Philippine Journal of Science
139 (2): 227-232, December 2010
ISSN 0031 - 7683

Research Note

Sequence Analyses of the Coconut Enolase 3' End Reveals a CACTG Motif Found Within the 3'- Untranslated Region

Marni E. Cueno[1*], **Rita P. Laude**[1], and **Antonio C. Laurena**[2]

[1]Institute of Biological Sciences, College of Arts and Sciences,
University of the Philippines Los Baños, College ,Laguna 4031
[2]Institute of Plant Breeding , Crop Science Cluster, College of Agriculture,
University of the Philippines Los Baños, College, Laguna 4031

As the coconut drupe matures, medium-chain fatty acids accumulate suggesting *de novo* fatty acid synthesis is active until drupe maturation is reached. This suggests that an active transcription of enolase mRNA takes place. Detection of coconut enolase transcripts from 4-5, 5-6, 6-7 and 7-8 month old coconut endosperms were made through 3' RACE. PCR products were cloned and sequence alignment showed higher homology between coconut and palm kernel enolase 3' CDS compared with that of rice and corn. Sequence alignment between the coconut and rice enolase 3' UTR revealed a common CACTG motif which is known to be conserved among the CACTA transposon family and has been speculated to insert regulatory elements. Folding pattern prediction of the coconut enolase 3' UTR revealed the occurrence of folding located immediately after the CACTG motif. We speculate this region to be the binding-site of regulatory elements.

Key Words: coconut drupe, coconut endosperm, enolase, transposable elements, 3' CDS, 3' UTR

INTRODUCTION

In the coconut plant, lipid accumulates in the coconut endosperm inside the coconut drupe. As the drupe matures, the coconut endosperm concurrently begins to thicken and readily stores oil (Harris 1994; Villalobos et al. 2001). There are nine known individual fatty acids in coconut oil that differ from each other in the number of carbon (C) atoms. 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). The most abundant form of fatty acids for coconut are the saturated medium-chain fatty acids (C6-C12) in which lauric acid predominates and accumulate in the coconut endosperm (Harries 1994; Knutzon et al. 1995) and is mainly driven by the acyl-ACP thioesterase enzyme (Jones et al. 1995). Another important enzyme that also plays an important role in *de novo* fatty acid synthesis is enolase.

Enolase is an essential and ubiquitous glycolytic enzyme that catalyzes the conversion of 2-phopphoglycerate to PEP (Forsthoefel et al. 1995; Van Der Straeten et al. 1991). In higher plants, the enolase enzyme is often present as multiple isoforms localized to the cytosol and to the plastids (Gottlieb 1982; Shih et al. 1986; Miernyk and Dennis 1984, 1992). In addition to its involvement in glycolysis, enolase plays specialized roles during plant development, in nonphotosynthetic tissues, and under conditions of anaerobiosis (Forsthoefel et al. 1995; Lal et al. 1998). Moreso, enolases together with polynucleotide phosphorylase (PNPase), a DEAD -box RNA helicase and RNAse E and possibly with other proteins form a multiprotein complex termed degradosome found to be involved in RNA degradation in *E. coli* (Komine et al. 2002). During artificially induced ripening, enolase

*Corresponding author: mecueno@uplb.edu.ph
marnicueno@yahoo.com

mRNA levels increased 30-fold and enolase activity increased 3-fold (Van Der Straeten et al. 1991). Similarly, exposure of plants to anaerobic stress causes a shift from an oxidative to a fermentative mode of carbohydrate metabolism, resulting in the increased expression of many enzymes involved in the glycolytic pathway, including enolase (Dennis et al. 1987; Lal et al. 1998). In the developing seeds of castor bean, it was observed that enolase activity levels of cytosol- and plastid-localized isozymes peak coordinately with maximal rates of storage lipid accumulation (Miernyk and Dennis 1982). Multiple isoforms of enolase have been previously reported in oilseeds (Miernyk and Dennis 1982, 1984, 1987) and the increased activity of plastid-localized isozymes reflects the high-demand for pyruvate for *de novo* fatty acid synthesis in tissues resulting in lipid accumulation in developing oilseeds (Miernyk and Dennis 1987).

Previous plant enolases already studied thus far include *Arabidopsis* and tomato (Van Der Straeten et al. 1991), castor bean (Blakeley et al. 1994), *Mesembryanthemum crystallinum* L. (Forsthoefel et al. 1995), *Echinochloa phyllopogon* (Fox et al. 1995), and oilseed rape (Zhao et al. 2004). Purification of the enzyme has been reported from potato tubers (Boser 1959), spinach (Sinha and Brewer 1984), *E. phyllopogon*, and *E. cruspavonis* (Mujer et al. 1995). To date, no molecular-based phylogenetic analyses study has been performed comparing the coconut *enolase* isolated from the coconut endosperm with other known *enolases*.

Here, we report a partial sequence analysis of the 3' end of the coconut *enolase* (ENO). Sequence alignment of the ENO 3' CDS (coding sequence) showed high homology with palm kernel compared with rice and corn. Sequence alignment of the ENO 3' UTR (untranslated region) with corn and rice showed low homology; however, a common CACTG motif was identified when ENO 3' UTR sequence was aligned with rice 3' UTR. The CACTG (or more commonly identified as CACTA) motif is unique within the members of the CACTA transposon family and is found to be highly conserved (Greco et al. 2005; Wicker et al. 2003) and commonly found among the members of the Triticeae family suggesting that certain elements of the CACTA transposon family may also exist in coconut.

MATERIALS AND METHODS

mRNA extraction

Coconut endosperm was collected from 4-5, 5-6, 6-7, and 7-8 month old coconut drupes of the Laguna Tall variety. mRNA was extracted using the Micro-FastTrack TM 2.0 Kit (Invitrogen) following manufacturer's recommendations with some modifications.

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 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 the coconut enolase 3' end

The forward primer used for this study, ENO-S (5'-ACRTTRTTNACRTGYTGR-3') was designed from the sequence alignment of other known plant *enolases*. In order to obtain the complete 3' end of the coconut *enolase*, the 3'RACE method was performed. The 3'end, composed of the 3' CDS and 3' UTR were cloned using the 3'RACE kit (Invitrogen) following the manufacturer's instructions. All components used were provided by the kit.

Briefly, 2 μL of 5 μg purified mRNA was mixed with 22 μL DEPC-water and 1 μL 3' adaptor primer. The cocktail was initially incubated in a PCR machine at 70°C for 10 min. Afterwards, a mixture pre-warmed at 50°C, containing 7.5 μL DEPC-water, 5 μL of 10x PCR buffer, 5 μL of 25 mM $MgCl_2$, 2.5 μL 10 mM dNTP, and 5 μL 0.1 M DTT were added to the cocktail and incubated at 50°C for 50 min. A 1 μL of a 200U/μL SuperScript II was added to the cocktail after the 50 min incubation and then incubated at 70°C for 15 min. It was then chilled on ice for 5 min upon which, 1 μL of RNAse was added and then incubated at 37°C for 30 min. The cocktail was then stored at -20°C for later use as PCR template.

A touchdown PCR procedure was then performed consisting of an initial denaturation at 95°C for 5 min proceeded by 5 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2min. This was followed by another 5 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min. The last set of cycles consists of 25 cycles 94°C for

1 min, 50°C for 1 min and 72°C for 2 min. A final 10 min extension at 72°C was performed before proceeding to cloning. Gel electrophoresis was used to determine the presence of coconut enolase among the varying cDNA template used. The detected PCR bands were immediately eluted and used in the TOPO TA Cloning® Kit (Invitrogen) following manufacturer's instructions. At least five randomly selected clones from each eluted product were purified for sequencing.

Sequence analyses

Purified plasmid DNA was commercially sequenced by the Division of the Biomolecular Research Facility, University of New Castle, Callaghen, Australia. *In silico* analysis of the sequences produced were analyzed using the Vector NTI Suite software and the web-based software mfold (www.bioinfo.rpi.edu/applications/mfold). Molecular phylogeny of the ENO gene was established through a partial molecular characterization of the ENO gene which includes sequence comparison of ENO 3' CDS with other known 3' CDS plant enolases. Comparison of the ENO 3' UTR to both corn and rice 3' UTRs was performed through the use of the Vector nti Suite software. The sequence for palm kernel 3' UTR was not available for comparison.

RESULTS AND DISCUSSION

To clone the 3' end of the coconut enolase gene, 3' RACE was performed. A PCR band (~400bp) was detected in all ages of the coconut endosperm used and the RACE product produced from the touchdown PCR performed (data not shown) was immediately used for cloning. This indicates that enolase (ENO) was actively being transcribed throughout the varying ages of coconut endosperm used. It has been previously reported that as the coconut drupe matures, the coconut endosperm also develops to readily store oil (Villalobos et al. 2001). This would mean that *de novo* fatty acid synthesis is active throughout endosperm development and would require a substantial amount of pyruvate which in turn requires an abundant amount of enolase enzyme (Miernyk and Dennis 1987) consistent with our results.

To verify that the cloned product is ENO, sequence comparison of the coconut, palm kernel, rice, and corn 3' CDS was performed. ENO 3'CDS was compared with: palm kernel (*Elaeis guineensis*) from the Palmae family, and corn (*Zea mays*) and rice (*Oryza sativa*) from the Triticeae family to establish the relatedness of the enolase cloned from coconut compared with palm kernel enolase (Palmae family), and also corn and rice enolases (Triticeae family).

Figure 1 shows the 3' CDS multiple sequence alignment from palm kernel, rice, corn, and coconut enolases. High sequence homology was observed confirming that the PCR product cloned was coconut enolase. Furthermore, a higher sequence homology was observed between that of coconut and palm kernel enolases compared with those from corn and rice, suggesting that enolases from Palmae and Triticeae are conserved within the family which is in agreement with the data presented by Piast et al. (2005). It has previously been shown that both rice and corn enolase genes have a higher homology between themselves compared with other known plant enolases, showing that the molecular evolution of these two enolases are not that far apart (Piast et al. 2005) consistent with the generated enolase phylogenetic tree wherein both rice and corn enolases are found on the same branch (Piast et al. 2005). It is also worth mentioning that the stop codon found among the coconut, palm kernel, rice, and corn enolases vary. Interestingly, both coconut and palm kernel enolase have a similar TAG stop codon, whereas both rice and corn have a similar TAA stop codon (Figure 1).

To establish any sequence similarities or differences among the enolase 3' UTRs, sequence comparison of the coconut, rice, and corn 3' UTRs were made. Alignment

Figure 1. Coconut enolase 3' CDS has higher homology with palm kernel (Palmae) than to rice (Triticeae) and corn (Triticeae) enolases. The PCR product produced through 3'RACE was immediately used for cloning and eventually sequenced. Identity of the cloned product was confirmed through multiple sequence alignment with other known plant enolases, particularly from the Palmae and Triticeae families.

of the 3' UTR sequences of coconut, rice, and corn was performed to identify any conserved regions. The 3' UTR sequence for palm kernel was not available. Sequence alignment between corn and coconut 3' UTRs revealed no conserved regions (data not shown). In contrast, sequence alignment between coconut and rice 3' UTRs revealed a common CACTG region (Figure 2) which we suspect to be part of the CACTG motif. We compared our proposed CACTG motif with previously published works (Table 1). We found a 75% homology between the rice CACTG motif found in the 3'UTR while 50% homology was detected between sorghum and maize CACTG motifs found in the CDS. The relatively high sequence homology of our suspected coconut CACTG motif with our known plant CACTG motifs further prove that the CACTG region identified (Figure 2) is indeed part of the CACTG motif. In effect, this establishes the presence of coconut CACTG motifs, in particular, in the 3' UTR. It is worth mentioning that the sequence from both the coconut and rice CACTG motifs were from the 3' UTR whereas both the sorghum and maize CACTG motifs were obtained from the CDS. Furthermore, there was a higher homology between the coconut and rice CACTG motifs (at 75%) compared with both sorghum and maize CACTG motifs (at 50%) suggesting that CACTG motifs found in the 3' UTRs are somewhat conserved among themselves compared with those found in the CDS.

The CACTG (or commonly known as CACTA) motif has been found to be conserved in rice (Panaud et al. 2002; Wang et al. 2003) and is a sequence element conserved within the CACTA transposon family which is regarded as a class II transposon (Wicker et al. 2003). Unlike the class I transposon (also called retrotransposon or the copy and paste transposon) where the elements integrate into the host genome via RNA intermediates using element-encoded reverse transcriptase leading to abundance of copies in the host genome (Kumar and Bennetzen 1999; Wicker et al. 2007), the class II transposons (or the cut and paste transposon) integrate into the host genome via DNA intermediates generally resulting in relatively low copy numbers in the host genome (Kunze et al. 1997; Wicker et al. 2007).

The function and possible benefit of repetitive elements for the host plant is a hotly debated question (Wicker et al. 2003). Repetitive elements are believed to contribute regulatory sequences that may alter gene expression (Zhang et al. 2000) and it was suggested that CACTA elements follow a similar role (Wicker et al. 2003). Considering the works of Zhang et al. (2000) and having located the CACTG motif in the ENO 3' UTR, regulatory regions were somehow inserted. In organisms ranging from viruses to humans, protein- or RNA-mediated interactions between transcript termini, either the 5'- or 3' UTR, result in the formation of folding (or more specifically an RNA loop) which may provide a significant potential for regulation originating at the 3' UTR (Mazumder et al. 2003).

To confirm the occurrence of an RNA loop or folding, mfold was used. The mfold software is useful in predicting all possible folding patterns that may occur in a given sequence. As seen in Figure 3, immediately downstream of the CACTG region, a predicted folding pattern is shown wherein the remaining 15-bps of the proposed coconut

Figure 2. Sequence alignment of coconut and rice enolase 3' UTRs reveal a common CACTG-motif usually associated with the CACTA transposon family. The 3' UTR of the cloned PCR product was aligned with corn and rice enolase 3'UTRs. Pairwise alignment of coconut and rice enolase 3' UTRs show a common CACTG region found to be part of the 20-bp CACTG motif. No similar motif was identified when coconut and corn enolase 3' UTRs were aligned.

Table 1. Sequence homology of known plant CACTG motifs

Plant origin	Location	Sequence	Homology relative to coconut (%)	Reference
Coconut	3' UTR	CACTGTTTTCTGCACGTGTA	-	-
Rice	3' UTR	CACTGTTGTGTGCATGGGTT	75	Greco et al. 2005
Sorghum	CDS	CACTATGTGAAAAAAAGCTTA	50	Chopra et al. 1999
Corn	CDS	CACTATGTGAAAAAAAGCTTA	50	Lee et al. 2005

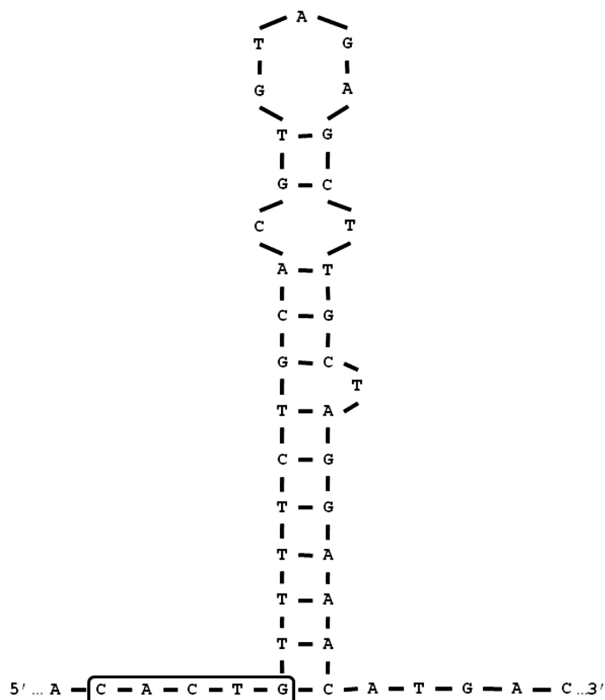

Figure 3. Predicted RNA loop formation found downstream of the coconut enolase CACTG region of the CACTG motif suggestive of regulatory element sequence insertion. Prediction of coconut enolase 3' UTR folding patterns was performed through mfold.

CACTG motif makes up the RNA loop, further adding to the notion proposed by Wicker et al. (2003) where regulatory sequences may have been inserted in the ENO 3' UTR through the CACTG motif. Furthermore, as seen in Figure 3, the CACTG motif may actually contain the regulatory sequences considering that the CACTG region serves as the base and the remaining 15-bps composes the loop. We speculate that the folding pattern observed is the binding region for regulatory elements, such as miRNAs, though substantial experimentation is recommended to further prove this point.

SUMMARY AND CONCLUSION

We have partially cloned, sequenced, and indentified the coconut enolase 3' end. The sequence obtained from the ENO 3' CDS showed high homology between coconut and palm kernel compared with corn and rice, suggesting that enolase is conserved within the respective phylogenetic family. The sequence obtained from ENO 3' UTR identified a CACTG motif commonly found in rice and the Triticeae family, and is associated with class II transposable elements, implying the existence of CACTG/CACTA transposons in coconut. Furthermore, the observed folding pattern found immediately downstream of the CACTG region concurs with the notion that regulatory sequences were inserted, though additional data is required to prove this point.

ACKNOWLEDGEMENTS

This study was funded by the UPLB-PCARRD-DOST project entitled: Cloning of Important Genes from Coconut. We also thank the Biochemistry Laboratory of the Institute of Plant Breeding, UPLB for the staff's invaluable help and permission to use most of the laboratory equipment.

REFERENCES

BLAKELEY SD, DEKROON C, COLE KP, KRAML M, DENNIS DT. 1994. Isolation of a full-length cDNA encoding cytosolic enolase from *Ricinus communis*. Plant Physiol 105: 455-456.

BOSER H. 1959. Zur heterogenitat von enzymen. I. uber enolase aus kartoffelknollen. Z Physiol Chem 315: 163-170.

DENNIS ES, WALKER JC, LLEWELLYN DJ, ELLIS JG, SINGE K, TOKUHISA JG, WOLSTENHOLME DR, PEACOCK WJ. 1987. The response to anaerobic stress: transcriptional regulation of genes for anaerobically induced proteins. In: D von Wettstein D, Chua NH, eds, Plant Molecular Biology. New York: Plenum. p. 407-417.

FORSTHOEFEL NR, CUSHMAN MAF, CUSHMAN JC. 1995. Posttranscriptional and posttranslational control of enolase expression in the facultative crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. Plant Physiol 108: 1185-1195.

FOX TC, MUJER CV, ANDREWS DL, WILLIAMS AS, COBB BG, KENNEDY RA, RUMPHO ME. 1995. Identification and gene expression of anaerobically induced enolase in *Echinochloa phyllopogon* and *Echinochloa crus-pavonis*. Plant Physiol 109: 433-443.

GOTTLIEB LD. 1982. Conservation and duplication of isozymes in plants. Science 216: 373-380.

GRECO R, OUWERKERK PBF, PEREIRA A. 2005. Suppression of an atypically spliced rice CACTA transposon transcript in transgenic plants. Genetics 169: 2383-2387.

HARRIS HC. 1994. The coconut palm and its importance. Coconuts 11:5-11

JONES A, DAVIES HM, VOELKER TA.1995. Palmitoyl-ACP thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. The Plant Cell 7: 359-371

KNUTZON DS, LARDIZABAL KD, NELSON JS, BLEIBAUM JL, DAVIES HM, METZ JG. 1995. Cloning of a coconut endosperm cDNA encoding a 1-acyl-sn-glycerol-3-phosphate acyltransferae that accepts medium chain length substrates. Plant Physiol 109: 999-1006.

KOMINE Y, KIKIS E, SCHUSTER G, STERN D. 2002. Evidence for *in vivo* modulation of chloroplast RNA stability by 3'-UTR homopolymeric tails in *Chlamydomonas reinhardtii* . Proc Natl Acad Soc 99: 4085-4090.

KUMAR R, BENNETZEN JL. 1999. Plant retrotransposons. Ann Rev Genet 33: 479-532.

KUNZE R, SAEDLER H, LONNIG WE. 1997. Plant transposable elements. Adv Bot Res 27: 331-370.

LAL SK, LEE C, SACHS MM. 1998. Differential regulation of enolase during anaerobiosis in maize. Plant Physiol 118: 1285-1293.

MAZUMDER B, SESHADRI V, FOX PL. 2003. Translational control by the 3'-UTR: the ends specify the means. Trends in Biochemical Sciences 28(2): 91-98.

MIERNYK JA, DENNIS DT. 1982. Isozymes of the glycolytic enzymes in endosperm from developing castor oil seeds. Plant Physiol 69: 825-828.

MIERNYK JA, DENNIS DT. 1984. Enolase isozymes from *Ricinus communis*. Partial purification and characterization of the isozymes. Arch Biochem Biophys 233: 643-651.

MIERNYK JA, DENNIS DT. 1987. Enolase isozymes from developing oilseeds: a survey. J Plant Physiol 128: 351-359.

MUJER CV, FOX TC, WILLIAMS AS, ANDREWS DL, KENNEDY RA. 1995. Purification, properties and phosphorylation of anaerobically induced enolase in *Echinochloa phyllopogon* and *E. crus-pavonis*. Plant Cell Physiol 36:1459-1470.

PANAUD O, VITTE C, HIVERT J, MUZLAK S, TALAG J. et al. 2002. Characterization of transposable elements in the genome of rice (*Oryza sativa* L.) using Representational Difference Analysis (RDA). Mol Genet Genomics 268: 113-121.

PIAST M, KUSTRZEBA-WOJCICKA I, MATUSIEWICZ M, BANAS T. 2005. Molecular evolution of enolase. Acta Biochemica Polonica 52(2): 507-513

SINHA S, BREWER JM. 1984. Purification and comparative characterization of an enolase from spinach. Plant Physiol 74: 834-840.

VAN DER STRAETEN D, RODRIGUES-POUSEDA RA, GOODMAN HM, VAN MONTAGU M. 1991. Plant enolase: gene structure, expression and evolution. Plant Cell 3: 719-735.

VILLALOBOS AL, DODDS PF, HORNUNG R. 2001. Changes in fatty acid composition during development of tissues of coconut (*Cocos nufera* L.) embryos in the intact and *in vitro*. J Exp Bot 52: 933-942.

WANG G, TIAN P, CHENG Z, WU G, JIANG J, et al. 2003. Genomic characterization of *Rim2/ Hipa* elements reveals a CACTA-like transposon superfamily with unique features in the rice genome. Mol Genet Genomics 270: 234-242.

WICKER T, GUYOT R, YAHIAOUI N, KELLER B. 2003. CACTA transposon in Triticeae. A diverse family of high-copy repetitive elements. Plant Physiol 132: 52-63.

WICKER T, SABOT F, HUA-VAN A, BENNETZEN JL, CAPY P, CHALHOUB B, et al. 2007. A unified system for eukaryotic transposable elements. Nat Rev Genet 8: 973-982.

ZHANG Q, ARBUCJLE J, WESSLER SR. 2000. Recent, extensive and preferential insertion of members of the miniature iverted-repeat transposable element family *Heratbreaker* into genic regions of maize. Proc Natl Acad Sci 97:1160-1165.

ZHAO J-Y, ZUO K-J, QIN J, TANG K-X. 2004. Molecular cloning and characterization of enolase from oilseed rape (*Brassica napus*). Acta Bot Sin 46(10): 1226-1233.