Transcriptome and Physiological Analysis of Germination in Gibberellic Acid-primed Tobacco Seeds

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Abstract

Seed priming with gibberellic acid (GA3) could promote seed germination in tobacco; however, the molecular mechanism is poorly understood. In this study, the physiological development and transcriptional regulatory network of tobacco seeds primed with GA3 or water, and non-primed seeds were studied during seed germination. When compared with non-primed or hydro-primed seeds, tobacco seeds primed with 100 mg/L GA3 solution for 24 h showed increased germination speed and uniformity under low temperatures (10°C). GA3+ levels and β-1,3-glucanase activity were significantly higher at the germination starting and pre-endosperm–rupture stages respectively. The transcriptome between the GA3-primed seeds and hydro-primed seeds was much similar than to that of the non-primed seeds. The transcripts for which their expression patterns were significantly up- or downregulated in both the GA3-primed and hydro-primed seeds relative to non-primed seeds were the focus of the study, 93.90% of which after priming and 83.89% of which during radicle expansion were regulated in the same manner, respectively. GA3 priming promoted seed germination that was associated with 67 differentially expressed transcripts after priming, 64 of which were associated with radicle expansion. Of these, ABA receptors PYL1 and PYL7, ABA biosynthesis genes ABI1, NCED6, NCED9, and AAO3, ABA negative signal regulators ABI2, AHG3, ABI3, ABI4, and ABI5, auxin response factor ARF10, ARF16; and ARF17, and light receptors PHYB and negative signal regulators HIF1, and XERICO were induced by GA3 priming. This study, thus, preliminarily revealed the physiological and molecular mechanism of gibberellin-priming promoting seed germination. © 2018 Friends Science Publishers

Keywords: Seed priming; Imbibition; Embryo development; Endosperm burst; Transcriptional network

Introduction

Seed germination is an important developmental stage during the life cycle of spermatophyte. Water uptake is an essential requirement for the starting of seed germination, with the radical breakthrough of seed coat generally considered to be the end of seed germination (Bewley, 1997). Water uptake by tobacco seed is triphasic, during which there is rapid initial water uptake within 3.0 h in stage I, a leveling off in stage II, and further increases in stage III, after which the radicle emerges (Manz et al., 2005). The embryo in the seed of tobacco is encircled by endosperm and testa, both of which restrain seed germination (Manz et al., 2005), with the endosperm being the main germination-limiting tissue (Kucera et al., 2005). Class I β-1,3-glucanase (βGlu I) is a target enzyme for endosperm rupture that is induced after testa rupture in tobacco seeds (Leubner-Metzger and Meins, 2000; Leubner-Metzger, 2003).

Seed priming, as a seed preliminary treatment before sowing that can promote germination, had been wildly used in many crops, such as rice (Farooq et al., 2010; Yari and Sheidai, 2011), maize (Foti et al., 2008; Jannamohammadi et al., 2008), wheat (Akbari et al., 2007; Wahid et al., 2007), cotton (Casenave and Toselli, 2007), tomato (Nawaz et al., 2011), and barley (Rashid et al., 2006). Various approaches of priming were reviewed recently (Jisha et al., 2012), the advanced germination uniformity, accelerated germination, and improved abiotic stress tolerance were attributed to seed priming (Jisha et al., 2012). During priming, the seeds are subjected to controlled hydration, and the molecular mechanism is “memorized” at the later dehydrated state so that primed seeds can quickly germinate (Rajjou et al., 2012). Some of the stored proteins and/or mRNAs might have been shown to function (Rajjou et al., 2004; Kimura and Nambara, 2010), early repair mechanisms were performed (Balestrazzi et al., 2011; Kibinza et al., 2011), and antioxidant activity was enhanced (Chen and Arora, 2011).
In recent years, high-throughput sequence methods emerged as useful tools that were used in plant omics studies, which also promoted the development of the molecular mechanisms involved in seed priming. Although various omics methods had been used for study in seed-priming (Ghareeb et al., 2011; Fercha et al., 2013; Kubala et al., 2015), however, none for GA\textsubscript{3} priming. In recent years, GA\textsubscript{3} priming has been widely used in tobacco seed in China, mainly for increased uniformity of seedling emergence and enhanced resistance to cold stress. In addition, tobacco is an important model plant for the germination study of endospermic seeds (Leubner-Metzger and Meins, 2000; Leubner-Metzger, 2003; Manz et al., 2005), however, the physiological and molecular mechanisms of tobacco seed priming have not been explored.

In the present study, GA\textsubscript{3}-primed, hydro-primed, and non-primed tobacco seeds were comparatively studied for their differences in dynamic physiological development during seed germination as follows: (i) imbibition, (ii) radicle expanding, (iii) endosperm rupture, (iv) radicle emergence, and (v) early seedling established stages. In addition, two metabolic statuses were separately studied at the transcriptome level as follows: (i) at the end of the priming treatment and (ii) before radicle emergence.

Materials and Methods

Seed Priming and Germination

Tobacco seeds from variety of Nanjiang3 were acquired from Guizhou Academy of Tobacco Science. Seeds were soaked for 24 h at 26°C in GA\textsubscript{3} or ddH\textsubscript{2}O solution, and the weight ratio between the seeds and solution is about 1:5. This seed priming protocol (26°C, 100 mg/L GA\textsubscript{3}, 12 h light/dark cycle) were optimized in the pre-experiments, based on national standard (GB/T26240-2010). After priming, the seeds were rinsed three times in distilled water. GA\textsubscript{3}-primed and hydro-primed seeds were dehydrated at room temperature to a moisture content of ~10%, as with the non-primed seeds used for germination and other tests.

Germination tests were performed on three replicates of 100 seeds. Seeds were germinated in a 12 h light/dark alternant cycle at 26 or 10°C. Germination was notarized as the length of observed radicle approximately equal to that of the seed. After 156 h, 10 radicles from the each replicate were randomly sampled, their weight were tested using a 10'\textsuperscript{3} g precise scale, and their length and surface area were measured with WinRHIZO that produced by Seiko Epson Corporation (Nagano, Japan) (Li et al., 2015). Moreover, germination potential or rate was recorded at 7 or 14 days’ respectively in the test. Mean germination time=\(\sum(Daster n)/\sum n\), and ‘n’ was calculated as hour or day respectively at 10 or 26°C (Zhu et al., 2018).

Transcriptomic Analyses

Transcriptomic analyses were conducted on seeds collected at crucial points of GA\textsubscript{3}-priming and hydro-priming as follows: (i) at the end of priming and (ii) radicle expansion before emergence (72 h of germination). Analyses were also performed on non-primed seeds and after germinating for 72 h. The germinating seeds from (i) and (ii) were collected, freezed in liquid nitrogen and stored in a -80°C refrigerator for RNA-seq analysis.

Extraction, Purification, and Quantification of the Phytohormones and βGlu I Activity in Seeds

The 0, 36, 72, 108, and 144 h germinating seeds were collected for quantizing βGlu I activities and 0, 72, and 144 h’ s for quantizing the content of phytohormones GA\textsubscript{1+3}, ABA and IAA. The procedures for protein extraction were performed as description of Leubner-Metzger (Leubner-Metzger et al., 1995). The method of extraction, purification, and quantification for phytohormones were performed as described by Wang (Wang et al., 2012). ELISA kits used for measuring βGlu I activity were purchased from R&D Systems (Minneapolis, MN, USA), and those for measuring hormonal levels were purchased from China Agricultural University (Beijing, China).

RNA Extraction and Transcriptome Sequencing

The total RNA of the samples were extracted by using the TRK-1001 Total RNA Purification Kid (LC Sciences, Houston, TX, USA). The quality of the total RNA and the standard for database building as our published requirements (Li et al., 2016). Using the Invitrogen Dynabeads mRNA Direct kit, mRNAs were enriched from 5.0 ug total RNA. Then, the enriched mRNA was fragmented on blocks at 95°C for 2.0 min. After purifying by Qiagen PCR Purification Kits, first-strand cDNA and double cDNA were synthesized by using SMARTscript II reverse transcriptase and SMARTeroligos-dNTPs, respectively. cDNAs were purified with gel and performed as templates to generate sequencing libraries. Quantitative polymerase chain reaction (qPCR) were used for the quality checking and concentration calculating of these libraries. Finally, the libraries were sequenced by using the Illumina HiSeq 2000 platform, and each sample yielded 10 Gb data. Base calling was finished by using CASAVA 1.8 software (Illumina, San Diego, CA, USA).

Transcriptome Assembly and Functional Classification

The raw reads from the Illumina Genome Analyzer (Illumina, San Diego, CA, USA) were cleaned by removing the adapter sequences and low-quality bases at the 3’ end. The clean reads were then mapped to the tobacco genome by TopHat (Kim et al., 2013), the parameters of which...
Gene Expression Difference and Enrichment

To quantify the expression of transcripts of all samples, Bowtie2 was used for mapping clean reads of each sample to the unique transcripts. eXpress, which allowed us to correct multiple mapped reads, was used to calculate the read count of transcripts. To identify the differentially expressed transcripts between the two treatments, we used R DESeq (Anders and Huber, 2010) for analysis, in which the nbinomTest function was used for no replicate comparison; p-values were corrected using the Benjamin and Hochberg FDR method.

Gene ontology enrichment analysis of differentially expressed transcripts was performed using the BiNGO plugin (Maere et al., 2005) in Cytoscape with a significance level of an FDR adjusted p-value < 0.05. Whole annotated transcripts were used as the reference set. The KEGG pathway enrichment analysis was calculated using the GSEA(KEGGHyperGParams) function in R GOstats (Falcon and Gentleman, 2007) with an FDR adjusted p-value <0.05.

Real-time qPCR

RT qPCR comprised two steps—reverse transcription (RT) and PCR. RT reactions were completed by using a GeneAmp® PCR System 9700 (Applied Systems, University Park, IL, USA) in a 10-μL volume in which was added 0.5 μg RNA, 2.0 μL PrimerScript Buffer, 0.5 μL oligo dT, 0.5 μL random 6mers, and 0.5 μL Primer Script RT Enzyme Mix I (TaKaRa Biosystems Inc, Kusatsu, Japan). PCR reactions were finished by using LightCycler® 480 II Real-time PCR Instrument (Roche, Basel, Switzerland) with a 10-μL PCR reaction system that consisted of 1.0 μL cDNA, 5.0 μL of 2× LightCycler® 480 SYBR Green I Master (Roche, Basel, Switzerland), 0.2 μL forward primer, 0.2 μL reverse primer, and 3.6 μL nuclease-free water. Samples were all run in triplicate for analysis. After the PCR cycles, melting curve analyses were conducted to validate the specific generation of the expected PCR products. The primer sequences were designed in the laboratory and synthesized based on the mRNA sequences obtained from RNA-seq assembled transcripts. The relative expression levels of the mRNAs were normalized to L25, and the results were calculated by using the 2-ΔΔCt method.

Results

GA3 Priming Improved Seed Germination

The germination speed and uniformity for the GA3-primed seeds were faster than that of the hydro-primed and non-primed seeds at low (10°C) temperatures (Fig. 1). The physiological effect of GA3 priming on seed germination were studied during the developmental stage, including water uptake, embryo development and endosperm burst. The results indicated that the seed vacuoles primed in GA3 solution unfolded similar to those in ddH2O (Fig. 2). The consistency and lengths of the emerged radicles in the GA3-primed seeds were superior to that in the non-primed seeds, but there were no significant differences in radicle weights and superficial areas among all the three seeds (Fig. 3A and B). The expression level of βGluc I enzyme in GA3-primed seeds was significantly higher than that of hydro-primed seeds and non-primed seeds at 72 h, the time just before radicle emergence (Fig. 4).

GA3 Priming Rebalanced the Hormone Ratio

The effects of GA3 priming on endogenous hormones, including ABA, GA3, and IAA, were measured during seed germination at the start of germination (0 h), radicle expansion (72 h), and early seedling establishment (144 h). ABA contents of GA3-primed seeds were significantly lower than that of non-primed seeds at the radicle expansion stage (Fig. 5B). GA3 content of the GA3-primed seeds was significantly higher than that of hydro-primed and non-primed seeds (Fig. 5D). The IAA content was not significantly different among three kinds of seeds at all three stages (Fig. 5G, H and I).

Transcriptomic Analysis of GA3 Priming Promoted Seed Germination

RNA sequencing was used to investigate the transcriptome of GA3-primed, hydro-primed, and non-primed seeds at the end of priming and at the radicle-expansion stage. Six paired-end libraries were constructed and sequenced on the Illumina HiSeq 2000 platform. The read counts and genomic mapping situations of the six libraries are listed in Table 1. There were 72.61~91.43% clean reads that were mapped on the N. tabacum L. reference genome (Siervo et al., 2014). Principal component analysis (PCA) was performed to investigate the gene expression patterns of these six samples, and the results displayed that the six
samples could be clustered into three nearly groups based on the spatial distribution of the points (Fig. 6) as follows: (i) UNP; (ii) H_2O_24 and GA_3P_24; and (iii) UNG_72, H_2O_G_72, and GA_3G_72. These results suggested that the samples could be obviously distinguished at the transcriptome level, which is in accordance with the physiological processes during seed germination, including the status of seed quiescence, imbibition, and radicle expansion. In addition, the transcriptomes of the two primed seeds were more similar to each other than to the non-primed seeds not only at the end of priming, but also at the subsequent radicle development stage.

At the end of priming, gene differential expression analyses were conducted on the GA_3-primed seeds (GA_3P_24), hydro-primed (H_2O_24) seeds, and non-primed (UNP) seeds.

### Table 1: Number of reads sequenced and mapped with Tophat

<table>
<thead>
<tr>
<th>Sample</th>
<th>Seed status</th>
<th>Total reads</th>
<th>Total mapped reads</th>
<th>Reads mapped in proper pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNP</td>
<td>Dry</td>
<td>39,344,236</td>
<td>28,566,421 (72.61%)</td>
<td>25,845,450 (66.09%)</td>
</tr>
<tr>
<td>H_2OP_24</td>
<td>Imbibition</td>
<td>39,795,012</td>
<td>35,183,360 (88.41%)</td>
<td>29,258,962 (73.52%)</td>
</tr>
<tr>
<td>GA_3P_24</td>
<td>Imbibition</td>
<td>32,346,324</td>
<td>25,276,690 (78.14%)</td>
<td>20,228,860 (62.53%)</td>
</tr>
<tr>
<td>UNG_72</td>
<td>Radicle expanding</td>
<td>33,792,314</td>
<td>30,897,092 (91.43%)</td>
<td>24,978,136 (73.91%)</td>
</tr>
<tr>
<td>H_2OG_72</td>
<td>Radicle expanding</td>
<td>35,593,768</td>
<td>31,962,893 (89.80%)</td>
<td>26,621,694 (74.79%)</td>
</tr>
<tr>
<td>GA_3G_72</td>
<td>Radicle expanding</td>
<td>36,098,848</td>
<td>31,023,702 (85.94%)</td>
<td>25,310,022 (70.11%)</td>
</tr>
</tbody>
</table>

The number of unique mapping reads plus multi-mapped reads equals the total number of alignments. UNP, H_2O-primed seeds or GA_3-primed seeds. UNG_72, H_2O-germinated seeds or GA_3-primed seeds. Numbers 24 or and 72 stand for primed or germinated time.

**Fig. 1:** Gibberellic acid (GA_3) priming promotes seed germination under low temperatures. Germination of seeds after being primed in 100 ppm GA_3 solution, ddH_2O for 24 h, or not primed and then germinated at suitable (26°C) or low (10°C) temperatures.

**Fig. 2:** Water uptake of tobacco seed primed in 100 ppm gibberellic acid (B) solution or ddH_2O (A) for 24 h at 26°C.

**Fig. 3:** Radicle emergence (A) and traits (B) of tobacco seed primed in 100 ppm gibberellic acid (GA_3) solution, ddH_2O for 24 h, or not primed and then germinated at 156 h at 26°C. Letters indicate significant differences according to the Duncan test (means ± SD, p < 0.05), as the same in below figures.
Similarly, 968 up- and 522 down-regulated transcripts between hydro-primed germinating (H$_2$OG$_{72}$) and UNG$_{72}$ seeds, and 673 up- and 1,043 down-regulated transcripts between GA$_3$G$_{72}$ and UNG$_{72}$ seeds were identified.

According to the results from all comparisons, there were less differentially expressed transcripts of the radicle-development-stage groups. This revealed that the difference among the three kinds of seeds in the radicle-development stage was smaller than that after priming, which was in accord with the results of PCA.

Similarity in Gene Expression Profiles between GA$_3$-Primed and Hydro-primed Seeds

To further study the effects of GA$_3$ priming on seed germination, the transcriptome difference between the two types of primed seeds was further studied. The non-primed seeds were used as the control, and the transcript expression patterns of the GA$_3$-primed and hydro-primed seeds relative to the non-primed seeds were calculated. Four patterns were obtained (Table 2). We identified 39,025 transcripts at the end of priming and 24,261 transcripts at the radicle expansion stage whose expression levels were significantly altered (more than a two-fold change, the transcript counts with p-value ≤ .05 were also listed) in the both types of primed seeds. Interestingly, 93.90% of these genes were regulated in the same manner between the two types of primed seeds. These results indicated that the germination of the two types of primed seeds might not be similar in only germination phenotype (Fig. 3), but also in transcriptomes (Fig. 6), especially after the priming stage.
Table 2: A table of genes whose expression is altered during seed primed with H₂O or GA₃

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Comparative gene expression</th>
<th>After priming</th>
<th>Radicle expanding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hydroprimed seeds/unprimed GA₃ primed seeds/unprimed GA₃ primed seeds</td>
<td>Percentage</td>
<td>Transcript</td>
</tr>
<tr>
<td>I</td>
<td>39.36%</td>
<td>1536</td>
<td>694</td>
</tr>
<tr>
<td>II</td>
<td>3.09%</td>
<td>1206</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>3.01%</td>
<td>1174</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>54.54%</td>
<td>21283</td>
<td>1449</td>
</tr>
</tbody>
</table>

Genes whose expression level was significantly up-regulated or down-regulated by more than 2-fold after seed priming and radicle expansion were grouped according to their expression behavior relative to unprimed seeds. Arrows facing up or down represent up-regulated or down-regulated genes, respectively. Superscript FC or P respectively stand for fold change > 2 or P-value < 0.05.

Fig. 7A, B: Venn diagrams representing the cross comparison of differentially expressed transcripts (with more than a two-fold change and p-value ≤ .05) in priming and post-priming germination of tobacco seeds. (A) Seeds after priming. (B) Seeds at the radicle expansion stage. UNP, H₂O₃P₂₄, and GA₃P₂₄, respectively, represented non-primed seeds, ddH₂O- primed seeds, or gibberellic acid (GA₃)-primed seeds. UNG₇₂, H₂O₃G₇₂, and GA₃G₇₂, respectively, represent germination for 72 h of non-primed seeds, ddH₂O-primed seeds, or GA₃-primed seeds.

GA₃-Responsive Genes in the Priming and Germination of N. tabacum L. Seeds

Although the transcriptome between GA₃-primed and hydro-primed seeds were similar, there remained a small number of differentially expressed transcripts. To further study these GA₃-responsive genes in the priming and germination stages, we chose the intersected transcript sets of the three groups of differential expressions (Fig. 7A and B) for functional analysis. Differentially expressed transcripts at such intersections could be deemed to be the main factors that result in the difference molecular mechanisms between GA₃-primed and hydro-primed seeds relative to non-primed seeds.

At the end of priming, we identified 67 differentially expressed transcripts at the intersection of the three groups (Fig. 7A, UNP vs. GA₃P₂₄ vs. H₂O₃P₂₄). A GO enrichment analysis of the transcript sets was performed using a hypergeometric test, which is related to the hormone signaling pathway, amino acid metabolism (glutamine family amino acid metabolic process, arginine biosynthetic process and peptidyl-histidine phosphorylation), and glutathione metabolism (glutamine-hydrolyzing activity, glutathione transferase activity and lactoylglutathione lyase activity) (Table 3). KEGG pathways enriched in were enriched in amino acid metabolism (alanine, aspartate, and glutamate metabolism), glutathione metabolism, etc. (Table 4).

Using the same methods at the radicle expansion stage, we detected 64 differentially expressed transcripts at the intersection of the three groups (Fig. 7B, UNG₇₂ vs. GA₃G₇₂ vs. H₂O₃G₇₂). There was significant enrichment of GO terms related to activities such as photosynthesis, light signals, methionine metabolism, and redox signaling (Table 3). KEGG pathways enriched in photosynthesis, carbon metabolism, and glyoxylate cycle (Table 4).

GA₃-Responsive Genes in Important Signaling Pathways

The interactions of ABA and GA (Rajjou et al., 2012), light, ABA, and GA (Vanderaelen and Benkova, 2012) and auxin and ABA (Shu et al., 2016) were the most important signaling pathways that controlled seed dormancy and germination. Other dormancy regulators were also reviewed (Graeber et al., 2012). The gene expression difference in the GA₃-primed seeds was studied in these important signaling pathways and regulators. First, homologous genes for tobacco in these pathways and groups were identified based on the gene functional annotation results. Then, combined with the results of gene differential expression analyses, the transcripts of both the up- or down-regulated genes relative to that of the non-primed and hydro-primed seeds were the focus, as shown in Table 5.
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Table 3: Representative significant enriched GO terms of the differential expressed transcripts

<table>
<thead>
<tr>
<th>Seed status</th>
<th>Category</th>
<th>GO term</th>
<th>Reference set</th>
<th>Query set</th>
<th>P-value (FDR)</th>
<th>Representative transcript</th>
<th>DE Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP BP</td>
<td></td>
<td>Hormone-mediated signaling pathway</td>
<td>1891 10</td>
<td>5.70E-05</td>
<td>TCONS_00081290</td>
<td>Ras-related protein RAB8-1</td>
<td>TCONS_0107714</td>
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<tr>
<td></td>
<td></td>
<td>Peptidyl-histidine phosphorylation</td>
<td>83</td>
<td>4.26E-02</td>
<td>TCONS_0003961</td>
<td>Ethylene receptor ERS homolog</td>
<td>TCONS_0005259</td>
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<tr>
<td></td>
<td></td>
<td>Glutamine family amino acid metabolic process</td>
<td>338 3</td>
<td>4.43E-02</td>
<td>TCONS_00066165</td>
<td>Putative carbamoyl phosphate synthase large subunit, Glutamate synthase 1 [NADH]</td>
<td>TCONS_00105308</td>
</tr>
<tr>
<td>MF</td>
<td></td>
<td>Glutathione transferase activity</td>
<td>96</td>
<td>1.34E-08</td>
<td>TCONS_0036199</td>
<td>Glutathione-S-transferase U3-like</td>
<td>TCONS_00066166</td>
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<tr>
<td></td>
<td></td>
<td>Glutamine-hydrolyzing activity</td>
<td>29</td>
<td>1.06E-02</td>
<td>TCONS_0066166</td>
<td>Carbamoyl phosphate synthase large subunit</td>
<td>TCONS_000102454</td>
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<td>RE BP</td>
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<td>Translation elongation factor activity</td>
<td>273 3</td>
<td>2.92E-02</td>
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<td>Elongation factor-1 alpha</td>
<td>TCONS_000102454</td>
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<td></td>
<td></td>
<td>photosynthesis, dark reaction</td>
<td>63 9</td>
<td>5.46E-16</td>
<td>TCONS_00102454</td>
<td>Ribulose bisphosphate carboxylase small subunit protein precursor</td>
<td>TCONS_000102454</td>
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<tr>
<td></td>
<td></td>
<td>photosynthesis, light harvesting</td>
<td>141</td>
<td>7.60E-06</td>
<td>TCONS_0004152</td>
<td>Chlorophyll a-b binding protein 7</td>
<td>TCONS_0004152</td>
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<tr>
<td></td>
<td></td>
<td>response to red light</td>
<td>285 6</td>
<td>8.24E-06</td>
<td>TCONS_0107704</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase B</td>
<td>TCONS_00008250</td>
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<td></td>
<td></td>
<td>response to far red light</td>
<td>324 6</td>
<td>1.48E-05</td>
<td>TCONS_00008250</td>
<td>Ribulose bisphosphate</td>
<td>TCONS_00008250</td>
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<td></td>
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<td>response to blue light</td>
<td>372 6</td>
<td>1.48E-05</td>
<td>TCONS_00008250</td>
<td>Carboxylase/oxygenase activase 2</td>
<td>TCONS_00008250</td>
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<td></td>
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<td>cysteine biosynthetic process</td>
<td>547 5</td>
<td>2.35E-03</td>
<td>TCONS_0032837</td>
<td>Chloroplast pigment-binding protein CP26</td>
<td>TCONS_00065605</td>
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<td></td>
<td></td>
<td>regulation of hydrogen peroxide metabolic process</td>
<td>287</td>
<td>1.69E-02</td>
<td>TCONS_00065605</td>
<td>Beta-carboxy anhydride</td>
<td>TCONS_00065605</td>
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<td></td>
<td>regulation of oxygen and reactive oxygen species metabolic process</td>
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<td>TCONS_00034183</td>
<td>Phosphoribulosokinase</td>
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<td>CC</td>
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<td>Ribulose-bisphosphate carboxylase activity</td>
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<td>1.13E-15</td>
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<td>Ribulose bisphosphate carboxylase small subunit protein precursor</td>
<td>TCONS_000102454</td>
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<td></td>
<td>Protochlorophyllide reductase activity</td>
<td>51</td>
<td>1.94E-06</td>
<td>TCONS_00055160</td>
<td>NADPH protoclorophyllide oxidoreductase</td>
<td>TCONS_00055160</td>
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<td></td>
<td></td>
<td>Chlorophyll binding</td>
<td>99</td>
<td>1.94E-06</td>
<td>TCONS_00055160</td>
<td>Chlorophyll a-b binding protein 21</td>
<td>TCONS_00055160</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase activity</td>
<td>8</td>
<td>1.94E-06</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase B</td>
<td>TCONS_00055160</td>
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<td>1.94E-06</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase B</td>
<td>TCONS_00055160</td>
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<td></td>
<td></td>
<td>Fruuctose-bisphosphate aldolase activity</td>
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<td>4.54E-03</td>
<td>TCONS_00063742</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase A</td>
<td>TCONS_00063742</td>
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<td></td>
<td></td>
<td>NADP or NADPH binding</td>
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<td>4.78E-03</td>
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<td>24K germin like protein</td>
<td>TCONS_0008412</td>
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<td></td>
<td>Superoxide dismutase activity</td>
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<td>4.78E-03</td>
<td>TCONS_0008412</td>
<td>24K germin like protein</td>
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</tr>
</tbody>
</table>

After priming (AP), Radicle expanding (RE)

Table 4: Significant enriched KEGG pathways of the difference express transcripts

<table>
<thead>
<tr>
<th>Seed status</th>
<th>KEGG ID</th>
<th>Reference set</th>
<th>Query set</th>
<th>P-value (FDR)</th>
<th>KEGG Pathway</th>
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<tbody>
<tr>
<td>Imbibition</td>
<td>ko05204</td>
<td>6 109</td>
<td>4.25E-07</td>
<td>Chemical carcinogenesis</td>
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<tr>
<td>(Quenched)</td>
<td>ko00980</td>
<td>6 131</td>
<td>4.67E-07</td>
<td>Metabolism of xenobiotics by cytochrome P450</td>
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<tr>
<td></td>
<td>ko00480</td>
<td>6 270</td>
<td>1.75E-05</td>
<td>Glutathione metabolism</td>
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<td></td>
<td>ko04152</td>
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<td>0.026481</td>
<td>AMPK signaling pathway</td>
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<tr>
<td></td>
<td>ko00250</td>
<td>3 278</td>
<td>0.039827</td>
<td>Alanine, aspartate and glutamate metabolism</td>
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<td>Radicle expansion</td>
<td>ko0710</td>
<td>13 290</td>
<td>1.06E-15</td>
<td>Carbon fixation in photosynthetic organisms</td>
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<td></td>
<td>ko01200</td>
<td>13 1041</td>
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<td></td>
<td>ko00630</td>
<td>8 252</td>
<td>1.33E-08</td>
<td>Glyoxylate and dicarboxylate metabolism</td>
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<td></td>
<td>ko00195</td>
<td>6 154</td>
<td>4.20E-07</td>
<td>Photosynthesis</td>
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<tr>
<td></td>
<td>ko00196</td>
<td>4 78</td>
<td>2.19E-05</td>
<td>Photosynthesis - antenna proteins</td>
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<td>ko00860</td>
<td>4 180</td>
<td>4.91E-04</td>
<td>Porphyrin and chlorophyll metabolism</td>
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</tbody>
</table>

For crosstalk of ABA and GA, ABA biosynthesis genes N Ced9 and AAO3 were down-regulated, ABA receptors PYL1 was up-regulated, and ABA negative signal regulator ABI4 was up-regulated after GA3 priming. At the radicle expansion stage, ABA receptors PYL1 and PYL7 were down-regulated, ABA negative signal regulators ABI2, AHG3, ABI3, ABI4, and ABI5 were down-regulated, and ABA biosynthesis genes ABA1 and NCED6 were down-regulated. In the interaction of ABA and AUX, auxin response factors ARF10 and ARF17 were down-regulated after priming, and ARF16 was up-regulated during the radicle expansion stage. During the crosstalk among light, ABA, and GA, PHYB was up-regulated and PIF1 and XERICO were down-regulated after seed priming, and COP1, PIF1, and XERICO were down-regulated during radicle expansion. In addition, other dormancy regulators, such as EFS and KYP were up-regulated after priming, DOG1 and MFT were down-regulated during radicle expansion, and AtHB-20 was up-regulated both after priming and during radicle expansion.

Validation RNA-seq Results by RT-qPCR

Eleven unigenes were randomly selected for validation RNA-seq results by using RT-qPCR. The results showed...
a significant positive correlation between them (Fig. 8). Pearson correlation: $p = 1.05E-4$, $r = 0.75$; Spearman correlation: $p = 4.96E-4$, $p = .71$, indicating that the RNA-seq expression analysis performed was highly reliable.

**Discussion**

As a common priming method, GA$_3$ priming could promote seed germination was well known (Harb, 1992; Bisht et al., 2009). In the present study, we uncovered a previously unrecognized transcriptional regulatory network of the germination for GA$_3$-primed seed. Through transcriptome studies, we speculate that GA$_3$ priming promotes seed germination was associating with: (i) phytohormone metabolism and/or signaling, (ii) special amino acid metabolism and protein synthesis potential, (iii) photosynthesis, (iv) redox signaling; (v) glutathione metabolism, and (vi) glyoxylate cycle. Of these, (ii) and (iv) had been identified as important metabolisms that regulated
by polyethylene glycol-priming during rape seeds germination (Kubala et al., 2015); (ii) and (v) regulated by ascorbate-priming during durum wheat seeds germination (Fercha et al., 2013); (i), (ii) and (v) regulated by IAA-priming during tobacco seeds germination (Li et al., 2016).

Of them, phytohormone metabolism and/or signaling pathways were the most important pathways induced by GA3 priming. GA and ABA were the key hormones during seed germination with GA3 promoting and ABA inhibiting it (Graeber et al., 2012). In this study, GA13 level was increased after GA3 priming compared with non-primed and hydro-primed seeds. Nakaue et al. (2012) showed similar results in tomato seeds, GA14 levels increased during seed germination after salt priming. Two mutually beneficial physiological functions for GA in seed germination have been recognized: First, GA increases the growth momentum of the embryo; Second, GA is needed to break through the mechanical constrain conferred by the endosperm surrounding the radicle (Hillorst, 1995; Bewley, 1997; Bewley, 1997; Koummef et al., 2002). In this study, only second function could be confirmed, which was determined over the activity changes of βGlu I, a enzyme that was prerequisite for endosperm rupture during seed germination of tobacco (Leubner-Metzger et al., 1995; Leubner-Metzger et al., 1996; Leubner-Metzger et al., 1998). In contrast to GA level changed, ABA content was not regulated by GA3 priming in this study. Similar results were obtained in tomato, ABA content was also not influenced by salt priming (Nakaue et al., 2012). However, ABA biosynthesis genes NCED9 and AAO3 were downregulated in primed seeds, ABA1 and NCED6 downregulated in germinating seeds respectively. Therefore, GA3 priming might have resulted in an increase in total GA levels after priming treatment, which might have further altered the ratio of GA/ABA that promoted seed dormancy release and germination.

Recently, auxin was discovered as a secondary phytohormone that controls seed dormancy and germination (Liu et al., 2013). Similar to ABA, IAA content was not regulated by GA3 priming in this study, which was confirmed by the expression pattern of metabolic genes. Auxin performing function need to recruit the auxin response factors ARF10 and ARF 16 during seed germination (Liu et al., 2013). In this study, ARF10 and ARF17 were down regulated after priming, and ARF 16 was upregulated at the germination stage. Therefore, we speculate that the auxin and GA signaling might also have a crosstalk during seed germination.

The molecular mechanisms, crosstalk between light, GA and ABA during seed germination, had been elucidated recently (Vanstraelen and Benkova, 2012). Light signals are received by the phytochromes, which are the main light receptors regulating seed germination. PHYA and PHYB are two key phytochromes that induce seed germination (Shinomura et al., 1994, Shinomura et al., 1996). PIF1 is an important transcription factor downstream of PHYB that through control the expression of metabolism genes for GA and ABA, mutually regulates the levels of GA and ABA (Kim et al., 2008; Gabriele et al., 2010). PIF1 strongly inhibits seed germination in the dark situation (Oh et al., 2009). XERICO is a negative regulatory factor on seed germination (Ko et al., 2006). In this study, PHYB was upregulated, and PIF1 and XERICO were downregulated after priming and in the radicle expansion stage. In addition, GO terms of the GA3-primed seed were the significant enrichment in light signals. Therefore, we propose that light signals are stimulated or partly replaced by GA3 priming during seed germination.

**Conclusion**

GA3 priming promotes seed germination under low temperature. Phenotypically, germination speed and uniformity of radicle emerging were raised by GA3 priming. Physiologically, GA3 priming resulting in improving of the ratio of GA/ABA and promoting endosperms burst. On the molecular biology, light signals pathways are stimulated or partly replaced by GA3 priming during seed imbibition.

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References


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