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Article

Involvement of Abscisic Acid in Transition of *Pisum sativum* L. from Germination to Post-Germination Stage

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Abstract: Transitions from seed to seedling represent a critical developmental step in the life cycle of higher plants, dramatically affecting plant ontogenesis and stress tolerance. The release from dormancy to acquiring the germination ability is defined by a balance of phytohormones, with a substantial contribution of abscisic acid (ABA), which inhibits germination. We studied the embryonic axes of *Pisum sativum* L. before and after radicle protrusion. Our previous work compared RNA sequencing-based transcriptomics in the embryonic axes isolated before and after radicle protrusion. The current study aims to analyze ABA-dependent gene regulation during the transition of embryonic axes from germination to post-germination stage. First, we determined the levels of abscisates (ABA, phaseic acid, dihydrophaseic acid, and neo-phaseic acid) using the ultra-high-performance liquid chromatography-tandem mass spectrometry. Second, we made a detailed annotation of ABA-associated genes using RNA sequencing-based transcriptome profiling. Finally, we analyzed the DNA methylation pattern in the promoters of the *PsABI3, PsABI4, and PsABI5* genes. We showed that changes in the abscisate profile are characterized by the accumulation of ABA catabolites, and the ABA-related gene profile is accompanied by the upregulation of genes controlling the seedling development and the downregulation of genes controlling the water deprivation. The expression of *ABI3, ABI4* and *ABI5*, which encode the crucial transcription factors during late maturation, was downregulated by more than 20-fold, and their promoters exhibited a high level of methylation already at the late germination stage. Thus, although ABA remains important, other regulators seems to be involved in the transition from seed to seedling.

Keywords: abscisic acid; DNA methylation; embryonic axes; ABA-associated genes; *Pisum sativum* L.; seed-to-seedling transition

1. Introduction

In higher plants, seed production is crucial to the species survival. Most seeds enter dormancy during late maturation and maintain this state until environmental conditions become favorable for germination [1,2]. The transition from dormancy to germination is influenced by a balance of phytohormones and significant environmental factors such as temperature, water availability, and light [1,3]. This transition, occurring at the end of germination, involves extensive transcriptome reprogramming and signaling pathway alterations, leading to the silencing of seed maturation genes and activation of those for vegetative growth [4–9].

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Whether seeds acquire the ability to germinate or remain dormant depends on the phytohormones balance [10–12]. Notably, abscisic acid (ABA) promotes seed dormancy and inhibits germination, while gibberellins (GAs) breaks seed dormancy and induce germination [13–18]. During early embryogenesis, ABA prevents seed abortion and promotes embryo growth, initially provided by the maternal tissues and later produced by the seeds themselves [18,19]. Consequently, ABA levels rise sharply late in embryogenesis, counteracting GAs and suppressing embryo growth [19].

As the embryo develops, it enlarges through cell elongation and accumulates storage compounds. ABA regulates the transport of monosaccharides and amino acids from maternal tissues and their conversion into stored forms, like polysaccharides and proteins. In late maturation, metabolic processes slow down, seeds desiccate, and enter dormancy [20,21].

Numerous studies have shown that decreasing ABA levels is crucial for dormancy release and germination [7,12,19,22]. ABA degradation occurs through hydroxylation and conjugation, with ABA 8'-hydroxylases playing a key role in rapid ABA level decline during seed imbibition [17,23–26]. However, ABA's signaling role during the seed-to-seedling transition remains unclear.

Key player in the seed transition from dormancy to germination include the LAFL regulatory network, comprising LEAFY COTYLEDON1 (LEC1) and LEC1-LIKE (L1L) of the NF-YB family transcription factors (TFs), and ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEC2 (LEAFY COTYLEDON2) of the B3-AFL gene family [27–29]. The LAFL network, originating in a common ancestor of bryophytes and vascular plants, acts as a positive regulator of seed maturation genes but suppresses germination [30–32]. This network allows orthodox seeds to maintain desiccation tolerance during dormancy and germination [33–36]. Radicle protrusion marks the transition to the post-germination stage, with seeds becoming seedlings and losing desiccation tolerance [9,33,37]. This stage is typically associated with LAFL network silencing [5,32,34,38,39].

Our previous transcriptomic profiling of *P. sativum* embryo axes before and after radicle protrusion revealed unexpected findings [4]. Although we anticipated the expression of LAFL network genes before radicle protrusion and their subsequent silencing, only *PsABI3* showed significant expression in seed axes. We also observed the expression of other ABA-related genes (*PsABI4* and *PsABI5*). Given that *ABI3*, *ABI4*, and *ABI5* are central transcriptional factors in seed-specific events, including maturation, dormancy, longevity, germination, and post-germination growth [16,40,41], we propose that *PsABI3*, *PsABI4*, and *PsABI5* also play a role in regulating the *P. sativum* seed-to-seedling transition [4,9].

Germination-related repression of the LAFL transcriptional network is due to epigenetic regulation of gene expression through DNA methylation and post-translational modifications of histones [5,8,32,42–44]. DNA methylation patterns change throughout seed development, germination and seedling establishment [8,45–53]. This study analyzes ABA metabolite profiles, ABA-associated gene expression, and DNA methylation in the promoters of *PsABI3*, *PsABI4*, and *PsABI5* in embryonic axes of germinated pea seeds before and after radicle protrusion. We discuss these findings in the context of ABA-dependent gene regulation during the seed-to-seedling transition.

2. Materials and Methods

2.1. Plant Material

Pea seeds of the commercial cultivar “Prima” were sourced from the N.I. Vavilov All-Russian Institute of Plant Genetic Resources, St. Petersburg, Russia. Seeds were imbibed for 72 hours between layers of moist filter paper, then visually divided into two batches: (a) before embryonic root growth initiation (before radicle protrusion) and (b) post-initiation of root growth (after radicle protrusion). Seed axes from both batches were isolated, frozen in liquid nitrogen, homogenized, and stored at −80°C before use in biochemical experiments.

2.2. Quantitation of ABA and ABA-related metabolites

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The selected plant hormones in the embryonic axes were quantified using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). The sample preparation and the analysis was performed according to the modified protocol by Šimura and co-workers [54]. For the quantitation of ABA and ABA-related metabolites, 15 mg (fresh weight) of the homogenized plant material were extracted in 1 mL 60% (v/v) acetonitrile (ACN) with the addition of 5 pmol of [2H6]ABA as the internal standard. Four zirconium oxide 2.0 mm extraction beads (Next Advance, Troy, NY, USA) were added into the liquid sample. The sample was shaked in a Retsch MM400 bead mill (Retsch, Haan, Germany) at 27 Hz for 5 minutes, sonicated for 3 minutes and incubated for half an hour at 4 °C. Afterwards, the sample was centrifuged at 20,000 rpm for 10 minutes at 4 °C (Allegra 64R benchtop centrifuge, Beckman Coulter, USA). Supernatant was loaded onto Oasis® HLB 30 mg/1 cc extraction cartridge (Waters, Milford, USA). The cartridge was washed subsequently with 0.5 mL 60% (v/v) ACN and 0.5 mL 30% (v/v) ACN. All fractions (the flow-through and both washes) were collected and dried under the reduced pressure using SpeedVac concentrator (RC1010 Centrivap Jouan, ThermoFisher, USA). The sample was reconstructed in 40 µL of 25% (v/v) ACN and 5 µL of the sample were injected into Acquity UPLC CSH C18 RP 150 × 2.1 mm, 1.7 µm chromatographic column (Waters, Milford, USA). The UHPLC separation was performed using Acquity UPLC I-Class System (Waters, Milford, USA) coupled to a triple quadrupole tandem mass spectrometer Xevo TQ-XS equipped with an electrospray ionization (Waters, Manchester, UK). The gradient elution and the MS/MS working in the multiple reaction monitoring (MRM) mode followed the previously published conditions described in Šimura et al, 2018 [54]. The obtained chromatographic peaks were evaluated in MassLynx V4.2 software (Waters, Manchester, UK). The targeted compounds were quantified by isotope dilution method.

2.3. Annotation of ABA-Associated Genes

ABA-associated genes were annotated based on RNA sequencing-based transcriptome profiling [4]. Annotation utilized the Ensembl BioMart tool (https://plants.ensembl.org/biomart/martview) and the URGI database (https://urgi.versailles.inra.fr/Species/Pisum). Gene ontology (GO) terms, InterPro domains (https://www.ebi.ac.uk/interpro), and *Arabidopsis thaliana* orthologs were identified for each gene [55]. Clustering was performed using the k-means algorithm, and the optimal number of clusters was determined using the Elbow method.

2.4. DNA Extraction and Sodium Bisulfite Treatment

Total genomic DNA from seeds at two developmental stages (before and after radicle protrusion) was extracted using the DNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions (www.qiagen.com). Sodium bisulfite treatment of 1 µg genomic DNA from each sample was conducted with the EpiTect Fast Bisulfite Kit (QIAGEN, Germany).

2.5. Primer Design and in silico Analysis

Primers for amplifying bisulfite-treated DNA were designed against cytosine-converted sequences using SnapGene 6.1.2 (https://www.snapgene.com). Prediction of CpG islands in *PsABI3*, *PsABI4*, and *PsABI5* promoter sequences utilized Meth-Primer 2.0 (https://www.urogene.org/methprimer2) and PlantPAN 3.0 (http://plantpan.itps.ncku.edu.tw/index.html). Promoter mapping for transcription factor binding sites was performed using PlantPAN 3.0 and PCBase (http://pcbase.itps.ncku.edu.tw/index), followed by filtering for stress and hormone response motifs at Similar Score = 1.

2.6. PCR, Electrophoretic Analysis, Extraction, and Purification

To amplify genomic and bisulfite-treated DNA, the PCR was performed in a 50 µL mixture containing 70 ng of DNA template, 10 pM of each primer, and BioMaster HS-Taq PCR kit (2×) (BioLabMix, Russia) or Tersus Plus PCR kit (Evrogen, Russia) according to the manufacturer's instructions. The PCR conditions included an initial denaturation step at 94 °C for 5 min, followed by

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35 cycles of denaturation at 94 °C for 1 min, annealing 50 °C for 1 min, and extension at 72 °C for 2 min, and a final elongation step at 72 °C for 5 min. The PCR screening of colonies was performed in a 25 μL mixture containing 10 pM of M13F and M13R primers (Evrogen, Russia), 0.25 mM of each dNTP, 1x reaction buffer (67 mM TrisHCl, pH 8.8; 2 mM MgCl2; 18 mM (NH4)2SO4; 0.01% Tween 20) and 0,5 U Taq polymerase (Syntol, Russia). After an initial denaturation at 95°C for 15 minutes, 35 cycles were performed at 94°C for 20 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by a final elongation at 72°C for 5 minutes. Electrophoretic analysis was performed on a 1% agarose gel (Helicon, Russia) prepared on a TAE buffer (Sigma-Aldrich, USA) with ethidium bromide (VWR (Amresco), USA). The amplified fragments were extracted from the gel using the MinElute Gel Extraction Kit (QIAGEN, Germany).

2.7. Cloning and Sequencing the Amplified PCR Fragments

Freshly prepared PCR products were ligated with a vector using the Quick-TA kit (Evrogen, Russia), which included the pAL2-T vector, Quick-TA T4 DNA Ligase, buffer, M13 Forward primer, and M13 Reverse primer according to the manufacturer's instructions. Chemical transformation of competent *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 DH10B cells was then performed. Transformed colonies carrying inserts of the expected size were selected on selective LB medium (DIA-M, Russia) with 100 μg/ml of ampicillin (BioChemica, PanReac Applichem, Spain). The purified amplified fragments were sequenced in both directions using M13 primers and the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems™, USA) on a 3500 Applied Biosystems Genetic Analyzer. For DNA methylation analysis, at least 10 clones were sequenced for each amplicon. The alignment of sequences was carried out using SnapGene 6.1.2 (https://www.snapgene.com/).

2.8. Statistical Analyses

Two-tailed t-tests (alpha = 0.05) compared means of ABA-related metabolites. Analysis was performed using MS Excel add-in, with data representing mean ± standard error of nine biological replicates.

3. Results

3.1. Quantitation of ABA and ABA-related metabolites in Pea Embryonic Axes Before and After Radicle Protrusion

To delve deeper into ABA homeostasis, we examined the levels of ABA and its metabolites in embryonic axes from germinated pea seeds, both before and after radicle protrusion. These axes encompass the first true leaves, epicotyl, hypocotyl, and root (Figure 1).

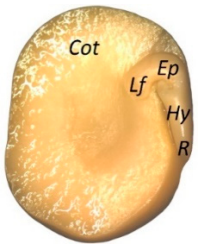

Figure 1. Image of *P. sativum* mature embryo. Embryo includes cotyledons (*Cot*), first true leaves (*Lf*), epicotyl (*Ep*), hypocotyl (*Hy*), and root (*R*). Embryonic axe includes *Lf*, *Ep*, *Hy* and *R*.

We analyzed levels of abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), neo-phaseic acid (neoPA), and 7′-hydroxy ABA (7′-OH-ABA). Notably, we observed an accumulation of PA and DPA, which are key products of ABA catabolism, against a backdrop of decreasing ABA levels (Figure 2). Intriguingly, 7′-OH-ABA was not detected in embryonic axes before or after radicle protrusion.

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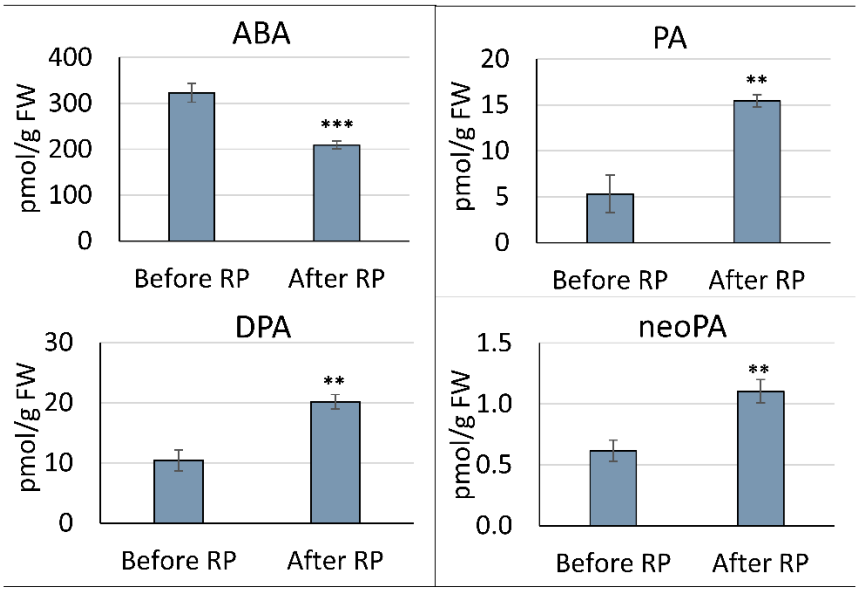

Figure 2. The contents of abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA), and neo-phaseic acid (neoPA) observed in embryonic axes of *P. sativum* before and after radicle protrusion (RP). The data represent the mean ± standard error of nine biological replicates. The statistical analysis relied on two-tailed t-test with a critical alpha value of 0.05. Significant differences between the mean values are indicated (*** $p \leq 0.001$, ** $p \leq 0.005$).

3.2. Categorization and Functional Annotation of ABA-Associated DEGs in Pea Embryonic Axes Before and After Radicle Protrusion

In our previous work we performed RNA sequencing of isolated embryonic axes before and after radicle protrusion [4]. Here, we provide a more detail profiling of ABA-associated differentially expressed genes (DEGs) annotated using the Pea Genome Assembly *v1a* from the UGRI server as an main annotation source [56]. A total of 70 ABA-associated DEGs were annotated in embryonic axes. Among these, 46 genes showed higher expression and 24 genes lower expression after radicle protrusion by more than 4-fold ($|\log FC| > 2$) (Figure 3, Figure S1, Table S1).

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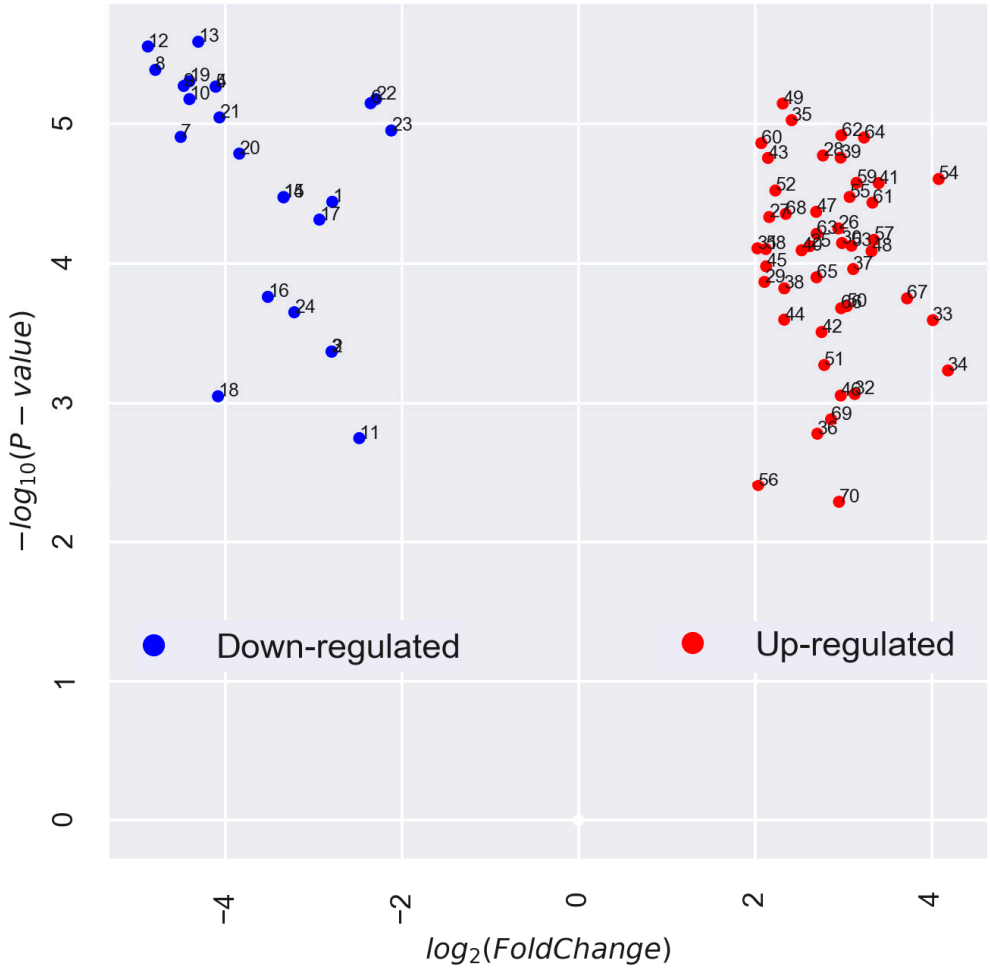

Figure 3. Volcano plot representing 70 differentially expressed genes (DEGs). The X-axis indicates the log2-transformed gene expression fold changes between seed axes before and after radicle protrusion. The Y-axis indicates the log10-transformed p-value. Significant DEGs with lower expression are highlighted in blue (№ 1-24). Significant DEGs with higher expression are highlighted in red (№ 25-70). See Table S1 for the full description of the down-regulated and up-regulated genes.

ABA-dependent DEGs up-regulated in seed axes after radicle protrusion included those related to cellular signaling and stress resistance, membrane transporters and TFs regulating developmental programs (Table S1). These comprised genes encoding serine-threonine/tyrosine protein kinases CRK29 (*Psat6g212040*) and CIPK17 (*Psat0s2012g0280)*, protein phosphatase 2C family member (*Psat7g017080*), *α*-subunit of G-protein (*Psat6g097080*), and inositol polyphosphate-related phosphatase (*Psat4g078320*). The expression of genes associated with water deprivation response, antifungal proteins, and calcium-signaling also increased significantly. The expression of *Psat6g199400* encoding a protein RD29B/LTI65 increased 4.5-fold, *Psat5g266320* encoding an antifungal protein ginkbilobin-2 – 5-8-fold, and *Psat4g146960* encoding a calcium-signaling protein ANNEXIN4 – 9-fold. Genes responsible for the synthesis of membrane transporters included *Psat4g117800* (encoding P-ATPase) and *Psat4g184760* (encoding potassium channel AKT2/3). The expression of *Psat2g121520* (encoding the TCP15 protein) increased 8-fold.

Conversely, downregulated DEGs included key ABA-response genes like ABI5 (Psat3g033680), ABI3 (Psat3g142040), ABI4 (Psat2g031240), LTI65 (Psat0s2227g0040), LTP4 (Psat7g227120), HVA22E (Psat5g052360), and RD22 (Psat6g033920 and Psat6g033960) (Table S1). These genes are highly conserved across functional domains, with ABI4, ABI5, and HVA22E exhibiting sequence homology in various drought-tolerant species [4]. These findings suggest these genes play a crucial role in dehydration tolerance during the transition from seed germination to seedling establishment.

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3.3. DNA Methylation in the Promoters of the PsABI3, PsABI4, and PsABI5 Genes

We selected the *PsABI3* gene along with newly identified drought-responsive genes *PsABI4* and *PsABI5* for epigenetic analysis. These genes were identified in the *P. sativum* genome and sequenced from the commercial cultivar "Prima" (Table S2).

In silico analysis of the promoters and first exons (including 5'-UTR) of *PsABI3*, *PsABI4*, and *PsABI5* revealed low GC composition (29%, 34%, and 23% respectively), with only individual CpG sites predicted and no CpG islands detected (Figure S2). Considering that plant DNA methylation can occur at CpG, CpHpG, and CpHpH sites, we designed primers for bisulfite sequencing (with conversion of unmethylated C to T) of both CpG and non-CpG sites (Table S2).

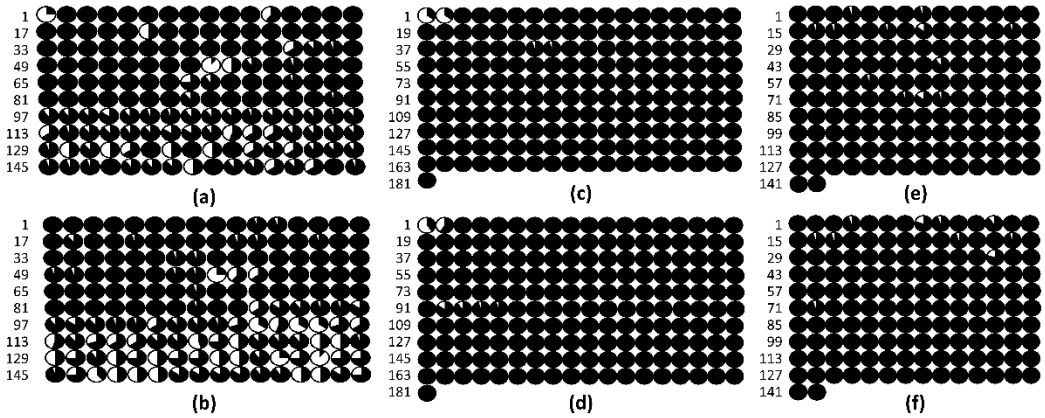

Figure 4. Methylation of the ABA-related gene promoters in embryonic axes of germinated *P. sativum* seeds before and after radicle protrusion (RP). (**a**) The *PsABI3* gene promoter before RP. (**b**) The *PsABI3* gene promoter after RP. (**c**) The *PsABI4* gene promoter before RP. (**d**) The *PsABI4* gene promoter after RP. (**e**) The *PsABI5* gene promoter before RP. (**f**) The *PsABI5* gene promoter after RP. The length of the analyzed segment *PsABI3* promoter is 1057 bp with the number of cytosines is 160, for the PsABI4 promoter is 721 bp with 181 cytosines, and for the PsABI5 promoter is 1231 bp with 142 cytosines. Circles represent cytosines, with methylated bases shown in black and unmethylated bases in white. See Figure S3, S4 and S5 for mapping of the *PsABI3*, *PsABI4* and *PsABI5*, accordingly.

To analyze the methylation profile of the promoter and the beginning of the first exon of the *PsABI3*, *PsABI4* and *PsABI5* genes, we performed the amplification of bisulfite-treated DNA using designed primers (Table S3). Bisulfite-treated DNA amplification and subsequent cloning revealed methylation in the promoters of *PsABI3*, *PsABI4*, and *PsABI5* already before radicle protrusion (Figure 4).

Additionally, we mapped the promoters of these genes to compare potential methylation sites and binding sites for TFs (Table S4). Notably, the *PsABI4* promoter had the lowest number of TFs binding sites, while *PsABI5*'s promoter contained numerous potential LAFL protein binding sites, along with motifs associated with responses to cold and water deprivation.

4. Discussion

4.1. ABA catabolism

ABA plays vital roles in seed development and maturation, encompassing the accumulation of storage compounds, acquisition of desiccation tolerance, induction of dormancy, and suppression of precocious germination [12,17–19,57–59]. However, to break dormancy and initiate germination, ABA needs to be catabolized, primarily through hydroxylation and conjugation. The primary ABA hydroxylation route is the ABA catabolic pathway (Figure 5), which relies on the activities of CYP707A cytochromes P450, notably ABA 8'-hydroxylases [60].

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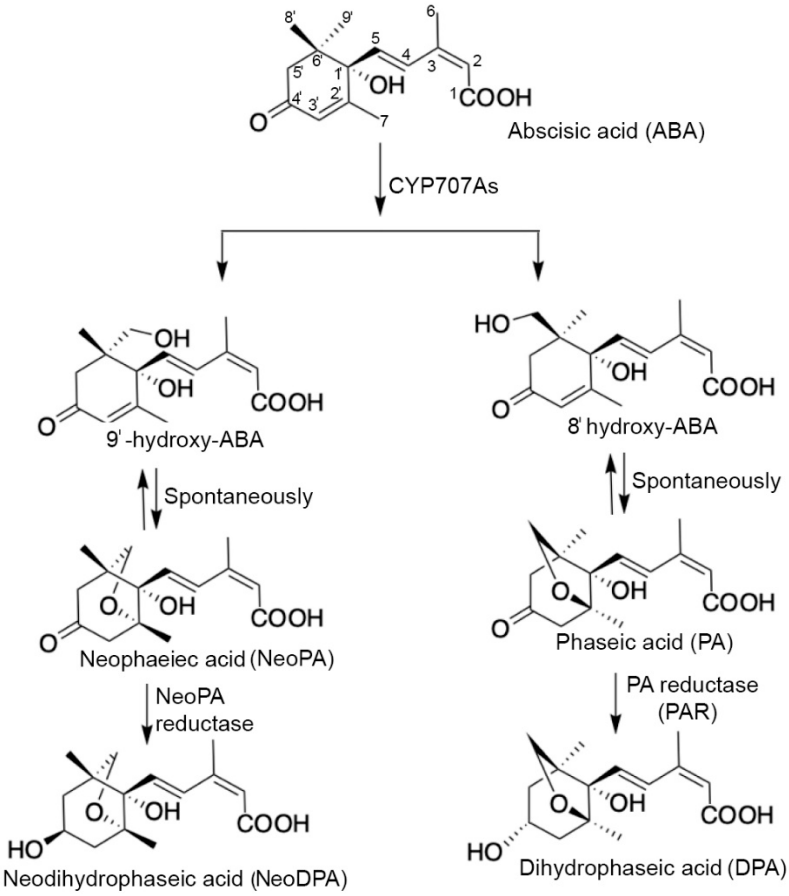

Figure 5. Oxidative pathways of ABA catabolism: CYP707s – – cytochrome P450 monooxygenases; PA – phaseic acid; neoPA – neophaseic acid; DPA – dihydrophaseic acid; neoDPA – epi-neodihydrophaseic acid; PAR – PA reductase.

Initially, ABA is catalyzed by 8'-hydroxylase, converting it to 8'-hydroxy ABA (8'-OH ABA), an unstable intermediate [61,62]. This intermediate is then spontaneously rearranged into PA and subsequently reduced by PA reductase (PAR) to DPA [24,63]. The 9'-hydroxylation pathway, similar to 8'-hydroxylation, involves CYP707As enzymes and converts 9'-hydroxy ABA (9'-OH ABA) to neoPA with both 8'-C and 9'-C hydroxylations catalyzed by the same enzyme [16]. Recently, Bai et al. (2022) [24] identified a downstream catabolite of neoPA in the 9'-hydroxylation pathway as epi-neodihydrophaseic acid (*epi*-neoDPA) and discovered the responsible enzyme, neoPA reductase 1 (NeoPAR1) (Figure 5).

Our study examined ABA and ABA-related catabolites in embryonic axes of *P. sativum* seeds before and after radicle protrusion. We found a decline in ABA content with a concurrent rise in its catabolites (PA, DPA, and neoPA) (Figure 2). Intriguingly, PA, similar to ABA, can regulate stomatal closure and suppress seed germination [64,65]. Weng et al. (2016) Weng *et al*. (2016) demonstrated that PA functions as a signaling molecule through ABA receptors. Similar ABA-like hormonal activity was observed for neoPA, but not for *epi*-neoDPA [24]. Additionally, altered seed germination patterns were noted in neo-PAR1 mutant and overexpression lines, implicating the ABA catabolic pathway as a critical regulatory mechanism during the seed-to-seedling transition [24]. Despite reduced ABA levels, the accumulation of its catabolic products (PA, neoDPA) in embryonic axes suggests a continued regulatory influence *via* ABA receptors.

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4.2. Annotation of ABA-associated DEGs

In our prior RNA sequencing-based transcriptomic analysis of pea embryonic axes isolated from seeds before and after radicle protrusion [4], we identified 24,184 DEGs, with 2,101 showing notably higher expression. This work extends that analysis by focusing on ABA-associated DEGs (ABA-DEGs). Of the 70 ABA-DEGs annotated, 46 genes up-regulated and 24 genes down-regulated more than 4-fold after radicle protrusion (Figure 3).

The up-regulated ABA-DEGs predominantly pertained to cellular signaling, stress resistance, membrane transporters, and transcription factors that regulate seedling development. For instance, *Psat6g199400*, encoding RD29B/LTI65 which responds to water deprivation, was up-regulated 4.5-fold. This gene's promoter region contains two ABA-responsive elements (ABREs) that required as *cis*-acting elements for the dehydration-responsive expression of RD29B/LTI65 [66,67] Similarly, *Psat4g146960*, encoding ANNEXIN4, a calcium-binding protein involved in drought and other stress responses [68,69], showed a 9-fold increase in expression (Table S1).

Among the up-regulated genes were those coding for membrane transporters like *Psat4g117800* (P-ATPase) and *Psat4g184760* (potassium channel AKT2/3). P-type ATPases play a role in ion transport across membranes, utilizing ATP for transmembrane conformational changes [70,71]. Additionally, *Psat2g121520*, encoding TCP15, a transcription factor implicated in cell expansion and proliferation [72,73] was up-regulated 8-fold. The TCP proteins, known as TEOSINTE BRANCHED 1 (TB1) in maize, CYCLOIDEA (CYC) in *Anthirrinum majus*, and PCF in rice [74] have been linked to various developmental processes, including light-induced cotyledon opening in *Arabidopsis* [75].

Conversely, **down-regulated ABA-DEGs** included genes central to ABA signaling (*ABI3*, *ABI4*, *ABI5*) and those involved in water deprivation response (*LEA14*, *RD22*, *HVA22*, *PER1*, and *LTI65*) (Table S1). Seed germination is governed by the antagonistic balance of ABA/GA, with ABA catabolism preceding GA synthesis and activation [5,7,17]. Key ABA signaling genes *ABI3*, *ABI4*, and *ABI5* encode the TFs featuring B3, AP2, and bZIP domains which control the expression of ABA-responsive genes crucial for seed maturation, dormancy, longevity, germination, and post-germination growth ([12,16,76–78]. .

ABI5 encodes a member of the basic leucine zipper TF family and involved in ABA signaling in seeds by acting as a signal integrator between ABA and other hormones [41,79,80]. The Arabidopsis *abi5* mutants have pleiotropic defects in ABA response, including reduced sensitivity to ABA, inhibition of germination, and altered expression of some ABA-regulated genes [81,82]. Notably, *Psat3g033680*, encoding ABI5, exhibited a 22-fold downregulation after radicle protrusion.

ABI4 was shown to be a key integration node for multiple signals, participating in critical transition steps during plant ontogenesis [83–85]. In dormant seeds, ABI4 acts as a repressor of ABA catabolism by binding to the promoter of CYP707A, being the main enzyme of ABA catabolism [86]. Thereby, ABA and GAs can antagonistically modify the expression and stability of *ABI4*, suggesting the existence of regulatory loops [83]. In germinating seeds, ABI4 can regulate both ABA synthesis and catabolism. Some authors suggest that ABI4 plays as a key regulator of the balance between ABA and GAs in seeds at post-germination stages [83,85]. In our study, the level of the *Psat2g031240* gene encoding ABI4 was decreased 21-fold.

ABI3 encoded AP2/B3-like transcriptional factor family protein [87]. ABI3 belongs to the LAFL regulatory network where interacts with LEAFY COTYLEDON1 (LEC1), ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEC2 [31,32]. The LAFL network is a positive regulator of seed dormancy and need to be suppressed for seed germination. Together, ABI3, FUS3, and LEC1 are involved in sensitivity of seeds to ABA, and regulate the expression of the 12S storage protein gene family [88]. In addition, both FUS3 and LEC1 positively regulate the ABI3 protein abundance in the seeds [89]. The expression of *Psat3g142040* encoded ABI3 was decreased 21-fold.

We also found the downregulation of ABA-dependent genes involved in response to water deprivation (*LEA14*, *RD22*, *HVA22*, *PER1* and *LTI65*) (Table S1). In accordance with our findings, *Psat7g085840* encoding peroxiredoxin1 (PER1), *Psat0s2227g0040* encoding protein LTI65/78, and *Psat0s2780g0040* encoding late embryogenesis abundant (LEA) protein were down-regulated 20-30-fold. Peroxiredoxins are thiol-dependent antioxidants containing one (1-Cys) or two (2-Cys)

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conserved Cys residues [90]. *PER1* encodes a 1-Cys peroxiredoxin (PER1) protein that accumulates during seed development but rapidly disappears upon germination [91]. PER1 is involved in the quenching reactive oxygen species (ROS) during late maturation, dormancy, and early germination, thereby maintaining seed viability [91–93]. The low-temperature-induced (LTI) protein family is associated with responses to abiotic stresses. In Arabidopsis, the homologous genes *RD29A (LTI78)* and *RD29B (LTI65)* are induced by cold, drought, salt, and abscisic acid [66]. Most LEA genes have ABA response elements in promoters and their expression can be induced not only by ABA, but also by cold or drought. The desiccation-related protein LEA14 belongs to the group II LEA proteins, also known as dehydrins [94]. LEA14 was induced in response to salt and low temperature [95].

4.3. Epigenetic regulation of PsABI3, PsABI4, and PsABI5 genes based on DNA promoter methylation.

Major transitions in the plant life cycle require fine-tuned regulation at the molecular and cellular levels. Epigenetic regulation, particularly DNA methylation, is crucial for maintaining genome stability in plants by inhibiting transposable element movement and modulating gene expression during development and stress responses [8,96]. DNA methylation patterns in seeds undergo significant changes during development and germination [8,46,47,50,52,97,98]. DNA methylation (mC) occurs in three sequence contexts (CG, CHG, and CHH) and refers as addition a methyl group to the C5 position of cytosine to form 5-methylcytosine [96]. Methylation of CHH sites notably increases from early to late stages of seed development, then decreases during germination [8,47,48]. Two DNA methylases, RdDM (RNA directed DNA methylation) and CMT2 (DOMAINS REARRANGED METHYLTRANSFERASE 2), responsible for methylating CHH sites in developing seeds, are inactivated during germination [51,52]. In contrast, CG and CHG methylation patterns are relatively stable throughout seed development [46,98,99]. Therefore, monitoring the level of 5-methylcytosine (m5C) is considered as a universal marker for seeds at the different stages of their ontogenesis [97].

Our study reveals that during the transition from germination to post-germination, expression of key ABA signaling pathway genes (*ABI3, ABI4*, and *ABI5*) is markedly suppressed. We analyzed the DNA promoter methylation profiles of *PsABI3, PsABI4*, and *PsABI5* to understand their epigenetic regulation. Contrary to our expectations of low promoter methylation levels based on their expression before radicle protrusion [4], we observed high methylation levels both before and after this developmental stage (Figure 4). Notably, approximately one-third of the *PsABI3* gene promoter region showed reduced methylation. However, this region might belong to the 5'-UTR as per the Pea Genome International Consortium version 1a (Figure S3, pink).

We further investigated the coincidence of epigenetic marks with transcription factor binding sites in the promoters of these genes, using PlantPAN 3.0 and PCBase, focusing on stress and hormone response motifs. *PsABI5* showed numerous potential binding sites for LAFL network proteins, along with motifs associated with cold and water deprivation responses (Figure S4). This finding aligns with the role of ABI5 as a major regulator of seed maturation and longevity in legumes [41]. Our results suggest that epigenetic modifications, impacting the binding ability of *ABI3*, *ABI4*, and *ABI5* to DNA promoters, occur prior to the initiation of the seed transition from germination to post-germination.

Thus, our study provided an insight into the involvement of ABA in the transition in *P. sativum* from germination to the post-germination stage when seeds turn into seedlings. The initiation of embryonic axis growth corresponds with changes in the abscisate profile: a decrease in ABA levels and an accumulation of its catabolites (PA, DPA, and neoPA), which possess hormonal activity similar to ABA [24,100]. Our in-depth analysis of ABA-DEGs revealed 46 up-regulated and 24 down-regulated genes with more than 4-fold changes. Most up-regulated ABA-DEGs were related to the regulation of seedling development. Most notably, the expression of *ABI3, ABI4*, and *ABI5* was significantly downregulated, and their promoters exhibited a high level of methylation both before and after radicle protrusion. While ABA continues to be important, other regulators appear to be involved in the seed-to-seedling transition.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Expression heatmap of ABA-associated DEGs in embryonic axes of *P. sativum* before and after radicle protrusion (RP); Figure S2: Predicted methylation sites for CpG motifs in the sequences of the seed resistance to dehydration genes in the *P. sativum* genome; Figure S3: Mapping of the *PsABI3* gene promoter; Figure S4: Mapping of the *PsABI4* gene promoter; Figure S5: Mapping of the *PsABI5* gene promoter; Table S1: Annotation of the ABA-associated genes in the transcriptome of *P. sativum* embryonic axes; Table S2: The ABA-dependent genes of *P. sativum* seeds selected for analysis of DNA methylation in the promoters; Table S3: Primers used for amplification of gene promoter regions from bisulfite-treated DNA isolated from embryonic axes of P. *sativum*; Table S4: Functions of transcription factors, binding sites of which were identified in gene promoter regions *PsABI3*, *PsABI4* и *PsABI5*.

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