CHARACTERIZATION OF 0.58 kb DNA STILBENE SYNTHASE ENCODING GENE FRAGMENT FROM MELINJO PLANT (*Gnetum gnemon*)

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ABSTRACT

Resveratrol is a potent anticancer agent resulted as the main product of enzymatic reaction between common precursor in plants and Stilbene Synthase enzyme, which is expressed by sts gene. Characterization of internal fragment of Stilbene Synthase (STS) encoding gene from melinjo plant (Gnetum gnemon L.) has been carried out as part of a larger work to obtain a full length of Stilbene Synthase encoding gene of the plant. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) was performed using two degenerated primers to amplify the gene fragment. Ten published STS conserved amino acid sequences from various plant species from genebank were utilized to construct a pair of GGF2 (5' GTTCCACCTGCGAAGCAGCC 3') and GGR2 (5' CTGGATCGCACATCC TGGTG 3') primers. Both designed primers were predicted to be in the position of 334-354 and 897-916 kb of the gene respectively. Total RNA isolated from melinjo leaves was used as template for the RT-PCR amplification process using two-step technique. A collection of 0.58 DNA fragments was generated from RT-PCR amplification and met the expected results. The obtained DNA fragments were subsequently isolated, refined and sequenced. A nucleotide sequence analysis was accomplished by comparing it to the existed sts genes available in genebank. Homology analysis of the DNA fragments with Arachis hypogaea L00952 sts gene showed high similarity level. Taken together, the results are evidence that the amplified fragment obtained in this study is part of melinjo sts gene

Keywords: *Resveratrol, stilbene synthase gene (sts), RT-PCR, Gnetum gnemon L.*

INTRODUCTION

Resveratrol is a class of stilbenoid that shows great effectiveness as anticancer agents in several most prevalent cancers such as servix cancer [1], pancreatic cancer [2], gastrous cancer [3], and liver cancer [4]. It is a natural polyphenol and an antioxidant, which is part of plant's secondary metabolites. The compound is synthesized by a specific group of plants including *Vitis vinifera* [5], *Arachis hypogaea* [6-7], *Gnetum gnemon* and *Pinus strobes* due to several factors including stress, pathogen attack, UV radiation or wound.

Resveratrol with an IUPAC name of (E)-5-(4-hydroxystyryl)benzene-1,3-diol [8] has a relatively low molecular weight [9]. In room temperature, the physical appearance of the compound is white powder. The Isolation process of resveratrol compound usually is carried out from tissues of resveratrol producing plant. After a certain methanol treatment and undergoing chloroform extraction method, impure resveratrol can be obtained as yellowish powder from melinjo's bark. Once the impure mixture is separated using LVC (liquid vacuum chromatograph) technique, pure resveratrol can be resulted. UV spectroscopy UV-VIS, IR spectrophotometer and NMR spectrometer NMR are the common methods used to identify resveratrol. However, those direct isolation techniques normally result in only very low amount of resveratrol.

In plants, resveratrol biosynthesis is highly controlled by the action of STS enzyme [10]. STS enzyme is extremely responsible to the formation of resveratrol's backbond structure from common precursors. Therefore, STS encoding gene has been the target of investigation as high production of STS enzyme will likely increase the production of resveratrol. Genetic modification to accelerate the production of STS enzyme has been intensively investigated [11]. The initial part of such works is the characterization of STS encoding gene from specific resveratrol producing plants.

The STS cDNAs from several group of plants including *Vitis vinifera* and *Arachis hypogaea*, have been successfully characterized [12-13]. More advanced attempts to accelerate the production of STS enzyme in order to escalate the synthesis of resveratrol have also been reported [14]. Several genetically

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modified plants are able to produce STS enzyme that induce high production of resveratrol [15].

Among resveratrol producing plants, melinjo shows a convincing ability to produce resveratrol due to the presence of numerous derivatives of resveratrol found in the plant's tissues [16]. However, due to limited information about sts gene in melinjo, its potential for genetic modification has not been explored.

This study is aimed to obtain information about the STS cDNA of melinjo as the main ingredient in STS gene over expression works. The initial characterization of the full length of melinjo STS resulted only 0.40 kb fragment [17] of more than 1.00 kb of nucleotides expected for a full length of plants' sts genes in average. For that reason, further characterization of Stilbene Synthase encoding gene fragment from melinjo (*Gnetum gnemon*) needs to be done.

This study reports new 0.58 kb characterized sts gene fragment of melinjo plant. The fragment is proven to be part of the full length of melinjo STS gene aside of the 0.40 one from previous study. The new sts gene nucleotide sequence shares high similarity with *sts genes*.

EXPERIMENTAL SECTION

Materials

Materials used for this study were leaf tissues of melinjo plant, Genebank database of amino acid and nucleotide sts gene sequences of various plants (http://www.ncbi.nlm.nih.gov). Trizol reagent (Invitrogen), isoprophanol (Merck), chloroform (Merck), ethanol 75% (Merck), RNAse *free water* (Gibco TM), liquid nitrogen, *transcriptor first strand* cDNA synthesis kit (Roche) [oligo (dT) primer, random hexamer primer, transcriptor RT-reaction buffer 5X, protector RNAse inhibitor, deoxynucleotide mix (dNTP), transcriptor RT], illustraTM Ready To Go PCR bead (GE Healthcare), PureLinkTM Quick Gel Extraction Kit (Invitrogen) [gel solubilization buffer (L3), wash buffer (W1), elution buffer (E5), quick gel extraction column, wash tubes, and recovery tubes], agarose (Invitrogen), loading buffer (Invitrogen), TAE buffer, ethidium bromide (EB) (Invitrogen), DNA marker (Invitrogen), tris base (Merck), acetic acid glacial (Merck), EDTA (Merck) pH 8, dH2O (Merck), sterile aquadest (LPPT).

Instrumentation

The apparatus used in this work were Denver AA-250 digital balance, Hiramaya HL 36 AE autoclave, OSK Seiwa Reiko water bath incubator, Sorvall Biofuge centrifugator, Nuaire laminar flow, Barnstead vortex, Bio Rad Thermal cycler PCR, Gilson micro pipettes, Sanyo microwave, Bio Rad (Wide Minisub(R) cell GT) electrophoresis, Shimadzu Probe UV-Vis Spectrophotometer, Bio-Rad UV lamp, ABI PRISM 310DNA sequencer.

Procedure

A pair of primers used to amplify the sts gene fragment was designed based on conserved regions of sts genes from other plants that have already been published in the genebank site (http://www.ncbi.nlm.nih.gov). Ten amino acid sequences of sts genes used in primer designing stage are *Arachis hypogaea* (Access Number NCBI: L00952 and DQ124938) *Vitis vinifera* (DQ459351, AF274281, DQ366302 DQ366301) *Vitis riparia* (AF128861), *Polygonum cuspidatum* (DQ900615 and DQ459349) *Rheum tataricum* (AF508150). Clustalw software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used in the primer designing process to check the homology of amino acid sequences of Stilbene Synthase protein available in the genebank. Conserved amino acid regions were back translated to obtain conserved amino acid by considering melinjo preference codon. The designed primers were then chosen based on generally accepted primer parameters: GC content of 45-55%, primer length of 18-22 pb and with zero possibility to form hairpin structures. Fragment target length of 500-700 pb was set based on primers relative positions to sts gene of *Arachis hypogaea*. The defined primers were then synthesized at 1stBASE (Singapore) to become ready for use.

The initial work of melinjo sts gene fragment characterization was the isolation of total RNA from melinjo leaf tissues using Trizol reagent and liquid nitrogen according to reagent's protocol. The next part was running RT-PCR using mRNA molecules available in the obtained total RNA as template. Gel isolation was conducted to isolate single band that refers to 0.58 kb fragment. DNA fragment sequencing, analysis to the resulted DNA sequence and homology analysis were performed to check whether the obtained fragment was truly part of melinjo sts gene. If homology test results in not less than 60% similarity level to any sts gene sequence available in genebank, then the fragment is strongly believed to be part of sts gene.

RESULT AND DISCUSSION

A preliminary homology study of ten amino acid sequences of sts genes using Genefisher2 software was not able to produce degenerated primers as expected. Different approach using the homology of Amino acids sequences was then applied to design primers and it showed 65-70% similarity level. The

Table 1. A pair of designed primers and their targeted region in amplification process

No	Primer pair	Targeted region for amplification	Fragment size	Tm (°C)	% GC
1	GGF2: 5’ AAGGGCATCAAGGAGTGGGG 3’	334-914	580	64.5	60
2	GGR2: 5’ CACCAGGATGTGCGATCCAG 3’	334-914	580	64.5	60

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L00952Arachishypogaea          -MVSVSGIRKVQRAEGPATVLAIGTANPPNCIDQSTYADYYFRVTNSEHM 49
DQ124938Arachishypogaea        -MVSVSGIRKVQRAEGPATVLAIGTANPPNCIDQSTYADYYFRVTNSEHM 49
DQ366301Vitisvinifera          -MASVEEFRNAQRAKGPATILAIGTATPDHCVYQSDYADYYFKVTKSEHM 49
AF128861Vitisriparia           -MASVEEIRNAQRAKGPATILAIGTATPDHCVYQSDYADYYFKVTKSEHM 49
DQ459351Vitisvinifera          -MASVEEFRNAQRAKGPATILAIGTATPDHCVYQSDYADFYFRVTKSEHM 49
DQ366302Vitisvinifera          -MASVEEFRNAQRAKGPATILAIGTATPDHCVYQSDYADYYFRVTKSEHM 49
AF274281Vitisvinifera          -MASVEEFRNAQRAKGPATILAIGTATPDHCVYQSDYADYYFRVTKSEHM 49
DQ900615Polygonumcuspidatm     -MAA--STEEMTKALTAATVLAIGTANPPNCYYQADFPDFYFRATNSDHL 47
AF508150Rheumtataricum         -MAPEESRHAETAVNRAATVLAIGTANPPNCYYQADFPDFYFRATNSDHL 49
DQ459349Polygonumcuspidatum    MSKLLQDIRNSQKATGPATVLAIGTAVPPTCYPQSEYPDFYFRMCKSEHM 50
                                    .    .    .**:****** *   *  *: :.*:**:  :*:*:

L00952Arachishypogaea          TDLKKKFQRICERTQIKNRHMYLTEEILKENPNMCAYKAPSLDAREDMMI 99
DQ124938Arachishypogaea        TDLKKKFQRICERTQIKNRHMYLTEEILKENPNMCAYEAPSLDAREDMMI 99
DQ366301Vitisvinifera          TALKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPSLNIRQEIIT 99
AF128861Vitisriparia           TALKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPSLNIRQEIIT 99
DQ459351Vitisvinifera          TALKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPSLNIRQEIIT 99
DQ366302Vitisvinifera          TELKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPSLNIRQEIIT 99
AF274281Vitisvinifera          TELKKKFNRICDKSMIKKRYIHLTEEMLEEHPNIGAYMAPSLNIRQEIIT 99
DQ900615Polygonumcuspidatum    THLKHKFKRICEKSMIEKRYLQLTEDILKENPNIGAYEAPSLDVRHEIQV 97
AF508150Rheumtataricum         THLKQKFKRICEKSMIEKRYLHLTEEILKENPNIASFEAPSLDVRHNIQV 99
DQ459349Polygonumcuspidatum    AQLKSKMKRICDRSGIRQRFMFHTEENLSQNSNMCKFKAPSLDARQEMLI 100
                               : ** *::***::: *.:*.:  **: *.:.*:  :  ****: *.::

L00952Arachishypogaea          REVPRVGKEAATKAIKEWGQPMSKITHLIFCTTSGVALPGVDYELIVLLG 149
DQ124938Arachishypogaea        REVPRVGKEAATKAIKEWGQPMSKITHLIFCTASGVALPGVDYELIVLLG 149
DQ366301Vitisvinifera          AEVPKLGKEAALKALKEWGQPKSKITHLVFCTTSGVEMPGADYKLANLLG 149
AF128861Vitisriparia           AEVPKLGKEAALKALKEWGQPKSKITHLVFCTTSGVEMPGADYKLANLLG 149
DQ459351Vitisvinifera          AEVPKLGKEAALKALKEWGQPKSKITHLVFCTTSGVEMPGADYKLANLLG 149
DQ366302Vitisvinifera          AEVPRLGRDAALKALKEWGQPKSKITHLVFCTTSGVEMPGADYKLANLLG 149
AF274281Vitisvinifera          AEVPRLGRDAALKALKEWGQPKSKITHLAFCTTSGVEMPGADYKLANLLG 149
DQ900615Polygonumcuspidatum    KGVAQLGKEAALKAMQEWGQPKSKITHLIVCCIAGVDMPGADYQLTKLLD 147
AF508150Rheumtataricum         KEVVLLGKEAALKAINEWGQPKSKITRLIVCCIAGVDMPGADYQLTKLLG 149
DQ459349Polygonumcuspidatum    MEVPKLGVEAAGKAIQEWGQDRSKITHLIFCTTTSNDMPGADYQFARLFG 150
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L00952Arachishypogaea          LDPCVKRYMMYHQGCFAGGTVLRLAKDLAENNKDARVLIVCSENTAVTFR 199
DQ124938Arachishypogaea        LDPCVKRYMMYHQGCFAGGTVLRLAKDLAENNKDARVLIVCSENTAITFR 199
DQ366301Vitisvinifera          LEPSVRRVMLYHQGCYAGGTVLRTAKDLAENNAGARVLVVCSEITVVTFR 199
AF128861Vitisriparia           LEPSVRRVMLYHQGCYAGGTVLRTAKDLAENNAGARVLVVCSEITVVTFR 199
DQ459351Vitisvinifera          LEPSVRRVMLYHQGCYAGGTVLRTAKDLAENNAGARVLVVCSEITVVTFR 199
DQ366302Vitisvinifera          LETSVRRVMLYHQGCYAGGTVLRTAKDLAENNAGARVLVVCSEITVVTFR 199
AF274281Vitisvinifera          LETSVRRVMLYHQGCYAGGTVRRTAKDLAENNAGARVLVVCSEITVVTFR 199
DQ900615Polygonumcuspidatum    LNSSVKRFMFYHLGCYAGGTVLRLAKDIAENNKGARVLIVCSEMTPICFR 197
AF508150Rheumtataricum         LQLSVKRFMFYHLGCYAGGTVLRLAKDIAENNKEARVLIVRSEMTPICFR 199
DQ459349Polygonumcuspidatum    LSPKVNRTMVYQQGCFAGGTVLRLVKDIAENNRGARVLVVCSEIVAFAFR 200
                               *.   *.* *.*: **:***** * .**:****  ****:* **  .  . **

L00952Arachishypogaea          GPSETDMDSLVGQALFADGAAAIIIGSDPVPEVEKPIFELVSTDQKLVPG 249
DQ124938Arachishypogaea        GPSETDMDSLVGQALFADGAAAIIIGSDPVPEVEKPIFEIVSTDQKLVPG 249
DQ366301Vitisvinifera          GPSEDALDSLVGQALFGDGSAAVIVGSDPDISIERPLFQLVSAAQTFIPN 249
AF128861Vitisriparia           GPSEDALDSLVGQALFGDGSAAVIVGSDPDISIERPLFQLVSAAQTFIPN 249
DQ459351Vitisvinifera          GPSEDALDSLVGQALFGDGSAAVIVGSDPDISIERPLFQLVSAAQTFIPN 249
DQ366302Vitisvinifera          GPSEDALDSLVGQALFGDGSSAVIVGSDPDVSIERPLFQLVSAAQTFIPN 249
AF274281Vitisvinifera          GPSEDALDPLVGQALFGDGSSAVIVGSDPDVSIERPLFQLVSAAQTFIPN 249
DQ900615Polygonumcuspidatum    GPSETHIDSMVGQAIFGDGAAAVIVGANPDLTVEKPIFELISTAQTIIPE 247
AF508150Rheumtataricum         GPSETHIDSMVGQAIFGDGAAAVIVGANPDLSIERPIFELISTSQTIIPE 249
DQ459349Polygonumcuspidatum    GPHEDHIDSLIGQLLFGDGAAALVVGCDPDLRDENPIFQIMSASQTTIPD 250
                               ** *   :*.::** :*.**::*:::*.:*      *.*:*:::*: *. :*

L00952Arachishypogaea          SHGAIGGLLREVGLTFYLNKSVPDIISQNINDALNKAFDPLGISDYNSIF 299
DQ124938Arachishypogaea        SHGAIGGLLREVGLTFYLNKSVPDIISQNINDALSKAFDPLGIFDYNSIF 299
DQ366301Vitisvinifera          SAGAIAGNLREVGLTFHLWPNVPTLISENVEKCLTQAFDPLGISDWNSLF 299
AF128861Vitisriparia           SAGAIAGNLREVGLTFHLWPNVPTLISENVEKCLTQAFDPLGISDWNSLF 299
DQ459351Vitisvinifera          SAGAIAGNLREVGLTFHLWPNVPTLISENIEKCLTQAFDPLGISDWNSLF 299
DQ366302Vitisvinifera          SAGAIAGNLREVGLTFHLWPNVPTLISENIEKCLTQAFDPLGISDWNSLF 299
AF274281Vitisvinifera          SAGAIAGNLREVGLTFHLWPNVPTLISENIEKCLTQAFDPLGISDWNSLF 299
DQ900615Polygonumcuspidatum    SDGAIEGHLLEVGLSFQLYQNVPALISNSIGTCLSEAFTPLNISNWNSLF 297
AF508150Rheumtataricum         SDGAIEGHLLEVGLSFQLYQTVPSLISNCIETCLSKAFTPLNISDWNSLF 299
DQ459349Polygonumcuspidatum    TLHTMQLHLTEAGLTFHLTKEVPQVIADNMERVMVEAFMPLGITDWNSIF 300
                               :  ::    * *.**:* *   ** :*:: :      : :** **.* ::**:*

DQ124938Arachishypogaea        WIAHPGGRAILDQVEQKVNLKPEKMKATRDVLSNYGNMSSACVFFIMDLM 349
L00952Arachishypogaea          WIAHPGGRAILDQVEQKVNLKPEKMKATRDVLSNYGNMSSACVFFIMDLM 349
DQ366301Vitisvinifera          WIAHPGGPAILDAVEAKLNLDKKKLEATRHVLSEYGNMSSACVLFILDEM 349
AF128861Vitisriparia           WIAHPGGPAILDAVEAKLNLDKKKLEATRHVLSEYGNMSSACVLFILDEM 349
DQ459351Vitisvinifera          WIAHPGGPAILDAVEAKLNLDKKKLEATRHVLSEYGNMSSACVLFILDEM 349
DQ366302Vitisvinifera          WIAHPGGPAILDAVEAKLNLEKKKLEATRHVLSEYGNMSSACVLFILDEM 349
AF274281Vitisvinifera          WIAHPGGPAILDAVEAKLNLEKKKLEATRHVLSEYGNMSSACVLFILDEM 349
DQ900615Polygonumcuspidatum    WIAHPGGPAILDHVEATVGLNKEKLKATRQVLNDYGNMSSACVFFIMDEM 347
AF508150Rheumtataricum         WIAHPGGRAILDDIEATVGLKKEKLKATRQVLNDYGNMSSACVFFIMDEM 349
DQ459349Polygonumcuspidatum    WQVHPGEKAILDKIEEKLELDAGKLRDSRYILSEYGNLTSACVLFVMDEM 350
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Fig 1. Relative position of amino acid conserve regions of sts gene sequences used to construct degenerated primers GGF2 and GGR2

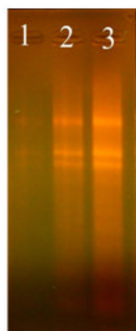

Fig 2. Total RNA visualized in electrophoresis agarose Gel (1) 1.12 µg, (2) 3.36 µg, and (3) 5.60 µg Total RNA of melinjo leaves

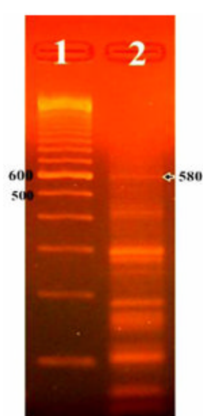

Fig 3. RT-PCR products using GGF2 and GGR2 degenerated primers visualized in Electrophoresis Agarose Gel (2), DNA marker (1) (scales in bp)

similarity level was much higher than that from nucleotide sequences. Among several conserved regions available in the amino acids sequences of sts gene, two regions located in the position of 112-118 = KAIKEWG, 299-305 = LDRTSWC, were selected and modified to generate GGF2 and GGR2 primers used to amplify melinjo sts gene. The length of ideal oligonucleotide used as primers generally contains 20-30 nucleotides and 50-60% G+C. Our primers complied with the rule. The designed primers GGF2 and GGR2 and their properties are shown in Table 1.

Total RNA was isolated from young leaf tissues of melinjo relied on previous researches on *Vitis vinifera* proving that the highest *sts gene expression* is in the plant's leaves. The aim of total RNA isolation is to obtain an adequate amount of Stilbene Synthase mRNAs to be used as templates for reverse transcription-polymerase chain reaction (RT-PCR).

Around 0.25 mg/mg fresh weight tissue of total RNA was recovered using Trizol kit (Invitrogen) according to written protocol of the reagent's manufacturer. Trizol kit isolation has similar principle to common phenol-chloroform isolation. The obtained melinjo total RNA was then diluted in RNase *free water* for storage and ready for further step. The quality of the result was checked both qualitatively and quantitatively.

Qualitative assay to the obtained total RNA was done using gel electrophoresis technique with 1% agarose gel in TAE solution. Melinjo total RNA obtained using electrophoresis technique was photographed and showed in Fig. 2.

Quantitative assay to the isolated total RNA was performed based on absorbance measurement using UV spectrophotometer at 254 nm. Total RNA isolate was diluted by dissolving 5 µL of it in 995 µL sterile aquadest. Solution absorbance was measured using UV spectrophotometer at two different wavelengths: λ 260 and λ 280 wait sterile aquadest as blank solution. The R260/280 ratio of obtained total RNA isolate was 1.75, which was higher than the expected value (below 1.60) showing there were contaminations from protein and other organic compound in the result.

RT-PCR process in two steps was carried out using mRNAs contained in total RNA as templates. The first step involves single chain synthesis of cDNA (reverse transcription, RT stage). The second step is common PCR stage. RT step was conducted using Transcriptor First Strand cDNA Synthesis Kit (Roche). Reaction mixture was made from 5.4 µL total RNA (template), 1 µL oligo-dT primer, 2 µL random hexamer primer and 4.6 µL RNase free water to obtain a total volume of 13 µL. 20 µL mineral oil was added to the mixture and followed by short centrifugation (spin) for 10 sec. The template-primer mixture was heated in PCR machine at 65 °C for 10 min and then cooled in an ice bath. 4 µL transcriptor RT reaction buffer 5X was added to the template-primer mixture along with 2 µL dNTP 0.5 µL protector RNAse inhibitor and 0.5 µL transcriptor RT to obtain a final total volume of 20 µL. The mixture was carefully homogenized and spinned for 10 sec. Final reaction mixture was incubated at 25 °C for 10 min, heated in PCR machine at 55 °C for 30 min and then at 85 °C for 5 min.

The second step of RT-PCR work was the PCR process, which was accomplished using illustraTM Ready To Go (RTG) PCR bead (GE Healthcare). The step was started by diluting RTG bead in micro tube PCR with 21 µL RNase free water. 1 µL primer forward (GGF2), 1 µL primer reverse (GGR2) and 2 µL cDNA template resulted from RT step were added to obtain a total final volume of 25 µL. In a micro tube, the mixture was incubated in a room temperature until steady and then homogenized for 10 min by spinning it. 25 µL mineral oil was added before the mixture was placed in PCR and reacted with condition of denaturing at 95 °C for 1 min, annealing at 57 °C for 1 min, polymerizing at 72 °C for 2 min within 35 cycles. The last cycle at 72 °C was extended for 10 min to make sure that elongation

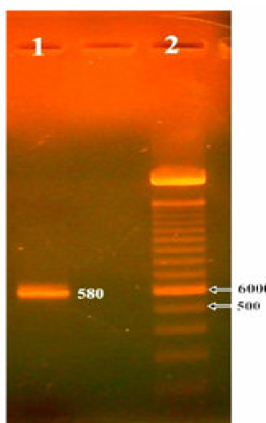

Fig 4. Gel isolated product of RT-PCR fragment using GGF2 and GGR2 primers (2), DNA marker (1) (scales in bp)

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5'TAAACAGGGGGCTCAAATCT
TTTCTACCCTGAGGGCCCCAA
ATCATAAGATGCCTATGACATA
ACAACTGCAAGTAGTTAGTAAT
CATGATAACCAATTGTCACCCT
TAAACATGGATTTCTGGGTCT
GGTCTCTCTCTTTTCTAGCCCT
CCCGGGCCCCCCTTTTATTTG
CCTTTGACATCTCCTCTTCTCC
TCTTTAGTATCGTGATACCTAA
ATGGTTCATTCAACCTGCTCTA
TAACTTCATTTATAAAATCTCT
CATCCTT3'
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Fig 5. Nucleotide sequence of DNA fragment obtained from RT-PCR using GGF2 primer from the reading of sequencing process' result

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L00952Arachis ATGGTGTCTGTGAGTGGAATTCGCAAGGTTCAAAGGGCAGAAGGTCCAGCAACTGTATTG 60
Gnetum gnemon ------------------------------------------------------------
L00952Arachis GCAATTGGAACAGCAAATCCACCGAACTGTATTGATCAGAGTACATATGCAGATTATTAT 120
Gnetum gnemon ------------------------------------------------------------
L00952Arachis TTTAGAGTAACCAATAGCGAACACATGACTGATCTCAAGAAGAAATTTCAGCGCATCTGT 180
Gnetum gnemon ------------------------------------------------------------
L00952Arachis GAGAGAACACAGATCAAGAACAGACATATGTACTTAACAGAAGAGATACTAAAAGAAAAT 240
Gnetum gnemon ------------------------------------------------------------
L00952Arachis CCTAACATGTGTGCATACAAGGCACCGTCATTGGATGCAAGAGAAGACATGATGATCAGG 300
Gnetum gnemon ------------------------------------------------------------
L00952Arachis GAGGTACCAAGAGTTGGAAAAGAGGCTGCAACCAAGGCCATCAAGGAATGGGGCCAGCCA 360
Gnetum gnemon ------------------------------------------------------------
L00952Arachis ATGTCTAAGATCACACATTTGATCTTCTGCACCACCAGCGGCGTTGCGTTGCCTGGCGTT 420
Gnetum gnemon ------------------------------------------------------------
L00952Arachis GATTACGAACTCATCGTACTTTTAGGGCTGGACCCATGCGTCAAGAGGTACATGATGTAC 480
Gnetum gnemon ------------------------------------------------------------
L00952Arachis CACCAAGGTTGCTTCGCTGGTGGCACTGTCCTTCGTTTGGCTAAGGACTTGGCTGAAAAC 540
Gnetum gnemon ------------------------------------------------------------
L00952Arachis AACAAGGATGCTCGTGTACTTATCGTTTGTTCTGAGAATACCGCAGTCACTTTCCGCGGT 600
Gnetum gnemon --------------------------------TAAACAGGGGGCTCAAATCTTTTCTACC 28
                                              * *  *    **    *  **

L00952Arachis CCTAGTGAGACAGACATGGATAGTCTTGTAGGACAAGCATTGTTTGCCGATGGAGCTGCT 660
Gnetum gnemon CTGAGGGCCCCAAATCATAAGATGCCTATGACATAACAACTGCAAGTAGTTAGTAATCAT 88
              *  ** *   ** *     * *  * * *   * **  * **   *  * * *   *  *

L00952Arachis GCGATTATCATTGGTTCTGATCCTGTGCCAGAGGTTGAGAAGCCTATCTTTGAGCTTGTT 720
Gnetum gnemon GCAACTATAACCAATTGTCACCCTTAAACATGGATTTCTGGGTCGATGGTCTCTCTCTTT 143
              ** * *** *    ** * * ***    **  * **     * * **  *    **  **

L00952Arachis TCGACCGATCAAAAACTTGTCCCTGGCAGCCATGGAGCCATCGGTGGTCTCCTTCGTGAA 780
Gnetum gnemon TCTAGCCCTCCCGGGCCCCCCTTTTATTTGCCTTTGACATCTCCTCTTCTCCTCTTTAGT 203
              ** * *  **     *    *  *      * *    *      *  ******   *

L00952Arachis GTTGGACTTACATTCTATCTTAACAAGAGTGTTCCTGATATTATTTCGCAAAATATCAAT 840
Gnetum gnemon ATCGTGATACCTAAATGGTTCATTCAACCTGCTCTATAACTTCATTTATAAACACTCTCT 263
               * * * * * * * * ** ** * ** ** *** ** *

L00952Arachis GACGCGCTCAATAAAGCTTTTGATCCATTGGGTATTTCTGATTATAACTCAATATTTTGG 900
Gnetum gnemon CATCCTT----------------------------------------------------- 270
               *  *

L00952Arachis ATTGCACATCCTGGTGGGCGTGCAATTTTGGACCAGGTTGAACAGAAGGTGAACTTGAAG 960
Gnetum gnemon ------------------------------------------------------------
L00952Arachis CCAGAGAAGATGAAAGCCACTAGAGATGTGCTTAGCAATTATGGTAACATGTCAAGTGCC 1020
Gnetum gnemon ------------------------------------------------------------
L00952Arachis TGTGTGTTCTTCATTATGGATTTGATGAGGAAGAGGTCTCTTGAAGAAGGACTTAAAACT 1080
Gnetum gnemon ------------------------------------------------------------
L00952Arachis ACCGGAGAAGGACTTGATTGGGGTGTGCTTTTTGGCTTTGGTCCTGGTCTCACTATTGAA 1140
Gnetum gnemon ------------------------------------------------------------
L00952Arachis ACTGTCGTTCTCCGCAGTGTGGCCATATAA 1170
Gnetum gnemon ------------------------------
```

Fig 6. Alignment result of DNA fragment from RT-PCR process with sts gene of *Arachis hypogaea* using CLUSTAL 2.0.12 software

process had completely finished.

RT-PCR amplification result was identified using electrophoresis technique, which is similar to that for total RNA qualitative assay in buffer TAE 1X at 100 V for 40 min but using 1.5% agarose gel. Visualization was done using UV light at 254 nm and photographed using digital camera and the result is shown in Fig. 3.

Since the visualization of the electrophoresis RT-PCR results shown in Fig. 3 did not show a single band, and then gel isolation was carried out to get the targeted sts DNA fragment of melinjo. Fragment isolation was done by cutting out targeted band that was available along with other PCR products seen in the agarose gel. Using *PureLink™ Quick Gel Extraction kit* (Invitrogen) the targeted fragment DNA can be obtained in a *recovery tube*. Further qualitative analysis to isolated DNA from gel was carried out using electrophoresis with 1.5% agarose gel at 50 volt in 90 min shown a single DNA band (Fig. 4).

Quantitative analysis to the targeted DNA isolated from gel was carried out using UV spectrophotometer by diluting 5 µL isolate in 995 µL aquadest and measuring the solution's absorbance with aquadest as blanko at two different wavelengths of λ 260 and λ 280. Absorbance ratio (R260/280) was calculated to determine the purity of DNA fragments obtained. Sequencing process to DNA fragments using primers of GGF2 and GGR2 was carried out using ABI PRISM 310 sequencer and was conducted by *first BASE* at Singapore.

Based on the reading sequencing results, the nucleotide sequence of sts gene fragment of melinjo obtained in our work is shown in Fig. 5.

The characterization of nucleotide sequences obtained from sequencing analysis to fragment resulted from RT-PCR process includes homology test of it with sts gene of *Arachis hypogaea* (access code: L00952) using CLUSTAL 2.0.12 software of multiple sequence alignment. Sequence alignment is part of homology analysis to the obtained 0.58 kb sequence to make sure that the RT-PCR product is truly part of melinjo sts gene. The alignment result between the obtained DNA fragment and *Arachis hypogaea* sts gene is shown in Fig. 6.

Result from the reading of sequencing process' result was 270 bp nucleotide sequence of RT-PCR product. In such alignment reading, the star signs showed similar amino acid in all fragments used in alignment process. Alignment of the obtained DNA fragment in our work with sts gene from *Arachis hypogaea* showed similarity. From that result, we can conclude that our DNA fragment is truly part of melinjo sts gene and the sequence obtained from sequencing process is also truly part of sts gene sequence.

CONCLUSION

This study demonstrates the isolation of a DNA fragment as part of melinjo sts gene that encodes the synthesis of Stilbene Synthase enzyme. The enzyme plays the key role in resveratrol biosynthetic pathway of melinjo. Based on the homology analysis on the obtained nucleic acid sequence with the existed sts genes in genebank, we make conclusion that the sts DNA fragment we obtained was part of sts gene that encodes melinjo Stilbene Synthase as evidenced by their high similarity to other plant stilbene synthase genes. To characterize the full length of melinjo sts gene, further work is ongoing.

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