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Glyphosate effects on the gene expression of the apical bud in soybean (*Glycine max*)

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ABSTRACT

Glyphosate is a broad spectrum, non-selective herbicide which has been widely used for weed control. Much work has focused on elucidating the high accumulation of glyphosate in shoot apical bud (shoot apex). However, to date little is known about the molecular mechanisms of the sensitivity of shoot apical bud to glyphosate. Global gene expression profiling of the soybean apical bud response to glyphosate treatment was performed in this study. The results revealed that the glyphosate inhibited tryptophan biosynthesis of the shikimic acid pathway in the soybean apical bud, which was the target site of glyphosate. Glyphosate inhibited the expression of most of the target herbicide site genes. The promoter sequence analysis of key target genes revealed that light responsive elements were important regulators in glyphosate induction. These results will facilitate further studies of cloning genes and molecular mechanisms of glyphosate on soybean shoot apical bud.

1. Introduction

Glyphosate is one of the most widely used nonselective herbicides in the world. Glyphosate inhibits5-enolpyruvylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) in the shikimic acid pathway, which is essential for the biosynthesis of aromatic compounds (tryptophan, phenylalanine, tyrosine) in plants [1]. In soybean, our research showed that the shoot apical bud was the most sensitive organ which progress in severe chlorosis and it could not recover at the early stage of glyphosate treatment in common cultivars. But for resistant ecotypes, the phenomenon of "yellow flash" on shoot apical bud and young leaves have been observed which were transitory, and the plants could recover from damage [2].

In recent years, the physiological and biochemical mechanisms of glyphosate, including genes involved in the shikimate acid pathway, have received extensive attention. However, little is known about the molecular and gene transcriptional responses toglyphosate, except for the well-known resistance/tolerance to glyphosate provided by the overexpression, amplification or mutation of *EPSPS* gene [3,4]. Currently, *EPSPS* (*Agrobacterium* sp. Strain CP4) which applies the mechanism of glyphosate target resistance gene is being used successfully in the commercial crops. Several other studies showed that the *GAT* (glyphosate N-acetyl transferase gene) [5], *GOX* (glyphosate oxidoreductase) [6] and *Escherichia coli yhhS* [7] genes, which were involved in glyphosate metabolism and degradation, could also confer glyphosate resistance. Most of the research on the molecular mechanisms of glyphosate on plants by using microarray technology including studies on *Arabidopsis thaliana*, *Brassica napus*, *Festuca arundinacea*, and *Glycine max,* but only recognized the genes already utilized in gene chip [8–10]. In recent years, next-generation sequencing-based digital gene expression tag profiling (DGE) has been widely applied in high throughput identification of differentially expressed genes. Compared with microarray, DGE gave high percentage of transcripts as microarray, but could detect more scare transcripts. Consistent analysis also showed that DGE had excellent correlation with qRT-PCR [11,12].

Cis-Elements in the upstream regions of genes play an important role in gene expression at the transcription level. Since glyphosate acts as an inducer which is capable of directly or indirectly activating transcription of genes, some *cis*-Elements which are known to be involved in gene transcription regulation in responding to glyphosate could be used to identify genes of interest during glyphosate inducing process. Therefore, upsteam region

Abbreviations: cDNA, complementary deoxyribonucleic acid; FC, fold change; FDR, false detection rate; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; DGE, digital gene expression tag profiling; 26 DGEs, 26 differentially expressed cross talk genes at three time points; 12 SHAPs, 12 genes participated in shikimate acid pathway.

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analysis of glyphosate inducilble genes could provide further information for the gene function analysis.

The molecular mechanisms of glyphosate treatment on soybean shoot apical bud in various stages are still unknown. In order to study how glyphosate is affecting specific gene expression and related pathways in soybean shoot apical bud, DGE was used to study the changes of gene expression. These results provided insights on the transcription level of the roles for the key genes in the process of glyphosate defense. Analysis of genes expression level and identification of the gene regulated pathways also provided a better understanding of the action and mechanism of the sensitivity of soybean shoot apical bud to glyphosate.

2. Materials and methods

2.1. Plant materials and glyphosate treatment

Soybean seeds (variety Zhonghuang 13) were germinated and grown in soil. The seedlings were grown for 30 days until the three trifoliate leaves were fully developed, and then treated with 2.10 a.i.kg·ha^{-1} glyphosate (Monsanto). The apical buds of 20 uniform plants were harvested separately before (CK) and after 6, 24, and 72 h of treatment (6, 24, and 72hpt). The plant tissues were frozen in liquid nitrogen and conserved under −80 °C.

2.2. Solexa/Illumina sequencing and gene annotation

Four libraries (CK, 6, 24, and 72 hpt) were constructed and used for sequencing in BGI–Shenzhen (http://www.genomics.cn/index). DGE tags were aligned to gene models predicted from the soybean reference genome of cv. Williams 82 available from Phytozome v7.0 (http://www.phytozome.net/).

2.3. Real-time quantitative RT-PCR (qRT-PCR) analysis

The soybean 18S RNA gene (GenBank Accession: M16859.1) was used to normalize gene expressions. Primers of candidate genes for qRT-PCR were listed in Additional Table S1. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method [13].

2.4. Promoter analysis of differentially expressed genes

1500 bp Upstream sequences from translational start sites of 26 differentially expressed genes (26 DGEs) at three time points and 12 genes participated in shikimate acid pathway (12 SHAPs) were analyzed using the PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) databases.

3. Results

3.1. Digital gene expression tag profiling (DGE) library sequencing

DGE analysis was performed to identify genes of soybean shoot apical bud involved in glyphosate treatment. Based on phenotyping, progressive symptoms were observed after 6, 24, and 72 hpt of glyphosate application. In total, about 3.5–3.8 million raw tags were generated for each of the four samples. After the transformation of raw sequences into clean tags, the total number of tags per library ranged from 3.3 to 3.6 million and the number of tag entities with unique nucleotide sequences ranged from 106,908 to 126,709 (Additional Table S2).

Heterogeneity and redundancy are two significant characteristics of mRNA expression. Categories designated by smaller numbers had a very high abundance of mRNA, while the majority of the tags were expressed at very low levels. The distribution of clean tag expression can be used to evaluate the normality of the whole data. The majority of the total clean tags were high-expression tags with copy numbers larger than 100, but low-expression tags with copy numbers smaller than five occupy the majority of tag categories. The distribution of total and distinct tags over different tag-abundance categories was similar for all four DGE libraries (Additional Fig. S1).

3.2. Identification, functional annotation and clustering of differentially expressed genes

Glyphosate induces strong gene responses and changes in soybean shoot apical bud expression profiles as shown by transcriptional analysis. Genes showing ⩾twofold changes were extracted for further analysis. Using these criteria, a total of 6413 genes (2661 genes up-regulated and 3752 down-regulated) showed evidence of changes in transcription after glyphosate treatment (P value < 0.01, variation coefficient was 0.1). Glyphosate treatment induced severe symptoms in plants and, as might be expected, gene expression changes increased over time after treatment (Fig. 1). As a control, the expression of 14 house-keeping genes in soybean was investigated [14] and all were evenly expressed ($p > 0.05$) (Additional Table S3), which indicated that the coefficient of variation was appropriate. To validate the expression profiles obtained throughDGE analysis, the expression of 16 consistently differentially expressed genes was confirmed by quantitative real time RT-PCR (qRT-PCR). The gene expression detected by qRT-PCR correlated well with that of DGE analysis (Additional Table S1).

Of all the differentially expressed genes, glyphosate treatment significantly affected 26 genes across the three time points, of which 18 were up-regulated and eight were down-regulated (Fig. 1; Additional Table S4). Genes that are differentially expressed in the glyphosate-treated plants over the entire time courses as compared to controls are likely to be genes involved in basal defense mechanisms.

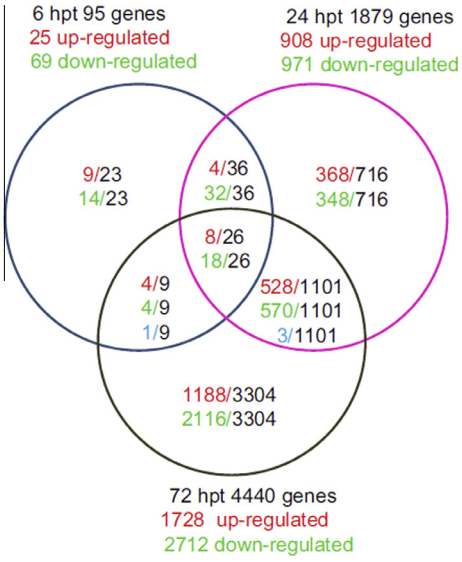

Fig. 1. A Venn diagram of differentially expressed genes from three treated samples (6, 24, and 72 hpt). Gene number significantly differentially expressed in comparison with the control was shown. Red and green colors represented up-regulated and down-regulated genes, respectively. Blue color represented differential gene expression found in only one time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cluster analysis of the expression profiles of all of the differentially expressed genes showed time-specific patterns. By average linkage of hierarchical cluster analysis, ten clusters of gene expression profiles could be defined using DGE data (Fig. 2). Cluster 5 contained the highest number of differentially expressed genes (1281), followed by cluster 7 (784), and cluster 8 (743). There were diverse patterns of differential gene expression in response to glyphosate application. These results indicated the complexity of the molecular responses within the soybean shoot apical bud to glyphosate, or these genes might play various roles in response to glyphosate and/or general stress physiology.

GO terms defines the functional roles of the genes and the pathways in which they might be involved. There were 28 GO terms overrepresented (Fisher's adjusted $p < 0.05$) in genes with high levels of expression specificity (15 for "biological process", five for "cellular component" and eight for "molecular function"; Additional Fig. S2, S3 and S4). Among these GO terms, metabolic process, cell, and catalytic activity were the largest categories with 348, 376, and 313 members respectively. These indicated that glyphosate could induce a large number of enzymes involved in basal metabolic pathways.

Furthermore, to identify the biological pathways in the glyphosate-treated apical bud of soybean, the annotated sequences were mapped to the reference canonical pathways in KEGG. Thirteen KEGG pathways were significantly enriched with differentially expressed genes due to glyphosate treatment (Additional Table S5). The pathways represented by the highest number of unique sequences were 'metabolism pathways' (462 members), 'biosynthesis of secondary metabolites' (262 members), and 'amino sugar and nucleotidesugar metabolism' (46 members). In our study, the transcription of genes involved in flavones and isoflavones biosynthesis were also influenced by glyphosate, which was previously reported in the leaf and root of soybean response to glyphosate [10].

3.3. Effects of glyphosate on herbicide target-site genes and shikimate acid biosynthetic pathway

Inhibiting a single biosynthetic pathway can specifically induce expression of genes not only in that pathway but also in cross-pathway metabolic regulation [15]. The mechanisms of herbicide-resistance can be target site based or non-target site based [16]. The modes of action corresponding to the herbicides mechanisms include the microtubule system,synthetic auxins, carotenoid biosythesis, cellulose biosynthesis, lipid synthesis, chlorophyll, photosystem I (PSI), photosystem II (PSII), acetolactate synthase (ALS), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), 4-hydroxyphenylpyruvate dioxygenase (HPPD), phytoene desaturase (PDS), and glutamine synthetase (GS), etc. These herbicide target sites have close relation (Fig. 3).

In the current study, the impact of glyphosate on the shikimate pathway is also extended to other related pathways and differentially expressed genes related to herbicide target enzymes genes. Herbicide target site genes include those related to inhibition of amino acid synthesis such as *ALS*, *GS*, *ASNS*, *DHPS* and eleven of twelve tubulin (with the exception of *Glyma16g04420*) genes were all down-regulated at 24 hpt (Additional Table S6). The PDS protein has been the main target for herbicides that inhibit the carotenoid biosynthetic pathway and participates in light harvesting. The *PDS* gene *Glyma14g03410* was up-regulated in soybean apical bud at 24 hpt and 72 hpt in response to glyphosate. At the transcriptional level, among the 18 differentially expressed genes related to photosynthesis, 15 genes were down-regulated in response to glyphosate at 24 hpt (Additional Table S6). Besides the genes involved in the light reactions, one gene (*Glyma03g26740*) encoding protochlorophyllide oxidoreductase A (PorA) was also down-regulated.

Auxin is critical for plant growth and developmental processes. Indole-3-acetic acid (IAA) is recognized as the key auxin which issynthesized both from tryptophan and indolic tryptophan precursor. Auxin responses are initiated through activation of a transcriptional response mediated by the TIR1/AFB family as well as the AUX/IAA and ARF families of transcriptional regulators [17]. Differential expression of genes involved in tryptophan and auxin metablism pathways were observed. Seven genes related to auxin response factor (ARF) were found and three of them were up-regulated. Proteins in the auxin-responsive family (AIR) were also influenced by glyphosate and five genes were differentially expressed. Two IAA genes were regulated by glyphosate. Fifteen

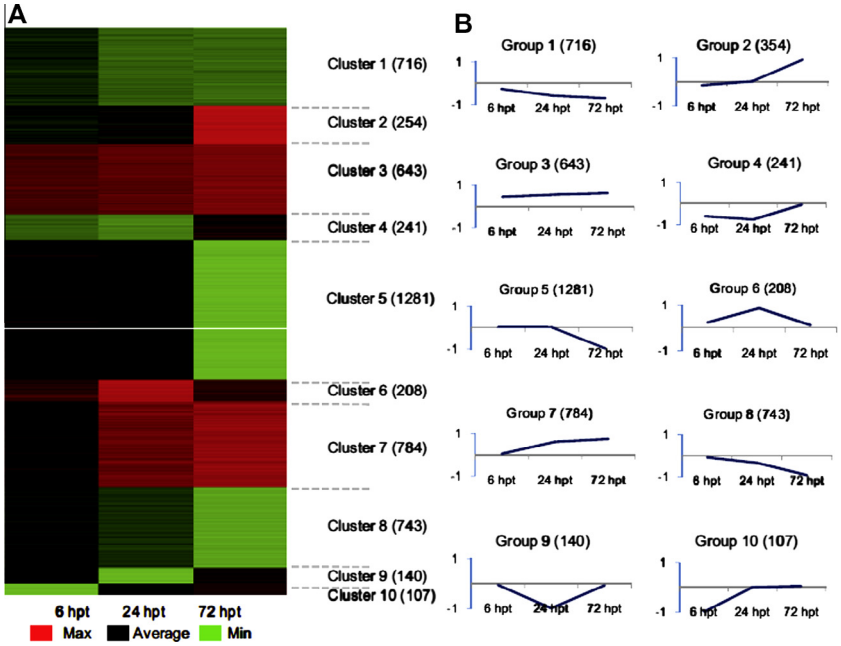

Fig. 2. Expression mode of differential expression genes. (A) Clustered expression profiles of 5,215 genes taken 2.0-fold cutoff criterion from 6 hpt to 72 hpt. (B) Mean expression values of genes (log 2) located in the defined clusters.

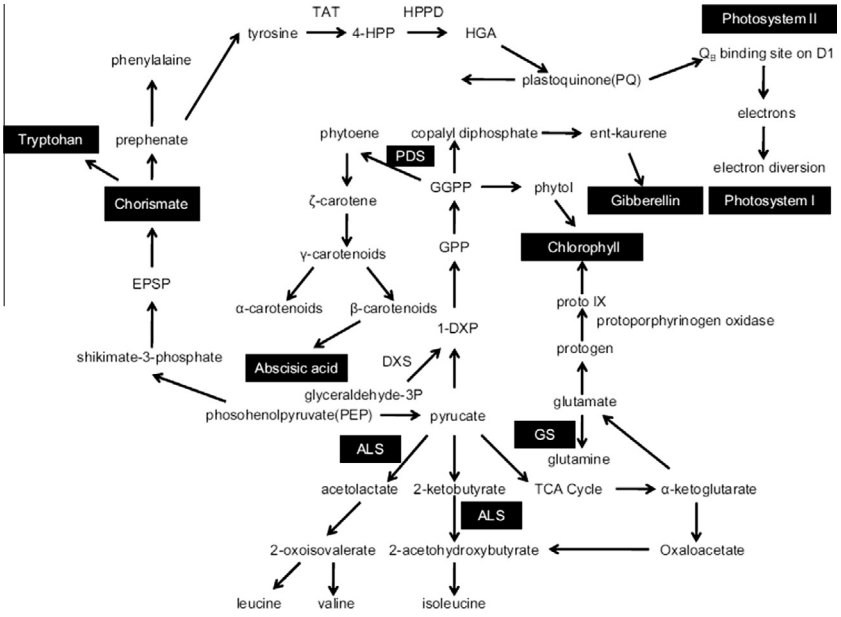

Fig. 3. Illustration summarizing the relationship between herbicide mechanisms of action. The pathway is obtained and intergrated from references [6,26]. TAT, tyrosine aminotransferase; HPPD, p-hydroxyphenylpyruvate dioxygenase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GS, glutamine synthetase.

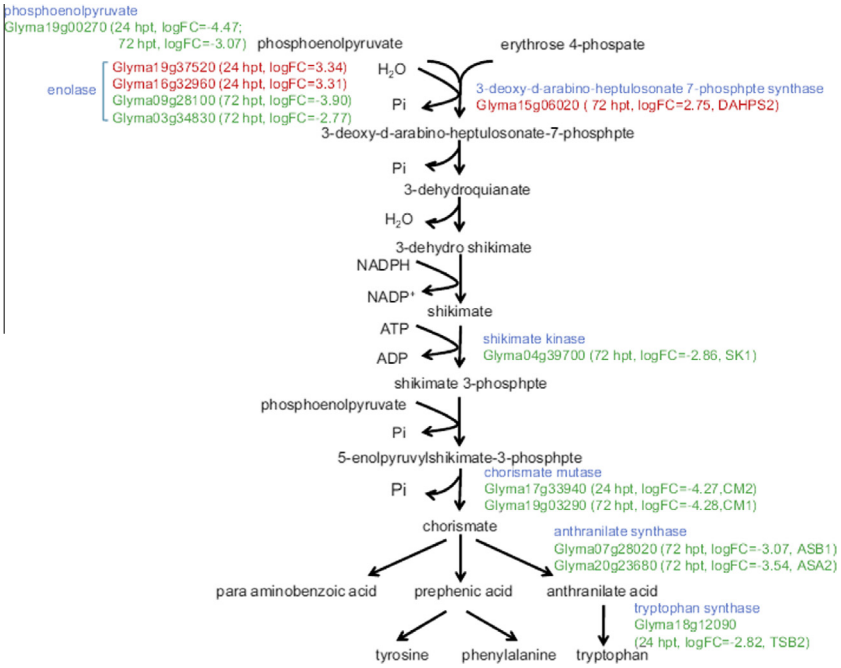

Fig. 4. Differentially expressed genes in the shikimate acid pathway. Genes in red and green indicate up-regulated and down-regulated genes, respectively. The pathway is obtained and modified from references [27]. (For interpretation of color in Fig. 4, the reader is referred to the web version of this article.)

genes encoding AUX/IAA, GH3 (Auxin-responsive GH3 family protein), and SAUR (SAUR-like auxin-responsive protein family) respectively were differentially expressed at either 24 or 72 hpt. These genes were participated in tryptophan metabolism and the effects of glyphosate on these genes may perturb cell enlargement and plant growth (Additional Table S7).

The endogenous level of abscisic acid (ABA) is increased with glyphosate treatment of yellow nutsedge (*Cyperus esculentus*) [18], indicating ABA is regulated by glyphosate in this species. In the current study, gene involved in the abscisic acid pathway such as one pyrabactin resistance like PYL (Glyma13g30210, PYR1-like 6), fourteen *PP2C* (Protein phosphatase 2C family protein) genes, and one ABF (abscisic acid responsive elements-binding factor) gene were differentially expressed at 24 hpt (Additional Table S8).

Glyphosate is well known to interfere with the shikimate acid biosynthetic pathway. In this study, the expression levels of 12 genes involved in the shikimate acidpathway were significantly increase or decrease (Fig. 4 and Additional Table S9). Moreover, four enolase genes of >95% of sequence identity were observed but their expression patterns was different. Evidence of the inhibition of shikimate pathway in the current study was supported by a significant increase in transcript levels of the 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DHS/DAHP) synthase gene *Glyma15g06020* at 72 hpt. We hypothesize that decreasing levels of tryptophan

activates DAHPS activity [19]. Therefore, the gene expression of DAHPS was up-regulated and tryptophan synthase was down-regulated. It has been documented that a number of cellular metabolites including Phe, Tyr, and Trp can feedback activate orinhibit the branch point enzymes anthranilate synthase and chorismate mutase [19]. Therefore, it is not surprising that transcript levels of anthranilate synthase (AS), the committed step in tryptophan biosynthesis, and tryptophan synthase (TS), the last step of tryptophan biosynthesis, decreased significantly with glyphosate treatment (Fig. 4).

3.4. Promoter regions of differentially expressed key target genes

Promoters in the upstream region of genes play key roles in conferring developmental and/or environmental regulation of gene expression. In this study, *cis*-Element analysis and element frequency distribution were used to elucidate the roles of transcriptional regulation in 26 DGEs and 12 SHAPs. A total of 92 and 58 types of *cis*-regulatory elements were identified among the promoters of 26 DGEs and 12 SHAPs, respectively, which do not contain the common eukaryotic regulatory elements, such as TATA-box, CAAT-box and TA-rich region. The names, functions and amounts of these 92 and 58 motifs are shown in Additional Table S10. The number of *cis*-Elements in all the 26 DGEs and 12 SHAPs are shown in additional Fig. S5. The box 4 was found at high frequency (90 times and 61 times) in the promoters of 26 DGEs and 12 SHAPs.

In the analysis of the promoters of 26 DGEs, more than 30 times occurrence of *cis*-Elements were related to light, defense, stress, circadian and drought and for 12 SHAPs, occurance of *cis*-Elements more than 20 times were related to light and heat, etc. Among 26 DGE spromoter, half of the genes contained the elements related to component, condition and light responsiveness. On the other hand, among 12 SHAPs promoter, half of the genes contained the elements similar to 26 DGEs, which indicated that these genes shared part of the *cis*-Elements in common and played important roles in the glyphosate regulation.

4. Discussion

In the shikimate acid pathway, no differentially expressed genes were observed at 6 hpt, which indicated that it would take longer time for glyphosate to reach its target site in soybean shoot apical bud. The number of differentially expressed genes increased with the glyphosate treatment time (five at 24 hpt and seven at 72 hpt). All of the differentially expressed genes decreased at 72 hpt, and significant symptoms were observed at this time. The results indicated that injury symptoms might be caused by the decrease of genes expression.

In this study, the inhibition of tryptophan biosynthesis in the shikimic acid pathway is possibly main mechanism of glyphosate in soybean apical bud. Previous study showed that only tryptophan decreased quickly and remained low afterwards when glyphosate inhibited bud elongation [20], which suggested that the inhibition of bud elongation was due to the repression of tryptophan synthesis. In the current study, tryptophan is the only effected aromatic amino acid, and this may be due to decreased expression of tryptophan synthase which has the similar mechanism as previous results [20].

Integration analysis of differentially expressed genes in this study and previous studies were conducted. A total of 20 genes in *A.thaliana*, *G. max*, and *F. arundinacea* were found, and among them, 11 homology genes were observed in the current study (Additional Table S11). There were three genes differentially expressed in *A. thaliana* and *G. max* in our study, and the homologies were all above 70%. Two ATP-binding cassette (ABC) transporter genes may participate in the glyphosate induced pathway, leading to a decrease in herbicide toxicity. One GTP-binding protein gene *Rab6*, was found in both *F. arundinacea* and *G. max* in the current study.

Compared with the study of glyphosate on soybean, 26 DGEs were regulated in our study but no cross talk genes with the other report [10]. This may be due to different time courses and glyphosate concentration between the current and previous study. Interestingly, eight genes including cytochrome P450, enolase, protein synthesis translation initiation factor 6, signaling calcium Ca^{2+}-binding EF-hand, caleosin related protein, and defense disease resistance protein-like MsR1, were identified by low concentrations of glyphosate treatment from genotype D5852641 [10], and were also found by high concentrations of glyphosate treatment from genotype Zhonghuang 13 in our study. This result provides us gene informations for discovering candidate genes to elucidate the molecular mechanisms of glyphosate on soybean apical bud.

Light is a predominant factor in the control of various biological processes in plants and a high frequency elements responsive to light in the upstream sequences providing a link to the light and stress mediated signaling in plant defenseresponses [21]. Most of the high frequency *cis*-Elements in the promoters of 26 DGEs and 12 SHAPs were related to light, indicating that light is also an important regulator in glyphosate induction. Although some studies have focused on glyphosate induced changes in physiological parameters that might have a direct or indirect effect on photosynthesis, none of them focused on the expressions of glyphosate inducible genes and its *cis*-Elements. A key promoter region of *TsVP1* gene contained the light response *cis*-Element G-box was identified to respond to salt stress [22]. G-box has an important role in the regulation of genes activated by environmental cues [23]. In the promoter region of *Smhppd* gene responses toplant defense, light responsive *cis*-Elements were the most abundant elements, which were similar to our study [24]. It is suggested that light related *Cis*-Element played an important role in abiotic stress. It is also reported that synthesis of phenylpropanoid compounds in the light is linked with a light-enhanced activity of the shikimate pathway [25]. Therefore, as an abiotic stress, glyphosate induced abundant of light responsiveness *cis*-Elements in 26 DGEs and 12 SHAPs were found in this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.112.

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