RNA-sequencing for transcriptomic analysis of chili peppers induced by *Serratia plymuthica* strain UBCF_13

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Abstract. Non-pathogenic bacteria are used as biocontrol agents in plants because of their capability to induce the plant’s resistance system through the ISR [Induction Systemic Resistance] mechanism. Rhizobacteria are known for their ability to signal the biosynthesis of jasmonic acid and ethylene, also stimulate an increased expression level of resistance genes and auxin in plants. RNA-sequencing can be used to analyze the transcriptomic profile of plants. In this study, we used the bacteria *Serratia plymuthica* strain UBCF_13 to induce the resistance system of chili plants. This study aimed to obtain information on the transcriptome level of chili after being induced by the bacteria. This study is expected to be a useful reference for generating transcriptome data of bacteria UBCF_13 as a biocontrol agent. For RNA sequencing, we used two different plants, namely plants without and with the induction of bacteria UBCF_13. The bacteria UBCF_13 [OD₆₀₀ 1] was applied 12 days after planting in the root area. Total RNA was isolated from plant roots after 14 days of treatment. Differential gene expression, GO enrichment, and KEGG analysis showed that the resistance genes expression increases. Genes involved in hormone signal transduction like tryptophan metabolism is affected by the induction of bacteria UBCF_13. The AUX1 [Auxin Influx Carrier], SAUR [Small Auxin Up RNA], and GH3 [Gretchen Hagen 3] genes family in the tryptophan metabolism become upregulated.

Keywords: AUX1, bacteria UBCF_13, GH3, RNA Sequencing, SAUR,

1. Introduction
The use of biological agents is one of the techniques for controlling disease in plants. Several studies have identified biological agents that have antagonistic abilities against fungi and geminiviruses. *Bacillus* spp. and *Pseudomonas* spp. produce phosphate and IAA compounds that can inhibit fungal growth. Besides, bacterial isolates of the *Enterobacter* sp. and *Bacillus cereus* groups produce the chitinase enzyme to lyse pathogenic fungi [1].

Bacteria have been applied as antagonistic agents to control plant pathogens. One of the bacteria that widely used is rhizobacteria. De Vleesschauwer et al [2]. tested *Serratia plymuthica* on the roots of rice plants. The study showed that colonization of *Serratia plymuthica* strain IC1270 affected oxidative reactions in plant defense against *Magnaporthe oryzae*, *Rhizoctonia solani*, and *Cochliobolus miyabeanus*. *Serratia plymuthica* strain UBCF_13 showed fungal suppressive activity
against Colletotrichum gloeosporioides, Fusarium oxysporum, and Sclerotium rolfsii. Because S. plymuthica interacts with plant roots to provide this potential, S. plymuthica becomes a bacteria promising for plant resistance in the agricultural sector [3].

Information about gene expression level can be analyzed using RNA-sequencing [RNA-Seq] technology. RNA-Seq can find out the genes that are involved and expressed in different mechanisms and organs or plant cells at the transcription level [4]. So, the genes that contribute to the response to the environment [biotic and abiotic] can be known earlier. RNA-seq provides much higher coverage and higher resolution from the dynamic properties of the transcriptome.

This study aimed to determine the effect of Serratia plymuthica strain UBCF_13 induction on the transcription levels of genes that are responsive at the molecular level of chili plants. So that it provides information that becomes the basis for the utilization of the biological agent Serratia plymuthica strain UBCF_13.

2. Materials And Methods

2.1. Chili plants preparation
In this study, we used seeds of chili [Capsicum annuum L.] var. Lotanbar. The seeds were sterilized by immersing them in 15% sodium hypochlorite for 15 minutes. After the sterilization process, the seeds were soaked for 24 hours in distilled water. Then the seeds were sown in planting medium [sterilized soil and manure] in a 25 x 20 cm polybag. After 14 days, the seedlings that have an average height are selected to proceed to the bacterial application stage [5]. One polybag contains one plant and each treatment has four replications.

2.2. Application of bacteria UBCF_13 to the chili plant
S. plymuthica strain UBCF_13 bacteria [bacterial collection of Laboratorium of Biotechnology of Agricultural Faculty, Andalas University] was cultured in liquid Luria Broth [LB] in a 250 ml Erlenmeyer flask. The culture was incubated with agitation at about 160 rpm for 24 hours at a shaker incubator. Then, bacterial cultures were harvested with centrifugation for 10 minutes at 10,000 rpm. The bacterial pellets were suspended with 15 ml of new liquid LB medium. After homogeneous, 15 mL suspension were transferred into a new tube and then were added with 0.9% NaCl until the OD600 value became 1. Bacterial suspension and control [only 0.9% NaCl] was applied into the plant roots as much as 10 ml per plant by pour to the roots [5]. This treatment was done when the chili seedlings are 12 days old.

2.3. Total RNA isolation and sequencing
The roots from chili plants 14 days after treatment were collected from all plants from each treatment with or without UBCF_13 as samples for RNA extraction. The total RNA was extracted using the Geneaid Total RNA Mini Kit [Plant] [Geneaid, Taiwan] following its manual protocol. The concentration of extracted RNA more than 50 ng/µL and RNA Integrity Number [RIN] more than 5, so it means the RNA’s quality is good. The RNA then be used for the RNA-sequencing [Illumina, Inc.]. Data analyzed were done by several software namely Illumina CASAVA v1.8, HTseq, and DEGseq for differential expression gene [6] [7].

3. Results and Discussion

3.1. RNA-sequencing and reads quality control
Analysis of the differential expression genes in the cells can use the RNA-sequencing [RNA-seq] technique. Illumina is one of the platforms used for RNA sequencing. Raw reads of the control plant [RL_CK] and bacteria UBCF_13 treated plant [RL_CP] samples were classified in Figure 1. RL_CK had 98.84% clean reads, 0.86% adapter, 0.20% low-quality reads, and 0.09% N. While RL_CP have 98.94% clean reads, 0.67% adapters, 0.20% low-quality readings and 0.19% N. A total of 86,717,122
raw reads were obtained from RL_CK plants and 87,094,984 from RL_CP. After the adapter and low-quality reads were removed, we got 85,715,364 from RL_CK and 86,173,486 from RL_CP. The net readings obtained represented the total base mean of each sample of 12.9 G. The quality of the reads controlled for the distribution of error rates, percentage Q20, percentage Q30, and GC content [shown in Table 1].

**Figure 1.** Raw Reads Classification. Clean reads are readings that have passed the quality control stage. The adapter is contaminated reading with the adapter. Containing N is readings with an uncertain nucleotide base of more than 10% of reads. Low-quality reads are readings with more than 50% lower quality nucleotides of reads. [A] control plant [RL_CK]. [B] bacteria UBCF_13 treated plant [RL_CP].

**Table 1.** Data Quality Control Summary

| Sample  | Raw reads   | Clean reads | Error rate [%] | Q20 [%] | Q30 [%] | GC [%] |
|---------|-------------|-------------|----------------|---------|---------|--------|
| RL_CK   | 86,717,122  | 85,715,364  | 0,03           | 97,55   | 93,03   | 42,48  |
| RL_CP   | 87,094,984  | 86,173,486  | 0,03           | 97,30   | 92,41   | 42,59  |

Table 1 shows the results of the reads quality control analysis. The error rate criteria vary depending on the platform used. The percentage error rate using the Illumina platform must be below 0.1%. So, the data can be used for gene expression analysis [8]. The distribution of the error rate in the readings of the two samples is 0.03%. This value indicates that the distribution of the reading error rate is low. The graph of the error level distribution is shown in Figure 2.
The relationship between the error rate and base quality seen in the quality of the Phred score [Q score]. This method is proven to be very accurate in seeing the quality of the reading data obtained. The Q score is the logarithmic value of the probability of base-calling errors. The percentage of Q20 used to see base-calling that is wrong 1 in 100 times, while Q30 is 1 in 1000 times. A high percentage of Q scores indicates a small error rate. Then a low percentage of the Q score results in a false variant that the reads cannot be used for further analysis. The percentage of Q20 above 95% and Q30 above 85% can be said to be well, which is the error rate is low [9]. RL_CK plants have a percentage of Q20 97.55% and Q30 93.03%. RL_CP plants have a percentage of Q20 97.30% and Q30 92.41%. The accuracy of the results reads of the two samples was very high. The level of reading error for the two samples based on the Q score is low.

In this study, we used chili [Capsicum annuum L.] var. Lotanbar. The reads of each plant are aligned with the reference genome, Capsicum chinesense [accession number: ASM227189v2]. The total readings mapped against the plant reference genome of the RL_CK plant was 74.39% and RL_CP of about 75.8%. A percentage above 70% meets the exact genome reference criteria and there are no contaminants [10]. A total of 77.3% and 79.7% reads were mapped to exons of the reference genome from RL_CK and RL_CP.

3.2. Expression Quantitative and KEGG Analysis
The distribution of differential expression gene shown through a volcano plot based on the log2 threshold [Fold Change] > 1 and q-value <0.005 [Figure 3]. Fold change describes the change in the expression level based on the ratio. A positive fold change value indicates that a gene has increased in expression [up-regulated] and minus means a decrease in expression [down-regulated]. The distribution of up-regulated genes is more numerous than for down-regulated genes and genes that are not significantly different. The genes that were up-regulated were 629 and down-regulated of about 252.

The results of KEGG analysis showed that plants induced by bacteria UBCF_13 [RL_CP] had increased gene expression in various metabolic processes. Plant hormone signal transduction up-regulated genes are involved in the metabolism of tryptophan. Tryptophan has an important role in regulating the development and response to plant resistance. Tryptophan is a precursor to indoleacetic acid [IAA] and a hormone for cell expansion [11]. Auxins, the auxin-responsive GH3 gene family, and SAUR [small auxin up RNA] protein are involved in tryptophan metabolism. Auxins regulate plant growth and development. Auxin protein-coding genes [AUX/1] namely BC332_25865, SAUR [BC332_08650, BC332_08651] and auxin-responsive GH3 family [BC332_22348] became up-regulated. The pathway could be seen in Figure 4.
The **AUX1** protein family [**auxin influx carrier**] is the main carrier of functional auxins to plants. **AUX1** regulates root gravitropism, root hair development, and phyllotaxis. **AUX1** is also involved in the response of plant resistance to stress [11]. As **AUX1** increases, the expression of the **SAUR** coding gene increases. **SAUR** is the largest family of early auxin response genes. **SAUR** has implications for the regulation of cellular, physiological, and plant development processes [12]. The **auxin-responsive** **GH3** gene family has been shown to play a role in plant resistance responses. **GH3** acts as a bifunctional modulator in auxin, JA [Jasmonic Acid], and SA [Salicylic Acid] signaling. Increased **GH3** results in increased accumulation [SA] and expression of **PR-1** [**pathogenesis-related-1**] genes. The expression of auxiliary gene families such as **GH3** and IAA affects homeostasis and the formation of reactive oxygen species [13].

**Figure 3.** Volcano plot of differential expression gene. Differential distribution graph of control plant [RL_CK] and bacteria UBCF_13 treated plant [RL_CP].

**Figure 4.** KEGG pathway of the upregulated gene in tryptophan metabolism. The red box is upregulated gene.

**4. Conclusion**
The treatment of **Serratia plymuthica** strain UBCF_13 on Lotanbar chili induced the expression of several genes. One of them is increasing the **AUX1**, **SAUR**, and **GH3** gene expression in tryptophan metabolism. Increased expression of these genes is expected to improve the plant defense systems.

**Acknowledgments**
This research was funded by the General Directorate of Higher Education through Post-Doctoral Grant No. SP DIPA-042.06.1.401516/2019 dated 5 December 2018.

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