JJBS

Volume 14, Number 2, June 2021
ISSN 1995-6673
Pages 279 – 283
https://doi.org/10.54319/jjbs/140212

Jordan Journal of Biological Sciences

Analysis of APXs and HSPs genes responsible to respond to heat stress in tomato plants cultivated in Central Sulawesi

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Received: May 14, 2020; Revised: September 11, 2020; Accepted: September 12, 2020

Abstract

Central Sulawesi is one of the areas in Indonesia located on the equator which has hot temperatures. This temperature greatly affects the growth of tomato plants. However, this plant can grow well. It is not yet known what crucial genes are responsible for the heat tolerance. Therefore, a study was conducted to reveal some genes; *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90*. Those genes are responsible for the heat tolerance. The study was carried out by the PCR method to determine the *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes. The DNA of these genes was isolated from the leaves and then cDNA was made to determine gene expression at the transcription level. Also, a qualitative analysis based on the NCBI search was used to describe the structure and function of each observed gene. The results showed that the genes; *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* were expressed in different levels in tomato plants from the Central Sulawesi region. Thus, it can be suggested that these genes have an important role in heat tolerance in the tomato plant.

Keywords: tomato, tolerant, genes, temperature, heat, temperature

1. Introduction

Tomato is one of the important food plants on earth because it functions as one of the buffering needs for humans and animals. To increase the growth and production of these plants, appropriate environmental factors are needed. Two factors including internal and external factors are very influential to limit or increase the growth and production of the tomato plants. Internal factors such as the ABA factor regulate plant growth through PGPR (Plant growth-promoting rhizobacteria) regulation (Porcel *et al.*, 2014), sugar molecules regulate metabolism and growth in wheat plants (Martínez-Barajas *et al.*, 2011). External factors also affect plant growth and development; for example, red light increases the growth and flowering of tomato plants (Cao *et al.*, 2016), fertilization using humic acid increases the productivity and quality of tomato plants (Yildirim, 2007). Meanwhile, increasing temperatures decrease biomass and metabolic activity of tomato plants (Rivero *et al.*, 2003). pH affects the absorption of nutrients in tomato plants (Wang *et al.*, 2000).

An increase in temperature results in a decrease in plant growth and development Giorno *et al.*, (2010). Furthermore, high temperatures inhibit the development of pollen, reduce pollen germination and pollen tube elongation (Astija, 2017; Firon *et al.*, 2006; Hedhly *et al.*, 2005). An important mechanism for how temperature affects the growth and development of plant cells is carried out by regulating the expression of genes (Wilson and Zang, 2009: Cordoba *et al*., 2015). Lately, this mechanism has become an interesting concern that needs to be revealed. Disclosure of the mechanism of environmental factors such as temperature regulating growth and development and productivity is clearly illustrated by Cabello *et al.*, (2014) through a cascade signal regulation mechanism that results in changes in stress, changes in hormones and changes in ROS signals. The result, changes in these signals will stimulate the modification of protein compounds such as enzymes from post-translational results (Huang and Xu, 2008; Baniwal *et al*., 2004). If explored, these protein compounds or enzymes carry out activities to decipher specific compounds. The results of the enzyme activity can then affect its own activity or act as an inhibitor or even a back signal to stop the enzyme formation process (Baniwal *et al*., 2004). Stopping enzyme productivity will inhibit the process of gene expression at the translation and transcription stages.

As for the temperature factor, many researchers have reported that temperature signals from outside will be transmitted to regulate metabolism. According to Pressman *et al.* (2012), temperature regulates growth of tomato plant through inhibition of sugar metabolism due to decreased activity of enzymes that play a role in carbohydrate metabolism. Temperature greatly influences changes in sugar metabolism and subsequently the molecules from the results of metabolism act as signals to regulate the expression of genes (Ponnu *et al*., 2011). Later, Krasensky and Jonak (2012) revealed that temperature can reduce plant growth and productivity because it changes morphological and physiological adaptations through changes in gene expression. Also, a research conducted by Frank *et al.* (2009) had explained that temperature can increase the activity of HSPs protein (heat shock proteins). HSPs are proteins that play a role in responding to heat so that plants survive. These HSPs are

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produced from the expression of genes from the HSPs family. The results of studies have found many genes from this family such as *HSP17* (Rang *et al.*, 2011), *HSP60* (Sheoran *et al.*, 2007), *HSP70* (Ge *et al.*, 2011), *HSP90* (Astija, 2015). Genes from other families that also play a role in responding to heat stress are the peroxidase gene (APXs), for example genes belonging to the peroxidase family is widely studied being *APX1, APX2* (Astija, 2015).

Recently, the characteristics of the development of tomato plants in the vegetative phase have been studied (Astija and Musdalifah, 2018). Likewise, the development of reproduction in the development of pollen was investigated (Astija, 2017). Another thing that has been investigated is that many genes have been identified and proven to play a role in responding to heat stress in tomato plants, such as *HSP 17.4-CII, HSP 17.6-CII, HSP70, HSP90, APX1, APX2* genes (Bita and Gerats, 2013). However, it is not known yet whether the genes are identified in tomato plant cultivated in Central Sulawesi Region. Therefore, those are identified their presences in the tomato plant. The existence of these genes was analyzed regarding the structure of nucleotides and their amino acids and the similarity between one another. All of these are to be used as candidates for important genes for crops in Central Sulawesi that have hot temperatures. Furthermore, this project can be used as a model of food crops suitable for cultivation in Central Sulawesi or to be developed into GMO crops that are resistant to heat.

2. Method

This study used a sample consisting of three leaf pieces of tomato plant each, originating from the Sidera Region. The sample was taken using "Pore Cut". Leaf sample was stored in the "Ice Box" for extract. The extraction process was conducted at the Biotechnology Laboratory of The University of Indonesia Education. The steps for extraction, gene identification by PCR and measurement of mRNA are described below.

2.1. Genomic DNA extraction and Genotific determination of DNA using PCR

Genomic DNA was extracted from leaves. This was to verify and to determine the target genes. The procedure was done using CTAB (hexadecyltrimethylammonium bromide) as described by (Astija, 2017).

2.2. Expression assay of APXs and HSPs genesRNA extract

RNA was isolated from 5-10 mg of material/sample and work procedures were carried out following the instructions set out in the "Qiagen RNeasy Plant Mini Kit (50)". The total RNA concentration was measured by a spectrophotometer (NanoDrop 1000, Thermo scientific) on waves of 230nm, 260 nm, and 280 nm. A total of 1 µg of total RNA per sample was used for cDNA synthesis.

2.3. Synthesis of cDNA

cDNA synthesis was begun with RNA extraction. The extraction is purified by removing DNA contamination by using DNase (Promega, Cat.M6101) by adding the following ingredients to the sample:

7 µL DEPC H_2O

1 µL RQ1 RNase-Free DNase 10X Reaction Buffer

1 µL RQ1 RNase-Free DNase (1 u / µL)

Then the solution was incubated at 25 ^oC for 15 minutes. Then 1 µL of 25 mM EDTA was added and incubated again at 65 ^oC for 10 minutes. A single cDNA chain was obtained from RNA synthesis by adding 1 µL oligo dT20 and 1 µL from 10 mM dNTP carried out at 65 ^oC for 5 minutes. Reverse transcription (Reverse transcription) is continued by adding a solution consisting of:

4 µL 5X First-strand Buffer

1 µL 0.1 M DTT

1 µL of SuperScript TM III RT (200 units / µl)

1 µL RNase OUT TM recombinant RNase inhibitor (Cat. 10777-019, 40 units / µl)

Reverse transcription is carried out at 50 ^oC for 45 minutes and continued at 70 ^oC for 15 minutes. The formed cDNA was confirmed using PCR using a reaction solution consisting of:

6.54 µL H_2O

1.05 µL50 mM $MgCl_2$

1.5 µL 10XPCR Buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl)

0.06 µL Taq DNA polymerase (5 u / µL)

0.75 µlLSYBGreen (1: 20000)

0.6 µL 5 mM dNTP Mix

0.375 µL FPrimer gene

0.375 µL RPrimer gene

3.75 µL cDNA

The cycle conditions that are treated include: initial denaturation at 94 ^oC for 10 minutes, denaturation at 94 ^oC for 20 seconds, attachment at 60 ^oC for 20 seconds, elongation at 72 ^oC for 20 seconds, final elongation at 68 ^oC for 1 second, and hold 16. cDNA is doubled by 45 cycles. The product is separated and measured by electrophoresis on 1.5% agarose gel stained with ethidium bromide. Electrophoresis was set at 100v for 15 minutes. The gel is visualized with the GelDoc XR Imaging System (BIORAD) and Quantity One Version 4.6.3 software.

2.4. Analysis of gene expression using Real-Time PCR

Quantitative PCR is performed using the same reaction mixture used in PCR as described above with the addition of primers from certain target genes including *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90*. The primers are designed using PRIMERE-BLAST software presented in table 1. The size of the resulting amplicon is then linked to the tomato sequence. Gene expression data were calculated with Corbett Rotor-Gene 6000. All amplifications were normalized to the CAC (Clathrin Adapter complex) gene.

Data from the average value of gene expression obtained from three technical replications and 3 plant biological replications were analyzed by one-way ANOVA from software XL-Stat 2018 or JMP V11.

Table1. Primers of the *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes designed using PRIMERE-BLAST software

Gene name	Product size (bp)	Accession number (http://www.ncbi.nlm.nih.gov/)	Primer sequence (5' – 3')	TM (°C)
HSP 17.4-CII	103	NM001247201	FP: ACTGTTCAGAAGCTGCCTCC	59.9
			RP: TCAAAACAGAGCAAGAAAACAGAGT	59.6
HSP 17.6-CII	151	NM001246984	FP: TCCTGAGCCAAAGAAACCCA	59.2
			RP: ACAGACGTGAAAACATAGCAGAA	58.6
HSP70	201	NM001247562	FP: AGTGTAAAGCAATGGCCGGA	59.9
			RP: GGGCGACCTGATTCTTAGCA	59.8
HSP90	205	NM001247510	FP: TCAGCAATTCTTCCGATGCTCT	60.1
			RP: TCCTTGGTTCCTGACCTTGC	59.9
APX1	288	NM001247853.1	FP: CACCTACTAGAACCCTTCTCTTTCT	59.2
			RP: AGAGTGCCATGCAAGACGGA	61.8
APX2	170	NM001247859	FP: TGCTGCGGATGAAGATGCC	60.8
			RP: AACGATATCCAACAATTCCAGCA	58.7

3. Result

Based on tracing through the NCBI website, we found that the *APX1* gene was located on chromosome 6 with the nucleotide position from 181740 to 185143 bp. The *APX1* gene has 1117 pairs of nucleotides that were translated into a protein with 250 amino acids. Similarly, the *APX2* gene was located in the same chromosome with the *APX1* gene, but the nucleotide was located from 170150 to 173336 bp. The *APX2* gene consists of 1197 pairs of the nucleotides and produces proteins with 250 amino acid numbers.

For the *HSPS*s group, we observed that the *HSP17.4* gene was located on chromosome 8 in which the nucleotide locus from 50976534 to 50977196 bp, or the number of nucleotides was 663 nitrogen base pairs. The *HSP17.4* gene expresses a protein with a total of 155 amino acids. Meanwhile, the *HSP17.6* gene was found on chromosome 6 at locus 47701113 to 47701916 bp so that it had 822 nucleotide pairs of nitrogen bases. The *HSP17.6* gene expresses a protein composed of 154 amino acids. Another *HSPs* is the *HSP70* gene that was found on chromosome 11 from 10036518 to 10040967 nucleotides or 1980 pairs of nitrogen bases. The gene can express a protein with 692 amino acids. The last *HSP* was the *HSP90* gene that was located at the same chromosome with *HSP17.6* but the location of the nucleotide was from 25893824 to 25897169 bp. The nucleotide sequence arranges a protein with 704 amino acids.

The *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* were then tested on samples of tomato plants cultivated in Central Sulawesi. The tests using PCR, it was found that DNA from the *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes were obtained as shown by the DNA band in Figure1.

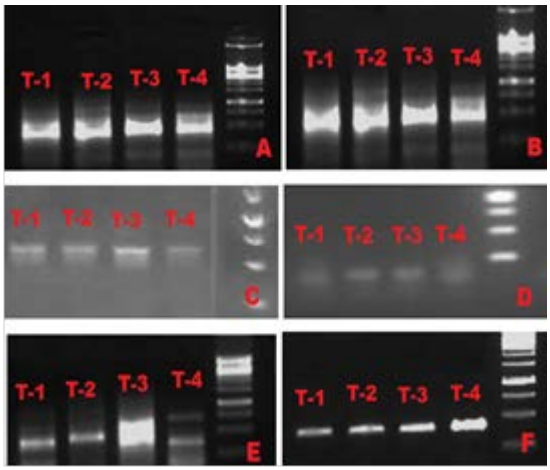
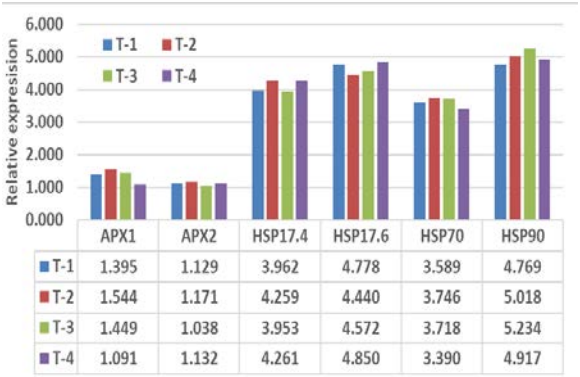


Figure 1. DNA from *APX1* (A), *APX2* (B), *HSP17.4* (C), *HSP17.6* (D), *HSP70* (E), *HSP90* (F) genes isolated from 4 tomato plants (T- 1, T-2, T-3, T-4) cultivated in Central Sulawesi.

Figure 1 shows that the four tomato plants cultivated by people in Central Sulawesi have genes that are responsible for heat tolerance including *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes. Furthermore, to find out whether the genes are expressed at the transcriptional stage in the form of mRNA, the gene expression testing is carried out by qPCR. In the cDNA testing phase, (unpublished data) the DNA bands were similar to the DNA bands in Figure 19.

The results of mRNA measurements via RT-qPCR of the six genes in all four plant samples are presented in Figure 2.



	APX1	APX2	HSP17.4	HSP17.6	HSP70	HSP90
T-1	1.395	1.129	3.962	4.778	3.589	4.769
T-2	1.544	1.171	4.259	4.440	3.746	5.018
T-3	1.449	1.038	3.953	4.572	3.718	5.234
T-4	1.091	1.132	4.261	4.850	3.390	4.917

Figure 2. Expression of *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes from four samples of tomato plants (T-1, T-2, T-3, T-4) cultivated in Central Sulawesi. The expression of peroxidase genes (*APX1, APX2*) is lower than that of *HSPs* genes.

4. Discussion

Central Sulawesi, located in equatorial regions that have extreme temperatures, has an average daily temperature of 37 ° C. Normally, plants that grow in areas with hot temperatures grow poorly as expressed by Krasensky and Jonak (2012) who reported that temperature can reduce plant growth and productivity because it changes morphological and physiological adaptations through changes in gene expression. However, different things happen in the Central Sulawesi, and tomato plants can grow and produce tomatoes properly. From the results of testing using the PCR method, it was found that tomato plants in the region have *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes. The six genes were identified based on the appearance of DNA bands through the electrophoresis process (Figure 1). DNA from all six genes was detected in all four plant samples from the region. Thus, it can be said that the DNAs are *APX1, APX2, HSP17.4, HSP17.6, HSP70, HSP90* genes.

DNA from the results obtained is convinced that the six genes are in tomato plants. The six genes have also previously been reported in other tomato plants (Sheoran *et al*., 2007). Besides, from search results on the NCBI website, it was found that the six genes were also found. The interesting thing about the search results is that the two genes of the peroxidase group (*APXs*), *APX1*, and *APX2* are located in tandem in one chromosome, chromosome 6. These two genes have different nucleotide sequences and sequences (NCBI Website). The composition and sequence of the nucleotides of the two genes have a similarity rate of 93%.

Tomato plants from the Central Sulawesi can grow well because they not only have two peroxidase genes (APXs), *APX1* and *APX2*, but both of these genes have been transcripted into mRNA and translated into proteins. The results were based on reverse transcription that is mRNA is reversed back into DNA to obtain cDNA from both genes. Furthermore, measurement of the transcript results using RT-qPCR obtained relatively similar mRNA expressions between the *APX1* and *APX2* genes (Figure 2). The relative value of the mRNA expression obtained illustrates that both genes have the same amount of transcription results from two different DNAs but are located in one chromosome, namely chromosome 6. From the results of further searching, the expression of these two genes does not stop until mRNA but the expression reaches a level of translation into protein. When comparing with relation to amino acids, the amino acids of the two genes have a slightly different arrangement of amino acids, which is a 96% similarity level (NCBI Website). Furthermore, the protein expressed from these two genes has reached post-transformation in the form of the enzyme ascorbic acid oxidase. The enzyme has been reported that an important substance that plays a role in oxidizing ascorbic acid to dehydroascorbic acid. Furthermore, dehydroascorbic acid is converted to 2,3 dico-1-gluconate acid. This is useful as a defense in responding to heat.

Tomato plants from the Central Sulawesi are also able to grow well because they have 4 genes responsible for responding to hot temperatures, including *HSP17.4, HSP17.6, HSP70*, and *HSP90* as shown by DNA bands from the four plants used as samples (Figure 1). These results indicate that tomato plants cultivated by people in the region have *HSPs* genes that play an important role in defending plants from heat. The role of HSPs in plant defense against heat has been widely reported (Wang *et al*., 2000; Chauhan *et al*., 2011; Al-Whaibi, 2011; Van Ooijen *et al*., 2010; Giorno *et al*., 2010; Baniwal *et al*., 2004). The mechanism by which HSPs play an important role in responding to hot temperatures is still unclear. However, Al-Whaibi (2011) has suggested that hot temperatures cause shrinkage of protein structures. Circumstances will then cause the protein to become damaged or not functioning. This event does not occur if there is a protein from the expression of *HSPs*. HSP proteins will be associated to prevent structural proteins from causing damage.

The results of this research are that all four genes have been expressed in all four plants (Figure 2). The gene expression shown is an expression at the transcription stage, which is a copy of one of the DNA chains of the genes into mRNA. The mRNAs from all four genes from all four plants exhibit similar levels of expression (Figure 2). Once compared with the level of expression of peroxidase genes, *HSPs* genes have higher levels of expression. This means that all four *HSPs* genes play a greater role in the defense of heat in tomato plants in the region compared to both genes of peroxidase. No comparison has been reported between the two gene groups, *APXs* and *HSPs*. Therefore, the results of this study are important to follow up on the important role of these *HSPs* genes and other *HSPs* genes. Based on the results of this study, it is also possible that the expression of the four *HSPs* genes has been expressed at the translation stage. Unfortunately, this study did not assay the proteins from all four genes. However, the results of study based on tracing through the NCBI website obtained amino acids from proteins in the four genes (NCBI Website). The number of amino acids for *HSP17.4, HSP17.6, HSP70*, and *HSP90* can be shown in the NCBI Website. Two genes, *HSP17.6* and *HSP90*, are known to be located on the same chromosome which is chromosome 6. This also means one chromosome with the *APX1* and *APX2* genes, while the two HSPs genes, *HSP17.4* and *HSP70*, are located on different chromosomes, being on chromosomes 7 and 10 (NCBI Website). Thus, chromosome 6 is important in tomato plants because it contains four important genes for the defense of the plant

to heat stress. Based on alignment dan phylogenetic using Clustal Omega of the four genes illustrated that *HSP17.4* and *HSP17.6* genes have a close relationship compared to the other genes. However, the two genes are located in a different chromosome in which *HSP17.6* gene is in chromosome 6 but *HSP17.4* is in chromosome 7. Thus, genes located on the same chromosome do not determine a relationship of the genes. In other words, the genes are independently expressed for certain responsibly.

5. Conclusion

The genes consisting of *APX1, APX2HSP17.4, HSP17.6, HSP70,* and *HSP90* are responsible genes for heat stress tolerance in tomato plants cultivated in Central Sulawesi in which those genes are expressed with different level of mRNA.

Acknowledgments

We thank FKIP Tadulako University for supporting and providing the facilities for the research.

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