Indian J Agric Biochem **33** (2), 146-153, 2020
doi 10.5958/0974-4479.2020.00022.2

Chlorophyll a/b Binding Protein (Cab) Gene Expression in *Hevea* Signifying Clone Rejuvenation through Somatic Embryogenesis

RG KALA*, R SUPRIYA, R JAYASHREE and A THULASEEDHARAN
Advanced Centre for Molecular Biology and Biotechnology, Rubber Research Institute of India, Kottayam, Kerala - 686 009, India

The natural rubber tree is highly heterozygous hence elite clones are propagated through bud grafting. It is well established that juvenile tissues are more responsive to *in vitro* propagation techniques. In *Hevea* also physiological maturity of clonal explants has a direct impact on induction of organogenesis and embryogenesis. The effect is modulated through expression of various genes associated with juvenility including Chlorophyll a/b binding protein gene (Cab gene). The gene was isolated and characterized from *Hevea brasiliensis* and showed 90 % sequence homology to Cab gene from *Ricinus communis*. In the present study, Cab gene expression with respect to *in vitro* culture response of leaf explants, harvested from *Hevea* plants of different maturity was studied. Leaves were harvested from seedlings, somatic plants bud grafted clonal plants and mature trees of clonal origin. A 525 bp Cab gene was differentially expressed in the leaves harvested from these sources. There was markedly more expression of this gene in explants harvested from young seedlings and clonal somatic plants than bud grafted plants and mature field grown trees of clonal origin. Northern analysis also gave similar results. Explants collected from physiologically juvenile sources such as seedlings and clonal somatic plants had higher expression of Cab gene and they also had better *in vitro* culture response. This indicated that somatic embryogenesis from clonal explants in *Hevea* resulted in physiological juvenility through clone rejuvenation.

Key words: Cab gene, differential expression RT-PCR, northern analysis

Hevea brasiliensis, a cross pollinated tropical tree belonging to the family *Euphorbeaceae* has been established as the major commercial source of natural rubber. In this tree crop, commercial propagation of the elite clones developed through conventional breeding is by bud grafting onto heterozygous seedling root stocks. Due to heterozygosity of seedling derived materials in *Hevea*, *in vitro* plant regeneration systems have to be developed with clonal materials having desirable secondary attributes. As a result of repeated vegetative propagation, the clonal materials attain physiological maturity which makes them highly recalcitrant to *in vitro* culture. In *Hevea* also, the explants collected from mature clonal materials are found to be highly recalcitrant to *in vitro* culture when compared to seedling derived materials. Somatic embryogenesis and plant regeneration systems have been developed in *H. brasiliensis* from different explants such as immature anthers, integuments, immature inflorescence and leaf (1-4) and these are now being used in developing *Hevea* transgenics (5). Though protocols have been developed for producing rooted clonal shoot tips, being a tree species with recalcitrance to tissue culture, the system could not be commercialized.

In *Hevea,* leaf explants can be collected from source plants of different physiological maturity such as seedling and somatic plants which are juvenile and bud grafted plants and field grown trees which are physiologically mature. Somatic embryogenesis and plant regeneration was achieved from leaf explants of six month old, glass house grown bud grafted plants of *Hevea* (6). When somatic embryogenesis was attempted in leaf explants collected from plants of different physiological maturity, variation was observed in embryogenic competence. The rate and time taken for embryogenic callus initiation and embryo induction varied with the source of the explant. The leaves collected from *in vitro* somatic plants and *in vitro* seedlings derived from cultured zygotic embryos which were physiologically juvenile gave good culture response compared to mature clonal materials such as six month old bud grafted plants grown in polybags in the glass house and ten year old field grown trees (7). It was observed that physiological age of the source of explant determines its embryogenic capacity and this

*Author for correspondence: Email: rgkala@rubberboard.org.in

was found to be reduced with maturity of the source plant. It has also been reported that the frequency and time taken for embryogenic callus initiation was found to be highly influenced by the explant source in *H. brasiliensis* (8). The developmental age of donor trees strongly affects the vigor and morphological traits of *in vitro* cultured tissues with respect to plant regeneration. All woody plants exhibit phase changes. These include morphological alterations, as exemplified by phyllotaxy or leaf form (9), and physiological phase changes, eg. differences in anthocyanin production (10). Other responses that show marked differences between juvenile and adult stages include inducibility of organogenesis and embryogenesis in tissue culture, with juvenile tissues such as embryonic stages and young seedlings being the most responsive (11). Loss of organogenesis and embryogenesis, whether progressive or sudden, reflects transition to the mature phase. A higher organogenic responsiveness on a wider range of culture media for physiologically juvenile tissues compared to mature ones was observed in other species (12,13), indicating that it may be a general phenomenon. *In vitro* regeneration is usually achieved by culturing tissues that are in a more juvenile, potentially regenerative state than most other tissues of the tree. The rapid growth of the juvenile phase is essential for the young tree to compete with other vegetation. Maturation in woody plants has recently received much attention because of the maturation-related decrease in the ability to clone selected individuals using explants from mature plants (14). The ability to reverse maturation will also be important for the propagation of specific mature trees via either rooted cuttings or tissue culture. Rooting potential has been used in several woody plants as a marker for juvenility (15, 16).

Several genes have been reported to be differentially expressed in juvenile and mature phase. The maturation-related changes in morphological and physiological phenotypes are associated with changes in gene expression (17). An analysis of gene expression in juvenile and mature trees found that the cab gene family is expressed at higher levels in juvenile plants than in mature plants in newly expanding short shoot needles. 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 (18). However, little is known about the physiological and metabolic mechanisms responsible for the differences between the juvenile and mature states of cells. Various workers have reported the effect of age on gene expression and particularly photosynthesis genes. Photosynthetic rates and related physiological attributes differ between juvenile and reproductively mature individuals in nearly every woody species examined (19). Changes in chlorophyll content may be reflected in more general changes in the photosynthetic apparatus. This would suggest that genes encoding elements of the photosynthetic apparatus would be differentially expressed between juvenile and mature plants. The largest class of juvenile-induced genes comprised of those involved in photosynthesis. Among the several genes differentially expressed during phase change, genes encoding elements of the photosynthetic apparatus such as the chlorophyll a-b binding protein (Cab) have also been reported. The Cab proteins of the light-harvesting complex of PSII 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 (20). Hutchison *et al.* (21) showed that the cab gene family is considerably more strongly expressed in light-grown juvenile shoots of larch than in those of adult shoots. The expression of sequences for the Cab gene decreases with maturation (23). 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 indicates that there are differences in gene expression between juvenile and mature shoots. Experiments showed difference in *in vitro* culture response of leaf explants which is also a major site of Cab gene function. Therefore, in an earlier attempt

isolation and characterization of chlorophyll a/b binding protein gene (Cab), whose major expression sites are the green leaves, was carried out from *Hevea brasiliensis* (clone RRII 105). In the present work, Cab gene expression in relation to phase change was studied using leaves collected from source plants of different maturity and this was related with the *in vitro* culture response of leaf explants.

Materials and Methods

Isolation and characterization of full length Cab gene from genomic DNA: As part of earlier experiments a 525 bp Cab gene sequence was amplified from genomic DNA which had maximum (91%) homology with *Ricinus communis* (Accession No.XM_002524570.1). Hence primers were designed from cDNA sequence of Cab gene reported in *Ricinus* in an attempt to amplify the full coding region of the sequence. The forward and the reverse primers contained the start and the 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.

Reverse Transcriptase – PCR: PCR was carried out with primers for 525 bp Cab gene amplification. First strand cDNA was synthesized from the isolated leaf RNA of the different sources by reverse transcription reaction with oligo-(dT) primers using the 'Improm-IITM Reverse Transcription System' (Promega, USA). 1ml of the first strand cDNA was used to amplify the 525 bp Cab gene. b-actin transcripts were also co- amplified as internal control. The PCR reaction products were separated on a 1.5% agarose gel, visualized with ethidium bromide staining under UV light and the image was captured using EDAS 290.

Differential expression of Cab gene: RNA was isolated from leaves collected from source plants of different physiological maturity. Leaves from *in vitro* raised seedlings derived from cultured zygotic embryos, *in vitro* somatic embryogenesis derived plants, six month old bud grafted plants grown in poly bags in the glass house and ten year old field grown mature trees of clone RRII 105 were used for the experiments. RNA was also isolated from leaf explants of different maturity such as immature (brownish), medium mature (light green, shiny) and mature (thick, dark green) collected from glass house grown bud grafted plants. The RNA was checked for DNA contamination and quality by agarose gel electrophoresis. Reverse Transcriptase – PCR was carried out with first strand cDNA synthesized from the different samples to studt differential expression of the Cab gene.

Northern hybridization: Electrophoresis of RNA samples was carried out in 1% agarose gel containing formaldehyde. The RNA was then blotted to nylon membrane (Hybond N^+, Amersham, UK) and hybridized using the 525 bp Cab cDNA as the probe. The Cab gene probe was radio labeled using 'Multiprime DNA labeling system' from Amersham, (UK). Hybridization of the labeled probe to the nylon membrane was also performed according to Sambrook (24). After stringent washing, the membrane was wrapped in a cling film and subjected to auto radiography with the phosphor image analyzer FLA 5000 (M/S Fujifilm, Japan) with laser beam (625 nm). The relative abundance of Cab protein gene mRNAs was determined by visualizing the net intensity of band in northern blots.

Results and Discussion

Isolation and characterization of Cab gene

PCR amplification of full length Cab gene from genomic DNA: An attempt has been made to amplify the full coding region of the sequence by designing primers from cDNA sequence of Cab gene reported in *Ricinus communis* that showed 91% homology (Accession No.XM_002524570.1). Using genomic DNA as template, specific amplification could be obtained using the primer designed for full length Cab protein gene, after optimization of PCR conditions. The PCR amplified product had size of approximately 0.8 kb (Fig.1.A-C). The fragment was cloned and sequenced. The amplified sequence contained 802 bps (Fig.2). The region amplified in the present study contains the full protein coding sequence. The start codon 'ATG' starts from the 4th position and TGA the stop codon starts at the 799th position. The number of coding DNA sequence contains

798 nucleotides including the stop codon. On comparison with the reported mRNA sequences for Cab gene from other species, the 802 bp full length coding region isolated in the present study also contains no introns. The sequence on comparison with the reported cDNA sequence of Cab gene from *Ricinus communis* (Accession. No: XM002524570.1) using CLUSTAL W (25) showed 91.1 % sequence homology. The sequence has been registered in NCBI with accession no. JN986719.

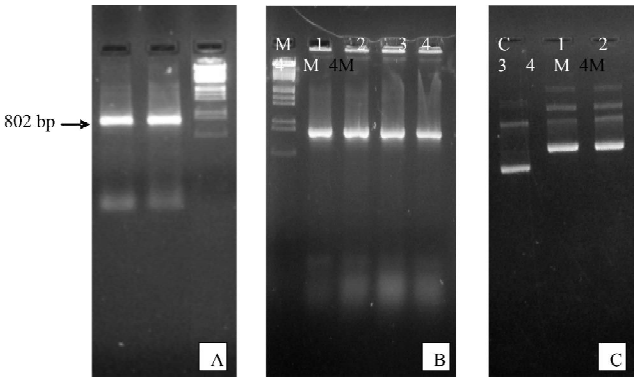

Fig. 1 (A-C): PCR amplification and cloning of full length Cab gene from genomic DNA
A.Cab 802 bp cab gene amplification, 1&2 Cab amplification, M-Marker
B. Colony PCR of transformed colonies
C. Isolation of recombinant plasmids; C- Control, 1&2- recombinant plasmids

5'
CAAATGGCTACCTCTACAATGGCCCTCTCCTCCCCCTCCT
TCGCCGGCAAGGCGGTGAAACTCACCCCATCTGCCCCTG
GGCTCATGGGCAATGCCCGTGTCTCAATGAGGAAATCTGT
TGGCAAGCCTGTTTCATCTGGAAGCCCATGGTATGGTCCA
GACCGTGTTAAGTACTTGGGTCCATTCTCTGGTGAGCCCC
CATCCTACTTGACCGGTGAGTTCCCTGGTGACTATGGCTG
GGACACTGCTGGTCTCTCTGCTGACCCAGAAACCTTTGCC
AAGAACCGTGAGCTCGAAGTGATCCACTGCAGATGGGCCA
TGCTTGGAGCCCTTGGGTGCGTCTTCCCCGAGCTCTTGGC
CCGCAACGGAGTCAAGTTCGGCGAGGCAGTGTGGTTCAA
GGCAGGAGCCCAGATCTTCAGCGAGGGTGGTCTTGACTA
CTTGGGTAACCCAAGCTTGATCCACGCACAAAGCATCTTG
GCCATCTGGGCCGTCCAGGTAGTGTTGATGGGTGCCGTT
GAAGGTTACAGAATTGCCGGTGGGCCGCTCGGTGAGGTC
ACAGACCCAATCTACCCAGGTGGAAGCTTTGACCCATTGG
GCTTGGCTGATGACCCAGAAGCATTTGCTGAGCTGAAGGT
GAAGGAGATCAAGAACGGCAGATTGGCTATGTTCTCCATG
TTCGGATTCTTTGTTCAGGCCATTGTGACAGGAAAGGGAC
CATTGGAGAACTTGGCTGACCACCTTGCTGACCCTGTCAA
CAACAATGCCTGGGCTTACGCCACAAAC**TTTGTCCCCGGA**
AAGTGAG 3'.

Forward primer- CAAATGGCTACCTCTACAATGG

Reverse primer- TTTGTCCCCGGAAAGTGAG

Fig. 2: Nucleotide sequence of full length Cab gene from genomic DNA

PCR amplification, cloning and sequencing of Cab cDNA: RNA was isolated from medium mature leaves of bud grafted plants and checked for DNA contamination and quality by agarose gel electrophoresis and found to be of very good quality (Fig.3A). First strand 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. After cloning and sequencing, it was observed that, the cDNA also contains a 525 bps as observed in the genomic DNA. On alignment with the genomic sequence 100% similarity was observed and no introns were present in the isolated region.

Differential expression of Cab gene

Reverse transcriptase – PCR: Cab gene amplification could be obtained from cDNA synthesized from RNA isolated from leaves of seedlings, somatic plants, six month old bud grafted plants and mature trees using the primer sequences used to amplify the 525bp Cab cDNA sequences. (Fig. 3A). Differential expression of the gene was also observed when RT-PCR was carried out with cDNA synthesized from different explant sources (Fig. 3B). Expression of the gene was found to be more in seedlings and *in vitro* derived plants which are juvenile and showed better *in vitro* culture response. With maturity of the plant, expression of the gene was found to be

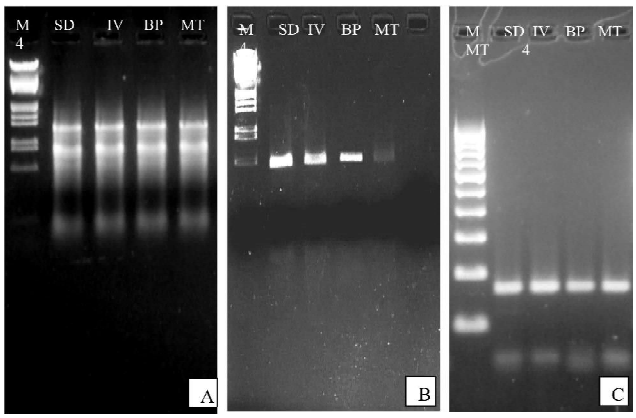

Fig. 3 (A-C): Differential expression of Cab gene
A. RNA of different samples (Lane 1-Marker, 2- Seedling, 3-Bud grafted plant, 4-Mature tree), B. RT-PCR showing differential Cab gene expression (Lane 1-Marker, 2- Seedling, 3-Bud grafted plant, 4-Mature tree), C. â-actin amplification in different samples (Lane 1-Marker, 2- Seedling, 3-Bud grafted plant, 4-Mature tree)

reduced, with the leaves from the mature trees showing the minimum expression. The â-actin gene was found to be constitutively expressed in all the samples which shows that equal amount of RNA has been loaded in all the wells (Fig. 3C).

Northern hybridization: When northern analysis was carried out using the RNA isolated from leaves of different sources such as seedlings, somatic plants, six month old bud grafted plants and mature trees using the 525 bp Cab cDNA as the probe, bands with different intensity was obtained although equal amount of RNA was loaded which indicates differential expression of the gene in different samples. The *in vitro* plants raised through somatic embryogenesis showed the maximum expression and the mature tree leaves showed the minimum expression as evident from the blots (Fig. 4. A & B). When northern analysis was conducted with leaves of three different stages such as immature, medium mature and mature leaves from the six month old bud grafted plants, maximum expression was observed in the medium mature leaves with immature and mature leaves showing slightly less activity (Fig. 4 C & D).

Juvenility of plant materials is a key factor in micro propagation because the regeneration ability of woody plants decreases with maturity (26). *In vitro* propagation of mature trees is generally more difficult than their juvenile counterparts. With mature trees of a few species, it has been possible to induce somatic embryogenesis in cultures derived from commonly used explants such as leaves and roots (27). They have reviewed the factors influencing rejuvenation in trees using methods of enhancing micro propagation through manipulations that involve application of osmotic, temperature or hormonal stress. Both micro propagation and somatic embryogenesis are influenced by phase change and the culture environment. The term maturation includes the transition from the juvenile to the mature phase, while the term aging includes loss of vigor associated with increased complexity of the plant. Maturation occurs in seedling development; whereas plants propagated vegetatively from sexually mature plants, unless rejuvenated, only undergo ageing (28). Micropropagation of some species is enhanced by the use of *in-vitro* derived

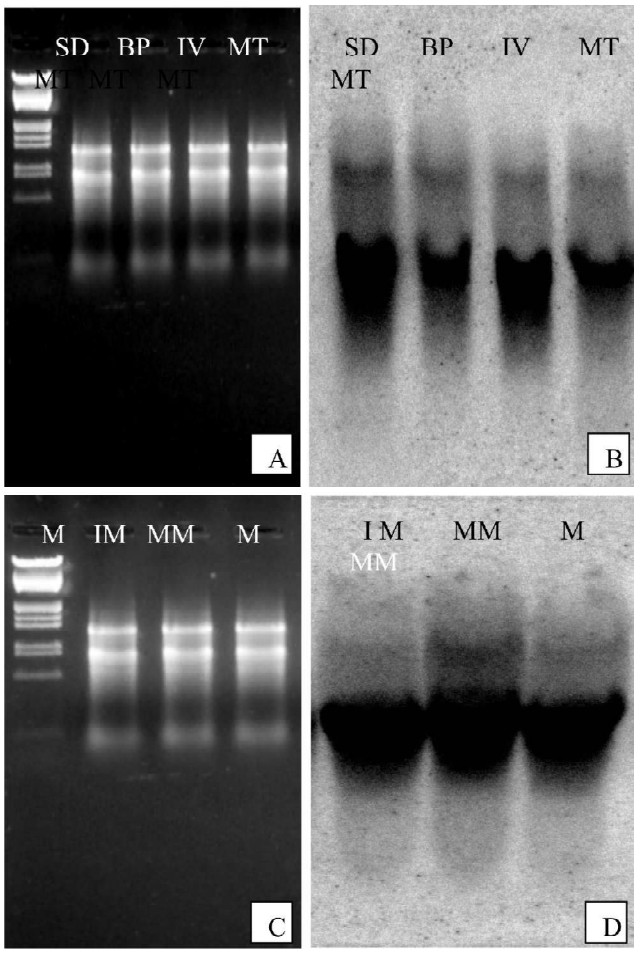

Fig. 4 (A&B): Northern blotting for differential Cab gene expression
A. RNA. (M-Marker, SD-Seedling, BP- Bud grafted plant, IV –*in vitro* plant, MT- Mature tree), B. Northern blot showing differential Cab gene expression in leaves of source plants of different maturity, C. RNA from leaves of different stages of budgrafted plants (M-Marker, IN- Immature, MM-Medium mature, M-Mature leaf), D. Northern blot showing differential Cab gene expression in leaves of different maturity

material. Seedlings and young plants derived from somatic embryos were more juvenile than their similarly aged zygotic counterparts (29). Accordingly in Picea abies (L.) Karst., for instance, *in-vitro* cultured somatic embryos showed higher ability for somatic embryogenesis than their similarly aged or older zygotic counterparts. Furthermore, somatic embryogenesis was more readily induced from mature plants derived by somatic embryogenesis than from plants of seedling origin (30). Previous studies have shown that physiologic aging negatively affects the micro propagation capacity of *H. brasiliensis* from microcuttings (31, 32). This phase change phenomenon has been observed in many studies

and is associated with a noticeable decline in the potential for micro propagation or somatic embryogenesis of most arborescent species (33-36). Explants with a high ability for somatic embryogenesis generally originate from reproductive organs (e.g., anthers, inner integument of the seed and zygotic embryos), young seedlings (37) and somatic embryogenesis derived plantlets. In Larix decidua Mill., somatic embryogenesis from cotyledons and needles has been successful only for tissues obtained from somatic embryogenesis derived plantlets or from immature and mature zygotic embryos (38).

The largest class of juvenility-induced genes 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 nearly every woody species examined (39). Maturation related changes in morphological and physiological phenotypes are associated with changes in gene expression. Two steady-state Cab mRNA levels are relatively higher in newly expanding short shoot foliage from juvenile plants compared to mature plants in Eastern larch, *Larix laricina* (40). Here juvenile and rejuvenated shoots also showed higher rates of photosynthesis and respiration, evidenced by faster O_2 evolution and consumption. In both larch and English ivy, a comparison of cDNA libraries made from RNA extracted from juvenile and mature foliage indicates that there are differences in gene expression between juvenile and mature shoots. The photosynthetic rates were associated with more chlorophyll, especially chlorophyll *a*, in the juvenile and the rejuvenated shoots. In tree species, the general trend is towards lower instantaneous or integrated photosynthetic rates in reproductively mature individuals, but the inverse has also been reported in several species. Basheer- Salimia (41) have identified that when the juvenile phase was compared to mature phase in olive trees; it was physiologically characterized by higher photosynthetic rate. Juvenile and rejuvenated shoots of *Sequoia sempervirens* also showed higher rates of photosynthesis and respiration, as evidenced by faster O2 evolution and consumption (42). In maize plants the largest class of juvenile-induced genes was comprised of those involved in photosynthesis, suggesting that they are primed for energy production early in vegetative growth (43).

Control of the differential gene expression in a relatively small percentage of the cells in the plant body can result in rather large differences in phenotype. Genes encoding elements of the photosynthetic apparatus such as the chlorophyll a/b binding protein have been found to be differentially expressed in juvenile and mature plants. Greenwood *et al.* (44) reported greater expression of chlorophyll a/b binding protein gene in Larix laricina in developing juvenile foliage than mature foliage. In both larch and English ivy, the expression of sequences for the chlorophyll a/b binding protein (cab) gene decreases with maturation (45, 46). Genes encoding elements of the photosynthetic apparatus would be differentially expressed between juvenile and mature plants. A similar observation was also obtained in the present experiments with higher expression of the Cab gene in leaves collected from juvenile source plants such as *in vitro* derived seedlings and somatic plants when compared with mature sources such as bud grafted clonal plants and field grown trees. This could be related with the *in vitro* culture response of leaf where explants from juvenile sources responded well in culture.

Both micro-propagation and somatic embryogenesis are influenced by the physiological phase changes and the culture environment. Most trees have marked physiological phase changes from juvenile to mature, that result in a decline in their potential for somatic embryogenesis or micro propagation. Phase change is a poorly understood phenomenon, particularly the reversal of phase change, from mature to juvenile, as occurs in the sexual process. By altering conditions of the source material *ex vitro*, or by changing *in vitro* conditions encountered by the explant, rejuvenation and increased propagation can sometimes be accomplished. In *Hevea,* clonal materials developed through conventional breeding and having high production potential combined with desirable secondary attributes are used in commercial propagation. Developing *in vitro* propagation systems for commercial propagation of these

clonal materials is difficult due to tissue recalcitrance as a consequence of their physiological maturity. In the present study, Cab protein gene expression was studied in relation to the *in vitro* culture response of leaf explants collected from source plants of different physiological maturity. Cab protein gene was found to be differentially expressed leaf collected from juvenile and mature plants of *Hevea.* It was observed that chlorophyll a/b binding protein gene expression was more in juvenile source plants such as seedlings and *in vitro* somatic embryogenesis derived plants whereas in mature source plants such as bud grafted plants and mature trees it was comparatively less. The results of the present study indicate that; for *in vitro* culture response, particularly in tree species, a physiological juvenility is essential. Higher Cab gene expression in clonal somatic plants also indicated the possibilities of clone rejuvenation by somatic embryogenesis.

Acknowledgement

The authors thank Dr. James Jacob, Director of Research, RRII for his support and encouragement.

Received November 11, 2020; accepted December 20, 2020

References

1 **Jayasree PK, Asokan MP, Sobha S, Sankari Ammal L, Rekha K, Kala RG, Jayasree R & Thulaseedharan A** (1999) *Curr Sci,* **76,** 1242.

2 **Carron MP, Enjarlic F & Deschamps A** (1985) *IRCA, CR, Coll Exp Phys Am Hevea IRRDB, IRCA, Monpellier,* 427-435.

3 **Sushamakumari S, Sobha S, Rehka K, Jayasree R & Asokan MP** (2000) *Indian Journal Natural Rubber Research,* **13,** 19.

4 **Kala RG, Kumari Jayasree P, Sushamakumari S, Jayashree R, Rekha K, Sobha S & Thulaseedharan A** (2005) *ICAR National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies*, Trichur, India, 105-106.

5 **Thulaseedharan A, Kala RG, Jayashree R, Rekha K & Sobha S** (2017) *Rubber: In Biotechnology of Plantation Crops (P. Chowdappa, Anitha Karun, M.K. Rajesh & S.V. Ramesh, eds.) Daya Publishing House, Astral International Pvt. Ltd, New Delhi*, 525-553.

6 **Kala RG, Kumari Jayasree P, Sobha S, Rajani Joseph & Thulaseedharan A** (2006) *International Symposium on Frontiers in Genetics and Biotechnology- Retrospect and Prospect, Jan. 8-10, Osmania University, Hyderabad*.

7 **Kala RG, Gimisha GC, Kumari Jayasree P, Sushamakumari S, Sobha S, Jayasree R, Rekha K & Thulaseedharan A** (2009) *Natural Rubber Research*, **22**(1&2), 117.

8 **Lardet L, Dessailly F, Carron MP & Monteuuis O** (2009) *Tree Physiology,* **29,** 291.

9 **Gupta PK, Timmis R & Mascarenhas AF** (1991) *In vitro Cell & Development Biology,* **27,** 159.

10 **Murray JR, Smith AG & Hackett WP** (1994*b*) *Planta,* **194**, 102.

11 **Greenwood MS** (1995) *Tree Physiology,* **15**, 433.

12 **Monteuuis O** (1987) *Tree Physiology,* **3**, 265.

13 **Monteuuis O** (2004) *Trees,* **18**, 77.

14 **Greenwood MS** (1987) *Plant Growth Regulation*, **6**, 1.

15 **Huang LC, Hsiao C, Lee S, Huang B & Murashige T** (1992a) *In Vitro Cell Developmental Biology,* **28**, 30.

16 **Huang LC, Lius S, Huang BL, Murashige T, Mahdi EFM & Van Gundy R** (1992b) *Plant Physiology*, **98**, 166.

17 **Hutchison KW, Sherman CD, Weber J, Smith SS, Singer PB & Greenwood MS** (1990) *Plant Physiology*, **94**, 1308.

18 **Hutchison KW, Singer PB & Greenwood MS** (1988) *In Molecular Genetics of Forest Trees. Petawawa National For. Res. Inst., Chalk River, Ontario, Canada, No.* P1-X-80, 26-33.

19 **Bond BJ** (2000) *Trends in Plant Science,* **5**, 349.

20 **Yun C, Chang & Linda L Walling** (1991) *Plant Physiology,* **97**, 1260.

21 **Hutchison KW, Sherman CD, Weber J, Smith SS, Singer PB & Greenwood MS** (1990) *Plant Physiology,* **94**, 1308.

22 **Hutchison KW, Singer PB & Greenwood MS** (1991) *In: Ahuja MR (ed) Woody Plant Biotechnology Plenum NY*, 69-75.

23 **Woo HH, Hackett WP & Das A** (1994) *Physiologia Plantarum*, **92**, 69.

24 **Sambrook J, Fritsch EF & Maniatis T** (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

25 **Thompson JD, Higgins DG & Gibson TJ** (1994) *Nucleic Acids Res,* **11**(22), 4673.

26 **Huang CL, Hsieh MT, Hsieh WC, Sagare AP & Tsay HS** (2000) *In Vitro Cellular and Developmental Biology- Plant*, **36**, 220.

27 **Bonga JM & von Aderkas P** (1992) In Clonal Forestry I: Genetics and Biotechnology. Eds. M.R. Ahuja and W.J. Libby. Springer-Verlag, Berlin, 182–199.

28 **Hackett WP** (1985) *Horticultural Reviews*, **7**, 109.

29 **Ruaud JN, Bercetche J & Paques M** (1992) *Plant Cell Reports,* **11**, 563.

30 **Pacques MJ, Bercetches & Harvengt L** (1997) *Proceedings of the Joint Meeting of IUFRO Working Parties 2.04.07 and 2.04.06*, Québec City, **4.**

31 **Lardet L, Enjalric F & Carron MP** (1990) *C.R. Academy of Science. Paris* 1990; **310**(Série III), 195-202.

32 **Perrin Y, Lardet L, Enjalric F & Carron MP** (1994) *Canadian Journal of Plant Science*, Kluwer Academic Publisher, The Netherlands, **74**, 623-630.

33 **Bonga JM** (1982) *In* Tissue Culture in Forestry. Eds. J.M. Bonga and D.J. Durzan. Martinus Nijhoff/Dr. W. Junk, The Hague, 387- 412.

34 **Hackett WP** (1985) Horticultural Reviews, **7**, 109.

35 **Monteuuis O** (1989) *In:* Gibson G.L., Griffin A.R., Matheson A.C. *editors.* Breeding Tropical Trees: Population Structure and Genetic Improvement Strategies in Clonal and Seedling Forestry; *Proc. Conference IUFRO*; *Pattaya, Thailand,* 106-118.

36 **Von Aderkas P & Bonga JM** (2000) *Tree Physiology*, **20**, 921.

37 **Dunstan DI, Tautorius TE & Thorpe TA** (1995) In In Vitro Embryogenesis in Plants. *Ed. T.A. Thorpe. Kluwer Academic Publishers, Dordrecht*, 471–538.

38 **Lelu MA, Klimaszewska K & Charest P** (1994), *Canadian Journal of Forestry Research,* **24**, 100.

39 **Bond BJ** (2000) *Trends in Plant Science,* **5**, 349.

40 **Hutchison KW, Sherman CD, Weber J, Smith SS, Singer PB & Greenwood MS** (1990) *Plant Physiology*, **94**, 1308.

41 **Basheer-Salimia R** (2007) *Hebran University Research Journal,* **3**(1), 17.

42 **Li-Chun Huang, Jui-Hsi Weng, Chiu-Hui Wang, Ching-I Kuo & Yuh-Jang Shieh** (2003) *Bot Bull Acad Sin,* **44**, 31.

43 **Strable J, Borsuk L, Nettleton D, Patrick SS & Irish EE** (2008) *The Plant Journal,* **56**, 1045.

44 **Greenwood MS, Hopper CA & Hutchison KW** (1989) *Plant Physiology,* **90**, 406.

45 **Hutchison KW, Sherman CD, Weber J, Smith SS, Singer PB & Greenwood MS** (1990) *Plant Physiology*, **94**, 1308.

46 **Woo HH, Hackett WP & Das A** (1994) *Physiologia Plantarum*, **92**, 69.