

# Characterization and expression of *Cm-AAT1* gene encoding alcohol acyl-transferase in melon fruit (*Cucumis melo* L.) 'Hikapel'

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**Abstract.** Wibowo WA, Fatkhurohman MI, Daryono BS. 2020. Characterization and expression of *Cm-AAT1* gene encoding alcohol acyl-transferase in melon fruit (*Cucumis melo* L.) 'Hikapel'. *Biodiversitas* 21: 3041-3046. Melon (*Cucumis melo* L.) is one of the horticulture commodities that have high economic value and its needs increase continuously. Many new melon cultivars have been assembled to produce a higher quality melon. Melon 'Hikapel' developed by the Laboratory of Genetics and Breeding, Faculty of Biology UGM has distinctive character in the form of a strong aroma. This aroma is a complex mixture of various kinds of volatile compound. One of the main determinant compounds is a volatile ester, synthesized by the alcohol acyl-transferase enzyme encoded by the *Cm-AAT1* gene. Characterization of *Cm-AAT1* began with isolation of melon rinds to get total RNAs. Synthesis cDNA was conducted with oligo-dT primer, followed by detection of *Cm-AAT1* using specific primers. A specific band was sequenced to perform phylogenetic tree. Gene expression from 4 melon cultivars, 'Hikapel', 'Hikadi', 'Sun Lady', and 'Luna' analysis was performed using relative quantitative Real-Time PCR. The results of this study showed that *Cm-AAT1* owned not only by aromatic cultivars 'Hikapel' and 'Hikadi', but also owned by non-aromatic cultivars 'Sun Lady' and 'Luna'. Phylogenetic analysis shows a high similarity between *Cm-AAT1* on 'Hikapel' and 'Hikadi'. Gene expression analysis on 'Hikapel' increases as the process of fruit ripening during the storage period and it is in contrast to 'Hikadi' at decrease when the fruit began to enter the decay process on day 7th. Expression of *Cm-AAT1* on 'Hikapel' was higher than 'Hikadi' at the peak of fruit maturity.

**Keywords:** Alcohol acyl-transferase, *Cm-AAT1*, *Cucumis melo*, Hikapel

## INTRODUCTION

Melon (*Cucumis melo* L.) is a horticultural commodity that has high economic value in Indonesia. Melon production in Indonesia from 2016 to 2018 increased (BPS 2019). Breeding efforts have been carried out to produce superior melon cultivars to meet the increasing needs of melon (Daryono et al. 2019). The superior character to measure the melon quality is a combination of soluble sugars, organic acids, pigments, and volatile aromatic compounds (Kyriacou et al. 2018).

The aroma of melons is composed of various kinds of complex volatile compounds that are highly dependent on the type and variety of melon plants (Oh et al. 2011; Condurso et al. 2012; Esteras et al. 2018). There are more than 250 compounds reported in melons that are influenced by genotype and fruit maturity (Lignou et al. 2014). Volatile compound biosynthesis involves the pathway between converting fatty acids and amino acids to aldehydes, aldehydes to alcohols, and alcohol compounds to esters (Portnoy et al. 2017)

In cantaloupensis melon varieties, esters have a significant role as a group of volatile compounds and are likely to be the key that contributes to the formation of the characteristic aroma present in ripe melons (Galaz et al. 2013; Galpaz et al. 2018). Esters represent 94% of the total

20% volatile compounds in physiologically mature pre-slip fruit (Vallone et al. 2013). The process of esters formation in fruit is catalyzed by the enzyme alcohol acyl-transferase (AAT) enzyme by transferring acyl-CoA to alcohol (Costello et al. 2013; Navarro-retamal et al. 2016). This enzyme can combine different types of alcohol and acyl-CoAs to produce various types of esters in the fruit. Several kinds of fruit have been known to have this type of enzyme, such as tomatoes (Goulet et al. 2015), bananas (Selli et al. 2012), strawberries (Cumplido-Laso et al. 2012), apples (Li et al. 2014), kiwi (Günther et al. 2011; Zhang et al. 2020), papaya (Balbontin et al. 2010; Morales-Quintana et al. 2013) and grapes (Ubeda et al. 2017; Qian et al. 2019).

In melons (*Cucumis melo* var. *Cantalupensis*), the enzyme alcohol acyl-transferase (AAT) is encoded by about four kinds of gene families, namely *Cm-AAT1*, *Cm-AAT2*, *Cm-AAT3*, and *Cm-AAT4* (Guo et al. 2017). The *Cm-AAT1* gene has the highest similarity to the *Cm-AAT2* gene compared to the other two genes (El-Sharkawy et al. 2005). The *Cm-AAT1* and *Cm-AAT2* genes have the same two transcription factors for gene expression activity (Rios et al. 2017).

The *Cm-AAT* gene found in melons was originally known as *MEL2* and was only expressed in fruit and not expressed in other organs in melon plants in the process of

fruit ripening (Gur et al. 2016). The expression of the *Cm-AAT1* gene in melons was higher than the three genes of its family (*Cm-AAT2*, *Cm-AAT3*, and *Cm-AAT4*) and it was also known that the expression of the *Cm-AAT3* gene was much lower than the others (Sharkawy et al. 2005). Spadafora et al. (2019) have also used the qRT-PCR method to look at the expression of *CmAAT1* and *CmAAT2* genes in melons with several treatment interactions between pre-harvest and post-harvest.

The 'Hikapel' melon has a strong aroma derived from its parents 'Gama Melon Perfume' (GMP). The 'GMP' melon has a very strong distinctive aroma and it is known that the ester compound dominates the aroma constituent components (Hasbullah et al. 2019). Of the four members of the gene family (*Cm-AAT1*, *Cm-AAT2*, *Cm-AAT3*, and *Cm-AAT4*), the *Cm-AAT1* is the most actively regulating the main process in the formation of aromatic volatile ester compounds in melons (Galaz et al. 2013; Freilich et al. 2015). Based on these points, this study aims to identify the *Cm-AAT1* gene found in 'Hikapel' melon and compare to several other melon cultivars to emphasize the characteristic aroma of 'Hikapel' melon.

## MATERIALS AND METHODS

### Plants materials

This study used fruit samples from 4 melon cultivars, namely, 'Hikadi' and 'Hikapel,' which are aromatic melons, and 'Sun Lady' and 'Luna' for the non-aromatic melons. Melon samples 'Hikadi' and 'Hikapel' were prepared from fruit skin extraction on days 1, 3, 5, 7, and 10 after harvest for characterization and gene expression study. While the samples of 'Sun Lady' and 'Luna' were directly extracted from the ripe fruit without storage. RNA was isolated from the rind sample using the RNA isolation kit (GeneJet Fermentas Thermo Scientific, USA). A total of 0.1 grams of fruit sample was ground with liquid nitrogen, put in a 1.5 ml tube, and then continued by procedure following the protocol from the manuals of the company. A total volume of 50  $\mu$ l total RNA was obtained and stored at -80 °C.

### Detection and characterization of *Cm-AAT1* gene

To synthesize cDNA, 1  $\mu$ L of total RNA was added with 1  $\mu$ L oligo-dT, and nuclease-free water 10  $\mu$ L. The mixed solution was incubated at 65 °C for 5 minutes. The mix (RT buffer 4  $\mu$ L, 10 mM dNTP mix 2  $\mu$ L, RiboLock RNase inhibitor 1  $\mu$ L) was then incubated at 42 °C for 2 minutes. The M-MLV Reverse Transcriptase enzyme was added 1  $\mu$ l and incubation was continued at 42°C for 50 minutes. The final stage is carried out by incubating at 70 °C for 15 minutes.

**Table 1.** Specific primers for the detection of the *Cm-AAT1* gene

Primers	The oligonucleotide sequence
Forward primer	5'- GTGCGGTTTCTTTGCGTAAT-3'
Reverse primer	5'- CAACGAGGCATGAACATTTG-3'

The cDNA obtained was further amplified using specific primers (Table 1) designed using Primer3 software. Gene sequence information was obtained from the CuGenDB melon database (DHL92) v3.6.1 genome (<http://cucurbitgenomics.org/>). Stages of cDNA synthesis included pre-denaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72°C for 1 minute. The amplification was run in 40 cycles. The results of the amplification were visualized by electrophoresis with SYBR Safe staining using agarose gel with a concentration of 2% and running gel with a voltage of 50 V for 45 minutes.

### *Cm-AAT1* gene sequencing and phylogenetic analysis

The sequencing was performed using Sanger methods. Sequencing data of the *Cm-AAT1* gene was constructed by a consensus sequence analyzed using BioEdit software. Contiguous sequences obtained were then carried out by the BLAST sequence analysis. The suitable BLAST results were inputted into a BioEdit program. The ClustalW Multiple Alignment was selected for sequence alignment. The results of sequencing were be used to compare 'Hikapel' and other cultivar melons. Phylogenetic tree was constructed using the MEGAX program with the neighbor-joining algorithm.

### Analysis of *Cm-AAT1* gene expression with real time-PCR

Real-Time PCR was performed using the KAPA SYBR® FAST qPCR Master Mix (2X) Universal kit (Sigma-Aldrich, Germany). The *ACTIN2* primers were used as control, i.e., CmActin2\_Forward [5'-GTGATGGTGTGAGTCACACTGTTC-3'] and CmActin2\_Reverse [5' -ACGACCAGCAAGGTCCAAAC-3']. The primer of *Cm-AAT1* gene, i.e., *Cm-AAT1*-1181 (F) [5'-CCACAGGGGCCAGAATTACA-3'] and *Cm-AAT1*-1284 (R) [5'-TGGAGGAGGCAAGCATAGACTT-3'] primers. The PCR program consisted of a preliminary stage at 50°C for 2 minutes and 95°C for 10 minutes. The PCR cycle was carried out for 40 times at 95°C for 15 seconds and 58°C for 1 minute. The qRT-PCR results were then analyzed using the Bio-Rad CFX Manager 2.1 software. The calculation of *Cm-AAT1* gene expression was carried out by the  $\Delta$ CT method using the  $2^{-\Delta\Delta C_t}$  formula (Derveaux et al. 2010; Kozera and Rapacz 2013; Navarro et al. 2015).

## RESULTS AND DISCUSSION

### Detection and characterization of *Cm-AAT1* gene phylogenetically

The *Cm-AAT1* was successfully amplified and visualized from all melon cultivars (Figure 1.A). It was found that all four melon cultivars had the *Cm-AAT1* gene with DNA band size  $\pm$  465 bp. This indicates that the aromatic and non-aromatic melon group (Figure 1.B) have the *Cm-AAT1* gene. Furthermore, other studies explain that there are differences in the aroma profile between the aromatic and non-aromatic melons. The aromatic melons

are dominated by ester-based (ethyl butanoate, methyl 2-methyl butanoate, and ethyl 2-methyl propanoate) while non-aromatic melons dominated by C9 aliphatic aldehydes (Verzera et al. 2014). In this case, differences in aroma synthesis activity will lead to differences in the expression of the genes involved. The presence of the *CmAAT1* gene plays an important role because it remains expressed despite there are differences in the metabolic pathways. Even qualitatively, the DNA bands obtained from 'Sun Lady' and 'Luna' are thicker than from 'Hikapel' and 'Hikadi' (Figure 1). DNA bands detected as fragments of the *Cm-AAT1* gene were then sequenced.

From the results of the gene sequence alignment, we utilize to construct the filogram (Figure 2) it can be seen that the *Cm-AAT1* gene fragment in the 'Hikapel' melon has high similarity of 98% with the 'Hikadi'. This could be because the two melons are derived from the reciprocal crossing originating from the same parent i.e., 'Gama Melon Perfume' (GMP) so that the gene coding for the aromatic compounds possessed by both melons is highly dominated by genes derived from GMP cultivars (Daryono and Maryanto 2017). This is also reinforced by the distant position of the Sun Lady cultivator in the dendrogram. Sun Lady cultivar include the elders from Hikadi and Hikapel but does not produce strong aromatic compounds like GMP (Rabbani 2015). Luna cultivar is a commercial melon cultivar developed by the same company as the Sun Lady cultivar and also does not have strong aromatic properties.

The presence of the *Cm-AAT1* gene in the four melon cultivars tested shows that this gene is active in fruit organs. The difference between the *Cm-AAT1* gene owned by 'Hikapel' and 'Hikadi' cultivars as the aromatic melon group and the 'Sun Lady' and 'Luna' as a non-aromatic group might cause differences in the activity or sensitivity of the resulting alcohol acyl-transferase enzyme. The enzyme has an active site that is sensitive to certain substrates and the enzyme activity is influenced by many

factors (Rodrigues et al. 2013). Changes of the active site might cause reduced activity or sensitivity of the enzyme.

#### Analysis of *Cm-AAT1* gene expression from melon cultivars 'Hikadi' and 'Hikapel'

Analysis of *Cm-AAT1* gene expression was carried out on 2 melon cultivars namely 'Hikadi' and 'Hikapel' which can be observed qualitatively changes in aromatic traits during the fruit storage process. At the beginning of the harvest, the two cultivars showed a special fragrance which then experienced an increase in the aroma during the fruit ripening process in the storage process and then decreased after passing the peak of fruit maturity. The activity of *Cm-AAT1* and *Cm-AAT2* genes in fruit organs involved in the process of fruit ripening, in contrast to both genes, *Cm-AAT3* was expressed in vegetative organs while *Cm-AAT4* expression was very low and could not be detected (Yano et al. 2018). In this research, it is known that the 'Hikadi' melon has matured faster, which on the 7th day it had begun to undergo decay, and on the 10th day, it had been rotten. While the 'Hikapel' melon lasted longer in storage, on the 10th day it still looked fresh and flavorful (Figure 3).

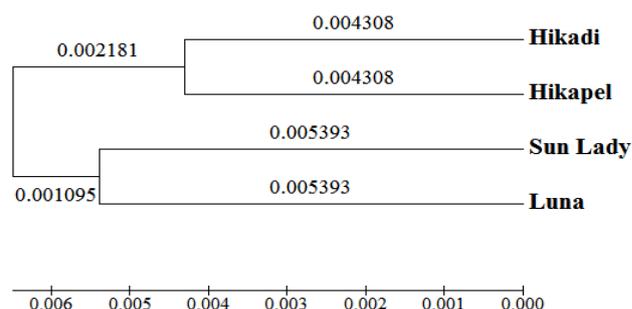


Figure 2. Filogram of the *Cm-AAT1* gene of 4 melon cultivars

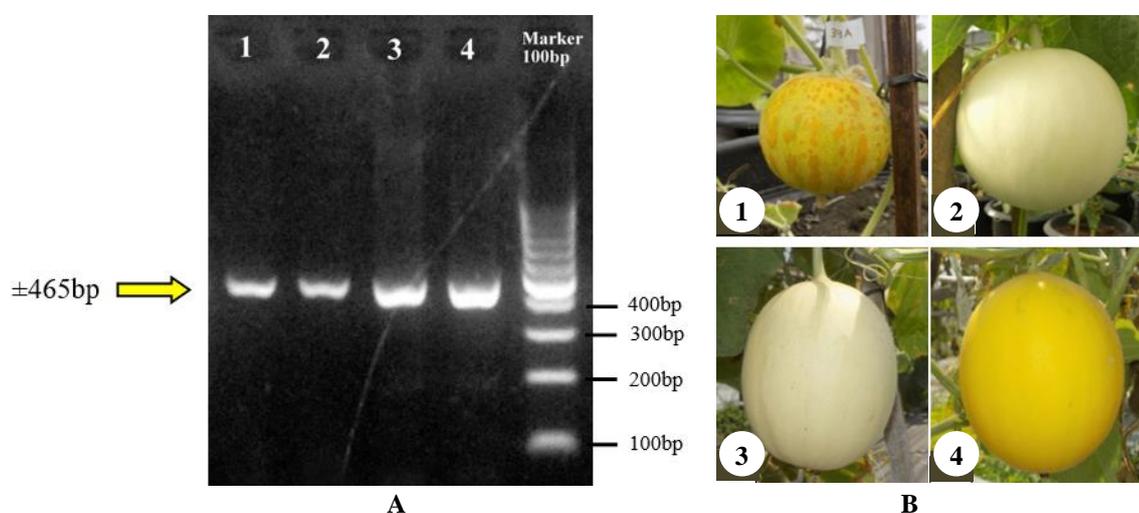


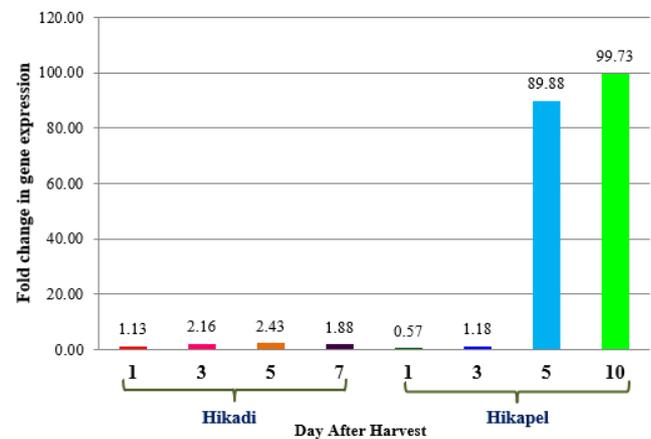
Figure 1. A. Visualization of DNA bands of *Cm-AAT1* gene fragments measuring  $\pm 465$ bp; B. Aromatic group (1-2) and non-aromatic group (3-4), from melon cultivars: 1. 'Hikadi', 2. 'Hikapel', 3. 'Sun Lady', 4. 'Luna'.

The expression of the *Cm-AATI* gene from the 'Hikadi' melon has increased slightly up to day 5 and has decreased on day 7 (Figure 4). While the 'Hikapel' melon the expression of the high *Cm-AATI* gene has increased on day 5 and still increasing until day 10. When compared to the maximum expression of the *Cm-AATI* gene between 'Hikadi' and 'Hikapel', the expression on 'Hikapel' is much higher than 'Hikadi'. On the contrary, the qualitative observation of the level of the aroma of the 'Hikadi' has a stronger aroma than the aroma of the 'Hikapel' melon. This could be due to many factors that influence the synthesis of volatile compounds during fruit development. Although esters are the main volatile compounds, there are complex mixtures that form volatile organic compounds (VOCs) in fruit (Gur et al. 2016). Variations in the aroma of unique expression on each type of melon originating from various metabolic pathways and involving many genes and enzymes (Gonda et al. 2016). The different types and composition of volatile compounds present in 'Hikapel' and 'Hikadi' melons are the cause of the expression of the *Cm-AATI* gene in the 'Hikapel' melon that is higher than the 'Hikadi' melon but the resulting aroma is stronger in the 'Hikadi' melon.

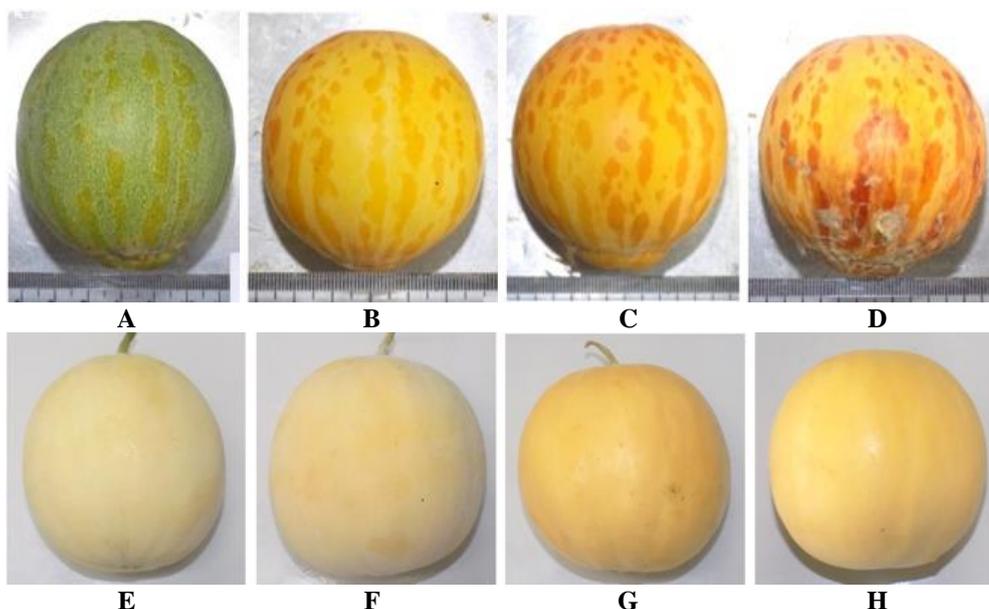
Meanwhile, the aroma character does not only depend on the expression of the *Cm-AAT* gene which plays a role in the synthesis of esters. Tang et al. (2015) reported that the enzyme Lipoxygenases (LOXs) produced by the *Cm-LOX* gene have higher activity in the formation of volatile organic compounds in aromatic melons than non-aromatic melon cultivars. *LOX* gene plays a role in converting fatty acids into aldehydes which are the main substrates in the formation of alcohol.

Aromatic compounds in melons can also originate from an overhaul of carotenoids oxidatively producing aromatic volatile apocarotenoids (Ibdah et al. 2006). The process is catalyzed by the enzyme Carotenoid Cleavage Dioxygenase (CCD) which is regulated by the *Cm-CCDI* gene. Some types

of apocarotenoid compounds have a very strong aroma although only a small amount is produced in the fruit, the aroma produced can be clearly felt by the ability of the human olfactory. In addition, the flesh of orange melons produced more apocarotenoid compounds than the flesh of melons which were green and white even though all types of melons expressed the *Cm-CCDI* gene (Milind 2011). Phenotypically in both morphology and aroma, the 'Hikadi' melon is more similar to the 'GMP', namely by the discovery of hues on the fruit skin and a more orange fruit skin color (Maryanto 2013; Retnaningati 2015), so that it indicates that the fruit skin of the 'Hikadi' contains more carotenoids than 'Hikapel'. Therefore, the stronger aroma of the 'Hikadi' melon is likely to be influenced by the carotenoid reshuffle activity to be greater apocarotenoids than the 'Hikapel'.



**Figure 4.** *Cm-AATI* gene expression of 'Hikadi' and 'Hikapel' fruits during the shelf life after harvest; The x-axis represents fruit storage of melon Hikadi (1, 3, 5, and 7 days) and Hikapel (1, 3, 5, and 10 days) after harvest.



**Figure 3.** Fruit morphology during storage, 'Hikadi' (A-D) and 'Hikapel' (E-H), AE. 1 day; BF. 3 days; CG. 5 days; D. 7 days; and H. 10 days

The 'Hikadi' is a type of climacteric melon, indicated by fast-ripening and short shelf-life. The shorter shelf life of melon 'Hikadi' rather than 'Hikapel' showed the climacteric nature of 'Hikadi' which is stronger. Pathway biosynthesis of volatile aromas begins from 5 DAA (Day After Anthesis) to 35 (DAA) and the *Cm-AAT* gene is expressed very strong at the end of fruit development (Guo et al. 2017; Yano et al. 2018). The expression of *Cm-AAT1* and *Cm-AAT2* genes was reported to decrease with storage time (Spadafora et al. 2019) and this was observed in the 'Hikadi' *CmAAT1* gene, while in 'Hikapel' the *CmAAT1* gene expression is still increased until the 10th day during storage (Figure 3). This indicates a delay in the process of biosynthesis of ester compounds so that the aroma of the 'Hikadi' melon dominates qualitatively at the time of measurement. The 'Hikapel' melon begins to enter decay on the 14th day of storage and within the range of days 10 to 14. The qualitative measurement of the 'Hikapel' melon scent was very high but the *Cm-AAT1* gene expression data was not successfully displayed. The data of this study also directed the 'Hikapel' melon as an aromatic melon with a long shelf life so that it was categorized as a semi-climacteric melon.

To conclude, the *Cm-AAT1* gene can be detected in four types of melons used in research ('Hikadi', 'Hikapel', 'Sun Lady', and 'Luna'). The expression of the *Cm-AAT1* gene in 'Hikadi' and 'Hikapel' melons increases with the fruit maturation process which is indicated by the appearance of aromas and has close evolutionary relationships as a type of aromatic melon. The 'Hikapel' melon has a long shelf life with high *Cm-AAT1* gene expression pattern so that it was categorized as a semi-climacteric melon.

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