agronomy

MDPI

*Article*

# Genomic Insights into Seed Germination Differences in Buffalobur (*Solanum rostratum* Dunal) under Contrasting GA and ABA Availability

**Zhaoxia Chen †, Longlong Li †, Kaidie Wu, Dandan Zhao, Long Yang, Hongjuan Huang *, Zhaofeng Huang and Shouhui Wei ***

State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China; chenzhaoxia@norsyn.com (Z.C.); 82101215277@caas.cn (L.L.); 82101235351@caas.cn (K.W.); njzxcb_2022@163.com (D.Z.); tjsnyzhxzzfzdzfzhs@tj.gov.cn (L.Y.); huangzhaofeng@caas.cn (Z.H.)

* Correspondence: hjhuang@ippcaas.cn (H.H.); weishouhui@caas.cn (S.W.)

† These authors contributed equally to this work.

**Abstract:** Buffalobur (*Solanum rostratum* Dunal) is an invasive species that seriously endangers crop production and the ecological environment. Seeds are the primary source of infestation; therefore, understanding the molecular basis of buffalobur seed dormancy, and germination is crucial for precision weed management. In this study, high-throughput RNA-Seq was performed on buffalobur seeds, which imbibed under 0.35 mmol/L giberellic acid (GA) and 0.35 mmol/L abscisic acid (ABA). In total, 3658 differentially expressed genes (DEGs) were identified during seed germination. Gene annotation revealed that the DEGs were significantly enriched during the protein metabolic process, as well as the macromolecular complex and cytoplasmic part for ABA versus GA. Pathway analysis predicted that the DEGs were associated with metabolic pathways, the biosynthesis of secondary metabolites and ribosome. Nine germination-related genes involved in the biosynthesis and metabolism of the phytohormones and encoding of the endo-β-mannanase (EBM) were identified. Gene expression indicated that GA upregulated *GA3OX1* and *MAN2* expression to increase the EBM activity, which caused the endosperm cap to weaken and lowered the puncture force to trigger the germination of buffalobur. The obtained results would be helpful to clarify the regulation of seed dormancy and the germination of buffalobur, and could serve as a valuable resource when unravelling the genetic basis of seed biology of this weed species.

**Keywords:** seed dormancy and germination; RNA-Seq; phytohormone; endo-β-mannanase; endosperm cap



**Citation:** Chen, Z.; Li, L.; Wu, K.; Zhao, D.; Yang, L.; Huang, H.; Huang, Z.; Wei, S. Genomic Insights into Seed Germination Differences in Buffalobur (*Solanum rostratum* Dunal) under Contrasting GA and ABA Availability. *Agronomy* **2024**, *14*, 212. https://doi.org/10.3390/agronomy14010212

Academic Editor: Daniela Romano

Received: 26 December 2023
Revised: 13 January 2024
Accepted: 16 January 2024
Published: 18 January 2024



## 1. Introduction

Buffalobur (*Solanum rostratum* Dunal) is an exotic weed species of Solanaceae family, which is native to North America and spreads to nearly 20 countries and regions in the world [1,2]. It is a noxious weed, which has strong adaptability and is fast-growing, and livestock may experience poisoning symptoms or even death if it is eaten by mistake [3]. In addition, it is also an intermediate host of destructive pests and diseases, which seriously endangers agricultural production [4,5].

Seeds are an important media for the dispersal and infestation of buffalobur [6]. Germination is a critical stage for control in their life-cycle. Seeds can adjust their germination timing through dormancy, allowing for plants to complete their vegetative and reproductive growth under more favorable conditions [7,8]. Phytohormones are important factors that affect the transition of seeds from dormancy to germination. Abscisic acid (ABA) and gibberellic acid (GA) are two classical phytohormones, and many reports indicate that they antagonize each other to regulate seed dormancy and germination [9]. ABA plays crucial roles in the

maintenance and induction of seed dormancy. Previous reports showed that the binding of ABA to its receptors could cause interactions that inhibit *PP2C* activity and thus relieve *SnRK2* to initiate downstream genes that respond to ABA [10–13]. The 9-cis-epoxycarotenoid dioxygenase (NCED) is a vital enzyme, responsible for ABA biosynthesis, which is widely reported in *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.), tomato (*Solanum lycopersicum* L.), tobacco (*Nicotiana tabacum* L.) and wheat (*Triticum aestivum* L.) [14–17]. Expression analysis proved that the *CYP707A* gene could play a mediating role in vivo, determining the contents of ABA under imbibition and stress conditions [18]. GA plays an important role in promoting germination and breaking seed dormancy. The gibberellin-insensitive gene (*GAI*) is one of the DELLA proteins that negatively regulates the gibberellin signaling pathway. The upregulation of *GAI* inhibits the process of seed germination [19–22]. GA 20-oxidase (*GA20OX*) and GA 3-oxidase (*GA3OX*) are crucial enzymes involved in gibberellin biosynthesis, and their upregulation can effectively promote the breaking of dormancy, while GA 2-oxidase (*GA2OX*) is involved in gibberellin catabolism, and its overexpression affects the process of seed germination [23–26].

Buffalobur belongs to the same genus as tomato, a typical species in studies of seed germination [27]. The endosperm cap of Solanoideae-type seeds is the place of radicle protrusion [28,29], which constitutes a major barrier for the germination of tomato seeds [30]. Endo-β-mannanase (EBM; E.C.3.2.1.78) is a key enzyme for the hydrolysis of mannan-rich cell wall, and thus weakens the endosperm cap. The cell walls of the solanaceous seed endosperm contain approximately 60% mannans, and the EBM is involved in the degradation of mannan polymers [28]. The EBM activity showed a negative correlation with the force needed to penetrate the endosperm cap. The weakening of the endosperm cap by EBM may possibly provide a pre-requisite for radicle protrusion [31]. In tomato seeds, ABA could repress germination by inhibiting phase II of endosperm cap-weakening without suppressing the EBM activity [31]. GA could trigger the weakening of endosperm cap and induce cell-wall hydrolysis, which allows the radicle protrude to pass through the endosperm cap [30].

Transcriptome and bioinformatics are important tools for gene-mining and a comprehensive analysis of molecular regulation in many species. Our objectives are to: (1) use high-throughput RNA-Seq to analyze the gene expression profiles of the seed germination of buffalobur under GA and ABA treatments; (2) unveil the interaction between GA and ABA in terms of their effects on puncture force and EBM activity during the seed germination of buffalobur; and (3) mine potential genes that might be used in the regulation of seed dormancy and germination for the integrated management of buffalobur.

## 2. Materials and Methods

### *2.1. Plant Material*

Mature buffalobur berries were collected from multiple plants from a corn (*Zea mays* L.) field in Beijing, China (longitude E 116.84°, latitude N 40.40°), in early December 2021. Berries were broken and seeds were separated using a sieve under tap water, before being rinsed and dried at a temperature of 25 ± 3 °C for 7 d, and then kept in paper bags at 5 °C for further use.

### *2.2. Seed Germination Assays*

Buffalobur seeds were placed in petri dishes (the diameter = 9 cm) on two layers of filter paper, which were moistened with 7 mL of dd$H_2O$ or a test solution of 0.35 mmol/L GA (giberellic acid, Sinopharm, Beijing, China) and 0.35 mmol/L ABA (abscisic acid, Sinopharm, China). Each of these three treatments contains three biological replicates with 100 seeds. All seeds were incubated for 7 d at 30 ± 0.5 °C with constant darkness. The number of germinated seeds was recorded and removed daily from the 1st to the 7th day during imbibition. The germination index (GI) was estimated using the following equation: $GI = \sum \frac{Gt}{Dt}$, where *Gt* is the germinated seed number on day *t*, and *Dt* is the corresponding days to *Gt*. The seeds from the same treatment were put together, and three biological

replicates were collected at 0 h, 2 h, 12 h, 24 h, 72 h, 120 h and 168 h after seed imbibition. Samples were immediately placed in liquid nitrogen and stored at −80 °C.

### *2.3. RNA Extraction and Quality Evaluation*

Extractions of total RNA were performed with TRIzol-A+ reagent (TIANGEN BIOTECH, Beijing, China) and RNase-free DNase I (TaKaRa Bio Inc., Beijing, China). RNA quantities were determined by the Nanodrop Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). RNA quality was measured using an Agilent Bioanalyzer Model 2100 (Agilent Technologies, Santa Clara, CA, USA). Three biological samples of each treatment with an RNA integrity number (RIN) larger than 7.5 were accepted by the requirements during the Illumina transcriptome sequencing protocol.

### *2.4. Library Generation and RNA-Seq*

A pooled cDNA library of 21 seed samples, which correspond to 7 treatments (0 h, 24 h and 72 h of $ddH_2O$ imbibition; 24 h and 72 h of GA imbibition; 24 h and 72 h of ABA imbibition), were analyzed to obtain comprehensive transcripts of buffalobur. The library for sequencing was constructed with NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) according to the manufacturer's recommendations. The library fragments were treated with AMPure XP system (Beckman Coulter, Beverly, MA, USA) and the qualities were evaluated using Agilent Bioanalyzer 2100 system. Construction of the mRNA pool was conducted following the methods reported by Wang et al. [32]. The constructed cDNA libraries were sequenced by Illumina Hiseq 4000. An assembly of clean reads was performed in Trinity. Unigenes were annotated using seven databases [33,34]: NCBI non-redundant protein sequences database (NR), NCBI nucleotide sequences database (NT); KEGG orthology database, gene ontology (GO), and clusters of orthologous groups of proteins (COG/KOG). The expression of the unigene was evaluated as fragments per kilobase of transcript per million reads mapped (FPKM) [35]. FPKM value was estimated by RSEM [36].

### *2.5. Differentially Expressed Gene Analysis*

DESeq2 ver. 1.26 was used to perform the differential expression analysis [37]. The obtained $p$ value was adjusted using the previously reported approach to control the false discovery rate (FDR) [38]. The genes with a $p$ value < 0.05 detected by DESeq were denoted as differentially expressed genes (DEGs). The expressions of each gene between sample pairs were compared (GA vs. ABA, GA vs. $H_2O$, ABA vs. $H_2O$). Gene annotation of enrichment analysis was conducted by GOseq R packages ver. 1.22 with Wallenius non-central hyper-geometric distribution [39]. DEGs enriched in KEGG pathways were analyzed with KOBAS software 3.0 [40].

### *2.6. Quantitative Real-Time PCR (qPCR)*

DEGs were further evaluated by qPCR. Three RNA samples of each treatment were obtained from buffalobur seeds, which were treated with 0.35 mmol/L GA, 0.35 mmol/L ABA or $ddH_2O$ for 0, 2, 12, 24, 72, 120 and 168 h. This was carried out using first-strand cDNA synthesis instruction manual to reverse transcription. Primers for qPCR were designed using the Oligo 7 (Molecular Biology Insights Inc., Colorado Springs, CO, USA). The qPCR was conducted using the 7500 RealTime PCR System (Applied Biosystems, Waltham, MA, USA). The reaction system and cycling conditions were set by the method used by Chen et al. [41]. The expressions of DEGs, normalized to reference gene *PP2Acs* (GeneBank ID: MK181639), were analyzed using the $2^{-\Delta\Delta Ct}$ method [42,43].

### *2.7. EBM Extraction and Determination*

Seeds of buffalobur were imbibed in 0.35 mmol/L ABA, 0.35 mmol/L GA or $ddH_2O$, and incubated under the conditions described in Section 2.2. Three biological replicates of each treatment were sampled daily for EBM extraction. The volume of Hepes buffer used

to grind the seeds was 300 mL. The subsequent extraction of EBM and determination of EBM activities were performed using the methods reported by Pinto et al. [28].

### *2.8. Puncture Force Measurement*

Buffalobur seeds were treated with 0.35 mmol/L ABA, 0.35 mmol/L GA or dd$H_2O$, and incubated under the conditions described in Section 2.2. Three biological replicates (30 seeds each) of each treatment were sampled daily to assess the force needed to puncture the seed endosperm cap. The measurement of puncture force was conducted using the methods described by Pinto et al. [28]. The puncture force, measured in Newtons (N), was used to compare the mechanical resistance of seed endosperm caps under different treatments.

### *2.9. Statistical Analyses*

The data obtained in this experiment were analyzed using Excel 2019 and SPSS Statistics 22.0 (IBM, Armonk, NY, USA). One-way ANOVA and Duncan's multiple range test were used to determine significant differences ($p \leq 0.05$, 0.01 or 0.001).

## 3. Results

### *3.1. Buffalobur Seed Germination under GA and ABA Imbibition*

Different phytohormones have contrasting effects on the seed germination of buffalobur. A dramatic increase in seed germination occurred 4 d after imbibition with 0.35 mmol/L GA, but the seeds imbibed with ABA remained ungerminated (Figure 1). The results also showed that the seeds undergoing GA imbibition germinated earlier and faster, and the germination index of seeds imbibed with GA and ABA were 15.05 and 0.00, indicating a significant difference in germination speed between these treatments.

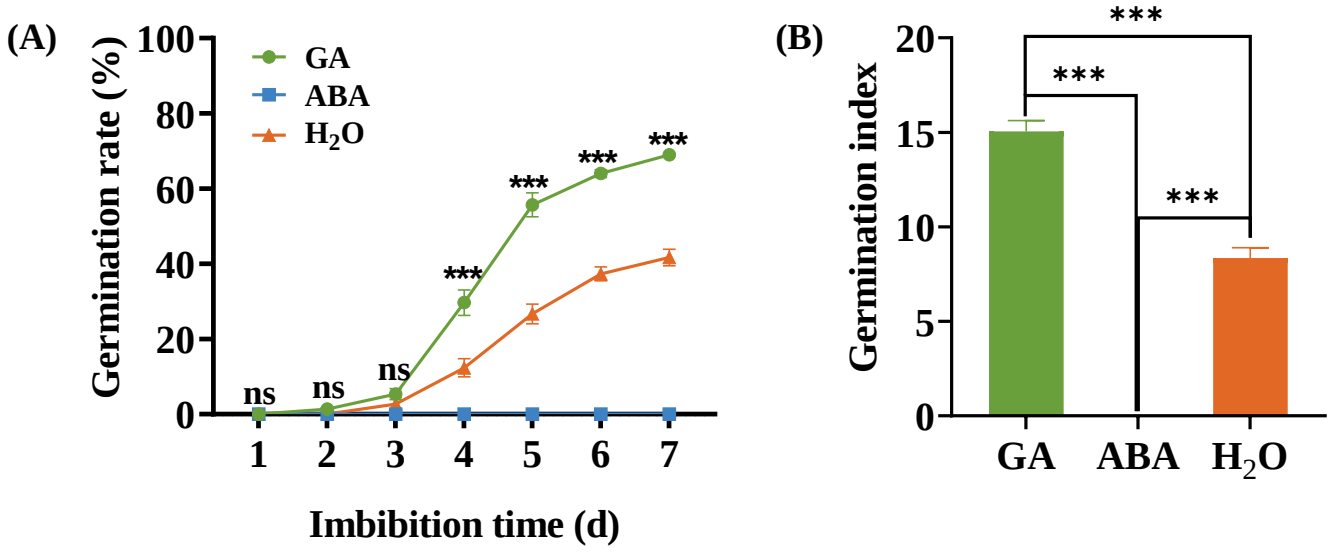


**Figure 1.** Effect of GA and ABA on seed germination of buffalobur. (**A**) Germination rate of buffalobur; (**B**) germination index of buffalobur. Vertical bars represent the standard error of the means. *** represent significant differences at $p \leq 0.001$ level; ns represents no significant differences.

### *3.2. Unigenes and Functional Annotation*

To further elucidate the molecular basis of phytohormone on seed germination, RNA-Seq were performed using the buffalobur seeds under GA and ABA imbibition. A total of 336,057 unigenes, which ranged from 201 to 16,961 bp and with an N50 of 818 bp, were obtained from the transcriptome sequencing of 21 sample datasets (Table 1). The species that were mapped as containing the most unigenes were *Solanum tuberosum* L. (11.04%), *Alternaria alternata* (Fr.) Keissl. (7.12%) and *S. pennellii* Correll (3.89%). There were 224,740

(66.88%) unigenes annotated in these databases, 18,134 (5.40%) of which were present in all seven databases. The unigenes annotated in NR were the most numerous (47.56%). A total of 101,187 unigenes were assigned to 42 functional categories in the GO database. A total of 75,203 unigenes were categorized into 26 groups in KOG, and 63,579 unigenes were classified into 129 pathways in KEGG (Table 1).

**Table 1.** Transcriptome sequencing and gene annotation for buffalobur.

| | **Statistic Value** |
|---|---|
| Total clean reads | 23,047,819–26,014,113 |
| Unigenes | 336,057 |
| Min sequence length | 201 |
| Median sequence length | 336 |
| Max sequence length | 16,961 |
| N50 length | 818 |
| N90 length | 248 |
| Annotated in NR | 159,827 (47.56%) |
| Annotated in NT | 122,783 (36.54%) |
| Annotated in KEGG | 63,579 (18.92%) |
| Annotated in SwissProt | 97,319 (28.96%) |
| Annotated in Pfam | 126,338 (37.59%) |
| Annotated in GO | 101,187 (30.11%) |
| Annotated in COG/KOG | 75,203 (22.38%) |

### *3.3. Identifying DEGs and qPCR Validations*

Differentially expressed genes (DEGs) were analyzed, and a total of 3658 DEGs was identified (Figure 2A). A comparison with $H_2O$, ABA and GA revealed 718 (128 upregulated and 590 downregulated) and 2765 genes (1406 upregulated and 1359 downregulated), respectively, that were differentially expressed. There were 1281 DEGs between ABA- and GA-treated seeds; 490 genes were downregulated in ABA and 791 genes were upregulated in GA. Nineteen DEGs were detected in three comparison groups, which indicates that they may play a role in all three treatments. However, 2008, 210 and 353 DEGs were only differentially expressed in GA vs. $H_2O$, ABA vs. $H_2O$ and ABA vs. GA groups (Figure 2B), which also indicates that these genes may be responsible for the changes in the different germination states.

All DEGs were annotated, and the classification of GO and KEGG is shown in Figure 3. In GO, for ABA vs. GA, the top three classifications of gene function terms were the protein metabolic process, macromolecular complex and cytoplasmic part; for GA vs. $H_2O$, the top three processes were the cellular component, cell part and cell; for ABA vs. $H_2O$, the top three terms were the primary metabolic process, macromolecule metabolic process and protein metabolic process (Figure 3A). In KEGG, for all sample pairs, DEGs are enriched in the metabolic pathways, the biosynthesis of secondary metabolites and ribosome. For ABA vs. GA, the top three pathways were the same as the overall annotations. In addition, carbon metabolism, protein processing in endoplasmic reticulum and the biosynthesis of amino acids are also important, leading to at least 64, 59 and 46 DEGs being enriched (Figure 3B).

To evaluate the reliability of transcriptome sequencing, 20 DEGs were randomly selected to determine their relative expression through qPCR. The correlation between FPKM in RNA-Seq and qPCR is presented in Figure 4. Primers for expression analysis of 20 DEGs in buffalobur seeds can be found in Table S1. Primers of reference gene and genes related to seed dormancy and germination in buffalobur seeds can be found in Table S2. Although some variations appeared due to the different quantitative analysis methods, the expression of about 80.8% RNA-Seq data was in accordance with that of the qPCR results, indicating that the RNA-Seq data were reliable for subsequent analyses.

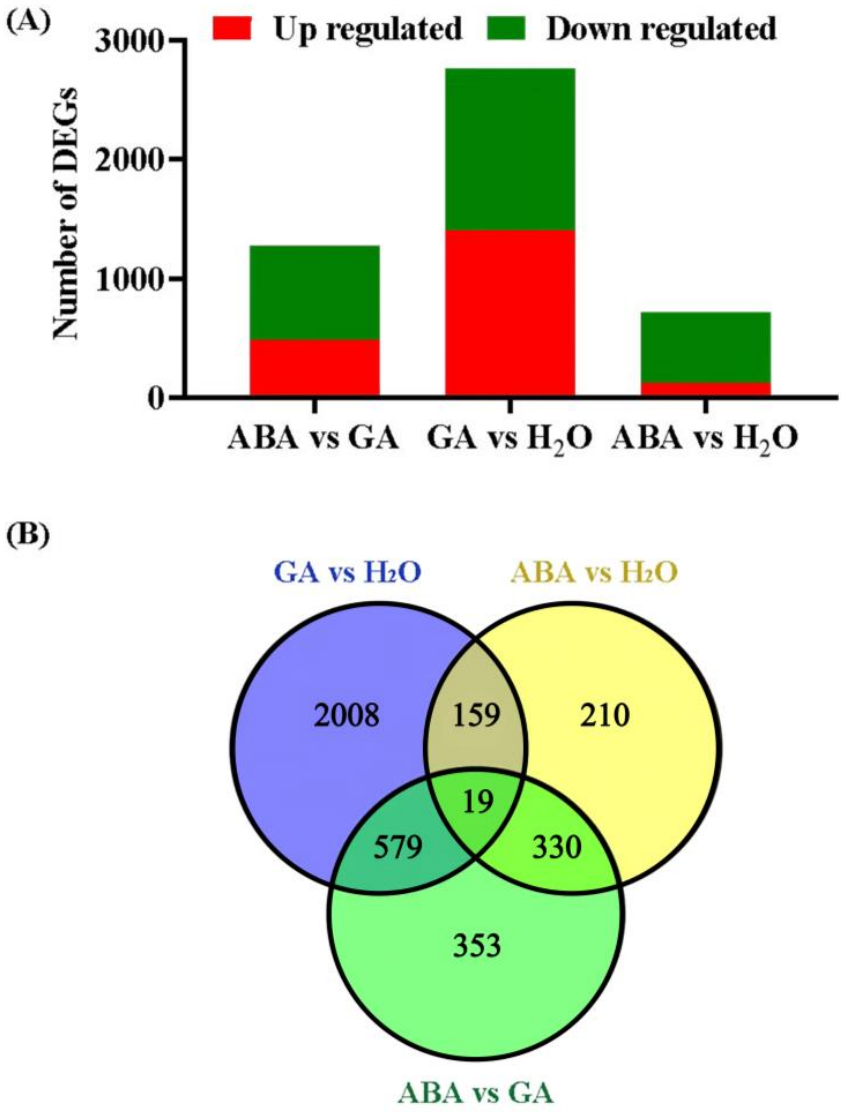


**Figure 2.** The number of differentially expressed genes (DEGs) of three sample pairs in buffalobur seeds. (**A**) Bar chart of DEGs among three sample pairs; GA, ABA and $H_2O$ represent the samples treated with 0.35 mmol/L GA, 0.35 mmol/L ABA or dd$H_2O$. The bar marked with green indicates upregulated genes, and the red indicates downregulated genes. (**B**) Overlapping Venn diagram of DEGs. All differentially expressed genes occurred in different differential combinations; numbers denote DEGs included in each subset.

### *3.4. Expression Analysis of Germination-Related DEGs*

To unravel the molecular mechanisms underlying the germination differences that occur under different hormone conditions, nine seed-dormancy- and germination-related DEGs were identified (Table 2). Three genes are involved in the GA signaling pathway (*GA3OX1*, *GA2OX2* and *GAI*), three genes are associated with the ABA pathway (*NCED6*, *CYP707A* and *PYL2*) and three genes do not directly participate in hormone signaling pathways (*MAN2*, *MAN5* and *MAN7*). Here, we examined the relative expression of these nine genes within 7 d under different treatments (Figure 5).

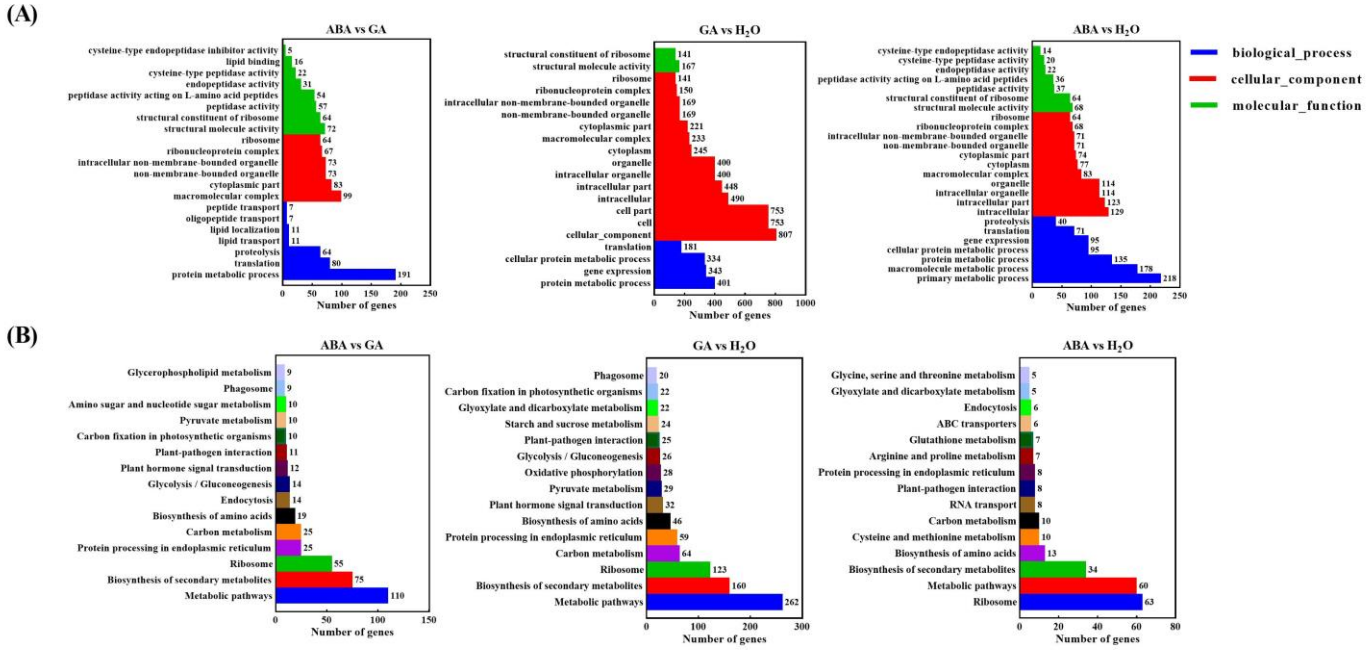


**Figure 3.** GO and KEGG classification of DEGs of three sample pairs in buffalobur seeds. (**A**) DEGs annotated in GO; (**B**) DEGs annotated in KEGG. GA, ABA and $H_2O$ represent the samples treated with 0.35 mmol/L GA, 0.35 mmol/L ABA or dd$H_2O$.

**Table 2.** KEGG annotation of DEGs relevant to the seed germination of buffalobur.

| Gene ID | Gene Name | Gene Symbol | KEGG Annotation | | |
|---|---|---|---|---|---|
| | | | Pathway ID | KO ID | KO Description |
| c155529_g1 | GA-insenstive | *GAI* | - | - | |
| c228847_g2 | gibberellin 3-beta-dioxygenase 1 | *GA3OX1* | ko00904 | K04124 | gibberellin 3-beta-dioxygenase |
| c233593_g3 | gibberellin 2-oxidase 2 | *GA2OX2* | ko00904 | K04125 | gibberellin 2-oxidase |
| c178034_g1 | pyrabactin resistance-like 2 | *PYL2* | ko04075 | K14496 | abscisic acid receptor PYR/PYL family |
| c227970_g1 | 9-cis-epoxycarotenoid dioxygenase 6 | *NCED6* | ko00906 | K09840 | 9-cis-epoxycarotenoid dioxygenase |
| c197117_g2 | abscisic acid 8′-hydroxylase | *CYP707A* | ko00906 | K09843 | (+)-abscisic acid 8′-hydroxylase |
| c208761_g1 | β-mannanase 2 | *MAN2* | - | - | - |
| c233052_g1 | β-mannanase 5 | *MAN5* | - | - | - |
| c194246_g2 | β-mannanase 7 | *MAN7* | - | - | - |

*GA3OX1* is the gene-encoding key enzyme involved in GA biosynthesis [24–26]. Its expression was significantly upregulated in GA-treated seeds (with the highest relative expression of 48.44) compared to $H_2O$-treated seeds (2.00), while ABA significantly downregulated its expression. This shows that the endogenous GA synthesis increases during imbibition, and the application of GA promotes more endogenous GA synthesis, while the application of ABA inhibits the endogenous GA synthesis. *GA2OX2*, which encodes key enzymes involved in GA catabolism [44,45], was significantly upregulated in GA-treated seeds (18-fold at 72 h), whereas, in ABA-treated seeds, its expression was significantly downregulated. That is to say, in the process of imbibition, the external application of GA can promote the metabolism of GA, while the external use of ABA has no obvious impact on the metabolism of GA. GAI is a conserved suppressor of GA [46,47]. The relative

expression of GAI for seeds with GA treatment was the highest at 24 h. No significant difference was observed in the expression of GAI under the three treatments.

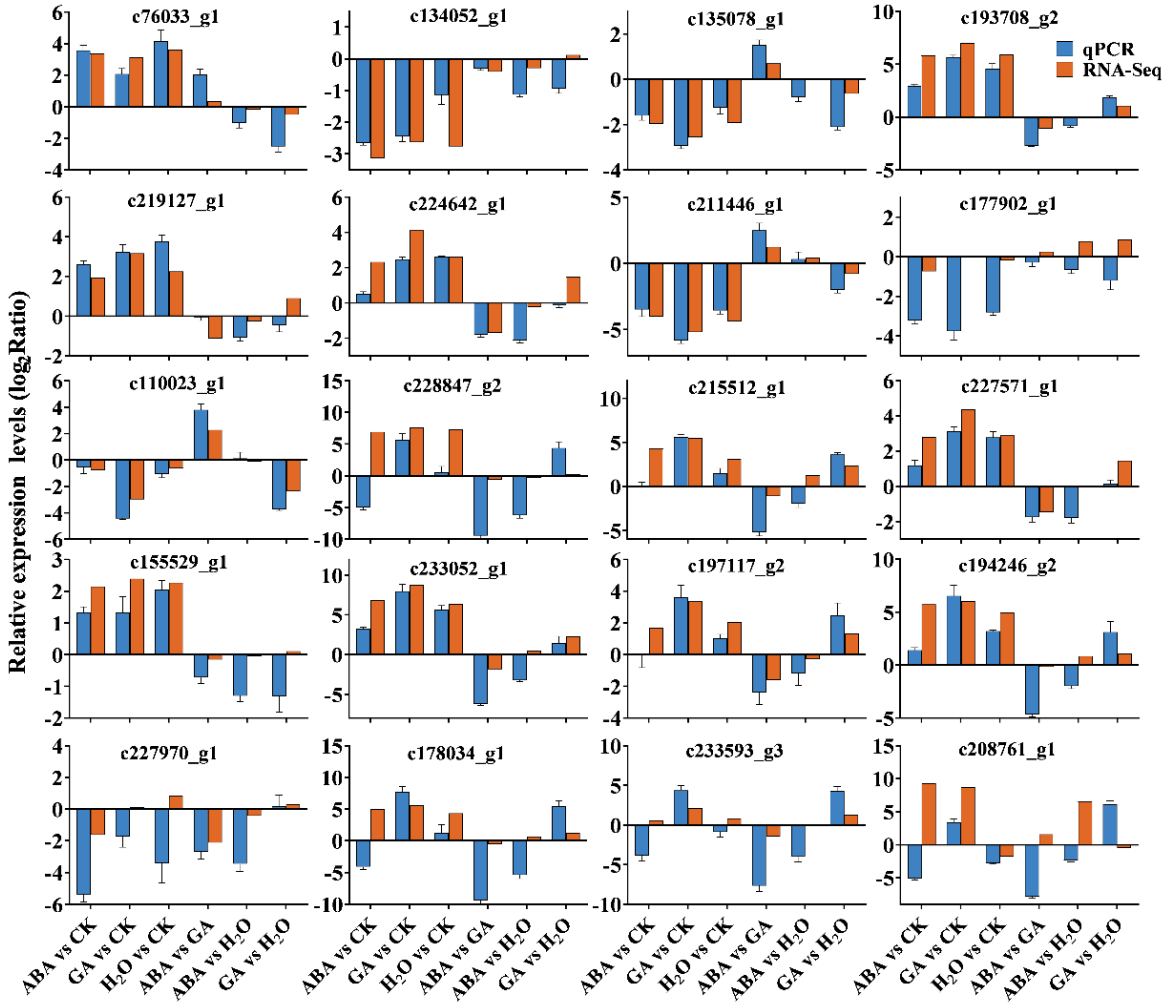


**Figure 4.** Gene expression of 20 DEGs in different sample pairs using RNA-Seq and qPCR. GA, ABA and $H_2O$ represent the samples treated with 0.35 mmol/L GA, 0.35 mmol/L ABA or dd$H_2O$. Negative indicates downregulation; positive indicates upregulation. Vertical bars represent the standard error of the means.

*NCED6* is the ABA biosynthetic gene [48–50]. GA promoted the *NCED6* expressions for from 2 to 24 h of imbibition. Within 2 h of imbibition, *NCED6* was rapidly downregulated, and the external application of GA delayed the downregulation of the gene compared with other treatments. *CYP707A*, which plays an essential role in ABA catabolism, was significantly upregulated in GA-treated seeds compared to $H_2O$-treated seeds. During imbibition, the dynamic expression of *CYP707A* promotes the decomposition of endogenous ABA. Under the treatment of exogenous GA, the expression of *CYP707A* reached the highest level at 2 h, preparing for the seed to break dormancy and start germination. However, exogenous ABA has no significant effect on *CYP707A* expression. For receptor *PYL2*, expression was significantly upregulated after GA treatment (with a relative expression of 134.33 at 72 h) and downregulated after ABA (0.09 at 12 h) and $H_2O$ treatment (4.05 at 72 h). For the expression of *PYL2*, the application of GA was significantly different from that of ABA and water, reaching its peak at 72 h.

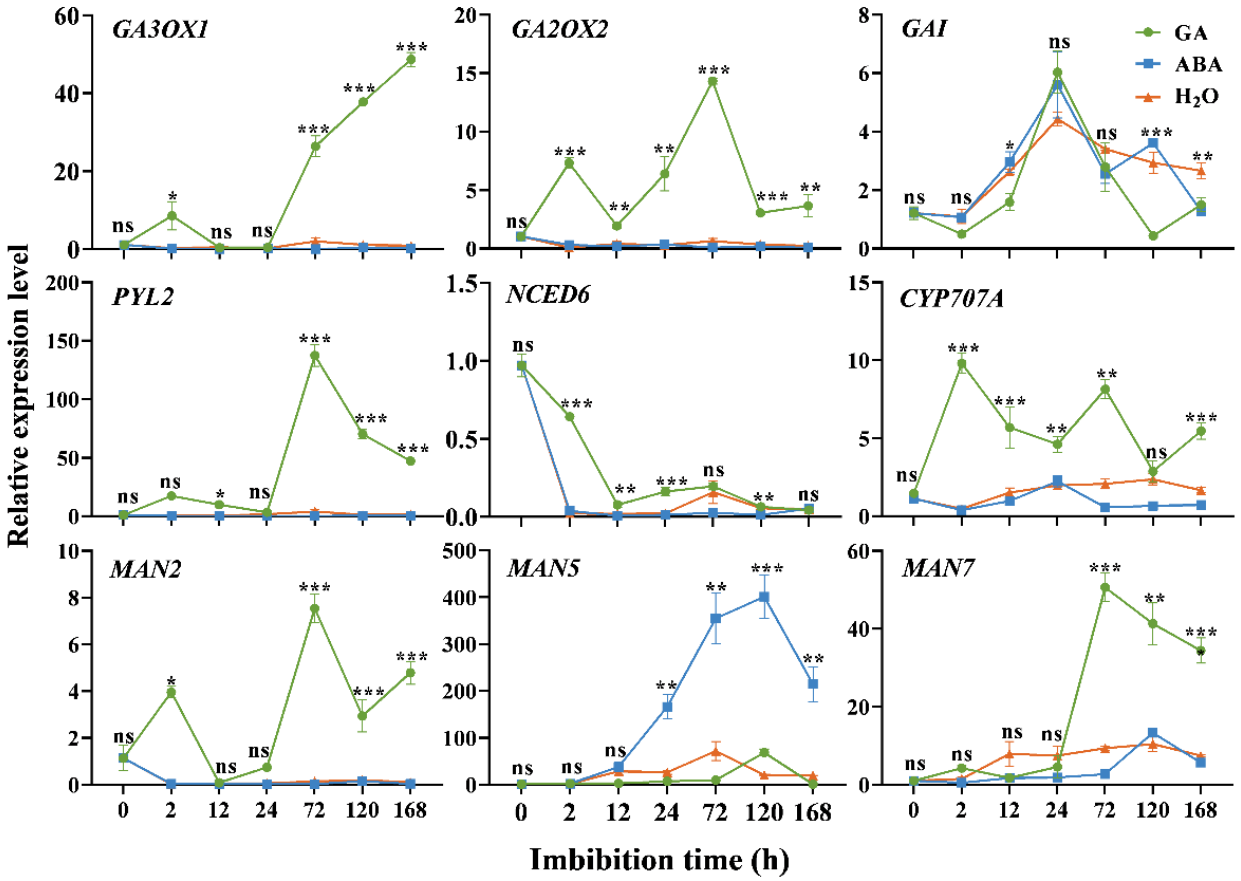


**Figure 5.** Expression of genes related to the dormancy and germination of buffalobur seeds in response to exogenous gibberellin and abscisic acid. The numbers 0, 2, 12, 24, 72, 120 and 168 represent the time after samples were treated with 0.35 mmol/L GA, 0.35 mmol/L ABA or dd$H_2O$. Vertical bars represent the standard error of the means. *, **, and *** represent significant difference at $p \leq 0.05$, 0.01, and 0.001, respectively; ns represents no significant differences.

*MAN2*, *MAN5*, and *MAN7* are associated with the degradation of cell walls containing mannan in the seed endosperm and are favorable for seed germination [51]. Compared with $H_2O$-treated seeds, the expressions of *MAN2*, *MAN5* and *MAN7* were significantly up-regulated significantly in GA-treated seeds, while the *MAN2* and *MAN7* of ABA treatment did not significantly reduce their expression compared to $H_2O$ treatment. This indicated that GA participated in the regulation of *MAN5* expression. The different expression patterns of these three β-mannanase genes under different treatments reveal that their functions in degrading endosperm cap tissue are not exactly the same, and the function of *MAN5* is possibly independent.

### 3.5. Activities of EBM

In the endosperm cap of buffalobur seeds imbibed in $H_2O$, the activities of EBM significantly increased at 3 d and peaked at 5 d (Figure 6). In the seeds imbibed in ABA, which peaked at 3 d, the EBM activity showed a similar trend to seeds imbibed in $H_2O$ but was relatively lower in these samples. EBM activity gradually increased from 2 d to 5 d when imbibed in GA, and decreased after 7 d of imbibition, but the activity was still higher than that imbibed in $H_2O$ and ABA.

### 3.6. Puncture Force Measurements

Among the seeds imbibed in water, 0.35 mmol/L ABA and 0.35 mmol/L GA during seed germination, the puncture force needed to penetrate the endosperm cap showed a

downward trend during the germination process, and significant differences were observed following the three treatments at 5 d and 6 d (Figure 7). GA immersion rapidly decreased the puncture force to 1.65 N on the 5th day, compared with 2.15 N at 4 d, indicating that 5 d imbibition was a critical period for the germination of the buffalobur. On the 6th day, the puncture force of ABA immersion seeds tended to be stable, which was significantly higher than that achieved with imbibition in water and GA.

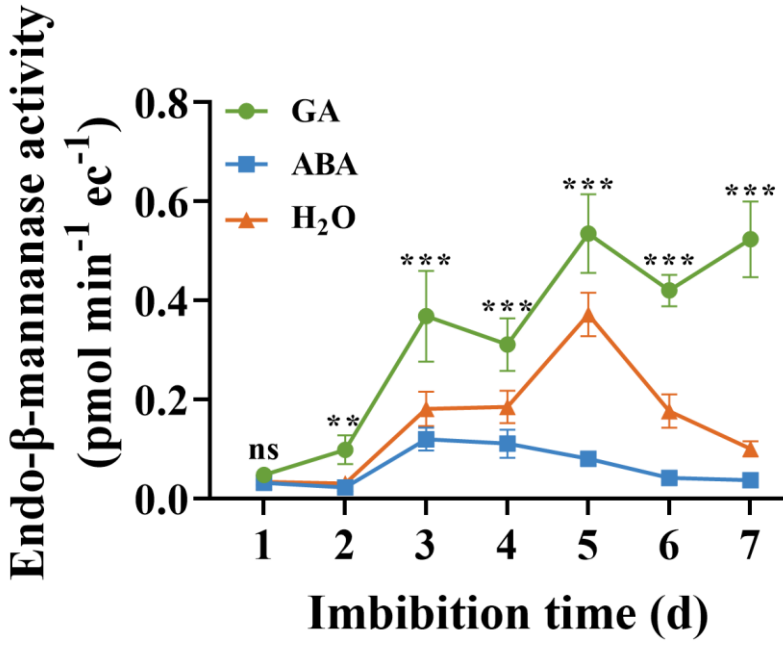


**Figure 6.** The activities of endo-β-mannanase (EBM) in buffalobur seeds treated with $H_2O$, 0.35 mmol/L ABA and 0.35 mmol/L GA. Vertical bars represent the standard error of the means. **, and *** represent significant difference at $p \leq 0.01$, and 0.001, respectively; ns represents no significant differences.

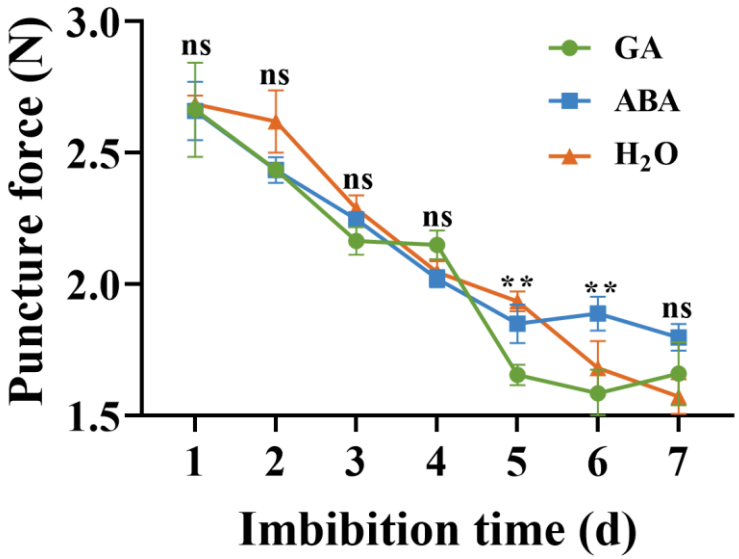


**Figure 7.** Changes in punture force of endosperm cap of buffalobur seeds imbibed in $H_2O$, 0.35 mmol/L ABA and 0.35 mmol/L GA. Vertical bars represent the standard error of the means. ** represent significant difference at the $p \leq 0.01$ level; ns represents no significant differences.

## 4. Discussion

The invasion of buffalobur in China has a serious impact on agricultural production and the ecological environment, and seeds are an important media for its survival and spread in the habitat. Buffalobur has a stong seed dormancy, which means that it could maintain its vitality for nearly 15 years in the soil and is difficult to eradicate using current weed management systems. It is important to gain knowledge regarding the molecular basis of seed dormancy and germination in buffalobur, which is fundamental to developing effective weed prevention and control strategies. In this study, buffalobur seeds showed a distinct germination behavior during imbibition with GA, ABA and $H_2O$. Seed germination could be significantly enhanced by exogenous GA availability.

Using RNA-Seq, 520 million clean-read pairs were generated and 336,000 unigenes were assembled for buffalobur seeds with GA and ABA imbibition. These unigenes were mapped to 45 terms in GO and 129 pathways in KEGG. A total of 28,874 DEGs were identified using DESeq. The expression levels of 20 DEGs with regard to seed dormancy and germination were analyzed by qPCR and were basically consistent with the results of transcriptome sequencing.

### *4.1. Response of Genes in GA Pathway during Seed Imbibition*

The expressions of *GA3ox1* and *GA2ox2* were significantly upregulated in GA-treated seeds within 7 days; *GA3ox1* was significantly downregulated after ABA imbibition; and *GAI* expression was significantly upregulated for both treatments. During the imbibition process, exogenous GA can promote the biosynthesis and degradation of endogenous GA, and exogenous ABA inhibits the biosynthesis of endogenous GA. According to Baskin et al., the downregulation of *LeGAI* was not necessary for tomato seed germination [52]. Therefore, the changes in exogenous hormones might only affect the synthesis and metabolism of endogenous GA, with little effect on *SrGAI*.

### *4.2. Response of Genes in the ABA Pathway during Seed Imbibition*

*NCED6* expression sharply reduced within 2 h in buffalobur seeds during imbibition, which indicated that the biosynthesis of ABA was greatly reduced. GA significantly upregulated *CYP707A*, which meant that exogenous GA promoted the degradation of endogenous ABA. Compared with $H_2O$ treatment, GA and ABA had a positive and negative effect on the expressions of *PYL2* in buffalobur seeds, respectively. This indicates that, at the imbibition stage of seed germination, the expression of an ABA receptor is required for seeds to complete some preparations related to seed germination.

### *4.3. Response of MAN2, MAN5 and MAN7 during Seed Imbibition*

The expressions of *MAN2*, *MAN5* and *MAN7* in buffalobur seeds were significantly upregulated after GA imbibition; the highest expression was obtained for *MAN5*, followed by *MAN7* and *MAN2*, but there was no significant difference when imbibed with ABA. A previous report showed that *MAN2*, *MAN5* and *MAN7*, which encode β-mannanase degrad endosperm tissue, were expressed before the radicle protrusion of *Arabidopsis* seeds through the seed coating [53]. Our results were in accordance with the report stating that the germination of tomato *gib-1* seeds depends on exogenous GA to induce *MAN2* expression was induced by GA, and in *Sisymbrium officinale* (L.) Scop. and *Brassica rapa* L., *MAN5* and *MAN7* expressions were also induced with seed imbibition [51,54].

### *4.4. Puncture Force and EBM Activity*

Seed germination can be divided into three phases, in accordance with water absorption and weight changes [55,56]. In phase I, the initial puncture force of endosperm cap of buffalobur seeds following imbibition in $H_2O$, GA, and ABA was 2.69, 2.66 and 2.66 N, respectively. This indicated the lowering puncture force could not be attributed to embryo expansion, but may be caused by the coat rupture. Like other solanaceous species, including tobacco, petunia (*Petunia hybrida* E. Vilm.) and lobeira (*Solanum lycocarpum* St.

Hill), coat rupture happens prior to the radicle protrusion resulting from water uptake and embryo swelling [28]. A change in the imbibition of buffalobur embryo will not occur until phase III of seed germination begins. This shows that the coat rupture that occurs in phase II may reduce the puncture force in seeds imbibed in $H_2O$ and GA. The puncture force prior to phase II of seeds imbibed in water and GA after 7 d was 1.66 N, while the puncture force of seeds imbibed in ABA remained stable during the whole incubation period (approximately 1.80 N). Therefore, the second reduction in puncture force in buffalobur seeds may be fundamental to radicle protrusion and seed germination.

The relative expression of *MAN2* and *MAN7* under GA imbibition was higher than that of the other two treatments, which was consistent with the trend of EBM activity, while the relative expression of *MAN5* gradually increased under ABA imbibition, but the EBM enzyme activity did not show a similar trend. The EBM activity of buffalobur seeds imbibed in ABA reaches its maximum on the third day and then gradually declines. However, the EBM activity of seeds imbibed in GA began to significantly increased on the second day, reaching a maximum on the fifth day, while the EBM activity was relatively higher than that of the other imbibed seeds. Combining the results of puncture force and EBM activity showed that the force of puncture decreases with the increase in EBM activity, and there was a certain correlation between the EBM activity and puncture force of buffalobur seeds during imbibition. The trend of increasing EBM activity, along with the decreasing puncture force, indicates that the EBM plays an essential role in the weakening of the endosperm coating around the radicle.

## 5. Conclusions

In summary, this study was conducted to investigate the transcriptome of buffalobur seeds under exogenous hormone to elucidate the molecular mechanisms of phytohormones on the seed germination of buffalobur. The exogenous hormone GA stimulates the synthesis of GA and the metabolism of ABA in buffalobur, so that seed germination occurs earlier. However, under exogenous ABA treatment, both ABA and GA synthesis and metabolism-related genes were expressed at low levels, preventing the seeds from germinating normally. Furthermore, the endosperm cap weakening is indispensable to the germination of buffalobur, and *SrMAN* plays a vital role in seed germination, of which GA promotes the upregulation in the expression of *SrMAN2* and *SrMAN7*, and ABA promotes the downregulation in the expression of *SrMAN5*. The EBM activity is negatively correlated with the puncture force, while the contrasting germination responses in buffalobur under GA and ABA are associated with distinct endosperm cap weakness. This is the first report on seed germination transcriptomics in buffalobur. The knowledge achieved by this study could facilitate future efforts to elucidate the molecular mechanisms that regulate seed dormancy and germination, and serve as a valuable resource for the genetic studies of seed biology of buffalobur.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010212/s1, Table S1: Primers for expression analysis of 20 DEGs in buffalobur seeds; Table S2: Primers of reference gene and genes related to seed dormancy and germination in buffalobur seeds.

**Author Contributions:** Conceptualization, S.W. and H.H.; literature search, Z.C. and K.W.; methodology, D.Z. and L.Y.; investigation and data curation, Z.C., L.L., K.W., D.Z. and L.Y.; writing—original draft preparation, Z.C. and L.L.; writing—review and editing, S.W., H.H. and Z.H.; project administration, S.W. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Beijing Natural Science Foundation (6212027), Natural Science Foundation of China (31572022), and the Sci-Tech Innovation 2030 Agenda (2022ZD04021).

**Data Availability Statement:** Primers for the expression analysis of 20 DEGs in buffalobur seeds are available in Table S1, and the primers of nine candidate genes in buffalobur seeds are available in Table S2.

**Conflicts of Interest:** The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## References


1. Wei, S.; Zhang, C.; Li, X.; Cui, H.; Huang, H.; Sui, B.; Meng, Q.; Zhang, H. Factors affecting buffalobur (*Solanum rostratum*) seed germination and seedling emergence. *Weed Sci.* **2009**, *57*, 521–525. [CrossRef]
2. Mora-Carrera, E.; Castaneda-Zarate, M.; Fornoni, J.; Boege, K.; Dominguez, C.A. On the adaptive value of monomorphic versus dimorphic enantiostyly in *Solanum rostratum*. *Ann. Bot.* **2019**, *123*, 205–212. [CrossRef] [PubMed]
3. Liu, Z.; Wang, M.; Tian, M.; Yuan, L.; Yu, B.; Qu, B.; An, T.; Feng, Y. Pyrrole alkaloids from *Solanum rostratum* and their chemical defense function against *Henosepilachna vigintioctomaculata*. *Fitoterapia* **2021**, *155*, 105031. [CrossRef] [PubMed]
4. Zhang, Y.; Guo, W.; Yuan, Z.; Song, Z.; Wang, Z.; Gao, J.; Fu, W.; Zhang, G. Chromosome-level genome assembly and annotation of the prickly nightshade *Solanum rostratum* Dunal. *Sci. Data* **2023**, *10*, 341. [CrossRef] [PubMed]
5. Wang, C.; Hawthorne, D.; Qin, Y.; Pan, X.; Li, Z.; Zhu, S. Impact of climate and host availability on future distribution of Colorado potato beetle. *Sci. Rep.* **2017**, *7*, 4489. [CrossRef]
6. Mayumi, V.; Lislie, S.; Mario, V.; Leonardo, D.A.; Jesús, F.G. Reproductive strategy of an invasive buzz-pollinated plant (*Solanum rostratum*). *S. Afr. J. Bot.* **2023**, *162*, 342–352. [CrossRef]
7. Penfield, S. Seed dormancy and germination. *Curr. Biol* **2017**, *27*, R874–R878. [CrossRef] [PubMed]
8. Klupczyńska, E.A.; Pawłowski, T.A. Regulation of seed dormancy and germination mechanisms in a changing environment. *Int. J. Mol. Sci.* **2021**, *22*, 1357. [CrossRef]
9. Shu, K.; Liu, X.D.; Xie, Q.; He, Z.H. Two faces of one seed: Hormonal regulation of dormancy and germination. *Mol. Plant* **2016**, *9*, 34–45. [CrossRef]
10. Fujii, H.; Chinnusamy, V.; Rodrigues, A.; Rubio, S.; Antoni, R.; Park, S.-Y.; Cutler, S.R.; Sheen, J.; Rodriguez, P.L.; Zhu, J.-K. In vitro reconstitution of an abscisic acid signalling pathway. *Nature* **2009**, *462*, 660–664. [CrossRef]
11. Lin, Z.; Li, Y.; Wang, Y.; Liu, X.; Ma, L.; Zhang, Z.; Mu, C.; Zhang, Y.; Peng, L.; Xie, S.; et al. Initiation and amplification of *SnRK2* activation in abscisic acid signaling. *Nat. Commun.* **2021**, *12*, 2456. [CrossRef] [PubMed]
12. Chen, K.; Li, G.; Bressan, R.A.; Song, C.; Zhu, J.; Zhao, Y. Abscisic acid dynamics, signaling, and functions in plants. *J. Integr. Plant Biol.* **2020**, *62*, 25–54. [CrossRef]
13. Ye, Y.; Zhou, L.; Liu, X.; Liu, H.; Li, D.; Cao, M.; Chen, H.; Xu, L.; Zhu, J.-K.; Zhao, Y. A novel chemical inhibitor of ABA signaling targets all ABA receptors. *Plant Physiol.* **2017**, *173*, 2356–2369. [CrossRef] [PubMed]
14. Grappin, P.; Bouinot, D.; Sotta, B.; Miginiac, E.; Jullien, M. Control of seed dormancy in *Nicotiana plumbaginifolia*: Post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta* **2000**, *210*, 279–285. [CrossRef]
15. Thompson, A.J.; Jackson, A.C.; Symonds, R.C.; Mulholland, B.J.; Dadswell, A.R.; Blake, P.S.; Burbidge, A.; Taylor, I.B. Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. *Plant J.* **2000**, *23*, 363–374. [CrossRef]
16. Martínez-Andújar, C.; Ordiz, M.I.; Huang, Z.; Nonogaki, M.; Beachy, R.N.; Nonogaki, H. Induction of 9-cis-epoxycarotenoid dioxygenase in *Arabidopsis thaliana* seeds enhances seed dormancy. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 17225–17229. [CrossRef] [PubMed]
17. Zheng, L.; Abe, F.; Nonogaki, M.; Kanno, Y.; Seo, M.; Nonogaki, H.; Kawakami, N. Modulation of wheat grain dormancy by introducing the recombinant abscisic acid-stimulated abscisic acid biosynthesis gene. *Plant Biotechnol.* **2023**, *40*, 31–41. [CrossRef]
18. Okamoto, M.; Kushiro, T.; Jikumaru, Y.; Abrams, S.R.; Kamiya, Y.; Seki, M.; Nambara, E. ABA 9′-hydroxylation is catalyzed by CYP707A in *Arabidopsis*. *Phytochemistry* **2011**, *72*, 717–722. [CrossRef]
19. Lee, S.; Cheng, H.; King, K.E.; Wang, W.; He, Y.; Hussain, A.; Lo, J.; Harberd, N.P.; Peng, J. Gibberellin regulates *Arabidopsis* seed germination via *RGL2*, a *GAI/RGA*-like gene whose expression is up-regulated following imbibition. *Genes Dev.* **2002**, *16*, 646–658. [CrossRef]
20. Li, C.; Dong, S.; Beckles, D.M.; Miao, H.; Sun, J.; Liu, X.; Wang, W.; Zhang, S.; Gu, X. The *qLTG1.1* candidate gene *CsGAI* regulates low temperature seed germination in cucumber. *Theor. Appl. Genet.* **2022**, *135*, 2593–2607. [CrossRef]
21. Hauvermale, A.L.; Ariizumi, T.; Steber, C.M. Gibberellin signaling: A theme and variations on DELLA repression. *Plant Physiol.* **2012**, *160*, 83–92. [CrossRef] [PubMed]
22. Yu, F.; Li, M.; He, D.; Yang, P. Advances on post-translational modifications involved in seed germination. *Front. Plant Sci.* **2021**, *12*, 642979. [CrossRef] [PubMed]
23. Fukazawa, J.; Nakata, M.; Ito, T.; Matsushita, A.; Yamaguchi, S.; Takahashi, Y. bZIP transcription factor RSG controls the feedback regulation of *NtGA20ox1* via intracellular localization and epigenetic mechanism. *Plant Signal. Behav.* **2011**, *6*, 26–28. [CrossRef] [PubMed]
24. Song, J.; Guo, B.; Song, F.; Peng, H.; Yao, Y.; Zhang, Y.; Sun, Q.; Ni, Z. Genome-wide identification of gibberellins metabolic enzyme genes and expression profiling analysis during seed germination in maize. *Gene* **2011**, *482*, 34–42. [CrossRef] [PubMed]
25. Gabriele, S.; Rizza, A.; Martone, J.; Circelli, P.; Costantino, P.; Vittorioso, P. The Dof protein DAG1 mediates PIL5 activity on seed germination by negatively regulating GA biosynthetic gene *AtGA3ox1*. *Plant J.* **2010**, *61*, 312–323. [CrossRef] [PubMed]
26. Hedden, P. The current status of research on gibberellin biosynthesis. *Plant Cell Physiol.* **2020**, *61*, 1832–1849. [CrossRef]

27. Hilhorst, H.W.M.; Groot, S.P.C.; Bino, R.J. The tomato seed as a model system to study seed development and germination. *Acta Bot. Neerl.* **1998**, *47*, 169–183.
28. Pinto, L.V.; Da Silva, E.A.; Davide, A.C.; De Jesus, V.A.; Toorop, P.E.; Hilhorst, H.W. Mechanism and control of *Solanum lycocarpum* seed germination. *Ann. Bot.* **2007**, *100*, 1175–1187. [CrossRef]
29. Finch-Savage, W.E.; Leubner-Metzger, G. Seed dormancy and the control of germination. *New Phytol.* **2006**, *171*, 501–523. [CrossRef]
30. Chandrasekaran, U.; Zhao, X.; Luo, X.; Wei, S.; Shu, K. Endosperm weakening: The gateway to a seed's new life. *Plant Physiol. Biochem.* **2022**, *178*, 31–39. [CrossRef]
31. Toorop, P.E.; van Aelst, A.C.; Hilhorst, H.W. The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA. *J. Exp. Bot.* **2000**, *51*, 1371–1379. [CrossRef] [PubMed]
32. Wang, M.; Qu, H.; Zhang, H.; Liu, S.; Li, Y.; Zhang, C. Hormone and RNA-seq analyses reveal the mechanisms underlying differences in seed vigour at different maize ear positions. *Plant Mol. Biol.* **2019**, *99*, 461–476. [CrossRef]
33. Burks, D.J.; Azad, R.K. RNA-Seq data analysis pipelinpe for plants: Transcriptome assembly, alignment, and differential expression analysis. *Methods Mol. Biol.* **2022**, *2396*, 47–60. [CrossRef] [PubMed]
34. Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q.; et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **2011**, *29*, 644–652. [CrossRef]
35. Trapnell, C.; Williams, B.A.; Pertea, G.; Mortazavi, A.; Kwan, G.; van Baren, M.J.; Salzberg, S.L.; Wold, B.J.; Pachter, L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **2010**, *28*, 511–515. [CrossRef] [PubMed]
36. Li, B.; Dewey, C.N. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* **2011**, *12*, 323. [CrossRef]
37. Li, D.; Zand, M.S.; Dye, T.D.; Goniewicz, M.L.; Rahman, I.; Xie, Z. An evaluation of RNA-seq differential analysis methods. *PLoS ONE* **2022**, *17*, e264246. [CrossRef]
38. Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. B.* **1995**, *57*, 289–300. [CrossRef]
39. Young, M.D.; Wakefield, M.J.; Smyth, G.K.; Oshlack, A. Gene ontology analysis for RNA-seq: Accounting for selection bias. *Genome Biol.* **2010**, *11*, R14. [CrossRef]
40. Mao, X.; Cai, T.; Olyarchuk, J.G.; Wei, L. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics* **2005**, *21*, 3787–3793. [CrossRef]
41. Chen, J.; Huang, Z.; Huang, H.; Wei, S.; Liu, Y.; Jiang, C.; Zhang, J.; Zhang, C. Selection of relatively exact reference genes for gene expression studies in goosegrass (*Eleusine indica*) under herbicide stress. *Sci. Rep.* **2017**, *7*, 46494. [CrossRef] [PubMed]
42. Zhao, D.; Wang, X.; Chen, J.; Huang, Z.; Huo, H.; Jiang, C.; Huang, H.; Zhang, C.; Wei, S. Selection of reference genes for qPCR normalization in buffalobur (*Solanum rostratum* Dunal). *Sci. Rep.* **2019**, *9*, 6948. [CrossRef] [PubMed]
43. Harshitha, R.; Arunraj, D.R. Real-time quantitative PCR: A tool for absolute and relative quantification. *Biochem. Mol. Biol. Educ.* **2021**, *49*, 800–812. [CrossRef] [PubMed]
44. Xiao, J.H.; Zhang, J.H.; Zhang, Y.Y.; Wang, T.T.; Chen, R.G.; Li, H.X.; Ye, Z.B. Isolation and expression of GA 2-oxidase2 in tomato. *DNA Seq.* **2007**, *18*, 474–479. [CrossRef]
45. Zhang, X.; Wang, B.; Zhao, Y.; Zhang, J.; Li, Z. Auxin and GA signaling play important roles in the maize response to phosphate deficiency. *Plant Sci.* **2019**, *283*, 177–188. [CrossRef]
46. Ariizumi, T.; Hauvermale, A.L.; Nelson, S.K.; Hanada, A.; Yamaguchi, S.; Steber, C.M. Lifting della repression of *Arabidopsis* seed germination by nonproteolytic gibberellin signaling. *Plant Physiol.* **2013**, *162*, 2125–2139. [CrossRef]
47. Wang, Y.; He, S.; Wei, Y.; Dong, C.; Liu, L.; Jue, D.; Shi, S.; Li, W. Molecular and functional characterization of two DELLA protein-coding genes in *litchi*. *Gene* **2020**, *738*, 144455. [CrossRef]
48. Kanno, Y.; Jikumaru, Y.; Hanada, A.; Nambara, E.; Abrams, S.R.; Kamiya, Y.; Seo, M. Comprehensive hormone profiling in developing *Arabidopsis* seeds: Examination of the site of ABA biosynthesis, ABA transport and hormone interactions. *Plant Cell Physiol.* **2010**, *51*, 1988–2001. [CrossRef]
49. Weng, J.K.; Ye, M.; Li, B.; Noel, J.P. Co-evolution of hormone metabolism and signaling networks expands plant adaptive plasticity. *Cell* **2016**, *166*, 881–893. [CrossRef]
50. Yang, D.; Zhao, F.; Zhu, D.; Chen, X.; Kong, X.; Wu, Y.; Chen, M.; Du, J.; Qu, L.J.; Wu, Z. Progressive chromatin silencing of ABA biosynthesis genes permits seed germination in *Arabidopsis*. *Plant Cell* **2022**, *34*, 2871–2891. [CrossRef]
51. Carrillo-Barral, N.; Matilla, A.J.; Rodríguez-Gacio, M.D.C.; Iglesias-Fernández, R. Mannans and endo-β-mannanase transcripts are located in different seed compartments during Brassicaceae germination. *Planta* **2018**, *247*, 649–661. [CrossRef]
52. Baskin, J.M.; Baskin, C.C. A classification system for seed dormancy. *Seed Sci. Res.* **2004**, *14*, 1–16. [CrossRef]
53. Iglesias-Fernández, R.; Matilla, A.J. Genes involved in ethylene and gibberellins metabolism are required for endosperm-limited germination of *Sisymbrium officinale* L. seeds. *Planta* **2010**, *231*, 653–664. [CrossRef]
54. Yu, L.L.; Xu, F. *MAN5*, a glycosyl hydrolase superfamily protein, is a key factor involved in cyanide-promoted seed germination in *Arabidopsis thaliana*. *Genes* **2023**, *14*, 1361. [CrossRef] [PubMed]

55. Bewley, J.D. Seed germination and dormancy. *Plant Cell* **1997**, *9*, 1055–1066. [CrossRef] [PubMed]
56. Mirwais, M.Q. Environmental regulation of weed seed dormancy and germination. *Seeds* **2023**, *2*, 259–277. [CrossRef]