A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Identification of the soybean genes responsible for UV-B stress by genome and transcriptome analyses

BY

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DECEMBER 2016

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GENERAL ABSTRACT

The depletion of the ozone layer in the stratosphere has led to a dramatic spike in ultraviolet B (UV-B) intensity and increased UV-B light levels. The direct absorption of high-intensity UV-B induces complex abiotic stresses in plants including excessive light exposure, heat, and dehydration. However, UV-B stress signaling mechanisms in plants including soybean (*Glycine max* [L.]) and quantitative loci (QTLs) responsible for UV-B resistance remain poorly understood. Therefore, we surveyed the overall transcriptional responses of two soybean genotypes, UV-B-sensitive Cheongja 3 and UV-B-resistant Buseok, to continuous UV-B irradiation for 0

(control), 0.5, and 6 h using RNA-seq analysis to identify signaling genes responsible for UV-B. Homology analysis using UV-B-related genes from *Arabidopsis thaliana* revealed differentially expressed genes (DEGs) likely involved in UV-B stress responses. Functional classification of the DEGs showed that the categories of immune response, stress defense signaling, and reactive oxygen species (ROS) metabolism were over-represented. UV-B-resistant Buseok utilized phosphatidic acid-dependent signaling pathways (based on subsequent reactions of phospholipase C and diacylglycerol kinase) rather than phospholipase D in response to UV-B exposure at high fluence rates, and genes involved in its downstream pathways, such as ABA signaling, mitogen-activated protein kinase cascades, and ROS overproduction, were upregulated in this genotype. In addition, the DEGs for TIR-NBS-LRR and heat shock proteins are positively activated. These results suggest that defense mechanisms against UV-B stress at high fluence rates are separate from the photomorphogenic responses utilized by plants to adapt to low-level UV light.

Furthermore soybean (*Glycine max* [L.]) has evolved to survive under abiotic and biotic stress conditions by utilizing multiple signaling pathways. Although several studies have revealed shared defense signaling pathways in plants, the majority of components at the convergence points of signaling pathways triggered by both abiotic and biotic stress remain poorly understood. In chapter II, we profiled the overall transcriptional responses of

soybean to two different types of stress using the UV-B-resistant cultivar, Buseok, and the UV-B-sensitive cultivar, Cheongja 3, as well as two near isogenic lines (NILs) carrying bacterial leaf pustule (BLP) disease-resistant and -susceptible alleles. We compared transcript abundance and identified genes that commonly respond to UV-B stress and BLP disease. In addition, we surveyed the co-localization of differentially expressed genes (DEGs) and their paralogs with abiotic and biotic stress-related quantitative trait loci (QTLs) on the soybean genome. Among 14 DEGs that respond to both stresses, five DEGs are involved in the jasmonic acid (JA) metabolic pathway, encoding Jasmonate ZIM (Zinc-finger protein expressed in Inflorescence Meristem) domain-containing protein 1 (JAZ 1), a negative regulator of JA signaling. Two DEGs for JAZ 1 were co-localized with biotic stress-related QTLs. One DEG encoding the stress-induced protein starvation-associated message 22 (SAM 22) and its two paralogs were co-localized with both abiotic and biotic stress-related QTLs.

In Chapter III, we investigated phenotypic data of UV-B treated 174 F_6 RIL population derived from Cheongja 3 and Buseok, and we tried to construct genetic map by genotype-by-sequencing to identify QTLs and candidate genes responsible for UV-B resistance. High degree of phenotypic variations was shown in response to UV-B irradiation. Frequency distribution of leaf damage degree for UV-B treatments were ranged between 10 and 100%. The mean range of damage leaf degree was 50.3%, paternal UV-B

resistance Buseok showed 26.8% damage degree, and maternal UV-B sensitive Cheongja 3 exhibited 62.4 damage degrees (%).Total high quality 2,291 SNPs were obtained and used for construction of genetic map using Joinmap 4.1. The newly detected two QTLs in this study are *UVBR14-1* and *UVBR17-1*. The one gene on chr 14 is K+ efflux antiporter 6 (KEA 6) (Glyma.14g093900) which may contribute towards K+ acquisition and homeostasis under saline conditions. The four candidate genes on chr 17 are Leucine rich repeat F-box/RNI-like superfamily protein (TIR1) (Glyma.17g247500), inositol polyphosphate 5-phosphatase 11 (Glyma.17g247600), Adaptor protein rnigma and related PDZ-LIM proteins (DA1) (Glyma.17g247700) and Leucine rich repeat receptor-like protein kinase (Glyma.17g247800). The biological function of KEA 6 on chr 14 and InsP5-ptase on chr 17 need to be examined more as UV-B responsible gene under UV-B light as multiple stressors including heat and hydration.

Finally, the results of this comprehensive study elucidate not only soybean signaling mechanism under UV-B stress but also identification of the candidate genes responsible for UV-B resistance. These results will help develop high adoptable soybean breeding under UV-B irradiation.

Key words: UV-B stress, Bacterial leaf pustule disease, soybean transcriptomic profiling, phosphatidic acid, diacylglycerol kinase, TIR-NBS-

LRR, JAZ 1, SAM 22, Shared defense signaling, Genotyping-by-sequencing, KEA 6, InsP5-ptase

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phosphatase; PLC: Phospholipase C; DG: Diacylglycerol; DGK: Diacylglycerol kinase; PA: Phosphatidic acid; PAB: Phosphatidic acid binding protein; ABI1: ABA insensitive phosphatase 1; IPK2: Inositol 1,3,4-trisphosphate 5/6-kinase family protein; IP6: inositol hexaphosphate; Cu/Zn SOD: Copper/Zinc superoxide dismutase; Fe- SOD: Iron-superoxide dismutase; Cyt P450: Cytochrome P450 family protein; F3H: Flavanone-3-hydroxylase; C4H: Cinnamate-4-hydroxylase; MAPK: Mitogen-activated-protein kinase; HSP 70: Heat shock protein 70; TIR-NBS-LRRs: Toll/interleukin-1 receptor nucleotide binding site leucine-rich repeat.

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NAC 3; NAC domain-containing protein 3, SAM 22; starvation-associated message 22, JAZ 1; Jasmonate ZIM domain-containing protein 1, PCSK; Protein convertase subtilisin/kexin

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LIST OF ABBREVIATIONS

BLP	Bacterial leaf pustule disease
Cu/Zn SOD	copper/zinc superoxide dismutase
DEG	Differentially expressed gene
DGK	Diacylglycerol kinase
FPKM	Number of fragments per kilobase of exon per million fragments mapped
GBS	Genotyping-by-sequencing
HSP	Heat shock protein
JA	Jasmonic acid
JAZ 1	Jasmonate ZIM (Zinc-finger protein expressed in Inflorescence Meristem) domain-containing protein 1
MAPK	Mitogen-activated protein kinase
NAC TF	(NAM, ATAF1, ATAF2, and CUC2) transcription factor (TF) family
NGS	Nest generation sequencing
NIL	Near-isogenic line
PCR	Polymerase chain reaction
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PLC	Phospholipase C
qRT-PCR	Quantitative reverse-transcription PCR
QTL	Quantitative trait loci
RIL	Recombinant inbred line
ROS	Reactive oxygen species
TIR-NBS-LRRs	toll/interleukin-1 nucleotide-binding-site leucine-rich repeat

LITERATURAL REVIEWS

Plant response to stress

The stress response can be defined as the interaction between plants and extreme environmental changes (Borkotoky et al. 2013). These environmental changes are generally induced by abiotic and biotic factors: temperature, light, ultraviolet radiation, osmotic stress, and physical wounding are abiotic stress inducers, while pest and pathogen attack are biotic stress inducers. Abiotic stress can cause accumulation of reactive oxygen species (ROS) in the cell. The ROS which act as destructive molecules that affect both DNA and proteins, are common transducers of abiotic and biotic stress signals (Apel and Hirt 2004; Fujita et al. 2006; Simon et al. 2000; Torres and Dangl 2005). NADPH oxidases (Rbohs for respiratory burst oxidase homologs) are an important ROS-generating system in plants under stress condition (Foreman et al. 2003). There were supporting evidences which are accumulations of ROS under heavy metal and salt stress (Leshem et al. 2007; RODRÍGUEZ-SERRANO et al. 2006). OsNAC6, a member of the NAC (NAM, ATAF1, ATAF2, and CUC2)

transcription factor (TF) family, functions as a transcriptional activator that responds to both abiotic and biotic stress. In soybean, *GmNAC2* was reported to be a drought inducible gene and was shown to be an induced by cold and high salinity (Pinheiro et al. 2009; Tran et al. 2009). In addition, this *GmNAC2* was induced by jasmonic acid(JA) and mechanical wounding (Jin et al. 2013).

Recent studies have revealed plant crosstalk between abiotic and biotic stresses (Rejeb et al. 2014). The effects of combination of multiple stresses can be divided into negative or additive effects and antagonistic effects (Suleman et al. 2001; Ton et al. 2009). One example of additive effects was that drought stress treated common bean showed more symptoms when infected by *Macrophomina phaseolina* (Suleman et al. 2001). On the other hand, antagonistic effects revealed that ABA increases the susceptibility of wild type tomato to *Botrytis cineria* (Audenaert et al. 2002). Over the past few years, several studies on the crosstalk between abiotic and biotic stress responses in soybean have been performed (Kulcheski et al. 2011; Zhang et al. 2009). Expressing the soybean Ethylene Response Factor 3 (GmERF3) gene in transgenic tobacco lines induced pathogen-related (PR) genes and conferred resistance to

tobacco mosaic virus and salt stress (Zhang et al. 2009).

Plant signaling mechanism

Plants have established defense signaling mechanisms that allow them to adapt to and survive under abiotic and biotic stress. The first crucial step in this process is to perceive stress in a rapid and efficient way (Rejeb et al. 2014). After the stress is recognized, complex defense signaling cascades operate, such as the activation of specific ion channels and kinase cascades and the accumulation of ROS and phytohormones such as abscisic acid (ABA), salicylic acid (SA), ethylene (ET), and JA (AbuQamar et al. 2009). These secondary messengers mediate the transduction of signals to the corresponding signaling pathways against abiotic and biotic stress (Wang et al. 2016). The transduced signals activate TFs that regulate genes for morphological and physiological responses to stress (Bhargava and Sawant 2013; Fujita et al. 2006). Calcium-dependent protein kinases (CDPKs) have also been implicated as Ca2+ signaling regulators of abiotic and biotic stress responses (Kudla et al. 2010; Ludwig et al.

2004), as shown by the transcriptional response of LeCDPK 1 to both wounding and fungal stress in tomato (Chico et al. 2002).

Next generation sequencing

In recent years, next generation sequencing (NGS) methods were hired to whole genome sequencing and transcriptome at a substantially reduced cost and higher accuracy (Grada and Weinbrecht 2013; Shendure and Ji 2008). These increased efficiency and accessibility allow to construct high resolution density maps and genetic diversity analysis on cultivars, landraces, and wild species (Van et al. 2013). First plant genome scale sequencing using NGS technology was applied to hybridization-based resequencing of *A.thaliana* (Clark et al. 2007). *De novo* plant genome sequencing has been performed using NGS for plant such as potato, pigeon pea and chickpea (Consortium 2011; Varshney et al. 2012; Varshney et al. 2013) and the applications of reference sequence data based re-sequencing analysis of related plant genome were increased (Nowrousian 2010). Through NGS technology, plant transcriptome

analysis was also increased as well as whole genome sequencing. In soybean, *de novo* whole genome sequencing of *G.max* was performed by whole genome shotgun methods and wild soybean (*G.soja*) also applied to resequencing approach (Kim et al. 2015; Schmutz et al. 2010).

Based on the primary objectives of functional genomics in agriculture, which are to connect phenotype and genotype and to make phenotypic prediction, genotyping-by-sequencing (GBS) has been developed as a rapid and robust approach for reduced-representation sequencing of multiplexed samples that combines genome-wide molecular marker discovery and genotyping (Poland and Rife 2012). This GBS, which uses enzyme-based complexity reduction coupled with DNA barcoded adapters to produce multiplex libraries have been applied to soybean for NGS sequencing (Iquira et al. 2015).

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CHAPTER I

Transcriptomic profiling of soybean in response to high-intensity UV-B irradiation reveals stress defense signaling

ABSTRACT

The depletion of the ozone layer in the stratosphere has led to a dramatic spike in ultraviolet B (UV-B) intensity and increased UV-B light levels. The direct absorption of high-intensity UV-B induces complex abiotic stresses in plants, including excessive light exposure, heat, and dehydration. However, UV-B stress signaling mechanisms in plants including soybean (Glycine max [L.]) remain poorly understood. Here, we surveyed the overall transcriptional responses of two soybean genotypes, UV-B-sensitive Cheongja 3 and UV-B-resistant Buseok, to continuous UV-B irradiation for 0 (control), 0.5, and 6 h using RNA-seq analysis. Homology analysis using UV-B-related genes from Arabidopsis thaliana revealed differentially expressed genes (DEGs)

likely involved in UV-B stress responses. Functional classification of the DEGs showed that the categories of immune response, stress defense signaling, and reactive oxygen species (ROS) metabolism were over-represented. UV-B-resistant Buseok utilized phosphatidic acid-dependent signaling pathways (based on subsequent reactions of phospholipase C and diacylglycerol kinase) rather than phospholipase D in response to UV-B exposure at high fluence rates, and genes involved in its downstream pathways, such as ABA signaling, mitogen-activated protein kinase cascades, and ROS overproduction, were upregulated in this genotype. In addition, the DEGs for TIR-NBS-LRR and heat shock proteins are positively activated. These results suggest that defense mechanisms against UV-B stress at high fluence rates are separate from the photomorphogenic responses utilized by plants to adapt to low-level UV light.

INTRODUCTION

Sunlight contains energy-rich ultraviolet (UV) light, which is divided into three classes: UV-A (320–400 nm), UV-B (290 to 320 nm), and UV-C (100 to 280 nm). Of these, UV-A and only a small proportion of UV-B reach the Earth's surface because short-wavelength UV-C is completely absorbed by the stratospheric ozone layer (Caldwell et al., 1989). UV-B generally accounts for less than 0.5% of the total amount of light energy reaching the Earth's surface. However, the depletion of the ozone layer in the stratosphere has led to increased UV-B levels over the past 30 years (Austin and Wilson, 2006; Searles et al., 2001). Although the UV-B exposure has been stabilized since the mid-1990s, sporadic ozone depletion is causing periodic or unpredictable spikes in UV-B intensity in the polar and temperate zones (Kerr and McElroy, 1993), and the recovery of the ozone layer to pre-1980 levels may require several decades(McKenzie et al., 2011). UV-B photons can function as both environmental stressors and developmental signals in plants(Britt, 1996). Since sunlight is required for photosynthesis, increased UV-B intensity inevitably threatens plant viability, as most vascular plants cannot adapt to such an influx of UV-B(Jordan, 2002; Paul and Gwynn-Jones, 2003). UV-B radiation causes physiological damage, such as

reduced photosynthetic capacity and impaired pollen fertility, as well as morphological changes including plant stunting, leaf discoloration, and reduced biomass and seed yields (Frohnmeyer and Staiger, 2003; Lytvyn et al., 2010).

Despite the complex effects of UV-B radiation on plants, recent studies have revealed a series of components implicated in UV-B-specific photomorphogenic (non-damaging) regulation in the circadian rhythm pathway (Ulm and Nagy, 2005). These components include the UV-B photoreceptor UV RESISTANCE LOCUS8 (UVR8), the E3 ubiquitin ligase (transducin/WD40 repeat-like superfamily protein) CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1), the basic leucine-zipper transcription factor ELONGATED HYPOCOTYL5 (HY5), and its interacting partner LONG HYPOCOTYL5-LIKE (HYH) (Ulm, 2003). In addition, chalcone synthase (CHS) is positively activated in response to UV-B radiation (Heijde and Ulm, 2012). On the other hand, UV-B radiation, like other environmental stresses, elicits nonspecific (genotoxic) damage responses that trigger stress defense signaling. In Arabidopsis thaliana, reactive oxygen species (ROS) production and the biosynthesis of secondary metabolites (e.g., flavonoids and phenylpropanoids) are induced by UV-B irradiation. A previous study with transcript profiling has suggested that stress responses are less diverse than the actual stressors, and stress receptors and intracellular signaling pathways overlap (Stratmann, 2003). However, the mechanism by which

UV-B triggers the intracellular defense signaling pathway remains poorly understood.

Annual soybean (Glycine max (L.) Merr.) is one of the most important crops that were used as food, energy, and industrial resources worldwide. Only a few studies have compared the morphological, anatomical, and biochemical differences between UV-B-sensitive and -resistant soybean cultivars (Essex vs. Williams) in response to enhanced UV-B radiation (Murali et al., 1988). Recently, quantitative trait loci associated with resistance to supplementary UV-B treatment were localized between Satt495 and Satt238 on chromosome 19 using a recombinant inbred line population of Keunol (UV-B sensitive) x Iksan10 (UV-B resistant) (Lee et al., 2016; Shim et al., 2015).

In the present study, we surveyed the overall transcriptional responses of two soybean genotypes, UV-B-sensitive Cheongja 3 and UV-B-resistant Buseok, to continuous UV-B irradiation for 0 (control), 0.5, and 6 h at high fluence rates. We compared the transcript abundance between the se genotypes in response to supplementary UV-B irradiation, as well as between control and UV-B treatments in each genotype. The differentially expressed genes (DEGs) were subjected to further filtering using a set of UV-B related genes from A. thaliana to identify DEGs that are most likely involved in UV-B stress defense. Furthermore, the biological functions of

several such DEGs were confirmed using A. thaliana knock-out mutants. The results of this study provide insights into the molecular basis of the capacity for plants to tolerate UV-B stress at high fluence rates.

MATERIAL AND METHODS

Plant materials and growth conditions

UV-B-sensitive Cheongja 3 and UV-B-resistant Buseok (IT162669), which were identified as the most sensitive and resistant soybean genotypes to supplementary UV-B irradiation, respectively (Kim et al., 2015), were used in this study. Under 6 hours of UV-B treatment, Buseok showed little leaf damage and vigorous growth, whereas in Cheongja 3, most leaves turned yellow with red spots, ultimately leading to defoliation (Figure I-S1). Seeds of both soybean genotypes were planted in plastic pots (18 cm diameter, 20 cm deep) in a 1:1:1 mixture of field soil, desalinated sand, and commercial peat soil in a greenhouse at Seoul National University Experimental Farm. One plant per pot was grown under a natural photoperiod of 11.5–14.5 h per day using standard cultivation methods.

UV-B treatment

For UV-B treatment, soybean plants were divided into three groups; no UV-B treatment (0 h) as a control, 0.5- and 6-hour UV-B treatments). Supplementary UV-B irradiation was conducted at V4 growth stage as previously described (Kim et al., 2015). UV-B

irradiation began at 9:00 am, and the plants were exposed to prolonged UV-B stress for 0.5 and 6 h. The intensity of 0.5 h UV-B irradiation was equivalent to 11.5 kJ/m2 daily soybean UV-B biological effective dose (UV-BBE), and the 6 h treatment was equivalent to a dose of irradiation that was 12-times higher than daily UV-BBE (Caldwell, 1971). For Illumina RNA sequencing, the uppermost trifoliate leaves were collected from UV-B-treated and non-treated plants. At each sampling time, the leaf tissues from four pots (four replicates, one pot per replicate) per group of each genotype were pooled together as one biological replicate, frozen immediately in liquid nitrogen, and stored at -80 until use. Thus, a total of six leaf samples (three treatments [0, 0.5, and 6 h] x two genotypes [Cheongja 3 and Buseok]) were collected for RNA-seq analysis.

Figure I-S1 Morphological changes of UV-B-resistant Buseok and UV-B-sensitive Cheongja 3 after 6 h UV-B irradiation. Buseok showed fewer damaged leaves and vigorous growth, whereas Cheongja 3 showed more yellow leaves with red spots, ultimately leading to defoliation.

RNA isolation and RNA sequencing using Illumina HiSeq

Total RNA was extracted from the leaf samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA library for Illumina HiSeq 2000 sequencing was constructed using an mRNA-seq sample preparation kit (TruSeq® RNA Sample Prep Kit v2, Cat.8207217, Illumina Inc., San Diego, CA, USA), including mRNA purification, cDNA synthesis, end-repair of cDNA, adaptor ligation, and cDNA amplification. The cDNA libraries were applied to an Illumina paired-end flow cell, and 100 bp sequence reads were obtained using the Illumina HiSeq 2000 platform.

Read alignment and RNA-Seq analysis

The 100 bp paired-end sequence reads were mapped to the soybean reference genome (Glyma v1.1) from Phytozome (http://www.phytozome.net/soybean) using Bowtie (http://bowtie-bio.sourceforge.net/index.shtml) and TopHat (http://tophat.cbcb.umb.edu/) in default modes (Langmead et al., 2009; Trapnell et al., 2009). The Cufflinks program was used to assemble gene transcripts and to normalize transcript abundance in terms of fragments per kilobase pair of transcript per million mapped reads (FPKM) (Trapnell et al., 2012). The Cuffdiff program

(http://cufflinks.cbcb.umd.edu/index.html) was used to test statistically significant differences in transcript expression in seven pairs of comparisons, which consisted of three comparisons of Cheongja 3 vs. Buseok under control conditions and two UV-B treatments, and four comparisons of control vs. UV-B treatments in the two genotypes (Figure I-S2). Significant DEGs were detected using the following criteria: (i) absolute fold-change > 1 and (ii) q-value (false discovery rate [FDR]) < 0.05 and p-value < 0.004. Hierarchical cluster analysis of DEG expression and the heatmap construction were performed using R software (http://www.r-project.org).

Survey of A. thaliana UV-B related genes

UV-B related genes from A. thaliana were surveyed using Gene Ontology (GO) (http://amigo.geneontology.org/amigo/search/ontology?q=UV-B) and The Arabidopsis Information Resource (TAIR) (https://www.Arabidopsis.org/) database. In addition, a list of A. thaliana genes shown to be involved in the UV-B response was compiled from the literature; 194 UV-B-related A. thaliana genes were ultimately obtained (Figure I-S2). To detect genes that function in networks with the

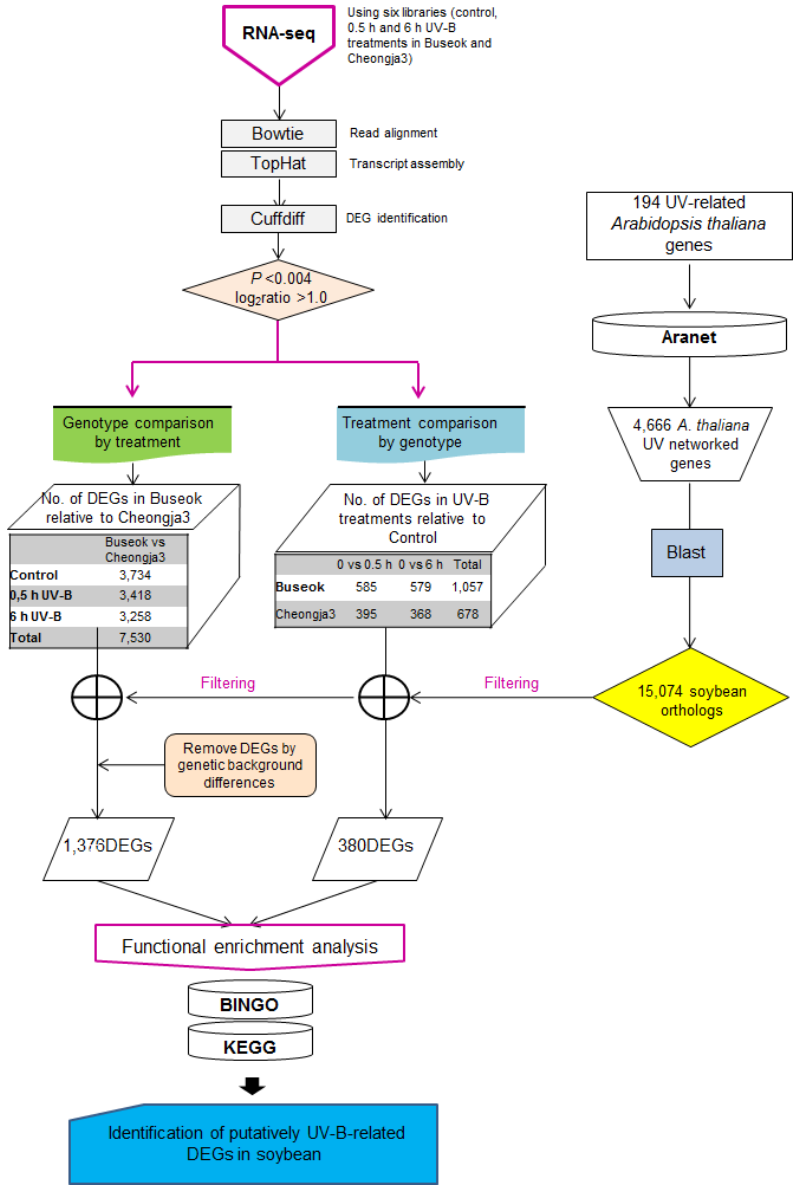

Figure I-S2 The workflow of two-way transcriptomic comparisons. Genotype comparison: the comparisons between Cheongja3 and Buseok under 0 (control), 0.5, and 6 h UV-B treatments. Treatment comparison: the comparisons between control and 0.5 & 6 h UV-B treatments in Buseok and Cheongja 3.

UV-B related genes, the set of selected UV-B related genes was analyzed with Aranet (http://www.functionalnet.org/aranet/), a probabilistic functional gene network for A. thaliana, resulting in the identification of 4,666 genes predicted to respond directly or indirectly to UV-B (Figure I-S2). A total of 15,074 soybean homologs of these A. thaliana genes were identified using Blast analysis. Based on this soybean gene list, DEGs putatively involved in the defense response to UV-B stress were identified (Figure I-S2).

Functional classification of DEGs by BINGO and KEGG

To better understand the biological functions of the DEGs in response to UV-B, enrichment of GO categories among the DEGs was assessed using BINGO software (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) (Maere et al., 2005). Significantly over-represented GO categories were visualized in Cytoscape (http://www.cytoscape.org). The biochemical pathways involving the DEGs were predicted using Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/).

Quantitative reverse-transcription PCR validation of DEGs

Gene-specific primers were designed based on the nucleotide sequences of the chosen DEGs for qRT-PCR analysis using Primer3 software. Total RNA from six samples (Cheongja 3 and Buseok; control, 0.5 h, and 6 h UV-B treatments) was used to synthesize cDNA using a Bio-Rad iScriptTM cDNA Synthesis Kit (Cat. 170-8891, Hercules, CA, USA). The synthesized cDNAs were used for qRT-PCR with a Bio-Rad iQTM SYBR Green Supermix Kit (Cat. 170-8882) using a LightCycler® 480 (Roche Diagnostics, Laval, QC, Canada). The qRT-PCR reaction mixtures (total volume of 20μl) contained 100 ng of cDNA, each primer at 300 μM, 8μl of sterile water, and 10μl of Bio-Rad iQTM SYBR Green Supermix. The amplification conditions were as follow: 5 min denaturation at 95°C followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. The samples were analyzed in triplicate to ensure statistical significance, and the tubulin gene was used as a reference gene for normalization of target gene expression in soybean. Data were analyzed based on the stable expression level of the reference gene according to the method of Livak and Schmittgen (Livak and Schmittgen, 2001).

Functional validation of DEGs using Arabidopsis knock-out mutants

Seeds of knock-out mutants for the target genes were obtained from the Arabidopsis Biological Resource Center (ABRC, http://abrc.osu.edu/) at Ohio State University, USA. Seven mutant lines were utilized, including mutants for five members of TIR-NBS-LRR family (AT1G64070.1; SALK_042846C, AT5G17680.1; SALK_004241C, AT5G36930.1; SALK_124056C, AT5G41540.1;SALK_034471C, and AT4G36150.1; SALK_084909C), one DGK mutant (AT5G07920.1; SALK_033664C), and one PIP5K mutant (AT1G34260.1; SALK_047604C). To identify homozygous mutants for the target genes, seeds incubated at 4°C were sown in commercial peat soil and grown under 16/8 h light conditions in a growth chamber for 14 days. Homozygous mutant plants were confirmed by RT-PCR using primers designed based on the inserted T-DNA and target gene DNA sequences, resulting in the identification of only four knock-out mutants. Both knock-out mutant and Columbia ecotype (Col-0) seeds were sterilized and incubated at 4°C in the dark for 4 days to synchronize germination and to ensure uniform growth, followed by cultivation under 16 h white fluorescent light for 7 days in a growth chamber. As a pilot UV-B irradiation test to determine suitable irradiation times for A. thaliana, wild-type (Col-0) plants were subjected to different UV-B treatments (1, 2, 4, and 8 h); 4 h UV-B treatment was determined to be suitable. Seven-days-old

knock-out mutants were treated with 4 h UV-B irradiation at 22°C. For qRT-PCR analysis, rosette leaves were collected from UV-B-treated and non-treated mutant plants, frozen immediately in liquid nitrogen, and stored at -80°C until use. The specific primers for qRT-PCR were designed using primer 3 (http://primer3plus.com/web_3.0.0/primer3web_input.htm).

RESULTS

RNA-seq analysis and DEG identification related to UV-B stress in Cheongja 3 and Buseok

To compare transcriptomic variation in the soybean lines in response to UV-B stress, Cheongja3 and Buseok leaves were collected after consecutive UV-B exposure at a high fluence rate for 0 (control), 0.5, and 6 h, with a total of six samples. Using Illumina HiSeq 2000 system, 0.3 billion 100 bp paired-end reads were produced, ranging from 47 to 62 million reads per sample (Table I-S1), which were mapped against the soybean reference sequence. The RNA-seq analysis workflow depicted in Figure S2 was implemented to analyze the sequencing data. Of the total reads, 78% were properly mapped to the reference sequence, resulting in approximately 25-fold average coverage (Table I-S1).

To identify DEGs related to UV-B stress, we compared transcript abundances among the six samples from two soybean genotypes differing in UV-B tolerance based on FPKM values. Two-way comparison data analysis was used to investigate transcriptomic variations, representing the comparison (i) between Cheongja 3 and Buseok by UV-

Table I-S1 Summary of RNA-seq data derived from Illumina Hiseq2000

Sample	Number of reads	Sum of read length	Number of properly mapped read	Total mapped depth	Total mapped position	Mapping coverage
Buseok-control	47108676	4710867600	36206146 (76.86%)	4250337952	183775226	23.1
Buseok-0.5 h	50348574	5034857400	39428414 (78.31%)	4721972620	195522924	24.1
Buseok-6 h	55178917	5517891700	43393678 (78.64%)	5343938022	200021200	26.7
Cheongja3-control	50972823	5097282300	39156576 (76.82%)	4756405618	195574728	24.3
Cheongja3-0.5 h	62332947	6233294700	49042114 (78.68%)	5853644374	203376470	28.8
Cheongja3-6 h	52252434	5225243400	40819278 (78.12%)	4989506691	200589198	24.9

100 bp paired-end RNA sequencing
Insert size: 500 bp
Six samples in 1 lane

Mapping reference: Gmax_109.Chloro.fa (975Mb)
Reference CDS length: 87 Mb

B treatment and (ii) between control and UV-B treatments by genotype (Figure I-S2). To further identify DEGs in response to supplementary UV-B irradiation, we then utilized a set of 4,666 A. thaliana genes predicted to be implicated in UV-B stress defense, either directly or indirectly, via Aranet (Figure I-S2). Homology comparative analysis revealed that 1,875 DEGs detected in the Cheongja 3 vs. Buseok comparison were orthologs of UV-B-related Arabidopsis genes (Figure I-S2). In the control vs. treatment comparison, 228 DEGs in Buseok and 129 in Cheongja 3 were found to be homologous to the UV-B related Arabidopsis genes (Figure I-S2).

Using the final set of DEGs, we generated a Venn diagram to identify treatment- or genotype-specific DEGs and common DEGs (Figure 1a). A larger number of DEGs was specific to each genotype and each UV-B treatment time. In the Cheongja 3 vs. Buseok comparison, 499 DEGs specific to the controls were considered to be due to differences in the genetic backgrounds between the genotypes and were thus excluded from further functional classification. Of the 1,376 DEGs in Buseok relative to Cheongja 3, 511 were upregulated and 345 were downregulated under 0.5 h UV-B treatment (Figure 1b). Under 6 h UV-B treatment, the number of up- and downregulated DEGs was similar. The control vs. treatment comparisons by genotype showed that Buseok had more DEGs than Cheongja 3 under all UV-B treatments (Figure 1b).

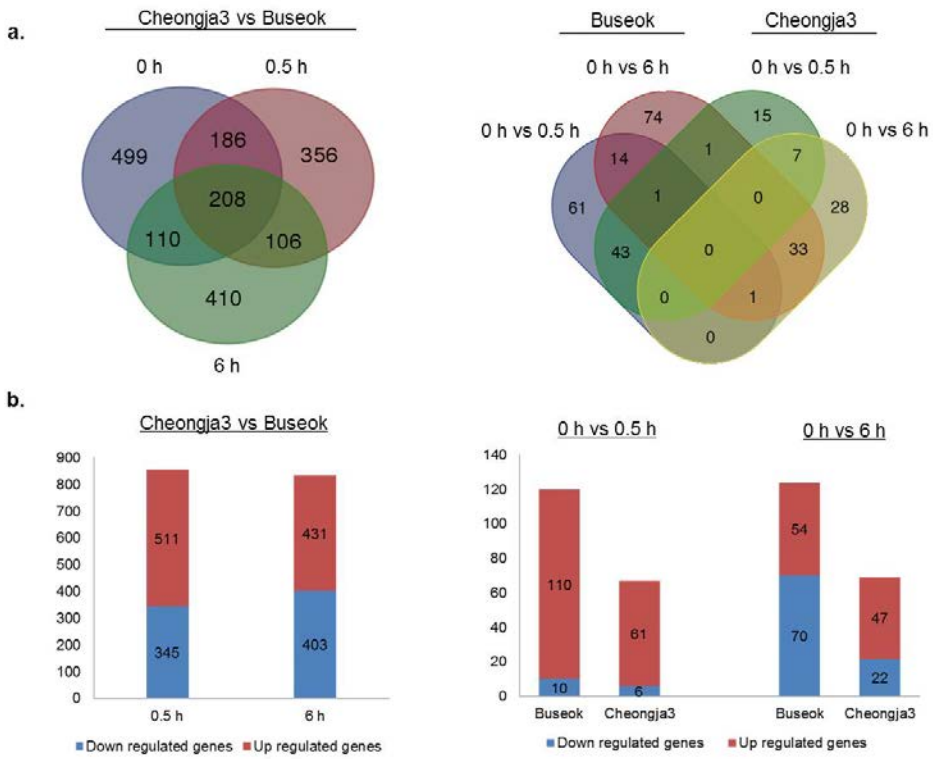

Figure I-1 Number of differentially expressed genes (DEGs) (a) Left: DEGs between Cheongja 3 and Buseok under control, 0.5 h, and 6 h UV-B treatments. Right: DEGs between the control and UV-B treatments in Cheongja 3 and Buseok. (b) Up- and down-regulated DEGs detected in each comparison.

There were 149 and 50 Buseok- and Cheongja 3-specific DEGs, respectively (Figure 1a). Most DEGs in both genotypes were upregulated by 0.5 h UV-B treatment but more strongly downregulated by 6 h UV-B exposure (Figure 1b).

Functional classification of DEGs by BINGO and KEGG

We investigated the biological significance of the changes in transcript abundance provoked by UV-B irradiation stress in soybean. To address the functional distribution of the identified DEGs, we performed GO term enrichment using Cytoscape plug-in BINGO. GO assignment of 690 DEGs among the 1,376 DEGs identified in the Cheongja 3 vs. Buseok comparison by UV-B treatment resulted in significant overrepresentation of 31 GO terms, including 17 in the biological process category and 14 in the metabolic process category (Figure 2). In the biological process category, over-represented GO terms were mainly classified into four clusters, as shown in Figure 2, including cell morphogenesis, immune response, signaling, and apoptosis. A large portion of the DEGs involved in the GO clusters immune response, signaling, and apoptosis were overlapping, i.e., toll/interleukin-1 nucleotide-binding-site leucine-rich repeat (TIR-NBS-LRR) genes.

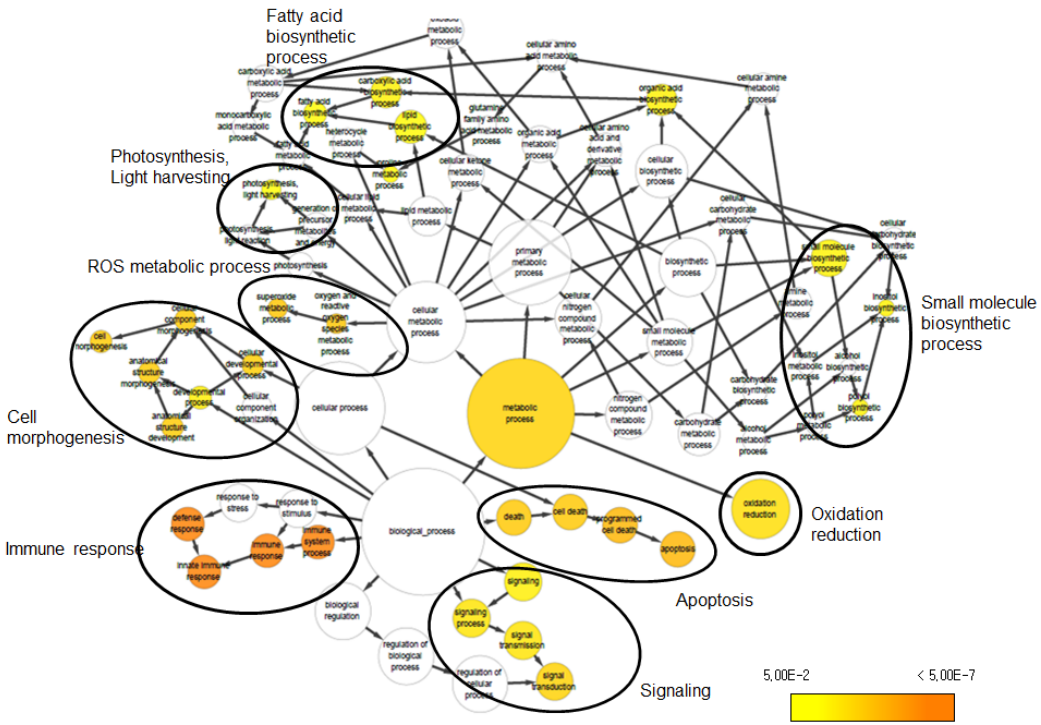

Figure I-2 Over-represented GO terms of DEGs that were identified from the comparison between Cheongja 3 and Buseok under UV-B treatments.

The GO terms over-represented in metabolic process were primarily divided into five clusters including light harvesting system, fatty acid biosynthetic process, small molecule biosynthetic process, ROS metabolic process, and oxidation reduction. From the control vs. treatment comparisons in UV-B-resistant Buseok, 176 DEGs induced by 0.5 h UV-B treatment were grouped into six GO clusters, including biological regulation, immune response, signaling, apoptosis, ion transport, and polysaccharide metabolic process. However, no GO term was over-represented by the 124 DEGs in Buseok in response to 6 h UV-B treatment. In the control vs. treatment comparisons in UV-B-sensitive Cheongja 3, 9 DEGs under 0.5 h UV-B treatment were grouped into the GO term metal ion transport (GO:0030001) and 26 DEGs under 6 h UV-B treatment were grouped into oxidation reduction (GO:0055114). According to BINGO analysis, the GO terms involved in immune response, cell death, and signaling were the most dominant.

KEGG pathway mapping of the 1,376 DEGs identified in the Cheongja 3 vs. Buseok comparison under UV-B treatments revealed the involvement of 101 biological pathways. The 228 and 129 DEGs in the control vs. treatment comparisons in Buseok and Cheongja 3 were assigned to 28 and 52 KEGG pathways, respectively. Interestingly, phosphatidylinositol signaling system (gmx04070) and mTOR signaling pathway (map04150), which are involved in environmental information

processing and signal transduction, were also identified.

Based on the results of BINGO and KEGG analysis, we specifically focused on DEGs in over-represented GO and KEGG terms such as cell death and immune system, stress defense signaling, and ROS metabolism, as described below. We also investigated the expression patterns of genes in the photosystem and circadian rhythm categories.

Photosystem and circadian rhythm

Supplemental UV-B radiation downregulates genes encoding several key photosynthetic proteins, including small subunits of Rubisco, subunits of ATP synthase, and chlorophyll a/b binding protein of the light harvesting antenna complex of photosystem II (Jenkins, 2009). In the present study, both Buseok and Cheongja 3 showed little significant UV-B-induced changes in the expression of photosynthetic genes compared to the control. However, differences between the two soybean genotypes were observed in the transcript abundance of chlorophyll a/b binding protein and photosystem light harvesting complex genes under both control and elevated UV-B conditions. Inherent variations in the expression of two genes encoding light harvesting complex subunits were detected between Buseok and Cheongja 3 under ambient light (control). In

addition, the expression patterns of six genes associated with photosystem during UV-B irradiation significantly differed between genotypes. These results indicate that the regulation of photosynthetic genes in response to supplementary UV-B irradiation is dependent on the genetic backgrounds of soybean genotypes.

Photomorphogenic UV-B responses are specifically mediated by (but are not independent of) other aspects of light signaling, including photoreceptors and circadian rhythms. We observed significant differences in the transcript abundance of genes involved in UV-B specific photomorphogenic pathways between UV-B-resistant Buseok and UV-B-sensitive Cheongja 3. Specifically, HY5 (Glyma08g41450.1) and COP1 (Glyma14g05430.1) were upregulated by 6 h UV-B treatment. Most DEGs encoding CHS proteins, which were expressed at lower levels in Buseok compared to Cheongja 3 under control conditions, were upregulated by UV-B irradiation. Additional key genes involved in the circadian rhythm pathway, such as PHYTOCHROME A, GIGANTEA PHYTOCHROME INTERACTING FACTOR 3, and PSEUDO-RESPONSE REGULATOR, were also differentially expressed in response to supplementary UV-B exposure. In the control vs. treatment comparisons by genotype, UVR8, HY5, and COP1 were not detected among DEGs (data not shown).

Cell death and immune response

To investigate which components in defense signaling pathways are activated in response to UV-B stress, we investigated significant differences in the expression of genes participating in programmed cell death and the immune system. The comparison of Cheongja 3 vs. Buseok under UV-B treatments revealed differential expression of 20 TIR-NBS-LRR genes, 11 of which were more highly expressed in Buseok than in Cheongja 3 (Table I-1; Figure I-3). Seven additional TIR-NBS-LRR and 2 coiled-coil-nucleotide-binding-site leucine-rich repeat (CC-NBS-LRR) genes were induced by 0.5 h UV-B irradiation only in Buseok (Table I-1), while none of these genes were induced by 6 h treatment. Indeed, TIR-NBS-LRRs were recently shown to also be activated by abiotic stresses such as heat and drought, leading to apoptotic-like programmed cell death as a relatively early defense response (Nawkar et al., 2013; Prasch and Sonnewald, 2013).

Nine members of the heat shock 70 (HSP70) protein family were differentially expressed in response to UV-B irradiation in Buseok relative to Cheongja 3, six of which were down-regulated (Table I-1; Figure I-3). However, there was no significant difference in the expression patterns of HSP70 genes between control and 0.5 h or 6 h UV-B treatment in Buseok or Cheongja 3.

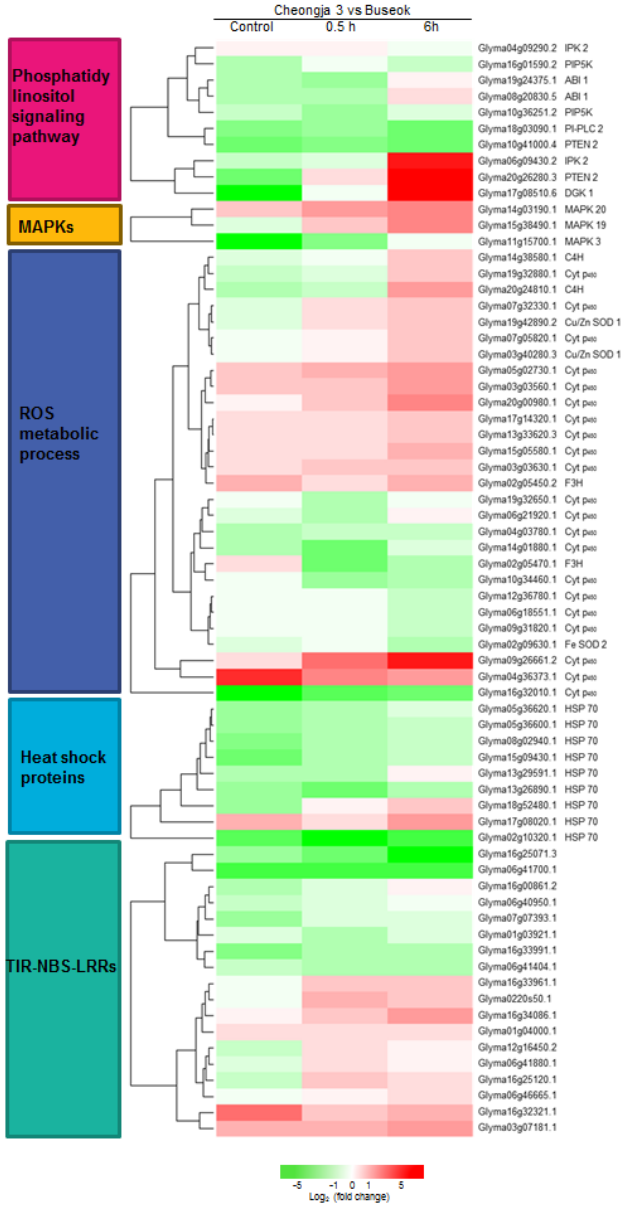

Figure I-3 Heatmap showing the differential expression levels of genes encoding components involved in the over-represented GO and KEGG terms between Cheongja 3 and Buseok under control, 0.5 h and 6 h UV-B treatments.

Table I-1 Differential expression of TIR/CC-NBS-LRRs and heat shock protein genes in the comparison between Cheongja 3 and Buseok by UV-B treatment and between control and UV-B treatments by genotype.

Gene ID	Chromosome position	Gene definition	Log_2 (fold change)*			A.thaliana homolog
Cheongja3 vs Buseok			**Control**	**0.5 h**	**6 h**	
Glyma01g03921.1	Gm01:3390102-3396781	disease resistance protein (TIR-NBS-LRR class), putative	-1.38	-3.62	-1.62	AT5G17680.1
Glyma01g04000.1	Gm01:3487914-3494204	disease resistance protein (TIR-NBS-LRR class), putative	1.79	1.85	1.56	AT5G17680.1
Glyma0220s50.1	scaffold_220:14-5602	disease resistance protein (TIR-NBS-LRR class) family	-0.56	3.09	2.29	AT5G36930.2
Glyma03g06285.1	Gm03:6480791-6481760	disease resistance protein (TIR-NBS-LRR class) family	-	-	-5.18	AT5G44510.1
Glyma03g07181.1	Gm03:7546779-7575160	disease resistance protein (TIR-NBS-LRR class) family	3.19	3.53	3.96	AT5G36930.2
Glyma06g40950.1	Gm06:44230032-44239212	disease resistance protein (TIR-NBS-LRR class), putative	-2.51	-1.76	-0.87	AT5G17680.1
Glyma06g41404.1	Gm06:44686411-44690459	disease resistance protein (TIR-NBS-LRR class) family	-2.81	-3.31	-3.64	AT5G45220.1
Glyma06g41700.1	Gm06:44984922-44988575	disease resistance protein (TIR-NBS-LRR class), putative	-7.63	-7.85	-7.94	AT5G17680.1
Glyma06g41880.1	Gm06:45152031-45155033	disease resistance protein (TIR-NBS-LRR class), putative	-1.63	1.84	0.29	AT5G17680.1
Glyma06g46665.1	Gm06:49244725-49251384	disease resistance protein (TIR-NBS-LRR class) family	-0.61	0.81	1.78	AT5G36930.2
Glyma07g07393.1	Gm07:6067981-6072205	disease resistance protein (TIR-NBS-LRR class), putative	-4.23	-1.34	-1.52	AT5G17680.1
Glyma12g16450.2	Gm12:15730001-15734533	disease resistance protein (TIR-NBS-LRR class), putative	-2.39	1.28	0.86	AT5G17680.1
Glyma13g03770.3	Gm13:3846577-3852431	disease resistance protein (TIR-NBS-LRR class), putative	-2.38	0.65	2.81	AT5G17680.1
Glyma16g00861.2	Gm16:516813-521800	disease resistance protein (TIR-NBS-LRR class) family	-3.12	-1.57	0.04	AT5G41540.1
Glyma16g25071.3	Gm16:28995807-29006764	disease resistance protein (TIR-NBS-LRR class), putative	-4.12	-5.93	-11.37	AT5G17680.1
Glyma16g25120.1	Gm16:29058174-29061521	disease resistance protein (TIR-NBS-LRR class), putative	-2.62	2.22	1.81	AT5G17680.1
Glyma16g32321.1	Gm16:35526836-35530790	disease resistance protein (TIR-NBS-LRR class), putative	6.40	2.55	3.62	AT5G17680.1
Glyma16g33961.1	Gm16:36692020-36696860	disease resistance protein (TIR-NBS-LRR class), putative	-0.24	2.06	2.19	AT5G17680.1
Glyma16g33991.1	Gm16:36712977-36715288	disease resistance protein (TIR-NBS-LRR class) family	-4.80	-3.78	-3.00	AT5G36930.2
Glyma16g34086.1	Gm16:36774650-36776469	disease resistance protein (TIR-NBS-LRR class), putative	0.28	2.08	3.83	AT5G17680.1
Glyma02g10320.1	Gm02:8186067-8188789	heat shock protein 70	-3.81	-6.06	-4.16	AT3G12580.1
Glyma05g36600.1	Gm05:40426888-40430895	heat shock protein 70 (Hsp 70) family protein	-2.39	-1.55	-1.05	AT3G12580.1
Glyma05g36620.1	Gm05:40443106-40447303	heat shock protein 70	-2.33	-1.59	-0.84	AT5G42020.1
Glyma08g02940.1	Gm08:2029877-2033833	heat shock protein 70 (Hsp 70) family protein	-2.63	-1.73	-1.06	AT5G02500.1
Glyma13g26890.1	Gm13:30070997-30076596	heat shock protein 70B	-2.18	-3.25	-1.66	AT3G12580.1
Glyma13g29591.1	Gm13:32478807-32481336	heat shock protein 70B	-1.85	-1.99	0.08	AT1G16030.1
Glyma15g09430.1	Gm15:6739539-6741346	heat shock cognate protein 70-1	-3.19	-1.93	-1.03	AT5G42020.1
Glyma17g08020.1	Gm17:5928338-5930881	heat shock protein 70	1.94	1.00	2.45	AT1G16030.1
Glyma18g52480.1	Gm18:61075241-61082432	heat shock protein 70B	-2.17	0.12	1.14	AT5G42020.1
Control vs treatment in Buseok			**C vs 0.5 h**		**C vs 6 h**	
Glyma05g17460.1	Gm05:20185056-20190951	disease resistance protein (CC-NBS-LRR class) family	2.96		2.56	AT5G66900.1
Glyma17g21240.1	Gm17:20538055-20543536	disease resistance protein (CC-NBS-LRR class) family	3.78		2.00	AT5G66900.1
Glyma03g14888.1	Gm03:19079981-19089860	disease resistance protein (TIR-NBS-LRR class) family	4.46		1.16	AT5G36930.2
Glyma06g40690.1	Gm06:43857935-43861836	disease resistance protein (TIR-NBS-LRR class), putative	1.55		4.35	AT5G17680.1
Glyma06g40740.1	Gm06:43913599-43918380	disease resistance protein (TIR-NBS-LRR class) family	3.84		4.14	AT4G12010.1
Glyma06g41880.1	Gm06:45152031-45155033	disease resistance protein (TIR-NBS-LRR class), putative	3.41		0.72	AT5G17680.1
Glyma12g16450.1	Gm12:15730001-15734533	disease resistance protein (TIR-NBS-LRR class), putative	2.94		1.74	AT5G17680.1
Glyma16g25120.1	Gm16:29058174-29061521	disease resistance protein (TIR-NBS-LRR class), putative	5.39		2.23	AT5G17680.1
Glyma16g33590.1	Gm16:36465825-36471050	disease resistance protein (TIR-NBS-LRR class) family	4.71		3.03	AT5G36930.2

*Dash (-) indicates uncalculated log_2 (fold change) values due to FPKM value = 0 in Cheongja 3.

Stress defense signaling

Genome-wide transcript analysis in response to supplemental UV-B irradiation revealed DEGs involved in the phosphatidylinositol signaling pathway (Table I-2), which may function upstream of defense-related responses, such as the activation of mitogen-activated protein kinase (MAPK) cascades and oxidative bursts. The phosphatidylinositol signaling pathway generates phosphatidic acid (PA) from structural phospholipids as a secondary messenger. Under UV-B stress, transcripts for phosphatidylinositol-4-phosphate 5-kinase (PIP5K) family protein were more abundant in Buseok than in Cheongja 3 (Table I-2); this protein generates phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] as a substrate of phospholipase C (PLC) from phosphatidylinositol 4-phosphate (Ptdins4P) in the plasma membrane(Mueller-Roeber and Pical, 2002). Also, PtdIns(4,5)P2 is produced from the phosphorylation of phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3] by phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN 2), for which one gene (Glyma20g26280.3) was upregulated in Buseok under UV-B irradiation (Table I-2). PtdIns(4,5)P2 is hydrolyzed into inositol 1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG) by phosphatidylinositol-specific PLC (PI-PLC) proteins. One DEG (Glyma18g03090.1) encoding PI-PLC was

upregulated in Cheongja 3 under 6 h UV-B treatment relative to Buseok (Table I-2). In UV-B-resistant Buseok, PI-PLC genes were expressed at low levels and independent of UV-B treatment. Meanwhile, two DEGs (Glyma17g08510.6 and Glyma06g39760.1) for diacylglycerol kinase (DGK) family proteins were upregulated in Buseok by UV-B stress (Table I-2); these proteins convert DAG produced by PI-PLC to PA (Laxalt and Munnik, 2002). The coupling of DGK to the activation of PI-PLC may be induced in response to UV-B stress in Buseok, leading to increased levels of PA in the cells, thereby activating several downstream defense responses.

InsP3 generated via the hydrolysis of PI-PLC diffuses into the cytosol and is involved in the release of Ca2+ from intracellular stores (Ruelland et al., 2015). Owing to a lack of InsP3 receptors in plants, InsP3 is converted into the more phosphorylated forms of inositol, i.e., tetra, penta, and hexaphosphates (InsP4, InsP5, and InsP6), through further phosphorylation steps involving at least two types of inositol polyphosphate 2-kinase (IPK1) and inositol polyphosphate kinase 2 (IPK2, synonym for inositol 1,3,4-trisphosphate 5/6-kinase) (Munnik and Vermeer, 2010; Sparvoli and Cominelli, 2015; Zhou et al., 2012). Among these enzymes, Buseok exhibited enhanced expression of two IPK2 family genes under 0.5 and 6 h UV-B irradiation compared to Cheongja 3 (Table I-2). Therefore, in UV-B-resistant Buseok, the genes for four

Table I-2 Differential expression of genes coding for five enzymes of the PA signaling pathway in the comparison between Cheongja 3 and Buseok by UV-B treatment and between control and UV-B treatments by genotype.

* Dash (-) means uncalculated log_2 (fold change) values due to FPKM value =0 in Cheongja3
** Vertical bars (|) represent "either/or".

Gene ID	Chromosome position	Gene definition	Reaction	Log_2 (fold change)*			A.thaliana homolog
Cheongja 3 vs Buseok				**Control**	**0.5 h**	**6 h**	
Glyma10g36251.2	Gm10:44448484-44458944	phosphatidylinositol-4-phosphate 5-kinase family protein (PIP5K)	PtdIns4P→PtdIns(4,5)P_2	0.78	-0.22	1.24	AT1G34260.1
Glyma16g01590.2	Gm16:1161766-1173675	phosphatidylinositol-4-phosphate 5-kinase family protein (PIP5K)	PtdIns4P→PtdIns(4,5)P_2	0.30	2.03	1.04	AT3G14270.1
Glyma10g41000.4	Gm10:48199551-48208068	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN 2)	PtdIns(3,4,5)P_3→PtdIns(4,5)P_2 | PtdIns3P	-1.18	-1.00	-1.31	AT3G19420.1
Glyma20g26280.3	Gm20:35776384-35782697	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase (PTEN 2)	PtdIns(3,4,5)P_3→PtdIns(4,5)P_2 | PtdIns3P	-1.21	3.01	-	AT3G19420.1
Glyma18g03090.1	Gm18:2033237-2038137	Phosphoinositide-specific phospholipase C 2 (PI-PLC 2)	PtdIns(4,5)P_2→ DAG + InsP_3	-0.70	0.04	-1.56	AT3G08510.1
Glyma17g08510.6	Gm17:6296131-6303862	diacylglycerol kinase1 (DGK 1)	DAG→PA	-4.30	2.04	-	AT5G07920.1
Glyma04g09290.2	Gm04:7455432-7461028	Inositol 1,3,4-trisphosphate 5/6-kinase family protein (IPK 2)	InsP3→InsP6	2.38	2.75	1.93	AT4G08170.2
Glyma06g09430.2	Gm06:6950627-6957813	Inositol 1,3,4-trisphosphate 5/6-kinase family protein (IPK 2)	InsP3→InsP6	0.76	1.42	-	AT4G08170.2
Control vs treatment in Buseok				**C vs 0.5 h**		**C vs 6 h**	
Glyma06g39760.1	Gm06:42662480-42670185	diacylglycerol kinase 5 (DGK 5)	DAG→PA	4.69		2.33	AT2G20900.2

enzymes involved in the PA-dependent signaling pathway were upregulated in response to UV-B stress (Figure I-3).

We also identified some DEGs encoding putative target proteins that interact with PA, including two DEGs (Glyma08g20830.5, Glyma19g24375.1) encoding protein phosphatase 2 family proteins, which were upregulated in Buseok under 0.5 h UV-B treatment (Figure I-3); these proteins carry a PA binding motif. One example of a functionally characterized protein phosphatase targeted by PA is the protein phosphatase 2C ABI1 (ABA insensitive 1), which is bound by PA to negatively regulate ABA signaling (Zhang et al., 2004). We detected differential transcript accumulation of three genes encoding MAPKs in response to UV-B stress between Cheongja 3 and Buseok (Figure I-3); MAPK 19 (Glyma15g38490.1) and MAPK 20 (Glyma14g03190.1) were upregulated in Buseok under 6 h UV-B treatment and MAPK 3 (Glyma11g15700.1) was upregulated in Cheongja 3 under 0.5 h UV-B treatment (Figure I-3). In both Buseok and Cheongja 3, however, no significant difference in MAPK expression was detected in the control vs. UV-B treatment comparison.

ROS production and scavenging

Abiotic stresses including UV-B stress induce ROS production and scavenging. Four genes encoding copper/zinc superoxide dismutase (Cu/Zn SOD) family proteins, which catalyze the dismutation of superoxide anion (O2• -) to oxygen (O2) and hydrogen peroxide (H2O2), were upregulated in Buseok in response to UV-B stress, while in Cheongja 3, the iron (Fe)-SOD gene was upregulated in response to 6 h UV-B treatment. Among the genes assigned to the GO term “oxidation reduction”, 21 cytochrome P450 (Cyt P450) family genes were identified, 12 and 9 of which were upregulated in Buseok and Cheongja 3, respectively (Figure I-3). Six of eight Cyt P450 DEGs were downregulated in Cheongja 3 under 6 h UV-B treatment compared to the control. NAD(P)H-dependent electron transport involving cytochrome P450 produces O2• - in the endoplasmic reticulum (Sharma et al., 2012). Genes of the Cyt P450 family 76 subfamily C2 and the Cyt P450 family 706 subfamily A are induced by UV-C irradiation (Narusaka et al., 2004). To avoid injury from ROS overproduction, ROS scavenging or detoxification is performed by antioxidative systems consisting of both nonenzymatic and enzymatic antioxidants (Sharma et al., 2012). In Buseok, we identified upregulated DEGs involved in flavonoid biosynthesis to produce phenolic compounds with antioxidant properties,

such as anthocyanidin and tannin, including genes encoding flavanone-3-hydroxylase (F3H) and cinnamate-4-hydroxylase (C4H). By contrast, a gene encoding the enzymatic oxidant ascorbate peroxidase was upregulated in Cheongja 3. Under 6 h UV-B treatment, genes encoding glutathione peroxidase were also upregulated only in Cheongja 3 compared to the control.

Transcription factors

Several transcription factor (TF) genes were differentially expressed only in Buseok under 0.5 h UV-B irradiation compared to the control. The WRKY and NAC (no apical meristem) TF families were the two largest families responding to UV-B stress. We identified seven members of the WRKY TF family exhibiting increased expression, as well as eight members of the NAC TF family. In addition, some TFs genes and genes encoding transcriptional regulators that interact with phytohormones including ethylene, cytokinin, and IAA were differentially expressed in Buseok under 0.5 h UV-B treatment. Genes in other TF families such as GATA, basic helix-loop helix (BHLH), and basic leucine zipper (bZIP) showed enhanced expression as well. Furthermore, some Ca2+/calmodulin (CaM)-binding TF genes were upregulated under these conditions, including genes encoding signal responsive 1 and calcium-

binding EF-hand protein.

RNA-seq validation by qRT-PCR

Using qRT-PCR, we confirmed that five genes (Glyma03g07121.1, Glyma06g41880.1, Glyma12g16450.1, Glyma16g34086.1, and Glyma19g07650.1) in the TIR-NBS-LRR family were upregulated, mainly in Buseok, in response to 0.5 h UV-B irradiation (Figure I-4a-e). The expression patterns obtained by qRT-PCR were similar to those obtained by RNA-seq . Interestingly, little transcript accumulation of Glyma03g07121.1 (TIR-NBS-LRR) was observed in Cheongja 3 by qRT-PCR or RNA-seq. Additionally, the expression profiles of individual DEGs encoding IPK2 (Glyma06g09430.2), PIP5K (Glyma10g36251.2), DGK 1 (Glyma17g08510.6) and DGK 5 (Glyma06g39760.1) in the PA signaling pathway obtained by qRT-PCR were similar to the RNA-seq results (Figure 4f-i). Finally, we investigated the expression patterns of three DEGs encoding Cyt P450 (Glyma06g21920.1), Cu/Zn SOD (Glyma11g19840.2), and C4H (Glyma14g38580.1), which are involved in ROS production and scavenging, using qRT-PCR (Figure I-4j-l). All of the gene expression patterns obtained by RNA-seq and qRT-PCR were similar, indicating the high reliability in our RNA-seq analysis.

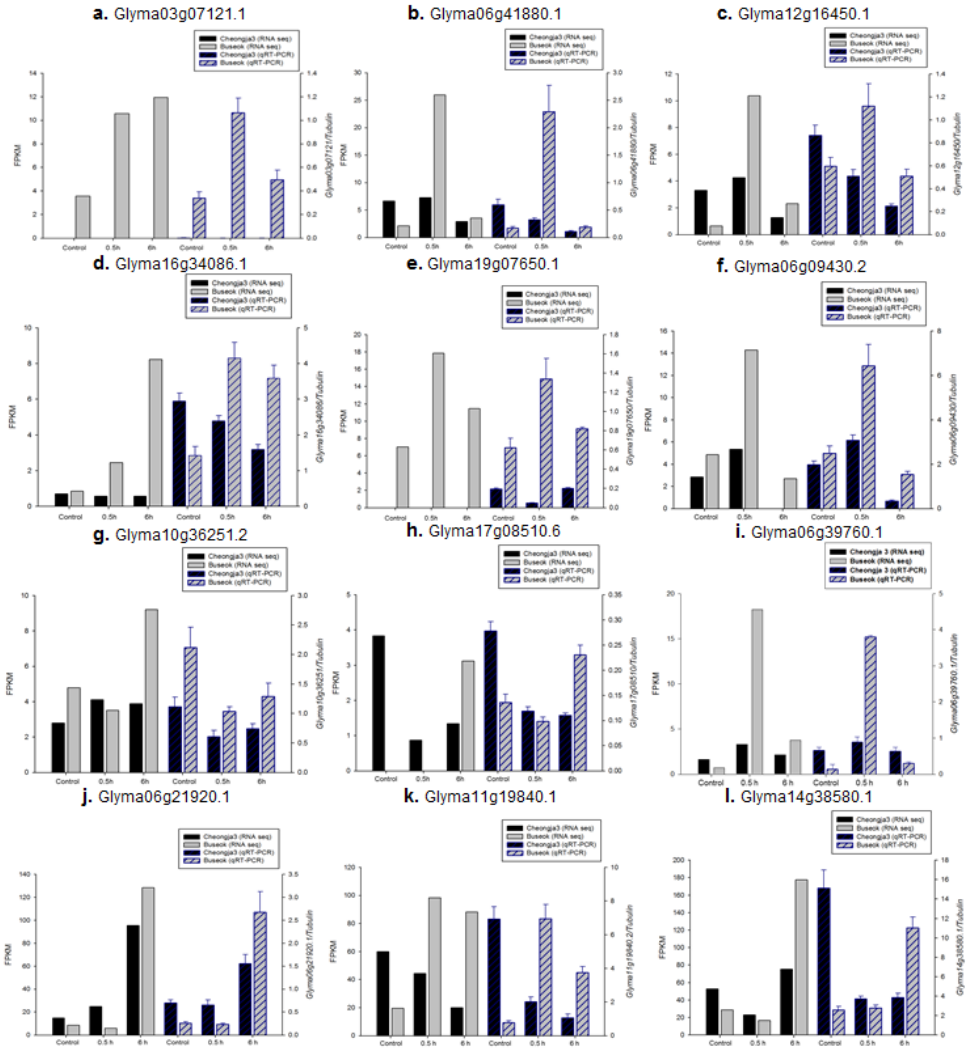

Figure I-4 Expression validations via qRT-PCR for UV-B related DEGs identified between UV-B-sensitive Cheongja 3 and UV-B-resistant Buseok. Left and right Y-axes represent relative transcript abundance from RNA-seq and qRT-PCR results, respectively. Black and gray bars indicate Cheongja 3 and Buseok, respectively.

Functional validation of UV-B stress defense signaling genes using A. thaliana knock-out mutants

We identified four A. thaliana knock-out mutant lines, including two for TIR-NBS-LRR (AT5G36930:SALK_124056C and AT4G36150: SALK_084909C), one for DGK (AT5G07920:SALK_033664C), and one for PIP5K (AT1G34260:SALK_047604C) (Figure I-5). There were few morphological differences between the knock-out mutant lines and wild type (Col-0) under normal conditions, suggesting that these genes do not play vital roles in basic growth and development. However, 2 days after 4 h UV-B stress, three of the mutant lines (AT5G36930:SALK_124056C, AT5G07920:SALK_033664C, and AT1G34260:SALK_047604C) showed severe chlorosis and stagnant growth, indicating increased sensitivity to UV-B stress (Figure I-5). By contrast, the wild type and the remaining mutant line (affected in the TIR-NBS-LRR gene) exhibited continued growth despite the presence of leaf curling and slight wilting. We measured the expression patterns of the four target genes in the wild type and mutant lines in response to 4 h UV-B irradiation by qRT-PCR. In the wild type, all of the genes except AT5G36930 (TIR-NBS-LRR) were induced by 4 h UV-B irradiation. As expected, the expression levels of all target genes were highly reduced in the corresponding mutant lines under both control and UV-B treatment. Functional validation using the A. thaliana knock-out mutants revealed that some genes implicated in the

phosphatidic acid signaling pathway and immune response play key roles in UV-B stress defense.

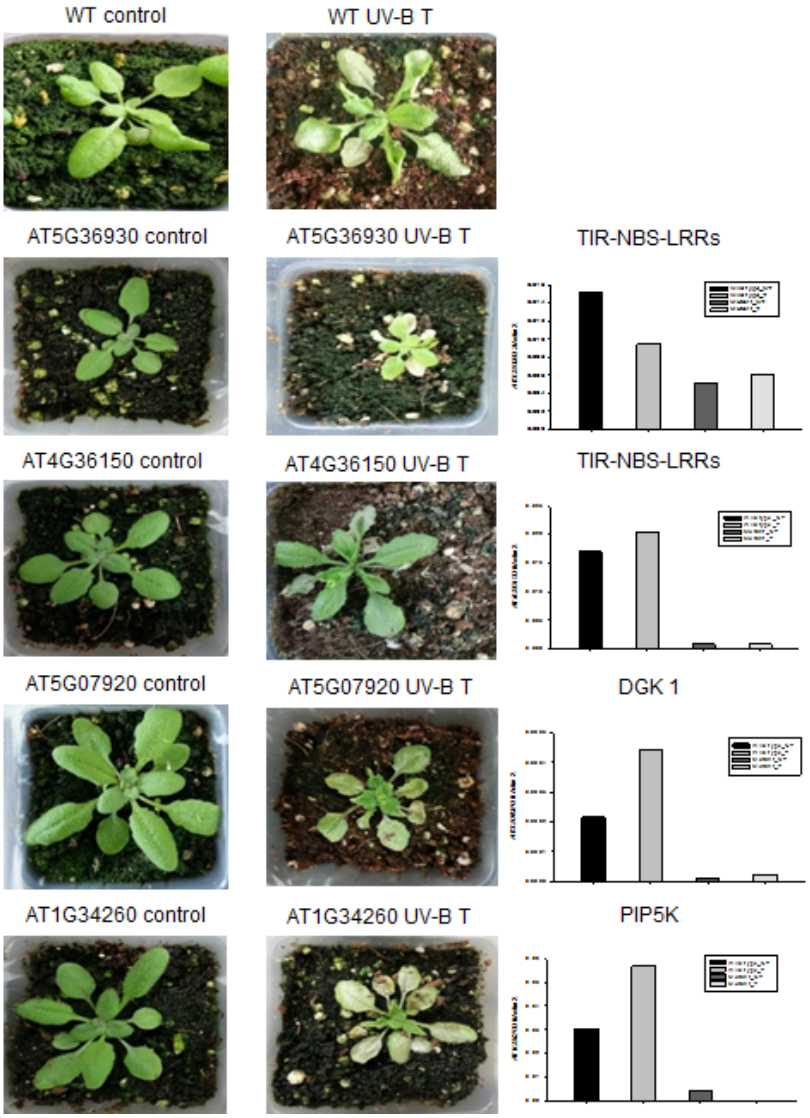

Figure I-5 Wild type and knock-out A. thaliana mutants after UV-B treatment. Left: morphological changes of the wild type and knock-out mutants after 4 h UV-B treatment. Right: expression levels of the target genes (TIR-NBS-LRR, DGK, and PIP5K) that were measured by qRT-PCR.

DISCUSSION

Two approaches are often used to investigate the responses of plants to UV-B radiation. One approach is to observe the damage and subsequent recovery of plants at specific times after UV-B irradiation (Biedermann and Hellmann, 2010; Kilian et al., 2007; Safrany et al., 2008), and the other is to investigate the accumulated responses of plants exposed to continuous UV-B treatment (Casati and Walbot, 2004; Gruber et al., 2010); both were utilized in the present study. A series of studies on the intracellular responses of maize have been conducted at different irradiation times from 5–90 min up to 6 h (Casati et al., 2011a; Casati et al., 2011b; Casati et al., 2011c), which were primarily focused on understanding plant acclimation to UV-B. In the current study, we surveyed differences in whole transcript abundance in response to supplementary UV-B exposure for 0.5 and 6 h using RNA-seq. Our transcriptome analysis provided evidence for the notion that intracellular photomorphogenic responses for adaptation to low UV-B levels are separate from defense mechanisms against UV-B stress at high fluence rates (Figure I-6). While the responses to UV-B stress are considered to be mediated by signaling pathways not specific to UV-B and are also induced by other stresses, our understanding of how plants activate components of defense signaling pathways against UV-B stress remains

limited.

The UV-B photoreceptor UVR8, which was isolated in A. thaliana, was not identified as a DEG in response to 0.5 and 6 h UV-B treatment in the current study, but other UV-B-specific photomorphogenic signaling components, including HY5, COP1, and CHS, were upregulated in UV-B-resistant Buseok compared to Cheongja 3. In addition, key genes involved in the circadian rhythm, such as PHYTOCHROM A, GIGANTEA, and PHYTOCHROM INTERACTING FACTOR 3, were upregulated by UV-B irradiation (Figure I-6). Low levels of UV-B are perceived by UVR8 followed by several downstream signaling pathways (Frohnmeyer and Staiger, 2003; Heijde and Ulm, 2012). The threshold UV-B doses that initiate photomorphogenic responses are much lower than those that induce stress defense gene expression (Boccalandro et al., 2001; Brown and Jenkins, 2008; Jenkins, 2009). In the current study, is unclear if UVR8 expression was upregulated instantly (≤1–2 min) after the start of UV-B irradiation to activate downstream photomorphogenic signaling and other light signaling pathways, followed by a return to the ground state. However, soybean is not presumed to have another UV-B photoreceptor in addition to UVR8 for photomorphogenic responses.

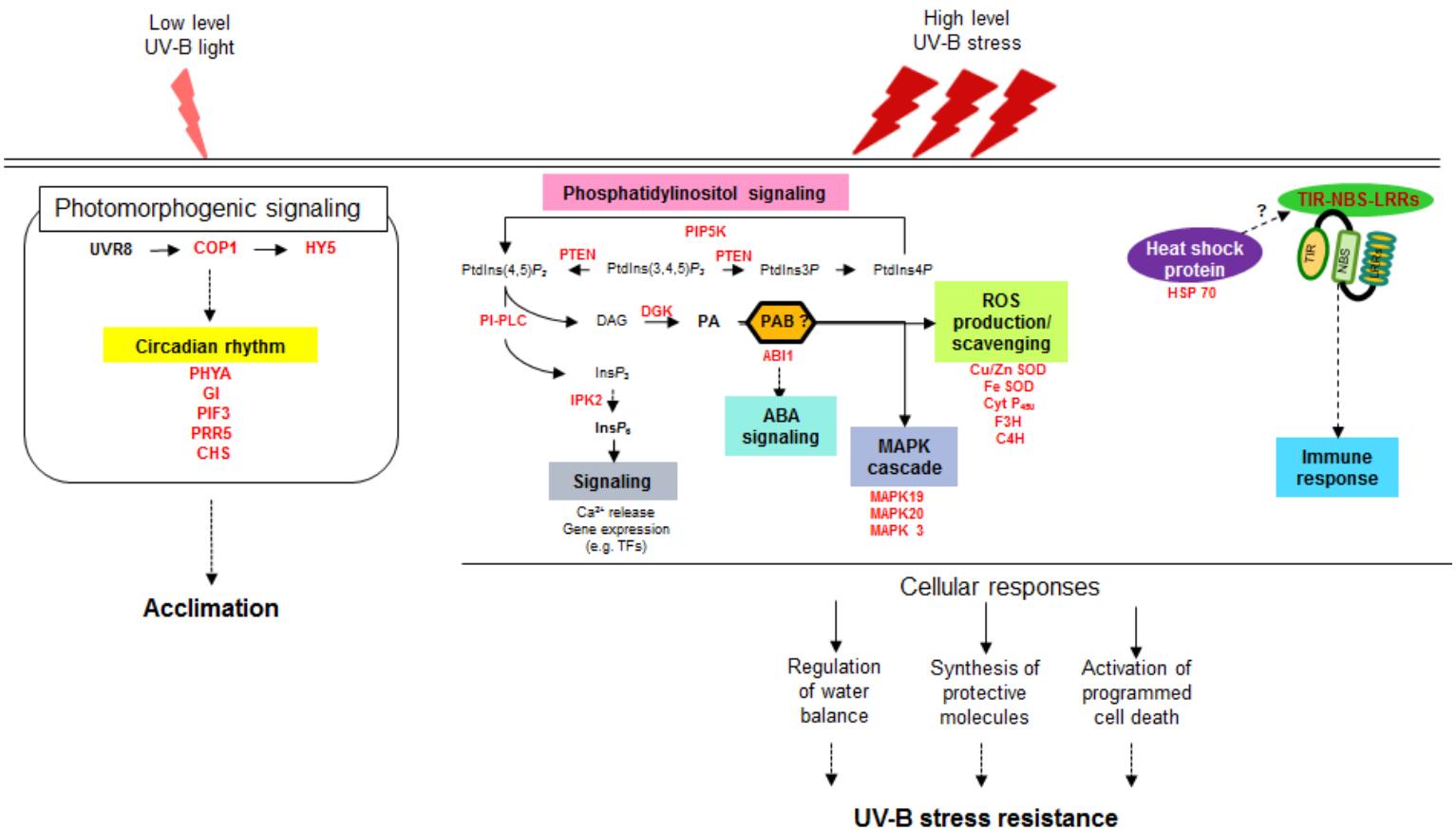
Low level
UV-B light
High level
UV-B stress
Photomorphogenic signaling
UVR8
COP1
HY5
Circadian rhythm
PHYA
GI
PIF3
PRR5
CHS
Acclimation
Phosphatidylinositol signaling
PIP5K
PtdIns(4,5)P2
PTEN
PtdIns(3,4,5)P3
PTEN
PtdIns3P
PtdIns4P
PI-PLC
DAG
DGK
PA
PAB ?
ABI1
InsP3
IPK2
InsP6
Signaling
Ca2+ release
Gene expression
(e.g. TFs)
ABA signaling
MAPK cascade
MAPK19
MAPK20
MAPK 3
ROS production/ scavenging
Cu/Zn SOD
Fe SOD
Cyt Pe5u
F3H
C4H
Heat shock protein
HSP 70
?
TIR-NBS-LRRs
TIR
NBS
Immune response
Cellular responses
Regulation of water balance
Synthesis of protective molecules
Activation of programmed cell death
UV-B stress resistance

Figure I-6 Schematic diagram of distinct signaling pathways depending on UV-B intensity. UVR8: UV Resistance Locus 8; COP1: Constitutively Photomorphogenic 1; HY5: Elongated Hypocotyl 5; PHYA: Phytochrome A; GI: Gigantea; PIF3: Phytochrome interacting factor 3; PRR5: Pseudo-response regulator 5; CHS: Chalcone and stilbene synthase family protein; PIP5K: phosphatidylinositol-4-phosphate 5-kinase family protein; PTEN: Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; PLC: Phospholipase C; DG: Diacylglycerol; DGK: Diacylglycerol kinase; PA: Phosphatidic acid; PAB: Phosphatidic acid binding protein; ABI1: ABA insensitive phosphatase 1; IPK2: Inositol 1,3,4-trisphosphate 5/6-kinase family protein; IP6: inositol hexaphosphate; Cu/Zn SOD: Copper/Zinc superoxide dismutase; Fe- SOD: Iron-superoxide dismutase; Cyt P450: Cytochrome P450 family protein; F3H: Flavanone-3-hydroxylase; C4H: Cinnamate-4-hydroxylase; MAPK: Mitogen-activated-protein kinase; HSP 70: Heat shock protein 70; TIR-NBS-LRRs: Toll/interleukin-1 receptor nucleotide binding site leucine-rich repeat.

Once plants recognize that irradiated UV-B fluence rates are beyond their adaptive capacity and are stressors, they immediately operate stress defense mechanisms for survival. The UV-B dosages used in our study are not low, and they represent dramatic spikes in UV-B intensity within short periods of time. Our results suggest that defense mechanisms against UV-B stress are provoked through PA-dependent signaling pathways via the sequential actions of PI-PLC and DGK, and that cell death and immunity systems also operate during this response (Figure I-6). Significant advances have been made in elucidating stress-induced PA signal transduction during the past several years, as described in recent reviews (Hong et al., 2016; Hou et al., 2016; Ruelland et al., 2015; Singh et al., 2015). PA is the common product of the phospholipase D (PLD) and PLC/DGK pathways, which use different phospholipids as substrates. The PLD pathway is involved in a wide range of responses to external stresses, such as cold, salinity, drought, and pathogen attack(Hong et al., 2016). Nonetheless, we found that UV-B stress triggered only a PLC/DGK-mediated PA signaling pathway in the current study. PLC is induced by the osmotic stress caused by salt and dehydration (Darwish et al., 2009). Since UV-B stress is a complex environmental stress comprising heat and dehydration as well as excessive light exposure, our finding is in line with previous results.

UV-B-resistant Buseok showed higher transcript abundance for four key

enzymes implicated in PI-PLC/DGK-mediated signaling cascades in plants (Table I-2; Figure I-3 and I-6), resulting in the production of polyphosphoinositols (membrane lipids) and inositol polyphosphates (water-soluble, IPP). These enzymes include PIP5K, PTEN2, DGK, and IPK2, exclusive of PI-PLC. In plants, the cellular levels of PtdIns(4,5)P2, which is produced from PtdIns4P by PIP5K and functions as a subtract for PI-PLC, are extremely low (30–100 fold lower than in mammalian cells) (Munnik and Vermeer, 2010). Nevertheless, Buseok had significantly higher levels of PIP5K transcript under both normal conditions (UV-B untreated) and UV-B exposure compared to Cheongja 3 (Table I-2). Increased PtdIns(4,5)P2 levels by concurrent activation of PIP5K might lead to earlier upregulation of PI-PLC in Buseok vs. Cheongja 3 (under 0.5 h UV-B treatment), which is in agreement with the results obtained for plant tissues exposed to salt or osmotic stress (Darwish et al., 2009) as well as heat stress (Mishkind et al., 2009). In the current study, the Arabidopsis mutant defective in PIP5K showed increased sensitivity to UV-B stress compared to wild type (Figure I-5). Upregulation of PI-PLC was observed only in Cheongja 3 under 6 h UV-B treatment (Table I-2) and Buseok showed stable expression patterns of PI-PLC independent of UV-B treatment. However, the activation of DGK, which catalyzes ATP-dependent DAG phosphorylation for PA biosynthesis, is more specifically regulated in response to UV-B stress and more dependent on genotype.

In Buseok, DGK was downregulated under control conditions and upregulated by UV-B treatment (Table I-2). By contrast, in Cheongja 3, DGK was not significantly upregulated by UV-B treatment. Thus, the regulation of DGK is likely more critical in the PI-PLC/DGK-dependent PA signaling pathways in response to UV-B stress and appears to be transcriptionally mediated, even though the type of TF that binds to its promoter region is currently unknown. A study performed more than a decade ago revealed that a UV-B light insensitive (uli) T-DNA insertional Arabidopsis mutant, which displays hyposensitivity to low-fluence UV-B irradiation, is defective in DGK (Suesslin and Frohnmeyer, 2003). However, in the current study, an Arabidopsis DGK knock-out mutant showed increased damage to high UV-B intensity (Figure I-5). Although whether DGK mediates different signaling pathways according to UV-B fluence rates is currently unclear, DGK is likely a specific component involved in UV-B-induced signal transduction in plants.

Increases in cytosolic Ca2+ levels in plant cells are the hallmark of stress defense responses (Singh et al., 2015). In mammalian systems, InsP3, the other product of PI-PLC hydrolysis of PtdIns(4,5)P2, releases Ca2+ from intracellular reservoirs by binding to ligand gated-calcium channels (Munnik and Nielsen, 2011). However, no InsP3 receptor has been identified in plants; instead, its multiple phosphorylated form InsP6, through stepwise phosphorylation by the IPP multikinases IPK2 and IPK1,

is thought to stimulate increases in Ca2+ levels and to function as a signaling molecule itself (Hou et al., 2016; Munnik and Vermeer, 2010). In the current study, the transcript levels of IPK2, which phosphorylates Ins(1,4,5)P3 to Ins(1,3,4,5,6)P5, were higher in Buseok than in Cheongja 3 (Table I-2).

PA binds to various target proteins to mediate downstream signal transduction during diverse defense responses such as ABA-mediated pathways, MAPK signaling cascades, and ROS over-accumulation (Hou et al., 2016). Among the dozens of identified effector proteins of PA in these cellular responses in plants (Hou et al., 2016; Ruelland et al., 2015), our RNA-seq analysis showed that ABI1, MAPK, and SOD were upregulated in Buseok under UV-B exposure (Figure I-3). PA binding to ABI1 helps tether it to the plasma membrane where it interact with ATHB6, a negative regulator of ABA signaling involved in stomatal closure in response to drought and salinity stress (Zhang et al., 2004). PA also can bind to and activate MAPK in the response of A. thaliana and soybean to salt stress (Im et al., 2012; Yu et al., 2010). Based on the current results, ROS generation induced by UV-B stress is more likely dependent on SOD than on NADPH oxidase, even though PLD-derived PA binds to and activates NADPH oxidases under environmental stress (Park et al., 2004; Zhang et al., 2009).

Another interesting result of the present study is that TIR-NBS-LRR

genes are upregulated in resistant Buseok under UV-B stress (Table I-1; Figure I-3). Plant NBS-LRR proteins can be divided into two major subfamilies based on the presence of TIR or CC motifs in their N-terminal domains (Göhre and Robatzek, 2008; Tameling and Joosten, 2007). Similarly, dozens of TIR-NBS-LRR genes, but no CC-NBS-LRRs, are differentially expressed in A. thaliana under heat and drought stress (Prasch and Sonnewald, 2013). A surprising role for TIR-NBS-LRRs has been proposed in the sensing of red light (Faigón-Soverna et al., 2006). A mutant of the constitutive shade avoidance (CSA1) gene in Arabidopsis is defective in red light-induced responses and produces a truncated protein with a structure similar to TIR.

We also observed increased accumulation of HSP70 transcripts in response to UV-B stress in Buseok (Table I-1 and Figure I-3). Indeed, in Arabidopsis, HSPs and heat shock factors are upregulated in response to pathogen infection, abiotic stresses including UV, and wounding (Swindell et al., 2007). HSPs including HSP90 are thought to regulate the function of NBS-LRR (Belkhadir et al., 2004). Similarly, in soybean, HSP70 is upregulated under high temperature stress (Ahsan et al., 2010). Therefore, the highly expressed TIR-NBS-LRR proteins and HSPs under UV-B stress likely play important roles in the UV-B resistance response.

The present study provides comprehensive insights into defense signaling pathways against high-intensity UV-B stress, from signal

transduction by second messengers to downstream defense-related gene expression (Figure I-6). It is currently likely indisputable that the generation of PPI-based signaling molecules such as PA and IP6 is the primary event in the signaling cascades from stress awareness to defensive metabolism. Based on our findings, further studies should be performed investigating how the key enzymes involved in PA signaling pathways are upregulated under UV-B stress and how the derived signaling molecules are integrated into downstream pathways, together with the identification of TFs specific to UV-B stress. Such studies will provide essential information for breeding resistant soybean genotypes that survive under high-intensity UV-B stress and can adapt to other adverse conditions for sustainable productivity in the future.

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CHAPTER II

Transcriptomic profiling of soybean in response to UV-B and *Xanthomonas axonopodis* treatment reveals shared gene components in stress defense pathways

ABSTRACT

Soybean (Glycine max [L.]) has evolved to survive under abiotic and biotic stress conditions by utilizing multiple signaling pathways. Although several studies have revealed shared defense signaling pathways in plants, the majority of components at the convergence points of signaling pathways triggered by both abiotic and biotic stress remain poorly understood. Here, we profiled the overall transcriptional responses of soybean to two different types of stress using the UV-B-resistant cultivar, Buseok, and the UV-B-sensitive cultivar, Cheongja 3, as well as two near isogenic lines (NILs) carrying bacterial leaf pustule (BLP) disease-

resistant and -susceptible alleles. We compared transcript abundance and identified genes that commonly respond to UV-B stress and BLP disease. In addition, we surveyed the co-localization of differentially expressed genes (DEGs) and their paralogs with abiotic and biotic stress-related quantitative trait loci (QTLs) on the soybean genome. Among 14 DEGs that respond to both stresses, five DEGs are involved in the jasmonic acid (JA) metabolic pathway, encoding Jasmonate ZIM (Zinc-finger protein expressed in Inflorescence Meristem) domain-containing protein 1 (JAZ 1), a negative regulator of JA signaling. Two DEGs for JAZ 1 were co-localized with biotic stress-related QTLs. One DEG encoding the stress-induced protein starvation-associated message 22 (SAM 22) and its two paralogs were co-localized with both abiotic and biotic stress-related QTLs. The results of this study help elucidate general responses to abiotic and biotic stress in soybean, thereby helping breeders improve stress-resistant soybean cultivars.

INTRODUCTION

The stress response can be defined as the interaction between plants and extreme environmental changes (Borkotoky et al. 2013). These environmental changes are generally induced by abiotic and biotic factors: temperature, light, ultraviolet radiation, osmotic stress, and physical wounding are abiotic stress inducers, while pest and pathogen attack are biotic stress inducers. Plants have evolved a wide range of molecular mechanisms to help them survive and adapt under both abiotic and biotic stress (AbuQamar et al. 2009). Recent studies revealed crosstalk between abiotic and biotic stress responses (Rejeb et al. 2014). Reactive oxygen species (ROS), which act as destructive molecules that affect both DNA and proteins, are common transducers of abiotic and biotic stress signals (Apel and Hirt 2004; Fujita et al. 2006; Simon et al. 2000; Torres and Dangl 2005). Calcium-dependent protein kinases (CDPKs) have also been implicated as Ca2+ signaling regulators of abiotic and biotic stress responses (Kudla et al. 2010; Ludwig et al. 2004), as shown by the transcriptional response of LeCDPK 1 to both wounding and fungal stress in tomato (Chico et al. 2002). OsNAC6, a member of the NAC (NAM, ATAF1, ATAF2, and CUC2) transcription factor (TF) family, functions as a transcriptional

activator that responds to both abiotic and biotic stress. Transgenic rice lines overexpressing OsNAC6 showed higher tolerance against salt stress and rice blast stress than the control (Nakashima et al. 2007).

Soybean (Glycine max [L.] Merr.) is one of the most economically important crops worldwide. Over the past few years, several studies on the crosstalk between abiotic and biotic stress responses in soybean have been performed (Kulcheski et al. 2011; Zhang et al. 2009). Expressing the soybean Ethylene Response Factor 3 (GmERF3) gene in transgenic tobacco lines induced pathogen-related (PR) genes and conferred resistance to tobacco mosaic virus and salt stress (Zhang et al. 2009). However, the majority of components at the convergence points of defense signaling under abiotic and biotic stress remain unknown.

Since the mid-1980s, increased intensity of ultraviolet (UV)-B due to depletion of ozone layer has caused plant physiological damage, such as reduced photosynthetic capacity and impaired pollen fertility, leaf discoloration, reduced biomass, and seed yields (Frohnmeyer and Staiger 2003; Lytvyn et al. 2010). UV-B radiation of high level can be considered as complex environmental stress including extreme light, heat and high temperature. Meanwhile, bacterial leaf pustule (BLP) disease, caused by Xanthomonas axonopodis pv. glycines (Xag), is one of the most serious diseases in soybean (Kim et al. 2011). Under

high temperatures and high humidity as effects of global warming, BLP reduces grain yield by 15–40%, primarily through chlorophyll degradation and premature defoliation.

The present study was performed to survey shared gene components in the defense mechanisms against abiotic and biotic stress. We profiled the transcriptional responses of two soybean genotypes (UV-B-resistant Buseok vs. UV-B-sensitive Cheongja 3) treated with UV-B and two near isogenic lines (NILs) carrying bacterial leaf pustule (BLP) disease-resistant and -susceptible alleles inoculated with Xag to identify common differentially expressed genes (DEGs) by comparing the transcript abundances between two genotypes under UV-B stress and BLP disease. To explore the functions of the newly identified common DEGs, we also investigated their proximity to previously reported abiotic and biotic stress-related quantitative trait loci (QTLs). Our findings help elucidate the general stress responses of soybean under abiotic and biotic stress.

MATERIAL AND METHODS

Plant materials

Four soybean genotypes were used in this study, including UV-B-resistant Buseok, UV-B-sensitive Cheongja 3, and BLP disease-resistant and BLP-susceptible NILs. Buseok and Cheongja 3 were characterized as the most resistant and sensitive soybean genotypes to supplementary UV-B irradiation, respectively, in our previous study (Kim et al. 2015). The NILs were generated by repeated backcrossing of the donor parent (BLP-resistant cultivar SS2-2) and recurrent parent (BLP-susceptible cultivar Taekwangkong) (Kim et al. 2011). Leaf samples from Buseok and Cheongja 3 treated with UV-B irradiation for 0.5 and 6 h were collected for RNA extraction and sequencing (RNA-seq). Illumina RNA-seq raw data from BLP-resistant and -susceptible NILs at 6 and 12 h after inoculation with Xag were obtained from a previous study (Kim et al. 2011).

RNA sequencing and data analysis

RNA-seq of UV-B-treated soybean was performed using Illumina

Hiseq 2000 (Illumina, Co, CA, USA), and the sequence data were retrieved through the standard Illumina pipeline with custom and default parameters. Additional filtering was performed by trimming adapter sequences and low quality sequences. Cleaned reads were mapped to the G. max reference genome (Glycine max Wm82.a2.v1) (https://phytozome.jgi.doe.gov/) using Bowtie2 aligner (Langmead and Salzberg 2012) and TopHat v2 (Kim et al. 2013). Gene expression values were calculated based on the number of fragments per kilobase of exon per million fragments mapped (FPKM) (Mortazavi et al. 2008) in Cufflinks (Roberts et al. 2011), and differentially expressed transcripts were identified using Cuffdiff (Trapnell et al. 2012). Significant DEGs were detected using the following criteria: (i) absolute log2 (fold change) > 1 and (ii) q-value (false discovery rate [FDR]) < 0.05 and p-value < 0.004.

Investigation of stress-related QTLs co-localized with common DEGs

Abiotic and biotic stress-related QTLs of soybean were obtained from the SoyBase website (http://soybase.org/). The QTLs were localized on soybean chromosomes using marker information from soybean map version 4.0 from SoyBase (Grant et al. 2009). A circular map of co-localized QTLs and common DEGs with their paralogous genes was constructed using Circos (http://circos.ca/).

RESULTS

Common DEGs responsive to UV-B stress and BLP disease in soybean

Illumina RNA-seq produced 0.3 billion 100 bp paired-end reads from the UV-B-treated cDNA libraries and 0.12 billion 76 bp single-end reads from Xag-inoculated RNA samples. Of these, 78% of UV-B-treated RNA-seq reads and 86% of Xag-inoculated RNA-seq reads were mapped to the reference genome.

To identify common DEGs responsive to UV-B stress and BLP disease, we first identified DEGs within the two UV-B-treated genotypes and two Xag-inoculated genotypes, respectively. We compared the gene expression values (FPKM) of transcriptome data from Cheongja 3 and Buseok at 0, 0.5, and 6 h. The DEGs detected at 0 h were filtered out because they were considered to be differentially expressed between the cultivars. In total, 2,888 DEGs were detected at the three time points, and 616 DEGs from the 0 h time point were removed as a non-treated control. Among the 353 DEGs detected between the BLP-resistant and -susceptible NILs at 0, 6, and 12 h, 244 DEGs detected at the 0 h time point were removed as a non-treated control. We finally identified 14

common DEGs that were responsive to both abiotic and biotic stress among a total of 2,381 DEGs (Table II-1 and Figure II-1), while there were 2,272 abiotic stress-responsive DEGs and 109 biotic stress-responsive DEGs.

Among the 14 DEGs, five homologous DEGs are involved in the jasmonic acid (JA) metabolic pathway, encoding Jasmonate ZIM (Zinc-finger protein expressed in Inflorescence Meristem) domain-containing protein 1 (JAZ 1), a negative regulator of JA signaling (Figure II-1). While all of these five JAZ1 were highly upregulated in UV-B- resistant cultivar Buseok (log2 fold change > 4.66 at 6 h), the expression of all of them except Glyma.01G204400 was downregulated or not significantly altered in the BLP-resistant NIL (Figure II-1 and Table II-1). Similarly, the DEG (Glyma.11G048300) coding for Ca2+ ATPase 2, which participates in Ca2+ signaling, showed up-regulation in Buseok but down-regulation in the BLP-resistant NIL (Figure II-1 and Table II-1).

There are three DEGs (i.e., Glyma.07G243500, Glyma.11G222300, and Glyma.02G059500) that showed upregulation in both Buseok and the BLP-resistant NIL by UV-B and Xag treatments, respectively (Table II-1 and Figure II-1). Stress-induced protein starvation-associated message 22 (SAM 22) encoded by Glyma.07G243500 is a major birch pollen allergen and a homolog of PR protein Betula verrucosa 1 (Bet v1).

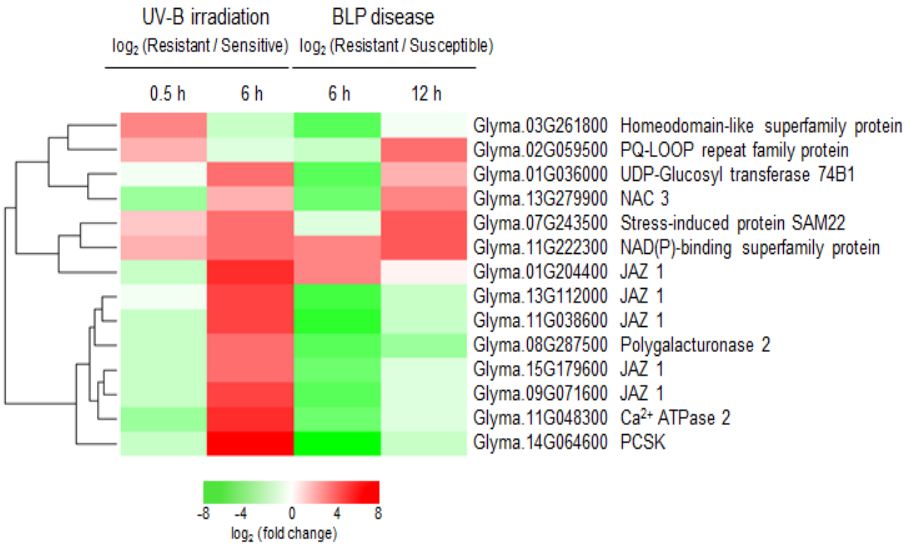

Figure II-1 Heatmap showing the differential expression levels of common 14 genes in response to UV-B stress and BLP disease. Red and green indicate upregulated and downregulated expression in UV-B-resistant Buseok and the BLP-resistant line vs the corresponding susceptible genotypes, respectively.

NAC 3; NAC domain-containing protein 3, SAM 22; starvation-associated message 22, JAZ 1; Jasmonate ZIM domain-containing protein 1, PCSK; Protein convertase subtilisin/kexin

Table II-1 Expression levels of 14 DEGs in response to UV-B irradiation as well as BLP inoculation.

Gene ID	UV-B-sensitive vs. -resistant									BLP-susceptible vs. -resistant								
	Control			0.5 h			6 h			Mock			6 h			12 h		
	FPKM		log_2 (fold change)	FPKM		log_2 (fold change)	FPKM		log_2 (fold change)	FPKM		log_2 (fold change)	FPKM		log_2 (fold change)	FPKM		log_2 (fold change)
	sensitive	resistant		sensitive	resistant		sensitive	resistant		susceptible	resistant		susceptible	resistant		susceptible	resistant	
Glyma.11G222300	0.42	0.76	0.87	0	0.72	-	0.24	6.1	4.68	0.38	6.29	4.06	0.28	4.62	4.05	0.27	11.52	5.4
Glyma.01G204400	9.26	583.75	5.98	5.28	3.55	-0.57	8.54	588.1	6.11	1.12	272.09	7.92	3.35	50.81	3.92	2.39	4.7	0.97
Glyma.02G059500	6.3	20.33	1.69	3.12	27.73	3.15	30.31	30.75	0.02	22.76	44.95	0.98	17.72	12.46	-0.51	1.44	37.9	4.71
Glyma.07G243500	29.43	11	-1.42	18.79	86.71	2.21	4.24	93.78	4.47	0	0.47	-	0.53	0.59	0.16	0.71	22.93	5.01
Glyma.13G279900	19.95	54.33	1.45	13.21	4.75	-1.47	4.53	31.28	2.79	0.9	133.55	7.21	26.62	4.38	-2.6	1.45	25.68	4.15
Glyma.03G261800	15.36	215.26	3.81	20.61	307.17	3.9	313.49	188.75	-0.73	100.78	331.63	1.72	69.84	6.29	-3.47	0.43	0.58	0.45
Glyma.01G036000	20.03	0.73	-4.77	0.85	1.38	0.7	0.92	18.27	4.31	0.87	7.29	3.07	22.28	2.12	-3.4	0.64	4.35	2.78
Glyma.08G287500	6.44	16.66	1.37	2.35	1.41	-0.73	1.06	27.53	4.7	3.66	55.22	3.91	21.76	2.44	-3.16	7.75	2.66	-1.54
Glyma.09G071600	28.07	1394.69	5.63	26.24	20.86	-0.33	18.75	1096.75	5.87	1.04	352.12	8.41	64.61	6.92	-3.22	7.77	9.84	0.34
Glyma.11G038600	3.36	642.01	7.58	4.09	2.78	-0.55	10.64	528.78	5.64	1.01	251.27	7.95	57.09	2.94	-4.28	2.01	1.47	-0.45
Glyma.11G048300	17.36	229.04	3.72	18.66	6.13	-1.6	2.76	232.11	6.4	1.19	65.43	5.78	14.17	2.36	-2.58	1.29	1.32	0.04
Glyma.14G064600	234.72	179.05	-0.39	8.65	6.65	-0.38	1.14	237.85	7.7	9.45	53.41	2.5	66.09	1.12	-5.88	171.04	136.35	-0.33
Glyma.13G112000	6.31	733.76	6.86	6.49	12.07	0.89	14.93	734.98	5.62	0.94	523.02	9.12	63.06	4.68	-3.75	4.38	3.59	-0.29
Glyma.15G179600	21	1103.76	5.72	31.62	20.11	-0.65	32.14	812.88	4.66	3.59	295.09	6.36	53.01	8.03	-2.72	9.29	11.34	0.29

The expression levels of SAM 22 significantly increased in Buseok (4.467 log2 fold change at 6 h) under UV-B stress and in the BLP-resistant NIL (5.01 log2 fold change at 12 h) after Xag inoculation compared to their counterpart genotypes, respectively (Table II-1). The expression level of the DEG encoding NAD(P)-binding Rossmann-fold superfamily protein (Glyma.11G222300) showed a 2.841 log2 fold change at 0.5 h in the UV-B- resistant Buseok and a 4.045 log2 fold change at 6 h in the BLP-resistant NIL. The DEG coding for a PQ-loop repeat family protein (Glyma.02G059500) also showed higher transcript abundance in Buseok (3.15 log2 fold change at 0.5 h) and the BLP-resistant NIL (4.714 log2 fold change at 12 h) than in Cheongja 3 and the BLP-susceptible NIL, respectively. The six remaining DEGs had different expression patterns in each dataset and at each time point (Figure II-1 and Table II-1).

Co-localization of common DEGs and their paralogous genes with stress-related QTLs

The G. max genome has undergone two large-scale whole genome duplications (WGD) and one small-scale duplication (SSD) (Schmutz et al. 2010). Among the 14 common DEGs, two genes, encoding a NAD(P)-binding Rossmann-fold superfamily protein (Glyma.11G222300) and protein convertase subtilisin/kexin (PCSK, Glyma.14G064600), are single

copy genes, whereas the 12 other DEGs are present in multiple (2–3) copies, for a total of 19 paralogs (Table II-2). For JAZ 1, six paralogous genes are present in the soybean genome, and four pairs show syntenic relationships (Table II-2). Moreover, soybean genome contains five duplicated genes of SAM 22: two paralogous pairs, Glyma.07G243500-Glyma.09G040400 and Glyma.07G243500-Glyma.15G145600 are present in the synteny blocks of Gm07:41726276..42773852-Gm09:2618477..4040200 (median Ks value: 0.6487, http://www.soybase.org/gb2/gbrowse/gmax2.0/) and Gm07:41726276..42394912-Gm15:11067544..12110387 (median Ks value: 0.5956), respectively, which were derived from the ancient WGD. The other paralogous pair Glyma.07G243500-Glyma.17G030200 is located in the synteny block of Gm07:41726276..44600634-Gm 17:17407..2796816 (median Ks value: 0.1246) which was generated by the recent WGD, where Glyma.17G030200 has a tandemly duplicated isoform of Glyma.17G030400 (Figure II-2, Table II-2).

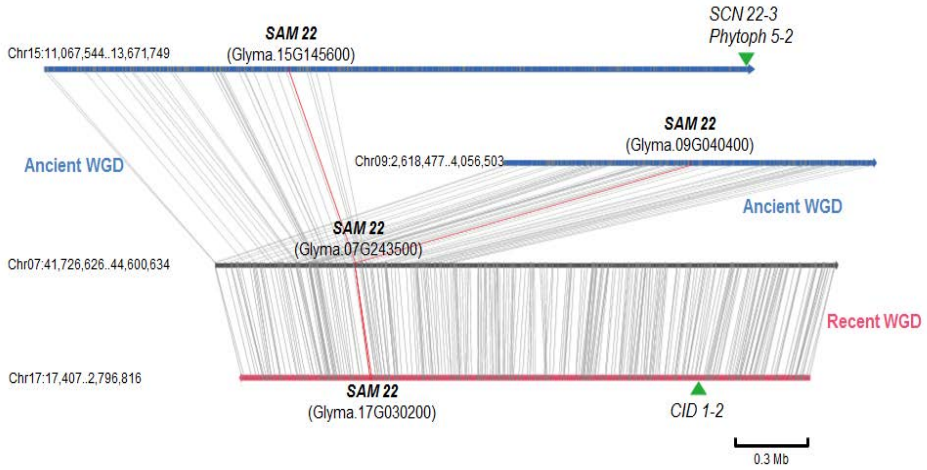

Figure II-2 Alignment of syntenic blocks on soybean chromosome 7 carrying SAM 22. The diagram includes horizontal lines representing soybean genome sequences showing syntenic relationships. Red dots indicate SAM 22 in each synteny block. Center line indicates soybean Chr 7, and the corresponding syntenic blocks are shown on Chr 15 (upper line, left) and Chr 9 (upper line, right), respectively, which were derived from the ancient WGD. One paralogous gene (Glyma.15G145600) of SAM 22 is co-localized with biotic stress QTLs SCN 22-3 and phytoph 5-2. Syntenic blocks between Chr 7 and Chr 17 (lower line) were generated by the recent WGD. The other paralogous gene (Glyma.17G030200) overlaps with abiotic stress QTLs (CID 1-2). SCN; soybean cyst nematode, reaction to Heterodera glycnes, phytoph; reaction of Phytophthora sojae infection, CID; carbon isotope discrimination

Table II-2 Information about 14 common DEGs under UV-B treatment and BLP inoculation. The paralogous genes and abiotic and biotic stress-related QTLs that are co-localized within 4 Mb regions around the genes are listed. Asterisks (*) indicate QTL-linked genes and their corresponding QTLs.

Gene ID	Position	Functional annotation	GO ID	GO term	Duplicated genes	Reported QTLs
Glyma.01G204400*	Chr01:53722328..53724096	Jasmonate ZIM domain-containing protein 1	GO:0043069	negative regulation of programmed cell death	Glyma.11G038600.1	Sclero 2-5*, Sclero 3-4*,Sclero 4-1*, Sclero 5-3*, SCN 39-11*, Whitefly resistance 1-1*
			GO:0009733	response to auxin stimulus		
			GO:0009753	response to jasmonic acid stimulus		
			GO:0009863	salicylic acid-mediated signaling pathway		
			GO:0009867	jasmonic acid-mediated signaling pathway		
			GO:0010363	regulation of plant-type hypersensitive response		
			GO:0009695	jasmonic acid biosynthetic process		
			GO:0009723	response to ethylene stimulus		
			GO:0009620	response to fungus		
			GO:0007165	signal transduction		
			GO:0009414	response to water deprivation		
			GO:0009611	response to wounding		
Glyma.09G071600	Chr09:7366510..7369241	Jasmonate ZIM domain-containing protein 1	GO:0042742	defense response to bacterium	Glyma.13G112000.1, Glyma.15G179600.1*, Glyma.17G047700.1	SCN 29-9*
			GO:0009867	jasmonic acid-mediated signaling pathway		
Glyma.11G038600	Chr11:2759949..2761506	Jasmonate ZIM domain-containing protein 1	GO:0009753	response to jasmonic acid stimulus	Glyma.01G204400.1*	Sclero 2-5*, Sclero 3-4*,Sclero 4-1*, Sclero 5-3*, SCN 39-11*, Whitefly resistance 1-1*
			GO:0009611	response to wounding		
Glyma.13G112000	Chr13:22541686..22545311	Jasmonate ZIM domain-containing protein 1	GO:0009753	response to jasmonic acid stimulus	Glyma.09G071600.2, Glyma.17G047700.1	
			GO:0009867	jasmonic acid-mediated signaling pathway		
			GO:0042742	defense response to bacterium		
Glyma.15G179600*	Chr15:17292780..17295404	Jasmonate ZIM domain-containing protein 1	GO:0009753	response to jasmonic acid stimulus	Glyma.09G071600.1	SCN 29-9*
			GO:0009867	jasmonic acid-mediated signaling pathway		
			GO:0042742	defense response to bacterium		
Glyma.07G243500	Chr07:42331425..42332476	Stress-induced protein SAM22	GO:0006952	defense response	Glyma.09G040400.1, Glyma.15G145600.1*, Glyma.17G030200.1**, Glyma.17G030400.1	SCN 22-3*, Phytoph 5-2*, carbon isotope discrimination 1-2**
			GO:0009607	response to biotic stimulus		
Glyma.13G279900*	Chr13:38124292..38125955	NAC DOMAIN-CONTAINING PROTEIN 3	GO:0006355	regulation of transcription, DNA-templated	Glyma.06G248900.1, Glyma.12G221500.1, Glyma.12G149100.1	Rag 2-2*
Glyma.11G222300	Chr11:31740260..31744799	NAD(P)-BINDING ROSSMANN-FOLD SUPERFAMILY PROTEIN	GO:0016491	oxidoreductase activity		
Glyma.02G059500	Chr02:5356619..5363076	PQ-LOOP REPEAT FAMILY PROTEIN /TRANSMEMBRANE FAMILY PROTEIN			Glyma.01G178800.3, Glyma.16G142300.1	
Glyma.03G261800	Chr03:45502100..45510812	Homeodomain-like superfamily protein	GO:0009753	response to jasmonic acid stimulus	Glyma.19g260900.1	
			GO:0009739	response to gibberellin stimulus		
			GO:0009651	response to salt stress		
			GO:0009751	response to salicylic acid stimulus		
			GO:0032922	circadian regulation of gene expression		
			GO:0009733	response to auxin stimulus		
			GO:0009737	response to abscisic acid stimulus		
			GO:0009723	response to ethylene stimulus		
Glyma.01G036000	Chr01:3770841..3773093	UDP-Glucosyl transferase 74B1	GO:0016758	transferase activity, transferring hexosyl groups	Glyma.02G029900.1	
Glyma.11G048300	Chr11:3601241..3607177	calcium ATPase 2	GO:0006754	ATP biosynthetic process	Glyma.05G105200.1, Glyma.17G161300.1*	pH sensitivity 1-2*
Glyma.08G287500*	Chr08:39862688..39865201	polygalacturonase 2	GO:0043481	anthocyanin accumulation in tissues in response to UV light	Glyma.01G030900.1, Glyma.02G034700.1	SCN 37-4*
Glyma.14G064600*	Chr14:5295129..5304967	PROPROTEIN CONVERTASE SUBTILISIN/KEXIN	GO:0006508	proteolysis		Sclero 8-2*
			GO:0043086	negative regulation of catalytic activity		

The co-localization of the DEGs and their paralogs with abiotic and biotic stress-related QTLs implies that they play a role in stress resistance in soybean. We detected 30 reported QTLs related to abiotic and biotic stress from SoyBase (http://soybase.org/). We selected 31 genes, including 14 DEGs and their 19 paralogs, and investigated whether the QTLs were located within a 4 Mb region surrounding these genes (Table II-2 and Figure II-3). We found that seven QTLs co-localized with eight stress-responsive genes (Figure II-3). These QTLs included two QTLs related to abiotic stress, carbon isotope discrimination (CID) and pH sensitivity (pH sens), and five QTLs related to biotic stress, reaction to Sclerotinia sclerotiorum infection (sclero), reaction to Heterodera glycines (SCN, soybean cyst nematode), reaction to Phytophthora sojae infection (phytoph), reaction to Bemisia tabaci (whitefly resistance), and reaction to Aphis glycines (Rag).

The DEG for calcium ATPase 2 (Glyma.11G048300) has two paralogous genes (Glyma.05G105200 and Glyma.17G161300) that co-localize with an abiotic stress-related QTL (pH sens 1-2). Two DEGs of JAZ 1 (Glyma.01G204400 and Glyma.15G179600) are localized near biotic stress-related QTLs: Glyma.01G204400 with sclero (Sclero 2-5, 3-4, 4-1, and 5-3), SCN 39-11 and whitefly resistance1-1, and Glyma.15G179600 with SCN 29-9 (Table II-2). Two DEGs encoding polygalacturonase 2 and NAC 3 (Glyma.08G287500 and Glyma.13G279900) are linked to SCN

37-4 and Rag 2-2, respectively. The single copy gene (Glyma.14G064600) for PCSK is located close to sclero 8-2. Interestingly, two paralogous genes of SAM 22 (Glyma.15G145600 and Glyma.17G030200), which were highly upregulated under both the abiotic and biotic stresses, were co-localized with abiotic and biotic stress-related QTLs, including Glyma.15G145600 with SCN 22-3 and phytoph 5-2, and Glyma.17G030200 with CID 1-2 (Figures II-2, II-3 and Table II-2).

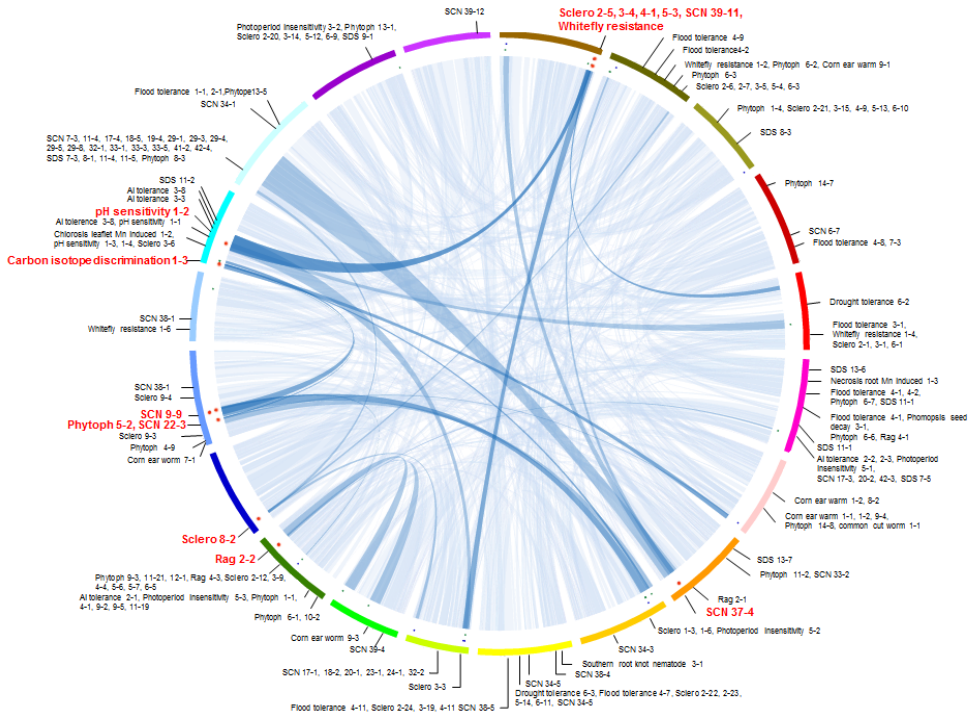

Figure II-3 The distribution of DEGs in response to UV-B stress and BLP disease and their paralogous genes with abiotic- and biotic stress-related QTLs.

The outer circle represents the Glycine max chromosomes. The green dots in the first layer inside the outer circle indicate common DEGs under both UV-B stress and BLP infection. The blue dots in the second layer indicate duplicated genes of common DEGs. Red dots indicate that the stress-related QTLs are co-localized within a 4 Mb region surrounding the genes. CID; carbon isotope discrimination, pH sens; pH sensitivity, sclero; reaction to Sclerotinia sclerotiorum infection, SCN; soybean cyst nematode, reaction to Heterodera glycines, phytoph; reaction to Phytophthora sojae infection, whitefly resistance; reaction to Bemisia tabaci, and Rag; reaction to Aphis glycines.

DISCUSSION

Plants have established defense signaling mechanisms that allow them to adapt to and survive under abiotic and biotic stress. The first crucial step in this process is to perceive stress in a rapid and efficient way (Rejeb et al. 2014). After the stress is recognized, complex defense signaling cascades operate, such as the activation of specific ion channels and kinase cascades and the accumulation of ROS and phytohormones such as abscisic acid (ABA), salicylic acid (SA), ethylene (ET), and JA (AbuQamar et al. 2009). These secondary messengers mediate the transduction of signals to the corresponding signaling pathways against abiotic and biotic stress (Wang et al. 2016). The transduced signals activate TFs that regulate genes for morphological and physiological responses to stress (Bhargava and Sawant 2013; Fujita et al. 2006).

In the current study, four UV-B sensitive/tolerant and Xag susceptible/resistant soybean genotypes have different genetic background and different experiment designs for UV-B irradiation and Xag inoculation were used. Even though the genotypes and the experimental designs ideally need to be synchronized and uniformed for concrete comparisons between expression profiles, it is not easy to find

soybean genotypes that display resistance or sensitivity commonly to varied types of abiotic and biotic stress. In addition, there has been little knowledge about how optimal treatment periods are determined for accurate transcriptomic comparisons according to the types of abiotic and biotic stress. Nevertheless, we detected several shared downstream genes affected by secondary messengers such as JA and Ca2+ after stress recognition under abiotic and biotic stress (Figure II-4). It supports an assumption that the shared molecular modules in the stress defense mechanisms exist across soybean genotypes.

Plants respond to biotic stress through JA biosynthesis and the activation of JA signaling (Santino et al. 2013). The JA signaling pathway is composed of two steps (Chini et al. 2007). The first step involves the formation of coronatine insensitive 1 (COI 1), containing Skip-Cullin-F-box (SCF) E3 ubiquitin ligase complexes, under stress. The SCFCOI1 complex activates TFs such as the basic-helix-loop-helix (bHLH) TF MYC 2 and ethylene response factor 1 (ERF 1) via ubiquitin-mediated degradation of the repressor JAZ (Boter et al. 2004; Lorenzo et al. 2003; Xu et al. 2002). The second step involves the regulation of JA-related genes by activated MYC 2 and ERF1 (Santino et al. 2013). JAZ genes are also transcriptional targets of MYC 2 TFs, and thus newly synthesized JAZ proteins regulate JA levels and repress other genes in the JA biosynthesis pathways based on a negative feedback regulatory loop

involving MYC 2 and JAZ. Among the common DEGs, we detected five JAZ 1 genes, which encode repressors of JA accumulation (Chung and Howe 2009). The BLP-resistant NIL had lower levels of JAZ 1 transcript than the BLP-susceptible NIL, indicating the presence of higher levels of JA in the BLP-resistant line. By contrast, in UV-B- resistant cultivar Buseok, all JAZ 1 genes were upregulated under UV-B treatment compared to Cheongja 3, resulting in reduced JA levels. In Nicotiana attenuate, specific UV-B radiation does not affect JA accumulation directly (Demkura et al. 2010). Moreover, UV RESPONSE LOCUS 8 (UVR8) and CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), which regulate UV-B-induced photomorphogenesis, do not induce the expression of genes controlled by JA (Favory et al. 2009; Jenkins 2009). These findings suggest that JA accumulation and JA signaling are necessary components for the activation of BLP disease resistance genes, whereas UV-B stress resistance genes appear to use other modes for JA accumulation.

MYC 2 as one of the regulatory modules in JA signaling also induces the up-regulation of NAC TFs (ANAC 055 and ANAC019 in A. thaliana) under biotic stress (Podzimska-Sroka et al. 2015). NAC TFs are plant-specific NAC domain-containing proteins that function in stress responses and development (Pinheiro et al. 2009). One of the 153 reported soybean NAC genes, GmNAC081 (Glyma.12G02540.1), is

induced by osmotic stress and functions in the programmed cell death response (Mendes et al. 2013). The up-regulation of NAC TFs induces the accumulation of PR proteins in plants after exposure to cold stress (Seo et al. 2010). The NAC 3 gene (Glyma.13G279900, synonym of ANAC055) was up-regulated in both Buseok and the BLP-resistant NIL, which was known to be a member of stress-responsive clade 3 of the abiotic stress-related GmNAC gene family (Le et al. 2011).

Another interesting result is that SAM 22 was up-regulated in Buseok and the BLP resistant NIL compared to the susceptible genotypes. SAM 22 is a well-known PR 10 protein that induces the hypersensitive response (HR) under both abiotic and biotic stresses such as wounding and treatment with SA, hydrogen peroxide, or sodium phosphate (pH 6.9) (Alkharouf et al. 2004; Hashimoto et al. 2004; Heath 2000). In addition, SAM22 is induced by SCN infection in resistant soybean (Crowell et al. 1992). We detected two paralogs of SAM 22, including one that overlaps with the abiotic stress-related QTLs CID1-2 and one that is localized near the biotic stress-related QTLs SCN 22-3 and phytoph 5-2. The finding that SAM 22 was up-regulated under UV-B treatment and Xag inoculation is consistent with the previous result that SAM 22 is an important resistance gene under both abiotic and biotic stress. Therefore, activation of NAC 3 under abiotic and biotic stress might induce the genes expression of PR proteins such as SAM 22,

subsequently leading to an increase in the HR (Figure II-4).

Many studies have been conducted on plant stress responses, but most have been limited to investigating the responses to individual stresses. The ongoing changes in plant growth conditions due to climate change emphasize the necessity of investigating the interactions of plant pathways for abiotic and biotic stress responses (Kissoudis et al. 2014). Major components of stress response regulatory pathways have been revealed by investigating current genomics data and performing functional characterization (Walley et al. 2007). We compared transcriptional changes from two different transcriptomic datasets under UV-B irradiation and Xag infection to detect common DEGs. Stress-related QTLs were located with the identified common DEGs and their paralogs. These results provide comprehensive information about the defense signaling mechanism that plants have evolved in response to abiotic and biotic stress, which could facilitate the development of highly adaptable soybean cultivars that can better cope with environmental changes.

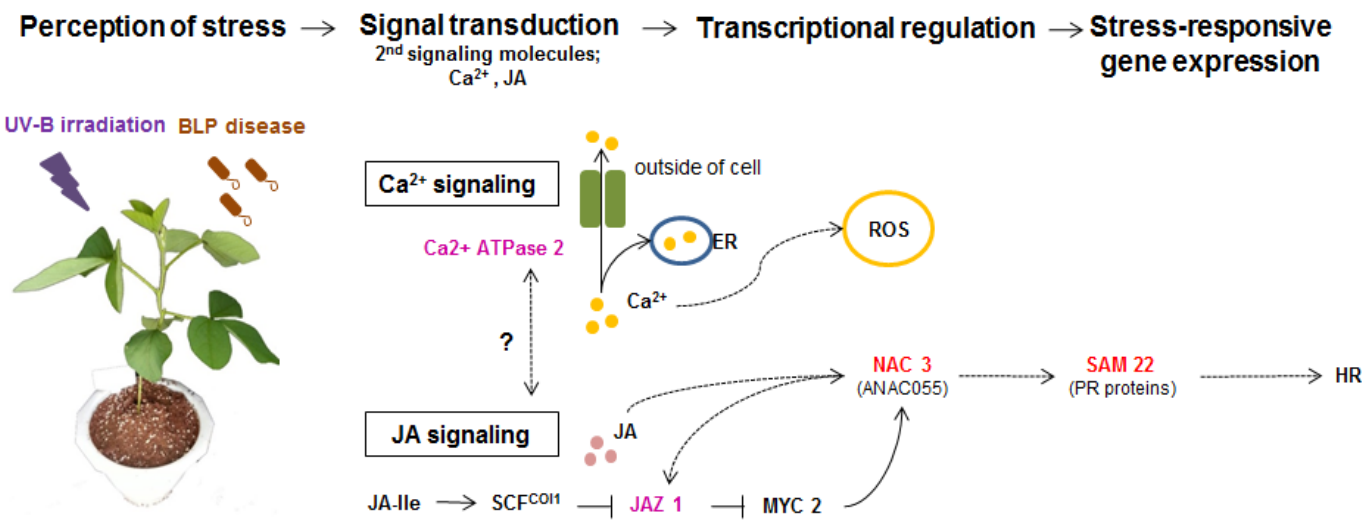

Figure II-4 Generic signaling pathway involved in UV-B stress and BLP disease responses. Ca2+ ATPase 2 and JAZ 1 (in pink) is up-regulated in UV-B- resistant Buseok but down-regulated in the BLP-resistant NIL. NAC 3 and SAM 22 (in red) are up-regulated in both of Buseok and the BLP-resistant NIL. ER; Endoplasmic reticulum, JA Ile: Jasmonoyl-isoleucine, SCFCOI1; coronatine insensitive 1 (COI 1) containing Skip-Cullin-F-box (SCF) E3 ubiquitin ligase complex. JAZ 1; Jasmonic acid ZIM domain-containing protein 1, NAC 3; NAM, ATAF1, ATAF2, and CUC2 transcription factor (TF) family 3, SAM 22; stress induced protein Starvation-associated message 22.

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CHAPTER III

QTL identification for UV-B resistance traits in soybean using genotype-by-sequencing

ABSTRACT

As one of the abiotic complex stressors, increased solar UV-B irradiation is induced by ozone depletion. Since the physiological damages and morphological changes are close to the reducing yields of soybean, it is important to investigate the responses in soybean under UV-B stress. Several studies tried to reveal quantitative trait loci (QTL) associated with resistance to supplementary UV-B treatment using soybean RIL population. However, QTLs and candidate genes responsible for UV-B stress resistance in soybean were still unclear. In the present study, we investigated phenotypic data of UV-B treated 174 F_6 RIL population derived from Cheongja 3 and Buseok and we tried to construct genetic map by Genotype-by-sequencing. High degree of phenotypic variations was shown in response to UV-B irradiation. Frequency distribution of leaf damage degree for UV-B treatments were ranged between 10 and 100%. The mean range of damage leaf degree was 50.3%, paternal UV-B resistance Buseok showed 26.8% damage degree, and maternal UV-B sensitive Cheongja 3 exhibited 62.4 damage degrees (%).Total high quality 2,291 SNPs were obtained and used for construction of genetic map using Joinmap 4.1. The newly detected two QTLs in this study are *UVBR14-1* and *UVBR17-1*. The one gene on chr 14 is K+ efflux antiporter 6 (KEA 6) (Glyma.14g093900) which may contribute towards K+ acquisition and homeostasis under saline conditions. The four candidate genes on chr 17

are Leucine rich repeat F-box/RNI-like superfamily protein (TIR1) (Glyma.17g247500), inositol polyphosphate 5-phosphatase 11 (Glyma.17g247600), Adaptor protein rnigma and related PDZ-LIM proteins (DA1) (Glyma.17g247700) and Leucine rich repeat receptor-like protein kinase (Glyma.17g247800). The biological function of KEA 6 on chr 14 and InsP5-ptase on chr 17 need to be examined more as UV-B responsible gene under UV-B light as multiple stressors including heat and hydration. It could be applied to breed high adaptable soybean under continuous climate changes in the future.

INTRODUCTION

As one of the abiotic stressors, increased solar UV-B irradiation is induced by ozone depletion. The ozone depletion with other climate factors such as wind patterns and warming may result in an increase of interaction between UV radiation and dehydration including drought and heat, which can cause physiological damage and morphological changes in plants as complex stressor (Bornman et al. 2015; Frohnmeyer and Staiger 2003; Lytvyn et al. 2010). The annual soybean (Glycine max (L.) Merr.) is one of the most important crops that were used as food, energy, and industrial resources worldwide. Under supplemental UV-B radiation condition, UV-B sensitive soybean showed the reduction of yield (Teramura et al. 1990). Since the physiological damages and morphological changes are close to the reducing yields of soybean, it is important to investigate the responses in soybean under UV-B stress (Baroniya et al. 2011, Rozema et al. 1997). There was a report that the comparisons of morphological, anatomical, and biochemical differences between UV-B-sensitive and -resistant soybean cultivars (Essex vs. Williams) in response to enhanced UV-B radiation (Murali et al. 1988). Recently, quantitative trait loci (QTL) associated with resistance to supplementary UV-B treatment were localized between Satt495 and Satt238 on chromosome 19 using a recombinant inbred line (RIL) population of Keunol (UV-B sensitive) x Iksan10 (UV-B resistant) (Shim et al. 2015). Also, there was study to identified UV resistance QTL using soybean SNP chip assay (Lee et al. 2016). They focused on the degree of leaf chlorosis, leaf shape and total plant damage of F8 RILs derived from Keunol x Iksan10 after UV-B treatments and narrowed down potential candidate gene of qUVBT1 QTL on chr 7. However, QTLs and candidate genes responsible for UV-B stress resistance in soybean were still unclear.

In recent years, next generation sequencing (NGS) methods were hired to whole genome sequencing and transcriptome at a substantially reduced cost and higher accuracy (Grada and Weinbrecht 2013; Shendure and Ji 2008). These increased efficiency and accessibility allow construct high resolution density maps and genetic diversity analysis on cultivars, landraces, and wild species (Van et al. 2013). Based on the primary objectives of functional genomics in agriculture, which are to connect phenotype and genotype and to make phenotypic prediction, genotyping-by-sequencing (GBS) has been developed as a rapid and robust approach for reduced-representation sequencing of multiplexed samples that combines genome-wide molecular marker discovery and genotyping (Poland and Rife 2012). This GBS, which uses enzyme-based complexity reduction coupled with DNA barcoded adapters to produce multiplex libraries have been applied to soybean for NGS sequencing (Iquira et al. 2015). A procedure for constructing GBS libraries based on reducing genome complexity with methylation-sensitive restriction enzyme (RE). By using methylation sensitive RE, repetitive regions of genome can be avoided and lower copy regions targeted with two to three fold higher efficiency (Elshire et al. 2011).

In the present study, we investigated phenotypic data of UV-B treated F6 RIL population between Cheongja 3 and Buseok and we tried to construct genetic map by GBS. These results can be compared to previous soybean study in response to UV-B irradiation using RNA-seq to identify UV-B resistant genes in soybean. Furthermore, the results of this study will help to elucidate the UV-B related QTLs of soybean and to develop high adoptable soybean breeding under UV-B irradiation.

MATERIAL AND METHODS

Plant materials

In this study, two soybeans were used as parents to develop a RIL population for genetic mapping and QTL analysis: Buseok was UV-B resistance soybean and Cheongja 3, the maternal parent, was UV-B sensitive soybean based on previous study (Kim et al. 2015). Crosses were performed in summer 2012 and 176 F6 RILs were generated from F2 seeds using single seed descendent (SSD) methods from winter 2012 to spring 2016. In August 2016, these soybean seeds were planted (4 seeds per pot) in 3 liter pots containing a 1:1 mixture of desalinated sand and commercial potting soil (Baroker, Seoul Bio Co., Ltd., Korea) in a greenhouse at experimental farm of Seoul National University, Suwon, Korea. Two to three weeks after emergence (V3-V4 soybean growth stages), supplemental UV-B radiation was treated to soybean population following previous study (Kim et al. 2015).

UV-B treatments and phenotypic evaluation

Supplemental UV-B radiation was provided by G40T10E UV-B lamps (Sankyo Denki, Japan) with 5.68±0.4 Wm-2 UV-B intensity. We investigated the phenotypic data of plant leaf samples which were collected after 4 days UV-B treatments (every 1 hour UV-B treatment at 11:00 AM from 17th August to 20th August). Plant leaf samples were collected three sets of unfolded trifoliate leaves (first and second trifoliate leaves above unifoliate leaves) from three replications of one line. To investigate leaf color changes and damages, the collected leaf samples were applied to WinDIAS 3 leaf image analysis

system (DELTA-T DEVICES LTD, UK). The degree of damage was scored on a scale of 1-10 [where 1=10%; damage / healthy x100 (%)].

Construction of GBS library and sequencing

To construct GBS library, young leaves from each of parents and RILs were collected and high quality gDNA were extracted by GeneAll® ExgeneTM Plant SV kit (GeneAll Biotechnology, Co., Ltd, Korea). Genomic DNA was quantified using Nanodrop 3000 spectrophotometer (Thermo Scientific, Wilmington, DE) and concentrations were normalized for library preparation. We followed steps based on GBS protocol described by Elshire et al. (2011). DNAs from the parents and progeny genotypes were digested individually with ApeKI restriction enzyme (RE), which recognizes a degenerate five base-pair sequence (GCWGC, where W is either A or T). Total 88 barcode adapters were applied to RE digested DNA fragments. Finally a total two ApeKI GBS libraries were constructed and each library includes 88 DNA samples. Single-end sequencing was performed on two lanes of an Illumina HiSeq2000 (at Macrogen, Korea).

Genetic map construction and QTL identification

The raw GBS data were applied to Bowtie v2.1 for alignment of sequenced reads with soybean reference genome (Langmead et al. 2012; Glaubitz et al. 2014). Two sequenced libraries produced total 254,777,458 and 250,611,830 reads, respectively, of which 78.4 % were good, barcoded reads. A total 271,000 ‘raw’ SNP and InDel were identified by variant calling in-house python scripts based on read depth, missing data in genotypes. After strict filtering of SNPs on the basis of reads depth, a final set of high quality 2,291

SNPs were obtained and used for construction of genetic map. The SNPs data were converted to the ‘a, b, h’ codes and genetic mapping of 138 F6 RIL population was performed using Joinmap ® 4.1 (https://www.kyazma.nl/index.php/mc.JoinMap). SNP markers were grouped using the Kosambi’s mapping function in Joinmap ® 4.1. Marker order and distance were calculated using the maximum likelihood mapping function with default mode. Segregation distortion of individual markers was calculated using X2 test in Joinmap ® 4.1. The QTLs for UV-B resistance were detected by inclusive composite interval mapping in 138 RILs derived from Cheongja 3 x Buseok for the trait degree of leaf damage using ICIMapping software (https://www.integratedbreeding.net/386/breeding-services/more-software-tools/icimapping). To determine QTL significance thresholds, a permutation test with 1,000 replications was performed with a logarithm of odds (LOD) value for each QTL in the range of 2.0–3.0 with 99% confidence.

RESULTS

Phenotype data analysis

From the parents and RILs generated by SSD from a cross between Cheongja 3 and Buseok, high degree of phenotypic variations was shown in response to UV-B irradiation. Frequency distribution of leaf damage degree for UV-B treatments were ranged between 10 and 100% (Figure III-1). The mean range of damage leaf degree was 50.3%, paternal UV-B resistance Buseok showed 26.8% damage degree, and maternal UV-B sensitive Cheongja 3 exhibited 62.4 damage degrees (%).

QTL identification for UV-B resistance

There were no reported QTLs related with UV-B resistance on chr 14 and chr 17 in soybean. The map positions and characteristic of QTLs identified are shown in Table 1. The newly detected two QTLs in this study are UVBR14-1 and UVBR17-1. The QTL (UVR-14-1) controlling resistance to UV-B in Buseok on Chr 14 was associated with the interval of marker (Chr14_8683294-Chr14_8806046) and had an LOD score 2.3 with 7.1 % of the phenotype variance. The interval of marker (Chr17_40197341-Chr17_40412430) carried another QTL (UVR-17-1) on Chr 17 with an LOD score 2.9 and 8.4 % of the phenotype variance (Table 1).

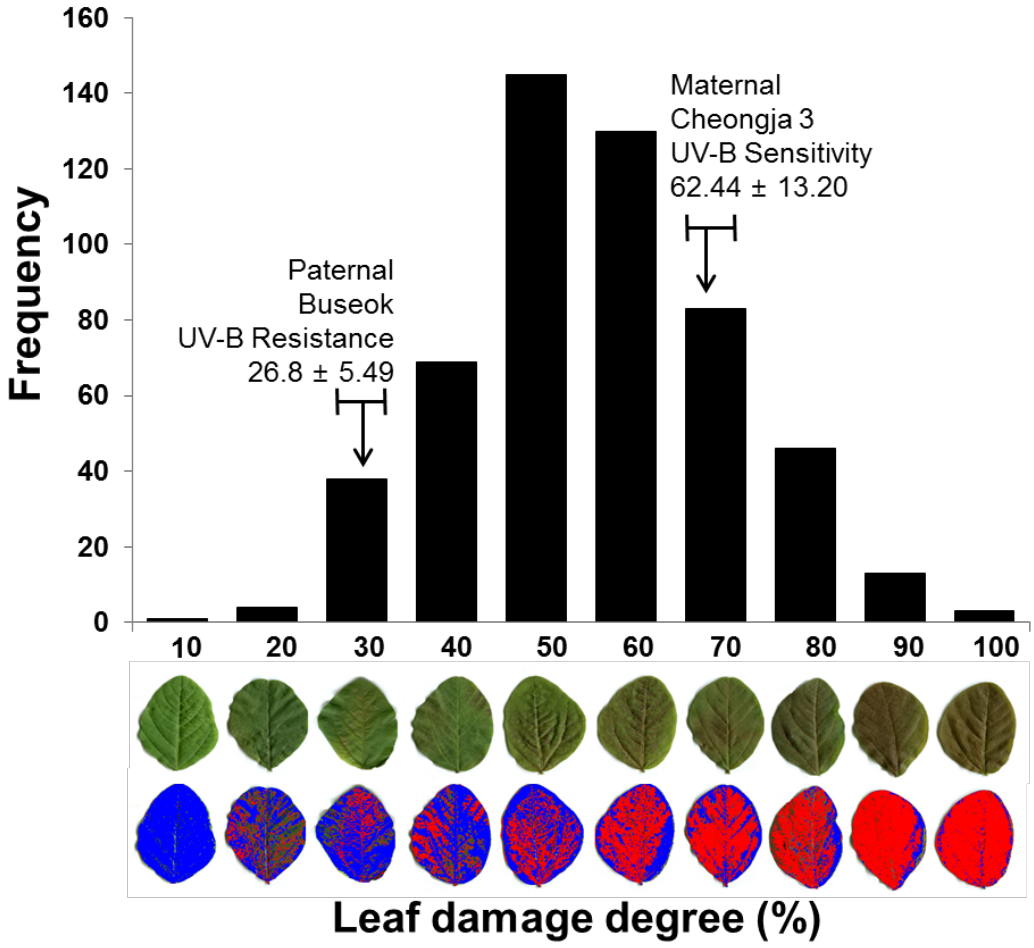

Figure III-1 The frequency of leaf damages under UV-B irradiation.

Table III-1 The major QTL for UV-B resistance identified by inclusive composite interval mapping in 138 RILs derived from Cheongja 3 x Buseok for the trait degree of leaf damage

Trait	Chromosome	Locus	Left Marker	Right Marker	Position[a] (cM)	LOD[b]	Add[c]	R^{2d} (%)	Number of gene
UV	14	*UVR-14-1*	Chr14_8683294	Chr14_8806046	5	2.3	-0.3	7.1	1
	17	*UVR-17-2*	Chr17_40412430	Chr17_40197341	3.8	2.9	-0.4	8.4	4

[a] Peak position of QTL in the linkage map developed in the present study

[b] Maximum-likelihood LOD score for the individual QTL

[c] The allelic genetic effect

[d] Phenotypic variance explained by the QTL

Candidate genes for UV-B resistance in soybean

The physical distances between markers of UVBR14-1 and UVBR17-1 are 122 kb and 215kb, respectively (Figure III-2). There are only one annotated gene on chr 14 and 4 genes were anchored on chr 17 according to the soybean reference genome database (soybase, https://www.soybase.org/) (Table III-2). The one gene on chr 14 is K+ efflux antiporter 6 (KEA 6) (Glyma.14g093900) which may contribute towards K+ acquisition and homeostasis under saline conditions (Cellier et al. 2004; Shabala and Cuin 2008). The four candidate genes on chr 17 are Leucine rich repeat F-box/RNI-like superfamily protein (TIR1) (Glyma.17g247500), inositol polyphosphate 5-phosphatase 11 (Glyma.17g247600), Adaptor protein rnigma and related PDZ-LIM proteins (DA1) (Glyma.17g247700) and Leucine rich repeat receptor-like protein kinase (Glyma.17g247800) (Table III-2).

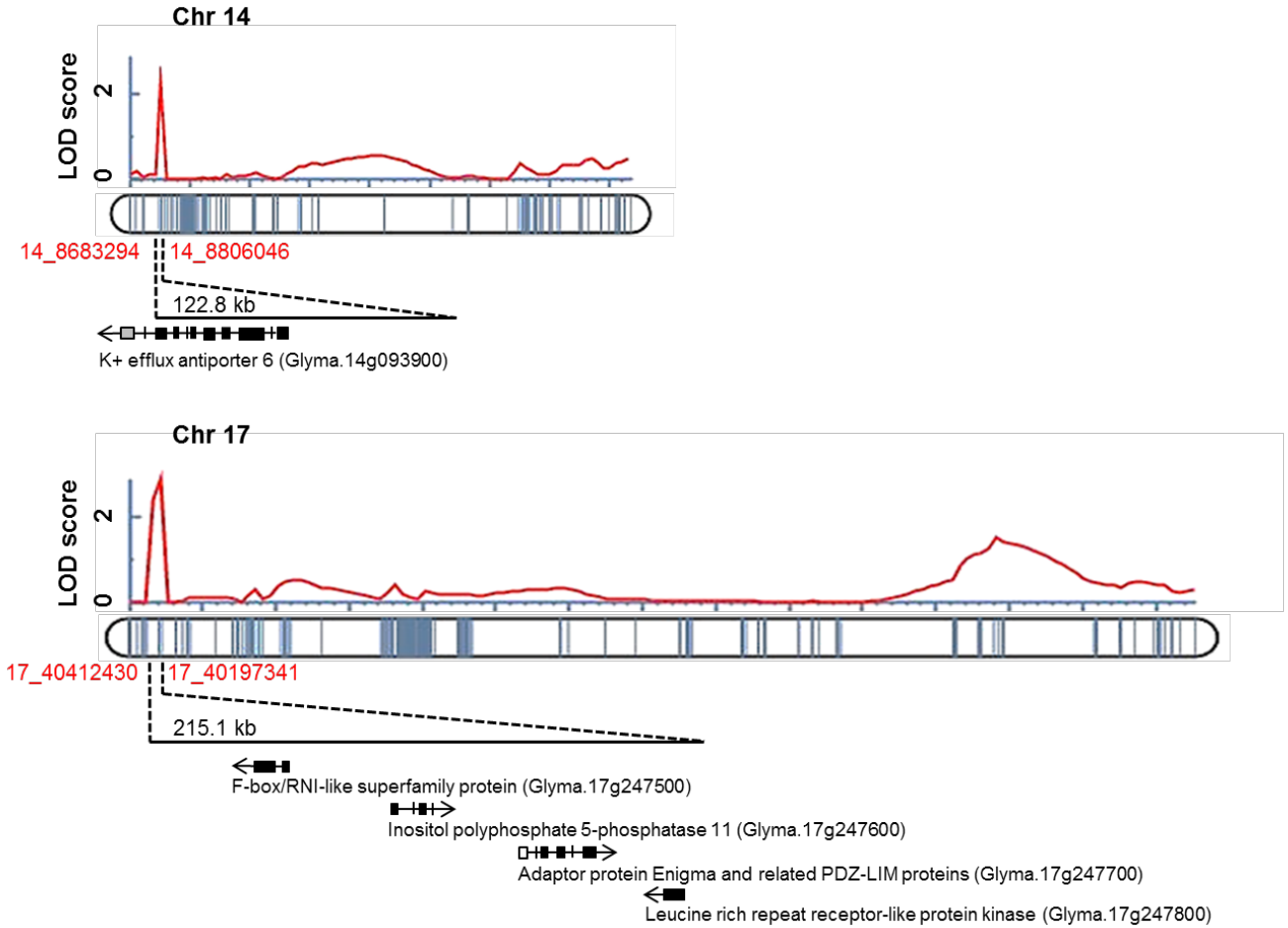

Figure III-2 The QTL regions and candidate genes responsible for UV-B resistance

Table III-2 Candidate genes responsible for UV-B resistance on QTL regions on chr 14 and 17.

Gene ID	Gene position	Gene annotation	A.Thaliana ID
Glyma.14g093900	Gm14:8732611..8743843	K+ efflux antiporter 6 (Potassium/proton antiporter-related)	AT5G11800.1
Glyma.17g247500	Gm17:40293548..40296483	Leucine rich repeat (F-box/RNI-like superfamily protein)	AT1G13570.1
Glyma.17g247600	Gm17:40301281..40304976	inositol polyphosphate 5-phosphatase 11 (Inositol polyphosphate 5-phosphatase and related proteins)	AT1G47510.1
Glyma.17g247700	Gm17:40307962..40314691	Adaptor protein Enigma and related PDZ-LIM proteins (DA1)	AT1G19270.1
Glyma.17g247800	Gm17:40315006..40316907	Leucine rich repeat receptor-like protein kinase	AT2G23770.1

Discussion

This study employed a QTL mapping approach using GBS to examine the UV-B resistance trait in soybean. To identify the genetic elements responsible for UV-B resistance in soybean, we used UV-B resistance soybean Buseok, which was shown less damaged rate and lowest changes of dry weight in previous study (Kim et al. 2015). We also developed an F6 RIL population of Cheongja 3 (UV-B sensitive cultivar) x Buseok and observed wide range of phenotypic variation under UV-B irradiation. The incidences of UV-B in frequency distribution indicate that there is quantitative regulation of resistance to UV-B in Buseok by two or more major genes (Figure III-1).

In this study, total 5 candidate genes for UV-B resistance are anchored on two QTL regions. One of the candidate genes is inositol polyphosphate 5-phosphatase (InsP5-ptase) which can be divided to two types. One is mammalian type I InsP5-ptase specifically hydrolyzes soluble inositol phosphates and thus, interferes InsP3 and InsP6 signaling in Arabidopsis thaliana (Barbaglia and Hoffmann-Benning, 2016; Perera et al., 2008). InsP5-ptase acts as negative regulators in plant cell through the reduction of the Ca2+ signaling following salt and cold-stress. However, there was a result that InsP5-ptase is closely related with guard cell opening under drought stress condition (Perera et al., 2008). InsP5-ptase control the opening the guard cell via increased sensitivity of abscisic acid (ABA) induced closure. The other one is type II InsP5-ptase which regulates the level of phosphoinositides. FRAGILE FIBER3 (FRA3) gene of Arabidopsis thaliana, which encodes a type II InsP5-ptase, plays an essential role in the secondary wall synthesis in fiber cells and xylem vessels (Zhong et al., 2004). Detected InsP5-ptase (Glyma.17g247600) in our study might responsible gene for drought stress under UV-B irradiation with heat.

The potassium (K+) is abundant cation in cytosol which has important functions such as osmoregulation, electrical neutralization (Very and Sentenac, 2003). Six genes named KEA1 to KEA6 encoding putative K+/H+ exchangers of the cation proton antiporters 2 (CPA 2) family, which are actually cation/proton antiporters have been identified in Arabidopsis thaliana (Mäser et al., 2001). In plants, these might play an important role in K+ homeostasis by K+ loading into the vacuoles or other acidic compartments. Another study suggested that at least one member of CPA2 family: the root and leaf-expressed AtCHX17, may contribute towards K+ homeostasis under saline conditions, as its transcript level increases under salinity (Cellier et al., 2004; Maathuis, 2006). Recent study of Arabidopsis thaliana, KEA3 localized in the thylakoid membrane was reported as key component for photosynthetic efficiency under fluctuating light condition (Armbruster et al. 2014). They suggested that the KEA 3 is correlated with photosynthesis related genes and accelerates photosynthetic acclimation from high to low light condition. The biological function of KEA 6 on chr 14 need to be examined more as UV-B responsible gene related with photosynthesis and osmotic regulators under UV-B light as multiple stressors including heat and hydration.

In this study, we tried to identify the major QTLs responsible for UV-B resistance in soybean. To identify the major QTLs, removed low reads GBS data in our results need to be added for further study. More functional validation for 5 candidate genes will elucidate UV-B resistance in soybean and it could be applied to breed high adaptable soybean under continuous climate change in the future.

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국문초록

성층권의 오존층의 감소는 자외선 (UV-B)의 극적인 증가를 유도한다. 강한 세기의 UV-B의 직접적인 흡수는 식물에의 강한 빛의 투사, 수분 증발 등의 복합적인 비생물학적 스트레스로서 작용된다. 그러나 콩을 포함하는 두과 작물에의 UV-B에 대한 신호 전달 메커니즘 및 자외선 저항성 관련 유전자 좌 및 유전자에 대한 내용은 아직 많은 부분이 미흡하다. 먼저, 본 논문에서는 UV-B에 저항성을 보이는 재래종인 부석과 감수성을 보이는 청자 3호의 전사체 반응을 살펴 보기 위해서 비처리 (0), 0.5시간 그리고 6시간의 UV-B 처리를 진행한 후 전사체 분석 (RNA-seq)을 수행하였다. 애기장대와의 상동분석 (homology analysis)를 통해 UV-B 스트레스에 반응하는 발현적 차이를 보이는 유전자 (Differentially expressed genes; 이하 DEGs) 들을 동정하였고, 이러한 DEG들이 면역 반응, 스트레스 신호, 활성 산소 (ROS) 메커니즘에 두드러지게 포함됨을 확인하였다. UV-B 저항성인 부석에서는 Phosphatidic acid (PA) 신호 전달 메커니즘에 포함된 유전자들인 Phospholipase C (PLC)와 Diacyglycerol kinase (DGK)가 자외선에 유의미하게 발현 됨을 확인하였고, 이는 이어지는 ABA 신호 전달, mitogen-activated protein kinase (MAPK) 반응 기작, 그리고 ROS 과생산 기작에 대해 영향을 미치는 것을 확인 하였다. 또한 많은 DEG들 중에 TIR-NBS-LRRs 과 Heat shock protein 이 과 발현됨을 확인 하였다. 이러한 결과들은 낮은 수준의 자외선에의 적응하는 광 형태형성 반응 (photomorphogenic response)과는 차이를 보이는 강한 UV-B에의 방어기작으로 설명될 수 있다.

더 나아가서, 생물학적, 그리고 비 생물학적 스트레스 환경에서 콩은 다양한 신호 기작의 조절을 통해 적응해왔다. 비록 여러 기존 연구들이 식물의 방어기작을 연구해 왔지만, 생물학적, 그리고 비생물학적인 스트레스에의 신호 전달 기작의 접점에의 주요인자들에 대해서는 아직 미흡한 것이 사실이다. 본 논문의 두번째 연구는 앞서 설명한 UV-B에의 저항성인 부석, 감수성인 청자 3호를 통해 비 생물학적 스트레스에 대한 전사체 분석을 다루었고, 또한 불마름병을 알려진 bacterial leaf pustule (BLP)에 저항성과 감수성 대립형질 (alleles)을 갖는 NILs을 통하여 생물학적 스트레스에 대한 전사체 분석을 함께 다루었다, 두 종류의 전사체 분석을 비교

함으로써, 생물학적, 그리고 비생물학적 스트레스에 공통적으로 반응하는 유전자들을 살펴 보았고, 더 나아가 발현의 차이를 보이는 DEG들과 co-localization 양상을 보이는 quantitative trait loci (QTLs) 분석을 진행하였다. 그 결과, 두 스트레스에 공통적으로 발현하되 저항성과 감수성 사이의 차이를 보이는 14개의 DEG들이 동정하였다, 그 중 5개의 유전자는 Jasmonic acid (JA) 신호 전달기작의 조절유전자인 Jasmonate ZIM (Zinc-finger protein expressed in Inflorescence Meristem) domain-containing protein 1 (JAZ 1)이며, 이 JAZ1은 생물학적 스트레스 관련 QTLs과 co-localization 됨을 확인 하였다. stress-induced protein starvation-associated message 22 (SAM 22) 인코딩하는 한 개의 DEG의 paralog는 생물학적, 그리고 비생물학적 스트레스 관련 QTLs 에 교차하여 위치 하고 있음을 확인하였다.

세 번째 논문 내용으로는 청자 3호와 부석사이의 교배를 통해 얻은 F6 174 RIL 집단을 이용하여 자외선 처리를 한 표현형 조사를 진행 하였으며, 동시에 자외선 저항성과 관련있는 유전자좌 (QTL)을 알아보기 위해 GBS 를 이용한 genetic map을 구축하였다. 자외선 처리에 대하여 집단 내에서의 큰 표현형적 차이를 보였고, 차이의 정도를 10-100% 단위로 분류하였다. 평균 피해 정도는 50.3%를 나타내었으며, 저항성 부본 부석의 경우에는 26.8% 그리고 감수성 모본인 청자 3호의 경우에는 62.4%의 피해 정도를 확인 할 수 있었다. GBS 분석 결과 전체 2,291개의 SNP들을 확보하여 genetic map을 구축하였으며, UVBR14-1 와 UVBR17-1 QTL을 동정하였다. 후보 유전자로서 K+ efflux antiporter 6 (KEA 6) (Glyma.14g093900)가 14번 염색체에 존재하고, inositol polyphosphate 5-phosphatase 11 (Glyma.17g247600)을 포함하는 4개의 후보 유전자들이 17번 염색체에 존재함을 확인하였다. 위의 두 후보유전자들의 기능적 확인을 통하여 보다 자외선 저항성 관련 유전자로서의 탐색이 향후 과제로 남아 있으며, 이를 이용하여 지속적으로 증가 유입되는 자외선에 높은 적응성을 보이는 대두 육종으로의 방향을 기대할 수 있을 것이다.

주요어: 자외선B (UV-B), 불마름병, 대두 전사체 분석, Phosphatidic acid, Diacylglycerol kinase, TIR-NBS-LRRs, JAZ 1, SAM 22, 공통 신호 전달 기작, KEA 6, InsP5-ptase

학번: 2011-30328