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CLONING AND CHARACTERISATION OF ACC OXIDASE GENE FROM MAS (AA) BANANA

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Abstract Considerable experimental attention has been given to amplify Mas (AA) ACC oxidase gene from Mas (AA) banana (Genbank accession number: EU106081). ACO gene from Mas (AA) has been compared with other homologous gene by using bioinformatics tools to study the characteristics of the gene such as evolutionary history, binding sites, predicted 3D structure and consensus region of the homologous proteins. The finding shows that Mas ACO is member of iron/ascorbate-dependent oxidase superfamily which requires ascorbate and iron for full activity so that it will meet the all the criteria requires as it is expressed *in vivo* to produce ethylene.

Abstrak Kaedah tertentu telah digunakan untuk mengamplifikasikan gen Mas (AA) ACC oksidase dari pisang Mas (AA) (Nombor Akses Genbank: EU106081). Gen ACC oksidase adalah dari Mas (AA) telah dibandingkan dengan sepuluh gen homologus yang lain dengan menggunakan peralatan bioinformatik untuk mengkaji ciri-ciri, sejarah evolusi, tapak pengikatan, ramalan struktur 3D dan konsensus protein homologi. Berdasarkan dari penemuan ini, didapati bahawa Mas ACO merupakan ahli keluarga oksidase yang bergantung kepada besi/askorbat untuk menjalankan akitiviti sepenuhnnya yang lengkap sepertimana ianya dihasilkan secara '*in vivo'* untuk menghasilkan etilena.

(**Keywords**: ACC Oxidase (ACO), Ethylene, Mas (AA) banana, *Musa acuminata*, bioinformatics)

INTRODUCTION

Banana is a member of the genus *Musa* of the family *Musaceae* where there are two main wild species- *Musa acuminata* and *Musa balbisiana*. Ethylene produced endogenously exerts a major role in plant growth, development and senescence apparently at regulatory levels of metabolism and can also be described as plants simplest hormone (Lieberman, 1979). The pathway of ethylene biosynthesis proceeds from methionine, through S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene and that the two main enzymes are ACC synthase (ACS) and ACC oxidase (ACO) (Yang and Hoffman, 1984) and (Abeles *et al*., 1992). ACO is a type of an oxidative enzyme which carries out the conversion of ACC to ethylene. The first authentic ACO activity *in vitro* was demonstrated by extracting melon fruit and assaying the enzyme under the conditions for flavanone-3-hydroxylase and this preparation needs Ferum (II) ion and ascorbate for full activity (Ververidis and John, 1991) and the failure of previous attempts of performing *in vitro* ACO activity was due to the loss of these factors during the enzyme extraction (Fernandez-Maculet and Yang, 1992). CO_2 has been identified as the species responsible for the activation of ACO and proved that CO_2 forms a carbamate with an amino group of the enzyme (Fernandez-Maculet *et al*., 1993). The conservation of histidine residues among the ACO contains critical histidine residues at the active site (Christoffersen *et al*., 1993). This work focuses on cloning and characterising ACO gene from Mas (AA) banana.

MATERIALS AND METHODS

Banana material and RNA extraction

Pulp tissues from dessert variety Mas (AA) banana was used for total RNA extraction (Liu *et al*., 1998). The RNA sample was dissolved in 100µl of SDW before being stored at -80°c.

Quantification and estimation of nucleic acid concentration

An aliquot (2-5µl) of RNA sample was taken made up to 1ml with SDW to a known dilution (200-500 folds) and placed in a quartz cuvette. Absorbance was measured at 200-320nm using a spectrophotometer (Pharmacia Ultraspec II) against SDW as blank with

maximum reading at 260nm (A_{260}). The concentration was calculated as follows:

RNA concentration (µg. μl^{-1}): 40 x dilution factor x A_{260}

Reverse Transcription Polymerase Chain Reaction (RT-PCR) of ACC Oxidase

The ACO primers (BACCO) were designed from the conserved region of banana (Pathak *et al*., 2003) and were commercially made. The sequences of the two primers are as follows:

BACCO Forward
5' TTTCCGGTTATCGACATGGAGAAGC 3'

BACCO Reverse
3'AATATTGTAGGTAATTACTATGGG 5'

Template preparation

2 µl of total RNA (1 µg μl^{-1}) were used for the amplification and the reverse transcriptase polymerase chain reaction (RT-PCR) was carried out in a volume of 50 µl in a 0.5 ml Eppendorf tube using the Promega AccessQuick™ RT-PCR system. The reaction mixture was thoroughly mixed and 1 µl of AMV Reverse Transcriptase was added by gentle vortexing. RT-PCR was started on a GeneAmp 9600 Thermcycler (Perkin Elmer) with amplification cycles as described: reverse transcription (1cycle) 45°c for 45 min, denaturation (1cycle) 95°c for 2 min, annealing (40 cycles) 95°c, 30 sec, 60°c, 1 min, 72°c, 2 min, extension (1cycle) 72°c, 7min, soak cycle (hold) 4°c, overnight.

Purification of RT-PCR product

Obtanied cDNA was purified using Perfectprep Gel cleanup system. Gel slice of PCR product was taken and weighed in a micro centrifuge tube. Equal volume of Binding buffer was added and incubated at 50ºc for 5 to 10 minutes in an Eppendorf Thermomixer at 1000rpm. Equal volume of isopropanol to the original gel slice was mixed by inversion to the dissolved gel slice. 800µl of sample was added to a spin column and centrifuged at 6000rpm for 1 minute filtrate was discarded and 750µl of diluted wash buffer was added to the spin column and centrifuged at 6000rpm for 1 minute. Filtrate was discarded and the spin column was centrifuged at 6000rpm for an additional minute to remove any residual diluted wash buffer. 30µl of elution buffer was added to the centre of the spin column and centrifuged at 6000rpm for 1 minute. Spin column was discarded and purified cDNA was stored at -80ºc.

Sequencing

cDNA sequencing of PCR was carried out commercially through BioSynTech Sdn Bhd.

Bioinformatics analysis

Most of the bioinformatics tools and databases were obtained and accessed through NCBI homepage. Sequence analysis and proteomics tools were accessed on ExPASy (Gasteiger *et al*., 2003) where conversion of nucleotide sequence to protein sequence and characterization of the sequence was done here. RasMol (Sayle and Milner-White, 1995) protein structure viewing software was used to view the protein's 3D structure generated by Quick Phyre web server (Kelley and Sternberg, 2009).

RESULTS

RT-PCR with the BACCO primers yielded a single product with a size of 1040 bp. Clear products were visible in 1.2% agarose gel for Mas (AA) RNA. The sequence analysis was done from the complete nucleotide sequence of ACC oxidase from Mas banana. The nucleotide is 100 bp long and includes the entire ACC oxidase coding region consensus pattern (P-V-I-[DN]-M-E-K-L-x-G-x-E-R-[AEGS]-x-[AT]-M-E-x-[IL]-x-D-A-C-E-x-W-G-F-F-E-x(0,1)-L-x(0,1)-N-H-G-I-[PS]-x(2)-L-[LM]-D-[ET]-V-E-[KR]-[LMV]-[NT]-K). The ATG beginning at position 1 is most likely to be the translation initiation codon and the TGA beginning at position 967 is the in-frame termination codon. Prosite scan was done to the protein sequence against PROSITE database to search for biologically relevant sites and signatures.

The scanning process revealed that there are 337 amino acids and further analysis on the sequence revealed that it comprises about 40 negatively charged residues (Asp+Glu) and 28 positively charged residues (Arg+Lys). The Mas ACC oxidase gene sequence has four N-glycosylation site (at residues N111-E114, N165-I168, N199-F202, N308-C311), three protein kinase C phosphorylation site (T7-K9, T132-R134, T167-R169), four Casein kinase II phophorylation site (S91-D94, T132-E135, S240-D243, S252-D255), one amidation site (T7-K10) and one N-myristoylation site (G137-S142).

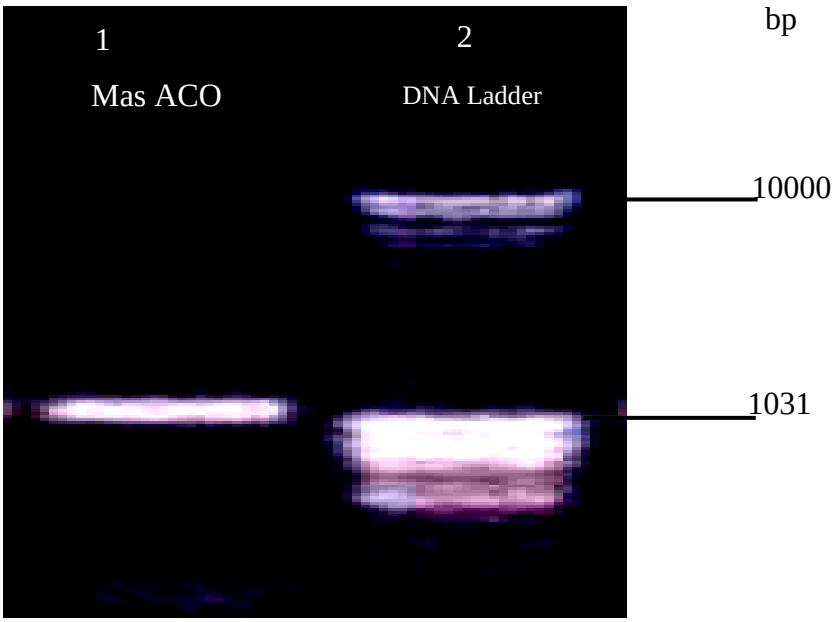

Figure 1: RT-PCR results with BACCO primer for Mas. In Lane 2 is GeneRuler DNA Ladder Mix (Fermentas). Lane 1 is PCR product of 1009 bp generated using primers BACCO for total RNA from Mas (AA).

BLAST (Altschul *et al*., 1990) homology search revealed the total of 109 blast hits homology. Ten most significant sequences were displayed to show significant to the query sequence Mas ACO gene:

1. 414/427 positive identities (96%) with *M. acuminata* ACO
2. 414/427 positive identities (96%) with *M. acuminata* ACO
3. 414/427 positive identities (96%) with *M. acuminata* ACO
4. 409/421 positive identities (97%) with Robusta cultivar ACO
5. 408/422 positive identities (96%) with *M. acuminata* ACO
6. 410/423 positive identities (96%) with *M. acuminata* ACO
7. 339/344 positive identities (98%) with *M. acuminata* ACO
8. 280/296 positive identities (94%) with *Mangifera indica* ACO
9. 125/125 positive identities (100%) with *M. acuminata* ACO
10. 125/125 positive identities (100%) with *M. acuminata* ACO

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Banana 1    ----------MDSFPVIDMEKLLGRERGAAMEILRDACEKWGFFEIL 37
Banana 2    ----------MDSFPVIDMEKLLGRERGAAMEILRDACEKWGFFEIL 37
Banana 3    ----------MDSFPVIDMEKLLGRERGAAMEILRDACEKWGFFEIL 37
Banana 4    ----------MDSFPVIDMEKLLGRERGAAMEILRDACEKWGFFEIL 37
Banana 5    ----------MDSFPVIDMEKLSGGERGAAMEILRDACEKWGFFEIL 37
Kiwi        ----------MEAFPVIDMEKLNGEERAPTMEKIKDACENWGFFELV 37
Mas-ACO     PRKYFATGKKERVMDSFPVIDMEKLLGRERGAAMEILRDACEKWGFFEIL 50
              :****:**** * **  :** :.****:*****::

Banana 1    NHGISHDLMDEVEKVNKEQYNKCREQKFNEFAN-KALENADSEIDHLDWE 86
Banana 2    NHGISHYLMDEVEKVNKEQYNKCREQKFNEFAN-KALENADSEIDHLDWE 86
Banana 3    NHGISHDLMDEVEKVNKEQYNKCREQKFNEFAN-KALENADSEIDHLDWE 86
Banana 4    NHGISHDLMDEVEKVNKEQYNKCREQKFNEFAN-KALENADSEIDHLDWE 86
Banana 5    NHGISHDLMDEVEKVNKEQYNKCREQKFNEFAN-KALENADSEIDHLDWE 86
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Kiwi        NHGISHELMDTVERLTKEHYNKCMEQRFKEMVATKGLEAVQSEINDLDWE 87
Mas-ACO     NHGISHDLMDEVEKVNKEQYNKCREQKFNEFAN-KALENADSEIDHLDWE 99
            ****.  *:* **::.**:*.** *::*:::.  *.**   *: .:***

Banana 1    STFFLRHLPVSNISEIPDLDDQYRKAMKEFAAAIEKLAERLLDLLGENLE 136
Banana 2    STFFLRHLPVSNISEIPDLDDQYRKAMKEFAAAIEKLAERLLDLLGENLE 136
Banana 3    STFFLRHLPVSNISEIPDLDDQYRKAMKEFAAAIEKLAERLLDLLGENLE 136
Banana 4    STFFLRHLPVSNISEIPDLDDQYRKAMKEFAAAIEKLAERLLDLLGENWK 136
Banana 5    STFFLRHLPVSNISEIPDLDDQN--AMKEFAAEIEKVAERLLDLLGENLG 134
Kiwi        STFFLRHLPVSNISEIPDLEQDHRKAMKEFAEKLEKLAEQLLDLLCENVG 137
Mas-ACO     SSFFLRHLPVSNISEIPILMTSIGKALKVFAATIREAGTSVCSTCWVITV 149
            *:*** .** **:*:: : .  .:* **  :. . : .


Banana 1    LEKGYLKK-AFSNGSKGPTFGTKVSSYPPCPRP-DLVKGLR--AHTDAGG 182
Banana 2    LEKGYLKK-AFSNGSKGPTFGTKVSSYPPCPRP-DLVKGLR--AHTDAGG 182
Banana 3    LEKGYLKK-AFSNGSKGPTFGTKVSSYPPCPRP-DLVKGLR--AHADAGG 182
Banana 4    LEKGYLKK-AFSNGSKGPTFGTKVSSYPPCPRP-DLVKGLR--AHTDAGG 182
Banana 5    LEKGYLKK-AFSNECKGPTFGTKVSSYPPCPRP-DLVKGLR--AHTDAGG 180
Kiwi        LEKGYLKK-AFY-GSKGPTFGTKVSNYPPCPRP-ELIKGLR--AHTDAGG 182
Mas-ACO     MVEVFPDEYPPLMDSNGTIRLGNCLQFPPHVLPRTLYRACRSSTYSSNGN 199
            : : : .: .  .:*.  :  :**  * * :. *  :::. *.

Banana 1    IILLFQDDQVSGLQFLKDGEWLDVPPMRHAIVVNLGDQLEVITNGKYKSV 232
Banana 2    IILLFQDDQVSGLQFLKDGEWLDVPPMRHAIVVNLGDQLEVITNGKYKSV 232
Banana 3    IILLFQDDQVSGLQFLKDGEWLDVPPMRHAIVVNLGDQLEVITNGKYKSV 232
Banana 4    IILLFQDDQVSGLQFLKDGEWLDVPPMRHAIVVNLGDQLEVITNGKYKSV 232
Banana 5    IILLFQDDQVSGLQFLKDGEWLDVPPIRHAIVVNLGDQLEVITNGKYKSV 230
Kiwi        IILLFQDNKVSGLQLLKDGEWIDVPPMKHSIVINIGDQLEVITNGKYKSV 232
Mas-ACO     FSFFFQDN---PLLVILAFPALTSPVIFRTSISLPTFFVDRTISPQDLDV 246
            : ::***:   * .:   : * : :: :    ::  . : .:

Banana 1    VHRVVAQTDGNRMSIASFYNPGSDAVIFPAPALVEKEA---EEKKEVYPR 279
Banana 2    VHRVVAQTDGNRMSIASFYNPGSDAVIFPAPALVEKEA---EEKKEVYPR 279
Banana 3    VHRVVAQTDGNRMSIASFYNPGSDAVIFPAPALVEKEA---EEKKEVYPR 279
Banana 4    VHRVVAQTDGNRMSIASFYNPGSDAVIFPAPALVEKEA---EEKKEVYPR 279
Banana 5    VHRVVAQTDGNRMSIASFYNPGSDAVIFPAPALVEKEA---EEKKEVYPR 277
Kiwi        MHRVIAQPDGNRMSIASFYNPGSDAVMYPAPALVDKEE---DQQKQVYPK 279
Mas-ACO     NDIAHSDPDSDVLYLLCLFSPMILSLHISSSCFLLISLYAPLCDLYPFSG 296
            . . : .*. : : .::.*  :: .:. :: .      :.

Banana 1    FVFEDYMKLYVGHKFQAKEPRFEAMKAMEAVAT-HPIATS- 318
Banana 2    FVFEDYMKLYVGHKFQAKEPRFEAMKAMEAVAT-HPIATS- 318
Banana 3    FVFEDYMKLYVGHKFQAKEPRFEAMKAMEAVAT-HPIATS- 318
Banana 4    FVFEDYMKLYVGHKFQAKEPRFEAMKAMEAVAT-HPIATS- 318
Banana 5    FVFEDYMKLYVGHKFQAKEPRFEAMKAMEAVAT-HPIATS- 316
Kiwi        FVFEDYMKLYAGLKFQAKEPRFEAMKAMENAVNLGPIATI- 319
Mas-ACO     TLSRVHMLECCNSTCLQNAFRVESSDFSSHSNYLQYYFTSS 337
            : . :*    .  : *.*: .        *
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Figure 2: Multiple sequence alignment was done for the deduced amino acid sequence of Mas ACC oxidase protein and sequences encoding ACC oxidase from banana 1 (Hsien Jin Chiao TrEMBL code Q43547), banana 2 (Hsien Jin Chiao, Q6LC46), banana 3 (Grand nain, O49892), banana 4 (Q70BE4), banana 5 (Q70Y37) and kiwi (*Actinidia chinensis*, P31237) from CLUSTALW from www.ebi.ac.uk/clustalw.

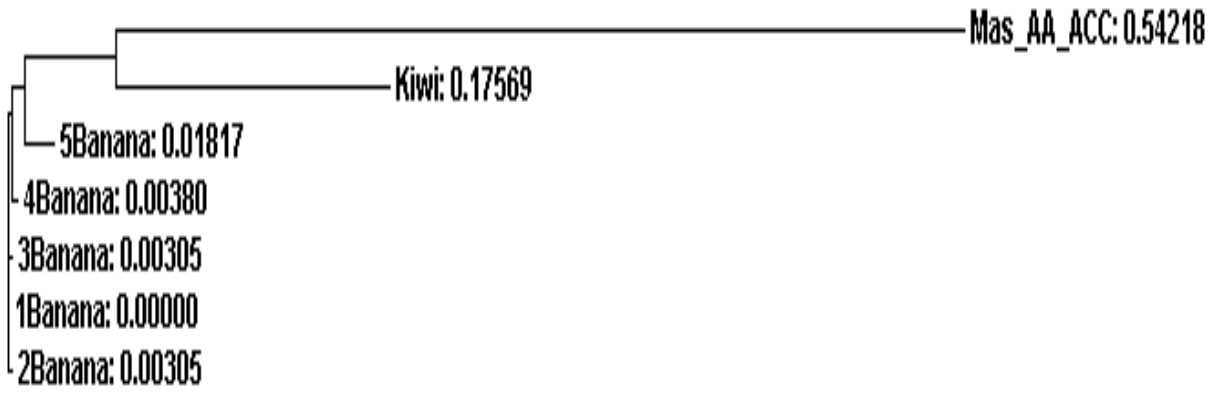

Figure 3: Phylogenetic tree shows the relationship between Mas ACC oxidase and ACC oxidase from significant homologies.

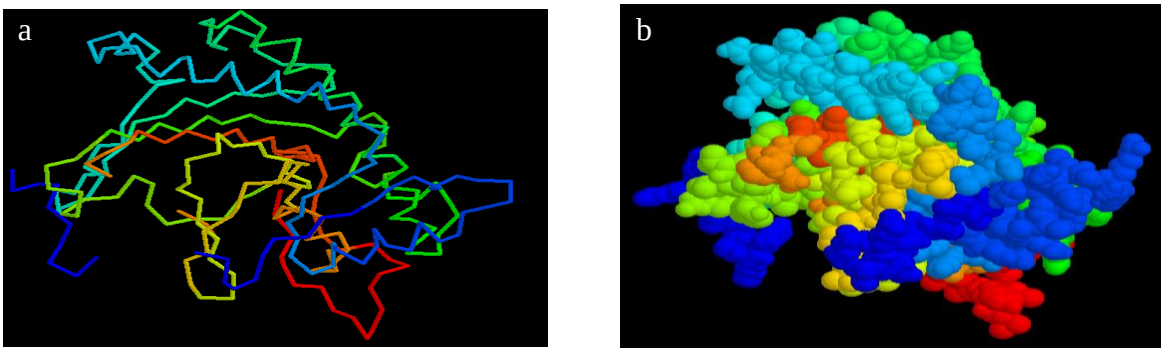

Figure 4: 3-D backbone (a) and spacefill model (b) structure of Mas ACO protein generated by a 3- state prediction (Helix/Strand/Coil).

DISCUSSION

A genomic sequence encodes ACO was isolated by RT-PCR approach from the Mas (AA) diploid. The ACO has been shown to require ascorbate and iron as cofactors. As *in vivo*, the purified enzyme requires CO_2 for activity and the enzyme displayed an absolute requirement for Ferum (II) ion and ascorbate. The stoichiometry of the enzymatic reaction was determined as ACC + ascorbate + $O_2 \rightarrow$ C_2H_4 + HCN + CO_2 + dehydroascorbate + $2H_2O$. ACO also can be isolated in soluble form and these discrepancies can be reconciled by the interpretation that native ACO is located in both the cytosol (major compartment) and membranous fractions (minor compartment). Further analysis on Mas (AA) ACO protein sequence through InterProScan showed that the sequence contains a region which encodes a protein from iron/ascorbate-dependent oxidase superfamily. This finding supports that Mas (AA) ACO needs Ferum (II) ions and ascorbate as co-factor and concurrently oxidized to dehydroascorbate in an amount equivalent to that of ethylene produced. Homology comparisons for Mas (AA) ACO protein sequence using BLASTp shows that it has more than 90% significant homologous gene which is from Banana 1 (Hsien Jin Chiao TrEMBL code Q43547), Banana 2 (Hsien Jin Chiao, Q6LC46), Banana 3 (Grand nain, O49892), Banana 4 (Q70BE4), Banana 5 (Q70Y37) and Kiwi (*Actinidia chinensis*, P31237). Multiple gene alignment was done using ClustalW to obtain consensus for the sequence homologous gene which is (P-V-I-[DN]-M-E-K-L-x-G-x-E-R-[AEGS]-x-[AT]-M-E-x-[IL]-x-D-A-C-E-x-W-G-F-F-E-x(0,1)-L-x(0,1)-N-H-G-I-[PS]-x(2)-L-[LM]-D-[ET]-V-E-[KR]-[LMV]-[NT]-K) where this consensus gives out two transmembrane helices. This finding was strengthened where ACO on *Arabidopsis* binds ethylene with the help of a copper cofactor that is bind in the transmembrane domain (Schaller, 2003).

CONCLUSION

Based on this finding we can deduce that most of the ACO in climacteric fruit is genetically conserved which requires Ferum (II) ions and ascorbate as co-factor to be part of the ethylene biosynthesis.

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