTCTCCGTCCTACTTGACTGGAGAG 219
Fagus           GACCGTGTCAA<b>GTACTTGGGCCCATTCTC</b>TGGTGAGCCCCCATCTTACCTTACTGGTGAA 260
Vigna           GACCGTGTCAA<b>GTACTTGGGCCCATTCTC</b>CGGCGAGCCCCCGTCCTACCTCACCGGCGAG 268
Brassi          GAGAGAGTCAA<b>GTACTTGGGCCCATTCTC</b>CGGCGAGCCACCGAGCTACCTTACCGGACAG 291
ara             GACCGTGTCAA<b>GTACTTGGGTCCATTCTC</b>TGGCGAATCACCGAGCTACCTTACCGGAGAG 285
Zea             GAAAGAGTCAA<b>GTACCTAGGTCCATTCTC</b>TGGCGAGCCACCGAGCTACCTTACCGGAGAG 276
                **  * ** ****** * ** ******** ** **  * **    *** * ** **  *

tobacco         TTTCCTGGTGATTACGGGTGGGACACTGCCGGACTTTCAGCTGATCCAGAAACATTCGCC 1257
Pisum           TTCCCCGGTGACTACGGTTGGGACACTGCCGGACTCTCTGCTGACCCAGAGACATTCTCC 279
Fagus           TTCCCTGGTGACTATGGCTGGGACACTGCTGGGCTATCAGCTGACCCAGAAACCTTTGCT 320
Vigna           TTCCCAGGTGACTACGGTTGGGACACTGCTGGGCTTTCCGCTGACCCAGAGACCTTCGCC 328
Brassi          TTCCCCGGAGACTACGGATGGGACACCGCAGGTCTCTCAGCCGTACCCGAGACGTTCGTG 351
ara             TTCCCCGGAGACTACGGATGGGACACCGCCGGACTTTCAGCTGACCCCGAGACATTCGCA 345
Zea             TTCCCTGGTGACTACGGATGGGACACCGCCGGTCTATCAGCCGACCCCGAGACCTTCGCC 336
                ** ** ** ** ** ** ******** ** ** ** ** ** *  ** ** ** **

tobacco         AAGAACCGTGAGTTGGAGGTGATCCACTGCAGATGGGCCATGCTTGGAGCTCTTGGTTGT 1317
Pisum           AAGAACCGTGAGCTTGAAGTCATCCACTCCAGATGGGCTATGTTGGGTGCTTTGGGATGT 339
Fagus           AAGAACCGTGAGCTTGAAGTGATCCACTCCAGATGGGCCATGCTTGGAGCTCTTGGGTGT 380
Vigna           AGGAACCGTGAGTTGGAAGTCATCCACTCCAGGTGGGCCATGCTGGGAGCCTTGGGCATC 388
Brassi          AGGAACCGTGAGCTAGAAGTTATCCACTGCAGGTGGGCCATGCTCGGTGCCCTAGGCTGC 411
ara             AGGAACCGTGAACTAGAAGTTATCCACAGCAGGTGGGCCATGCTCGGAGCCCTAGGCTGC 405
Zea             AGGAACCGTGAGCTAGAAGTTATCCACTGCAGGTGGGCCATGCTCGGAGCCCTAGGCTGC 396
                * *********  * ** ** ******  *** ***** *** * ** **  * **

tobacco         GTCTTCCCCGAACTCTTGGCACGTAATGGTGTCAAGTTCGGTGAAGCTGTATGGTTCAAG 1377
Pisum           GTCTTCCCAGAGCTTTTGTCTCGCAACGGTGTTAAATTCGGTGAAGCTGTGTGGTTCAAG 399
Fagus           GTCTTCCCTGAGCTCTTGGCCCGTAACGGGGTTAAGTTTGGTGAGGCCGTTTGGTTCAAG 440
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Forward primer site ←

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Vigna           GTCTTCCCCGAACCCTTGTCCCGCAACGGTGTGAAATTCGGCGAGGCCGTCTGGTTCAAA 448
Brassi          GTCTTCCCGGAGCTGTTGGCCAGGAACGGAGTCAAGTTCGGAGAGGCGGTTTGGTTCAAG 471
ara             GTCTTCCCTGAGCTTTTGGCTAGAAACGGAGTCAAGTTCGGAGAGGCGGTTTGGTTCAAG 465
Zea             GTTTTCCCTGAGCTCTTGGCTCGTAACGGAGTCAAGTTCGGAGAAGCGGTTTGGTTCAAG 456
                ** ***** ** *  *** *  * ** ** ** ** ** ** ** ** ** ********

tobacco         GCTGGATCCCAAATTTTTAGCGAGGGTGGACTTGACTACTTGGGCAACCCAAGTTTGGTC 1437
Pisum           GCAGGATCTCAAATCTTTAGCGAGGGTGGACTTGACTACTTGGGTAACCCAAGCTTGGTC 459
Fagus           GCTGGAGCCCAAATTTTCAGTGAAGGTGGGCTTGACTACTTGGGTAACCCAAGCTTGATC 500
Vigna           GCCGGTTCCCAGATCTTCAGCGAGGGTGGGCTCGACTACTTGGGCAACCCAAGCCTCATC 508
Brassi          GCCGGTTCGCAGATCTTCAGCGAAGGAGGACTTGATTACTTGGGAAACCCTAGCTTGGTT 531
ara             GCCGGTTCACAGATCTTCAGCGATGGAGGGCTCGATTACTTGGGAAACCCTAGCTTGGTT 525
Zea             GCTGGTTCACAGATCTTCAGCGAAGGAGGACTCGACTACTTGGGCAACCCGAGCTTGGTC 516
                ** **  * ** ** ** ** ** ** ** ** ** ******** ***** **  *  *

tobacco         CACGCACAAAGCATCTTGGCCATTTGGGCTTGCCAAGTTGTGTTGATGGGAGCTGTCGAA 1497
Pisum           CATGCACAAAGCATCCTTGCCATCTGGGCTACTCAAGTTATCTTGATGGGAGCTGTCGAA 519
Fagus           CATGCACAAAGCATTTTGGCCATCTGGGCCACACAGGTTATCTTGATGGGTGCTGTTGAG 560
Vigna           CACGCTCAGAGCATCCTCGCCATCTGGGCCACCCAAGTCATCCTCATGGGCGCCGTCGAG 568
Brassi          CACGCTCAGAGCATCTTGGCTATTTGGGCTACTCAAGTGATCTTGATGGGAGCCGTTGAA 591
ara             CACGCTCAGAGCATTTTGGCCATTTGGGCCACACAAGTTATTTTGATGGGAGCCGTTGAA 585
Zea             CACGCTCAGAGCATCTTAGCCATTTGGGCTACTCAGGTGATCCTCATGGGAGCTGTTGAG 576
                ** ** ** *****  * ** ** *****    ** **  *  * ***** ** ** **

tobacco         GGTTACCGTGTTGCTGG---TGAACCTCTCGGTGAGGTTGTTGACCCACTTTACCCCGGT 1554
Pisum           GGTTACCGTATTGCCGG---TGGCCCTCTTGGTGAGGTGGTTGACCCACTTTACCCAGGT 576
Fagus           GGTTACCGTATTGCTGG---TGGGCCACTTGGTGAGGTGACTGACCCACTTTACCCAGGT 617
Vigna           GGTTACCGTATTGCCGG---TGGGCCTCTCGGTGAGGTCACTGACCCCACTTACCCCGGT 625
Brassi          GGTTACAGAGTCCCAGGAGATGGGCCGTTGGGAGAGGCCGAGGACTTGCTTTACCCAGGT 651
ara             GGCTACAGAGTCGCAGGAAATGGGCCATTGGGAGAGGCCGAGGACTTGCTTTACCCCGGT 645
Zea             GGTTACAGAGTTGCCGGAGAGGGACCATTGGGAGAGGCCGAGGACTTGCTTTACCCAGGA 636
                ** *** *  *  * **    *  **  * ** ****     ***     ****** **

tobacco         GGCAGCTTCGACCCATTGGGCCTTGCTGAAGATCCAGAGGCTTTCGCTGAGCTCAAGGTA 1614
Pisum           GGTAGCTTTGACCCATTGGGCTTAGCTGACGACCCAGAAGCATTCGCAGAATTGAAGGTG 636
Fagus           GGAAGCTTTGACCCATTAGGCCTTGCTGATGACCCAGAGGCTTTTGCTGAGCTTAAGGTG 677
Vigna           GGCAGCTTCGACCCATTGGGTCTCGCTGACGACCCAGAGGCTTTCGCCGAGCTCAAGGTC 685
Brassi          GGCAGCTTTGACCCGTTGGGTTTGGCTACTGACCCAGTGGCCTTCGCGGAGTTGAAGGTG 711
ara             GGCAGCTTCGACCCATTGGGTTTGGCTACCGACCCAGAGGCATTCGCTGAGTTGAAGGTG 705
Zea             GGCAGCTTTGACCCATTGGGTCTCGCTACCGACCCAGAAGCTTTCGCCGAGTTGAAGGTG 696
                ** ***** ***** ** **  * ***   ** ****  ** ** ** **  * *****

tobacco         AAGGAAATCAAGAATGGTAGACTTGCTATGTTCTCCATGTTT GGATTCTTTGTTCAGGC T 1674
Pisum           AAGGAACTCAAGAACGGTAGATTAGCTATGTTCTCTATGTTT GGATTCTTTGTTCAAGC T 696
Fagus           AAGGAGCTCAAGAATGGAAGACTGGCTATGTTCTCTATGTTT GGATTCTTTGTTCAGGC C 737 Reverse primer
Vigna           AAGGAACTCAAGAACGGTAGGTTGGCCATGTTCTCCGTGTTC GGGTTCTTCGTCCAGGC C 745 sitc
Brassi          AAGGAGATCAAGAACGGAAGATTGGCTATGTTCTCTATGTTT GGATTCTTTGTTCAAGC T 771
ara             AAGGAGCTCAAGAACGGAAGATTGGCTATGTTCTCTATGTTT GGATTCTTCGTTCAAGC C 765
Zea             AAGGAGATCAAGAACGGAAGGTTGGCTATGTTCTCTATGTTT GGATTCTTTGTCCAGGC T 756
                *****  ******* ** **  * ** ********  **** ** ***** ** ** **

tobacco         CTCGTCACCGGAAAGGGTCCATTGGAGAACCTTGCTGACCACCTTGCTGACCCGGTTAAC 1734
Pisum           ATTGTGACCGGAAAGGGACCTTTGGAGAATCTTGCTGATCATCTTTCCGACCCAGTTAAC 756
Fagus           ATTGTGACTGGAAAGGGACCCATTGAGAACCTTGCTGACCACCTTGCTGACCCAGTCACC 797
Vigna           ATTGTCACCGGAAAGGGACCATTGGAGAACCTCGCCGACCACCTTGCTGACCCAGTCAAC 805
Brassi          ATTGTCACTGGTAAGGGACCTTTGGAGAATCTTGCTGACCATTTGGCTGATCCAGTTAAC 831
ara             ATCGTCACTGGTAAGGGACCGATAGAGAACCTTGCTGACCATTTGGCCGATCCAGTTAAC 825
Zea             ATTGTCACTGGAAAGGGACCGTTGGAGAATCTCGCCGACCATTTGGCTGATCCAGTCAAC 816
                 * ** ** ** ***** **  * ***** ** ** ** **  *  * ** ** ** * *

tobacco         AACAACGCCTGGTCTTATGCCACTAACTTTGTTCCCGGAAAGTGAAG-ATCTTAAATACG 1793
Pisum           AACAATGCATGGTCATATGCCACCAACTTTGTTCCCGGAAAGTGAGT-CGTCGTATTGTG 815
Fagus           AACAATGCTTGGGCTTATGCCACAAACTTTGTCCCCGGGAAATGAGC-ATAGGCAGAAAA 856
Vigna           AACAACGCCTGGGCCTACGCCACCAACTTCGTCCCCGGAAAGTGAGC-GTCAACAGAATG 864
Brassi          AACAATGCATGGGCCTTTGCTACCAACTTCGTTCCCGGAAAGTCAACGATGTTTTATCTG 891
ara             AACAACGCATGGGCCTTCGCCACCAACTTTGTTCCCGGAAAGTGAGCCAAGTTTTATCTG 885
Zea             AACAACGCATGGGCCTTCGCCACCAACTTCGTCCCCGGAAAGTGAAGCTTATGTGAGTGA 876
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Fagus           AAAA-TGCGAATTTTACTTTTTGTATTGATGATGATGTTGTAAATTATTTGCGAAAGTAA 915
Vigna           ACTTGTGCCCTCGAGAGTTTTAGATTTCAGTTTGTTGAAGTATATGTTAT-AAATTGCAA 923
Brassi          TAATTTGC----------TTCAGTATTTGCTTCTTGTGCGAGTGTAAAAGGAGAAAAAGA 941
ara             TATTTTGC----------TTCAGTCTTTGCTTC----GTGAGTGTGAGAGGAGAAAGAGA 931
Zea             GAGCCAGAGAAAGAGAAGTTTGTTTGTGGTCTTTCCTATGTAAATTTGTGCAATTTCCTT 936
                                 *

tobacco         ------------------------------------------------------------
Pisum           TGCAACTGAATGTTAGTGTTTTTTTCAGTGTTTAAAAAAAAA------------------ 916
Fagus           TGGAGATTACCTTTGGTCTTATAAAATGTTGTGTCGTGTAACTTGTAAAATTTCCATTAT 975
Vigna           TGTACTTCAGATAATGTGAATCATCTTGTGTATTCGATCCAAAAAAAAAAAA-------- 975
Brassi          TGTTGTTTACATGTAAAATTTGCAAGTATTTGTATGATTTACCATTAATCCAATAACTCT 1001
ara             GCTTGTTTAGATGTAAAATTTGCAAGACTTTGTATT-TTTTTCATTAATCAAATAACTCG 990
Zea             TGTCGTATCTTTGTATGATTCATCAGAAATCGAACCTCTTTTTCTCAACTGTGTTGGTGT 996


tobacco         ----------------------------------------------------
Pisum           ----------------------------------------------------
Fagus           TGCATCTCTACTTGTAAACTGCGAACTATGCCATG------------------- 1010
Vigna           ----------------------------------------------------
Brassi          TTGTCTTAGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA------------- 1042
ara             TTTTTCTCTCAAATTATCTGGCAATTACCAAATTGGATTCGTTTTATGTCCTTC 1044
Zea             TTTACCTGAAGACTCGAACTGAACTGAATAAAAAAAAAAAAAAAAAA------ 1044
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Fig. 1. Multiple sequence alignment for primer synthesis

Pisum (M64619.1), *Brassica* (AY288914.1) and *Arabidopsis* (NM_001036140) aligned by CLUSTAL W software at the EMBL EBI site (Thompson *et al*., 1994). The nucleotide sequence of the designed primers was as follows: forward primer-5′ GTA CTT GGG TCC ATT CTC 3′ and reverse primer -5′ GCC TGA ACA AAG AAT CC 3′ (Fig.1). The PCR was carried out in 20 ML reactions, which contained 1X buffer 1.5 mM $MgCl_2$ (pH 8.3), 100 ML each of dNTPs, 0.5 unit of *Taq* DNA polymerase (M/ S Bangalore Genei, India), 20 ng of template DNA and 250 nM of primers. The PCR conditions were: initial denaturation at 94°C for three minutes followed by 36 cycles with denaturation at 94°C for one min., annealing temperature of 60°C for one min. and extension at 72°C for one min. The final extension was carried out at 72°C for 10 min. Amplified products were separated in a one per cent agarose gel.

The amplified fragment was eluted out using the DNA Gel Band Purification Kit (M/S Amersham Pharmacia Biotech, USA) and after purification using strataclone PCR cloning kit (M/S Stratagene, USA) was used for cloning. The recombinant vector was transformed into chemically competent *E. coli* (DH5α) cells supplied along with the kit. The transformed cells were plated on the LB agar plates containing 50 μg ml^{-1} ampicillin, the positive colonies were selected and colony PCR was done to confirm the positive clones. Plasmids were isolated from positive clones using Perfect prep Plasmid mini Kit (M/S Eppendorf, USA). Further confirmation of transformation was done by amplification of the insert from the plasmids. About 2 ng of plasmid DNA was used as template in a PCR reaction of 30 cycles with the same primer used to amplify the *CAB* gene. Amplification was checked through 1.5 per cent agarose gel electrophoresis. The recombinant plasmids were sequenced at M/S Macrogen, Korea. Sequence was aligned using the CLUSTAL W software with the reported cDNA sequence of *CAB* gene (Accession no: M60274). The sequence

obtained was compared with the *CAB* gene sequences from different plants with the BLAST analysis programme of NCBI (National Center for Biological Information), USA (Altschul *et al.*, 1990).

PCR amplification of *CAB* cDNA

RNA was isolated from leaves of glass house grown bud grafted plants of *Hevea brasiliensis* (clone RRII 105) through a modified protocol of Kush *et al.* (1990) and checked for DNA contamination and quality by agarose gel electrophoresis. First strand cDNA was synthesized from the isolated RNA by reverse transcription reaction with oligo-(dT) primers using the 'Improm-II™ Reverse Transcription System' (Promega, USA). RT-PCR was carried out using gene specific primers of *CAB* gene used for genomic DNA amplification. The amplified product was cloned and sequenced as described earlier.

PCR amplification of full length *CAB* gene from genomic DNA

Since the sequence showed 91 per cent (maximum) homology with *Ricinus communis*, an attempt was made to amplify the full coding region of the sequence by designing primers from cDNA sequence of *CAB* gene reported in *Ricinus* (Accession No.XM_002524570.1). The forward and the reverse primers contained the start and stop codons, respectively and the sequences are as follows: Forward primer 5'- CAA ATG GCT ACC TCT ACA ATG G - 3' and Reverse primer - 5'- CTC ACT TTC CGG GGA CAA AG - 3'. The amplified product was cloned and sequenced as described earlier.

Amino acid sequence and protein model

The amino acid sequence was deduced using ExPASY translate tool (http: //web. expasy.org/ translate/). With the help of ProtParam tool (http://web.expasy.org/ protparam/) the characteristic features of the protein were identified. The Myristoylator tool (http:// web.expasy.org/ myristoylator) and Palmitoylator tool (http://web.expasy.org/ palmitoylator) predicted the myristoylation and palmitoylation sites, respectively. With the help of Plant-mPLoc tool (http:// www.csbio.sjtu.edu.in/bioinf/plant-multi), subcellular localization of the protein was virtually determined. The protein model was also predicted for the amino acid sequence. The protein sequences were submitted in the Protein Model Port (http://www.proteinmodelportal.org/) which generates protein models using Swiss-MODEL workspace (http:// swissmodel. expasy.org/workspace/).

RESULTS AND DISCUSSION

Cloning and characterization of *CAB* gene from genomic DNA

The genomic DNA (Fig.2a) isolated from the leaves was used for PCR amplification of the gene using the designed gene specific primers. Forward and reverse primers were designed and synthesized using the nucleotide sequences from the conserved regions after multiple sequence alignment of the *CAB* sequences reported earlier in other crops (Fig.1). A single band of approximately 0.5 kb was amplified (Fig. 2 b). The amplified product was ligated in Strata Clone™ vector (M/S Strataclone, USA) and transferred into DH5α competent cells. Positive transformants were identified through colony PCR (Fig. 2c) and these also showed amplification of the same 0.5 kb fragment. The isolated plasmids from the PCR positive colonies were sequenced after PCR confirmation of the

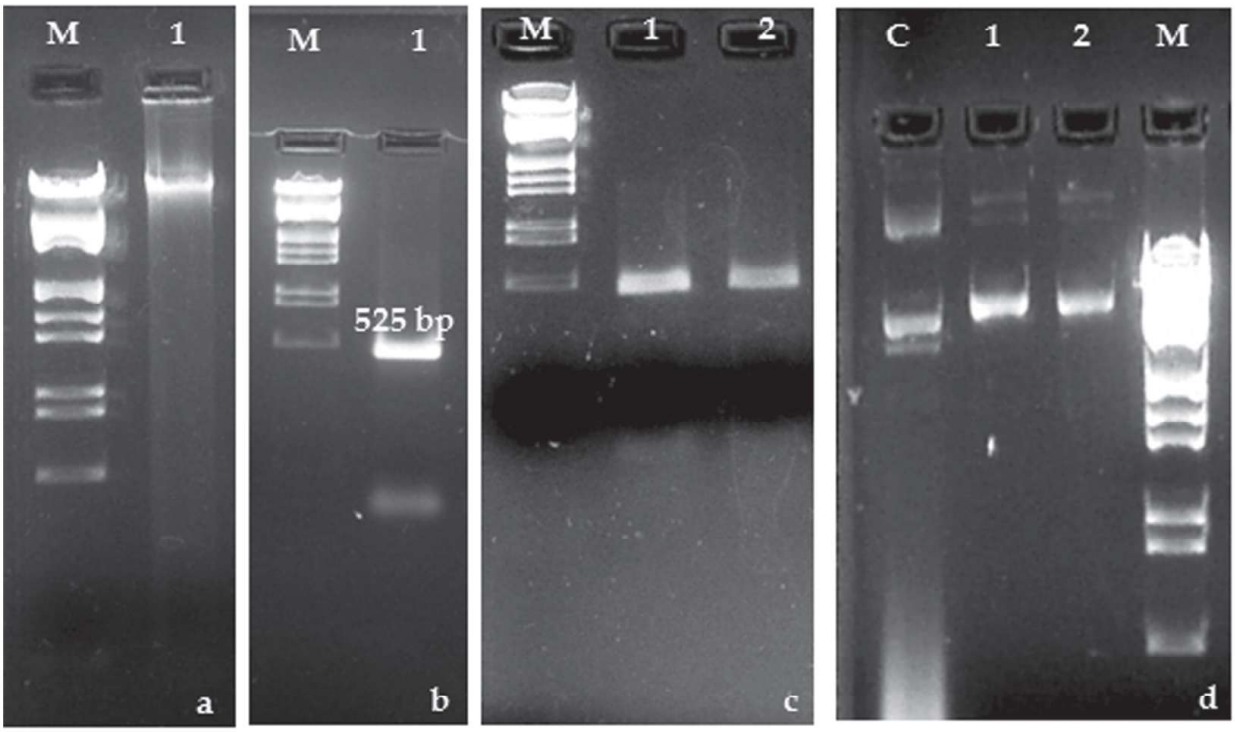


Fig. 2.(a- d) PCR amplification and cloning of the 525 bp *CAB* gene from genomic DNA

a. Genomic DNA (Lane 1-Marker,Lane 2-DNA)
b. PCR amplification of *CAB* gene. M-Marker,1-Cab gene
c. Colony PCR of the 0.5 kb CAB gene insert from the transformed colonies
d. Isolation of recombinant plasmids; C-Control, 1&2 recombinant plasmids, M- Marker

5'
GTACTTGGGTCCATTCTCTGGTGAGCCCCCATCCTACTTGACCGGTGAGTTCCC
TGGTGACTATGGCTGGGACACTGCTGGTCTCTCTGCTGACCCAGAAACCTTTGCC
AAGAACCGTGAGCTCGAAGTGATCCACTGCAGATGGGCCATGCTTGGAGCCCTT
GGGTGCGTCTTCCCCGAGCTCTTGGCCCGCAACGGAGTCAAGTTCGGCGAGGCA
GTGTGGTTCAAGGCAGGAGCCCAGATCTTCAGCGAGGGTGGTCTTGACTACTTG
GGTAACCCAAGCTTGATCCACGCACAAAGCATCTTGGCCATCTGGGCCGTCCAG
GTAGTGTTGATGGGTGCCGTTGAAGGTTACAGAATTGCCGGTGGGCCGCTCGGT
GAGGTCACAGACCCAATCTACCCAGGTGGAAGCTTTGACCCATTGGGCTTGGCT
GATGACCCAGAAGCATTTGCTGAGCTGAAGGTGAAGGAGATCAAGAACGGCAG
ATTGGCTATGTTCTCCATGTTC**GGATTCTTTGTTCAGGC** 3'

Forward primer- GTACTTGGGTCCATTCT
Reverse primer- GGATTCTTTGTTCAGGC

Fig. 3. Nucleotide sequence of the 0.5 kb *CAB* gene amplified

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
XM_002524570.1	*Ricinus communis* chlorophyll a/b binding protein, putative, mRNA	732	732	100%	0.0	91%	G
XM_002307689.1	*Populus trichocarpa* light-harvesting complex II protein Lhcb1 (Lhcb1-3), mRNA	719	719	99%	0.0	91%	UG
EF146739.1	*Populus trichocarpa* clone WS01213_B15 unknown mRNA	719	719	99%	0.0	91%	UG
XM_002519678.1	*Ricinus communis* chlorophyll a/b binding protein, putative, mRNA	715	715	99%	0.0	91%	G
XM_002306891.1	*Populus trichocarpa* light-harvesting complex II protein Lhcb1 (Lhcb1-2), mRNA	713	713	99%	0.0	91%	UG
EF147785.1	*Populus trichocarpa* clone WS0125_A05 unknown mRNA	713	713	99%	0.0	91%	UG
EF148774.1	*Populus trichocarpa* x *Populus deltoides* clone WS0137_M02 unknown mRNA	708	708	98%	0.0	91%	
EF148596.1	*Populus trichocarpa* x *Populus deltoides* clone WS0134_I20 unknown mRNA	708	708	98%	0.0	91%	
EF148766.1	*Populus trichocarpa* x *Populus deltoides* clone WS0137_J16 unknown mRNA	702	702	98%	0.0	91%	

Fig. 4. BLASTN analysis of *CAB* gene sequences producing significant alignment

plasmids (Fig.2d). Sequencing was done using M13 forward primer.

The sequence analysis showed that the amplified fragment had 525 base pairs after deleting the vector sequences (Fig. 3). The sequence comparison using BLAST N analysis of the isolated sequence with the reported cDNA sequence of *CAB* gene from *Ricinus communis* showed 91 per cent sequence homology in the 525 base pair region (NCBI

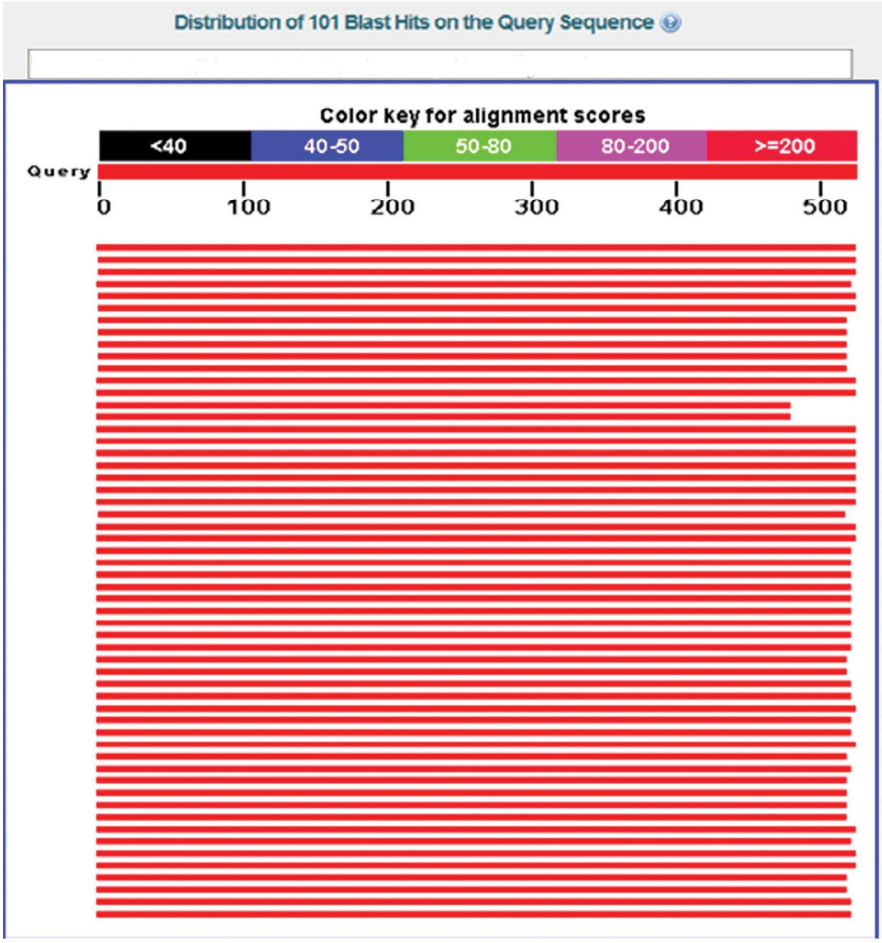


Fig. 5. BLASTN sequence alignment score of 525bp gene

accession No: M60274). BLAST N analysis also revealed sequence homology with *CAB* mRNA sequence from other species like *Manihot esculenta* (88%) and *Oryza sativa* (79%). Maximum sequence homology was obtained with *Ricinus communis* belonging to the same family, Euphorbiaceae (Fig. 4 & 5.). On comparison with cDNA sequence of *Ricinus communis*, no introns were observed in the amplified region. The genomic sequence has been registered in NCBI with the accession no: HM803119.1.

PCR amplification, cloning and sequencing of *CAB* cDNA

Good quality RNA was isolated from moderately mature leaves. First strand

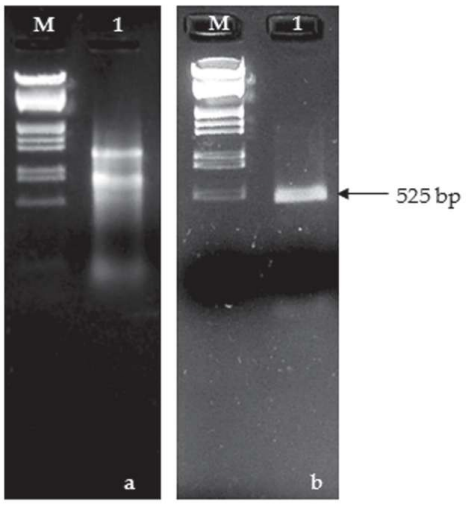


Fig. 6. (a&b) PCR amplification of *CAB* gene from cDNA
a. RNA isolation , Lane 1 M-Marker & 2 RNA
b. *CAB* gene amplification from cDNA

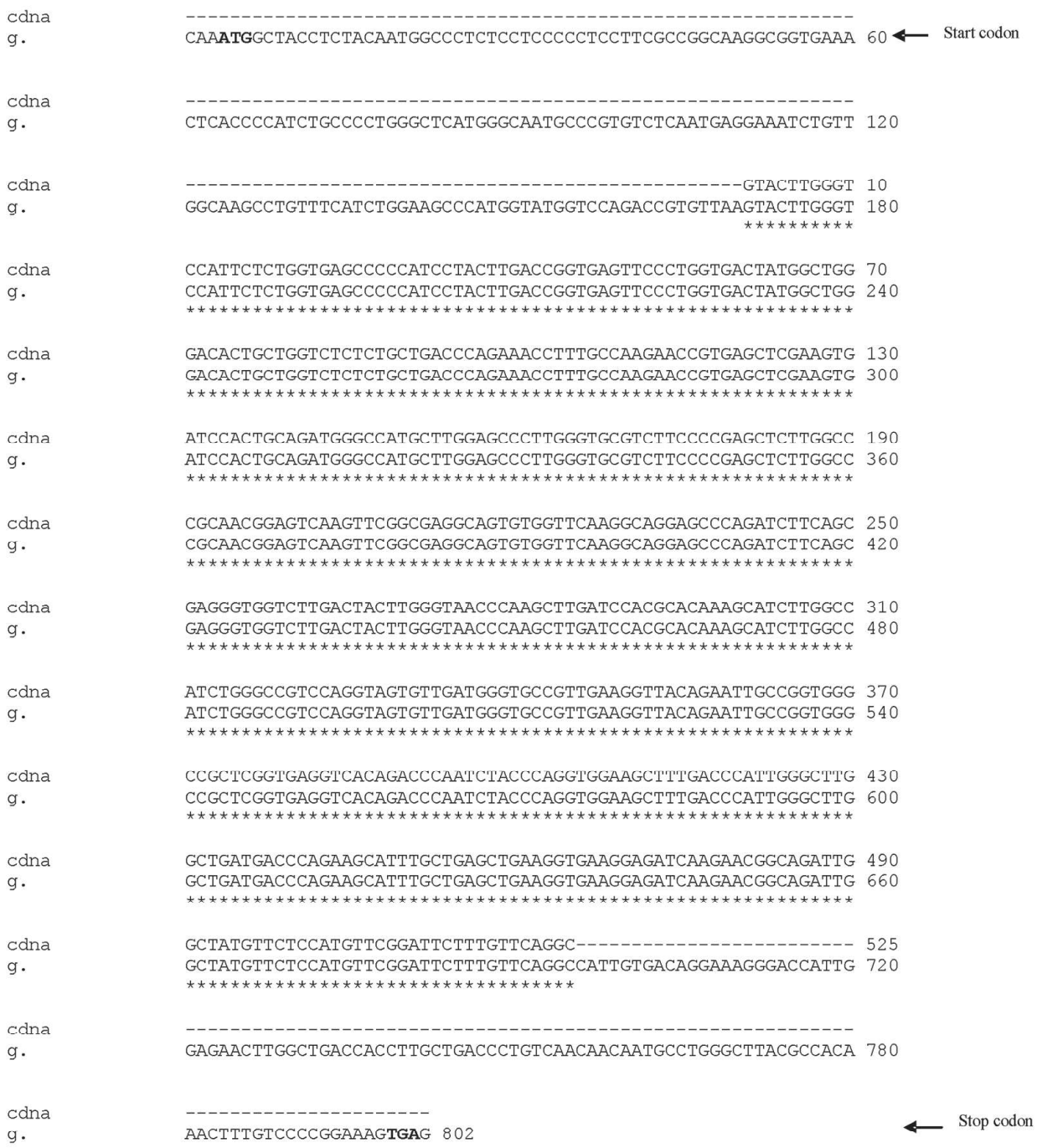


Fig. 7. CLUSTALW multiple sequence alignment of cDNA and genomic DNA sequences of *CAB* gene

cDNA synthesis was done from the isolated RNA by reverse transcription reaction. A 0.5 kb single band was amplified using the primer pairs used to amplify the 525 bp genomic sequence of the *CAB* gene (Fig. 6.). After cloning and sequencing, it was observed that the cDNA also contained 525 bps as observed in the genomic DNA. On alignment with the genomic sequence of *CAB* gene from *Hevea,* 100 per cent similarity

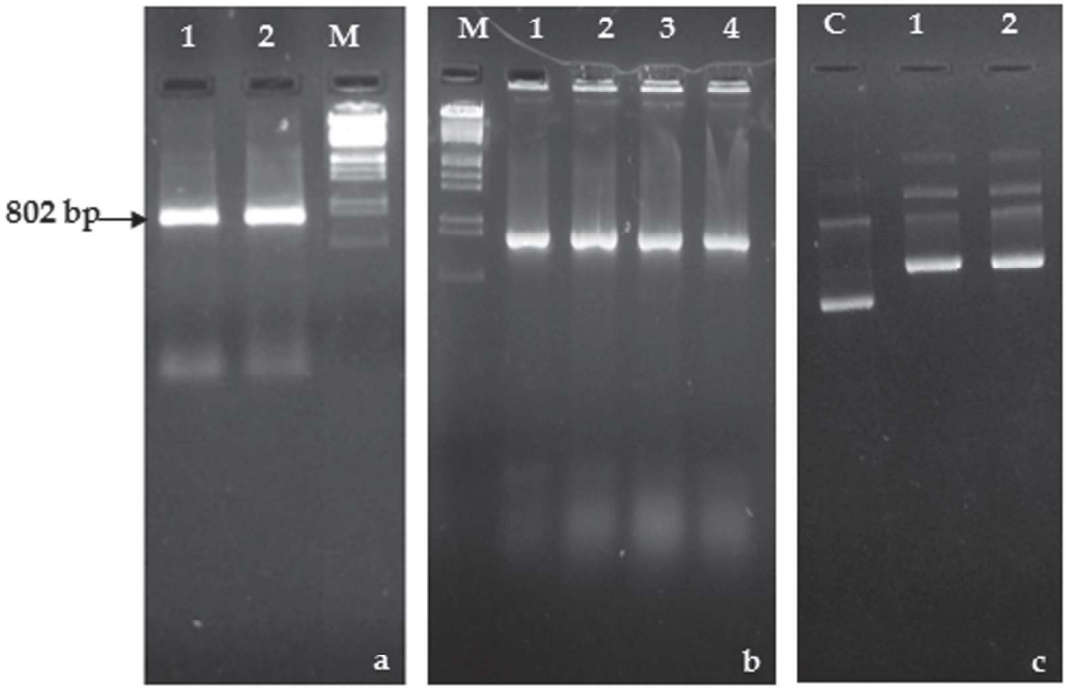


Fig. 8. (a-c). PCR amplification and cloning of full length *CAB* gene from genomic DNA
a. *CAB* 802 bp *CAB* gene amplification, 1&2 *CAB* amplification, M-Marker
b. Colony PCR of transformed colonies
c. Isolation of recombinant plasmids; C- Control, 1&2- recombinant plasmids

was observed and no introns were present in the isolated region (Fig. 7).

PCR amplification of full length *CAB* gene from genomic DNA

Using genomic DNA as template, specific amplification could be obtained, using the primer designed for full length *CAB* protein gene from cDNA sequence of *CAB* gene reported in *Ricinus communis*, after optimization of PCR conditions. The PCR amplified product had a size of approximately 0.8 kb (Fig. 8. a-c). The fragment was cloned and sequenced. The amplified sequence contained 802 bps (Fig. 9). The region amplified in the present study contained the full protein coding sequence. The start codon 'ATG' was at the 4th position and 'TGA' the stop codon was at the 799th position. The number of coding DNA sequence contained 798 nucleotides including the stop codon. Comparison with the reported mRNA sequences for *CAB* gene from other species showed that the 802 bp full length coding region isolated in the present study also contained no introns (Fig. 9).

The *CAB* protein genes are members of a multigene family and the 3' and 5' untranslated sequences of these genes diverge from each other. There exists a diversity of molecular species among the *CAB* proteins suggesting functional specialization which is brought about by the expression of a large set of different genes. Most of these genes contain no introns (Tyagi *et al.*, 2012). Possibly intron is not an essential factor of the *CAB* genes and it has later got introduced into the gene during evolution. *CAB* genes can also be grouped into sub-families based on the presence and

5'
CAAATGGCTACCTCTACAATGGCCCTCTCCTCCCCCTCCTTCGCCGGCAAGGCG
GTGAAACTCACCCCATCTGCCCCTGGGCTCATGGGCAATGCCCGTGTCTCAATGA
GGAAATCTGTTGGCAAGCCTGTTTCATCTGGAAGCCCATGGTATGGTCCAGACC
GTGTTAAGTACTTGGGTCCATTCTCTGGTGAGCCCCCATCCTACTTGACCGGTGA
GTTCCCTGGTGACTATGGCTGGGACACTGCTGGTCTCTCTGCTGACCCAGAAACC
TTTGCCAAGAACCGTGAGCTCGAAGTGATCCACTGCAGATGGGCCATGCTTGGA
GCCCTTGGGTGCGTCTTCCCCGAGCTCTTGGCCCGCAACGGAGTCAAGTTCGGC
GAGGCAGTGTGGTTCAAGGCAGGAGCCCAGATCTTCAGCGAGGGTGGTCTTGAC
TACTTGGGTAACCCAAGCTTGATCCACGCACAAAGCATCTTGGCCATCTGGGCCG
TCCAGGTAGTGTTGATGGGTGCCGTTGAAGGTTACAGAATTGCCGGTGGGCCGCT
CGGTGAGGTCACAGACCCAATCTACCCAGGTGGAAGCTTTGACCCATTGGGCTTG
GCTGATGACCCAGAAGCATTTGCTGAGCTGAAGGTGAAGGAGATCAAGAACGGC
AGATTGGCTATGTTCTCCATGTTCGGATTCTTTGTTCAGGCCATTGTGACAGGAAA
GGGACCATTGGAGAACTTGGCTGACCACCTTGCTGACCCTGTCAACAACAATGC
CTGGGCTTACGCCACAAAC**TTTGTCCCCGGAAAGTGAG** 3'.

Forward primer- CAAATGGCTACCTCTACAATGG
Reverse primer- TTTGTCCCCGGAAAGTGAG

Fig. 9. Nucleotide sequence of full length *CAB* gene from genomic DNA

absence of introns. The type 1 gene contains no introns and are numerous in several plant species and codes for longer polypeptides compared to the type II genes that contains one or more introns (Tyagi *et al.,* 2012). Intron containing *CAB* genes have also been identified in tomato (Pichersky *et al.,* 1989) The transit peptide of both type of genes are functional. Gene expression is regulated in a quantitative and qualitative manner and chimeric genes under control of the 5′ flanking region have been used to study light inducible as well as tissue specific expression. In pea about 400 bp of 5′ flanking region is reported to be sufficient to give tissue specific expression (Simpson *et al.,* 1982).

The sequence on comparison with the reported cDNA sequence of *CAB* gene from *Ricinus communis* (Accession. No: XM002524570.1) using CLUSTAL W (Thompson *et al.,* 1994) showed 91.1 per cent sequence homology (Fig. 10.). The sequence has been registered in NCBI with accession no. JN986719. The phylogenetic tree analysis showed the close relationship of the *CAB* gene of *Hevea* with *Ricinus communis* (Euphorbiaceae) and *Populus trichocarpa* apart from the other eukaryotic species like *Solanum*, *Gossypium*, *Nicotiana* etc. (Fig.11).

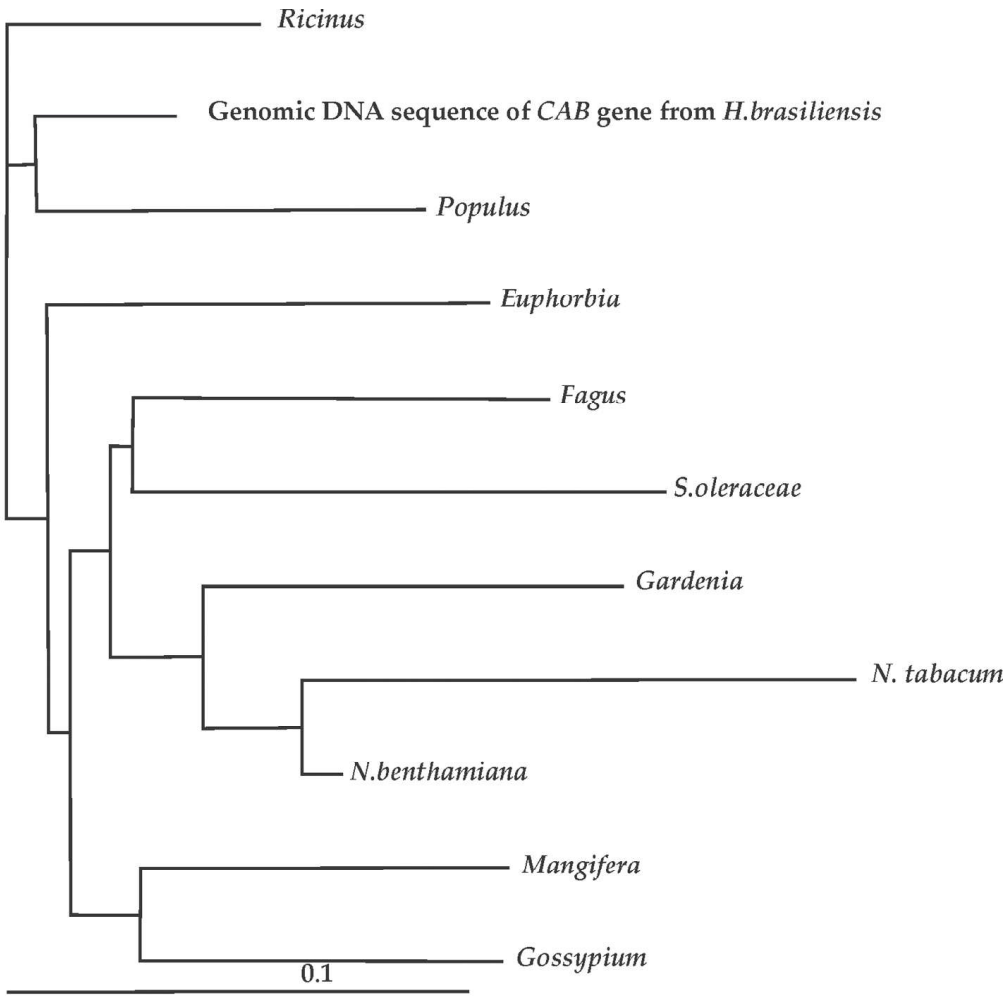
Amino acid sequence and protein model

The amino acid sequence deduced using the ExPASY translate tool spans an open reading frame of 265 amino acids (Fig. 12.a). The nucleotide sequence of *CAB* genes reported earlier also shows an open reading frame of 264-269 amino acids for the precursor protein (Tyagi *et al.,* 2012). The ProtParam tool predicted the molecular weight as 28 kDa and iso-electric point (pI)

as 5.45. With the help of myristoylator and palmitoylator tools, it was predicted that there are no myristoylation and palmitoylation sites, suggesting that the protein is not secretory, a finding in agreement with already reported chlorophyll a/b binding proteins from other plant species. The subcellular localization of the protein sequence was deduced using Plant-mPLoc tool predicting its site in

```
g.dna           ------------------------------------------------------------
ricinus         CTCCCTATAAGACACCCTCCAAACTCACTTCTATCTTCTACCGCTCTTAAACACCACTCC 60

g.dna           -----------------------------------CAAATGGCTACCTCTACAATGG 22   <- Start codon
ricinus         TACTCGTTCAAGTCTAAAACACTACTCCCTCATTTTTGACAATGGCTACCTCTACAATGG 120
                                                   ..********************

g.dna           CCCTCTCCTCCCCCTCCTTCGCCGGCAAGGCGGTGAAACTCACCCCATCTGCCCCTGGGC 82
ricinus         CCCTCTCCTCCCCTTCATTCGCTGGCAAGGCTGTGAAGCTCTCCCCTTCTGCCCCTGAGC 180
                ************* **.***** ******** *****.***:****:**********.**

g.dna           TCATGGGCAATGCCCGTGTCTCAATGAGGAAATCTGTTGGCAAGCCTGTTTCATCTGGAA 142
ricinus         TCATGGGCAATGGCCGTGTCTCAATGAGGAAAACCGCCGCCAAGAATGTTTCCTCCGGAA 240
                ************ ******************:* *  * ****..******.** ****

g.dna           GCCCATGGTATGGTCCAGACCGTGTTAAGTACTTGGGTCCATTCTCTGGTGAGCCCCCAT 202
ricinus         GCCCATGGTACGGCCCAGACCGTGTTAAGTACTTGGGTCCATTCTCCGGTGAGCCCCCAT 300
                ********** ** ********************************* *************

g.dna           CCTACTTGACCGGTGAGTTCCCTGGTGACTATGGCTGGGACACTGCTGGTCTCTCTGCTG 262
ricinus         CCTACTTGACTGGTGAATTCCCCGGTGACTACGGATGGGACACTGCTGGGCTCTCTGCTG 360
                ********** *****.***** ******** **.************** **********

g.dna           ACCCAGAAACCTTTGCCAAGAACCGTGAGCTCGAAGTGATCCACTGCAGATGGGCCATGC 322
ricinus         ACCCAGAGACCTTTGCCAAGAACCGTGAGCTCGAAGTCATCCACTGCAGATGGGCCATGC 420
                *******.***************************** **********************

g.dna           TTGGAGCCCTTGGGTGCGTCTTCCCCGAGCTCTTGGCCCGCAACGGAGTCAAGTTCGGCG 382
ricinus         TTGGAGCTCTTGGATGCGTCTTCCCTGAGCTCTTGGCACGCAACGGTGTTAAATTCGGTG 480
                ******* *****.*********** ***********.********:** **.***** *

g.dna           AGGCAGTGTGGTTCAAGGCAGGAGCCCAGATCTTCAGCGAGGGTGGTCTTGACTACTTGG 442
ricinus         AGGCTGTATGGTTCAAGGCTGGATCCCAGATCTTCAGCGAGGGTGGTCTTGATTACTTGG 540
                ****:**.***********:*** *************************** *******

g.dna           GTAACCCAAGCTTGATCCACGCACAAAGCATCTTGGCCATCTGGGCCGTCCAGGTAGTGT 502
ricinus         GCAACCCCAGCTTGATCCACGCACAAAGCATCTTGGCCATCTGGGCTTCCCAGGTTGTGT 600
                * ******.************************************   ******:****

g.dna           TGATGGGTGCCGTTGAAGGTTACAGAATTGCCGGTGGGCCGCTCGGTGAGGTCACAGACC 562
ricinus         TGATGGGTGCCGTTGAGGGTTACAGAGTTGCCGGCGGGCCACTAGGAGAGGTGACCGACC 660
                ****************.*********.******* *****.**.**:***** **.****

g.dna           CAATCTACCCAGGTGGAAGCTTTGACCCATTGGGCTTGGCTGATGACCCAGAAGCATTTG 622
ricinus         CAATCTACCCAGGTGGAAGCTTTGACCCATTGGGTTTGGCTGATGACCCAGAGGCATTCG 720
                ********************************** *****************.***** *

g.dna           CTGAGCTGAAGGTGAAGGAGATCAAGAACGGCAGATTGGCTATGTTCTCCATGTTCGGAT 682
ricinus         CTGAGTTGAAGGTGAAGGAGATCAAGAACGGTAGATTGGCTATGTTCTCTATGTTTGGAT 780
                ***** ************************ ***************** ***** ****

g.dna           TCTTTGTTCAGGCCATTGTGACAGGAAAGGGACCATTGGAGAACTTGGCTGACCACCTTG 742
ricinus         TCTTTGTTCAGGCCATTGTTACAGGAAAGGGACCATTGGAGAACTTGGCTGACCACCTTG 840
                ******************* ****************************************

g.dna           CTGACCCTGTCAACAACAATGCCTGGGCTTACGCCACAAACTTTGTCCCCGGAAAGTGAG 802   <- Stop codon
ricinus         CTGATCCCGTCAACAACAATGCCTGGGCATATGCCACAAACTTTGTCCCCGGAAAGTGAG 900
                **** ** ********************:** ****************************
```

Fig. 10. CLUSTALW multiple sequence alignment of the isolated genomic DNA sequence of chlorophyll a/b gene with *Ricinus communis*



Ricinus : 0.05469,
Genomic DNA from *Hevea* : 0.03006
Populus : 0.08465 : 0.00628,
Euphorbia.esula : 0.09579,
Fagus : 0.09021,
S.oleraceae : 0.11591: 0.00494,
Gardenia : 0.0913,
N.tabacum : 0.12005,
N.benthamiana : 0.00888,
Mangifera : 0.08004,
Gossypium : 0.0786:0.01517:0.0047:0.00891.

Fig: 11. Phylogenetic tree analysis of the isolated full length genomic DNA sequence of *CAB* gene of *H. brasiliensis* with other reported sequences from the eukaryotes.

chloroplast strengthening our claim of the isolated protein being the chlorophyll a/b binding protein present in chloroplast.

The protein model was also predicted for the amino acid sequence. The ribbon structures are ± - helix and the coils represent turns. The carbon ring structures represent ligand sites for binding (Fig.12.b). A total of 104 templates were identified and the most similar template models were identified as 24. Out of the 24 template designs, the list was filtered to a heuristic down to six templates among which the most important were 3jcu.1.G, 1rwt.1.A, 1vcr.1.A. The maximum similarity was found to the template 3jcu.1.G which has a sequence identity of 90.57. The reliability of the structures was determined by the QMEAN values of 0.80. These values are within the permissible limits of designing

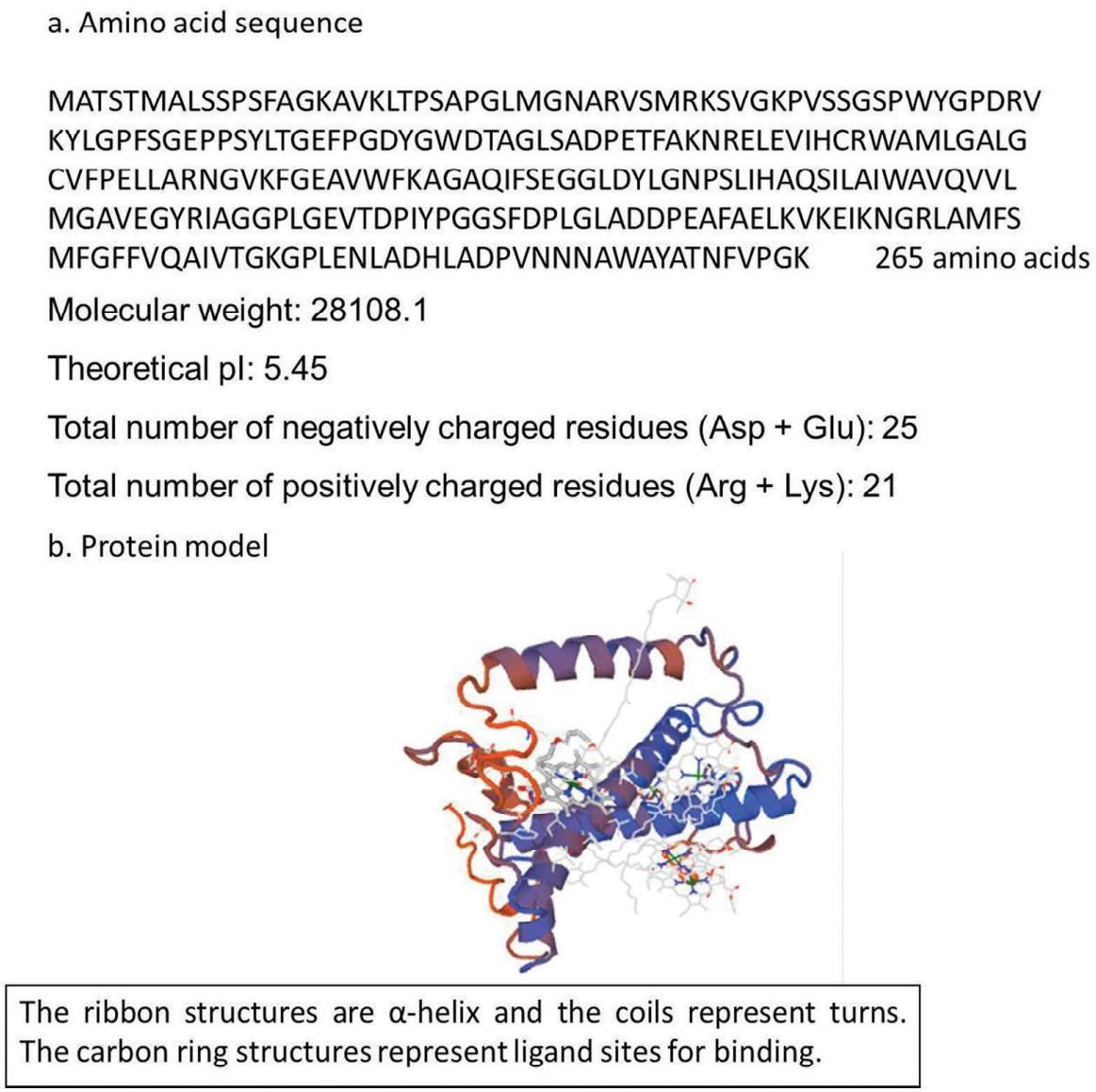


Fig. 12. a & b Amino acid sequence and protein model predicted from the sequence of chlorophyll a/b gene isolated from *H. brasiliensis*

protein models. The Qualitative Model Energy Analysis (QMEAN) score is a combination of six terms C-beta interaction energy, all-atom pairwise energy, solvation energy, torsion angle energy, secondary structure agreement and solvent accessibility energy. Reliable models have a total QMEAN score value between 0 and 1 (Benkert *et al.,* 2009).

CONCLUSION

Maturation related changes in morphological and physiological phenotypes are associated with changes in gene expression. The largest class of juvenility-induced genes are comprised of those involved in photosynthesis. It is also well established that photosynthetic rates and related physiological attributes differ between juvenile and mature plants. Juvenility of plant materials is also a key factor in micro- propagation because the regeneration ability of woody plants decreases with maturity. The same is observed in *Hevea* also with leaf explants collected from juvenile plants giving more response to *in vitro* culture. In the present study, a full length *CAB* gene has been cloned and characterized from *Hevea.* Hence expression analysis of the characterized *CAB* gene during juvenile - mature phase change can be carried out and this can be further related with the varied in *vitro* culture response of explants from juvenile and mature *Hevea* plants.

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