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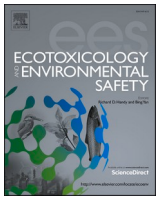

Transcriptome analysis revealed that jasmonic acid biosynthesis/signaling is involved in plant response to Strontium stress

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ABSTRACT

Strontium (Sr) has become an increasing global threat for both environment and human health due to its radioactive isotope, Sr-90 which can be found in the nuclear-contaminated soils and water. Although excessive Sr has been known to be toxic to plant growth and development, the molecular mechanisms underlying plant response to Sr stress, especially on the transcription level, remains largely unknown. To date, there is no published genome-wide transcriptome data available for the plant responses to Sr toxicity. Therefore, we aimed to gain insight on the molecular events occurring in plants in Sr toxicity condition by comparing the genome-wide gene expression profiles between control and Sr-treated plants using RNA-seq analysis. A total of 842 differentially expressed genes (DEGs) were identified in response to Sr stress compared to the control. Based on the analysis of DEGs using Gene Ontology (GO), DEGs were significantly enriched in the GO terms of response to salicylic acid (SA), response to jasmonic acid (JA), and defense response to bacterium. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis indicated that DEGs were mainly involved in metabolic processes including phenylpropanoid biosynthesis and alpha-linolenic acid metabolism, which is known as a precursor of JA biosynthesis. Furthermore, MapMan analysis revealed that a number of genes related to the biotic stress such as pathogenesis-related protein (PR) genes were highly up-regulated under Sr stress. Taken together, this study revealed that JA biosynthesis and/or signaling might be associated with plant response to Sr stress, and play important roles to maintain proper growth and development under Sr stress.

1. Introduction

Strontium (Sr) is one of abundant elements on earth's crust but a non-essential element for plant nutrition which can be toxic to plants at high concentrations. Since Sr is a member of Group II alkaline-earth metals with physicochemical properties similar to calcium (Ca), an essential element for plant growth and development, it can be easily absorbed by plant roots through Ca-transport systems and interfere with a variety of the cellular processes by competing and displacing with Ca, leading to Ca deficiency in plants (Burger et al., 2019). Previous studies have demonstrated that excessive Sr has negative effects on plant growth and development, such as an inhibition of root elongation, a reduction in biomass, a decrease in chlorophyll and carotenoid content, and a decrease of photosynthetic efficiency (Chen et al., 2012; Kalingan et al., 2016; Meena et al., 2013; Pyo et al., 2020; Sowa et al., 2014). Sr toxicity can also cause oxidative stress by the accumulation of reactive oxygen species (ROS), the induction of activity of several antioxidant enzymes, and increase of the production of secondary metabolites (Kalingan et al., 2016; Nagata, 2019; Wójciak-Kosior et al., 2016). In addition, Sr stress can lead to disruption of miRNA biogenesis (Pyo et al., 2020).

Recently, Sr has become a global environmental problem due to its

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radioactive isotope, Sr-90 which can be released into the environment through radioactive fallout from the explosion of nuclear weapons, nuclear waste disposal, and nuclear accidents such as in Chernobyl and Fukushima. Sr-90 is one of major concern in nuclear contamination due to relatively long half-life ($t_{1/2}$ = 28.8 years) and rapid incorporation into biological systems such as bones, leading to exposure of the internal organs to beta radiation during its radioactive decay and high risk to cancer (Burger et al., 2019). Since plants cannot distinguish significantly stable and radioactive Sr, Sr-90 can be readily absorbed by plant roots from the soil and accumulated in plant body, leading to serious problems to environment, agricultural production, and human health.

Jasmonic acid (JA) is a lipid-derived plant hormone that is well known to play important roles in regulating a variety of biological processes during growth and development including defense responses against biotic stresses such as herbivores and necrotrophic pathogens attacks (Acosta and Farmer, 2010). JA is synthesized from alpha-linolenic acid (C18:3), a fatty acid derived from the chloroplast membranes, through sequential reactions catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC) in chloroplasts, and 12-oxo-phytodienoic acid reductase (OPR3) in peroxisomes (Wasternack and Song, 2017). Once JA is produced, it is rapidly conjugated to isoleucine (Ile) to form bioactive JA-Ile by the Jasmonate-amino acid synthetase which is encoded by a *JASMONATE RESISTANT 1* (*JAR1*) (Fonseca and Brandi, 2010). Subsequently, the F-box protein CORONATINE INSENSITIVE 1 (COI1), a part of the E3 ubiquitin ligase complex SCF^{COI1}, directly binds to JA-Ile, leading to ubiquitination and degradation of transcriptional repressor JASMONATE-ZIM DOMAIN (JAZ) proteins (Wasternack and Song, 2017). This degradation leads to transcriptional activation or repression of JA-responsive genes. In addition to its role in defense to biotic stress, several studies have shown that JA is also involved in plant responses to abiotic stresses including cold, heat, drought, salinity, cesium, ozone and K^+ deficiency (Balfagón et al., 2019). Furthermore, recent studies have reported that JA is linked to plant response to heavy metals such as cadmium (Cd), arsenic (As), chromium (Cr), and lead (Pb) (Lei et al., 2020).

Previous studies have demonstrated that high levels of Sr play a negative effect on plant growth and development. In addition, Sr contamination to environment evidently become a serious threat to human health. Therefore, understanding the molecular mechanism of how plants sense and respond to Sr stress is important for environment and human health. However, the molecular mechanisms underlying plant response to Sr stress remains largely unexplored. In this study, we investigated the transcriptional changes under Sr stress using RNA-seq analysis to obtain information on the molecular basis in plant responses to Sr stress. Based on the analysis of DEGs using GO, KEGG, and MapMan, we found that genes related to JA biosynthesis and signaling were significantly up-regulated under Sr stress. Therefore, our results suggested that JA biosynthesis and/or signaling might be associated with plant responses to Sr stress, and play important roles in the plant growth and development under Sr stress. Our findings provide valuable information about the molecular basis underlying plant responses to Sr stress and several candidate Sr-responsive genes for plant growth and development under Sr stress.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. *Columbia*-0 ecotype (*Col*-0, ABRC stock center #: CS1902) were surface sterilized and incubated at 4℃for 3 days and then plated on half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie, Netherlands) containing 1% (w/v) sucrose and 0.8% (w/v) phyto agar (Duchefa Biochemie, Netherlands). Stratificated Col-0 seeds were then transferred to the growth chamber and grown for 10 days in long day (16 h light/8 h dark, LD) on MS medium alone or supplemented with 20 mM $SrCl_2$ (Cat. No.: 255521, Sigma-Aldrich, USA) in a growth chamber at 22 ℃ under cool-white fluorescent lamp (8890 lux).

2.2. Library preparation and RNA-seq analysis

Total RNA was isolated from 10-ᴅ-old seedlings grown on half strength MS medium alone (control) or supplemented with 20 mM $SrCl_2$ (Sigma-Aldrich, USA) using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The construction of RNA libraries with MS and $SrCl_2$-treated samples, and RNA sequencing were completed by Macrogen Inc., Korea (http://www.macrogen.com).

2.3. Identification of differentially expressed genes (DEGs)

The raw sequence reads were pre-processed for quality check using FastQC (Andrews, 2010), and removed adaptor sequences and low-quality reads using Trimmomatic (Bolger et al., 2014). The cleaned reads were mapped onto the Arabidopsis reference genome TAIR10 using HISAT2 (Kim et al., 2019) and mapped reads were counted using HTSeq-count (Anders et al., 2014). The raw read counts were imported into DESeq2 package to perform the differential gene expression analysis (Love et al., 2014). The DEGs were defined as genes with both an adjusted P-value < 0.05 and an absolute $\log_2$ fold change ≥ 1.

2.4. Functional analysis of DEGs

GO enrichment analysis of the DEGs was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009). The results from DAVID were further processed using the REVIGO to reduce redundant GO term categories (Supek et al., 2011). KEGG Pathway enrichment analysis of the DEGs was performed using KEGG pathway database (http://www.genome.jp/kegg/) and KOBAS (KEGG Orthology-based Annotation System) to identify significantly enriched metabolic pathways or signaling pathways (Xie et al., 2011). The MapMan was used to visualize the metabolic pathways of the DEGs (Thimm et al., 2004).

2.5. Quantitative real-time RT-PCR (qRT-PCR) analysis

The total RNA was extracted from 10-ᴅ-old seedlings grown on MS medium with or without 20 mM $SrCl_2$ using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA with oligo-dT primer using the M-MLV Reverse Transcriptase (Promega, USA). qPCR experiments were conducted using TB Green Fast qPCR Mix (Takara Bio, Japan) and a LightCycler 96 real-time PCR system (Roche, Switzerland). The reaction was performed using the following PCR cycle: 95 ℃ for 30 s, 40 cycles at 95℃for 10 s, 55 ℃ for 10 s, and 72 ℃ for 10 s *UBI10* was used as the internal reference gene. Each experiment was repeated with three independent biological samples. The primers used in this study are listed in Supplementary Table S1.

2.6. Statistical analysis

One-way ANOVA followed by Tukey's test were performed using SigmaPlot software (version 14.0, Systat Software Inc.). Significant differences were indicated by different letters above the bars ($p < 0.05$).

3. Results and discussion

3.1. Identification of DEGs in response to Sr stress

Previous our study showed that plant growth and development were clearly inhibited when plants were grown on MS medium containing

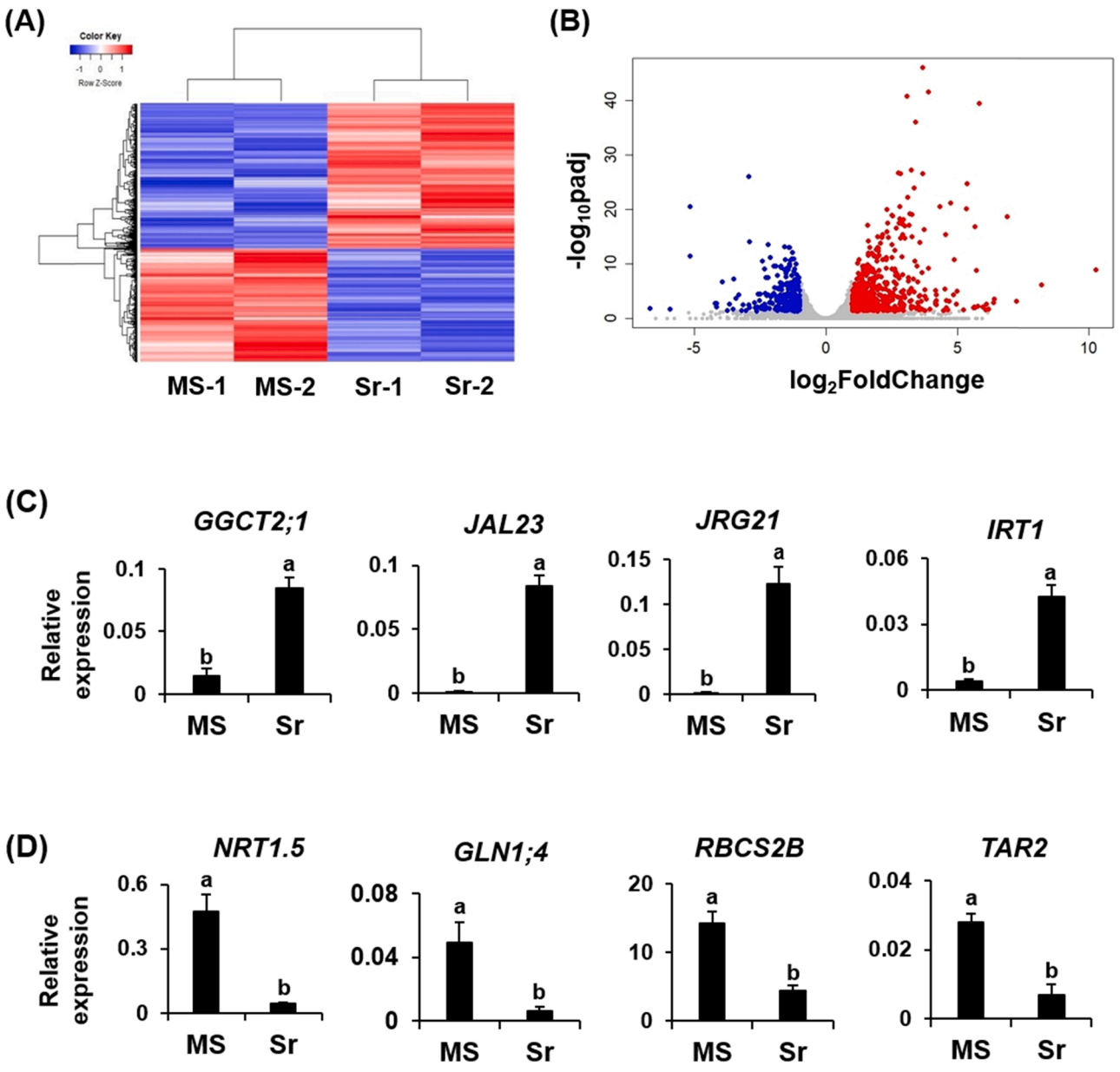


Fig. 1. Expression profiles of differentially expressed genes (DEGs) in response to Sr stress. **(A)** Heatmap and hierarchial cluster analysis of the DEGs between the control samples (MS) and the Sr-treated samples (Sr). Each column represents a sample, and each row represents a differentially expressed gene (DEG). Red and blue represents up-regulated and down-regulated genes, respectively. **(B)** Volcano plot of the DEGs between MS and Sr. X-axis represents the $\log_2$foldchange and Y-axis represents the $-\log_{10}$(adjusted p-value). The threshold in the volcano plot was adjusted P value < 0.05 and $|\log2\text{foldchange}| > 1$; Red and blue represents up-regulated and down-regulated genes, respectively. Grey represents genes with no significant difference. **(C)** qRT-PCR analysis of 4 selected DEGs (*GGCT2;1*, *IRT1*, *JAL23*, and *JRG21*) showing up-regulated expression in the Sr-treated samples (Sr) compared to the control samples (MS). **(D)** qRT-PCR analysis of 4 selected DEGs (*NRT1.5*, *GLN1;4*, *RBCS2B*, and *TAR2*) showing down-regulated expression in the Sr-treated samples (Sr) compared to the control samples (MS). For **(C)** and **(D)**, the relative expression was normalized to *UBI10*. The data represents the mean of the relative expression from three biological replicates with the error bars showing standard deviation ($n = 3$). One-way ANOVA followed by Tukey's test was applied to calculate the statistical significance. Significant differences were indicated by different letters above the bars ($p < 0.05$).

concentrations of 20 mM $SrCl_2$ or higher (Pyo et al., 2020). Therefore, 20 mM $SrCl_2$ was selected for RNA-seq analysis. To understand how plants sense and respond to Sr stress at the molecular level, we examined the global gene expression profile of 10-D-old Arabidopsis seedlings (ecotype *Col*-0) grown on MS medium with or without 20 mM $SrCl_2$ using RNA-seq analysis. After removing low quality, N-containing and adaptor-contaminated reads, 42,734,922 and 33,941,420 clean reads for control (MS) and 41,409,960 and 49,260,306 clean reads for Sr-treated samples (Sr) were obtained (Supplementary Table S2). All clean reads were mapped to the *Arabidopsis* reference genome TAIR 10 using HISAT2 (Kim et al., 2019). Approximately, 99.38% and 99.32% reads from MS and 99.35% and 99.32% reads from Sr were mapped to the Arabidopsis reference genome, respectively (Supplementary Table S2). HTSeq-count was then used to estimate the mapped reads per genes (Anders et al., 2014). In order to identify potential candidate genes related to plant responses to Sr stress, we analyzed the differentially expressed genes (DEGs) between the control and Sr-treated seedlings using the DESeq2 package (Love et al., 2014). Compared with control, we identified a total of 842 DEGs in response to Sr stress based on the criteria of adjusted P value < 0.05 and $|\log_2\text{FoldChange}| \geq 1$ (Fig. 1A and B). Among those DEGs, 559 genes were up-regulated, 283 genes were down-regulated in response to Sr stress (Supplementary

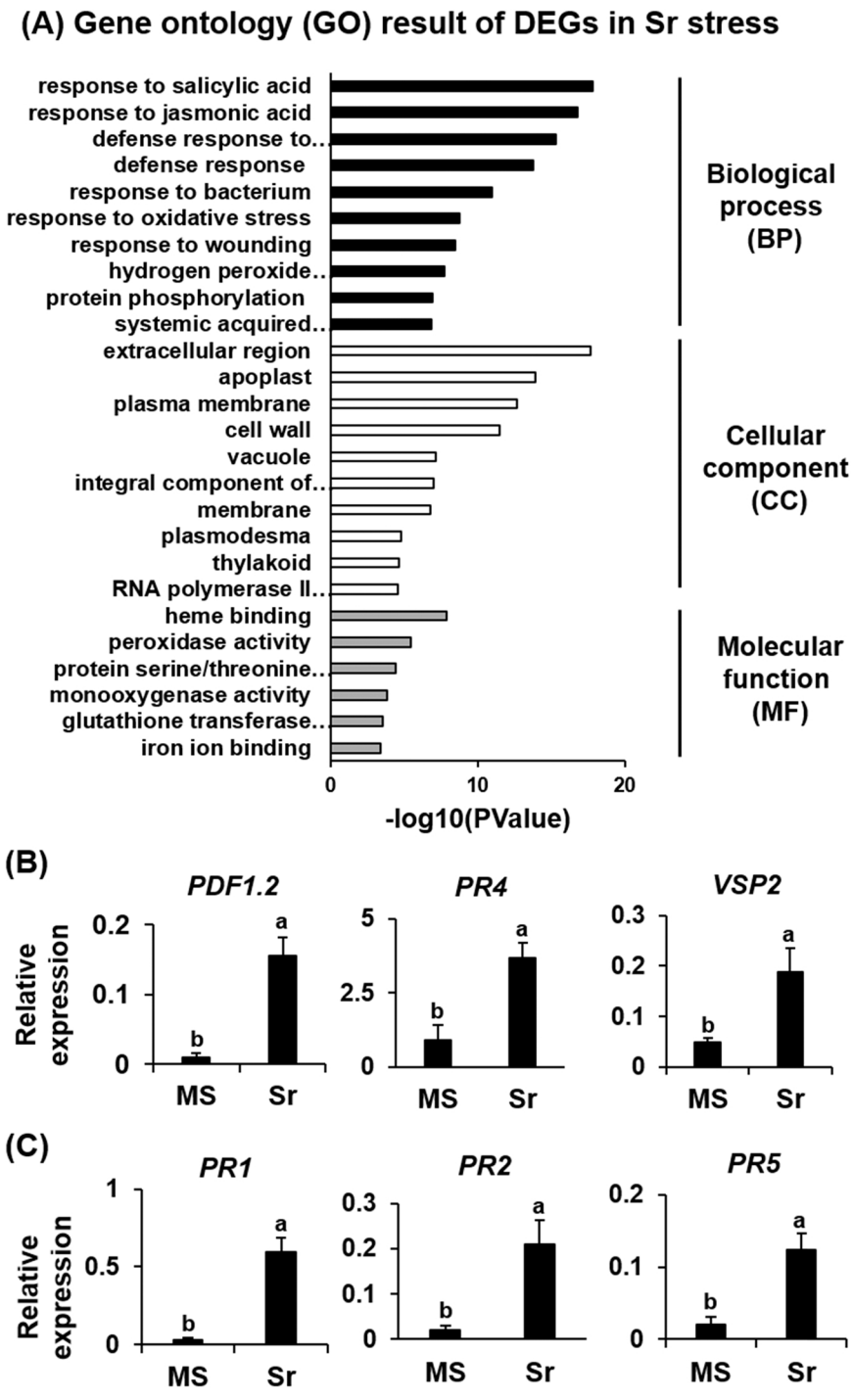


Fig. 2. Gene Ontology (GO) enrichment analysis of DEGs in response to Sr stress. **(A)** GO analysis of DEGs. The x-axis and y-axis represent $-\log_{10}$ (PValule) and the names of GO terms, respectively. GO enrichment analysis of the DEGs was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009). The results from DAVID were further processed using the REVIGO to reduce redundant GO term categories. **(B)** Expression of *PDF1.2*, *PR4*, and *VSP2* genes related to the jasmonic acid (JA) and **(C)** Expression of *PR1*, *PR2*, and *PR5* genes related to the salicylic acid (SA) in response to Sr stress. The relative expression was normalized to *UBI10*. For **(B)** and **(C)**, the data represents the mean of the relative expression from three biological replicates with the error bars showing standard deviation (n = 3).). One-way ANOVA followed by Tukey's test was applied to calculate the statistical significance. Significant differences were indicated by different letters above the bars ($p < 0.05$).

Fig. S1). Full lists of DEGs were shown in Supplementary Table S3.

To validate the DEGs determined by RNA-seq analysis, we chose 16 genes that were representing up-regulated and down-regulated, and compared the expression level between the control and Sr-treated seedlings using qRT-PCR assay. As shown in Fig. 1C, all tested genes showed similar expression patterns as those observed by RNA-seq analysis. The expression levels of 4 genes representing up-regulated DEGs, *GGCT2;1* (*GAMMA-GLUTAMYL CYCLOTRANSFERASE 2;1*), *IRT1* (*IRON-REGULATED TRANSPORTER 1*), *JAL23* (*JACALIN-RELATED LECTIN 23*), and *JRG21* (*JASMONATE-REGULATED GENE 21*)) were significantly increased in Sr-treated seedlings compared to those of the control seedlings. It was previously reported that GGCT2;1 is involved in the detoxification of toxic metals by ensuring GSH degradation during abiotic stress condition (Paulose et al., 2013). IRT1 is a metal transporter required for uptake of iron from the soil, which was up-regulated in the iron deficiency (Vert et al., 2002). In addition, it was recently shown that Jacalin-related lectin proteins play a role in the plant resistance against a diversity of stresses (Esch and Schaffrath, 2017), suggesting that *JAL23* encoding Jacalin-like lectin might also have a function involved in the plant stress response. A reactive oxygen species (ROS) marker gene, *JRG21* was previously shown to be induced by other stresses like pathogen and H_2O_2 (Balazadeh et al., 2012; Mehterov et al., 2012), implying that *JRG21* might be involved in a broad range of plant resistance in *Arabidopsis* plant. In contrast, the expression of 4 genes showing down-regulated DEGs, *GLN1;4* (*GLUTAMINE SYNTHASE 1;4*), *NRT1.5* (*NITRATE TRANSPORTER 1.5*), *RBCS2B*

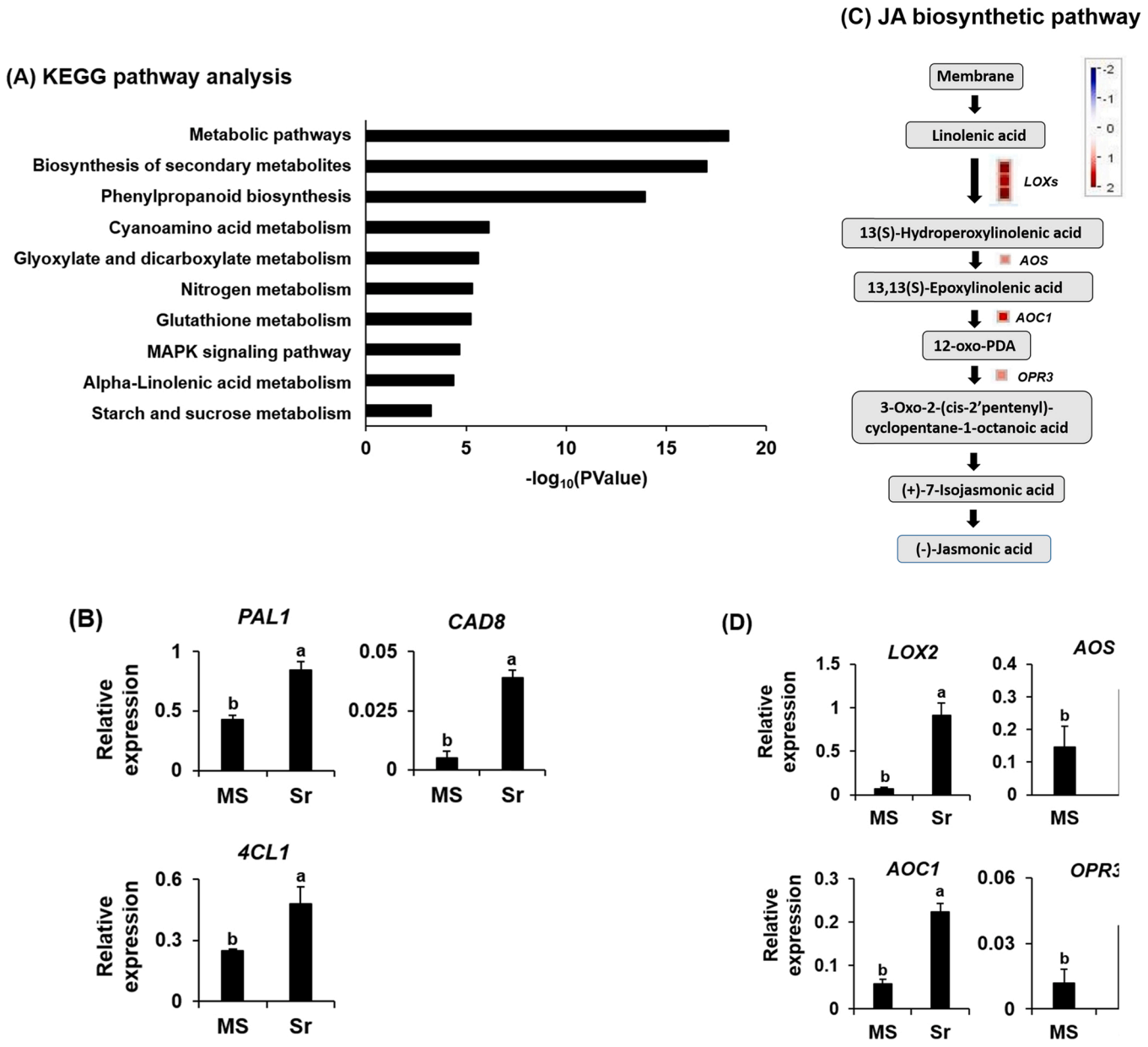


Fig. 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs in response to Sr stress. **(A)** KEGG pathway enrichment analysis of DEGs. The x-axis indicates $-\log_{10}$(P value) and the y-axis represents the pathway name. **(B)** Expression of genes (*PAL1*, *CAD8*, and *4CL1*) related to phenylpropanoid biosynthesis in response to Sr stress. The relative expression was normalized to *UBI10*. The data represents the mean of the relative expression from three biological replicates with the error bars showing standard deviation (n = 3).). One-way ANOVA followed by Tukey's test was applied to calculate the statistical significance. Significant differences were indicated by different letters above the bars ($p < 0.05$). **(C)** MapMan analysis of JA biosynthetic process in response to Sr stress. Boxes with red indicate up-regulated genes in response to Sr stress. **(D)** Expression profiles of genes (*LOX2*, *AOS*, *AOC1*, and *OPR3*) involved in JA biosynthesis under Sr stress by qRT-PCR analysis. The relative expression was normalized to *UBI10*. The data represents the mean of the relative expression from three biological replicates with the error bars showing standard deviation (n = 3).). One-way ANOVA followed by Tukey's test was applied to calculate the statistical significance. Significant differences were indicated by different letters above the bars ($p < 0.05$).

(*RUBISCO SMALL SUBUNIT 2B*), and *TAR2* (*TRYPTOPHAN AMINO-TRANSFERASE RELATED 2*) were significantly reduced under Sr stress. These results were consistent with the RNA-seq data, indicating that our RNA-seq data were reliable for further analysis. Collectively, we found that some stress-responsive genes like *GGCT2;1*, *IRT1*, *JAL23*, and *JRG21* play a positive role in the plant response against Sr stress, indicative of Sr-responsive marker genes in *Arabidopsis* plant.

3.2. Gene ontology (GO) enrichment analysis of DEGs in response to Sr stress

To elucidate the biological function of DEGs in response to Sr stress, we performed GO enrichment analysis using the DAVID and REVIGO (Huang et al., 2009; Supek et al., 2011). GO analysis revealed that the DEGs were significantly enriched in GO terms related to the defense responses (Fig. 2A and Supplementary Table S4). In terms of biological process (BP), we found that the most significant GO terms were response to SA and response to JA, implying that these hormones might play an important role in the plant response to Sr stress. For the cellular

component (CC) category, the DEGs in response to Sr stress showed a clear enrichment in 'extracellular region', 'apoplast', 'plasma membrane', and 'cell wall'. In the molecular function (MF) category, the major GO terms were 'heme binding' and 'peroxidase activities'.

To further dissect the plant response to Sr stress, we also carried out GO enrichment analysis separately for the up-regulated and down-regulated DEGs (Supplementary Fig. S2). In the BP category, GO terms of up-regulated DEGs was most enriched at the 'response to salicylic acid (SA)', and 'response to jasmonic acid (JA)' and 'defense response'. In the CC category, the up-regulated DEGs were significantly enriched in GO terms related with 'extracellular region', 'plasma membrane', and 'cell wall'. In the MF, the up-regulated DEGs were enriched into GO terms related with 'heme binding' and 'protein serine/threonine kinase'. For down-regulated DEGs, in the BP, response to 'red light', 'photosynthesis', and 'photosynthetic electron complex' were significantly enriched. In the CC, the down-regulated DEGs were significantly enriched into chloroplast-related GO terms, including 'thylakoid', 'chloroplast thylakoid membrane', and 'chloroplast'. In the MF, the down-regulated DEGs were enriched into GO terms of 'oxidoreductase activity' and 'ribulose-bisphosphate carboxylase activity'.

Our GO analysis suggested that Sr stress may affect JA- and SA-signaling pathways (Fig. 2A). To confirm that the JA- and/or SA signaling pathway are associated with plant response to Sr stress, we examined the expression level of several JA- and SA-responsive genes by qRT-PCR analysis. While the expression of *PDF1.2* (*PLANT DEFENSIN 2.1*), *PR4* (*PATHOGENESIS-RELATED 4*), and *VSP2* (*VEGETATIVE STORAGE PROTEIN 2*) are used as markers for JA-dependent pathway (Penninckx et al., 1998; Lorenzo et al., 2003), the expression of *PR1* (*PATHOGENESIS-RELATED 1*), *PR2* (*PATHOGENESIS-RELATED 2*), and *PR5* (*PATHOGENESIS-RELATED 5*) are considered as markers for SA-dependent pathway (Durrant and Dong, 2004). As shown in Fig. 2B and C, in consistent with RNA-seq analysis, the expression of all these genes were significantly induced by Sr stress, implying that both JA- and SA-signaling pathway may be activated in response to Sr stress. Besides JA-responsive genes, marker genes (*PR1*, *PR2*, and *PR5*) involved in SA pathway were strongly up-regulated under Sr stress in RNA-seq and qRT-PCR analysis (Fig. 2C), indicating that SA signaling is also involved in plant response to Sr stress. Thus, our result unveiled that plants respond to Sr stress by activation of defense response through JA and SA pathway. Because JA has been suggested to antagonize SA signaling, it remains to be investigated how both JA- and SA-signaling pathways contribute to the response against Sr stress.

Based on the GO analysis, a number of DEGs in response to Sr stress were involved in JA signaling pathway and many of them (28 of 35) were up-regulated under Sr stress (Fig. 2A). In addition, the expression of marker genes in JA signaling pathway (*PDF1.2*, *PR4*, and *VSP2*) was strongly up-regulated under Sr stress (Fig. 2B). These results suggest that JA signaling pathway might be activated under Sr stress, thus helping plants cope with Sr toxicity. It has been reported that endogenous JA level is induced under various abiotic stress conditions, such as salt, drought, cold, and heat stress (Wang et al., 2020). Thus, our results are in the line with the previous studies describing positive relationship between JA level and abiotic stresses. As a result, our study revealed that JA biosynthesis is induced under Sr stress and required for the plant response to Sr stress.

3.3. KEGG pathway enrichment analysis revealed that JA pathway might play a role in plant response to Sr stress

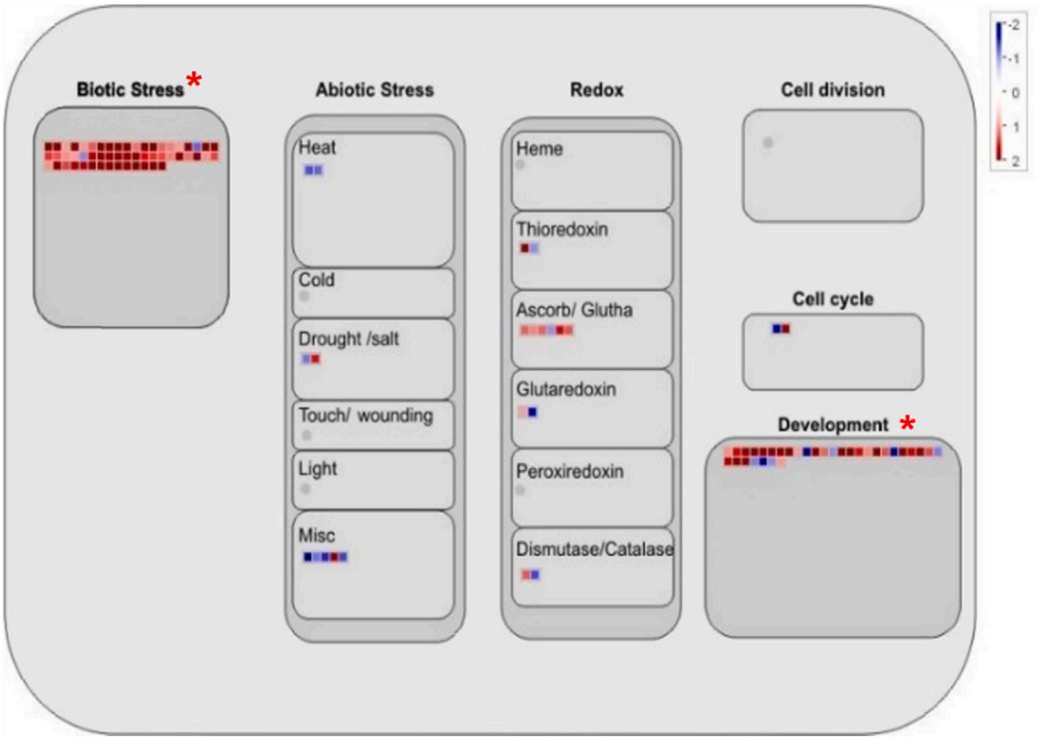
To further obtain biological information for the DEGs in response to Sr stress, we performed a pathway enrichment analysis using KEGG pathway database (https://www.genome.jp/kegg/) and KOBAS (Xie et al., 2011). KEGG analysis showed that 'metabolic pathways (133 genes)' were most significantly enriched, followed by 'biosynthesis of secondary metabolites' (84 genes), and 'phenylpropanoid biosynthesis' (29 genes), indicating that a variety of metabolic pathways were affected in the process of plant response to Sr stress (Fig. 3A and Supplementary Table S5). For instance, most of 'phenylpropanoid biosynthesis' category genes (26 out of 29) were up-regulated and only 3 DEGs were down-regulated under Sr stress (Supplementary Table S5). In addition, we noticed that the 'alpha-linolenic acid metabolism' pathway was showed in the top 10 significantly enriched KEGG pathways (Fig. 3A). Because this 'alpha-linolenic acid metabolism' pathway is involved in the production of precursors of a defense-related plant hormone, JA, it is likely that JA biosynthetic process might be stimulated by the presence of Sr stress.

To further dissect the plant response to Sr stress, we also analyzed pathway enrichment analysis separately for the up-regulated and down-regulated DEGs (Supplementary Fig. S3). In the up-regulated DEGs, most of genes are primarily participated in 'phenylpropanoid biosynthesis', 'metabolic pathway', 'biosynthesis of secondary metabolite', 'cyanoamino acid metabolism', 'alpha-linolenic acid metabolism', and 'glutathione metabolism'. This result coincides with the result of GO enrichment analysis shown in Fig. 3A (Supplementary Fig. S3A). The down-regulated DEGs were enriched in the pathways of 'glyoxylate and dicarboxylate metabolism', 'biosynthesis of secondary metabolite', 'metabolic pathway', 'carbon metabolism', 'carbon fixation in photosynthesis', and 'nitrogen metabolism' (Supplementary Fig. S3B). 'Metabolic pathway' genes were substantially affected by Sr stress either down-regulated or up-regulated. In addition, 'Photosynthesis' and 'Carbon fixation', 'Carbon metabolism' were uniquely found in the list of down-regulated DEGs. Since these pathway genes are involved in the energy production and plant growth, it is likely that plant growth were substantially reduced under Sr stress, which was demonstrated by our previous study (Pyo et al., 2020). This is also in the line with other studies, reporting that a diversity of stresses negatively affect the expression of genes involved in the 'photosynthesis' and 'carbohydrate metabolism', thus reducing plant growth (Bechtold and Field, 2018).

Our KEGG analysis showed that Sr stress affects 'phenylpropanoid biosynthesis' pathway genes (Supplementary Table S6). Phenylpropanoids are a group of secondary metabolites including lignin, anthocyanin and flavonoids, which function to elevate the plant resistance against a diversity of stresses (Fraser and Chapple, 2011; Tang and Tang, 2021; Vogt, 2010). Thus, we selected some of 'phenylpropapanoid' pathway genes and analyzed the expression levels of genes involved in phenylpropanoid pathway by qRT-PCR analysis (Fig. 3B). PAL1 (PHE AMMONIA LYASE 1), 4CL1 (4-COUMARATE:COA LIGASE 1), and CAD8 (CINNAMYL-ALCOHOL DEHYDROGENASE 8) are key enzymes in the phenylpropanoid biosynthesis. *PAL1* encodes PHE AMMONIA LYASE 1 which is a critical enzyme in the first step of the phenylpropanoid pathway (Zhang and Liu, 2015). 4CL1 and CAD8 are involved in lignin biosynthesis which produces monolignol and lignin monomers, respectively (Li et al., 2015; Raes et al., 2003). The expression of these genes were significantly induced by Sr stress, implying that the phenylpropanoid biosynthesis was activated and the increased secondary metabolites like lignin and anthocyanin might play a beneficial role in plant defense or growth under Sr stress.

Like the result of GO analysis, KEGG analysis also showed that 'alpha-linolenic acid' is affected by Sr stress (Fig. 3A). The JA biosynthesis are catalyzed by the enzymes like LIPOXYGENASE 2 (LOX2), ALLENE OXIDE SYNTHASE (AOS), and ALLENE OXIDE CYCLASE 3 (AOC3), OPR3 (OXOPHYTODIENOATE-REDUCTASE 3) (Stenzel et al., 2003). We found all six DEGs related to JA biosynthesis, such as *LOX2*, *LOX3*, *LOX4*, *AOS*, *AOC3*, and *OPR3* exhibited increased expression under Sr stress (Fig. 3C). Thus, we checked the expression of these JA biosynthetic pathway genes. Expression of *LOX2*, *AOS*, *AOC3*, and *OPR3* genes were significantly up-regulated under Sr stress (Fig. 3D), suggesting JA biosynthetic pathway is stimulated in response to Sr stress and might play a role in stress response against Sr metal.

(A) Cellular response overview



(B) Biotic stress overview

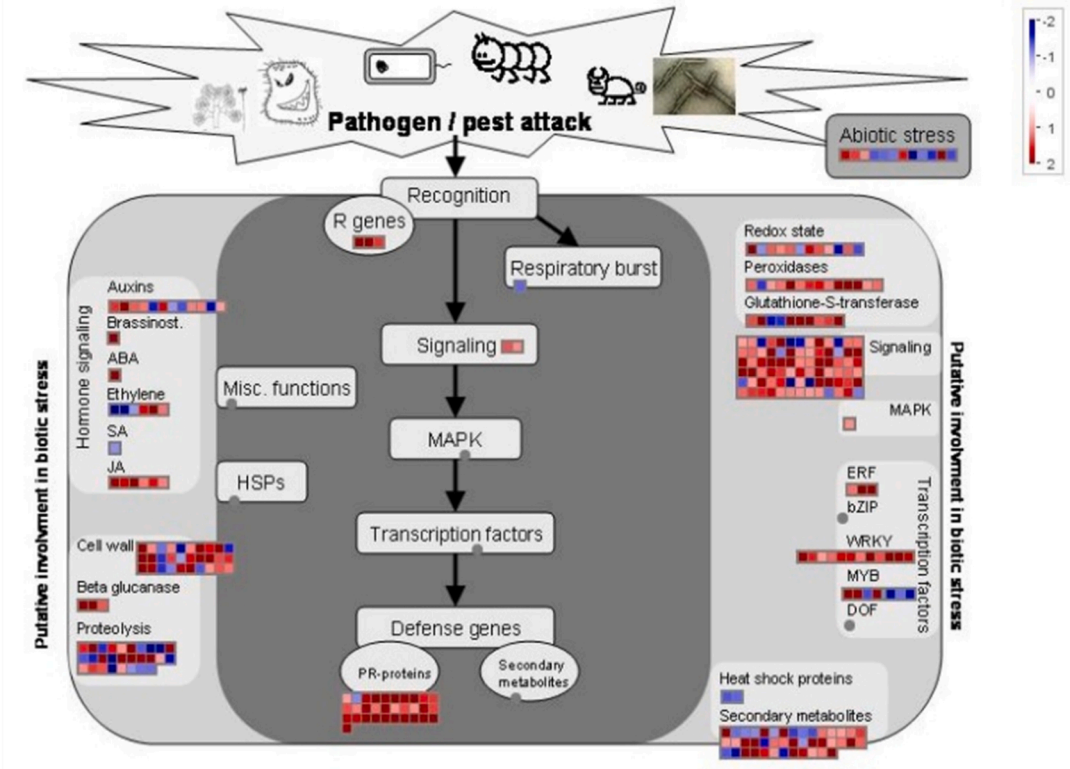


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Fig. 4. MapMan analysis of DEGs in response to Sr stress. **(A)** Within cellular response overview category, genes involved in the biotic stress and development are actively up-regulated upon Sr stress (marked with red asterisks). It indicates that many biotic stress responsive genes are involved in the plant response against Sr stress. In addition, plant development might be severely affected by the Sr stress. Boxes with red and blue indicate up-regulated and down-regulated genes in response to Sr stress, respectively. The graduation can be seen on the scale presented in the top right corner of each subfigure. The values are the $\log_2$ fold change. **(B)** Genes in biotic stress category was affected by Sr stress. Among hormone signaling, jasmonic acid (JA) signaling is highly induced by Sr stress. Many PR genes involved in biotic stress response are highly induced upon Sr stress, suggesting that biotic stress responsive PR genes might play a role in plant defense against Sr stress. In addition, many signaling genes are severely induced by Sr stress, indicating systematic defense system are turned on in Sr stress condition. Boxes with red and blue indicate up-regulated and down-regulated genes in response to Sr stress, respectively. The graduation can be seen on the scale presented in the top right corner of each subfigure. The values are the $\log_2$foldchange. MapMan software (ver. 3.6.0) was used to identify the enriched metabolic pathways (https://mapman.gabipd.org/mapman).

3.4. MapMan metabolic pathway analysis reveal that many biotic stress responsive genes are involved in the response to Sr stress

Since Sr stress affects plant metabolisms, we decided to further investigate the functional annotation and categorization of DEGs in response to Sr stress. Thus, we performed metabolic pathway analysis using MapMan (Thimm et al., 2004). Similar to results from the previous GO and KEGG analyses, in the 'secondary metabolism' overview, genes related to the 'cell wall' and 'secondary metabolism' such as 'phenylpropanoids', 'lignin and lignans', 'anthocyanins' as well as 'glucosinolates' were significantly up-regulated under Sr stress (indicated with red boxes in Supplementary Fig. S4). It indicates that plants actively produce a diversity of secondary metabolites to deal with Sr stress.

Previous studies have also indicated that Sr treatment could induce the production of secondary metabolites (Kalingan et al., 2016; Wójciak-Kosior et al., 2016). According to our results from KEGG and MapMan analyses, genes related to phenylpropanoid biosynthesis, one of the most significant secondary metabolism pathway in plants, were mainly enriched under Sr stress (Figs. 3 and 4). We found that the 29 genes involved in 'phenylpropanoids biosynthesis' were affected in response to Sr stress and most of these DEGs (26 of 29 genes) were up-regulated (Supplementary Table S6), implying that many phenylpropanoid biosynthesis-related genes may play important roles in plant responses to Sr stress. The qRT-PCR results were consistent with the transcriptome data, and showed up-regulated expression levels of *PAL1*, *4CL1*, and *CAD8* in response to Sr stress (Fig. 4). The 'phenylpropanoid biosynthesis' pathway has been reported to be involved in many biological processes, such as growth and development, as well as defense responses to biotic and abiotic stresses (Sadeghnezhad et al., 2019; Tang and Tang, 2021; Vogt, 2010). Therefore, these results indicate that 'phenylpropanoid biosynthesis' pathway is activated under Sr stress, leading to the accumulation of secondary metabolites which function in proper growth and development under Sr stress, as well as defense responses to Sr stress.

Next, we looked into the 'cellular response' overview using DEGs (Fig. 4A). DEGs were highly enriched with the 'biotic stress' and 'development' categories but not in the 'abiotic stress'. For instance, a total of 54 and 32 genes were associated with 'biotic stress' and 'development' categories, respectively. In case of 'biotic stress' category, most of them (52 of 54 genes) were up-regulated, whereas small portion of genes (2/54) were down-regulated in response to Sr stress (Fig. 4A and Supplementary Table S7), suggesting that *Arabidopsis* plants might deploy biotic stress defensive machinery to cope with the abiotic stress caused by Sr metal. In addition, 32 DEGs genes fell into the 'development' category (Fig. 4A and Supplementary Table S8). Among them, 25 genes (78%) were up-regulated and 7 genes (22%) were down-regulated under Sr stress. This data is in the line with the previous report that Sr stress negatively affect plant developmental program at least in part by inhibiting root growth, seedling development, and photosynthesis (Pyo et al., 2020).

Furthermore, because many of DEGs were involved in the 'biotic stress' response, we further dissected the DEGs found in the 'biotic stress' category (Fig. 4B). The enriched DEGs were associated with the 'signaling', 'secondary metabolites', 'proteolysis', 'cell wall', and 'PR genes'. Within the 'biotic stress' overview, we noticed that 78 genes were involved in the 'signaling' (Supplementary Table S9). Out of 78 signaling genes, 67 signaling genes were up-regulated and 11 genes down-regulated. Many of these signaling genes encode 'receptor kinases' and 'calcium-binding proteins' known to be involved in a diversity of plant defense mechanism. Many of PR defense genes were also highly up-regulated, consistently indicating that plants might deploy PR-mediated defense system to deal with Sr metal stress.

In consistency with GO and KEGG result, among plant hormones, we found that 41 JA signaling-related genes were substantially activated in response to Sr stress (Fig. 4B and Supplementary Table S10). It is likely that Sr stress triggers JA biosynthesis and/or signaling pathway, resulting in high endogenous JA level which in turn stimulates the production of secondary metabolites including phenylpropanoids.

In this study, we performed transcriptome analysis and identified a large number of candidate genes involved in plant response to Sr stress. Our results indicated that a number of Sr-responsive genes were related to the secondary metabolites. Our GO, KEGG, and MapMan analyses notified that among plant hormones, JA biosynthesis and/or signaling is significantly induced in the presence of Sr stress and JA play a pivotal role in plant response to Sr stress. Because many Sr-induced or repressed genes were identified in this study, these genes can be good Sr-responsive marker genes including JA-response genes verified in qRT-PCR in this study. In summary, our results not only provide useful information of the molecular basis in plant response to Sr stress but also identified candidate Sr stress marker genes (i.e. JA-responsive genes) required for plant defense against Sr stress.

CRediT authorship contribution statement

Youngjae Pyo: Conceptualization, Investigation, Writing-Original Draft. **Heewon Moon**: Investigation, Formal analysis. **Adji Baskoro Dwi Nugroho**: Validation, Formal analysis. **Seong Wook Yang**: Conceptualization, Resources. **Il Lae Jung**: Conceptualization, Funding acquisition. **Dong-Hwan Kim**: Conceptualization, Data curation, Writing-review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113552.

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