Msc Plant Breeding Thesis Report (PBR-80424):

THE ROLE OF CELL WALL DEGRADATION GENES IN THE POSTHARVEST SOFTENING OF PEPPER FRUIT

(Correlating Candidate Gene Expression to Postharvest Softening)

(Part of RC for Biotechnology, Indonesian Institute of Science (LIPI) project with a title: Elucidation of Molecular Basis on Fruit Softening Delay in Pepper Fruits to Reduce Post-Harvest Loses)

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The Role of Cell Wall Degradation Genes in the Postharvest Softening of Pepper Fruit – Correlating Candidate Gene Expression to Postharvest Softening

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Summary

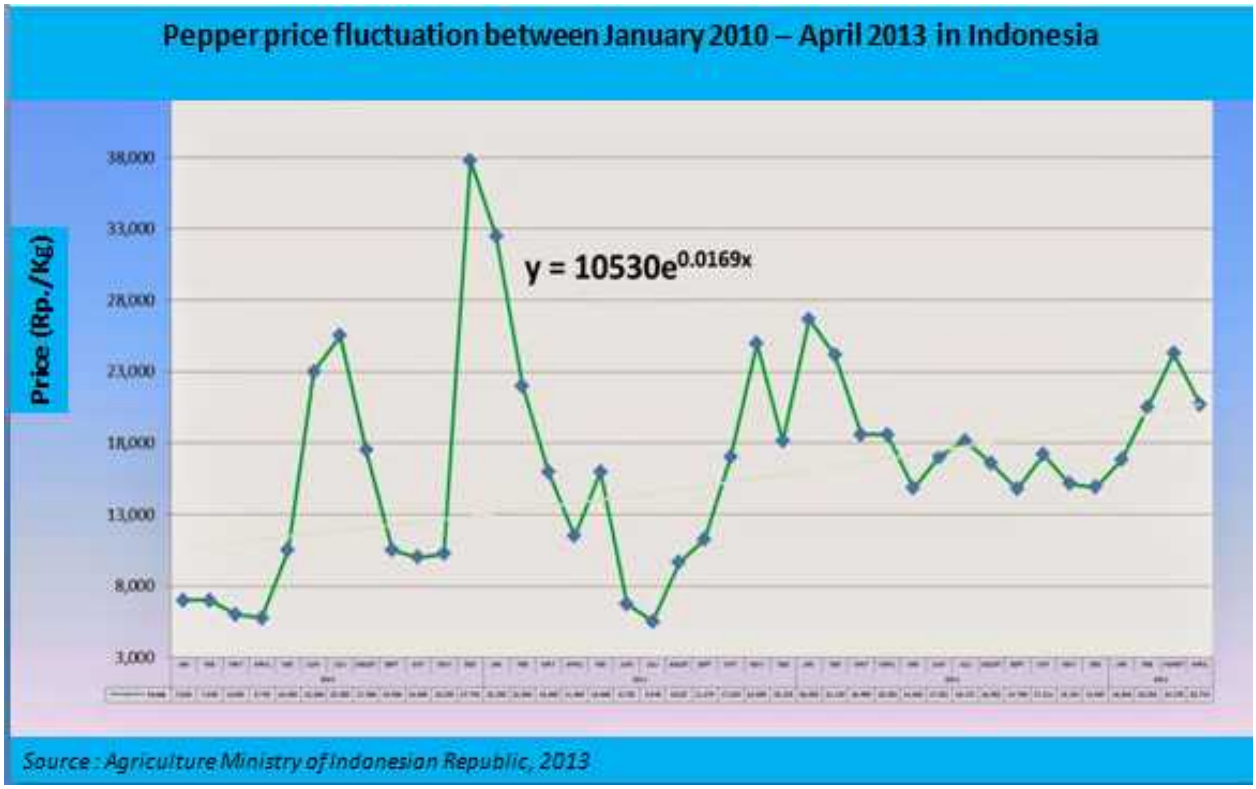
Fruit softening because of postharvest water-loss becomes one of the main problems in pepper (*Capsicum spp*.). Researchers suggested that fruit softening is strongly correlated with Cell Wall Degradation (CWD). The objective of this research is to find candidate genes that may play a role in regulating fruit-softening delay in peppers by linking the fruit-softening characters with relative expressions of Polygalacturonase (PG), Pectinesterase (PE) Pectate Lyase (PL), Beta-Galactosidase and Polygalacturonase Inhibitor Protein (PIP) in four accessions. The result showed that after 14 days of storage, around 38% - 60% of the fruit weight of four genotypes was lost. Respectively, 67%-83% fruit had lost its fresh weight after 30 days of storage. Generally, the relative expressions of six genes were different in unripe and ripe fruits in all genotypes. Two of pectinesterases genes showed different expression patterns. PIP roles were not so clear as well. Relative expressions of the PG gene in SSP were very low at unripe and ripe stages compared to Kopay and Princess. Also, Sret showed low levels of PG expressions. Overall, the relative expressions of Beta galactosidase and PL were not highly expressed compared to others. Pepper fruit softening is an accumulation result of cell to cell adhesion as a consequence of pectin degradation and cell wall dissolution. This mechanism is a complex process which involves various change reactions in different polymers. For every gene, significant differences among CWD mechanisms exist among species and even among cultivars from similar species. Although six genes in this experiment were expressed in both stages of all genotypes, they were expressed in different levels and patterns. Those differences may give a significant effect on the storage quality of each genotype.

Keywords: Fruit softening, Cell Wall Degradation, Polygalacturonase, Pectinesterase, Pectate Lyase, Beta-Galactosidase and Polygalacturonase Inhibitor Protein

1. Introduction

1.1. Pepper

Pepper (*Capsicum* spp.) is an agricultural crop belonging to the family *Solanaceae*. The cultivated type is classified into five major groups based on their fruit and flower characteristics: *C. annuum, C. fructescens, C. baccatum, C. chinense, and C. pubescens*[1]. It has a specific pungency and aroma which makes people favor them. Furthermore, a wide range of pepper is used as a fresh fruit, spice or coloring agent, creating a high commercial value for this fruit [2]. Pepper is positioned as one of the most important agriculture commodities worldwide, especially in tropic and sub-tropic countries. In 2012, Indonesia was among the top ten pepper producing countries, with a share of 13% of the total amount of pepper [3]. Despite this high productivity, pepper price was varying over years, especially in the rainy season. The main reason for that condition is low pepper production in rainy season could not fulfill stable pepper demand over season [4].



1.2. Pepper softening and Cell Wall Degradation (CWD) enzyme

Furthermore, fruit softening because of postharvest water-loss becomes one of the main problems in pepper (*Capsicum spp*.)[5]. Researchers suggested that fruit softening is strongly correlated with Cell Wall Degradation (CWD) [6]. Mostly, the CWD's research was focused on the pectin degradation enzyme. Several pectolytic enzymes have been implicated to play a role in fruit softening [7] such as Polygalacturonase (PG), Pectate Lyase (PL), Beta-Galactosidase (B.Gal) and Pectinesterase (PE) [8, 9]. Some of the underlying genes have been used in biotechnological approaches to delay the softening process.

The first enzyme was studied for a long time due to the assumption that this enzyme has a principle role in softening fruit [7]. PG activity was known to have a positive correlation with fruit softening initiation for some fruits such as papaya and guava[10, 11]. However, the PG pattern is still unclear. Formerly, a transgenic tomato fruit, resulting from antisense suppression of PG mRNA, still softened like a common fruit [12].

The second enzyme is an enzyme that has an important role in pectin degradation [13]. Previously, the PL enzyme was studied as a secreted pathogen enzyme which is used to macerate plant tissue [12]. However, the discovery of PL-like genes in the Arabidopsis genome led to the new idea that this enzyme might play an important role in fruit ripening as well [12].

The third enzyme is Beta-Galactosidase (B.Gal). It is a pectin-modifying enzyme that induced fruit softening as well. In bell pepper, B. Gal activity was increased up to five times during fruit ripening. This activity can lower fruit firmness through pectin hydrolysis [8].

Conversely, the fourth enzyme plays a different role regarding the fruit softening process. The PE enzyme induces a pectin demethylation process in the fruit cell wall. Through this process, calcium pectate is produced, leading to firmer fruit texture [9]. It was found that PE activity decreased during the ripening process in mango, guava and strawberry [10].

Although delaying fruit softening in pepper fruit is becoming an important breeding target, there is limited knowledge of the genetic regulation of the softening process in pepper fruit. Therefore, a genomic approach is needed to study this problem.

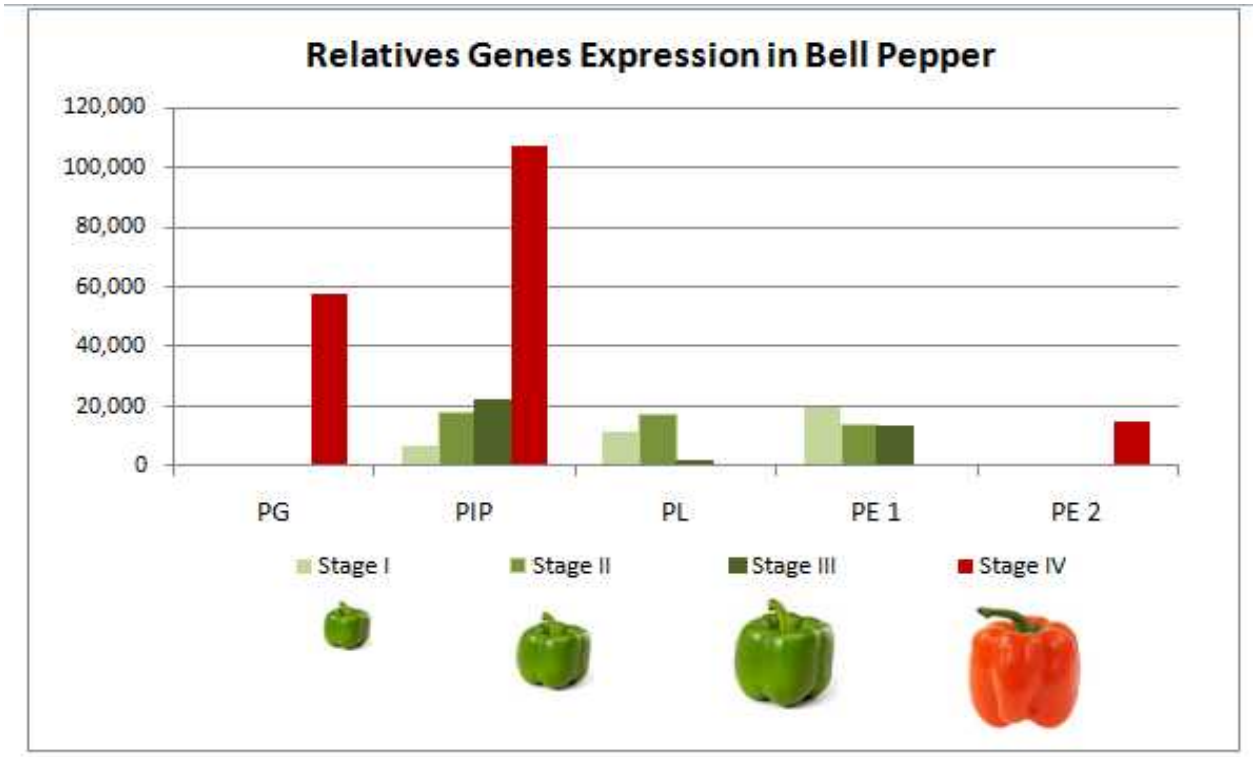


Figure 1. Relatives gene expression of five candidate genes of bell pepper in four ripening stages (Arnaud Bovy, press.com)

1.3. Pectin Pathway

A pectin is a group of complex polysaccharides, which are highly abundant in the plant's primary cell wall. Three main pectic polysaccharides were identified: homogalacturonan (HG), substituted galacturonan (SG) and rhamnogalacturonan- I (HG-I) [14]. During the cellulose metabolism process, pectin is degraded into pectate by pectinesterase. Subsequently, pectate degradation happens in two ways which have an opposite genes regulation to each other, i.e. into galacturonate by galactosidase and into 5-dehydro-4-deoxy-d-glucuronate by enzyme PL (figure 2.) [15].

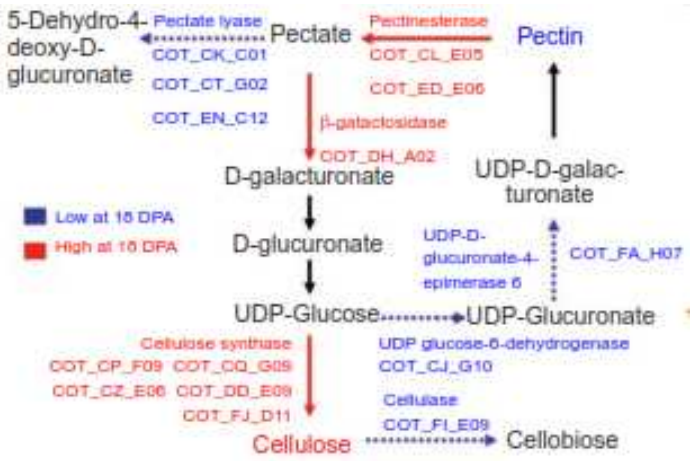


Figure 2.Pectin in cellulose metabolism pathway [15]

Aside from that, another compound named oligogalacturonide acid could be produced from pectin through PG's activities that digest homogalacturonan in the plant cell wall [16]. On the other hand, Polygalacturonase Inhibitor Protein (PIP) has been reported to provide a defense PG-effect. This enzyme has the capability to bind pectin in order to protect this compound from the degradation effect of PG [17, 18].

A preliminary study was done in RC for Biotechnology, at the Indonesian Institute of Science (LIPI). Some pepper accessions were observed for postharvest storage quality. One of the accessioned fruit stayed in a relatively firm condition up to 30 days after harvesting time.

1.4. Objective

The objective of this research is to find a candidate gene that may play a role in regulating fruit-softening delay in peppers by linking the fruit-softening characters with relative expressions of Polygalacturonase (PG), Pectinesterase (PE) pectate Lyase (PL), Beta-Galactosidase and Polygalacturonase Inhibitor Protein (PIP) in four accessions.

1.5. Hypothesis

1. At least one pepper accession have a higher storage quality compared to other accessions
2. At least one candidate gene playing a role in fruit softening delay

2. Material and Method

2.1. Location and time

This experiment was done in Laboratory of Plant Molecular Genetics and Biosynthetic Pathway Alteration, RC for Biotechnology (Indonesian Institute of Science (LIPI) from March 14 – June 17, 2016. It was located in Cibinong, West Java, Indonesia.

2.2. Designing Primers for Selected Genes

The appropriate primer is an important requirement for the RT-qPCR application. To assess that requirement, we design a gene – specific PCR primer using the Sole Genomic Network (SGN) and Primer3Plus online application using a gene list for pepper ripening from Bell pepper as the reference. We determine some standard criteria for the six targeted genes mentioned above i.e. primer Tm is between 55 and 65°C, GC content is between 40 and 60%, length is around 18 - 25 bp, Δ tm between forward and reverse primer is not more than 5°C. We also choose a primer which has a closer position to 3' end in order to obtain perfect base-pairing between the template sequence and the 3' end of the primer [19, 20].

2.3. Sample Collection

A total of four pepper genotypes from RC for Biotechnology (Indonesian Institute of Science (LIPI)) collection were selected as our samples. Two genotypes were selected based on their variation in firmness condition (SSP and Kopay) and another two (Princess and Sret) were selected from a new collection which has not been observed before. For the fruit firmness characteristic, we combine the data from the preliminary study which is done by Dr. Wahyuni with the new data that we got during this experiment. Subsequently, the fruit samples were harvested and collected in separated labeled plastic bags. We used fruit of three biological replicates for storage observation and 5 - 10 fruits for RNA isolation. The pepper plant of whichsamples were harvested,is shown in figure 3 and 4.

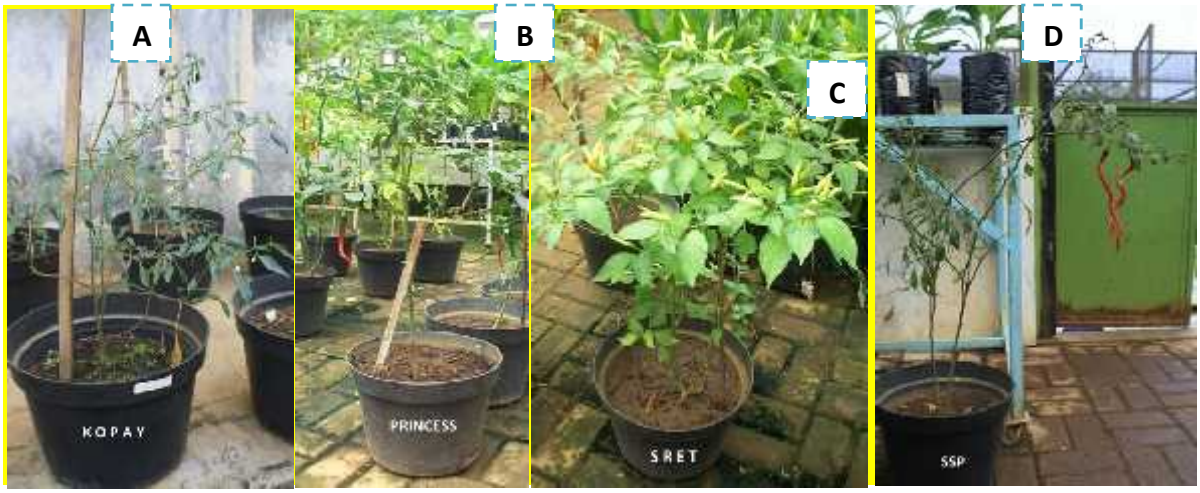


Figure 3. Pepper plant of four genotypes after sample collection: (A) Kopay, (B) Princess, (C) Sret and (D) SSP

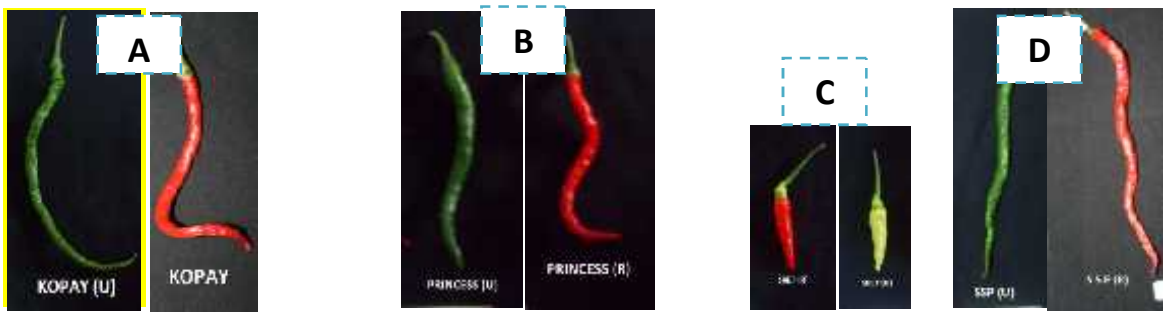


Figure 4. Pepper fruit of four genotypes after sample collection: (A) Kopay, (B) Princess, (C) Sret and (D) SSP

2.4. Fruit Weight Loss

In addition, we also performed analysis on water-loss in fruits post-harvest (Fig. 2). We use relative fruit weight to access the storage quality of the fruit. This aims to see the fruit weight losses/freshness byhow much water is lost at the post-harvest stage. Harvested fruits were washed with water, air-dried and placed in plastic boxes. Fruit weight loss was accessed as the post-harvest softening indicator. Fruit weight loss was observed in two different chamber conditions: under room temperature (± 27°C) and by 20 °C. Fruit weighing was accessed at 0, 14 and 30 days after harvest. The relative weight was accessed by comparing real time fruit weight to fruit weight at 0 days after harvest (%). The resulted data was confirmed using ANOVA and LSD.

2.5. Sample Preparation

Fruits were processed directly after harvest. Fruit tissue dissociation was started by opening the fruit and lifting the seeds from it. Afterwards, the part which was left,was sliced and frozen in a liquid

nitrogen storage. Tissues were solidified in fluid nitrogen promptly after harvest, grounded to a fine powder using mortar and pestle and stored in 50 ml corning tubes at - 80°C until utilized for the RNA isolation.

2.6. Total RNA Isolation

Total RNA was isolated from pericarp tissues from two different ripening stages i.e. unripe (U) and ripe (R) stages. Absolute RNA was isolated using the Geneaid tool kit as indicated by the manufacturer's instruction (appendix 1). The isolation was achieved from 50 mg solidified grounded pericarp of unripe and ripe stages which was replicated three times for each sample. The quality and amount of absolute RNA were measured by Nanophotometer from IMPLEN. The total RNA (500 ng/µl) from each sample was separated in 1.5% w/v agarose gel. (SYBR^{TM} safe DNA gel stain was used for staining RNA in gels). We used ATTO submerged mini type WSE-1710 which was set at 100 Volt for 30 minutes. From the combination result of Nanophotometer measurement and gel separation, we chose the best samples to be amplified at c DNA synthesize process. Isolated RNA was kept in 1,5 ml microtubes at - 80°C.

2.7. c DNA Synthesize

Verso cDNA synthesis kit from Thermo Scientific was used to synthesize cDNA from RNA (The kit contains RT enhancer which is used to remove DNA contamination). 500 ng//µl total RNA was processed as the starting material according to the manufacturer's instructions.

2.8. q RT - PCR Analysis for Selected Genes in Local Peppers.

Preliminary study

Before carried out the real Real-Time Quantitative Polymerase Chain Reaction (q RT – PCR), some preliminary study were conducted i.e. determine cDNA amount i.e. 1 µl, 2 µl, 3 µl and 4 µl. Besides that, the annealing temperature should be set for all of the gene primers. Three annealing temperatures alternatives were observed i.e. 51°C, 58.5°C and 57°C. The resulted DNA was run in agarose gel w/v 1%. Among the three temperatures only at 57°C, PCR reaction for the whole genes could perform. Two housekeeping genes i.e. Alfa-tubulin and Ubiquitin were included in two different sample stages analysis. Of two different housekeeping genes, Alfa-tubulin was chosen as references genes in order to normalized targeted gene expression. This preliminary observation results is a combination set of 2 µl cDNA, 57°C annealing temperature and Alfa-tubulin as reference genes. The thermal setting for RT-qPCR was shown in figure 5.

Data analysis

Relatives expressions of each candidate gene were analysed using multivariate analysis included at the SIMCA 14.1 software. Pre-treatment, quantitative RT-PCR was carried out using KAPA SYBR® FAST qPCR Kit Master Mix (2X) Universal and Eco Real-Time PCR from Illumina with the thermal setting as shown in figure 2.3. Gene expressions were calculated based on the resulted data by using Relative Gene Expression (RGE) formula:

$$RGE = 2^{-\Delta\overline{C_T}}$$ **[21]**

RGE value was transformed by log 2 and mean centering. The transformed data were subjected into Principal Component Analysis and Hierachical Cluster Analysis (HCA). Both the analysis methods were performed using the SIMCA software. To test the dendogram reliability produced by HCA, Jack-knife (JK) method was performed as well. Correlation analysis between fruit weight after 30 days of storage and RGE were performed by Pearson correlation coefficient.

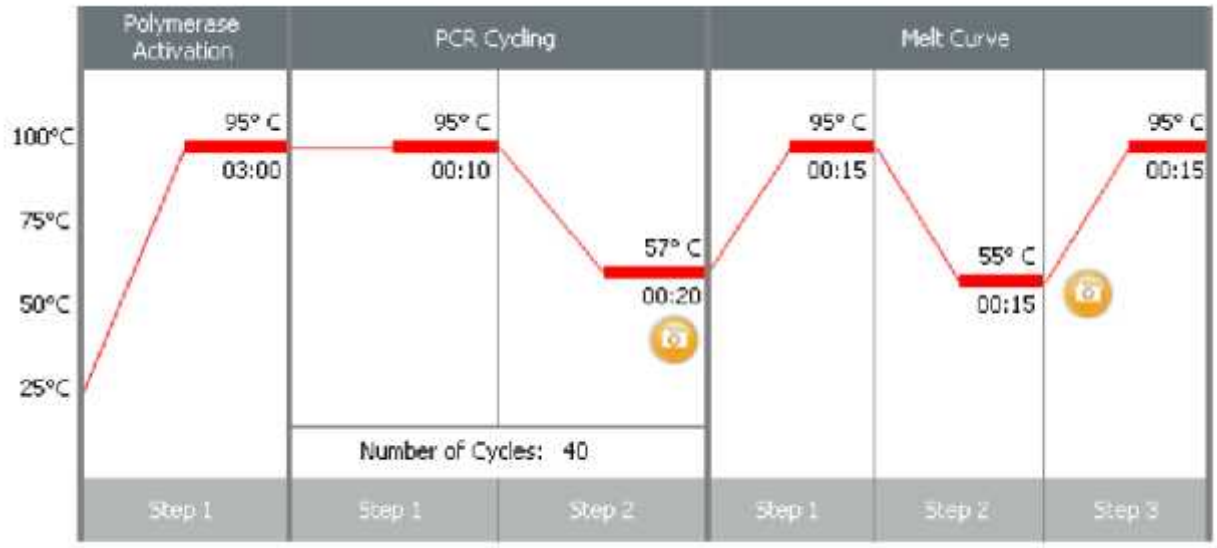


Figure 5. Thermal setting for q RT-PCR

3. Results and Discussions

3.1. Relative fruit weight after storage

The visualization of pepper fruit after 30 days of storage is shown in figure 6. The genotypes Sret and SSP have a fresher performance than others. The relative fruit weight 0, 14 and 30 days after harvest are shown in Figure 6. All genotypes showed weight losses at 14 and 30 days after harvest in both temperature treatments. Relative weight losses occur slowly upon storage treatment. After 14 days of storage, around 38% - 60% of the fruit weight of four genotypes was lost. Respectively, 67%-83% fruit had lost its fresh weight after 30 days of storage. Post hoc comparisons using the Fisher LSD test revealed that among all genotypes, only genotype Sret has the highest fruit fresh weight compared to others. In average, the fruit weight of this genotype remains at 31.469% point. Overall, the visualization of fruit performance and relative fruit weight after storage seems to show a positive correlation.

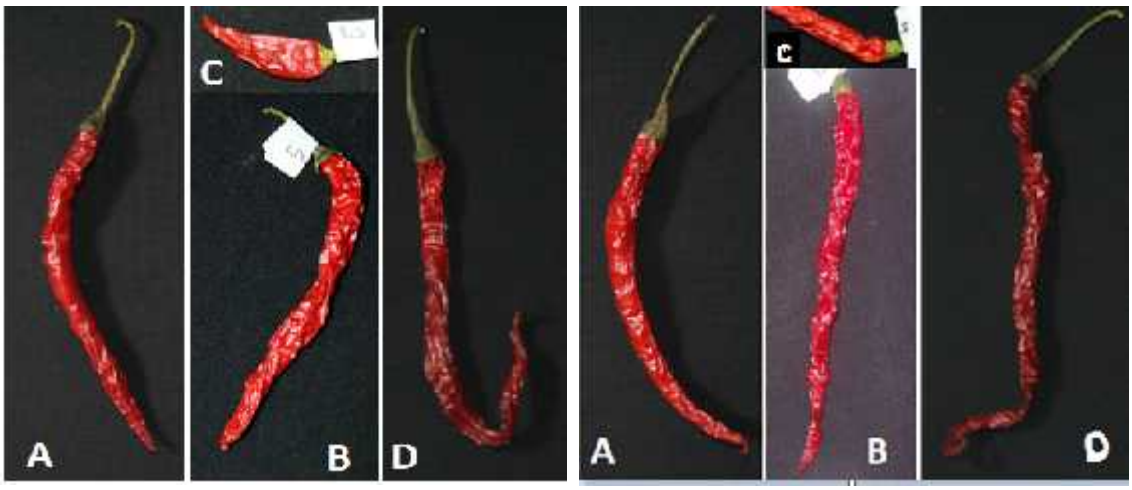


Figure 6. Pepper fruit of four genotypes after 30 days of storage in 20°C and 27°C. A = SSP, B= Princess,C= Kopay, D=Sret

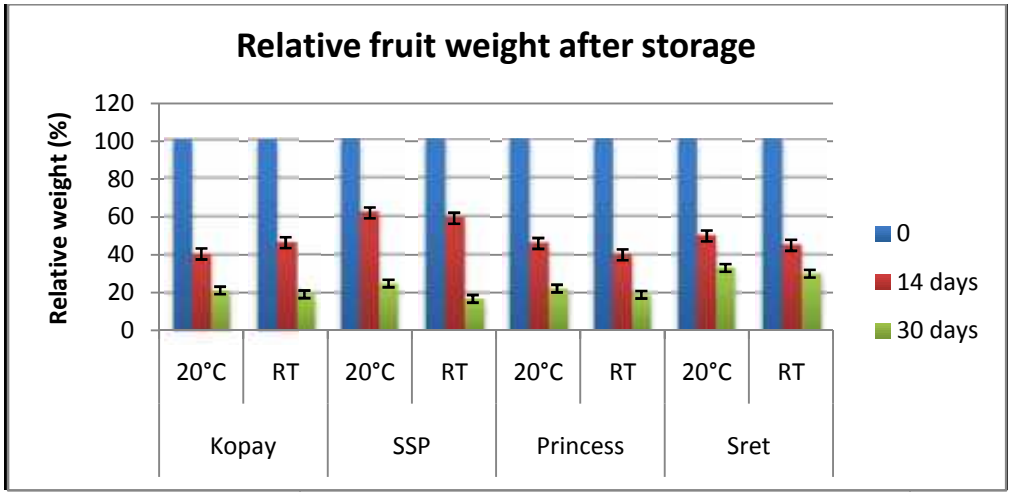


Figure 7. Relative fruit weight of four pepper accessions in two different storage temperatures

Table 1. Relative fruit weight mean of four pepper accessions in two different storage temperatures at 30 days after harvest

Genotype	Mean *
Kopay	20.108 a
SSP	20.755 a
Princess	20.493 a
Sret	31.469 b
Temperature	Mean *
20°C	25.208 b
Room Temperature	21.557 a

** Within each column values followed by a letter in common are not significantly different (at α = 5%) (LSD = 3.672)*

3.2. Isolated RNA

The electrophoresis gel results are shown in picture 8. Although not all of the migration patterns showed a good result, the gel pictures revealed that each of the samples has their non-degraded RNA. In the first gel we got smear visualization of RNA for the three samples of Sret Unripe, thus we repeated the isolation procedure and the result was captured at the third gel. We have chosen the RNA which has the clearest 18s and 28s bands for cDNA synthesis.

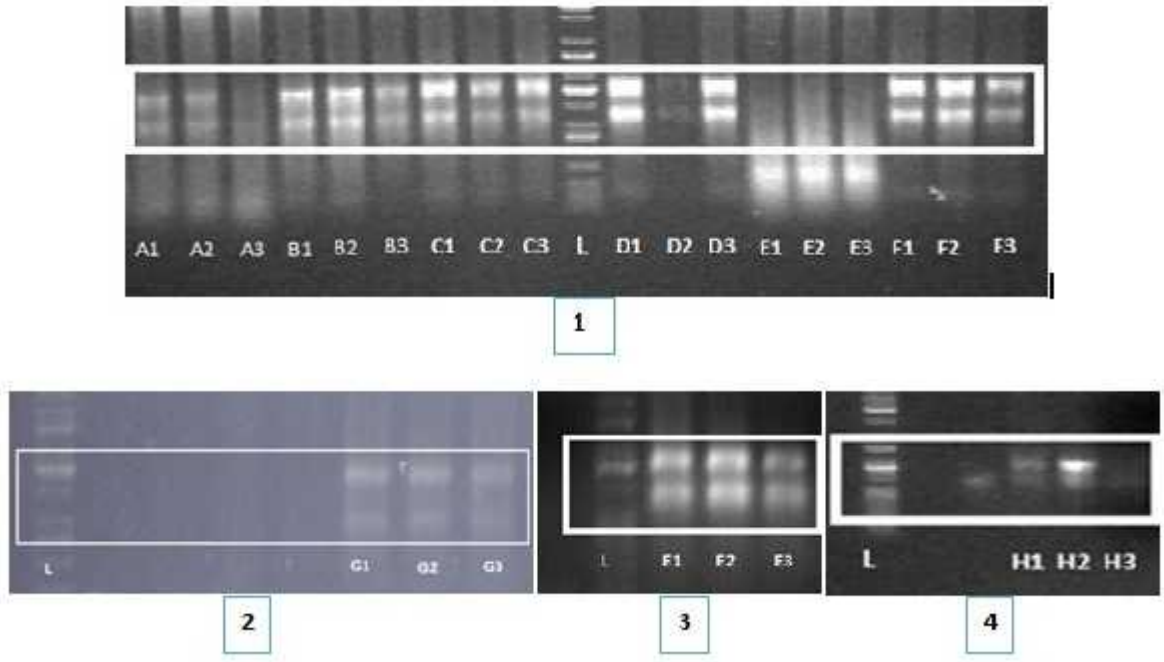


Figure 8. RNA picture of four genotypes in two different stages: **Gel 1:** (A)Kopay Ripe; (B) Princess Ripe; (C)SSP unripe; (D) SSP Ripe; (E) Sret Unripe; (F) Sret Ripe; **Gel 2 :** (G) Princess Unripe; **Gel 3:** (E) Sret Unripe and **Gel 4:** (H) Kopay Unripe. Number 1-3 indicated the number of sample replication

3.3. Gene Primers

We got six pairs of candidate genes which are suspected to play a role in the pepper fruit softening, i.e. Beta galactosidase, PIP, PL, PE and PGE. For PE genes we used two different pectinesterases which are suspected to have different expression trends during the ripening process. In bell pepper, PE 1 has a down-regulated expression at a later ripening stage, whilst Pectinesterase II has an upregulated expression.

Table 2. Primer List for 2 housekeeping genes and 6 candidate genes

Nr.	Primer ID		Sequence	Orientation	Length (kb)
	Code	Name			
1	CaATUB-F-1088	Alfa Tubulin (Housekeeping gene)	TTCCTGGAGGTGATCTTGCT	Forward	20
2	CaATUB-F-1278	Alfa Tubulin (Housekeeping gene)	AGCCAGATCTTCACGTGCTT	Reverse	20
3	192-CaUb-F	Ubiquinin	AATAAGGATGCAGGCTTCAAGGGC	Forward	24
4	193-CaUb-R	Ubiquinin	TGATGTCACGGGACCGAAGAAGAT	Reverse	24
5	CA08BG1701F	Beta-galactosidase	CCGTGGTGTGACAATTCAAG	Forward	20
6	CA08BG1860R	Beta-galactosidase	TCCTGGTGGAGGACCAGTTA	Reverse	20
7	CA09PIP0881F	Polygalacturonase inhibiting protein (PIP)	AGCAATCTTGGCAGCTGTTC	Forward	20
8	CA09PIP1022R	Polygalacturonase inhibiting protein (PIP)	CATTTACATGGTGGCAATGG	Reverse	20
9	CA11PL1024F	Pectate lyase	TGGAATTGGAGATCTGAAGGA	Forward	21
10	CA11PL1176R	Pectate lyase	AAGTGCACCAGCATTCATTG	Reverse	20
11	CA03PE3960F	Pectinesterase I	GGAGCATCAACCTCAGGAAG	Forward	20
12	CA03PE4100R	Pectinesterase I	ACCGAGAGAGAAGGGAAAGC	Reverse	20
13	CA10PG0786F	Polygalacturonase	CAAGATGTTGGCGTTATCCA	Forward	20
14	CA10PG0893R	Polygalacturonase	TCATTGCACACGATAAGCAAC	Reverse	21
15	CA12PE675F	Pectinesterase II	AGCACAAGGGAGATCAAACC	Forward	20
16	CA12PE839R	Pectinesterase II	TCCCAACCTTGAGGTGTTGT	Reverse	20

3.4. Relative Genes Expression

Relative gene expression of six targeted genes in four pepper genotypes at two ripening stages is shown in figure 9. Generally, the relative expressions of these six genes were different in unripe and ripe fruits in all genotypes. Two of pectinesterases genes showed different expression patterns. The first PE gene showed a regulated pattern during the ripe stage for all genotypes. At this point, it seems that PE1 is responsible for cellulose metabolism during ripening stages. However, we do not know the role of PE2 since the expression pattern of this gene was different in Sret. Similar with PE2, PIP roles were not so clear as well. Relative expressions of the PG gene in SSP were very low at unripe and ripe stages compared to Kopay and Princess. Also, Sret showed low levels of PG expressions. Overall the relative expressions of Beta galactosidase and PL were not highly expressed compared to others.

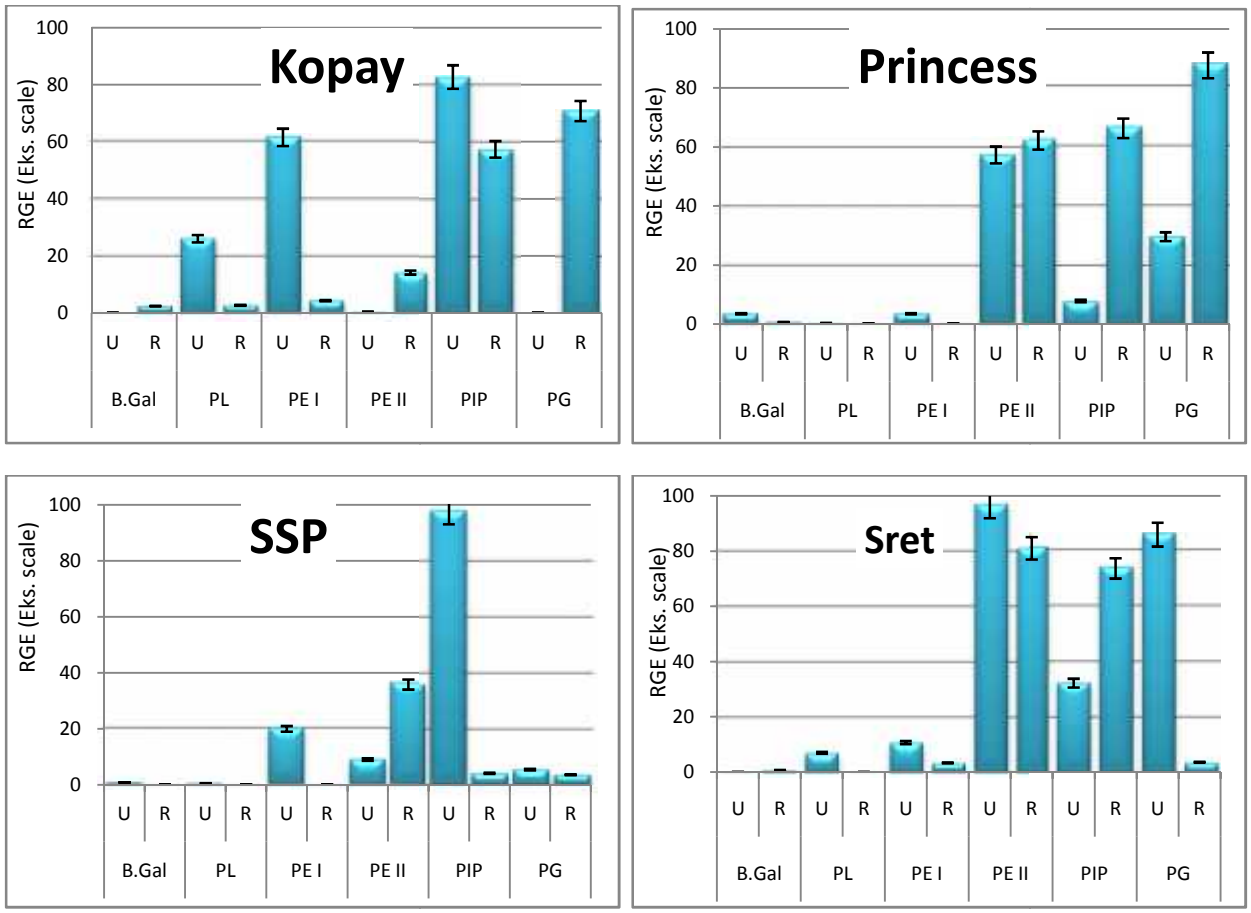


Figure 9. Relative Expression of six targeted genes in four pepper genotypes at two ripening stages (**U**=Unripe, R=Ripe; **B.Gal**=Beta Galactosidase; **PE I**=Pectinesterase I; **PE II**=Pectinesterase II; **PIP**= Polygalacturonase Inhibiting Protein; **PG**=Polygalacturonase)

3.5. Correlation between the targeted genes

HCA analysis and a heat map of gene to gene correlation reveal quite different results. Both methods were created from log 2 scale of RGE value. The reliability of the HCA graph is represented by the Jack knife value indicated in this graph. The main branch of HCA differentiated PIP of others. HCA showed that PG and PE-2 was clustered together, meanwhile PL and PE-1 were clustered in another group. This graph reveals evidence that two types of PE genes exist. On the other hand, the heat map of gene to gene correlation revealed that PE-1 has the closest correlation to PL, that was shown by Pearson correlation pointed at 0.91. In contrast, some negative correlations were built between PG and other genes i.e. PIP, PL and PE-1 (figure 10.).

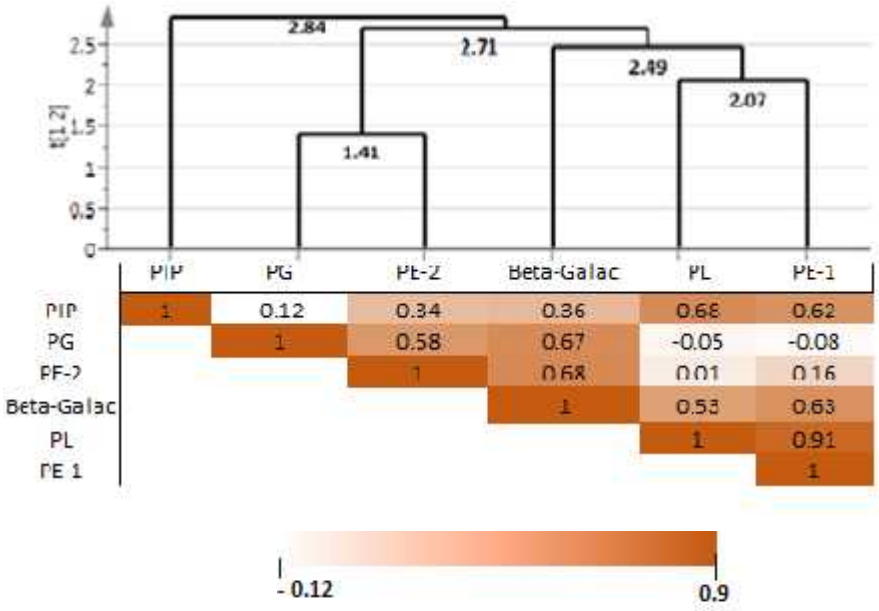


Figure 10. Gene to gene correlation, depicted in: (A) Hierarchical Clustering graph and (B) Heat map of gene to gene correlation based on Pearson coefficient correlation.

3.6. Correlation Analysis between relative weight and gene expression

The Biplot graph depicts the relative RGE position to genotypes at two stages based on PC1 and PC2 value. PIP was highly expressed during the unripe stage of SSP. This gene was also well expressed in ripe stages of SSP and Sret. Meanwhile, PE-2 and PG were well adapted in ripe stages of Princess and Sret. A high expression was shown in Kopay and Princess as well (figure 11).

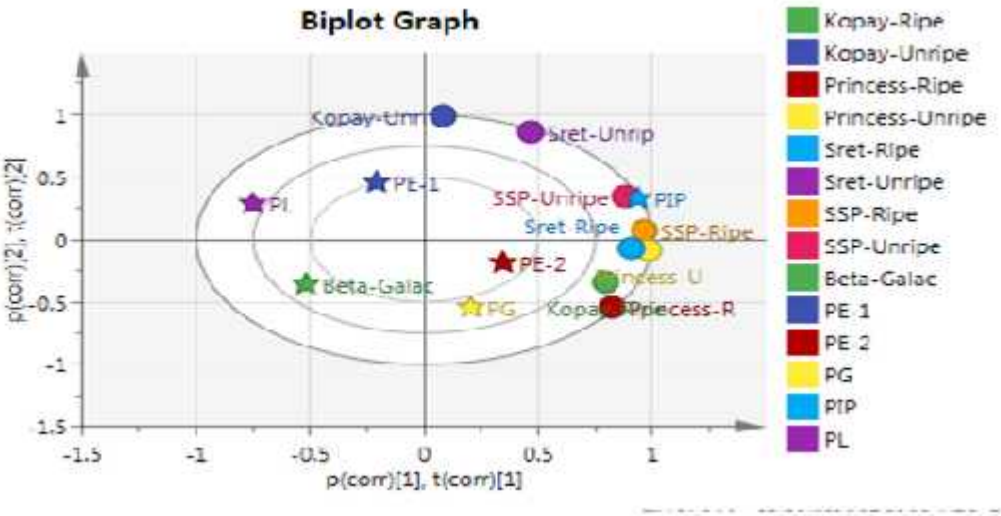


Figure 11. PC1 and PC2 Biplot Graph

Relative gene expression of PE 2 seems to have a highly negative correlation with fruit weight at 14 days in 20°C of storage. It means that these genes are playing a significant role to lowering fruit weight at the early stage of storage. On the other hand, a high expression of B.Gal, PIP and PG showed a negative correlation to fruit weight. Furthermore, gene expression of PL and PE1 after 30 days in 20°C of storage has a positive relation with fruit weight after 30 days of storage. Whilst, increasing value of PG expression after 14 and 30 days of storage in 20°C has a negative effect on fruit weight (figure 12). Overall, each gene has a significant correlation with fruit weight either in 14 days storage or in 30 days after storage in 20°C and 27°C.

TEMP.	WEIGHT	B.GAL	PE 1	PE 2	PG	PIP	PL
20 °C	14 days	-0.81	-0.12	0.85	-0.74	-0.87	-0.13
	30 days	-0.16	0.51	0.29	-0.69	-0.23	0.61
27 °C	14 days	-0.31	0.23	0.35	-0.44	-0.72	0.15
	30 days	0.01	0.33	0.07	-0.24	0.13	0.45

Figure 12. Heat map graph for correlation between relative weight after 14 and 30 days of storage and RGE

To get a better view on the pectin degradation mechanism, we tried to depict how the candidate genes are playing a role in fruit softening. Combining RGE (log 2 scale) of each genotype in a pectin degradation scheme could give a simple overview of that. The starting scheme showed that pectin is degraded into oligogalacturonide acid and pectate by PG and PE. Figure 13 shows that the candidate genes were highly expressed in earlier stages of the cellulose metabolism mechanism.

Meanwhile, PIP has a role to inhibit PG's activity. We assume that through this scheme we can see that every genotype has different RGE values in different positions that might play a role in determining pepper fruit weight after storage.

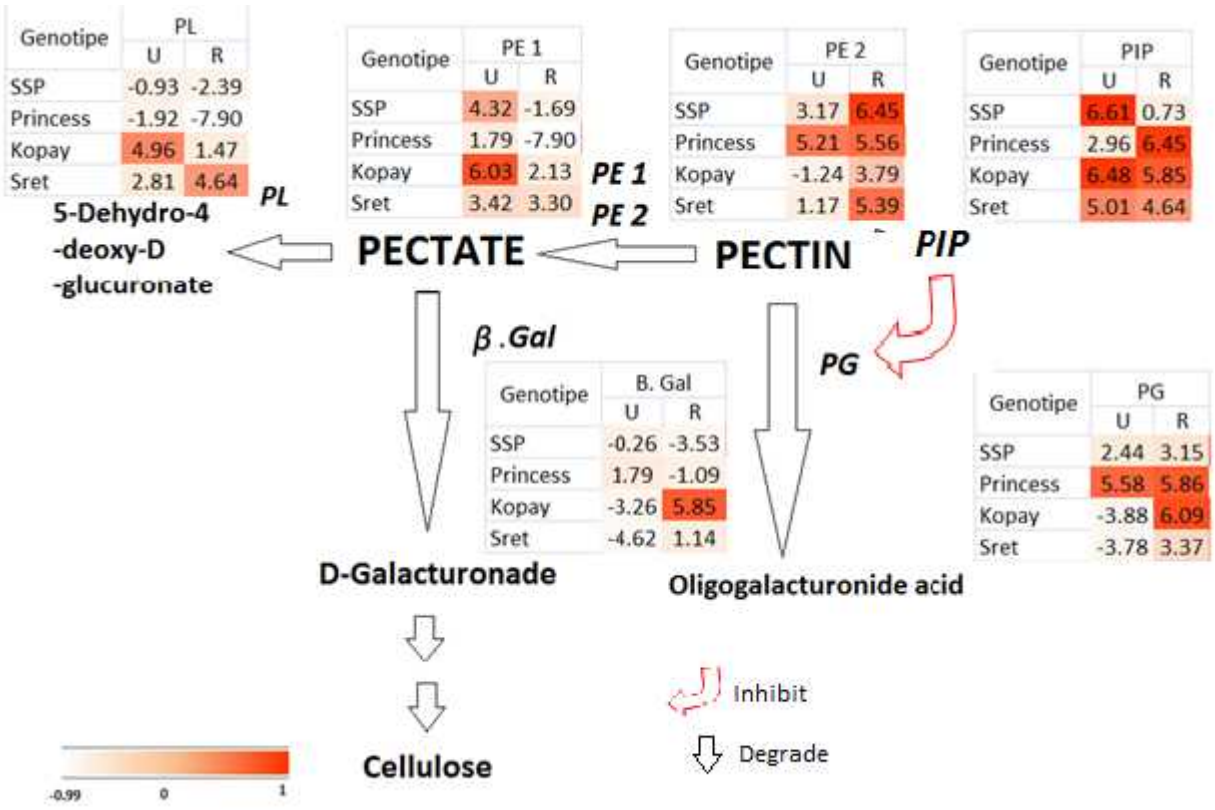


Figure 13. Pectin degradation scheme

Pepper fruit softening is an accumulation result of cell to cell adhesion as a consequence of pectin degradation and cell wall dissolution [22]. This mechanism is a complex process which involves various change reactions in different polymers. For every gene, significant differences among CWD mechanisms exist among species and even among cultivars from similar species [23, 24]. Although six genes in this experiment were expressed in both stages of all genotypes, they were expressed in different levels and patterns. Those differences may give a significant effect on the storage quality of each genotype.

Based on HCA and PCA analysis results, a strong correlation was found between PE-2 and PG genes. This aligns with a former study of PE and PG in Tabasco pepper, which was executed by Ramon and Carl in 2005[22]. They conclude that the effect of the PG genes on pectin degradation mechanism was enhanced by a PE gene. Meanwhile, a strong correlation was found between PL and PE-1 as well.

The research result shows clearly that PE 1 and PE 2 are different genes. However, It was not so clear which PE gene played a role in the pectin degradation pathway and how it works. It might have a correlation with the blast result from SGN were both genes were homolog to other genes from other species. In this case, PE 1 is homolog to threonine-protein kinase in *Solanum tuberosum.* Meanwhile,PE 2 is homolog to GCN5-related N-acetyltransferase family protein of *Populustrichocarpa*and Phospholipase D p1-like in *Solanum lycopersicum*.

Visualization of pepper fruit freshness after storage could be used as the first indication to identify the relative weight of pepper fruit which leads to a high storage quality. As indicated in this experiment, the most wrinkle pericarp texture was shown in Kopay,whilst the pericarp texture of Sret and SSP showed a fresher performance than Kopay. Phenotyping is an essential tool in plant breeding development. We must be conscious of certain agronomic traitsthat have a correlation with yield quality and quantity, so that undesirable traits can be avoided[25].

4. Conclusion

1. Among four pepper accessions, Sret and SSP shown the relative higher weight after storage compared to others.
2. Gene expression of PG, PE 2 and PIP could diferentiates between sample with a high storage quality and low quality. We suspect that PG,PE 2 and PIP has a role in fruit softening.
3. Major enzymes in 4 pepper accessions expressed in different levels and patterns. They were highly correlated with fruit weight in 20°C of storage.
4. Based on HCA and correlation analysis results, a strong correlation was constructed between PE-1- PL and between PG - PE2.
5. Pepper fruit softening process involve many genes that should be explored to get a better explanation about their role by following fruit development process.

5. Further study

The next plan for this study is gene sequencing. Gene sequencing analysis should be conducted to verify the gene identities. Furthermore, online alignment software and manual checks would be introduced to confirm whether the sequences acquired are identical to the targeted gene sequences. Further study is needed to get additional data for storage quality of unripe fruit in order to better understand the correlation between genes and fruit weight. Afterwards the data could be combined with the current experiment results. The new correlation coefficient analysis results could be used then.

As the next step we need to confirm the gene expression in the plant by applying mutagenesis. Afterwards, we can use TILLING technologies to identify the resulted mutant. We will choose the gene that has a high correlation value with fruit weight in this observation as the targeted genes. Additionally, silencing the right targeted gene using CRIPSR technology may result in delaying softening fruit process as well.

References:

1. Barboza, G.E. and B. De Bem, *Three new species of Capsicum (Solanaceae) and a key to the wild species from Brazil.* Systematic Botany, 2005. **30**(4): p. 863-871.
2. Di Cagno, R., et al., *Use of autochthonous starters to ferment red and yellow peppers (Capsicum annum L.) to be stored at room temperature.* International journal of food microbiology, 2009. **130**(2): p. 108-116.
3. FAO. 2013; Available from: http://faostat.fao.org/site/339/default.aspx.
4. Sayekti, A.L. and Y. Hilman, *DINAMIKA PRODUKSI DAN VOLATILITAS HARGA CABAI: ANTISIPASI STRATEGI DAN KEBIJAKAN PENGEMBANGAN.* Pengembangan Inovasi Pertanian, 2015. **8**(1): p. 33-42.
5. Smith, D.L., et al., *Influence of cultivar and harvest method on postharvest storage quality of pepper (Capsicum annuum L.) fruit.* Postharvest biology and technology, 2006. **42**(3): p. 243-247.
6. Osorio, S. and A.R. Fernie, *Biochemistry of Fruit Ripening*, in *The Molecular Biology and Biochemistry of Fruit Ripening*. 2013, Blackwell Publishing Ltd. p. 1-19.
7. Jia, Y.J., et al., *Polygalacturonase, Pectate Lyase and Pectin Methylesterase Activity in Pathogenic Strains of Phytophthora capsici Incubated under Different Conditions.* Journal of Phytopathology, 2009. **157**(10): p. 585-591.
8. Ogasawara, S., K. Abe, and T. Nakajima, *Pepper β-Galactosidase 1 (PBG1) Plays a Significant Role in Fruit Ripening in Bell Pepper (<I>Capsicum annuum</I>).* Bioscience, Biotechnology, and Biochemistry, 2007. **71**(2): p. 309-322.
9. Villarreal-Alba, E.G., et al., *Pectinesterase activity and the texture of Jalapeño pepper.* European Food Research and Technology, 2003. **218**(2): p. 164-166.
10. El-Zoghbi, M., *Biochemical changes in some tropical fruits during ripening.* Food Chemistry, 1994. **49**(1): p. 33-37.
11. Paull, R.E. and C. Nancy Jung, *Postharvest Variation in Cell Wall-Degrading Enzymes of Papaya (Carica papaya L.) during Fruit Ripening.* Plant Physiology, 1983. **72**(2): p. 382-385.
12. Marín-Rodríguez, M.C., J. Orchard, and G.B. Seymour, *Pectate lyases, cell wall degradation and fruit softening.* Journal of Experimental Botany, 2002. **53**(377): p. 2115-2119.
13. Fu, L., et al., *Characterization of Cell-Death-Inducing Members of the Pectate Lyase Gene Family in Phytophthora capsici and Their Contributions to Infection of Pepper.* Molecular Plant-Microbe Interactions, 2015. **28**(7): p. 766-775.
14. Leclere, L., P.V. Cutsem, and C. Michiels, *Anti-cancer activities of pH-or heat-modified pectin.* Front Pharmacol, 2013. **4**(128): p. 1-8.
15. Gou, J.-Y., et al., *Gene expression and metabolite profiles of cotton fiber during cell elongation and secondary cell wall synthesis.* Cell research, 2007. **17**(5): p. 422-434.
16. Ferrari, S., et al., *Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development.* Frontiers in Plant Science, 2013. **4**.
17. Kalunke, R.M., et al., *An update on polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein that protects crop plants against pathogens.* Frontiers in plant science, 2015. **6**: p. 146.
18. King, D., et al., *Use of amide exchange mass spectrometry to study conformational changes within the endopolygalacturonase II-homogalacturonan-polygalacturonase inhibiting protein system.* Biochemistry, 2002. **41**(32): p. 10225-10233.
19. Dieffenbach, C., T. Lowe, and G. Dveksler, *General concepts for PCR primer design.* PCR Methods Appl, 1993. **3**(3): p. S30-S37.
20. Udvardi, M.K., T. Czechowski, and W.-R. Scheible, *Eleven golden rules of quantitative RT-PCR.* The Plant Cell, 2008. **20**(7): p. 1736-1737.

21. Livak, K.J. and T.D. Schmittgen, *Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method.* Methods, 2001. **25**(4): p. 402-408.
22. Arancibia, R.A. and C.E. Motsenbocker, *Pectin methylesterase activity in vivo differs from activity in vitro and enhances polygalacturonase-mediated pectin degradation in tabasco pepper.* Journal of plant physiology, 2006. **163**(5): p. 488-496.
23. Hiwasa, K., et al., *European, Chinese and Japanese pear fruits exhibit differential softening characteristics during ripening.* Journal of Experimental Botany, 2004. **55**(406): p. 2281-2290.
24. Brummell, D.A., *Cell wall disassembly in ripening fruit.* Functional Plant Biology, 2006. **33**(2): p. 103-119.
25. Peleman, J.D. and J.R. van der Voort, *Breeding by design.* Trends in plant science, 2003. **8**(7): p. 330-334.

Appendix

Appendix 1

Time Schedule

Table S1. Time schedule of activities during internship period

No	Period	Activity
1	Week 1 (March 14 – March 18)	Discussion with supervisor, preparation
2	Week 2 (March 21 - March 25)	Designing primers for selected genes
3	Week 3 (March 28 - April 1)	Sample collection and ground the samples into fine powder for RNA extraction purpose
4	Week 4 (April 4 - April 8)	Field observation and sample collection for fruit srorage quality, weight loss observation at 0 days after harvest
5	Week 5 (April 11 – April 15)	mRNA extraction for six samples, RNA concentration measurement using nanophotometer and RNA separation on agarose gel
6	Week 6 (April 18 – April 22)	mRNA extraction for nine samples, RNA concentration measurement using nanophotometer and RNA separation on agarose gel and weight loss observation at 14 days after harvest
7	Week 7 (April 25 – April 29)	Preliminary experiment for cDNA synthesize
8	Week 8 (May 2 – May 6)	cDNA synthesize and weight loss observation at 30 days after harvest
9	Week 9 (May 9 – May 13)	Primers dilution
10	Week 10 (May 16 – May 20)	Preliminary observation for PCR setting and DNA separation in agarose gel
11	Week 11 (May 23 – May 27)	Preliminary observation for qRT - PCR
12	Week 12 (May 30 – June 3)	Preliminary observation for qRT - PCR
13	Week 13 (June 6 – June 10)	qRT - PCR analysis of selected genes in local peppers
14	Week 14 (June 13 - June 17)	Data analysis
15	Week 15 (June 20 – June 24)	Literatures review for report
16	Week 16 (June 27 – July 1)	Writing the report
17	Week 17 (July 4 – July 8)	Finalizing and send the report

Apendix 2

Total RNA Kit Mini Protocol

Step 1 Tissue Dissociation	• Cut off 50 mg (up to 100 mg) of fresh or frozen plant tissue. • Grind the sample (in liquid nitrogen) to a fine powder. Transfer the powder to a 1.5 ml microcentrifuge tube (RNase-free) (some plant samples can be ground without liquid nitrogen).
Step 2 Lysis	• Add **500 µl of RB Buffer (or PRB Buffer)** and 5 µl of ß-mercaptoethanol to the ground sample and mix by vortex. • Incubate at 60ºC for 5 minutes. • Place a **Filter Column** in a **2 ml Collection Tube** and transfer the sample mixture to the column. • Centrifuge for 1 minute at 1,000 x g. • Discard the **Filter Column** and proceed to Step 3 RNA Binding.
Step 3 RNA Binding	• Add a ½ volume of absolute ethanol to the clarified filtrate from Step 2 and shake vigorously (e.g. add 250 µl of absolute ethanol to 500 µl of filtrate). • Place a **RB Column** in a **2 ml Collection Tube**. • Transfer the ethanol-added mixture to the **RB Column**. • Centrifuge at 14-16,000 x g for 1 minute (if the mixture could not flow past the RB Column membrane following centrifugation, increase the centrifuge time until it passes completely). • Discard the flow-through and place the **RB Column** back in the **2 ml Collection Tube**. Optional Step 1: DNA Residue Degradation (see options above)
Step 4 Wash	• Add **400 µl of W1 Buffer** into the center of the **RB Column**. • Centrifuge at 14-16,000 x g for 30 seconds. • Discard the flow-through and place the **RB Column** back in the **2 ml Collection Tube**. • Add **600 µl of Wash Buffer** (ethanol added) to the center of the **RB Column**. • Centrifuge at 14-16,000 x g for 30 seconds. • Discard the flow-through and place the **RB Column** back in the **2 ml Collection Tube**. • Add **600 µl of Wash Buffer** (ethanol added) to the center of the **RB Column**. • Centrifuge at 14-16,000 x g for 1 minute. • Discard the flow-through and place the **RB Column** back in the **2 ml Collection Tube**. • Centrifuge at 14-16,000 x g for 3 minutes to dry the column matrix.
Step 5 RNA Elution	• Place the dried **RB Column** in a clean 1.5 ml microcentrifuge tube (RNase free). • Add **50 µl of RNase-free water** to the center of the column matrix. • Let stand for at least 2 minutes to ensure the **RNase-free water** is absorbed by the matrix. • Centrifuge at 14-16,000 x g for 1 minute to elute the purified RNA. If higher RNA concentration is required, repeat Step 5 using the final eluate. Optional Step 2: DNA Residue Degradation (see options above)

Source : www.geneaid.com

Appendix 3

Nanodrop results for isolated RNA

No	Genotype	Code	Stages	Replication	A 260/280	A 260/230	Concentration (ng/µl)
1	Kopay	G1	Unripe	I	2.000	1.774	183
2	Kopay	G1	Unripe	II	2.054	2.054	223
3	Kopay	G1	Unripe	III	2.021	2.068	184
4	Kopay	G1	Ripe	I	2.023	2.075	169
5	Kopay	G1	Ripe	II	1.918	2.008	227
6	Kopay	G1	Ripe	III	1.897	2.000	144
7	Princess	G2	Unripe	I	2.045	2.267	445
8	Princess	G2	Unripe	II	2.058	2.400	416
9	Princess	G2	Unripe	III	2.044	2.115	357
10	Princess	G2	Ripe	I	1.945	2.026	208
11	Princess	G2	Ripe	II	2.000	2.061	280
12	Princess	G2	Ripe	III	2.000	2.167	202
13	SSP	G3	Unripe	I	1.971	1.971	260
14	SSP	G3	Unripe	II	2.026	2.028	303
15	SSP	G3	Unripe	III	1.978	2.068	353
16	SSP	G3	Ripe	I	2.019	2.029	208
17	SSP	G3	Ripe	II	1.959	2.087	186
18	SSP	G3	Ripe	III	2.000	2.026	229
19	Sret	G4	Unripe	I	1.983	2.164	231
20	Sret	G4	Unripe	II	1.985	2.079	254
21	Sret	G4	Unripe	III	1.964	1.964	214
22	Sret	G4	Ripe	I	2.012	2.037	317
23	Sret	G4	Ripe	II	2.034	2.067	462
24	Sret	G4	Ripe	III	2.019	2.162	416

Appendix 4

Homogeneity and normality test result for storage quality

Test of Homogeneity of Variances

WEIGHT

Levene Statistic	df1	df2	Sig.
2.706	3	20	.073

One-Sample Kolmogorov-Smirnov Test

		Unstandardized Residual
N		24
Normal Parameters[a]	Mean	.0000000
	Std. Deviation	3.87276474
Most Extreme Differences	Absolute	.137
	Positive	.072
	Negative	-.137
Kolmogorov-Smirnov Z		.669
Asymp. Sig. (2-tailed)		.762

a. Test distribution is Normal.

Anova result for storage quality

Tests of Between-Subjects Effects

Dependent Variable:WEIGHT

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	678.124[a]	7	96.875	14.605	.000
Intercept	12924.892	1	12924.892	1.949E3	.000
GENOTIPE	547.456	3	182.485	27.511	.000
TEMPERATURE	96.143	1	96.143	14.494	.002
GENOTIPE * TEMPERATURE	34.525	3	11.508	1.735	.200
Error	106.131	16	6.633		
Total	13709.147	24			
Corrected Total	784.255	23			

a. R Squared = .865 (Adjusted R Squared = .805)

Reflection paper

During my internship, I worked 8,5 hours per day from 07.30 – 16.00 am at weekdays, as are common office hours in Indonesia. However, I had to work overtime when I did certain laboratory work since I had to share laboratory tools with other students or researchers. Some tools needed to be booked a few days in advance, since the ratio between the number of tools and researchers' activities were relatively low.

During the first discussion with the field supervisor, we agreed to focus on Polygalacturonase as the candidate gene for softening delay in pepper *(Capsicum annum)* fruit. The internship supervisor was very interested in that subject since he works with tomatoes, which have a close relation with pepper. Besides, he was the former promoter for my field supervisor during her PhD. He gave some suggestions related to several targeted gene alternatives which have an important role on CWD. Those suggestions became a new insight for this internship project. Afterwards, we decided to observe another five genes, as suggested by him, i.e. PE1, PE2, PIP, PL, and β. Gal. It was quite challenging since there was not sufficient information about the CWD mechanism in pepper.

Formerly, we were interested to observe gene expression in four different stages, i.e. unripe small, unripe big, turning, and full ripe stages. Unfortunately, we could not get those samples since the high proportion of pepper fruits was still in green stage by the time the internship period ended. Moreover, some plants were suffering from white flies (*Bemisia tabaci*) attacks which could lower fruit production. After discussing this problem with the field supervisor, we decided to focus on unripe and ripe stages in four genotypes.

During my field work, I faced some unpredictable problems which influenced the continuity of my experiment. The first problem came from the fruit storage quality. Usually, that trait could be approached with pericarp's firmness. As no penetrometer instrument was available, we used fresh weight loss during storage as the quality assessment. The second problem is related with RNA isolation for Kopay and Sret at unripe stages. I got degraded RNA results for both genotypes. Unfortunately, the washing buffer component normally included in a Geneaid kit was empty and it takes one month to reorder a new kit. I proposed to my field supervisor to substitute that component with 75% Et-OH. As she gave permission to me, I tried this alternative and it turned out to work. I could isolate my RNA without waiting for one month. The last main problem is related to software availability. In this institution we were not supported by adequate analysis software. So, I used free access software that supported a SPSS application and Microsoft Excel.

Learning from this experience, I realize that sometimes not all of our plans could be realized due to some unpredictable reasons. In the case of samples availability, I could not get the complete fruit stages from the targeted genotypes. It made me redesign my experiment, compile the data and use the starting material from the fine powder which was made by Mbak Yuni at her previous experiment.

For further study, I will start my experiment from preparing plant material and keep them in greenhouses to protect them from plant pathogens and disease attacks. I will replicate each genotype as my biological replication. I will use similar sample categories for quality storage and gene expression measurement, so I can create a complete correlation between both of them. I will observe unripe fruit weight loss during storage when I decide to observe the gene expression in unripe fruit. I will predict and calculate my chemical compound requisite and check the availability in laboratory before starting my experiment, so I can anticipate that deficit earlier.

As we work on a bimolecular experiment, instrument availability and good internet networks to access the latest research publications, become an important research requirement. Although some instruments were not available in the Laboratory of Plant Molecular Genetics and Biosynthetic Pathway Alteration, I could use instruments which are available in other laboratories i.e. Real Time PCR; nanodrop spectrophotometer, gel doc or UV transilluminator. The real problem did come from the complex bureaucracy system in RC for Biotechnology, Indonesian Institute of Science (LIPI). We were obliged to ask for an official permission for using certain instruments. Meanwhile, as an internship student, I could not get the free Wi-Fi access in this office due to some new rules in this office. I was helped by my home internet connection, so I could access these publications from WUR digital library to support my report. Besides that, my field supervisor also helped me by giving some recommendations for supporting journals. Those problems could be prevented by providing a better internet connection network. Through this solution, a simpler bureaucracy system for online applications could be built.