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Short communication

Expression Study of *LeGAPDH*, *LeACO1*, *LeACS1A*, and *LeACS2* in Tomato Fruit (*Solanum lycopersicum*)

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ABSTRACT

Tomato is a climacteric fruit, which is characterized by ripening-related increase of respiration and elevated ethylene synthesis. Ethylene is the key hormone in ripening process of climacteric fruits. The objective of this research is to study the expression of three ethylene synthesis genes: *LeACO1*, *LeACS1A*, *LeACS2*, and a housekeeping gene *LeGAPDH* in ripening tomato fruit. Specific primers have been designed to amplify complementary DNA fragment of *LeGAPDH* (143 bp), *LeACO1* (240 bp), *LeACS1A* (169 bp), and *LeACS2* (148 bp) using polymerase chain reaction. Nucleotide BLAST results of the complementary DNA fragments show high similarity with *LeGAPDH* (NM_001247874.1), *LeACO1* (NM_001247095.1), *LeACS1A* (NM_001246993.1), *LeACS2* (NM_001247249.1), respectively. Expression study showed that *LeACO1*, *LeACS1A*, *LeACS2*, and *LeGAPDH* genes were expressed in ripening tomato fruit. Isolation methods, reference sequences, and primers used in this study can be used in future experiments to study expression of genes responsible for ethylene synthesis using quantitative polymerase chain reaction and to design better strategy for controlling fruit ripening in agroindustry.

1. Introduction

Tomato (*Solanum lycopersicum*) is a fruit that has economic value as food stuff. Tomato fruit is also one of the top priorities of horticultural commodity in Indonesia, based on the decision of Ministry of Agriculture in 2008 (Hanindita 2009). Since 2000, tomato production in Indonesia has steadily increased and reached 950,385 tons in 2011 (Badan Pusat Statistik dan Direktorat Jenderal Hortikultura 2011; FAOSTAT 2013).

Climate change affects tomato production worldwide, especially in countries with only 2 seasons, such as Indonesia. Increasing global temperatures and erratic seasonal changes may have negative impacts on the production of tomatoes. These impacts can be direct on the vegetation or indirect, that is, the impact on factors that may impair or inhibit plant growth. One aspect of the tomato plants that is affected by climate change is fruit ripening. The increase of global temperature may affect ripening process of tomato fruits. Tomatoes that ripe at temperatures above 35°C showed decline in ethylene evolution (Picton and Grierson 1988).

Ethylene is a plant hormone that plays a key role in fruit ripening. During tomato fruit ripening, ethylene regulates expression of ethylene responsive genes which are responsible for ripening and senescence-related physiological changes, such as ACC synthase (*ACS*), ACC oxidase (*ACO*), polygalacturonase (*PG*), pectin methylesterase (*PME*), phytoene synthase (*PSY*), or *E4* and *E8* genes whose function is still explored (Cara and Giovanni 2008). Among several genes that play a role in ethylene biosynthesis, only *ACS* and *ACO* have been characterized extensively (Cara and Giovanni 2008). ACS is a pyridoxal-phosphate dependent enzyme which catalyzes change of S-adenosyl methionine to 1-aminocyclopropane-1-carboxylic acid (ACC) at the first stage of ethylene biosynthesis (Cara and Giovanni 2008; Barry *et al.* 2000), as well as a member of the superfamily aspartate aminotransferase (AAT_1). ACO is involved in the conversion of ACC to ethylene (C_2H_4) at the second stage of ethylene biosynthesis and is a member of oxygenase superfamily that requires Fe (II) as cofactor and ascorbate as cosubstrate [DIOX_N and 2OG-Fe (II)] (Jafari *et al.* 2000; Marchler-Bauer *et al.* 2013). Both of these enzymes are encoded by multigene families *LeACO* and *LeACS*, respectively. In tomato fruit four *LeACS* genes (*LeACS1A*, *LeACS2*, *LeACS4*, and *LeACS6*) and three *LeACO* genes (*LeACO1*, *LeACO3*, and *LeACO4*) are expressed differentially during ripening (Barry *et al.* 2000; Jafari *et al.* 2000).

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One approach in the study of tomato fruit ripening is characterization of ethylene biosynthesis genes' expression. These studies are usually performed using real-time quantitative polymerase chain reaction (qPCR) to obtain accurate data of the expression level of the genes. The initial step is to perform isolation and characterization of genes expressed during tomato fruit ripening. Because of their crucial role in fruit ripening, *LeACS* and *LeACO* genes become the main concern of this study.

The objective of this research is to amplify fragment of ethylene biosynthesis gene *LeACS*1A, *LeACS*2, and *LeACO*1 from a ripening tomato fruit. *LeACS*2 is expressed in climacteric phase of tomato fruit resulting in auto-catalytic ethylene characteristic of system 2, meanwhile the expression of LeACS1A is reduced in climacteric phase (Barry *et al.* 2000). LeACO1 is expressed since pre-climacteric phase of fruit ripening and the expression increased when the fruit enters climacteric phase. *LeACO1* is also a member of multigene family *LeACO* majorly expressed in tomatoes (Jafari *et al.* 2000). Character expression of each gene is expected to provide an overview of ethylene biosynthesis in tomato fruit that relates to ripening. In addition, in this research isolation and characterization of housekeeping gene *LeGAPDH* as an internal control for the study of gene expression analysis in tomatoes was also performed. Output of this research a resequence of references, methods, and primers that become a foundation for the study of ethylene synthesis gene expression during tomato fruit ripening in the future.

2. Materials and Methods

2.1. Tomato fruit

Tomato fruit used in this study is an Arthaloka variant. The seeds were obtained from Perseroan Terbatas (PT) East West Seed which was collected and planted in Cihanjuang, Cimahi. The fruit was collected at a "turning" phase, when 30% of the surface of the fruit had a yellow-pink color. After collection, the fruit was rinsed, preserved in liquid nitrogen and stored in -80°C for RNA extraction.

2.2. Primer design

Primer design was performed using BioEdit software and Primer-BLAST tool at National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The criteria for primers were based on primer criteria for optimal qPCR, with intent to use the primers in gene expression studies in the future. The *LeGAPDH*, *LeACO1*, *LeACS1A*, and LeACS2s' primer was designated based on complementary DNA (cDNA) sequences of *LeGAPDH* messenger RNA (mRNA) (NM_001247874.1), *LeACO1* (NM_001247095.1), *LeACS1A* (NM_001246993.1), and *LeACS*2 (NM_001247249.1), respectively. Primer of *LeACO1*, *LeACS1A*, and *LeACS*2 was designed to anneal at base sequence that are specific for the target gene; at the unconserved region of gene family.

2.3. cDNA amplification

Total RNA extraction was performed by method of Zhuang *et al.* (2006) with modification. RNA sample was purified by Fermentas Purification Kit (catalog number: #EN0521). cDNA synthesis was performed with iScript cDNA synthesis kit from BioRad (catalog number: 170-8890). Fragments of *LeGAPDH, LeACO1, LeACS1A,* and *LeACS2* were amplified from cDNA by PCR using GoTaq® Green Master Mix from Promega (catalog number: M7122). Gene fragment was separated by electrophoresis in agarose gel at 100 V. As reference, 100 bp Fermentas ladder was used (catalog number: #SM0321).

Gene fragments were purified from electrophoresis gel using Geneaid purification kit (catalog number #DF 100). The purified fragments were then inserted into cloning vector pGEM-T easy using T4 DNA ligase enzyme. Vector : insert molar ratio was calculated based on sample concentration which was estimated by using DNA 1 kb mass ruler as size reference. Vector : insert molar ratio used for fragment of *LeACS1A* and *LeACO1* is 3:1, whereas for fragment of *LeGAPDH* and *LeACS*2 is 5:1. Cloning vector containing inserted fragments was then used to transform competent cell of *E. coli* DH5α with heat-shock transformation. Recombinant bacteria colonies were identified with blue-white screening technique on colony PCR. Plasmid containing inserted fragments was extracted from recombinant bacteria using High-Speed Plasmid Mini Kit from Geneaid. The extracted plasmids were then submitted to Macrogen Inc., Seoul, Korea for sequencing.

2.4. Gene characterization

Nucleotide sequence of isolated gene fragments obtained from sequencing characterized using BLASTn. Refseq database was used as reference. Amino acid sequence was predicted using Expasy Translate tool (http://www.expasy.ch/tools/dna.html). Conserved motives in amino acid sequence were identified with BLAST at Conserved Domain Database (CDD) (Marchler-Bauer *et al.* 2013). Alignment of amino acid sequence of gene family was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

3. Results

cDNA fragments were successfully amplified using total RNA from tomato fruit at "turning" phase, which is when 30% of the surface of the fruit has a yellow-pinkish color. The result showed that *LeGAPDH*, *LeACO*1, *LeACS*1A, and *LeACS*2 were expressed during this stage. cDNA amplification was performed using oligonucleotide primers forward and reverse, designated from NCBI Refseq sequences (Table 1). A single PCR fragment of the expected size from each primer was generated, namely GA (143 bp), O1 (240 bp), S1A (169 bp), and S2 (148 bp), which are amplified by primer *LeGAPDH*, *LeACO1*, *LeACS1A*, and *LeACS*2, respectively (Figure 1).

3.1. LeGAPDH

Nucleotide BLAST (BLASTn) result showed that fragment GA had 100% similarity with *Solanum lycopersicum* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (NM_001247874.1) and *Lycopersicon esculentum* GAPDH mRNA, complete cds (U93208). This similarity suggested that the fragment GA was a strong candidate for *LeGAPDH*. BLASTn result also showed similarity with *LeGAPDH* from other plant species, such as potato (*Solanumtuberosum*), tobacco (*Nicotianatabacum*), and *Arabidopsis thaliana* with 89%-96% similarity. This indicated that GAPDH in tomato and other plant species had similarity in nucleotide sequence.

A reading frame consisting of 27 amino acid sequence was predicted from nucleotide sequence of fragment GA. Protein BLAST (BLASTp) result showed that the amino acid sequence had 100% similarity with GAPDH protein from tomato, soybean, and barrel cloverplant (*Medicago truncatula*), with the lowest e-value (1e-18) obtained at similarity with GAPDH from tomato plant. BLASTp results also showed specific hit with GAPDH, C-terminal domain (Gp_dh_C) protein superfamily in the NCBI CDD which indicated that the isolated fragment was a member of Gp_dh_C superfamily.

GAPDH is an NAD-binding enzyme which catalyzes phosphorylation of glyceraldehyde-3-phosphate in glycolysis pathway (Barber *et al.* 2005). In the predicted amino acid sequence of GA fragment, two motifs were found that belonged to Gp_dh_C superfamily (Figure 2).

3.2. LeACO1

Sequence analysis of O1 fragment (240 bp) was performed and showed 100% sequence similarity with *Solanum lycopersicum* 1-

Table 1. Sequence of Reverse Transcription - polymerase chain reaction (RT-PCR) primers used to amplify fragment *GAPDH*, *ACO1*, *ACS1A*, and *ACS2* from tomato cDNA

Sequence	Forward	Reverse	Product size (bp)	Tm (°C)
LeGAPDH	5'AAATTGCCTTGCTCCCTTGG3'	5'CAGCTCTTCCACCTCTCCAA3'	143	65
*LeACO*1	5'ACTCCTCAAAGACGAGCAATG3'	5'ACTTGTGTACTTTCCTCTGCCT3'	240	63, 5
*LeACS*1A	5'GACGAGACGATGAGGATT3'	5'GAGAGTGAGCTGGTGAGTT3'	169	65,5
*LeACS*2	5'TTCGGAGGTTCGTAGGTGTT3'	5'GGAATAGGTGACGAAAGTGGTG3'	148	63,8

cDNA = complementary DNA.

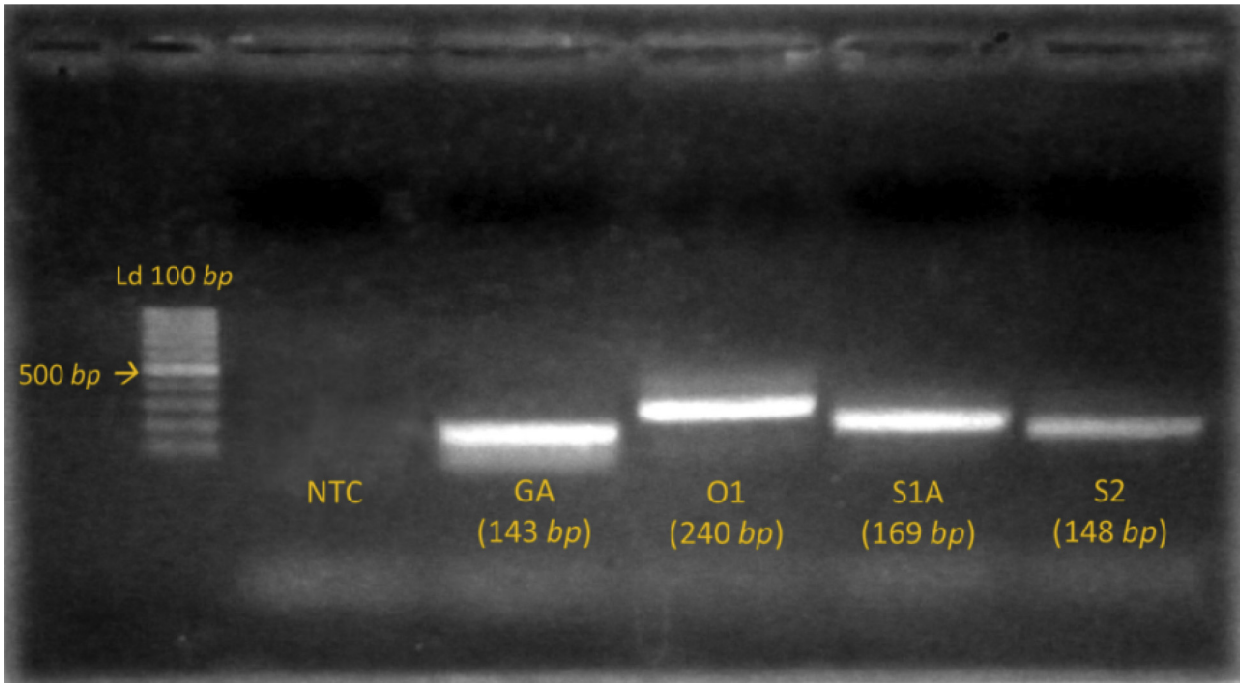

Figure 1. Electropherogram of PCR results of cDNA amplification using specific primers for LeGAPDH, LeACO1, LeACS1A, and LeACS2. Gene fragments were obtained with the expected product size of the corresponding primers. Left to right: ladder (Fermentas), non-template control (NTC), GA (primer LeGAPDH; 143 bp), O1 (primer LeACO1; 240 bp), S1A (primer LeACS1A; 169 bp), S2 (primer LeACS2; 148 bp). cDNA = complementary DNA; PCR = polymerase chain reaction.

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                    10        20        30        40        50        60        70        80        90       100
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAPDH[Solanum       ----------------------------------------MTYMFKYDSVHGQWKHHELKVKDEKTLLFGEKAVTVFGFRNPEETSMDRLGADYIV
GA                  ----------------------------------------------------------------------------------------
GAPDH[Arabidopsis   MADKKIRIGINGFGRIGRLVARVVLQRDDVELVAVNDPFITTEYMTYMFKYDSVHGQWKHHELKVKDDKTLLFGEKPVTVFGIRNPEDIPWGEAGADFVV
GAPDH               --MGKVKIGINGFGRIGRLVARVALQRDDVELVAVNDPFITTDYMTYMFKYDSVHGHWKHHDVTVKDEKTLLFGDKPVTIFGHRNPEEIPWGSTGADIIV
GAPDH[Medicago      MGGAKIKIGINGFGRIGRLVARVALKRDDVELVAVNDPFITTDYMTYMFKYDSVHGQWKNDELTVKDSKTLLFGEKPVTVFAHRNPEEIPWASTGADIIV
GAPDH[Zea           --MGKIKIGINGFGRIGRLVARVALQSEDVELVAVNDPFITTDYMTYMFKYDTVHGQWKHSDIALKDSKTLLFGEKPVTVFGIRNPEEIPWGEAGAEYVV
Clustal Consensus                                          ********:***:**. :: :**.******:* **:*. ****:      **: :*

                   110       120       130       140       150       160       170       180       190       200
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAPDH[Solanum       ESTGVFTDKDKPAAHLKGGAKKFIISAPSKDAPMFVVGVNEKEYNPELNIVSNASCTTNCLAPLAKVINDRFGIVEGLMTTVHSITATQKTVDGPSAKDW
GA                  ----------------------------------------------------------------------------MTTVHSITATQKTVDGPSAKDW
GAPDH[Arabidopsis   ESTGVFTDKDKAAAHLKGGAKKVVISAPSKDAPMFVVGVNEHE----------------------------FGIVEGLMTTVHSITATQKTVDGPSMKDW
GAPDH               ESTGVFTDKDKAAAHLKGGAKKVIISAPSKDAPMFVVGVNEHEYKPELDIISNASCTTNCLAPLAKVINDRFGIVEGLMTTVHSITATQKTVDGPSAKDW
GAPDH[Medicago      ESTGVFTDKDKAAAHLKGGAKKVIISAPSKDAPMFVVGVNEKEYKPEFDIISNASCTTNCLAPLAKVINDRFGIVEGLMTTVHSITATQKTVDGPSAKDW
GAPDH[Zea           ESTGVFTDKDKAAAHLKGGAKKVVISAPSKDAPMFVVGVNEDKYTSDVNIVSNASCTTNCLAPLAKVIHDNFGIIEGLMTTVHAITATQKTVDGPSAKDW
Clustal Consensus   *********** ********** .:*****************.:                              ***:********:************ ***

                   210       220       230       240       250       260       270       280       290       300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAPDH[Solanum       RGGRAASFNIIPSSTGAAKAVGKVLPALNGKLTGMAFRVPTVDVSVVDLTVRLEKEATYDEIKAAIKEESEGKLKGILGYTEDDVVSTDFVGDNRPSIFD
GA                  RGGRA-------------------------------------------------------------------------------------
GAPDH[Arabidopsis   RGGRAASFNIIPSSTGAAKAVGKVLPSLNGKLTGMSFRVPTVDVSVVDLTVRLEKAATYDEIKKAIKEESEGKMKGILGYTEDDVVSTDFVGDNRSSIFD
GAPDH               RGGRAASFNIIPSSTGAAKAVGKVLPALNGKLTGMAFRVPTVDVSVVDLTVRLEKEASYDEIKNAIKEESEGKLKGILGYTEDDVVSTDFIGDSRSSIFD
GAPDH[Medicago      RGGRAASFNIIPSSTGAAKAVGKVLPALNGKLTGMAFRVPTVDVSVVDLTVRLEKPATYDQIKAAIKEESEGNLKGILGYTEDDVVSTDFIGDTRSSIFD
GAPDH[Zea           RGGRAASFNIIPSSTGAAKAVGKVLPELNGKLTGMSFRVPTVDVSVVDLTVRIEKGASYEEIKKAIKAASEGPLKGIMGYVEEDLVSTDFTGDSRSSIFD
Clustal Consensus   ************************** ********:*****************:** *:*::** ***  *** :***:**.*:*:***** **.* ****

                   310       320       330       340
                    ....|....|....|....|....|....|....|
GAPDH[Solanum       AKAGIALSKNFVKLVAWYDNEWGYSTRVVDLIIHMSSVQ-
GA                  ----------------------------------------
GAPDH[Arabidopsis   AKAGIALSDKFVKLVSWYDNEWGYSSRVVDLIVHMSKA--
GAPDH               AKAGIALNKNFVKLVSWYDNEWGYSSRVIDLLVFVAKKSL
GAPDH[Medicago      AKAGIALNDNFVKLVSWYDNELGYSTRVVDLIVHIAKQL-
GAPDH[Zea           AKAGIALNDHFIKLVSWYDNEWGYSNRVVDLIRHMFKTQ-
Clustal Consensus   *******...*:***:***** ***.**:**: .: .
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Figure 2. Alignment of predicted amino acid sequence of GA fragment with GAPDH from some plant species (up to bottom): *Solanum lycopersicum*, *Arabidopsis thaliana*, *Glycine max*, *Medicago truncatula*, and *Zea mays*. GA fragment contained conserved motif (highlighted yellow) of Gp_dh_C superfamily which was found in GAPDH from other plant species. * = consensus sequence. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

aminocyclopropane-1-carboxylate oxidase 1 (ACO1), mRNA (NM_001247095.1) and 91% similarity with *Solanum lycopersicum* ethylene-forming enzyme (ACO4), mRNA (NM_001246999.1). This result indicated that the isolated fragment contained conserved nucleotide sequence of ACO multigene family that presents both in *LeACO1* and *LeACO4*. The BLASTp result showed that the 73 predicted amino acid sequence had 100% similarity with 1-aminocyclopropane-1-carboxylate oxidase 1 (*Solanum lycopersicum*) (NP_001234024.1). BLASTp result also showed specific hit with 2OG-Fe(II) oxygenase (20G-FeII_OXY; pfam03171) in CDD.

In predicted amino acid sequence, sequence that is specific to *LeACO1* and motifs conserved in *LeACO* was found, among them were motifs that belonged to 20G-FeII_OXY (Figure 3).

3.3. *LeACS1* and *LeACS2*

BLASTn result of S1A fragment showed 100% similarity with *Solanum lycopersicum* 1-aminocyclopropane-1-carboxylate synthase (ACS1A), mRNA (NM_001246993.1). In this nucleotide sequence a reading frame containing 57 amino acids was found. BLASTp result showed that these amino acids corresponded with 1-aminocyclopropane-1-carboxylate synthase (*Solanum lycopersicum*) protein (NP_001233922.1), with 100% similarity. Conserved domain was not identified.

BLASTn result of S2 fragment showed 99% similarity with *Solanum lycopersicum* ripening-related ACC synthase 2 (ACS2), mRNA (NM_001247249.1). In this nuclotide sequence, a reading frame containing 39 amino acids was found. These amino acids corresponded with *Solanum lycopersicum* ripening-related ACC synthase 2 (ACS2), mRNA (NM_001247249.1), with 96% similarity and 53, 9 alignment score, which was lower than the other isolated fragments. The S2 fragment also corresponded with ACS protein from other plants, such as *Capsicum chinense*, *Nicotiana tabacum*, and *Solanum tuberosum*, although with much lower alignment scores (<40). In this fragment, conserved domain was not identified.

Through multiple alignment of predicted amino acid of S1A and S2 fragments with other members of *LeACS* family, it was found that the isolated fragments had no conserved features of AAT_1, but constituted amino acid sequence was specific to *LeACS*1A and *LeACS*2, respectively (Figure 4).

4. Discussion

The result showed that *LeGAPDH*, *LeACO1*, *LeACS1A*, and *LeACS2* fragments were amplified from tomato fruit at "turning" phase, which is when 30% of the surface of the fruit has a yellow-pinkish color. The analysis result showed that the fragment GA was a strong candidate for *LeGAPDH*. GAPDH belongs to GAPDH, C-terminal domain (Gp_dh_C) superfamily. Two motifs of Gp_dh_C superfamily were found in the predicted amino acid sequence of GA fragment.

ACO is an Fe(II)-dependent oxidase which requires Fe(II) as cofactor and ascorbate as a cosubstrate (Jafari *et al.* 2000). LeACO and ACO in other plants have conserved motifs for binding the cofactor (H-X-D-X-H) and cosubstrate (R-X-S) (Jafari *et al.* 2000). Based on information from CDD (Marchler-Bauer *et al.* 2013), LeACO contained two conserved domains of oxygenase family: non-haem dioxygenase on N terminal (DIOX_N; pfam14226) and superfamily of 2OG-Fe(II) oxygenase (20G-FeII_OXY; pfam03171). In predicted amino acid sequence of O1 fragment, sequence that is specific to LeACO1 and motifs conserved in LeACO were found, among them were motifs that belonged to 20G-FeII_OXY. In the predicted amino acid sequence was also found a motif for binding cosubstrate (R-X-S), which is conserved in *ACO* gene family. This indicated that O1 fragment was likely a candidate for *LeACO1*.

ACC synthase is an enzyme that catalyzes the first step of ethylene synthesis (Barry *et al.* 2000). ACC synthase belongs to aspartate aminotranferase superfamily (AAT_1), which is pyridoxal-phosphate dependent (Cara and Giovanni 2008). Based on information in CDD (Marchler-Bauer *et al.* 2013), AAT_1

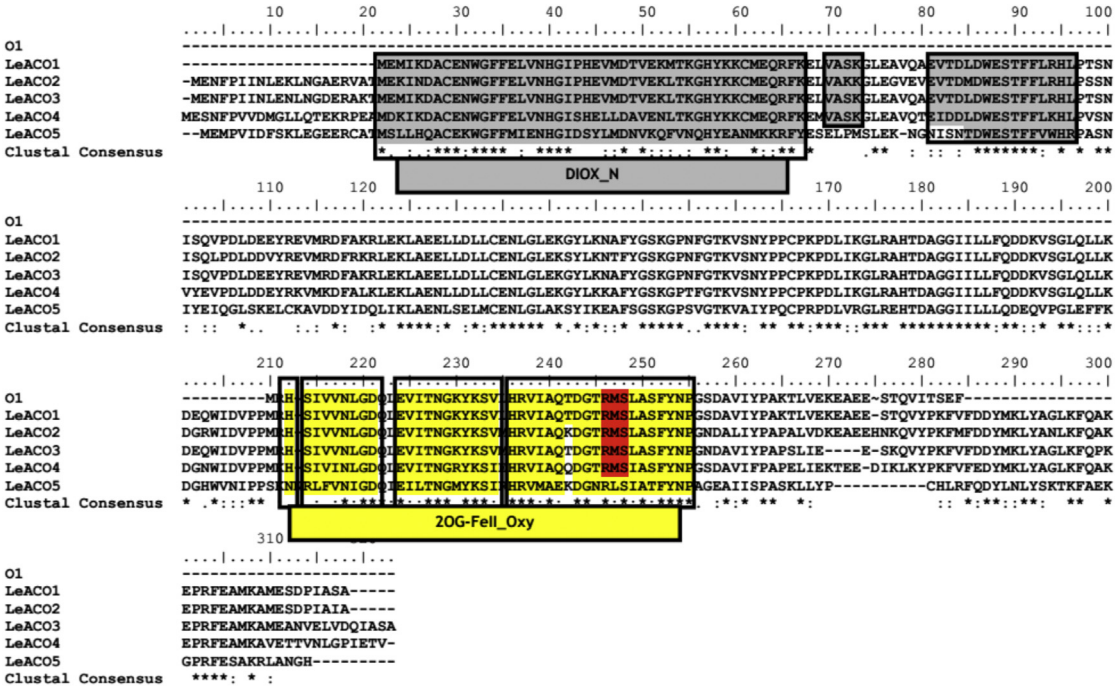

Figure 3. Alignment of predicted amino acid of O1 fragment (first row) with other *LeACO*, which contained domain of DIOX_N (highlighted with gray) and 2OG-FeII_Oxy (highlighted with yellow). Predicted amino acid of O1 fragment contained domain of 2OG-FeII_Oxy and conserved motif for binding cosubstrate (highlighted with red). Consensus sequence was annotated with *.

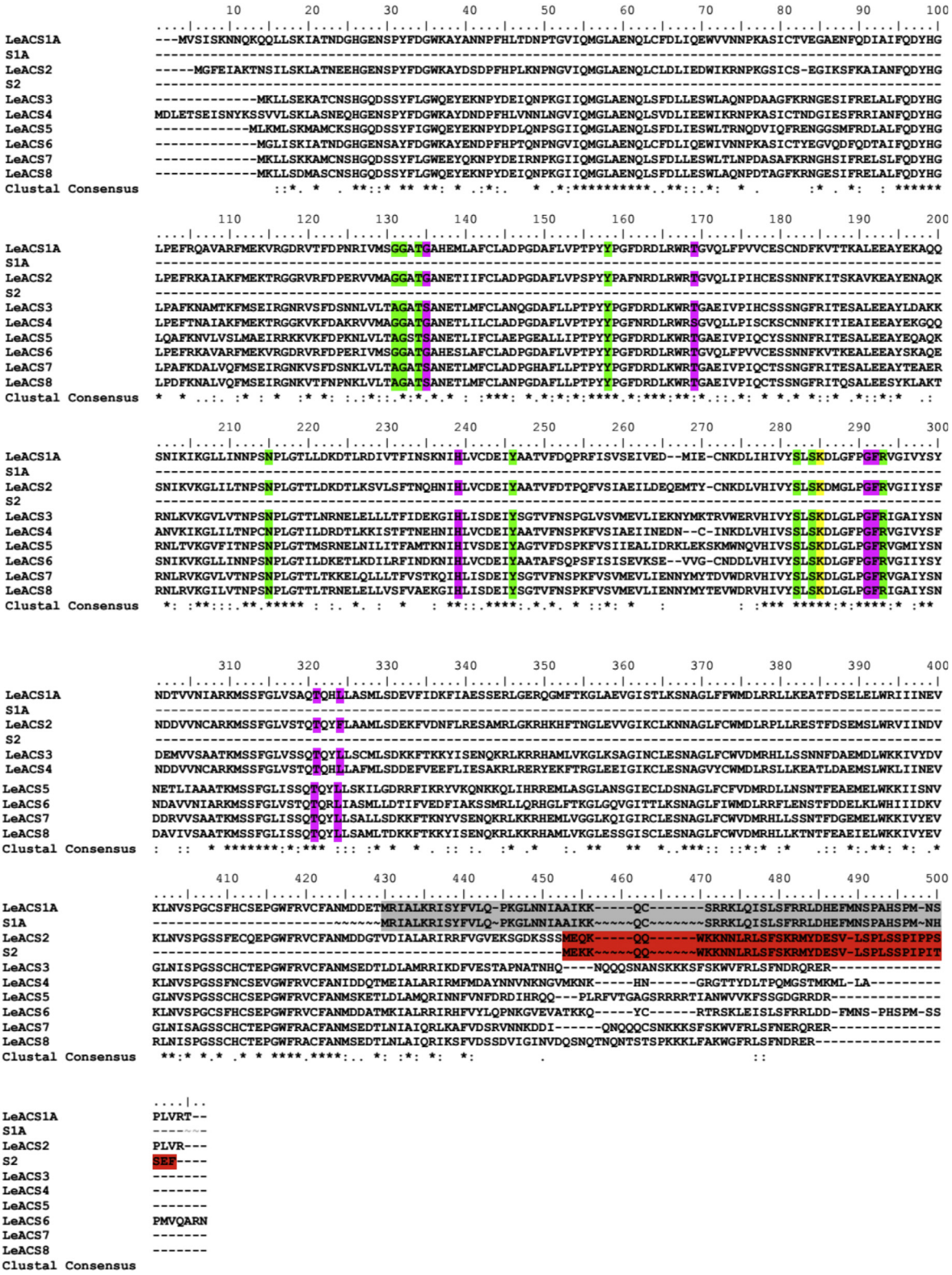

Figure 4. Alignment of predicted amino acid sequence of *LeACS* isolated fragments with other *LeACS* multigene family members. S1A fragment (isolated from *LeACS*1A; highlighted gray) and S2 (isolated from *LeACS*2; highlighted in red) did not have consensus amino acid sequences of *LeACS* family, only specific amino acid sequence for *LeACS*1A and *LeACS*2, respectively. The isolated fragments also lacked the conserved features of AAT_I superfamily: active site (yellow), pyridoxal-phosphate binding region (green), and polypeptide binding region (pink).

superfamily is known to have conserved features, i.e: (i) catalytic residue which is the active site of the enzyme, (ii) binding site of pyridoxal-phosphate, and (iii) binding site of polypeptide. Multiple alignment of predicted amino acid of S1A and S2 fragments showed that constituted amino acid sequence was specific to *LeACS1A* and *LeACS*2, respectively. This finding indicated that isolated fragments of S1A and S2 were also candidates for *LeACS1A* and *LeACS2* genes, respectively.

The result of study showed that *LeACS1A*, *LeACS2*, and *LeACO1* genes were expressed during fruit ripening and may have important roles in ethylene biosynthesis. Understanding their roles during ripening process will lead us to design better strategy for controlling fruit ripening in agroindustry.

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