Cord 2009, **25** (1)

Feasibility of Using the Expression of the Retinoblastoma Gene as a Marker for Assessing the Embryogenic Potential of Coconut Ovary Culture

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Abstract

Coconut is a monocotyledonous tree crop that is highly recalcitrant to *in vitro* culture conditions. Ovary culture is a promising technique for clonal propagation of coconut. A greater understanding of the fundamental aspects of somatic embryogenesis and plant regeneration is very important in achieving a break-through. Identification of tissues having a high embryogenic potential at an early stage is also very important to achieve a high regeneration efficiency and to avoid maintenance of non-responsive cultures for a prolonged period. *In situ* hybridization was employed to study the expression of CnRb gene in selected tissues, to identify a potential molecular marker to assess the embryogenic potential of coconut ovary cultures. The results revealed that *in situ* hybridization can be used to detect the expression of CnRb gene in the cells. It was possible to establish a relationship between the meristematic activity and expression of CnRb gene in the tissues tested. CnRb mRNAs were mainly localized in the meristematic cells and tissues such as calli and growing point of the developing shoots.

Keywords: Coconut (*Cocos nucifera* L.)**,** *In situ* hybridisation, Ovary culture, Retinoblastoma gene, Somatic embryogenesis.

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Introduction

Coconut is a monocotyledonous tree crop that is highly recalcitrant for *in vitro* culture. Although *in vitro* propagation of coconut has been researched for more than two decades, no protocol is yet available for reproducible *in vitro* propagation of clonal plants on a large scale. Development of an efficient protocol for *in vitro* plant regeneration in coconut is hampered by numerous constraints. Thus a greater understanding of the fundamental aspects of somatic embryogenesis and plant regeneration is very important in achieving a break-through, considering the highly recalcitrant nature of coconut under *in vitro* conditions. Furthermore, identification of tissue having a high embryogenic potential at an early stage is very important to achieve high regeneration efficiency and to avoid prolonged maintenance of non-responsive cultures. At present, evaluation of cultures is based only on visual and histological observations. Even though meristematic activity and embryogenic potential of *in vitro*-cultured coconut tissues have been studied by histo-chemical staining (Verdeil *et al*., 1994; Fernando *et al*., 2003; Perera *et al*., 2007), the expression of genes involved in these processes is yet to be understood.

Studies on molecular markers associated with embryogenic competence have been conducted in various crops. Molecular analysis of somatic embryogenesis has mostly been done by comparing genes and proteins expressed in embryogenic and non-embryogenic cells as well as in different stages of embryogenesis (Rao, 1996). Hecht *et al*. (2001) studied the expression of the somatic embryogenesis receptor kinase 1 *(AtSERK1)* gene in embryogenic and non-embryogenic structures in *Arabidopsis,* using seedling assay. According to the results, the seedlings that over-expressed the *AtSERK1 mRNA* exhibited a 3- to 4-fold increase in efficiency for initiation of somatic embryogenesis. The expression of *BABY BOOM* (Boutilier *et al*., 2002), viviparous 1 (*Pavp1*) and P34 ^{cdc2} protein kinase (*cdc2Pa*) genes (Footitt *et al*., 2003) during somatic embryogenesis have also been studied and related with different stages of somatic embryo development.

The poor response of coconut tissues under *in vitro* culture and the difficulty in maintaining the meristematic potential in these tissues has triggered an interest in studying fundamental aspects such as cell cycle control in coconut. Sandoval *et al*. (2003) studied the cell cycle status of *in vitro*-grown coconut cells, by flow cytometry, which allowed rapid assessment of the ability of tissues cultured *in vitro* to divide. A semi-quantitative RT-PCR analysis of CnRb expression in coconut shoot meristems (with active and retarded growth) and calli (with fast and slow growth) has implied a relationship between meristematic activity and the abundance of CnRb transcripts (Sandoval, 2002). CnRb is highly expressed in fast-growing calli and active shoot meristems when compared to slow-growing calli and inhibited shoot meristems. The above studies have implied the possibility of using CnRb as a potential marker for assessing the meristematic potential of *in vitro*-cultured coconut tissues. Localization of the CnRb transcript by *in situ* hybridization (ISH) would further support and confirm the results obtained by RT-PCR. However, RT-PCR may sometimes be inefficient, if the level of gene expression is low, and in such instances ISH, which is a more sensitive technique, can be employed. With ISH, it will also be possible to establish a correlation between the expression of CnRb and characteristic cytological features of meristematic tissues (e.g. high nucleo-cytoplasmic ratio).

Ovary culture is a promising technique for clonal propagation of coconut (Perera *et al*. 2007). Thus in the present study, *in situ* hybridization was employed to assess the expression of CnRb gene in selected tissues, with the ultimate objective of identifying a potential molecular marker to assess the embryogenic potential of coconut ovary cultures.

Materials and method

In situ hybridization

In situ hybridization was conducted as described by Abirached-Darmency et al. (2005) and Gutierrez et *al*., (2006). Callus (embryogenic and non-embryogenic), somatic embryos and shoot-like structures derived from unfertilized ovary explants (described in Perera *et al*. 2007) were used as experimental material and they were selected based on morphological features. After trimming the samples, they were immersed in chilled (4 ºC) fixative (10% w/v of paraformaldehyde, 10% v/v of 10x PBS buffer with DEPC-treated water) and kept under vacuum for 1 h. After adding a fresh solution of fixative, the tissues were incubated at 4 ºC overnight.

Preparation of tissues and sectioning

To remove aldehyde groups, the samples were rinsed in three changes of PBS-Glycine solution, 15 min in each followed by 2 changes in 1x PBS solution for 15 min at room temperature and overnight at 4 ºC. Then the samples were dehydrated in a graded ethanol series [2 changes (15 min each) in 50% v/v, two changes (30 min and 1 h) in 70% v/v, 2 changes (30 min and 1 h) in 95% v/v and 2 h in absolute ethanol]. This was followed by ethanol-butanol solution (1:1 v/v) for 1 h (butanol was used to facilitate further impregnation of paraplast) and 100% butanol for 3 days at 4 ºC. The samples were then transferred to 100% butanol/ Safesolve (safesolve is the solvent of paraplast) (1 h each in 2:1 (v/v) and 1:2 v/v solutions) followed by 2 changes in 100% Safesolve for 30 min. Wax infiltration was done afterwards by transferring the samples to Safesolve / melted Paraplast X-Tra (Tissue embedding medium, Ref. 8889 503002; 3:1 v/v for 1 h, 1:1 v/v for 2 h, 1:3 v/v for 2 h) and finally to 100% Paraplast for 3 days with 2 changes per day, at 58 ºC. Infiltrated samples were sealed in plastic molds with fresh, melted Paraplast. The wax-embedded samples were sectioned (15 µm in thickness) by using a rotary microtome (Microm). Sections were collected with a fine paint brush and floated on a drop of sterile distilled water on Silanized slides (Dako Cytomation, Ref. S3003, Carpinteria, CA 93013, USA). The slides were then placed on a hot plate at 42 ºC for several minutes and dried overnight in an oven at 42 ºC. The slides were stored at 4 ºC until further use.

Preparation of Psoralen-Biotin-labeled RNA probe

PCR amplification of the CnRb gene

Primer stock solutions (500 µM) were prepared according to the manufactures guidelines and the diluted (1:100) primers were stored at –20 ºC. PCR amplifications were performed in 50 µl reaction volumes each containing 1 µl of Plasmid DNA, 2µl of each 5 µM CnRb-T_7 forward primer (sense or anti-sense) and their reverse primers, 2 µl of 5 mM dNTPs, 1 µl of 5U/µl Taq DNA polymerase, 5 µl of standard 10x PCR buffer and 1.5 µl of 50 mM $MgCl_2$ and the final volume was adjusted with sterile water.

PCR was performed (as illustrated in Fig. 1) for 35 cycles [denaturation for 30 s (3 min for first cycle) at 95 ºC; annealing at 60ºC for 30 s and extension at 72 ºC for 1 min (7 min for final cycle at 72ºC) in a PTC- 100™ programmable thermal controller (MJ Research Inc. USA)].

The amplified product was diluted 1:100 with sterile water and used as the template for the second PCR. It was amplified with T_7 promoter and CnRbS/CnRbAS primers (Table 1) under similar thermal cycling parameters of PCR 1. Each reaction mixture (50 µl) for the second PCR contained 2 µl of template (product of PCR 1), 1 µl of forward primer (T_7) and 2 µl of reverse primer (CnRbS/ CnRbAS) (5µM), 4 µl of dNTPs (5 mM), 1.25 µl of Taq DNA polymerase (5U/µl), 5 µl of standard PCR buffer (10x) and 1.5 µl of $MgCl_2$ (50 mM) and the final volume was adjusted with sterile water. Several control PCR amplifications were done with known primers

Figure 1. DNA amplification in the fi

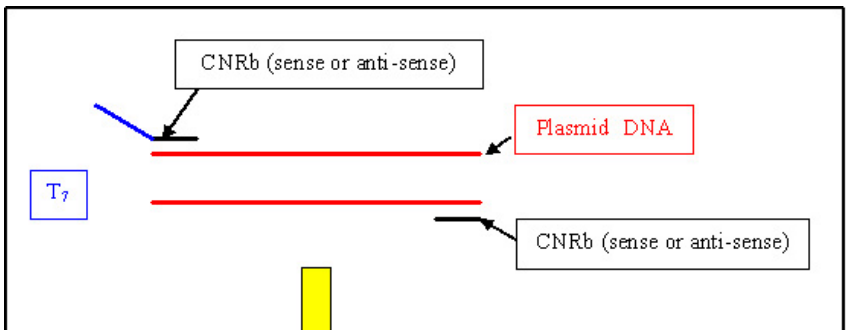


Table 1. Sequences of the primers used for PCR

Primer	Sequence
CnRbS (Sense)	ATTGAGAGGGAAGAGAGAGGGG
CnRbAS (Anti Sense)	GAACAAAATCATCGCCTGCC
T_7	**GCGAAATTAATACGACTCACTATAGGGCGAA**
CnRbS-T_7	**GCGAAATTAATACGACTCACTATAGGGCGAA**ATTGAG AGGGAAGAGAGAGGGG
CnRbAS-T_7	**GCGAAATTAATACGACTCACTATAGGGCGAA**GAACAA AATCATCGCCTGCC

provided by the CIRAD laboratory to check whether the conditions used were sufficient.

Aliquots (5 µl) of final reaction mixtures were electrophoresed through 1% agarose gels and stained with ethidium bromide. DNA was visualized by UV trans-illumination. The amplified DNA products were purified by ammonium acetate-isopropanol precipitation. Purified DNA was electrophoresed to check the presence of the required bands of correct size. The DNA was quantified by comparison with a series of standard DNA solutions (100, 75, 50,

25, 10 ng/µl) based on the intensity of color developed after ethidium bromide staining (data not shown).

Transcription and labeling of RNA probe

Transcription was performed with the Maxi-Script (Ambion) kit, with 1 µg of PCR product DNA in 20 µl reaction volumes containing 2 µl of 10x transcription buffer, 2 µl of RNA polymerase, 1 µl each of 10 mM stocks of ATP, CTP, GTP and UTP, and DEPC-treated water, for 1 h at 37 ºC. The reaction mixtures were incubated for 30 min at 37 ºC after adding 1 µl of DNase (2U/µl) to remove the DNA template. RNA purification and quantification were similar to the process of DNA purification.

Aliquots (1 µl) of purified RNA solutions were mixed with 10 µl of loading buffer (200 µl glycerol, 750 µl DEPC-treated water, 46 µl of saturated xylene cyanol) and incubated at 70 ºC for 2 min followed by quick cooling on crushed ice for 2 min.

The solutions were electrophoresed through 1% agarose gel (treated with 250 µl of 5 M NaOH for RNase free condition), stained with ethidium bromide and visualized by UV transillumination and photographed to check the size of the synthesized RNA.

RNA was labeled with Psoralen-Biotin Kit (Ambion ®, Bright Star TM, UK), according to the manufacture's instructions. For labeling, 100 µl of denatured RNA (at 65 ºC for 5 min followed by rapid cooling to avoid renaturation) was mixed with 10 µl of Psoralen-Biotin in the micro plate wells, and UV light (Ambion ®, UK) at 365 nm (4W) was applied directly to the plate for 45 min. After adding 200 µl of n-Butanol to the labeled solution, the mixture was vortexed and centrifuged at 1000 g for 1 min to remove the excess Psoralen. The supernatant was discarded and the labeled RNA probes (sense and anti-sense) were stored at –20 ºC.

Hybridization

The sectioned tissues (mounted on Silanized slides, Dako Cytomation, USA) were de-waxed by three rinses (10 min each) in Safesolv TM (a biodegradable paraffin clearing agent; Labonord Company, Templemars, France) and rehydrated with three rinses (10 min each) in 100% alcohol followed by 70% ethanol (2 min), 50% ethanol (2 min) and DEPC-treated water (2 rinses of 2 min). Enzymatic digestion of the sections was done by incubating in 200 µl Protinase K (5 µg/ml) (ROCHE, GmbH, Ref. 795723) solution at 37ºC for 30 min in a thermocycler for histology (for histological glass slide incubation) (Thermo hybaid, $HyPro_{20}$) under saturated atmosphere, to remove the proteins present in the sections. The digestion was completed by immersing the sections in buffer arrest -Protinase K solution for 2 times (5 min each) followed by 0.2% glycine in 1x PBS for 2 min and finally in 1x PBS for 2 times (1 min each). Sections were then dehydrated in a graded ethanol series (30%, 50%, 70%, 90% and 100%; 1 min in each) and air-dried for 2 h at room temperature.

A hybridization chamber (Eppendorf UK Ltd, Cambridge, UK) was placed on the slide to continue the procedure. Hybridization was performed in 100 µl volume containing, 50 µl de-ionized 100% formamide, 10 µl of 20x SSC buffer (3 M NaCl, 0.3 M Sodium citrate .$2H_2O$, pH 7.0), 20 µl of 50% Dextran sulphate, 2 µl of 100x Denhardt, 1 µl of t-RNA (10 mg/ml) and 200 ng of probed RNA for each slide at 45 ºC under saturated environment overnight. Post-hybridization washing was done in the following solutions; 2x SSC buffer for 5 min at room temperature, 2x SSC buffer for 45 min at 50 ºC, 1x NTE buffer (10 mM NaCl, 10 mM Tris.Cl, 1 mM EDTA, pH 7.5) for 5 min at room temperature, 1x NTE buffer for 5 min at 37 ºC, RNase A for 30 min at 37 ºC, 1x NTE buffer (2 times, 5 min in each) and 1x SSC buffer (2 times, 30 min in each) at 55 ºC.

Detection of hybridized, biotin-labeled RNA probe

The slides were air dried and incubated in freshly prepared 1% blocking solution (ROCHE, GmbH) in PBS at 37 ºC for 1 h. Vectastain ABC-AP kit (VECTOR Laboratories, USA) solution was added and kept for 15 min at 37 ºC and washed three times with 1x PBS buffer at room temperature each for 10

min. Revelation buffer bath was changed two times keeping in each for 5 min and 1 ml of revelation solution was added per slide and incubated at 37ºC over night. The slides were observed under the microscope (Leitz) and photographed. Positive reactions were indicated by deposition of an insoluble pink-purple stain on the surface of the section.

Acridine orange staining for RNA retention; Negative controls [in which all the RNA was destroyed by treating with RNase A (R 4875, Sigma)] to identify potential false positive or false negative results, and sense Rb probes were used as the controls.

Results

In the first PCR reaction, amplification of part of the CnRb cDNA sequence was performed to generate the target sequences of CnRb RNA probes of sense and anti-sense sequences, starting from the same plasmid. The plasmid contains the full sequence of CnRb cDNA that has been cloned from coconut. In this reaction, the T_7 promoter sequence is present in the target sequence as the T_7 promoter is necessary to convert the target cDNA sequence into RNA during the transcription step by RNA polymerase. Gel electrophoresis revealed that the amplified product of PCR 1 was of the correct size (517 bp) (Fig. 2a). However, some of the nucleotides in the T_7 promoter could be lost due to exo-nuclease activity. Initiation of transcription occurs only if the complete sequence of T_7 promoter is present since RNA polymerase will not recognize the target sequence if complete sequence of T_7 promoter is absent. Consequently, the concentration of the transcript (RNA) would be low. To avoid this, the second PCR was conducted, using the product of PCR 1 as the template and T_7 as the forward primer. This primer ensures that the second PCR product will contain the full sequence of T_7 promoter that is necessary for the transcription of the cDNA into RNA. Gel electrophoresis (Fig. 2b) revealed that the synthesized DNA fragment was of the correct size (approx. 0.4 Kb). After transcription, all the manipulations were necessarily conducted under RNase-free conditions to avoid contamination. Gel electrophoresis of the purified transcript revealed that the synthesized RNA was of correct size which ensured that the probe synthesis was in order (Fig. 2c).

In this study, the probe used was directed against biotin which is coupled with the enzyme, alkaline phosphatase. Thus once the substrate is added, a pink-purple product develops, that can be detected under the microscope. The control treatments were necessarily performed to confirm the expression of the CnRb gene in the tested tissue sections. After the tissue sections of ovary-derived structures were treated with RNA-binding fluorochome acridine orange, a light green fluorescence was emitted.

When sections were examined by the fluorescence microscope (Fig. 3a), it confirmed the presence of RNA in the embryogenic tissues used for the present experiment. The tissue sections, digested with RNase prior to hybridization, lacked the stain deposition due to the absence of RNA within the tissue (Fig. 3b). When *in situ* hybridization

Figure 2. Agarose gel electrophoresis of PCR p
Products of first PCR (lanes 1 & 8- 1 Kb DN
CNRbT_7S, lane 3- product of CNRbT_7AS, lanes 4
amplifications) **b.** Products of second PCR (lanes 1
2-7- CNRbS; 8-13- CnRbAS). **c**. Purified RNA (la
lanes 2-3- product of CNRbS-RNA probe, 4- prod
Arrow heads indicate the presence of bands of correc

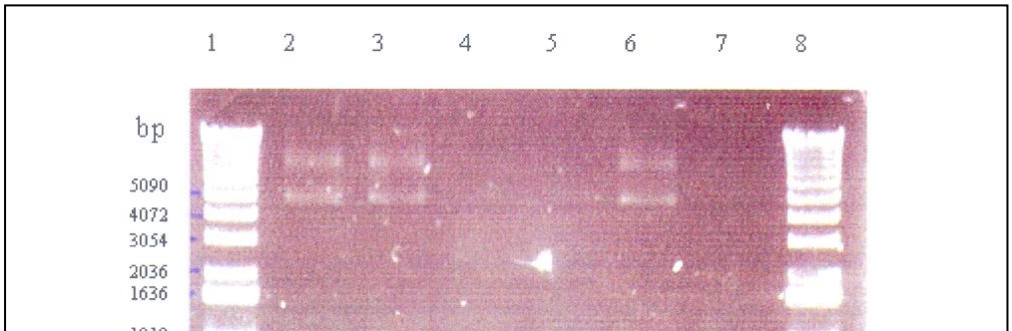
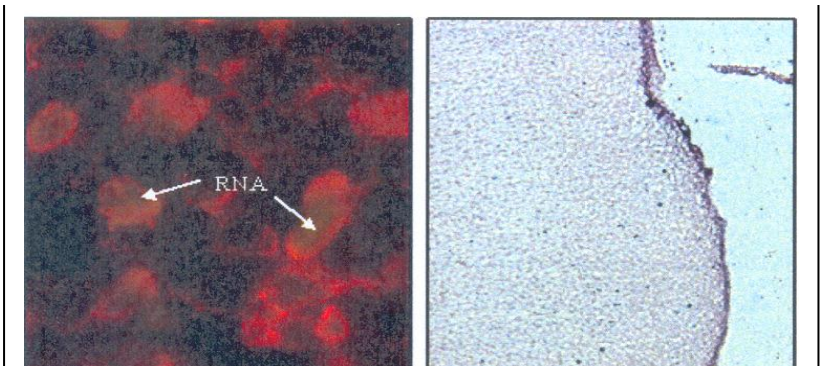


Figure 3. The Controls used in *in situ* hybridization. a. A callus section stained with acridine orange. Note the presence of RNA in green color *(bar=10 µm)* **b.** Negative control – hybridization after RNase digestion *(bar=50 µm)*. **c.** *In situ* hybridization with ovary-derived non-embryogenic callus by biotin-labeled CNRb anti-sense RNA probe *(bar=100 µm)* **d.** *In situ* hybridization with ovary-derived non-embryogenic callus by biotin-labeled CNRb sense RNA probe *(bar=100 µm)*. Absence of specific color development in the tissues indicates non-expression of the Rb gene.

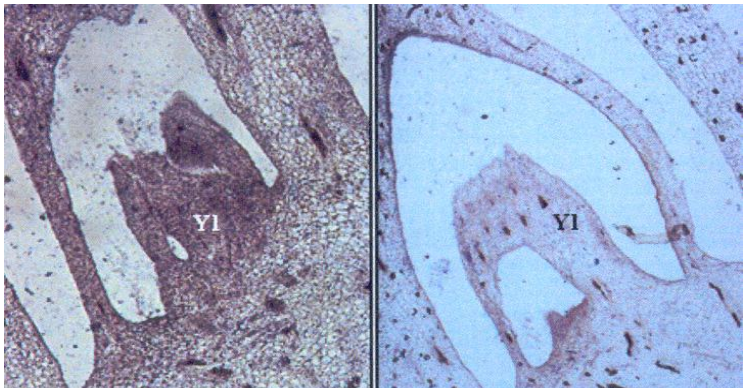


was performed with the non-embryogenic structures, they lacked any sign of colour development in any part of the tissue. It could be due to the non-expression of the CnRb gene within the cells of non embryogenic tissues (Fig. 3c, d).

The deposition of a pink colour stain confined to the young leaf primordia close to the meristem of the developing shoot (Fig. 4a) and the cambium like zone (CLZ) of the callus (Fig. 4c) indicated the CnRb expression in these regions. According to histological studies (Perera *et al.*, 2007), these areas of the tissues contain meristematic cells with high mitotic index. Colour deposition, restricted to such areas revealed that the expression of CnRb gene is higher in cells in which the cell cycle controlling genes are activated. Absence of a colour signal shows a lack of cell division in the rest of the parenchyma cells.

Normally, a signal should not be developed inside the tissue with the sense probe. In the present experiment, the colour signal was produced in similar areas with both sense and anti-sense probes (Fig. 4 b, d). However, the intensity of the color with anti-sense probe is higher than the sense probe. Deposition of the pink signals with sense probe

Figure 4. Expression of CnRb gene in ovary-derive *situ* hybridization a. Germinating shoot hybridized probe. Note that the color intensity is higher in the yo meristem. *(bar=200 µm)* **b.** Germinating shoot hybri probe *(bar=200 µm)* **c.** Embryogenic callus section hy RNA probe. Note the color development in the cells of callus section hybridized by biotin-labeled sense RNA of the signal is lower with the sense RNA probe.



might reflect the presence of slight RNA interference (RNAi) that is involved in post-transcriptional regulation by destruction of the RNA messengers. Therefore, it may be considered that the sense probe is not a good negative control.

Discussion

Cell division and growth depends on a series of coordinated events strictly regulated both temporally and spatially in individual cells. Investigation of links between cell cycle regulation and plant development can shed light on the importance of a strict balance between cell proliferation and differentiation during development (Boniotti and Gutierrez, 2001). One key cell cycle regulatory pathway depends on the plant retinoblastoma-related (RBR) protein and the E2F transcription factors (Gutierrez *et al*., 2006). RBR protein is required for cell proliferation arrest in cultured cells of tobacco (Gordon-Kamm *et al*., 2002) and during *Arabidopsis* gametophytic development (Ebel, *et al*., 2004). The importance of tobacco RBR protein in restricting cell division and endoreplication in leaf cells has been demonstrated by Park *et al*. (2005). Recent studies have shown a distinct response of different cells to RBR inactivation in terms of maintaining the balance between cell division and endoreplication during leaf development in *Arabidopsis thaliana* (Boniotti and Gutierrez, 2001; Desvoyes *et al*., 2006). In the present study, attempts were made to identify any correlation between CnRb expression and meristematic activity of *in vitro*-cultured coconut tissues.

In situ hybridisation provides cellular and sub-cellular resolution of mRNA levels within multi-cellular organisms and is widely used to provide spatial and temporal information on gene expression that often directly reflects functional involvement in developmental processes (Drea *et al*., 2005). In multi-cellular organisms, ISH complements northern blotting, RT-PCR and microarrays that provide a wealth of expression data for functional genomics, in which, the extraction of RNA from whole tissues invariably results in the loss of spatial resolution information (Drea *et al*., 2005). It has become an essential and powerful tool for understanding regulation of gene expression in cells and tissues of different organisms by localization of gene expression at a high

resolution level with multiple probes (Takechi *et al*., 1999). However, it is a tedious process that has to be handled carefully as it requires RNase-free conditions for precise expression of the target gene.

The study confirmed the previous results obtained by RT-PCR, showing that CnRb gene is highly expressed in active, fast growing tissues. It also established a correlation between the meristematic potential of the cells characterized by cytological features (i.e. a high nucleo/cytoplasmic ratio etc.) and the expression of CnRb gene. It showed that the transcript of CnRb is accumulated more in cells having characteristic cytological features of meristematic cells (i.e. dense cytoplasm, high nucleo/ cytoplasmic ratio etc.) compared to the already differentiated cells (i.e. parenchymatous cells). Thus CnRb transcript appears to be a good marker of meristematic cycling cells having a good potential to multiply before differentiation. This is an interesting and important fact for coconut in which maintaining *in vitro* cycling cells, necessary for callogenesis and embryogenesis induction is found to be difficult.

Conclusion

The results revealed that *in situ* hybridization can be used to detect the expression of CnRb gene in the cells, by comparing the sense and anti-sense probes. When the probe detects only the target mRNA sequence, it can be used as a marker to study the somatic embryogenesis pathway and to identify the good embryogenic structures derived from different explants. By *in situ* hybridization, it was possible to establish a relationship between the meristematic activity in the tissues tested and expression of the CnRb gene. It was seen clearly that CnRb mRNAs were mainly localized in the meristematic cells and tissues.

Acknowledgements

We gratefully acknowledge the financial assistance provided by the Institut de Recherche pour le Developpement (IRD), Montpellier, France. We are thankful to Mr. Jacques Escoute (CIRAD, Monpellier, France) for the extended assistance in photography.

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