Analysis the Action Mechanism of Pyrazole Derivatives EH-1 that Induces Triple Response in *Arabidopsis* Seedlings by Using RNA-Sequencing

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***Abstract*—We have previously reported the discovery of pyrazole derivative EH-1 displayed biological activity on inducing triple response in dark grown *Arabidopsis* seedlings. To improve our understanding of the action mechanism of EH-1, we conducted RNA-sequencing analysis of EH-1 treated *Arabidopsis* seedlings. Data present in this article for the first time demonstrated that EH-1 did not significantly induce ethylene responsive genes of ethylene receptors and/or ethylene signaling components in compared with none chemical treated control. However, we found that the expression levels of several genes were significantly increased by the treatment of EH-1 and ACC, the precursor of ethylene biosynthesis. This result indicates that the EH-1 induced the expression of some genes which are common to ethylene. We also found that there are several genes significantly induced by EH-1 but not induced by ACC. Our results suggest that the action mechanism of EH-1 shares with ethylene on inducing triple response in *Arabidopsis* seedlings but the primary site of action between EH-1 and ethylene are different.**

***Index Terms*—ethylene, triple response, RNA Seq, plant growth regulators**

I. Introduction

Plant hormones are natural occurring substances that involve in many physiological processes through regulating gene expression in plants. Ethylene is a gaseous plant hormone that plays key roles in plant growth and development [1]. Ethylene is an important plant hormone which involves in seed germination, flower development, fruit growth and ripening [2]-[5]. Ethylene also regulates plant defense gene expression against pathogen inflection as well as abiotic stress such as drought [6]-[8].

Because of the gaseous nature of the ethylene as well as its flammable property, it is difficult to handling ethylene as an agrichemical agent in agricultural industry for crop production. By now, efforts have been made to develop chemicals with ethylene activity. Ethephon (the IUPAC name is 2-Chloroethylphosphonic acid), which has been registered as pesticide for a number of food crops and some non-food crops, is a prodrug of ethylene. Ethephon is believed to degraded in plant tissues thereby produce ethylene as we as phosphoric acid. Because Ethephon produces strong acid during its degradation, chemical agent with ethylene activity and which covers the shortage of Ethephone is a good candidate of novel plant growth regulators. With this background, we carried out a systematic search for compounds that display ethylene activity through screening chemical library.

In the previous work, we reported the discovery of **EH-1**, a pyrazole derivative which induces triple response in *Arabidopsis* seedlings [9]. We named the compound **EH-1**. The chemical structure of **EH-1** is shown in Fig. 1.

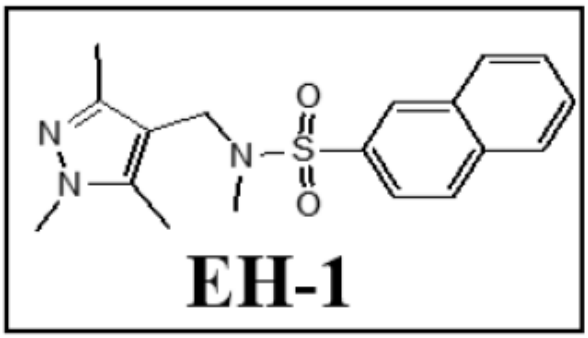

Figure 1. The chemical structrure of EH-1

Manuscript received September 1, 2018; revised February 13, 2019.

doi: 10.18178/ijpmbs.8.2.45-48

In the course of work, we used 'triple response' assay method to identify the hit compounds with ethylene-like activity in the chemical library screening. Triple response assay method has been widely used to determine the actions of ethylene in plants [10]. Use triple response assay to screening genetic mutants, several knockout mutants have been identified as ethylene signaling mutants. ETR1 mutant which is insensitive to ethylene treatment on inducing triple response have been characterized that the genes encode ETR1 is ethylene receptor [11], [12]. Similarly, CTR1 which is a negative regulator of the ethylene signaling pathway [13], and several other genes including ETR2 and EIN 4 were identified by using triple response assays [14], [15]. Based on these facts, the 'triple response' assay is useful in identification chemicals with ethylene activity in compound library screening.

As we reported in our previous work, **EH-1** displays promising activity on inducing triple response in *Arabidopsis* seedlings [9], the biological action mechanism of **EH-1** is remained to be characterized. In order to improve our understanding of the mode of action of **EH-1**, we carried out RNA-sequencing analysis. We have previously reported the number of genes that are commonly induced by **EH-1** and ACC, the precursor of ethylene biosynthesis. After extensive data analysis, we found several significant important results on RAN-sequencing analysis. In the present article, we report the effect of **EH-1** on the expression levels of ethylene signaling genes. We also report several genes that are significantly (above 20 fold in comparison of none chemical treated *Arabidopsis* seedlings) induced by **EH-1** but not by ethylene.

II. Materials and Methods

A. General Reagents

Chemicals were purchased from Kanto Chemicals Co. Ltd. (Tokyo, Japan) and Tokyo Kasei Co. Ltd. (Tokyo, Japan). Reagents were of the highest grade commercially available.

B. Plant Materials and Growth Conditions

Seeds of *Arabidopsis* (ecotype Columbia) were purchased from Lehle Seeds (Round Rock, TX, USA). Seeds used for the assay were sterilized in 1% NaOCl for 20 min and washed with sterile distilled water. Seeds (approximately 50) were sown on a 1% solidified agar medium containing 1/2 Murashige and Skoog (MS) salt added to 96-well plates (Fukaekasei Co., Ltd., Kobe, Japan) for library screening and to 24 well plates (Fukaekasei Co., Ltd.) for test the biological activity of synthesized compounds. The plates were wrapped with three layers of aluminum foil to keep the seed germination in a dark condition. After pre-incubation the plates at 4 ^{o}C for two days, plants were grown in a growth chamber at 25 ^{o}C for 5 days. Effect of the test compounds on growth of *Arabidopsis* seedlings were determined by measuring the hypocotyl length, root length and degree of the apical hook. Stock solutions of all of the chemicals were dissolved in DMSO and stock at -30 ^{o}C before use. The amount of DMSO was added below 0.1% (v/v) of growth media in all the experiments.

C. Isolation of Total-RNA

The seedlings were collected from the 90-mm diameter plastic petri dishes under the above growth conditions. Total RNA was extracted from the tissues using the RNeasy Plant Mini Kit (Qiagen,Tokyo, Japan). To avoid amplifying genomic DNA, each RNA fraction was treated with RNase-free DNaseI (RQ1; Promega KK, Tokyo, Japan). DNA elimination was confirmed by a usual RT-PCR with or without the reverse transcriptase. The isolated total RNA samples were stored -70 ^{o}C before use.

D. RNA-Seq Analysis

Triplicate cDNA libraries for each treatment were prepared and sequenced with 100 bp paired-end reads by the Illumina HiSeq 1000 platform at the Biotechnology Center in Akita Prefectural University.

Raw data were preprocessed by three programs (FASTX-Toolkit, Prinseq and Trimmomatic). RNA-seq analysis was carried out using the Tophat-Cufflinks pipeline [16], with the following versions: Tophat v2.0.14, Bowtie2 v2.2.6.0 and Cufflinks v2.2.1. The *Arabidopsis* TAIR10 genome and gene model annotation data were downloaded and used for reference. Differentially expressed genes (DEGs) were determined by applying the screening thresholds of 20-fold changes in FPKM (fragments per kilobase of exon per million fragments mapped) and FDR-adjusted p-value <0.05 using Cuffdiff tool.

E. Statistical Analysis

All measurements were carried out at least in triplicate. Data analysis (t-test and analysis of variance) was applied to determine the significant difference with the use of significance throughout the manuscript being based upon $p < 0.05$ unless stated otherwise.

III. Results and Disscossion

To determine the mode of action of **EH-1**, we conducted three independent trails of transcript expression analysis of RNA-Seq experiments [9].

Fig. 2 outlined the experiments carried out in the present work. *Arabidopsis* seedlings used for experiments were grown in half-strength MS agar medium containing **EH-1** (10 μM), ACC (10 μM) and without chemical treatment, respectively. Total-RNA extraction and Library preparation were carried out. Triplicate cDNA libraries for each treatment were prepared and sequenced with 100 bp paired-end reads by the Illumina HiSeq 1000.

Raw data were preprocessed by three programs as we previously described. RNA-seq analysis was carried out using the Tophat-Cufflinks pipeline, with the following versions: Tophat v2.0.14, Bowtie2 v2.2.6.0 and Cufflinks v2.2.1. The *Arabidopsis* genome and gene model were used for reference. Differentially expressed genes (DEGs) were determined by applying the screening thresholds of

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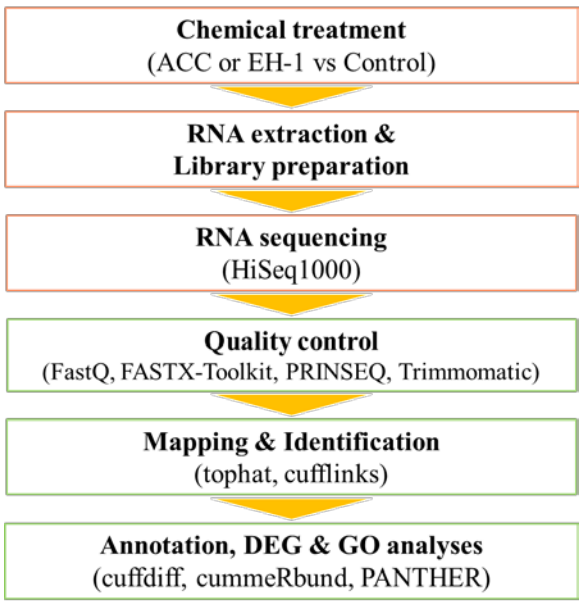

Figure 2. Experiment flow of RNA-seq Analysis the biological actions of EH-1.

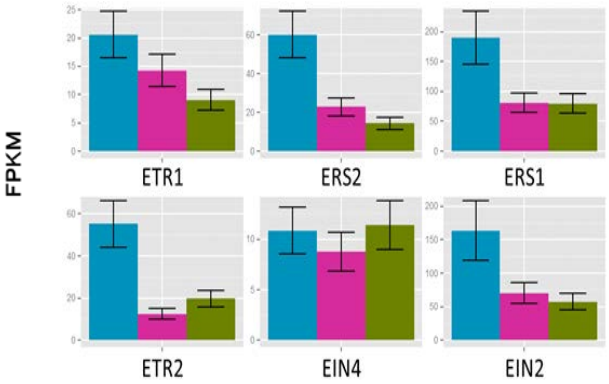

Figure 3. Expression Levels of Ethylene Responsive Genes. ETR1, ETR2, ERS1, ERS2 are ethylene receptors, EIN2 is a gene works in ethylene signaling, CTR1: a negative regulator of ethylene signaling. ACC treatment: blue bar; control: red bar; EH-1 green bar. Error bars represent standard deviation (n>P5). All the experiments were duplicated to establish repeatability.

Fig. 3 displayed the ethylene responsive genes including the ethylene receptor ETR 1 ETR2 and Several signal transduction genes such as EIN2 and EIN4. Our data indicated that **EH-1** did not promote the expression level of ETR1 and ETR2. As shown in Fig. 3, ACC (blue) promoted the expression levels of these genes while the expression levels of these genes were lower in **EH-1** treated plants (green) than that of control (red).

As shown in Fig. 4, several genes are significantly induced both by ACC (blue) and **EH-1** treatment (green) in comparison to that of control (red). Although the biological functions of these genes have not yet identified, our data indicated that these genes may working on the inducing of triple response in *Arabidopsis* seedlings.

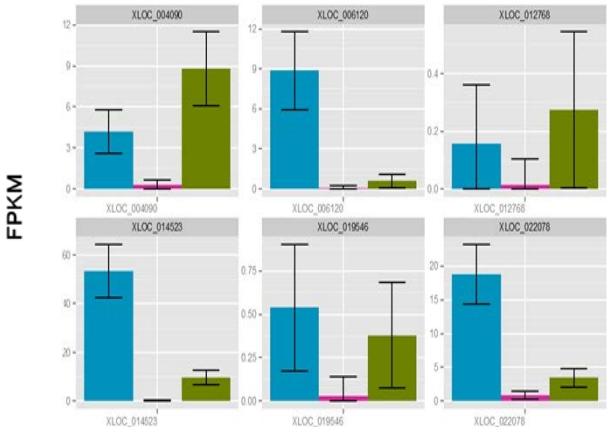

Figure 4. Genes Significantly Up-regulated by EH-1 and ACC. ACC treatment: blue bar; control: red bar; EH-1 green bar. Error bars represent standard deviation (n>P5). All the experiments were duplicated to establish repeatability.

Another line of evidence that indicated **EH-1** display a different action mechanism on inducing triple response in *Arabidopsis* seedling. As shown in Fig. 5, **EH-1** promoted the expression of several genes which are not induced by the treatment of ACC. Based on the RNA-seq analysis of **EH-1** in compared with ACC. Data obtained from present work clearly indicated that the primary site of the action of **EH-1** is different from ethylene. Further analysis the functions of the genes specifically induced by **EH-1** may provide new information about the triple response of *Arabidopsis.*

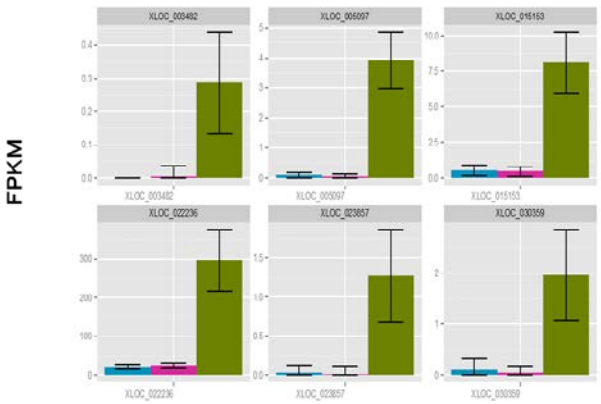

Figure 5. Genes Significantly Up-regulated by EH-1. ACC treatment: blue bar; control: red bar; EH-1 green bar. Error bars represent standard deviation (n>P5). All the experiments were duplicated to establish repeatability.

ACKNOWLEDGMENT

This research is supported by JSPS KAKENHI Grant Number JP16K01936 to Keimei Oh, Akita Prefectural University president research project, a grant of industrial-academic cooperation project of Akita Prefectural University and is partially supported by the Platform Project for Supporting in Drug Discovery and Life Science Research (Platform for Drug Discovery, Informatics, and Structural Life Science) from Japan Agency for Medical Research and Development (AMED). We thank the staff of the Biotechnology Center in Akita

Prefectural University for their efforts in sample preparing of RNA-seq experiment.

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