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Research Note

An Insight into the Ontogenetic Pattern Observed Among NQHVVN-Containing Transcripts Found in Varying Ages of the Coconut Endosperm

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Coconut oil starts to accumulate in the endosperm 6-7 months after pollination suggesting a time-dependent pattern of expression among fatty acid genes. NQHVVN-containing transcripts were readily amplified from varying ages of coconut endosperm following an ontogenetic pattern. Two amplified bands were detected at the 5-6- and 6-7-month old coconut endosperms whereas no bands were amplified at the 4-5- and 7-8-month old coconut endosperms following the perceived ontogenetic pattern. *In silico* analyses of the three sequenced 3'UTR variants show Iso1 possessing an IRES-like motif, Iso2a, a K-box-like and NUE and Iso2b, a Brd-box-like, and an IRES-like motif. K- and Brd-boxes are known sites for miRNA attachment insinuating the involvement of miRNAs in TE ontogenetic expression.

Key Words: coconut endosperm, *Cocos nucifera*, NQHVVN-containing transcripts, ontogenetic expression, 3' RACE, 3' UTR

INTRODUCTION

Coconut oil is a naturally saturated vegetable oil and, as such, it is desirable for food and industrial purposes. It is resistant to oxidative rancidity endowing foods prepared with the oil flavor stability. It also has its advantages in soap production, as solid coatings for confectionary and baked foods, and has medical applications as well (Banzon and Velasco 1982; Harris 1994). There are nine known individual fatty acids in coconut oil differing from each other in the number of carbon (C) atoms and these are: caproic acid (C6), caprylic acid (C8), capric acid (C10), lauric acid (C12), myristic acid (C14), palmitic acid (C16), stearic acid (C18), oleic acid (C18:1), and linoleic acid (C18:2) (Banzon and Velasco 1982). The most abundant form of fatty acid for coconut are the saturated medium-chain fatty acids (C6-C12) where lauric acid predominates and accumulate in the coconut endosperm (Banzon and Velasco 1982; Harries 1994).

In coconut, the solid endosperm develops at the earlier stages of development of the coconut drupe during which medium-chain fatty acids start to accumulate in the solid endosperm (Banzon and Velasco 1982; Harries 1994) suggesting that the expression of genes involved in fatty acid synthesis is regulated ontogenetically. In addition, enzymes necessary for fatty acid synthesis has already been shown to be absent among certain ages of coconut endosperm which has then been suggested that an ontogenetic expression pattern of fatty acid synthase genes in coconut occurs (Villalobos et al. 2001). This hypothesis, however, has never been proven to date.

One of the genes involved in fatty acid synthesis, the acyl-acyl carrier protein (ACP) thioesterase (TE), plays a crucial role in the distribution of *de novo* synthesized

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acyl groups by catalyzing the first committed step in eukaryotic lipid biosynthesis pathway in all plant cells (Jones et al. 1995). The most commonly found plant TE are specific for 18:1-ACP and, therefore, have been termed long-chain or "oleoyl"-ACP thioesterases. Encoded by nuclear genes, 18:1-ACP thioesterases are synthesized as preproteins in the cytoplasm and subsequently imported into the plastids (Kridl 1998). An isolated 12:0-ACP thioesterase from California bay trees (*Umbellularia californica*) was expressed in developing oil seeds of *Arabidopsis* (Hawkins and Kridl 1998; Kridl 1998). The bay enzyme redirects the resident long-chain fatty acid synthase to medium-chain (12:0) fatty acid production demonstrating for the first time the chain length determining role of TE during *de novo* fatty acid biosynthesis (Voelker et al. 1992) as the primary involvement of the coconut TE gene in medium-chain fatty acid synthesis and the start of medium-chain fatty acid accumulation in the coconut endosperm 6-7 months after pollination make the TE transcript (or a portion of the transcript) a good basis to establish the occurrence of ontogenetic expression in the coconut endosperm. Coincidently, the TE enzyme contains a conserved NQHVVN region which has been readily detected among plant TEs and has been previously used as an oligonucleotide primer designed to amplify the TE transcript (Aluru et al. 2003; Hawkins and Kridl 1998; Jones et al. 1995).

The crucial role of the untranslated region (UTR) is now widely acknowledged primarily due to the post-transcriptional regulatory pathway that control mRNA localization, stability and translation efficiency (Pesole et al. 2000; Rothnie 1996). Contrary to the 3' UTR of mammals, where a common ARE (AU-rich element) is found making it easy for 3'UTR research in mammals, plant 3' UTR research is hindered mainly by the absence of a common ARE among plant genomes (Barreau et al. 2006; Canadillas and Varani 2003; Hesketh 2004; Pesole et al. 2000) which is why most of the information derived from 3' UTRs come from the studies of vertebrate systems (Rothnie 1996). In addition to sequence analysis being the common method of analyzing 3' UTRs (Brockman et al. 2005; Pesole et al. 2000; Rothnie 1996) a study conducted by Hunt et al. (1987) showed that animal poly (A) signals are not properly recognized in plant cells suggesting functional differences in the *cis*-acting sequences controlling 3' end formation. This main feature suggests that the process of mRNA cleavage and polyadenylation in plants differ mechanistically from those characterized in vertebrate systems (Rothnie 1996).

The 3' UTR plays an important role in many aspects of RNA function and metabolism, particularly in mRNA translation and turnover (Barreau et al. 2006; Jackson and Standart 1990; Rothnie 1996; Sachs and Whale 1993). Post-transcriptional processing of mRNA is a fundamental step in eukaryotic gene expression. The primary transcript is capped at the 5'-end, introns are removed by splicing and the mature 3'-end is formed by an endonucleolytic cleavage followed by addition of a poly (A) tail to the 3'-end of the upstream cleavage product (Rothnie 1996). The 3' UTR is the site of several post-transcriptional controls such as RNA-protein interactions involving also multi-protein complexes, antisense RNA interactions and cytoplasmic polyadenylation elements (Pesole et al. 2000). A more thorough understanding of the 3' UTR is ideal, especially in plants, since it would allow for the deciphering of the control mechanisms ruling coordinated expression of genes ontogenetically based on the physiological requirements of the cell or in response to environmental stimuli.

In this paper, we established the occurrence of an ontogenetic pattern of our designated NQHVVN-containing transcript (NCT) found among certain ages of the coconut endosperm and we present an insight, through bioinformatic analyses, on the possible mechanism leading to its ontogenetic expression at varying ages of the coconut endosperm.

MATERIALS AND METHODS

mRNA extraction

Coconut endosperm tissues ages 4-5-, 5-6-, 6-7- and 7-8-month old were collected from the drupe of the coconut variety Laguna Tall. Coconut meat or the solid endosperm was freshly obtained for mRNA. The Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen, USA) was used to isolate pure mRNA from coconut endosperm following the manufacturer's instructions with some modifications. All chemicals and tubes were provided by the kit.

Briefly, powderized coconut endosperm samples were treated with lysis buffer and incubated at 45°C for 20 min before centrifuging. The supernatant was then transferred to a new tube and NaCl concentration adjusted to 0.5M concentration. Remaining DNA was sheared by quickly passing the lysate five times through a 1-mL pipette tip. The cell lysate was added to a tube of oligo(dT) cellulose and allowed to swell before placing the tube horizontally in a platform and incubated for 20 min at room temperature. The oligo(dT) cellulose, after centrifugation, was resuspended in binding buffer and again centrifuged. This process was repeated until the buffer was no longer cloudy. The resin was then resuspended in binding

buffer, transferred to a spin-column then centrifuged. After repeating this step thrice, low salt wash buffer was added to resuspend the resin before centrifugation. The spin-column was then placed into a new tube and 100 μL of elution buffer was added and mixed into the cellulose bed before centrifugation. This step was repeated twice. The mRNA was precipitated from the solution using glycogen carrier, sodium acetate, and ethanol placed in -80°C overnight. The mRNA was pelleted through centrifugation and resuspended in 10 μL elution buffer for use in this study.

Cloning coconut transcripts using a NQHVVN-based oligonucleotide primer

The designed forward primer, THIO-*S* (5'-AAYCARCAYGTNAAYAAYGTN-3'), used for the study was based on the highly conserved region, NQHVNN, as indicated in previously published works (Aluru et al. 2003; Hawkins and Kridl 1998; Jones et al. 1995). As a control, a forward primer ENO-S (5'-ACRTTRTTNACRTGYTGR-3') was designed for the coconut enolase gene. The 3' RACE (Rapid Amplification of cDNA Ends) procedure was carried out using the 3' RACE Kit (Invitrogen,USA) following the manufacturer's instructions. The TOUCHDOWN PCR condition performed consists of an initial denaturation at 95°C for 5min followed by five cycles of 94°C for 1min, 55°C for 1min and 72°C for 2min. This was followed by another five cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2min. The last set of cycles consists of 25 cycles 94°C for 1min, 50C for 1min and 72°C for 2min. A final 10min extension at 72°C was performed before proceeding to cloning.

Gel electrophoresis was used to determine the presence of amplified PCR products among the varying ages of coconut endosperm used as cDNA template. The detected PCR bands were immediately eluted and used in the TOPO TA Cloning® Kit (Invitrogen, USA) following manufacturer's instructions. At least five randomly selected clones from each eluted product were purified for sequencing.

Sequencing and Analysis

Purified plasmid DNA was commercially sequenced by the Division of the Biomolecular Research Facility, University of New Castle, Callaghen, Australia. Bioinformatic analysis of the sequences produced were analyzed using the Vector NTI Suite software and the web-based services, BLAST (www.ncbi.nlm.nih.gov/BLAST), Rfam (www.sanger.ac.uk/Software/Rfam), and UTRScan (www.ba.itb.cnr.it/BIG/UTRScan).

RESULTS AND DISCUSSION

Ontogenetic expression of NQHVVN-containing transcripts (NCT)

As the coconut drupe matures, several physiological changes are happening in the coconut endosperm. Among these changes, the coconut endosperm slowly starts to thicken and concurrently accumulate medium-chain fatty acids driven by the coconut TE enzyme (Banzon and Velasco 1982; Harries 1994). As previously mentioned, medium-chain oils only accumulate during the 6-7-month old after pollination, suggesting an ontogenetic expression of the coconut TE. To readily establish the ontogenetic expression in the coconut endosperm, 3' RACE was performed. As shown in Figure 1, NCTs are present in the 5-6- and 6-7-month old coconut endosperms with a single band in the 6-7-month old and two bands detected in the 5-6-month old. No band was detected in the 4-5- and 7-8-month old endosperms. The coconut enolase gene, which served as the control, is clearly shown to be present in all cDNA templates, confirming the quality of the cDNA template used. At this point, we classified the two distinct bands detected as NCT 1 for the transcript detected in the 5-6-month old (upper band) coconut endosperm only and NCT 2 for the transcripts detected in the 5-6-(lower band) and 6-7- month old coconut endosperms. The absence of PCR bands in the 4-5- and 7-8-month old coconut endosperms and the presence of PCR bands in the 5-6- and 6-7-month old coconut endosperms confirm the occurrence of ontogenetic expression in coconut. The results obtained are in agreement with previous observations made (Villalobos et al. 2001) where certain fatty acid enzymes were not detected at varying ages of the coconut endosperm. The absence of either isoform in the 4-5-month old coconut endosperm show that both isoforms have not been transcribed in the coconut endosperm and the absence of both isoforms in the 6-7-month old (for NCT 1) and 7-8-month old (NCT 2) coconut endosperms suggests NCTs were regulated which is in agreement with the findings of Villalobos et al. (2001). Considering the results obtained, we analyzed the 3' UTR sequences to provide a possible insight to the temporal pattern observed.

Sequence profiling of NCT 3'UTRs

To differentiate all three PCR bands produced, all three were cloned and sequenced (Figure 2) wherein only the 3' UTR sequences were analyzed. At this point, we designate Iso 1 to represent the UTR variant obtained from the 5-6-month old (upper band) coconut endosperm only; Iso 2a to represent the UTR variant obtained from the 5-6-month old (lower band) coconut endosperm; and Iso 2b to represent the UTR variant obtained from 6-7-month old coconut endosperm. The sequence profiles of all three

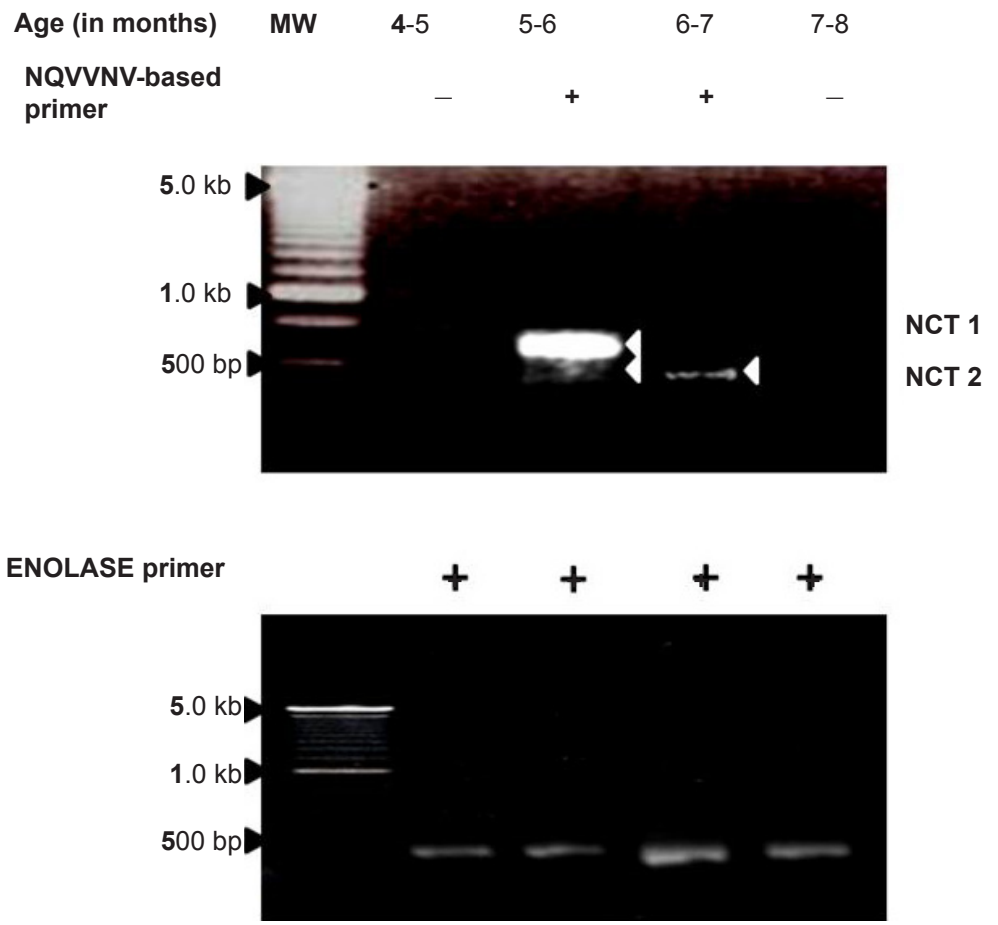

Figure 1. Ontogenetic pattern of NQHVVN-containing transcripts observed in varying ages of coconut endosperm. Touchdown PCR was performed using the four ages of coconut endosperm used are the: 4-5, 5-6, 6-7 and 7-8 months old as template where a NQHVVN-based oligonucleotide primer was used for the 3'RACE procedure and as control, the ENO-S primer was used to amplify an enolase housekeeping gene.

```
                1                                                                                                   100
Iso 1     (1) TAAAAGCGGACGACCCCGTTTGGTCGCACCTGGCGAACGACGTTGACAGAGTCCCGGTCCGCCACTTCCCGTTAGTCGACAACGGGCAGAGTGACCACTT
Iso 2a    (1) TAATGGCGGAAACTCAC------TCGACTATGGCGAGCCGGCGTCG---GCTTGCTGGCTCGCGTCGCTCAGTCACT------CG------CT----CCTT
Iso 2b    (1) TAATGGCGGAAACTCAC------TCGACTATGGCGAGCCGGCGTCG---GCTTGCTGGCTCGCGTCGCTCAGTCACT------CG------CT----CCTT

              101                                                                                                   200
Iso 1   (101) TTCTTTTTGGTGGGACCGCGGGTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTTTCG
Iso 2a   (76) CGCCTTCT-------C-GCGGGTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTTTCG
Iso 2b   (76) CGCCTTCT-------C-GCGGGTTATGCGTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGGGCTGACCTTTCG

              201                                                                                                   300
Iso 1   (201) CCCGTCACTCGCGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACAC
Iso 2a  (168) CCCGTCACTCGCGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACAC
Iso 2b  (168) CCCGTCACTCGCGTTGCGTTAATTACACTCAATCGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACAC

              301                                                                                                   400
Iso 1   (301) TCGCCTATTGTTAAAGTGTGTCCTTTGTCGATACTGGTACTAATGCGGTTCGAGAATT-ATGCTGAGTGATATCCCTTTCGACC-ATGCGGACGTC----
Iso 2a  (268) TCGCCTATTGTTAAAGTGTGTCCTTTGTCGATACTGGTACTAATGCGGTTCGATAA---ATCCACTGTGATATCTTATGAGTTCGATACGTAGTTCGAAC
Iso 2b  (268) TCGCCTATTGTTAAAGTGTGTCCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTC---GACCATGGC----

              401                                                                                                   491
Iso 1   (395) CATGGCCAG-GCCTTAAT----------------------------------------------------------------------------------
Iso 2a  (365) CATGCCTCGAGCCTACGTGATCATTGCCGCCGGTCACACGACCTTAAGCGGGAACCGGTGCGCAGCTGATCATGAAAAAAAAAAAAAAAAA
Iso 2b  (361) CCGGGGGGGAGCTCCAGCTGCCATAGCTATTCGA-ACTATAGCTTAAGGACGTCGGGCCCCCTAGGTG----------------------
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Figure 2. Sequence alignment of all three NCT 3' UTRs. Iso 1, Iso 2a and Iso 2b were aligned using the Vector nti Suite software. The boxed codon represents the putative STOP codon. The arrows indicate the area of the 233nt conserved nucleotide region. The underlined region represents the 10nt conserved region.

Table 1. Sequence profiles of NCT 3' UTRs.

	Iso 1	Iso 2a	Iso 2b
UTR Length	411nt	455nt	427nt
G+C%	52.06	51.99	55.08
Near-upstream Element (NUE) GATAAA[1]	(–)	(+) pos. 319-324	(–)
Far-upstream Element (FUE)	(–)	(–)	(–)
Transposable Element CACTG-motif	(–)	(+) pos. 327-332	(–)

[1]Sequence position relative to Iso 2a sequence found in Figure 3.

3'UTR variants (Table 1) show that all 3'UTRs sequenced are >400nt in length while the GC contents observed were >50%. It has been well established that the 3'UTR always have a longer nucleotide sequence compared to the 5'UTR (Pesole et al. 2000). This would mean that the length of the three coconut TE 3'UTRs sequenced are not unusually long since it does share the trend previously established among 3'UTRs. The length of the 3'UTR is important to consider since this may contain the various control mechanisms (Beelman and Parker 1995; Mazumder et al. 2003), especially in plants (Mazumder et al. 2003; Rothnie 1996). Noticeably, Iso 2a possesses a poly(A) tail while Iso 2b and Iso 1 has none which maybe associated to the presence of topoisomerase in the cloning vector used wherein it has a tendency to randomly cut and ligate gene sequences (Zhu and Schiestl 1996) which would then explain why no poly (A) tail was detected. Nevertheless, when all three 3'UTRs sequenced were aligned (Figure 2), a common 233nt conserved region was observed. Visual inspection further downstream of the 233nt region reveals a 10nt conserved region in all 3'UTRs. The presence of both conserved regions in all the 3' UTR sequence variants disproves the notion that the sequences obtained were formed as chimeric regions. Noticeably, the sequence in between the 233nt and 10nt region of all three 3'UTRs (Figure 2) varies among all three 3'UTRs. Interestingly, a CACTG motif was identified in Iso 2a (Table 1). The same CACTG motif was also identified in the coconut enolase 3'UTR sequence (unpublished data). The CACTG motif has been well associated to a family of the class II transposons and its presence has since been a hotly debated question (Wicker et al. 2003). It was suggested that its presence contribute to regulatory sequences that may alter gene expression (Zhang et al. 2000).

Further visual inspection and comparison for known plant upstream elements, the far-upstream element (FUE), and the near-upstream element (NUE) was done among all three TE 3'UTR variants since there is no existing software to search for either FUEs or NUEs. The FUE has been observed to enhance overall processing efficiency while NUE contains the plant poly(A) signal to which it is probably the target for a nuclear 3'-end processing factor (Loke et al. 2005; Rothnie 1996). No known FUE pattern has been observed while among the three sequenced TE 3'UTR variants, only the Iso 2a contained an identifiable NUE pattern (Table 1) which is located immediately downstream of the conserved 233nt region.

Bioinformatic analysis of the NCT 3'UTRs

Three web-based softwares were used to analyze the 3'UTR sequences obtained, namely: BLAST, Rfam, and UTRScan. The BLAST software would identify any similarities with other registered UTR sequences. The Rfam software would search and analyze for non-coding RNA families. The UTRScan software would identify known UTR patterns within the sequence. It is worth mentioning that though the UTRScan software is initially designed to search for animal UTR patterns within the UTRs which includes miRNA-binding regions, it was earlier proposed in a previous publication that both animal and plant miRNAs share some mechanistic similarities (Broderson et al. 2008). In the absence of software designed for analyses of plant 3' UTRs and considering the works of Broderson et al. (2008), we use the UTRScan software to identify established animal UTR patterns and miRNA-binding regions in the NCT 3' UTR variants.

Table 2 summarizes the results obtained from BLAST, Rfam, and UTRScan analyses. The UTRScan of Iso 1 identified an internal ribosome entry site (IRES)-like sequence motif which includes the downstream portion of the 233nt conserved region. The IRES is a sequence motif normally associated to the 5'UTR (Baird et al. 2006) and its presence in the 3'UTR has been shown to be involved in transcript circularization (Mazumder et al. 2003). There have been some circumstances in higher eukaryotes where the m^7GpppN cap found in the 5'UTR is bypassed by the IRES, allowing for transcript circularization between the 5' and 3'UTRs, a phenomena already observed (Mazumder et al. 2003). For the NCT 3'UTR, the detection of an IRES-like motif suggests that there is an IRES-mediated interaction between the 5'UTR and 3'UTR, suggestive of a transcript circularization

Table 2. Bioinformatic analyses of NCT 3'UTRs.

	Iso 1	Iso 2a	Iso 2b
BLAST	No significant similarity		
Rfam	No significant similarity		
UTRScan[a]			
IRES	pos. 345-411	(–)	pos. 290-427
K-box (CTGTGATA)	(–)	pos. 329-336	(–)
Brd-box (GAGCTTA)	(–)	(–)	pos. 320-327

[a]Sequence position relative to 3'UTR sequences found in Figure 3.

event. We emphasize that IRES are not well defined at the sequence level so computer predictions are not robust and require more experimental proof.

Performing UTRScan on Iso 2a identified a K-box-like motif downstream of the proposed NUE while UTRScan of Iso 2b identified a Brd-box-like motif. The K-box and Brd-box both mediate negative regulation and, in the case of the K-box, mainly decrease transcript levels, while the Brd-box affects transcript stability and translational efficiency (Lai 2002). Regulation of both the K-box and Brd-box are spatially and temporally ubiquitous and likely involves the formation of RNA:RNA duplexes (Lai 2002) where both boxes have already been established to be the site for miRNA attachment (Lai et al. 2004). Identification of similar and previously established UTR patterns using UTRScan found in all three coconut 3'UTR variants is in agreement with the works of Broderson et al. (2008) where it was suggested, as mentioned earlier, that both animal and plant miRNAs share some mechanistic similarity.

miRNAs are well known to inhibit the translation of mRNAs into protein and to promote mRNA degradation (Buchan and Parker 2007). As such, given the sequences we have obtained from the 3'UTR variants, the presence of the CACTG motif in Iso 2a which was suggested to insert regulatory sequence and the candidate motifs identified, our insight on the possible mechanism by which ontogenetic expression occurs in the coconut endosperm is through the involvement of miRNAs. Nevertheless, substantial experimentation is further recommended to further prove this point.

SUMMARY AND CONCLUSION

We established the occurrence of ontogenetic expression of an NCT which is in agreement with the works of Villalobos et al. (2001) and speculate on the possible mechanism through sequence analyses of the 3' UTR sequence variants. We hypothesize that this mechanism is in a way associated with the possible miRNA attachment to candidate Brd-box-like and K-box-like motifs found downstream an identified conserved 233nt region of the TE 3'UTR where both are associated with transcript downregulation and turnover, respectively. We also propose that transcript circularization, as suggested by the presence of the IRES-like motif, should be performed.

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