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Full Length Article

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

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Abstract

Seed priming with gibberellic acid (GA_3) 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 GA_3 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 GA_3 solution for 24 h showed increased germination speed and uniformity under low temperatures (10°C). GA_{1+3} levels and β-1,3-glucanase activity were significantly higher at the germination starting and pre-endosperm–rupture stages respectively. The transcriptome between the GA_3-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 GA_3-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. GA_3 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 *ABA1*, *NCED6*, *NCED9*, and *AAO3*, ABA negative signal regulators *ABI2*, *AHG3*, *ABI3*, *ABI4*, and *ABI5*, auxin response factor *ARF10*, *ARF 16*, and *ARF17*, and light receptors *PHYB* and negative signal regulators *PIF1*, and *XERICO* were induced by GA_3 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, 1997a). 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 Sheidaie, 2011), maize (Foti *et al*., 2008; Janmohammadi *et al*., 2008), wheat (Akbari *et al*., 2007; Wahid *et al*., 2007; Jafar *et al*., 2012), 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).

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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_3 priming. In recent years, GA_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 be explored.

In the present study, GA_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_3 or ddH_2O solution, and the weight ratio between the seeds and solution is about 1:5. This seed priming protocol (26°C, 100 mg/L GA_3, 12 h light/dark cycle) were optimized in the pre-experiments, based on national standard (GB/T25240-2010). After priming, the seeds were rinsed three times in distilled water. GA_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^{-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= $\sum(D * n)/\sum n$, 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_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 geminating seeds were collected for quantizing βGlu I activities and 0, 72, and 144 h' s for quantizing the content of phytohormones GA_{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 was 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 were as our published requirements (Li *et al*., 2016). Using the Invitrogen Dynabeads mRNA direct kit, mRNAs were enriched from 5.0 μg total RNA. Then, the enriched mRNA was fragmented on blocks at 95°C for 2.0 min. After purificating 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) was 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

allowed up to two mismatches and reported up to 40 alignments for multiple mapped reads. To assemble more refined transcripts, we combined the five previously published paired-end RNA-seq data (Li *et al.*, 2016) with the current six libraries. All Sequence Alignment Map files generated by TopHat were provided into Cufflinks (Trapnell *et al*., 2010) for transcript assembly, and the assembled gene transfer format (GTF) files from the six samples were input into Cuffcompare, in which we used the previously assembled GTF file as reference information to detect new transcript loci and isoforms. After the transcripts < 200 bp were removed, the newly identified transcripts were annotated using a non-redundant database with an E-value < 1.0 e-5, gene ontology (GO) by BLAST2GO (Gotz *et al*., 2008), and KEGG pathway using the online KAAS tool (Moriya *et al*., 2007).

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.

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 < .05. Whole annotated transcripts were used as the reference set. The KEGG pathway enrichment analysis was calculated using the GSEAKEGGHyperGParams 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

GA_3 Priming Improved Seed Germination

The germination speed and uniformity for the GA_3-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 GA_3 priming on seed germination was studied during the developmental stage, including water uptake, embryo development and endosperm burst. The results indicated that the seed vacuoles primed in GA_3 solution unfolded similar to those in ddH_2O (Fig. 2). The consistency and lengths of the emerged radicles in the GA_3-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 βGlu I enzyme in GA_3-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).

GA_3 Priming Rebalanced the Hormone Ratio

The effects of GA_3 priming on endogenous hormones, including ABA, GA_{1+3}, 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 GA_3-primed seeds were significantly lower than that of non-primed seeds at the radicle expansion stage (Fig. 5B). GA_{1+3} content of the GA_3-primed seeds was significantly higher than that both 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 GA_3 Priming Promoted Seed Germination

RNA sequencing was used to investigate the transcriptome of GA_3-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 (Sierro *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_2OP_{24} and GA_3P_{24}; and (iii) UNG_{72}, H_2OG_{72},

Table 1: Number of reads sequenced and mapped with Tophat

Sample	Seed status	Total reads	Total mapped reads	Reads mapped in proper pairs
UNP	Dry	39,344,236	28,566,421 (72.61%)	25,845,450 (65.69%)
H_2OP_{24}	Imbibition	39,795,012	35,183,360 (88.41%)	29,258,962 (73.52%)
GA_3P_{24}	Imbibition	32,346,324	25,276,690 (78.14%)	20,228,860 (62.53%)
UNG_{72}	Radicle expanding	33,792,314	30,897,092 (91.43%)	24,978,136 (73.91%)
H_2OG_{72}	Radicle expanding	35,593,768	31,962,893 (89.80%)	26,621,694 (74.79%)
GA_3G_{72}	Radicle expanding	36,098,848	31,023,702 (85.94%)	25,310,022 (70.11%)

The number of unique mapping reads plus multi-mapped reads equals the total number of alignments. UNP, H_2OP_{24} and GA_3P_{24} respectively stand for unprimed seeds, H_2O-primed seeds or GA_3-primed seeds. UNG_{72}, H_2OG_{72} and GA_3G_{72} respectively stand for germinating of unprimed seeds, H_2O-primed seeds or GA_3-primed seeds. Numbers 24 or and 72 stand for primed or germinated time

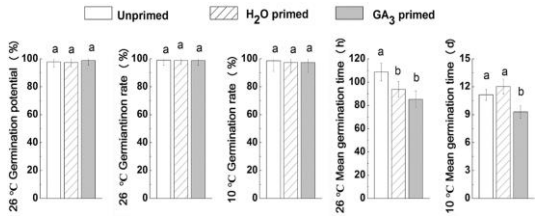

Fig. 1: Gibberellic acid (GA_3) priming promotes seed germination under low temperatures. Germination of seeds after being primed in 100 ppm GA_3 solution, ddH_2O for 24 h, or not primed and then germinated at suitable (26°C) or low (10°C) temperatures

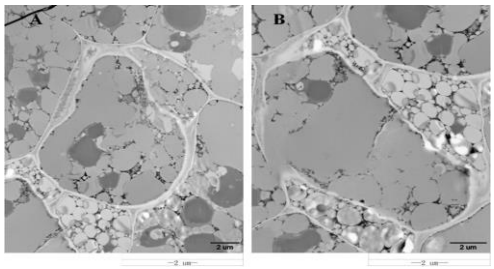

Fig. 2: Water uptake of tobacco seed primed in 100 ppm gibberellic acid (B) solution or ddH_2O (A) for 24 h at 26°C

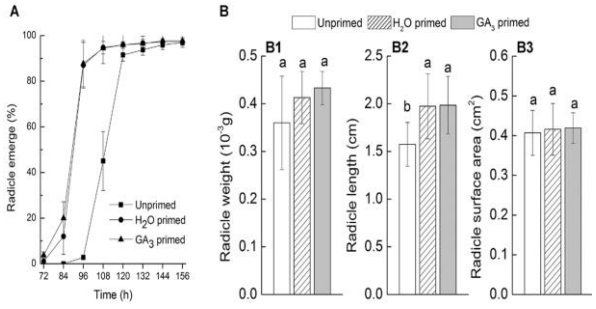

Fig. 3: Radicle emergence (A) and traits (B) of tobacco seed primed in 100 ppm gibberellic acid (GA_3) solution, ddH_2O for 24 h, or not primed and germinated for 156 h at 26℃. Letters indicate significant differences according to the Duncan test (means ± SD, $p < 0.05$), as the same

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_2OP_{24}) seeds, and non-primed (UNP) seeds. At the significant level of both $p \leqslant 0.05$ and fold change ≥ 2, 2,246 up-regulated and 1,644 down-regulated transcripts were identified as differentially expressed between the GA_3-primed and non-primed seeds. Under the same conditions, 2,507 up- and 1,677 down-regulated transcripts between the H_2OP_{24} and UNP seeds, and 1,839 up- and 1,924 down-regulated transcripts between the GA_3P_{24} and H_2OP_{24} seeds were identified.

As the above method, the differential analysis was conducted at the radicle development stage. In the comparison between GA_3-primed germinating (GA_3G_{72}) and non-primed germinating (UNG_{72}) seeds, 1,078 up- and 782 down-regulated transcripts were detected. Similarly, 968 up- and 522 down-regulated transcripts between hydro-primed germinating (H_2OG_{72}) and UNG_{72} seeds, and 673 up- and 1,043 down-regulated transcripts between GA_3G_{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

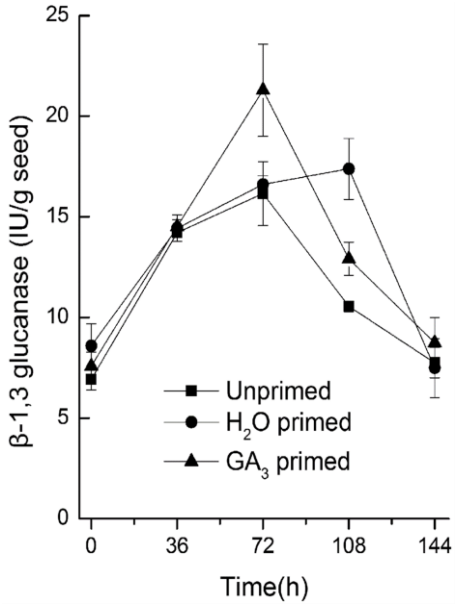

Fig. 4: β-1,3-glucanase activity of tobacco seed primed in 100 ppm gibberellic acid (GA_3) solution, ddH_2O for 24 h, or not primed and then germinated for 144 h at 26°C

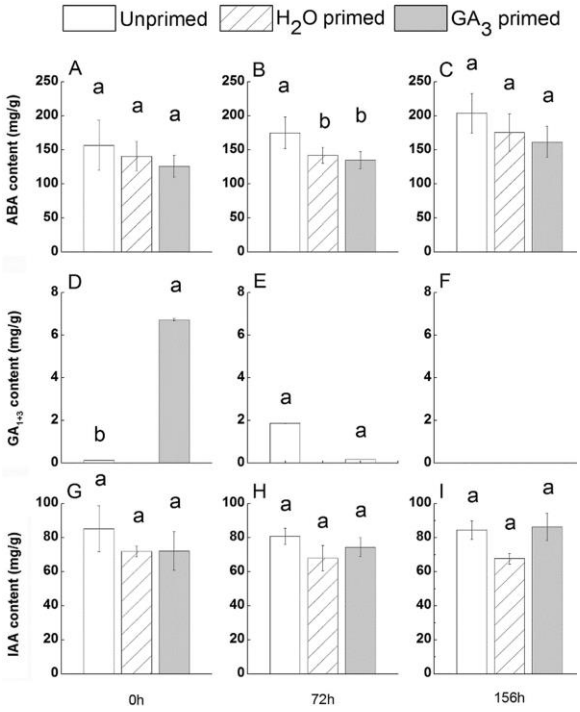

Fig. 5: ABA (a–c), GA_{1+3} (d–f), and IAA (g–i) hormonecontent of tobacco seed after being primed in 100 ppm gibberellic acid (GA_3) solution, ddH_2O for 24 h, or not primed and germinated for 144 h at 26°C

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, 83.89% of which were regulated in the radicle expansion stage. 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.

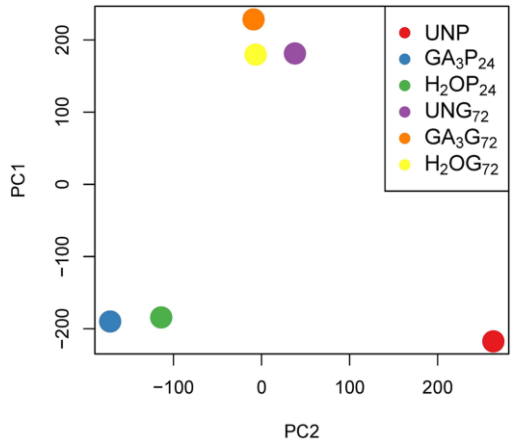

Fig. 6: Principal component analysis (PCA) exploring the relationship among the primed and germinated seeds. The more similar the samples, the more concentrated the spatial distribution of different color points. UNP, H_2OP_{24}, and GA_3P_{24}, respectively, represent the non-primed seeds, ddH_2O-primed seeds, or gibberellic acid (GA_3)-primed seeds. UNG_{72}, H_2OG_{72}, and GA_3G_{72}, respectively, represent germination for 72 h of non-primed seeds, ddH_2O-primed seeds, and GA_3-primed seeds

GA_3-Responsive Genes in the Priming and Germination of *N. tabacum* L. Seeds

Although the transcriptome between GA_3-primed and hydro-primed seeds were similar, there remained a small number of differentially expressed transcripts. To further study these GA_3-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 the main factors that result in the difference molecular mechanisms between GA_3-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.* GA_3P_{24} *vs.* H_2OP_{24}). 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).

Table 2: A table of genes whose expression is altered during seed primed with H_2O or GA_3

Pattern	Comparative gene expression		After priming			Radicle expanding		
	Hydroprimed seeds	seeds/unprimed GA_3 primed seeds/ unprimed seeds	Percentage^{FC}	Transcript^{FC}	Transcript^{P}	Percentage^{FC}	Transcript^{FC}	Transcript^{P}
I	↓	↓	39.36%	15362	694	35.29%	8561	109
II	↑	↓	3.09%	1206	0	6.50%	1577	1
III	↓	↑	3.01%	1174	0	9.61%	2332	8
IV	↑	↑	54.54%	21283	1449	48.60%	11791	344

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

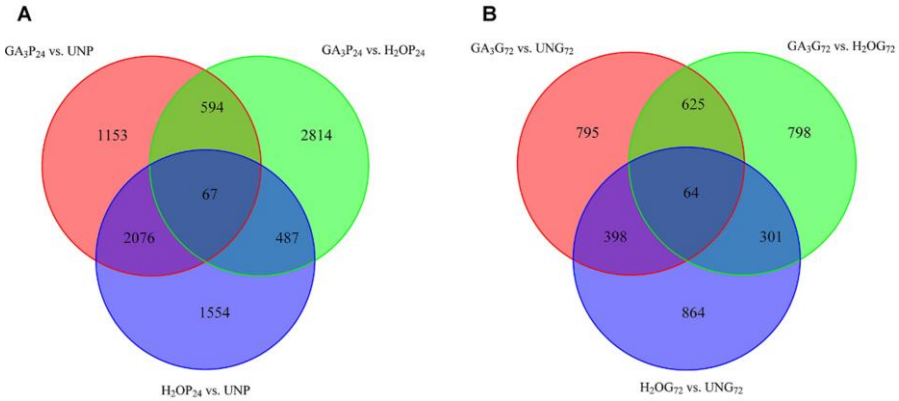

Fig. 7A, B: Venn diagrams representing the cross comparison of differentially expressed transcripts (with more than a two-fold change and p-value ≤ .05) in priming and post-priming germination of tobacco seeds. (A) Seeds after priming. (B) Seeds at the radicle expansion stage. UNP, H_2OP_{24}, and GA_3P_{24}, respectively, represented non-primed seeds, ddH_2O-primed seeds, or gibberellic acid (GA_3)-primed seeds. UNG_{72}, H_2OG_{72}, and GA_3G_{72}, respectively, represent germination for 72 h of non-primed seeds, ddH_2O-primed seeds, or GA_3-primed seeds

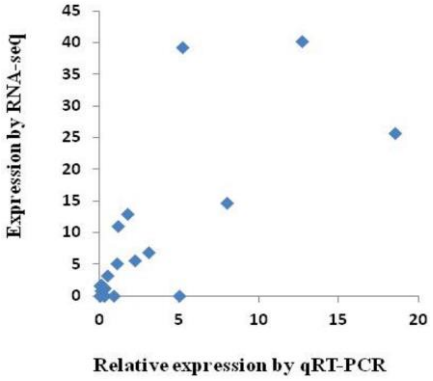

Fig. 8: The expression correlation between qRT-PCR and RNA-seq, and these two methods shown significant positive correlation (Pearson correlation p=1.05E-4, r=0.75; Spearman correlation p =4.96E-4, ρ=0.71)

Using the same methods at the radicle expansion stage, we detected 64 differentially expressed transcripts at the intersection of the three groups (Fig. 7B, UNG_{72} *vs.* GA_3G_{72} *vs.* H_2OG_{72}). 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).

GA_3-Responsive Genes in Important Signaling Pathways

The interactions of ABA and GA (Rajjou *et al*., 2012), light, ABA, and GA (Vanstraelen 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 GA_3-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

Table 3: Representative significant enriched GO terms of the differential expressed transcripts

Seed status	Category	Go term	Reference set	Query set	P-value *(FDR)*	Representative DE transcript	Function
AP	BP	Hormone-mediated signaling pathway	1891	10	5.70E-05	TCONS_00081290	Ras-related protein RAB8-1
						TCONS_00107714	Auxin response factor 5
		Peptidyl-histidine phosphorylation	83	2	4.26E-02	TCONS_00003961	Ethylene receptor ERS homolog
						TCONS_00005529	Histidine kinase cytokinin receptor
		Glutamine family amino acid metabolic process	338	3	4.43E-02	TCONS_00066165	Putative carbamoyl phosphate synthase large subunit, Glutamate synthase 1 [NADH]
						TCONS_00105308	
	MF	Glutathione transferase activity	96	6	1.34E-08	TCONS_00036199	Glutathione S-transferase U9-like
		Glutamine-hydrolyzing activity	29	2	6.42E-03	TCONS_00066166	Carbamoyl phosphate synthase large subunit
		Translation elongation factor activity	273	3	2.29E-02	TCONS_00061525	Elongation factor-1 alpha
RE	BP	Photosynthesis, dark reaction	63	9	5.46E-16	TCONS_00102454	Ribulose bisphosphate carboxylase small subunit protein precursor
		Photosynthesis, light harvesting	141	5	7.60E-06	TCONS_00045152	Chlorophyll a-b binding protein 7
		Response to red light	285	6	8.24E-06	TCONS_00107704	Glyceraldehyde-3-phosphate dehydrogenase B
		Response to far red light	324	6	1.48E-05	TCONS_00008250	Ribulosebisphosphate carboxylase/oxygenaseactivase 2
		Response to blue light	372	6	3.06E-05	TCONS_00034183	Phosphoribulokinase
		Cysteine biosynthetic process	547	5	2.35E-03	TCONS_00032837	Chloroplast pigment-binding protein CP26
		Regulation of hydrogen peroxide metabolic process	287	3	1.69E-02	TCONS_00065605	beta-carbonic anhydrase
		Regulation of oxygen and reactive oxygen species metabolic process	296	3	1.78E-02	TCONS_00034183	Phosphoribulokinase
	CC	ribulose-bisphosphate carboxylase activity	20	7	1.13E-15	TCONS_00102454	Ribulose bisphosphate carboxylase small subunit protein precursor
		Protochlorophyllidereductase activity	51	4	1.94E-06	TCONS_00055160	NADPH:proto chlorophyll ideoxido reductase
		Chlorophyll binding	99	4	1.78E-05	TCONS_00062043	Chlorophyll a-b binding protein 21
		Glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating) activity	8	2	1.94E-04	TCONS_00107704	glyceraldehyde-3-phosphate dehydrogenase B
		glyceraldehyde-3-phosphate dehydrogenase activity	26	2	1.86E-03	TCONS_00025843	Plastidicaldolase NPALDP1
		Fructose-bisphosphate aldolase activity	35	2	3.13E-03	TCONS_00097442	Glyceraldehyde-3-phosphate dehydrogenase A
		NADP or NADPH binding	201	3	4.54E-03	TCONS_00057435	Ferredoxin--NADP reductase
		superoxide dismutase activity	48	2	4.78E-03	TCONS_00008412	24K germin like protein

After priming (AP), Radicle expanding (RE)

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

Seed status	KEGG ID	Refence set	Query set	P-value *(FDR)*	KEEG Pathway
Imbibition	ko05204	6	109	3.25E-07	Chemical carcinogenesis
(Queience)	ko00980	6	131	4.67E-07	Metabolism of xenobiotics by cytochrome P450
	ko00480	6	270	1.75E-05	Glutathione metabolism
	ko04152	4	467	0.026481	AMPK signaling pathway
	ko00250	3	278	0.039827	Alanine, aspartate and glutamate metabolism
Radicle expansion	ko00710	13	290	1.06E-15	Carbon fixation in photosynthetic organisms
	ko01200	13	1041	5.90E-09	Carbon metabolism
	ko00630	8	252	1.33E-08	Glyoxylate and dicarboxylate metabolism
	ko00195	6	154	4.20E-07	Photosynthesis
	ko00196	4	78	2.19E-05	Photosynthesis - antenna proteins
	ko00860	4	180	4.91E-04	Porphyrin and chlorophyll metabolism

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. For crosstalk of ABA and GA, ABA biosynthesis genes *NCED9* and *AAO3* were down-regulated, ABA receptors *PYL1* was up-regulated, and ABA negative signal regulator *ABI4* was up-regulated after GA_3 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*,

Table 5: Fold changes of significant differential expressed transcripts revealed in GA_3 or H_2O-responsive seed priming and germination regulation by RNA-seq

Pathway	Transcript	Encoding protein	Biological function	GA_3P_{24}/ UNP	H_2OP_{24}/ UNP	GA_3P_{24}/ H_2OP_{24}	GA_3G_{72}/ GUN_{72}	H_2OG_{72}/ GUN_{72}	GA_3G_{72}/ GH_2O_{72}
ABA & GA	TCONS_00086103	GA2ox2	GA deactivating	0.516	0.222	2.364	0.989	0.934	1.058
	TCONS_00038628	GA3ox1	GA biosynthesis	13.955	14.053	1.009	0.823	1.449	0.568
	TCONS_00084330	CYP707A1	ABA deactivating	79.418	130.036	0.621	0.579	1.445	0.401
	TCONS_00061964	CYP707A2	ABA deactivating	6.515	6.812	0.972	0.826	1.300	0.635
	TCONS_00088749	CYP707A3	ABA deactivating	30.036	41.770	0.731	1.501	1.609	0.933
	TCONS_00041203	ABA1	ABA biosynthesis	0.646	0.712	0.922	0.467	1.013	0.461
	TCONS_00105851	ABA2	ABA biosynthesis	0.653	1.086	0.611	0.452	0.285	1.588
	TCONS_00072648	NCED2	ABA biosynthesis	0.415	0.840	0.502	4.943	6.538	0.756
	TCONS_00027758	NCED6	ABA biosynthesis	2.145	1.945	1.120	0.102	2.190	0.047
	TCONS_00047307	NCED9	ABA biosynthesis	0.255	3.402	0.076	0.883	2.057	0.429
	TCONS_00100200	AAO3	ABA biosynthesis	0.059	0.167	0.357	1.208	1.349	0.896
	TCONS_00039776	MCSU	ABA biosynthesis	1.018	0.756	1.368	0.494	0.467	1.058
	TCONS_00068807	PYL1	ABA receptors	11.200	0.756	15.053	0.076	1.221	0.062
	TCONS_00077460	PYL3	ABA receptors	7.709	9.612	0.815	0.488	0.735	0.664
	TCONS_00035674	PYL4	ABA receptors	30.545	14.364	2.161	3.954	1.535	2.577
	TCONS_00069450	PYL7	ABA receptors	154.763	271.412	0.579	0.310	3.004	0.103
	TCONS_00083359	ABI2	Protein phosphatase 2C	1.018	3.024	0.342	0.220	0.519	0.423
	TCONS_00025778	AHG3	Protein phosphatase 2C	0.630	0.731	0.875	0.337	1.515	0.222
	TCONS_00063906	ABI3	Protein phosphatase 2C	0.292	0.397	0.748	0.151	0.702	0.215
	TCONS_00034519	ABI4	Protein phosphatase 2C	2.764	1.296	2.167	0.330	2.179	0.151
	TCONS_00091263	ABI5	Protein phosphatase 2C	0.404	0.442	0.929	0.328	1.078	0.304
	TCONS_00026510	SnRK2.6	Protein kinase	0.039	0.058	0.684	0.187	0.025	7.409
	TCONS_00016966	SnRK2.1	Protein kinase	0.170	0.343	0.502	0.767	1.578	0.486
ABA & AUX	TCONS_00093569	TIR1	AUX receptors	0.486	0.520	0.949	0.966	1.145	0.844
	TCONS_00057561	ARF10	Auxin response factor	0.148	0.550	0.274	1.193	0.904	1.319
	TCONS_00064315	ARF16	Auxin response factor	1.018	0.756	1.368	3.954	0.934	4.234
	TCONS_00046509	ARF17	Auxin response factor	0.127	0.473	0.274	0.989	0.934	1.058
	TCONS_00107488	PIN3	Auxin efflux carrier	6.982	6.264	1.132	2.170	1.547	1.403
	TCONS_00053233	IAA14/SLR	Auxin signal transduction	7.035	7.973	0.897	2.087	1.106	1.888
	TCONS_00072463	AXR2/IAA7	Auxin signal transduction	3.394	2.604	1.324	1.494	1.213	1.232
Light & ABA & GA	TCONS_00090795	PHYA	Photoreceptors	1.045	0.121	8.793	1.749	1.105	1.583
	TCONS_00077134	PHYB	Photoreceptors	5.091	1.848	2.799	1.003	0.599	1.674
	TCONS_00001444	COP1	E3 ubiquitin-protein ligase	1.018	94.503	0.011	0.024	0.205	0.118
	TCONS_00071334	HY5	Transcription factor	2.061	3.613	0.580	1.030	0.912	1.129
	TCONS_00052394	PIF3	Phytochrome interacting factor	0.509	0.630	0.821	2.471	4.203	0.588
	TCONS_00055485	PIF4	Phytochrome interacting factor	1.018	0.756	1.368	0.989	35.495	0.028
	TCONS_00004377	PIL5/PIF1	Phytochrome interacting factor	0.058	0.151	0.391	0.387	1.218	0.318
	TCONS_00018854	XERICO	RING-H2 protein	0.034	0.245	0.142	0.089	0.491	0.181
	TCONS_00104432	DEELLA	DEELLA	10.691	9.450	1.149	1.256	0.303	4.145
	TCONS_00101768	GAMYB	Transcription factor	0.629	0.234	2.737	2.035	2.995	0.680
	TCONS_00106991	Alpha-amylase	Alpha-amylase	1.527	1.512	1.026	1.412	0.600	2.352
Epigenetic regulators	TCONS_00057450	HUB1	C3HC4 RING finger	4.276	2.419	1.796	1.095	0.924	1.185
	TCONS_00065033	HUB2	C3HC4 RING finger	4.020	3.102	1.317	2.778	3.028	0.918
	TCONS_00072327	EFS	Histone H3 methyltransferase	9.978	0.756	13.411	2.754	2.135	1.290
	TCONS_00068779	KYP	Histone methyltransferase	12.218	0.756	16.421	1.483	0.467	3.175
Dormancy-specific genes	TCONS_00071015	DOG1	Unknown protein	0.325	0.374	0.882	0.270	1.269	0.213
	TCONS_00046918	MFT	Phosphatidylethanolamine-binding protein	0.210	0.154	1.391	0.233	1.355	0.172
Release from dormancy	TCONS_00104313	AtHB-20	NADPH-oxidase	3.055	1.512	2.053	3.542	0.156	22.755
	TCONS_00076152	AtrbohB_1	NADPH-oxidase	1.018	0.756	1.368	5.190	9.107	0.570
	TCONS_00040697	PRT6	Targeted proteolysis	2.715	1.627	1.696	0.989	4.814	0.205

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, ρ = .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 GA_3 priming. GA and ABA were the key hormones during seed germination with GA_3 promoting and ABA inhibiting it (Graeber *et al*., 2012). In this study, GA_{1+3} level was increased after GA_3 priming compared with non-primed and hydro-primed seeds. Nakaune *et al.* (2012) showed similar results in tomato seeds, GA_{1+4} 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 (Hilhorst, 1995; Bewley, 1997b; Koornneef *et al*., 2002). In this study, only second function could be confirmed, which was determined over the activity changes of βGlu I, an 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 GA_3 priming in this study. Similar results were obtained in tomato, ABA content was also not influenced by salt priming (Nakaune *et al*., 2012). However, ABA biosynthesis genes *NCED9* and *AAO3* were downregulated in primed seeds, *ABA1* and *NCED6* downregulated in germinating seeds respectively. Therefore, GA_3 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 GA_3 priming in this study, which was confirmed by the expression pattern of metabolic genes. Auxin performing function need to recruite 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, 1996). PIF1is 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 stuation (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 GA_3-primed seed were the significant enrichment in light signals. Therefore, we propose that light signals are stimulated or partly replaced by GA_3 priming during seed germination.

Conclusion

GA_3 priming promotes seed germination under low temperature. Phenotypically, germination speed and uniformity of radicle emerging were rasied by GA_3 priming. Physiologically, GA_3 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 GA_3 priming during seed imbibition.

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