The Transition from Seed to Seedling

Edited by
Galina Smolikova and Sergei Medvedev

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Cover image courtesy of Galina Smolikova

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About the Editors

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Dr. Galina Smolikova has been an Associate Professor at the Department of Plant Physiology and Biochemistry of Saint Petersburg State University (SPBU), Russia, since 2015. She completed her undergraduate studies in Biology at the Belorussian State University, Belarus, in 1988 and received her Ph.D. at the V.F. Kuprevich Institute of Experimental Botany of the National Academy of Sciences of Belarus (IEB), in 1999. She worked at the IEB from 1990 to 2010 and became a Leader Researcher in 2003. She was the National Representative of the Federation of European Societies of Plant Biology (FESPB) from Belarus from 2003 to 2010. In 2010, she moved to Saint Petersburg State University, Russia. Currently, she is a member of the Russian Society of Plant Physiologists, the Federation of European Societies of Plant Biology, and the International Society for Seed Science. Her research is focused on the different aspects of seed maturation, dormancy, and germination, including acquisition and loss of desiccation tolerance and photosynthesis of developing seeds.

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Editorial

Seed-to-Seedling Transition: Novel Aspects

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Abstract: Transition from seed to seedling represents a critical stage in plants' life cycles. This process includes three significant events in the seeds: (i) tissue hydration, (ii) the mobilization of reserve nutrients, and (iii) the activation of metabolic activity. Global metabolic rearrangements lead to the initiation of radicle growth and the resumption of vegetative development. It requires massive reprogramming of the transcriptome, proteome, metabolome, and attendant signaling pathways, resulting in the silencing of seed-maturation genes and the activation of vegetative growth genes. This Special Issue discusses the mechanisms of genetic, epigenetic, and hormonal switches during seed-to-seedling transitions. Detailed information has also been covered regarding the influence of germination features on seedling establishment.

Keywords: seed; germination; post-germination; seedling establishment; gene expression; hormonal regulation

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1. Introduction

In higher plants' life cycle, transitions from seed to seedling represent a critical developmental step, dramatically affecting plant ontogenesis and stress tolerance. To establish seedlings at the appropriate time, seeds have evolved mechanisms to maintain dormancy until environmental conditions (temperature, moisture, and light) are favorable for germination [1–3]. Three phases of seed germination are recognized [4–6]. Phase I is primarily a physical process characterized by rapid water uptakes and the hydration of macromolecules. It results in the resumption of mitochondrial respiration and activation of DNA and membrane reparation. During phase II, the main metabolic processes, for example, the synthesis of new RNA, lipids, and proteins as well as the mobilization of reserve nutrients are activated. Phases I and II encompass important events that drive the seed from a quiescent to a germination state. Phase III represents the post-germination stage characterized by the progressive division and elongation of cells in the embryonic axes resulting in so-called "radicle protrusion." This ultimately results in the loss of desiccation tolerance occurring in orthodox seeds during late germination [7–9].

Before plants can resume vegetative development, a massive reprogramming of the transcriptome and attendant signaling pathways is required. This results in the silencing of seed maturation genes and activation of vegetative growth genes. These genes, as a rule, are methylated during seed development and demethylated during germination [10]. A network of transcription factors known as LAFLs (LEAFY COTYLEDON1 and 2, ABSCISIC INHIBITOR3, and FUSCA3) and DELAY OF GERMINATION1 (DOG1) was described as the negative regulators of seed germination and should be repressed before the initiation of seedling development [11,12]. Their repression is associated with chromatin-remodeling complexes Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2), as well as the PICKLE (PKL) and PICKLE-RELATED2 (PKR2) proteins [11,13,14].

2. Novel Aspects Discussed in the Special Issue

Smolikova et al. [15] reviewed the epigenetic and hormonal switches regulating seed-to-seedling transitions. Abscisic acid and gibberellins act as central endogenous regulators,

controlling seed dormancy and germination antagonistically. However, in recent years, other hormones such as ethylene, cytokinins, brassinosteroids, auxins, and jasmonates have also been shown to be involved. The main result of hormone activity is the suppression of genes controlling seed maturation and the activation of those involved in vegetative growth. As previously described, epigenetic signaling provides a multifactorial and robust basis for the regulation of plant development and adaptation [16–19]. Smolikova et al. [15], therefore, also described primary epigenetic mechanisms involved in seed development and transitions from seed to seedling, such as DNA methylation, posttranslational modification of histones, and interaction with non-coding RNAs.

Research conducted by Smolikova et al. [20] deals with RNA sequencing-based transcriptomics in the embryonic axes isolated from pea seeds just before and after radicle protrusion. Seed-to-seedling transition was shown to involve a loss of desiccation tolerance, the initiation of secondary metabolism, and the activation of genes involved in defense responses to biotic stress. Many genes associated with auxin, ethylene, salicylic, and jasmonic acids were upregulated, while genes involved in the synthesis of transcription factors ABI3, ABI4, ABI5, and LEA14 were downregulated. Importantly, among LAFL genes, only *ABI3* was expressed. Authors further observed the downregulation of ABA-related genes *HVA22E*, *LTI65/RD29B*, and *LTP4* involved in responses to water deprivation as well as *PER1* involved in the suppression of ABA catabolism and GA biosynthesis via reactive oxygen species elimination.

Next, Arif et al. [21] reviewed the genetic aspects of seed longevity and their relationship with seedling viability. The authors defined seed longevity as "the maximum time period during which seeds can germinate and produce viable seedlings capable of developing into healthy plants and bearing seeds for the next generation." The authors summarized the most relevant genetic studies on seed longevity performed in Arabidopsis and some crop species such as rice, barley, wheat, maize, soybean, tobacco, lettuce, and tomato. This review contains further information about collections of various crops available worldwide and new emerging technologies for research on seed longevity.

Another three research studies, conducted by Ducatti et al., Ribeiro-Oliveira et al., and Wang et al., paid attention to the role of germination on seedling establishment [22–24]. Ducatti et al. [22] analyzed gene expression patterns during the germination of soybean seeds with different vigor. The authors showed a high correlation between germination and vigor for twenty genes. Among the target genes were *EXPANSIN-LIKE A1*, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 22*, *65-KDA MICROTUBULE-ASSOCIATED PROTEIN*, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 2*, and *N-GLYCOSYLASE/DNA LYASE OGG1*. These transcripts are suggested for use as a tool for evaluating seed vigor.

Ribeiro-Oliveira et al. [23] showed that acceleration of water uptake during seed germination influences seedling vigor at the post-germination stage. The authors measured water dynamics in soybean seeds during germination, in seedlings during development, and clarified their relationship. They demonstrated that water dynamics are associated with embryo and seedling vigor and, thus, can help to predict seed vigor.

Wang et al. [24] selected six grape varieties' conditions for the most efficient seed germination and seedling development. The authors tested different seed treatments such as stratification, chemical substances, beak cutting, and pre-germination to find the optimal combination for each variety. They propose using the developed approach for characterizing the germination and post-germination growth of seeds obtained by an intraspecific hybridization of grape varieties.

3. Future Perspectives

Transitions from seed to seedling are associated with a complex temporal sequence of signals. Exciting prospects for studying this phenomenon lie in the avenue of the epigenetic regulation of metabolic rearrangements. Transitions between active and repressive chromatin states in germinating seeds are still under active investigation, and the under-

lying molecular mechanisms remain largely unknown. Our current knowledge mainly arises from studies in Arabidopsis. However, the mechanisms of seed germination and seedling establishment in another plant species may differ significantly. Comparative studies between Arabidopsis and other crops will certainly enrich practical applications. They will provide ample opportunities for dissecting the role of epigenetic variations in trait regulation, which can be utilized in crop improvement. The locus-specific manipulation of DNA methylation by epigenome-editing tools can facilitate the molecular breeding of important crop plants. We hope that the manuscripts from this Special Issue stimulate additional research on this critical topic.

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Review

Transition from Seeds to Seedlings: Hormonal and Epigenetic Aspects

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Abstract: Transition from seed to seedling is one of the critical developmental steps, dramatically affecting plant growth and viability. Before plants enter the vegetative phase of their ontogenesis, massive rearrangements of signaling pathways and switching of gene expression programs are required. This results in suppression of the genes controlling seed maturation and activation of those involved in regulation of vegetative growth. At the level of hormonal regulation, these events are controlled by the balance of abscisic acid and gibberellins, although ethylene, auxins, brassinosteroids, cytokinins, and jasmonates are also involved. The key players include the members of the LAFL network—the transcription factors LEAFY COTYLEDON1 and 2 (LEC 1 and 2), ABSCISIC ACID INSENSITIVE3 (ABI3), and FUSCA3 (FUS3), as well as DELAY OF GERMINATION1 (DOG1). They are the negative regulators of seed germination and need to be suppressed before seedling development can be initiated. This repressive signal is mediated by chromatin remodeling complexes—POLYCOMB REPRESSIVE COMPLEX 1 and 2 (PRC1 and PRC2), as well as PICKLE (PKL) and PICKLE-RELATED2 (PKR2) proteins. Finally, epigenetic methylation of cytosine residues in DNA, histone post-translational modifications, and post-transcriptional downregulation of seed maturation genes with miRNA are discussed. Here, we summarize recent updates in the study of hormonal and epigenetic switches involved in regulation of the transition from seed germination to the post-germination stage.

Keywords: desiccation tolerance; DNA methylation; epigenetics; germination; histone modification; hormonal regulation; miRNA; post-germination; seeds

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1. Introduction

Seed development is a critical step in the ontogenesis of higher plants. Obviously, it is crucially important in terms of plant survival and successful reproduction. Thereby, mature seeds are typically highly dehydrated and can be considered as units of dispersal and survival during the periods of unfavorable environmental conditions [1–3]. On the other hand, for successful propagation, germination of seeds needs to be associated with the periods of optimal water and temperature regime. To adjust growth of seedlings to environmental conditions, spermatophyte plants evolved an ability to control the time of germination [4]. This ability relies on the phenomenon of dormancy, i.e., a period or temporal inhibition of plant growth, which impacts on the prevention of germination under unfavorable conditions [5,6]. Thus, release from seed dormancy is controlled by such environmental factors as light, temperature, and duration of dry storage, whereas hormonal regulation, genetic, and epigenetic factors impact essentially on this phenomenon [7–12].

The network of four master regulators, which is usually referred to as LAFL, i.e., LEAFY COTYLEDON1 (LEC1), ABSCISIC INHIBITOR3 (ABI3), FUSCA3 (FUS3), and LEC2, is directly involved in coordination of seed maturation and inhibition of seed germination [10,13]. Thus, LAFL acts as a positive regulator of seed maturation genes, whereas the players involved in inhibition of germination are only partly addressed so far. The switch of the developmental program from maturation to germination is accompanied with suppression of LAFL genes and activation of those involved in vegetative growth [14,15]. It was shown that expression of LAFL genes is negatively regulated by two transcriptional repressors, namely HIGH-LEVEL EXPRESSION OF SUGAR INDUCIBLE GENE2 (HSI2) and HSI2-LIKE1 (HSL1), also often referred to as VP1/ABI3-LIKE1 (VAL1) and VAL2, respectively [16]. The mechanisms behind the germination-related repression of the LAFL transcriptional network by HSI2 and HSL1 rely on modification of chromatin in particular, the chromatin remodeling complexes POLYCOMB REPRESSIVE COMPLEX1 and 2 (PRC1 and PRC2) [17–20], as well as PICKLE (PKL) and PICKLE-RELATED2 (PKR2) proteins [21–23]. Alterations in chromatin structure underlying changes in gene activity can be related to post-translational modifications of histones—N-terminal acetylation, side-chain methylation, phosphorylation, ubiquitination, and SUMOylation. Thereby, acetylation and methylation of histone H3 at its lysine residues are the most critical contributors in epigenetic regulation of gene expression.

Importantly, the patterns of histone modifications, also referred to as histone code, serve as specific markers for recruitment of further players of the chromatin remodeling machinery [24–26]. Specifically, it was shown that the chromatin remodeling factor PKL directly blocks the *DELAY OF GERMINATION1* (*DOG1*) gene—the key player in induction and maintenance of seed dormancy [27–29]. The authors found that PKL physically interacted with LUX ARRHYTHMO (LUX), a player of the evening complex involved in the circadian clock. LUX was shown to bind directly to a specific coding sequence of DOG1. The loss of function of either PKL or LUX resulted in decreased levels of trimethylation at K27 in histone H3 in the *DOG*1 locus. Zha et al. suggested that LUX binds directly to a specific DNA sequence of *DOG*1 and recruits PKL to the *DOG*1 locus through their physical interaction [28]. This interaction increases the levels of the trimethyllysine at K27 in histone H3 (H3K27me3), representing the part of chromatin bound to DOG1, thereby repressing transcription of this gene and leading to reduced levels of seed dormancy [28].

Thus, before plants enter the vegetative phase upon germination, the rearrangements of signaling pathways and switching of gene expression programs occur. However, despite their importance for understanding the following plant development, the molecular mechanisms behind these switches remain mostly unknown. Therefore, here, we highlight the recent advances and provide a comprehensive overview of the recently reported experimental data, providing access to understanding of epigenetic and hormonal regulation of the seed-to-seedling transition.

2. Stages of Seed Germination

In general, the overall process of seed germination can be divided into two principal periods, which are often referred to as phases I and II [2,30–32] (Figure 1). Imbibition represents the first phase, which is characterized with fast water uptake, hydration, and softening of the seed coat due to degradation of seed coat polymers. Thereby, seed water content rapidly increases due to enhanced hydration of macromolecules. Recently, Dorone et al. identified the prion-like protein FLOE1, which forms condensates during imbibition and attenuates germination under water-limiting conditions in a dose-dependent manner [33]. Imbibition is accompanied with activation of respiration and, therefore, ROS production. On the other hand, this phase features repair of membranes, mitochondria, and DNA. In turn, phase II is characterized by activation of the principal metabolic processes associated with seed germination—mobilization of reserve substances, translation of stored mRNAs, transcription and translation of newly synthesized mRNAs, processing of proteins, and their co- and post-translational modification. It is important to note that both

phases I and II are critical for maintaining seed viability. Thus, enhanced respiration and water uptake result in dramatic upregulation of ROS production. To avoid accumulation of molecular damage, repair of DNA and proteins is enhanced during phases I and II [31].

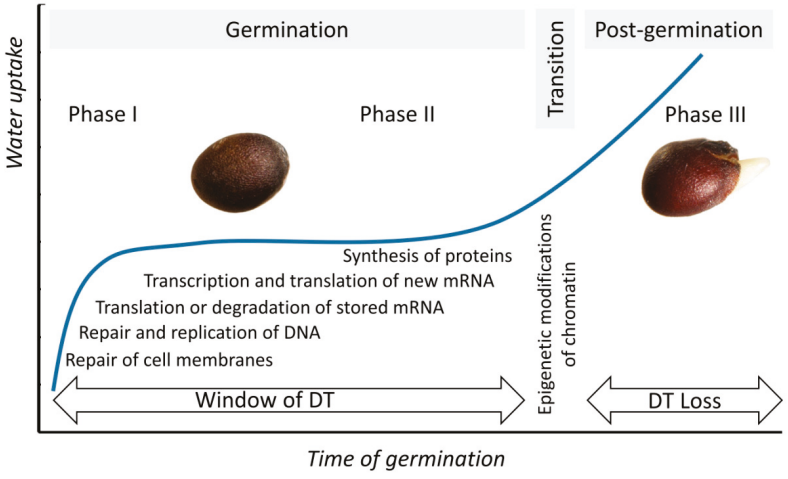

Figure 1. Time course of seed germination. The overall germination times vary from hours to weeks, depending on plant species and environmental conditions. Phase I is characterized by rapid water uptake, accompanied with enhanced hydration of macromolecules, activation of respiration, repair of membranes, mitochondria, and DNA. Phase II is characterized by mobilization of reserves, translation of stored mRNAs, transcription and translation of newly synthesized mRNAs, and activation of protein biosynthesis. The radicle protrusion is considered as the beginning of Phase III. Epigenetic changes (methylation of DNA, as well as trimethylation, ubiquitination, and acetylation of histones), occurring in this phase, result in silencing of the genes related to seed maturation and triggering expression of the genes responsible for vegetative growth of seedlings. "Window of DT (desiccation tolerance)" can be defined as the part of the overall germination period, when seeds can be dried back to their original water contents without a decrease of their viability. Transition from germination to the post-germination stage corresponds to loss of DT.

With respect to desiccation tolerance (DT), the seeds of vascular plants can be classified into orthodox and recalcitrant types [34–37]. During the last stages of maturation, the seeds of orthodox type acquire DT, which allows them to sustain unfavorable germination conditions in the metabolically inactive dry state [2,11,38,39]. The inhibited metabolic processes are resumed during germination afterwards. This ultimately results in the loss of desiccation tolerance at the stage of radicle protrusion [40,41] (Figure 1), which can be considered as the beginning of phase III—post-germination. Importantly, the part of the overall germination process before this time point is usually referred to as the "window of DT", i.e., the period when germinating seeds can be dried to their original water contents without loss of seed viability and any deterioration of their quality [42,43]. Already in the last decade, Buitink et al. showed that *Medicago truncatula* L. seeds with a radicle length of up to 1 mm can sustain a short-term dehydration, whereas the DT of the seeds was completely dependent on the degree of applied osmotic stress when the radicle length achieved 2.7 mm [40]. This fact illustrates the effect of osmopriming, which can extend a species-specific DT window [40,44]. It is known that DT of germinated *Arabidopsis thaliana* (L.) Heynh. seeds was, to a large extent, dependent on the presence of ABA in the germination medium [43,45,46]. Similar to PEG, ABA can extend the period of DT. The corresponding time window, when seeds are responsive to the introduction of ABA in medium, was described by Lopez-Molina et al. [47].

Phase III is characterized by progressing division and elongation of radicle cells, degradation of endosperm, and radicle protrusion [2,30]. Thereby, loosening of the cell wall in

the hypocotyl region (which underlies elongation of radicle cells) is mediated by ROS. The main source of the oxidative burst accompanying radicle elongation is the superoxide anon radical generated by NADPH oxidases of the plasma membrane [48,49]. The transition from phase II to phase III can be treated as the transition from seed to seedling.

3. Hormonal Regulation of Seed Germination

3.1. ABA and GAs Signaling

The release from dormancy and acquiring the germination ability are defined by the balance of phytohormones, with a strong contribution of environmental factors such as temperature, water availability, and light [50–53]. Thereby, ABA and GAs act as the main endogenous regulators, which control seed dormancy and germination in an antagonistic manner [6,8,50,54–59]. Specifically, ABA promotes seed dormancy and inhibits germination, whereas GAs, in contrast, disrupt seed dormancy and trigger germination. At the early stages of embryogenesis, low ABA contents, required for embryo development, are established by the maternal ovarian tissues, whereas the further developmental stages (seed maturation and dormancy) are controlled by ABA synthesized in the seed itself [54,57,60]. Finally, after the start of imbibition, the embryo switches to the synthesis of GAs. These hormones are transported to the aleurone layer, where they trigger expression of the genes encoding α-amylases and proteases [61].

Recently, a unique DOG1-dependent ABA signaling pathway was characterized in Arabidopsis seeds [27,62–64]. DOG1 is a master regulator of primary dormancy, onset of which follows seed maturation [29]. It was found that DOG1 interacts with negative regulators of ABA signaling and seed dormancy—phosphatases ABA-HYPERSENSITIVE GERMINATION 1 and 3 (AHG1 and AHG3)—preventing their involvement in the release of seed dormancy [65,66].

AHG1 and AHG3 belong to the group A type 2C protein phosphatases (PP2Cs) suppressing sucrose-non-fermenting-related kinases (SnRK2), which positively regulate the activity of the transcription factors ABI3 and ABI5 [65,66]. Binding of DOG1 to AHG1 and/or to AHG3 triggers the release of SnRK2, which phosphorylates ABI5 and ABI3 [11]. Thus, the DOG1-dependent signaling pathway results in the inhibition of PP2C family members, which, in turn, suppress the expression of ABA-responsive genes [67].

Developing seeds of some higher plants contain photochemically active chloroplasts and chlorophylls, which are typically destroyed at the late maturation stage [68–71]. Shanmugabalaji et al. showed that GAs control the biogenesis of chloroplasts in developing seedlings [72]. When the GA contents in germinating seeds are low, their negative regulator DELLA (RGL2) can accumulate in tissues [73]. DELLA blocks the conversion from pro-plastids to chloroplasts by promoting the degradation of TRANSLOCASE OF CHLOROPLAST159 (TOC159) via the ubiquitin/proteasome system [72]. TOC159 is known to mediate recognition of pre-protein and regulates its transport into plastids [74]. The increase of GA contents results in degradation of DELLA. This makes TOC159 available for assembly into the TRANSLOCON ON THE OUTER CHLOROPLAST MEMBRANE (TOC) complex, which accomplishes the import of photosynthesis-associated proteins into the chloroplast [72].

According to the current state of the knowledge, hydrogen peroxide (H_2O_2) acts as the master ROS-related secondary messenger involved in regulation of seed germination [75–78]. Specifically, it alters the balance between ABA and GAs by promoting the expression of *CYP707A2* genes involved in ABA degradation, and increasing the expression of *GA3ox1* genes involved in GA biosynthesis [79,80]. Not less importantly, enhancement of ROS generation triggers activation of GA signaling and promotes inactivation of ABA signaling [81]. During the recent decade, special attention was paid to the cross-talk between ROS- and phytohormone-mediated signaling pathways during seed germination [77]. Thus, Bailly et al. proposed that increased tissue ROS levels might shift the ABA/GA ratio in favor of GAs, i.e., the phytohormones triggering germination [81].

3.2. Role of Ethylene, Cytokinins, Brassinosteroids, IAA, and Jasmonates

Besides ABA and GA, other hormones, such as ethylene [82–85], cytokinins [8,86], brassinosteroids [8,87], IAA [8,55,88,89], and jasmonates [84,90], are also involved in the control of seed dormancy and germination.

Ethylene interferes with ABA- and GA-related signaling pathways, promoting seed germination in numerous species [8,83,91]. On the one hand, ethylene acts as an ABA antagonist by suppressing the regulation of ABA metabolism and signaling [6,8,92]. In some species (e.g., Brassicaceae), ethylene prevents the inhibitory effects of ABA by facilitating endosperm rupture of germinating seeds [6,60,92,93]. On the other hand, ethylene impacts the GA biosynthesis via modulation of *GA3ox* and *GA20ox* gene expression and GA signaling via DELLA proteins [94].

Cytokinins can also promote germination at the signaling level, acting as antagonists of ABA [86,95,96]. Specifically, ABA triggers downregulation of *Arabidopsis Response Regulator*s (*ARR*s), a family of genes induced by cytokinins during seed germination and cotyledon greening [86]. Among the type-A *ARR* family members, expression of *ARR*6, *ARR*7, and *ARR*15 was reported to be upregulated in ABA-deficient mutants. In turn, *ARR*6, *ARR*7, and *ARR*15 attenuated the ABA-mediated inhibition of germination. Application of exogenous ABA suppressed the type-A ARRs in Arabidopsis seeds and seedlings. Among the type-A *ARR* family members, expression of *ARR*6, *ARR*7, and *ARR*15 was upregulated in ABA-deficient mutants. In turn, *ARR*6, *ARR*7, and *ARR*15 proved to be negative regulators of ABA-mediated inhibition of germination. ABSCISIC ACID-INSENSITIVE4 (ABI4) plays the key role in ABA and cytokinin signaling by inhibiting transcription of type-A *ARR*s [86]. The ABI4 is a crucial regulator of the ABA signaling pathway during seed development, providing functional interactions between ABA and other hormones [86,97–100]. ABI4 modulates ABA and GA metabolism by targeting *CYP707A1, CYP707A2,* and *GA2ox7*. It is involved in the suppression of ethylene biosynthesis by targeting *ACS4* and *ACS8* [98,100]. A high level of ABA in dormant Arabidopsis seeds enhances the transcriptional activity of *ABI4*. In the presence of high ABA content, this factor blocks induction of ARR6/7/15, resulting in the suppression of cytokinin responses. After completion of germination, cytokinins stimulate accumulation of ARR4/5/6 [86].

Brassinosteroids (BRs) are ABA antagonists and, like GAs, can promote seed germination by enhancing the growth potential of the embryo [7,58,101–103]. In Arabidopsis, the BRs biosynthetic mutant *det2-1* and the BRs responsive mutant *bri1-1* were shown to be more sensitive to inhibition of ABA than the wildtype [101]. This observation indicates that the pathways of ABA and BR signaling might work as antagonistic regulators of seed germination. Recently, Sun et al. revealed that BRs signaling represses the accumulation of PIN-LIKES (PILS) proteins at the endoplasmic reticulum, thereby increasing nuclear abundance and signaling of auxin [104].

Auxin maintains a high level from fertilization to seed maturation by PIN carriers [105]. Auxin transport from endosperm is regulated by AGAMOUS-LIKE62 (AGL62), which is specifically expressed in the endosperm [106]. Auxins have recently emerged as essential players which modulate (in concert with ABA) different cellular processes involved in seed development, dormancy, and longevity [107–109]. Thereby, ABI3 appeared to be critical for cross-talk between auxin and ABA signaling [107,109]. In developing Arabidopsis embryos, the longevity-associated genes with promoters enriched in IAA response elements and ABI3 were induced by auxin [109], but the effect of exogenous auxin treatment was abolished in *abi3-1* mutants.

Recently, Hussain et al. showed that the auxin signaling repressor Aux/IAA8 accumulates and promotes seed germination. The IAA8 loss-of-function mutant iaa8-1 exhibited delayed seed germination. IAA8 was shown to suppress transcription of ABI3, a negative regulator of seed germination. Accumulation of IAA8 promotes seed germination by inhibiting AUXIN RESPONSE FACTOR (ARF) activity, which is accompanied by downregulating *ABI3* gene expression [89].

Treatment of wheat (*Triticum aestivum* L.) with *methyl jasmonate* inhibited expression of the ABA biosynthesis-related gene, *Ta9-cis-EPOXYCAROTENOID DIOXYGEN-ASE*1 (*TaNCED*1), which resulted in a decrease of seed ABA contents [110]. However, in Arabidopsis, jasmonate precursor (12-oxo-phytodienoic acid) inhibited seed germination, indicating that the role of jasmonates in dormancy varies between the species [111]. Xu et al. found that cold-induced germination of dormant embryos correlated with a drop of ABA contents and an increase of jasmonic acid (JA) levels, along with expressional enhancement of JA biosynthesis [90]. It was shown that the cold-induced increase in JA contents was required for the release of seed dormancy [90]. The increase of JA levels was, at least partly, mediated by the repression of two key ABA biosynthesis genes—*9-cis-EPOXYCAROTENOID DIOXYGENASE 1* and *2* in bread wheat *Triticum aestivum* L. (*TaNCED1* and *TaNCED2*). These genes encoded for 9-*cis*-epoxycarotenoid dioxygenase, catalyzing oxidative cleavage of *cis*-epoxycarotenoids—a critical step in ABA biosynthesis in higher plants [112].

3.3. The Effects of Light and Temperature

Light is a critical regulator of seed germination, especially for light-loving species with small seeds [113,114]. For most of the higher plants, seed germination is triggered by red and repressed by far red parts of the spectrum [54,115]. While far red light increases the tissue levels of ABA and suppresses GA biosynthesis, red light has an opposite effect [116,117]. Light is the key environmental signal, and phytochromes redundantly affect seed germination, with phytochrome B (PhyB) playing the major role in this process [114,116]. During the early stages of seed imbibition, Phy B mediates the R/FR photo reversible response to trigger germination. Phytochrome A (PhyA) is directly involved in irreversible photoinduction of seed germination via irradiation with low-fluence light in a broad spectral band from ultraviolet-A to the far red region of the spectrum [118,119].

The key element of the seed light-dependent signal transduction pathways is phytochrome-interacting factor 1 (PIF1), also known as PIF3-LIKE 5 (PIL5), which is known to strongly suppress seed germination in the dark via modulating the expression of GA- and ABA-related genes [114,120]. Indeed, PIF1 inhibits germination by suppressing GA biosynthesis and GA-related signaling, with a simultaneous activation of the ABA biosynthesis and signaling [116]. This inhibition is controlled by PhyB. Activation of this protein by red light leads to the degradation of PIF1. On the other hand, inactivation of PhyB by far red light results in stabilization of PIF1. Thus, light acts as a switch, affecting the balance between ABA and GA metabolism via a phytochrome-mediated mechanism, based on the PIF1 degradation and stabilization.

The temperature is another critical environmental cue affecting seed dormancy and germination timing [9,121]. Thus, application of low temperatures during seed imbibition typically stimulates seed germination (so-called stratification), whereas high temperatures inhibit it [1,117]. Cold stratification was shown to interrupt seed dormancy and to enhance germination by modulation of the balance between ABA and GAs. Recently, Yamauchi et al. found that a subset of GA biosynthesis genes was upregulated in response to low-temperature treatment [122]. This resulted in higher transcript abundances of GA-inducible genes in imbibed Arabidopsis seeds and increased tissue levels of bioactive GAs. On the other hand, ABA metabolism and signaling also underlie the release of seed dormancy after cold stratification. During cold imbibition, ABA seed contents decrease and the expression of ABA-responsive genes changes [32].

4. Epigenetic Mechanisms of the Seed-to-Seedlings Transition

All the major epigenetic mechanisms, which are generally known in eukaryotes to date, were successfully confirmed in plants [26,123,124]. Thus, DNA methylation, post-translational modification of histones, and interaction with non-coding RNAs provide a multifactorial and robust basis for epigenetic regulation of plant development and adaptation [125–128]. Thereby, stable allelic epigenetic inheritance efficiently complements

the hereditary role of DNA, representing an additional molecular mechanism underlying practically unlimited diversity [129].

4.1. DNA (de)Methylation

Generally, DNA methylation represents a covalent modification of the cytosine base, which is typically associated with the dinucleotide consensus CG. However, in plants, in contrast to other organisms, DNA methylation can also occur at cytosines localized to CHG and CHH consensus sequences, where H is A, C, or T (Bird, 1986; Finnegan et al., 1998). In coding sequences, methylation most commonly occurs at CG sites, while non-CG methylation (CHG and CHH) is much less common [130]. In angiosperms, CG methylation accounts for more than 50% of the total cytosine methylation [131]. However, independently from the specific consensus sequence, the overall methylation patterns of genomic DNA vary essentially among plant species. Thereby, the heterogeneity of CHG and CHH methylation is higher in comparison to the modification patterns, characteristic for the CG sites [131–133].

In plants, the occupation of potential methylation sites decreases upstream of the transcription start site (TSS) and around the transcription termination site (TTS), while the degree of methylation inversely correlates with gene expression levels in promoter regions [130,134]. On the other hand, moderately expressed genes are methylated in gene bodies [134,135]. Thus, in the Arabidopsis genome, 73% of DNA methylation sites are located in exons, whereas only 8% of them can be found in putative promoter regions, 3% in introns, and 16% in extragenic regions [51].

It is generally agreed that DNA methylation is an epigenetic modification underlying the silencing of transposable elements (TE) and directly involved in gene expression regulation. It plays a critical role in plant growth and development [130]. Accordingly, both seed development and germination are accompanied by dynamic reconfiguration of DNA methylation [136,137]. DNA methylation is represented by two forms—maintenance methylation and de novo methylation [138]. Maintenance methylation assumes recognition of the methylation marks on the DNA parental strand and transfers new methylation to the daughter strands after DNA replication. During de novo methylation, transfer of methyl groups to cytosines of DNA occurs independently from their previous methylation by DRM2, with the participation of the RNA-directed DNA methylation (RdDM) pathway [139–141]. It is de novo methylation that is involved in the rearrangement of methylation patterns during differentiation processes. Several distinct DNA methyltransferases are involved in generation (de novo) and subsequent maintenance of DNA methylation at three sequence contexts.

In Arabidopsis, DNA METHYLTRANSFERASE 1 (MET1) is the major enzyme involved in maintaining CG methylation [142–144]. In contrast, methylation at CHH and CHG sites typically relies on activities of two enzymatic systems—DNA CHROMOMETHYLASE 2 and 3 (CMT2 and CMT3) and DOMAINS REARRANGED METHYLTRANSFERASES 1 and 2 (DRM1 and DRM2) [145–150].

Recently, it was shown that precocious germination of *Solanum lycopersicum* L. seeds could be promoted by silencing of MET1 [151]. This was associated with a decrease in the contents of mRNAs encoding 9-*cis*-epoxycarotenoid-dioxygenase—a key enzyme of ABA biosynthesis.

As repression of TEs is required for stability of the plant genome, they are typically located in transcriptionally inactive regions [152]. Thus, potential methylation sites in long and gene-distal TEs are the typical targets for both CMT2 and CMT3 [153]. Maintenance of CHH methylation at short, gene-proximal TEs as well as at the edges of long TEs requires the mechanism of RNA-dependent DNA methylation (RdDM), which involves DNA-dependent RNA polymerases IV and V (Pol IV and Pol V) [152,154]. This pathway involves two main steps: an upstream small interference RNA (siRNA) biogenesis phase and a downstream methylation targeting phase. Pol IV produces short precursor RNAs that are processed into 24 nt small interfering RNAs (siRNAs) by a Dicer-like endonucle-

ase 3 (DCL3) and further loaded into ARGONAUTE 4 (AGO4), forming AGO4-siRNA complexes [155,156]. Pol V produces non-coding RNA transcripts that are proposed to act as a scaffold at sites of DNA methylation [157]. These scaffold transcripts are bound by Pol IV-dependent 24 nt siRNAs that recruit DRM1 and DRM2 to maintain DNA methylation [154,158].

DNA methylation patterns have a clearly dynamic character and are continuously changing during plant seed development [159–162]. Thus, the occupancy of the CHH methylation sites remarkably increases from the early to the late stages of seed development and gradually decreases later on during germination. Thereby, both RdDM and CMT2 are responsible for CHH methylation in developing seeds, although both these enzymes lose their activity upon germination [136,163]. In soybean (*Glycine max* (L.) Merr.), DNA methylation in the CHH context increased from 6% at the early stage of seed development to 11% at the late stage [161]. Thus, the dynamics of soybean and Arabidopsis seed methylomes were clearly similar, i.e., the levels of CHH methylation gradually increased during seed development from fertilization to onset of dormancy in all parts of the seeds [164]. In contrast to the CHH sites, the patterns of CG and CHG methylation remain, to a large extent, unchanged over the whole period of seed development (Figure 2A) [136,161–165].

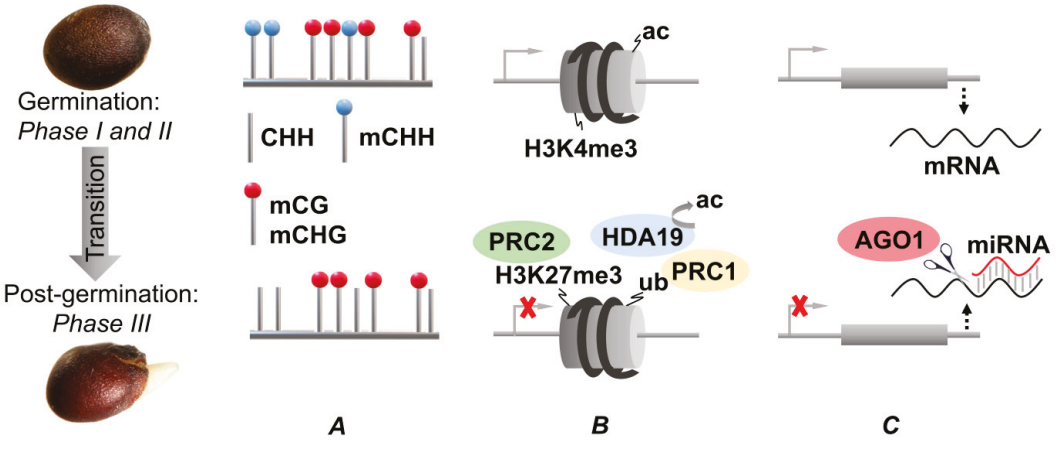

Figure 2. Epigenetic modifications accompanying the transition from germination to the post-germination stage of plant development. (**A**) Methylation of cytosines in DNA. CHH methylation (mCHH) is gradually lost during germination. In contrast, CG and CHG methylation (mCG and mCHG) mostly remain stable. (**B**) Histone post-translational modifications. The genes involved in seed maturation and dormancy are repressed by trimethylation of K27 in histone H3 by PRC2 and ubiquitination at K119 of histone H2A by monoubiquitin ligase PRC1. The repression of the target gene is initiated by recruitment of histone deacetylase 19 (HDA19) by VAL proteins to remove histone acetylation marks and PRC1 to incorporate the H2A ubiquitine marks. Upon its onset, stable repression of the target genes can be constantly maintained by PRC2-mediated trimethylation of K27 in histone H3. (**C**) Post-transcriptional downregulation of target genes. miRNAs (red wavy line) block the expression of the genes involved in development and dormancy genes by cleavage of their mRNAs with Argonaute1 (AGO1) proteins.

Later, during the seed maturation step, the levels of CHH methylation in TEs decrease drastically [163]. For example, it was shown for the Arabidopsis quadruple mutant *ddcc* (*drm1 drm2 cmt2 cmt3*), which was deficient in all methyltransferases required for all types of non-CG methylation [164]. The authors found that more than 100 TEs were transcriptionally derepressed in *ddcc* seeds. This might indicate reinforcement of TE silencing in developing seeds upon the upregulation of cytosine methylation in the CHH consensus sequence [164]. Thus, the proposed mechanism might underlie the constantly silent state of TEs, which, therefore, do not inactivate genes essential for seed development.

Multiple genes involved in seed development and germination are located in hypomethylated regions of the genome, known as DNA methylation valleys (DMVs, [166]). The DNA methylation status of these regions remains unchanged during the whole period of seed development, from fertilization to germination. Indeed, several genes encoding the enzymes of hormone biosynthesis (e.g., gibberellic acid oxidase *GmGA20Ox2*, *GmGA3Ox1*, *AtGm20Ox2*, and *AtGA3Ox1*), storage proteins (e.g., *GmGlycinin1*, *AtCruciferin1*), and some transcriptional regulators, are located within hypomethylated regions of the soybean and Arabidopsis genomes [166]. DMVs constitute an important part of the soybean seed genome, which does not vary significantly in the context of methylation status during seed development and early germination [166]. Moreover, genome regions hypomethylated during the plant lifecycle are enriched in genes encoding TFs, as well as in the genes critically impacting on seed formation (*LEC1*, *ABI3*, and *FUS3*) [10,166].

Seed germination is accompanied by silencing of the genes involved in seed development and activation of those controlling vegetative growth, mostly associated with cell division and cell wall organization. These genes are typically methylated throughout seed development and are later demethylated during germination [136,163]. Hypermethylation of genes in germinating seeds is reprogrammed mainly by passive CHH demethylation.

Demethylation of plant DNA can be either passive or active. Passive demethylation occurs when the new chain in the replicated DNA molecule is not involved in maintaining methylation. In this case, only the old (maternal) chain appears to be methylated [26]. In contrast, active demethylation relies on the activity of demethylases represented in Arabidopsis by four enzymes: DEMETER (DME), REPRESSOR OR SILENCING 1 (ROS1), DEMETER-LIKE 2 (DML2), and DEMETER-LIKE 3 (DML3) [167,168].

4.2. Modification of Histones

Different types of histone post-translational modifications have been described to date in the context of epigenetic regulation of seed development and germination. Thus, alterations in chromatin structure leading to gene expression changes can be underlined, at least by acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation of histones [24–26]. These modifications play an important role in control of seed maturation, dormancy, and germination [10,62]. Thereby, the patterns of histone modifications (so-called histone code) serve as the marks for attachment of other proteins, involved in remodeling of chromatin. Methylation and acetylation of lysine residues in histone H3 directly affect the expression of associated genes.

Thus, the cycles of histone acetylation and deacetylation are important elements in the regulation of genome activity [169]. Acetylation of lysine side chains affects the overall positive charge of histones and charge distribution on their surface [170,171]. It ultimately affects the interaction of histones with negatively charged phosphate groups of DNA and results in de-condensation of chromatin. The resulted relaxed structure is associated with its higher transcriptional activity. This relaxation can be reversed by the activity of deacetylases [172] (Figure 2B).

Trimethylation at K4 and K27 of histone H3 (H3K4me3 and H3K27me3), leading to activation or suppression of gene expression respectively, represent the most well-characterized examples of site-specific post-translational modifications of histones (Figure 2B). H3K27me3 plays a critical role in the regulation of genes involved in plant developmental control [10,26].

In plants, H3K27me3 is found in transcriptionally inactive regions of promoters and in transcribed regions of individual genes, whereas H3K4me3 is an antagonistic modification of histones in transcriptionally active regions [173]. Multiple DNA methylation valley (DMV) genes contain H3K4me3, H3K27me3, or bivalent marks that fluctuate during development [25,174]. The Arabidopsis H3K4me3 demethylases are also known as Arabidopsis trithorax (ATX) and Arabidopsis trithorax-related (ATXR) [175] (Table 1).

In mature embryos, *ABI3* and *LEC2* are associated with H3K4me3, which marks these genes as transcriptionally active. However, upon germination, these modifications

are replaced by antagonistic H3K27me3, which results in transcriptional deactivation of these genes [10]. Recently, Chen et al. revealed that H3K27me3 demethylase RELATIVE OF EARLY FLOWERING6 (REF6) directly upregulates the expression of abscisic acid 8′-hydroxylase 1 and 3 (*CYP707A1* and *CYP707A3*) involved in ABA catabolism in seeds [176] (Table 1).

Polycomb proteins form chromatin-modifying complexes that implement transcriptional silencing in higher eukaryotes. Thus, hundreds of genes can be silenced by Polycomb proteins, including dozens of those encoding crucial developmental regulators in organisms from plants to humans [177,178]. Gene suppression typically relies on the PRC1 and PRC2. Both PRC1 and PRC2 are represented by several families of related complexes, which target specific repressed regions [10]. Thus, PRC2 is responsible for the trimethylation at K27 of histone H3 [177,179], whereas PRC1 catalyzes mono-ubiquitination of K119 in histone H2A, yielding a transcriptionally inactive chromatin conformation. It is generally agreed that PRC2 is required for initial targeting of the genomic region to be silenced, whereas PRC1 impacts on stabilizing this silencing and underlies the cellular memory of the silenced region after cell differentiation. The activity of PRC2 in plants can be inhibited by treatment with 1,5-bis-(3-bromo-4-methoxyphenyl)-penta-1,4-dien-3-one, which affects both seed germination and radicle growth [179].

Although the PRC1 complexes differ significantly between animals and plants, some of their components, such as ring-finger proteins RING1 and BMI1, are rather conserved [180]. In Arabidopsis, the PRC1 core components, AtRING1 and AtBMI1, were shown to physically interact with the PHD domain H3K4me3-binding ALFIN1-like (AL) proteins. The loss of AL6 and AL7 by T-DNA insertion mutant analysis, as well as the loss of AtBMI1a and AtBMI1b, retards seed germination and causes transcriptional de-repression, accompanied by a switch of histone modification state from H3K4me3 to H3K27me3 [17]. Therefore, AL PHD-PRC1 complexes associated with histone H3 act as switchers from the H3K4me3-associated active to the H3K27me3-associated repressive transcription state of the genes involved in seed development.

In the regulatory pathways involved in control of seed development, maturation, and germination, transcription factors containing the B3 DNA-binding domain (DBD) play the key role [181]. The DBD is a highly conserved domain consisting of 100–120 amino acid residues, designated as B3, that was originally identified as the third basic region in the ABI3 and VP1 proteins [182]. Among them, the LAFL network of transcription factors is directly involved in the activation of seed maturation, whereas VAL (VP1/ABI3-LIKE) proteins suppress LAFL-related effects, i.e., initiation of germination and vegetative development [16,183]. As was already mentioned above, chromatin remodeling complexes PRC1 and PRC2 [17,18], as well as the PKL and PKR2 proteins [21,22], are involved in repression of the LAFL network of transcription factors during seed germination [160]. For example, during seed germination, LAFL genes are repressed by the Polycomb machinery via its histone-modifying activities: the histone H3 K27 trimethyltransferase activity of the PRC2 and the histone H2A E3 monoubiquitin ligase activity of the PRC1 [184–187] (Figure 2B). Specifically, VAL proteins recruit histone deacetylase 19 (HDA19) and PRC1 to the chromatin regions, which contain genes involved in regulation of development and dormancy (Table 1). Thereby, HDA19 removes histone acetylation marks, whereas PRC1 incorporates monoubiquitinated histone H2A (H2Aub) marks to initiate initial repression of the target gene [188]. Thus, the VAL proteins (which are required for the introduction of H2Aub gene marks in histone H3 molecules) appear to cause the initial repression of the seed development- and germination-related genes. Later on, this repression is maintained by PRC2-mediated trimethylation at H3K27 [188]. It is important to note that VAL1 was shown to interact with HDA19 and to repress LAFL gene expression during germination [189].

Table 1. A selected list of epigenetic modifications of histones involved in seed development and germination.

Enzymes	Function	Target Gene(s)	Trait Affected	Species	References
PRC2	Trimethylation of K27 on histone H3 (H3K27me3)	*LEC1, ABI3, FUS3, LEC2, DOG1,* repression	Germination	*A. thaliana*	[18,184,186,187]
HDA19	Removal of histone acetylation mark (H3K9ac)	*LEC1, ABI3, FUS3, LEC2, DOG1,* repression	Germination	*A. thaliana*	[10,160,189]
PRC1	Ubiquitination at K119 of histone H2A (H2Aub)	*LEC1, ABI3, FUS3, LEC2, DOG1,* repression	Germination	*A. thaliana*	[17,185,188]
ATXR	Trimethylation of K4 on histone H3 (H3K4me3)	*LEC1, ABI3, FUS3, LEC2, DOG,* etc., transcriptional activation	Seed development	*A. thaliana*	[17,175]
REF6	H3K27me3 demethylase	CYP707A1, CYP707A3, transcriptional regulation	ABA catabolism in seeds	*A. thaliana*	[176]

Two other factors, playing an important role in repression of the embryonic state, were identified in Arabidopsis: PICKLE (PKL), encoding for the putative chromatin-remodeling factor CHD3, and gibberellins. It was found that PKL acts throughout the seedling, repressing the expression of embryonic traits, and is required for GA-dependent responses in shoots [190].

4.3. miRNA-Target Modules

It is well-known that the plant genome contains both protein-coding and non-coding sequences [191,192]. Non-coding sequences are represented by regulatory non-coding RNAs—microRNAs (miRNAs), long non-coding RNAs (lncRNAs), short interfering RNAs (siRNAs), and circular RNA (circRNA) [193,194]. Small non-coding RNAs (sRNAs) are known as important regulators of gene expression, affecting almost all stages of the plant lifecycle [195,196]. The regulatory RNAs of this type act at the transcriptional and post-transcriptional levels and essentially impact on seed development and germination [160,197,198].

The major class of plant sRNAs is represented by miRNAs, which are involved in regulation of plant development at the post-transcriptional level (Figure 2C). The biogenesis of miRNAs is a multistep process, including transcription of miRNA genes, processing of primary miRNAs, and loading of mature miRNAs into ARGONAUTE (AGO) proteins to form the miRNA-induced silencing complex (miRISC). Plant miRNAs are involved in multiple regulatory mechanisms, including mRNA cleavage, repression of translation, and DNA methylation [193,196,199,200].

The tissue contents of individual miRNAs change dynamically throughout the whole stages of seed development and germination. Thereby, their abundance correlates well to the phases of seed development, maturation, and germination [165,197]. Thus, miRNAs block the expression of the genes involved in control of development and dormancy via cleavage of mRNA by AGO1 proteins [193] (Figure 2C). Comprehensive analysis of miRNAs in canola seeds showed that miR156 is involved in regulation of the transition to germination [201] (Table 2). It was also shown that DOG1 affected the levels of miR156 and miR172 and could therefore regulate seed dormancy in lettuce [202]. Thereby, suppression of the DOG1 expression enabled seed germination at high temperatures. This effect was accompanied by a decrease in miR156 and an increase in miR172 levels.

The small RNAs miR159 (targeting transcripts of the myeloblastosis family genes MYB33, MYB65, MYB101) and miR160 (targeting transcripts of the gene ARF10) also impact on seed germination (Table 2). Changes in the levels of these miRNAs or in the sensitivity of the target transcripts alter the response of germinating seed to suppression of ABA biosynthesis [198,203,204]. Five further miRNAs (ath-miR8176, ath-miR851-5p, ath-miR861-3p, ath-miR158a-5p, and ath-miR779.2) showed the highest expression level during germination. Among these RNAs, miR851 might target the pentatricopeptide repeat (PPR) gene family, which is also expressionally upregulated during germination [165]. As some

predicted targets of miR858a (MYB13, MYB65, and MYB93) are known as the regulators of germination, this RNA might also be involved in germination [165].

Table 2. The list of miRNAs involved in regulation of seed development and germination.

miRNAs	Target Gene(s)	Trait Affected	Species	References
miR160	*ARF10*	Germination, seedling growth	*A. thaliana*	[3]
miR858a	*MYB13, MYB65, MYB93*	Germination	*A. thaliana*	[4]
miR8176, miR851-5p, miR861-3p, miR158a-5p, miR779.2	Pentatricopeptide repeat (*PPR*) gene family	Germination	*A. thaliana*	[165]
miR156, miR172 (and high temperature)	*DOG1*	Dormancy, germination	*A. thaliana*, *L. sativa*	[202]
miR159	*MYB33, MYB65, MYB101*	Gibberellin, signaling pathway, germination	*A. thaliana*, *B. napus*	[1,2]
miR160, mi167	*ARF17, ARF8, ARF6, ARF10*	Auxin signaling pathway, germination	*A. thaliana*	[201]
7miR156	*BRI1*, *FUS3*, squamosa-promoter binding-like *(SPL)* gene family, etc.	Germination	*B. napus*	[201]
miR402	*DML3*	Germination	*A. thaliana*	[205]

To summarize, the epigenetic signals, such as the changes in DNA methylation, demethylation, histone post-translational modifications, and sRNA-related regulatory mechanisms, are the key modulators of seed development and the transition from seeds to seedlings. To date, the role of reversible DNA methylation and histone modifications accompanying seed germination is well-studied. However, specific miRNAs and their specific target genes are still mostly uncharacterized.

5. Conclusions

Due to complex temporal patterns of specific signals, deciphering the mechanisms behind the transition from seed to seedling represents a challenging task. Nevertheless, some exciting prospects for the future research in this area can be clearly seen. Thus, highly efficient comprehensive approaches to dissect these mechanisms at the epigenetic level will reveal gaps in our understanding of the transition from dormancy to germination. In this regard, the role of epigenetic modifications in the hormonal regulation of the transition from seed to seedling is of a particular interest. Obviously, detailed studies addressing the loss of desiccation tolerance during seed germination and aiming at identification of the involved genes, transcripts, proteins, and metabolites by means of comprehensive post-genomic techniques are still to be accomplished. Dynamics of chromatin, i.e., the transitions between its active and repressed states, are also poorly characterized in the context of seed germination, and the underlying molecular mechanisms remain mostly unknown. Physiological diversity of the seed to seedling transition is another issue to be addressed in the nearest future. Indeed, the mechanisms of seed dormancy and germination are mostly characterized for Arabidopsis as a model plant, whereas the crop plants remain to a high extent insufficiently addressed in this context. Thus, a comprehensive comparison of the mechanisms underlying the transition from dormancy to germination and from seed to seedlings in different species is strongly mandatory. The state-of-the-art methods of epigenomics research, such as bisulfite sequencing and 5-methylcytosine sequencing, might help to gain a deeper insight into the role of epigenetic variability in the formation of crop plant phenotype [206]. On the whole, the methods of genomics and post-genomic research provide a versatile instrument to probe the regulatory mechanisms behind the traits, promising in crop improvement programs. Finally, locus-specific modification of DNA methylation patterns by epigenome-editing tools might facilitate molecular breeding (epibreeding) of valuable crop plants.

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Review

Genetic Aspects and Molecular Causes of Seed Longevity in Plants—A Review

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Abstract: Seed longevity is the most important trait related to the management of gene banks because it governs the regeneration cycle of seeds. Thus, seed longevity is a quantitative trait. Prior to the discovery of molecular markers, classical genetic studies have been performed to identify the genetic determinants of this trait. Post-2000 saw the use of DNA-based molecular markers and modern biotechnological tools, including RNA sequence (RNA-seq) analysis, to understand the genetic factors determining seed longevity. This review summarizes the most important and relevant genetic studies performed in Arabidopsis (24 reports), rice (25 reports), barley (4 reports), wheat (9 reports), maize (8 reports), soybean (10 reports), tobacco (2 reports), lettuce (1 report) and tomato (3 reports), in chronological order, after discussing some classical studies. The major genes identified and their probable roles, where available, are debated in each case. We conclude by providing information about many different collections of various crops available worldwide for advanced research on seed longevity. Finally, the use of new emerging technologies, including RNA-seq, in seed longevity research is emphasized by providing relevant examples.

Keywords: seed longevity; genetics; quantitative trait loci; candidate genes; genebanks

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1. Introduction

Sustainable agriculture depends on the judicial use of natural resources, including use of crop varieties that are resistant to pests and diseases and do not require pesticide spraying, thus providing environmental benefits in addition to fulfilling the energy requirements of mankind. At present, >7000 plants are cultivated for food, shelter, and other purposes [1], and approximately 50% of human food comes from maize, wheat, and rice. Climate change has posed an extinction threat to 8% of the 250,000 species of flowering plants by 2025. To arrest this calamity, plant genetic resources are stored and regenerated in >1750 gene banks storing >7,000,000 accessions [2]. To successfully maintain such an extensive germplasm, a systematic evaluation of seed survival and longevity of the plant material stored is always under progress [3]. Because seeds are the prime storage material, research on seed longevity is of particular importance [4].

Seed longevity is defined as the maximum time period during which seeds can germinate [5–7] and produce viable seedlings capable of developing into healthy plants and bearing seeds for the next generation. In addition to many other features, seed longevity is influenced by pre-storage and storage conditions and the genetic and physiological storage potential of seeds. It is also affected by harsh conditions during or after seed development or damage prior to or during storage [8]. The long-term storage of seeds, particularly under unfavorable conditions, leads to the loss of viability, which is variable in nature. Loss

of viability is related to various seed properties, including color, weight, and membrane composition, which are often species or, in some cases, even variety specific [9].

Seed quality can be reduced in parental plants owing to adverse environmental conditions, premature germination [10] and pathogens [11]. Damage to seed quality can be categorized into either short-term deterioration (occurring in the field, such as deterioration of the mother plant) or long-term deterioration (occurring during storage). The latter includes membrane and genetic damage, changes in respiratory activity and enzymes and protein damage [9,10]. All parts of the seed deteriorate with time, the damage from which can be sustained by the chemical constituents of seeds and the way these compounds interact to form biological structures. Integrity of DNA, proteins, and membranes is especially important for maintaining seed viability [12].

Seed deterioration during storage may involve many physical and chemical changes, including disrupted intracellular integrity, decreased enzyme activity, lipid peroxidation, and nonenzymatic reactions [13,14]. Seed viability and vigor are dependent on the integrity of cellular macromolecules and orderly compartmentalization of the cell [13]. Aging is an inexorable trend to disorder. Defense mechanisms innate to the seed's structural and chemical features that are characteristic of a particular species may limit the rate of this decay [15].

Seed deterioration varies between different varieties of the same species. Even within a variety, the storage potential of individual lots varies, and within a seed lot, individual seeds have different storage potentials [9]. Broken, cracked, or bruised seeds deteriorate more rapidly than undamaged seeds [14,16]. Environmental stresses, including deficiency of minerals (including nitrogen, potassium, and calcium) [17]), water [18] and temperature extremes [19] during seed development and prior to physiological maturity can also reduce the longevity of seeds.

2. Mechanisms of Seed Ageing

A number of mechanisms of seed aging have been identified [20], including lipid peroxidation, which results in membrane damage and generation of toxic byproducts [21], oxidative damage to DNA and proteins [22], and loss of protein function during deterioration as a result of the formation of sugar–protein adducts [23]. In contrast, antioxidants, heat shock proteins (HSPs) and enzymes to repair protein damage are thought to be involved in mitigating the effects of ageing on seed longevity [24,25].

Genetic differences between species are also responsible for differential seed longevity; for example, seeds of *Canna* [26] and *Lotus* [27] are thought to be viable even after 1300 years. *Albizia* benth., *Cassia* L., *Goodia*, and *Trifolium* L. seeds can germinate after 100 years [28]. Seeds of other species are characteristically short-lived, including lettuce (*Lactuca sativa* L.), onions (*Allium cepa* L.), parsnips (*Pastinaca sativa* L.), and rye (*Secale cereal* L.) [29]. The influence of oil content on longevity under open storage conditions has also been addressed [29]; however, further analysis is required to arrive at a definitive conclusion. Species with similar chemical composition could also have significantly different storability. For example, chewing fescue (*Festuca rubra* subsp. *commutata* Gauidin) and annual ryegrass seeds have similar appearances and chemical compositions. However, ryegrass seeds were stored much better under comparable conditions [30]. Seed longevity can vary by as much as seven-fold depending on the genetic differences among cultivars of the same species [31].

Very little is known about the genetic basis of differences in seed quality because this trait is strongly affected by environmental factors during seed formation, harvest, and storage and is probably controlled by many genes. Therefore, seed longevity is a composite trait because in genetic studies of longevity [32], genetically identical seed lots of seeds, even when grown under identical conditions or derived from a single plant, lose their viability at different intervals after harvest. Seed longevity is a quantitative trait [12] and is strongly affected by the environment during seed formation, harvest, and storage [33,34] through a variety of mechanisms, whose understanding might enable us to greatly increase

the seed longevity of agriculturally important species and varieties and preserve plant genetic resources for generations [35].

This review focuses on the genetic determinants of seed longevity in a variety of plant species after describing classical genetic studies.

3. Classical Genetic Studies

Awareness of seed longevity dates back to ~2500 years ago (372 BC–287 BC) when Theophrastus discussed seed deterioration in his botanical writings [14,36] Similarly, in Fan Sheng-zhi Shu, an agricultural book of China written in the 1st century BC [37], the longevity of wheat and millet seeds is discussed. Their viability was maintained if they were kept as dry as possible, cool, and free from pests. It was also suggested that only large and solid ears of wheat should be chosen for sowing, and they should be dried as thoroughly as possible by the heat of the sun before storage.

Classical genetic studies were first initiated and probably the best characterized in maize (*Zea mays* L.). Maize seeds have been declared more susceptible to aging [38] if they carry homozygous alleles for either the *luteus 2* or *luteus 4* genes, although the physiological basis of this difference remains obscure. Later, two unrelated studies [39,40] demonstrated that the long-lived character of maize appeared to be dominant, although a non-cytoplasmic maternal plant influence was also identified. Thirty-seven years later, Rao et al. [41] transferred the same nuclear genotype used by Haber [40] to genetically different cytoplasmic types and concluded that cytoplasmic factors had a marked influence on seed storability. Later, single-cross hybrids were employed to investigate long- and short-lived lines of maize, which demonstrated the dominant character of the long-lived seeds [42]. After three selection cycles (based on resistance to aging using experimental procedures (42 °C and saturated humidity)), a reduction in sensitivity to aging was reported, which suggested that genetic improvement for storability is achievable [43].

The longevity of spring and winter wheat remains controversial. For example, according to Van der Mey et al. [44], winter wheat stores better than spring wheat over periods of 15–20 years at 5 °C. In contrast, Arif et al. [45] did not find any difference in longevity between spring and winter wheat after experimental aging. Furthermore, [46] reported no association between grain color and longevity in wheat.

In legumes, hard seeds within a particular seed lot retain viability for longer storage periods than their softer companions [47]. Seed color and coat thickness have also been reported to play role in seed longevity in chickpeas. For example, pale-seeded "Kabuli" chickpeas have also been reported to be shorter lived than "Desi" types with thicker, harder and darker coats [48]. Dark-seeded soybeans were also more resistant to storage under high humidity [49]. In *Phaseolus vulgaris*, a diallele cross-analysis was used to demonstrate that superior longevity was dominant in nature [50]. In soybean, reciprocal crosses revealed a strong maternal influence through the characteristics of the seed coat on the longevity of F_1 seeds [51] in addition to a minor influence of the seed's own genotype.

In a study of 55 accessions of barley stored in the Gatersleben gene bank since 1974, intraspecific variability in longevity was addressed [52]. Germination tests after 35 years of storage indicated intraspecific variability in seed longevity within barley owing to genetic determinants. A similar conclusion was drawn for *Brassica napus* L. [53] based on the results of 42 accessions. Hence, genotypic components are involved in determining seed viability. The same phenomenon of intraspecific variability towards longevity has been observed in *Sorghum bicolor* L., *Secale cereale* L., and *Linum usitatissimum* L. [54].

4. Genetic Studies in the 21st Century

Genetic mapping of seed longevity was initiated only in the 21st century when the molecular markers were readily available, and plant breeders started to use this new resource to decipher the genes and molecular mechanisms behind intraspecific variability in seed longevity in model, as well as crop, species [36].

4.1. Arabidopsis thaliana L.

The first detailed genetic analysis of longevity after natural aging was published in 2000 [55] while investigating raffinose family oligosaccharides (RFOs), including sucrose, raffinose, and stachyose. A recombinant inbred population (RIL) was developed, and subsequent quantitative trait loci (QTL) mapping revealed one major QTL for oligosaccharide OSs. Two candidate genes, *galactinol synthase* and *raffinose synthase*, were detected at the site of the major QTL. Additionally, four QTLs for storability were detected. QTLs for OS, however, lie at different positions from QTLs for storability, indicating different genetic controls of OS and longevity. Many years later, galactinol content was demonstrated to be highly correlated with seed longevity in *Arabidopsis*, and galactinol was identified as a suitable biomarker for predicting seed longevity [56].

Another detailed investigation was undertaken by Clerkx et al. [12] through the development of different RIL populations ('*Landsberg erecta*' × '*Shakdara*') to determine variation in seed longevity after various artificial ageing protocols. One or more QTLs were identified for various traits (dormancy, speed of germination, seed sugar content, and germination) after various treatments (controlled deterioration test (CDT), H_2O_2 treatment, and abscisic acid), with some QTLs for different co-locating traits. Using various *Arabidopsis* mutants, the same group of scientists concluded that *abscisic acid insensitive3* (*abi3*), *abscisic acid deficient1* (*aba1*), and *aberrant test shape* (*ats*) mutants have reduced longevity [57]. Consequently, the importance of genetic background was revealed through the analysis of "double" mutants. In the same year, the role of vitamin E (tocopherols) in seed longevity [58] in *Arabidopsis* was demonstrated using the same mutation approach used by [57]. The authors provided evidence that tocopherols play a role in seed longevity by limiting non-enzymatic lipid oxidation during storage. In contrast, Gerna et al. [59] found that an *A. thaliana* T-DNA insertional line (*Atfahd* (*Arabidopsis thaliana fumarylacetoacetate hydrolase domain) 1a-1*) had extended seed longevity, whereas metabolite profiling of dry *Atfahd1a-1* seeds showed low δ-tocopherol levels. This indicates that the role of vitamin E in extending seed longevity is complex. In another investigation using mutant technology in *Arabidopsis,* employing genotypes with altered expression of protein L-isoaspartyl methyltransferase (PIMT1), it was concluded that the PIMT repair enzyme system contributes to seed longevity in concert with other anti-aging pathways to improve seed longevity and vigor [60].

Subsequent experiments, based on RNA interference against the three seed-expressed dehydrins, viz. LEA14, XERO1, and RAB18 (responsive to abscisic acid 18), revealed that at least one of the three seed-specific dehydrins plays a role during long-term cold storage at low moisture content [61]. Further developments in our understanding of the genetic basis of longevity after both long-term storage and artificial aging (AA) in *Arabidopsis* was made one year later with the simultaneous analysis of six RIL populations where five loci were discovered (*germination ability after storage 1* (*GAAS1*) to *GAAS5*). Although *GAAS* loci co-located with dormancy loci (*delay of germination* (*DOG*)), a negative correlation between longevity and dormancy was observed [62].

Through mutant analysis and a forward genetics approach in a subsequent study, under both natural and accelerated aging treatments, a RING-type zinc finger putative ubiquitin ligase was identified as imparting a long life to *Arabidopsis* seeds [63] by enhancing responses to gibberellins (GAs). This gene was named *RSL1* (from the ring finger of Seed Longevity1). The role of GAs was further clarified in a similar study using the same mutant approach and it was concluded that GAs may act in seed coat reinforcement [64].

To understand the influence of selective environmental influences on seed longevity, 12 *Arabidopsis* mutants varying in different seed attributes were harnessed [65] and temperature was demonstrated to play a dominant role in seed longevity after AA treatments, whereas light affected plant traits more. Furthermore, individual genotypes responded differently to different environmental conditions. For example, low temperature increased longevity and decreased dormancy in two mutants. Likewise, low light intensity also increased and decreased dormancy and longevity, respectively, in two mutants, demonstrating that different molecular pathways are involved in longevity and dormancy [62].

Further developments to understand seed longevity in *Arabidopsis* after CDT were made when tonoplast intrinsic proteins (TIP3;1 and TIP3;2) were studied in *abi3-6* mutants, where TIP2 transcript and protein levels were significantly reduced in the mutants. It was concluded that TIP3s may help extend seed longevity under the expressional control of ABI3 during seed maturation. Thus, TIP3s are members of the ABI3-mediated seed longevity pathway, together with small HSPs and late embryogenesis abundant (LEA) proteins [66]. Simultaneously, a proteomics approach was employed to obtain a deeper mechanistic view of longevity in *Arabidopsis.* Aged dry seeds and after-ripened seed proteomes were markedly dissimilar and showed that antioxidant systems, including vitamin E, are essential for seed longevity. The abundance of seed storage proteins (SSPs) also indicated that they act as buffering agents to protect seeds against oxidative stress during storage [67].

A role for genome integrity has been critically addressed in seed longevity in *Arabidopsis* [68]. Using mutant resources of *Arabidopsis*, seeds of two mutants (*atm* and *atr)* were found to be highly resistant to aging. Therefore, *ATAXIA TELANGIECTASIA MUTATED* (*ATM*) and *RAD3-RELATED* (*ATR*) are important determinants of seed viability. Taken together, the physiological functions of sensor kinases, including *ATM* and *ATR*, in linking genome integrity to germination, which influence seed quality, are crucial for plant survival in the natural environment and sustainable crop production. The same year also witnessed the use of ectopic expression methodology to understand the role of 1-cys peroxiredoxin (1-Cys Prx aka *PER1*) in seed longevity after experimental ageing [69]. Seed-specific *PER1* protein from the sacred lotus (*Nelumbo nucifera* Gaertn.) NnPER1 is ectopically expressed in *Arabidopsis* and causes enhanced germination after aging. The main reason for this enhanced germination was the significantly lower levels of ROS release and lipid peroxidation.

The year 2017 saw the use of RNA sequencing technology (RNA-seq) to address seed longevity in *Arabidopsis* during priming after CDT [70]. In total, three QTLs were detected in 279 RILs derived from cross "Est-1 $\times$" Col-0". RNA-Seq analyses revealed that brassinosteroid (BR) biosynthesis/signaling and cell wall modification genes were differentially expressed in primed seeds with poor longevity. Positive BR signaling (to some extent) is thought to be the probable cause of increased permeability of the seed coat, resulting in poor seed longevity. The role of SSPs in longevity and seed germination has been investigated [71] and this study demonstrated that aspartic protease ASPG1 (ASPARTIC PROTEASE IN GUARD CELL 1) is also key factor in seed viability of *Arabidopsis* seeds. Using *Arabidopsis* mutants, they found that the SSPs during germination in *aspg1-1* mutants were very impaired in both naturally aged and artificially aged (after CDT) seeds, which led them to conclude that ASPG1 is an important player in seed longevity, dormancy, and germination, and acts via SSPs degradation and regulation of GA signaling.

Cytoplasmic genomes are an additional source of natural variation in seed longevity [72]. Cytoplasmic genomes include chloroplast and mitochondrial genomes, which differ from the nuclear genome [73]. A role for cytoplasmic genomes was identified when seed dormancy, longevity, and germination performance with natural and new genomic compositions were investigated to gain a deeper understanding of all three traits in *Arabidopsis*. Surprisingly, all traits were modified by cytonuclear reshuffling, with certain combinations providing favorable effects of novel cytonuclear combinations on longevity and other traits demonstrating the existence of suboptimal genetic combinations in natural populations for these traits. Furthermore, certain combinations exhibited a positive influence on longevity compared to natural combinations. In addition, to shed further light on the role of oxidative stress in seed longevity after subjecting seeds to CDT, another study [74] found that *Arabidopsis* seeds lacking functional NADP-MALIC ENZYME 1 (NADP-ME1) have reduced seed viability relative to the wild type. Furthermore, NADP-ME1 loss-of-function mutant seeds exhibited higher levels of protein carbonylation. NADP-ME1 catalyzes the oxidative decarboxylation of malate to pyruvate with the simultaneous production of CO_2 and NADPH, whose expression is increased in imbibed aged seeds compared with

non-aged seeds. Its activity during testa rupture promotes the normal germination of aged seeds. Hence, it was concluded that NADP-ME1 activity is required to protect seeds against oxidation during dry seed storage.

In addition to developments in genetics and genomics, the role of proteins in seed longevity after CDT has also been addressed. For example, [75] investigated the role of retromers (multi-protein complexes) in seed longevity using comparative proteomic and metabolomic analyses in the wild-type and the null-retromer mutant vacuolar protein sorting 29 (*vps29) Arabidopsis* mutant. Major changes were observed in the retromer mutant with respect to SSPs and synthesis of lipid reserves. These changes negatively altered vigor and longevity. It was concluded that retromers stimulate energy metabolism, including cell wall biogenesis, underlining the importance of retromer function in seed biology.

In 2020, natural variations of 270 ecotypes of *Arabidopsis* were used to map seed longevity loci by employing a genome-wide analysis, and several multiple genomic regions associated with variation in seed longevity were detected after subjecting seeds to various experimental aging conditions, as well as natural aging [76]. Furthermore, reverse genetics identified seven positive (*PSAD1*, *SSLEA*, *SSTPR*, *DHAR1*, *CYP86A8*, *MYB47*, and *SPCH*) and five negative (*RBOHD*, *RBOHE*, *RBOHF*, *KNAT7*, and *SEP3*) seed longevity genes. In addition, the protective role of the seed coat during seed aging was strengthened by cytochrome P-450 hydroxylase, CYP86A8, and transcription factors, *MYB47*, *KNAT7* and SEP3. The same group also reported the up-regulation of several peroxidase genes in an *Arabidopsis* mutant (*cog1-2D*) [77] with enhanced seed longevity. Furthermore, they found that seeds of double (*prx2 prx25)* and triple (*prx2 prx25 prx71)* mutants possessed reduced longevity because of low seed coat permeability. Altered polyphenolics were concluded to be the likely reason for low permeability (and hence reduced seed longevity).

More advancements took place in 2021, when it was demonstrated that the *AtHB25* transcription factor regulates seed permeability and longevity in naturally aged seeds as well as in seeds subjected to CDT by increasing the accumulation of lipid polyesters in the seed coat [78]. They further demonstrated that the AtHB25 binding target is the lipid polyester biosynthetic gene long-chain acyl-CoA synthetase 2 (*LACS2*). Its importance in seed longevity was also demonstrated by transferring *LACS2* into wheat and tomato, thus identifying AtHBD25 as a trans-species regulator of seed longevity. Finally, *AtFAHD1* [59] has also been nominated as an important agent influencing seed longevity in *Arabidopsis.*

4.2. Rice (Oryza sativa L.)

In crop plants, seed longevity studies were first initiated in rice in 2002 when three QTLs in 98 backcross inbred lines (BILs) on chromosomes 2 (*qLG-2*), 4 (*qLG-4*) and 9 (*qLG-9*) were identified after accelerated aging treatment [79]. Three years later, another 12 QTLs on chromosome 7 (one region) and chromosome 9 (two regions) in a set of 191 RILs after normal and experimental ageing treatments were reported [80]. Another three QTLs for storability in 127 doubled haploid (DH) lines on chromosomes 9 (*qLS-9*), 11 (*qLS-11*), and 12 (*qLS-12*) following AA treatments were subsequently identified the following year [81].

Using chromosome segment substitution lines (CSSLs), the role of hull, seed coat, and embryo on the effect of rice storability QTLs (*qLG-9*, *qLG-2*, and *qLG-4* QTLs) reported by [79] was investigated [82] after being subjected to CDT. No maternal effects of the hull or seed coat were detected in the case of *qLG-9.* Embryonic and/or endospermic factors were concluded to influence longevity. Consistent with previous reports, another three rice storability QTLs were detected on chromosomes 1, 3, and 9 [83] using artificial aging protocols. Thus, chromosome 9 was thought to be targeted for longevity gene cloning in rice, which was achieved in 2015 when the chromosomal location of *qLG-9* was fine-mapped in a 30 kb interval (defined by two markers, *CAPSb* and *CHPa12*) [84]. Furthermore, two genes ((encoding trehalose-6-phosphate phosphatase (TPP) (*Os09g0369400*) and an unknown protein (*Os09g0369500*)) were annotated in this region.

In 2008–2009, the rice community replicated research in *Arabidopsis* when the potential of mutants was used to understand seed longevity. It was demonstrated that a

rice aldehyde dehydrogenase (*OsALDH7*) plays an important role in maintaining seed viability after accelerated ageing treatments by detoxifying the aldehydes generated by lipid peroxidation [85]. Later, six longevity QTLs (with three QTLs (*qMT-SGC5.1, qMT-SGC7.2*, and *qMT-SGC9.1* on chromosomes 5, 7, and 9, respectively, in one RIL population) and three QTLs (*qDT-SGC2.1, qDT-SGC3.1*, and *qDT-SGC9.1* on chromosomes 2, 3, and 9 in another RIL population)) were detected after various storage periods [86]. Similar to previous studies, chromosome 9 was identified in both RIL populations. In another study, six QTLs, *qSS-2*, *qSS-3*, *qSS-4*, *qSS-6*, *qSS-9*, and *qSS-11*, on chromosomes 2, 3, 4, 6, 9, and 11, respectively, for rice storability were identified in a set of 182 BILs after 32 and 48 months of storage at 40–60% relative humidity, where *qSS-9* was the most stable [87] as it was detected in seeds from all environments and storage times, further supporting the idea of map-based cloning of *qSS-9* to gain an understanding of seed storability in rice and possibilities for its improvement. However, before the storability QTL on chromosome 9, *Os03g0700400* was identified as a candidate gene for a seed lipoxygenase (*sLOX3*) QTL on chromosome 3, after application of a map-based cloning strategy. It was determined that *sLOX3* negatively influences seed longevity, probably by facilitating the colonization of some seed pathogens [88,89]. Later, another member of the LOX gene family, *OsLOX2*, was found to act like *sLOX3* in rice seed longevity after accelerated aging [90].

Another related investigation used a set of 85 BILs to locate seed storability QTLs in rice under natural storage conditions and after AA [91]. The authors reported a total of 13 QTLs for seed storability on chromosomes 1, 2, 3, 4, 5, 7, 11, and 12, where two QTLs on chromosome 2 (*qSSh-2-1* and *qSSh-2-2*) were repeatedly detected in both treatment conditions, whereas the remaining four (*qSSh-4*, *qSSs-5-1*, *qSSs-5-2*, and *qSSh-12*) and seven QTLs (*qSSh-1*, *qSSh-3-1*, *qSSh-3-2*, *qSSh-3-3*, *qSSh-7-1*, *qSSh-7-2*, and *qSSh-11*) were detected only once in the natural and artificial aging treatments, respectively. The existence of several QTLs (*qSSh-1*, *qSSh3-1*, *qSSh-3-2*, *qSSh-3-3*, *qSSh-4*, *qSSh-7-1*, *qSSh-7-2*, and *qSSh-11*) was confirmed using CSSLs.

With the development of more sophisticated technologies to develop rice mutants in 2015, it was demonstrated that the *PIMT* gene in rice (*OsPIMT1*) increases seed longevity during AA, probably via its involvement in the repair of detrimental isoAsp-containing proteins that accumulate in acceleratory aged embryos [92].

Pioneer association mapping (AM), also known as genome-wide association study (GWAS), was initiated in 140 rice genotypes [93], and 10 associated markers were detected for longevity after experimental aging, on chromosome 1 *(RM283*, *RM81*, and *RM495*), chromosome 2 (*RM174*), chromosome 4 (*RM124*), chromosome 7 (*RM348*, *RM248)*, chromosome 8 (*RM433*, 337), and chromosome 9 (*RM160*). Additionally, a clarification of the role of tocopherols in seed longevity after aging at high temperatures in rice [94] was made when it was concluded that the specific ratio of tocopherol homologues is more important than the total tocopherol content in the seed longevity mechanism. Two years later, it was discovered that high γ-tocotrienol levels enhanced seed longevity, whereas a high proportion of β-tocopherol relative to δ-tocopherol reduced seed longevity [95]. A further development in understanding rice seed storability was made using transgenics when it was demonstrated that aldo-ketoreductases (*AKR1*) enhanced seed longevity by detoxifying toxic compounds and glycation products [96]. In 2019, the role of microRNAs (miRNAs) in seed longevity after AA treatment was addressed [97]. Up-regulation of *osa-miR164c* and down-regulation of *osa-miR168a* were observed in aged seeds. Concomitant changes in the cytomembrane permeability of seeds and the expression of *osa-miR164c* target genes (*OsPM27* and *OsPSK5*) and *osa-miR168a* target genes (*OsAGO1* and *OsPTR2*) under aging conditions coincided with changes in seed vigor induced by *osa-miR164c* and *osa-miR168a*. Hence, miRNAs can be targeted for future research and improvement in longevity.

Another genetic study mapped novel loci linked to seed longevity in rice using 172 RILs after natural aging. It uncovered two QTLs, *qSL-2* and *qSL-8*, on chromosomes 2 and 8, respectively, where the latter was regarded as a novel QTL for longevity [98]. The study of metabolites sheds further light on seed longevity in rice when it was dis-

covered that amino acid-related and sugar-related metabolites were active in seeds with poor storability. However, raffinose levels were lower in seeds with better storability [99], indicating that raffinose can be used as a marker for seed longevity. More loci for seed longevity have been detected on chromosomes 1, 3, 4, 9, and 11 through AM [100]. Chromosome 2 was demonstrated to be resident of a natural longevity QTL in a tropical japonica rice in a F_3 bi-parental population with 45 annotated genes potentially relevant to seed longevity located in that area [101]. AM was further convened in 456 rice core collections and produced nine QTLs (*SS1-1*, *qSS1-2*, *qSS2-1*, *qSS3-1*, *qSS5-1*, *qSS5-2*, *qSS7-1*, *qSS8-1*, and *qSS11-1*) [102] after accelerated aging treatments. They also confirmed that *qSS1-2* and *qSS8-1* colocalized with *the qSS1/OsGH3-2* and *OsPIMT1* loci. Finally, bulked segregant analysis of two BILs through whole-genome sequencing (BSA-seq) was used to locate potential longevity QTLs when subjected to AA treatments [103]. Two main genomic regions containing 18,550,000–20,870,000 bp on chromosome 4 and 7,860,000–9,780,000 bp on chromosome 9 were identified, and 448 annotated genes were predicted.

4.3. Barley (Hordeum vulgare L.)

Following the successful application of genetic studies in rice, investigations have started in other crops, including barley. Three DH mapping populations were employed by [52] viz. 'Steptoe' × 'Morex' (S × M) population (94 DHLs), the OWB population (94 DHLs) and the W766 population (100 DHLs), which were subject to both AA and CDT. For "S × M", a highly significant QTL was located on chromosome 5HL. In the case of OWB, three QTLs were detected on chromosomes 2H, 5H, and 7H. Finally, in W766, a single QTL on chromosome 7H was identified. The candidate genes on chromosome 7H were suggested to be the "nud" gene that segregates for the character hulled/naked caryopsis [104]. The hulled trait contributed to superior longevity. On chromosome 2HL, *Zeo1* (responsible for small plant stature with compact spikes, long awns, and reduced fertility) [104] was found to be responsible for differences in seed longevity. On chromosome 5H, one of the candidate genes was *Aleurain* (*Ale*) which is a barley vacuolar thiol protease whose expression is regulated by the plant hormones gibberellic acid and abscisic acid. On chromosome 2H, dehydration responsive element binding protein (DREB) was reported as a candidate gene, which, together with the ethylene-responsive element (ERE) binding factors, belong to the APETALA2/ethylene-responsive element-binding protein family that play an important role in the regulation of abiotic and biotic stress responses, respectively. DREB expression is activated by drought, cold or ethylene [105]. On chromosome 5H, other genes, including thaumatin-like proteins such as *Barperm1*, have been proposed as a candidate gene [106]. AM analysis was used by [107] and they detected 55 loci for normal seedling appearance and 36 loci for total germination in a set of 175 genotypes (122 landraces and 53 cultivars), which were mainly concentrated on chromosomes 2H, 5H, and 7H, thus confirming previous results [52]. The most important candidate genes on chromosome 2H included the DREB protein, stem rust resistance protein RPG1, putative gag-pol polyprotein, RNaseH (Ty1/Copia family), and ABC transporter C family member 10, whereas the candidate genes on chromosome 5H included the WD40-like beta propeller repeat family protein, thaumatin-like protein TLP5, *Barperm1,* heat shock cognate 70 kDa protein 2, and APETALA2-like protein. Enoyl-ACP reductase, ethylene-responsive element-binding factor 1 (EREBP-1), and sucrose synthase have been reported at the site of chromosome 7H loci.

More refined effort towards genetic understanding of seed longevity in barley was attempted by [108] through a combination of quantitative genetics and "omics" approaches in near isogenic lines (NILs) derived from crosses between the spring barley landraces "L94" from Ethiopia and "Cebada Capa" from Argentina. RNA-seq and total seed proteomic profiling identified the UDP-glycosyltransferase MLOC_11661.1 as a candidate gene for the quantitative trait locus on chromosome 2H, and the NADP-dependent malic enzyme (NADP-ME) MLOC_35785.1 as possible downstream target gene. This finding was validated using ectopic expression of the aforementioned genes in *Arabidopsis* under the control

of constitutive promoters. Both NADP-ME MLOC_35785.1 and UDP-glycosyltransferase MLOC_11661.1 were able to restore the nadp-me1 seed longevity phenotype.

Further developments revealed the association of miRNAs with seed aging in barley in an investigation that involved distinctive seed lots that belonged to a single genotype (cv. 'Damazy') which varied in viability after over 45 years of storage in a dry state [109]. The dry seeds carried 142 miRNAs, 81 of which were novel. Four conserved miRNA families (miR159, miR156, miR166, and miR168) were highly expressed. Surprisingly, almost all miRNA levels were similar in both highly viable and low viability seed lots, providing evidence that miRNAs remained unaffected during long-term storage. The authors also detected a novel miRNA, viz. hvu-new41, which could be used as an indirect marker to determine seed viability in barley.

4.4. Wheat (Triticum aestivum L.)

The first report addressing the genetic basis of longevity in bread wheat appeared in 2010 reporting a pilot investigation of seed longevity traits in wheat/*Aegilops tauschii* introgression lines following AA [110]. Overall, five QTLs for seed longevity were reported on *Aegilops* chromosomes 1D and 5D, indicating the existence of genetic variability in seed longevity in wheat. This was followed by a detailed investigation in 2012 of classical linkage mapping (performed on 114 RILs of the "International Triticeae Mapping Initiative" mapping population (ITMI/MP)) and GWAS was performed on 96 winter wheat accessions [111]. In ITMI/MP, longevity loci were detected on chromosomes 1A, 1D, 2A, 2D, 3B, 3D, 6B, and 7A using both CDT and AA. Interestingly, the loci detected after CDT and AA treatment were distinct. The region detected on chromosome 2A also harbors many pathogen defense response genes. In particular, the QTL is flanked by *Per2* (peroxidase) and *Wip* (wound-induced protein) genes [112], whereas the 3B locus is implicated in yield-related traits, including grains per ear and thousand grain weight [113]. The chromosome 1A locus has been mapped close to a QTL for spike compactness [114]. Longevity loci in ITMI/MP were reported for relative germination rates after the ageing treatments [111]. The authors of [115], using the unpublished data of [111] and some newly generated data in the same ITMI/MP, revealed some new loci, as well as those already reported in [111], for absolute germination before and after aging treatments. Seed longevity loci were located on chromosomes 1A, 1B, 2B, 2D, and 3D (two similar and one distinct loci) and 4A, 5D, 6B, and 7B (two similar loci), indicating the quantitative nature of longevity related traits in wheat.

The studies of [111,115] were performed with a basic genetic map consisting of 942 loci (mainly "single sequence repeats" and some "restriction fragment length polymorphism" markers) with limited coverage. Recently, a substantially more saturated map of ITMI/MP comprising 7584 single-nucleotide polymorphism (SNP) markers has been published [116]. This new information was utilized [117] to identify novel loci related to longevity using the phenotypic data of [111] and [115]. Overall, 31 loci were reported on chromosomes 1A, 1B, 3D, 4A, 4B, 5A, 5B, 5D, 6B, 6D, 7A and 7B for longevity. In addition, 14 other loci were detected for dormancy-related traits on chromosomes 1A, 2D, 4A, 4B, 5A, 6D, and 7D (Figure 1).

In addition to additive QTLs, epistatic QTL networks of seed longevity loci were reported for the first time in ITMI/MP [117], which was only made possible after the arrival of more sophisticated statistical tools [118] to elucidate genetic networks of the loci responsible for agronomically important and complex traits in polyploid species. The most important but novel candidate genes associated with seed longevity-related traits were detected by the authors of [117], including *ankyrin (ANK)-3-like isoform X1* and *60S ribosomal protein L10a*, *pentatricopeptide repeat-containing protein*, *geraniol 8-hydroxylase-like*, *ETHYLENE-INSENSITIVE 2-like* (*EIN2*) and *RDM16-like isoform X2*, and *carotenoid 9,10(9′,10′)-cleavage dioxygenase-like isoform X1* and *aspartyl protease family protein 2-like*. ANK proteins play a prominent role in plant immunity, development, and growth by being involved in protein–protein interactions [119], whereas members of the pentatricopeptide repeat (PPR) protein

family are sequence-specific RNA-binding proteins that are central to organelle RNA metabolism [120]. Geraniol 8-hydroxylase-like proteins are associated with *MAP kinase* signaling pathways [121]. *EIN2* governs stable miRNA164 expression during aging [122]. Likewise, *carotenoid cleavage deoxygenases* that cleave carotenoids and apocarotenoids are produced by their actions, which play various roles in the growth and development of plants [123]. Members of the aspartyl protease family regulate plant defense responses via different means [124]. Some of these genes have recently been reported to impart *Fusarium* head blight resistance [125].

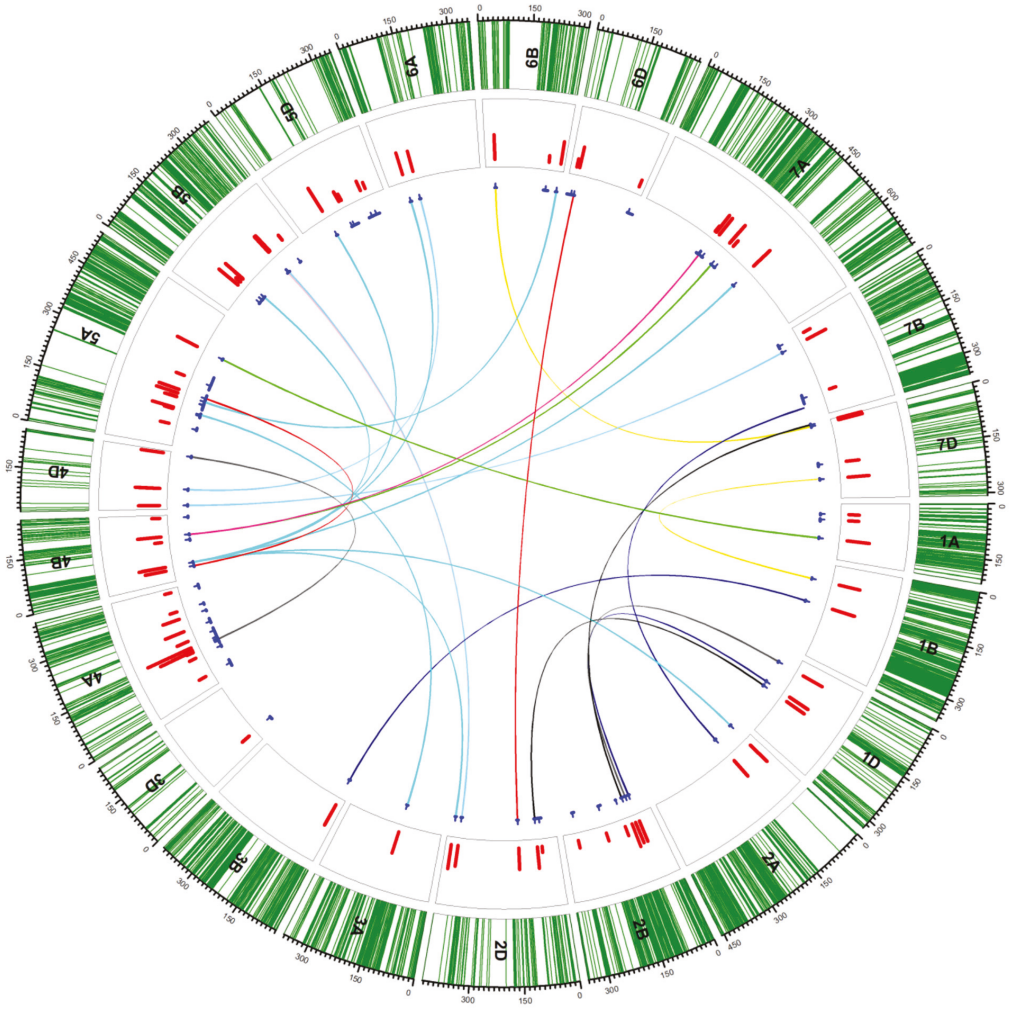

Figure 1. Circos diagram showing the presence of additive (unconnected blue lines in the inner circle) and epistatic (connected blue lines in inner circle) QTLs. Green lines in the outer track indicate the SNP positions on each chromosome; red bars in the second circle indicate the LOD values of QTLs. The blue lines under the track circle indicate the confidence interval of QTLs with small vertical lines point to the peak position of QTL. The colored lines linked different biallelic epistasic QTLs (yellow, pink, sky blue, navy blue, aqua, black, deep pink and red indicate traits related to seed longevity in either control or after various treatments and green and grey and grey indicate dormancy related loci. The figure is reproduced from [117].

Thirty-eight diversity array technology (DArT) markers out of a total of eight hundred and forty were reported to be associated with seed longevity-related traits in the GWAS analyses [111], and a number of candidate genes have been proposed. This population was later covered by a substantially higher number of SNP markers. Overall, 11,139 SNPs were mapped to this panel and the phenotypic data of [111] were reanalyzed in [7]. A more refined analysis uncovered 16 associations on chromosomes 1A, 2A (10 associations), 2D, 6A (3 associations), and 7A. The probable candidate genes reported at these loci included *rhomboid 19*, *disease resistance RPM1-like* and its *isoform X1*, *eukaryotic translation initiation factor 3 subunit M*, *subtilisin-like protease isoform X2*, the *ELMO CED-12 family*, and *Brahma 1*.

Another report in 2017 [45] attempted to define seed longevity loci from a collection of genebank accessions in wheat that were covered with 2134 polymorphic DArT markers. Altogether, 103, 74, and 97 loci for long-term cold storage aging, AA, and CDT, respectively, have been reported. Fifteen bins were identified as likely to contain genes that influence longevity. In addition, the loci linked with germination percentage following long-term low-temperature storage and lab treatments were largely dissimilar, probably because of the involvement of different mechanisms of deterioration [9]. This study was conducted on a collection containing both spring and winter wheat accessions [45], which could have possible implications on the detected loci, as flowering time influences the outcome of most development-related traits. In view of the above possibility, a filtered attempt was made by [7], who used a subset of spring wheat accessions reported in [45] and a new set of 9804 SNPs, combined with more stringent statistical significance. Their effort [7] revealed a total of 56 loci on chromosomes 1A, 1B (10 loci), 2A (2 loci), 2B (6 loci), 2D, 3A (2 loci), 3B, 4A (2 loci), 4B (16 loci), 5B (9 loci), 7A (3 loci), 7B (2 loci) and 7D which were confined to 20 QTLs, out of which eight QTLs were reported to be potentially novel which were located on chromosomes 2B, 3A, 4A, 5B (two QTLs), 7A, and 7D.

The data presented in [45,111] reported a large number of candidate genes, whereas the reanalysis of the data of these studies confined the probable candidate genes to 37 that can be used for future research [7]. Among them, the most important genes that have also been reported include the *stem rust resistance protein Rpg1* and *NBS-LRR resistance-like protein* [126]. Likewise, another common candidate gene was *FAR1-related sequence 6-like protein* w (also reported in [127]), which is expressed in hypocotyls, rosette and cauline leaves, inflorescence stems, and flowers, and is linked to positive regulation of the circadian rhythm and transcription. In addition, it is thought to be involved in ABA signal transduction and abiotic stress response pathways.

More reports emerged in 2018 and 2019 when loci for longevity were examined in-depth in an RIL population composed of 246 genotypes using the classical linkage mapping approach [126] and in a diverse panel of 166 varieties of wheat (144 indigenous Chinese and 22 foreign accessions) using the GWAS approach [127] where the wheat 90K iSelect SNP array (81,587 gene-associated SNPs) [128] was convened in both cases. Ninety-six QTLs were reported on all wheat chromosomes except 2B, 4D, 6D, and 7D, which were clustered into 17 QTL-rich regions on chromosomes 1AL, 2DS, 3AS (3), 3BS, 3BL (2), 3DL, 4AS, 4AL (3), 5AS, 5DS, 6BL, and 7AL, exhibiting pleiotropic effects in the former report [126]. The most promising candidate genes reported were *starch synthase 3*, *stem rust resistance protein Rpg1*, *NBS-LRR resistance-like protein*, *dolichyl-diphosphooligosaccharide-protein glycosyltransferase*, *glutaminyl-peptide cyclotransferase-like*, and *wheat alpha-Amy2/53*. In a latter report [127], 23 loci were reported on chromosomes 1A, 2A (2), 2B (3), 2D, 3A (2), 3B, 3D (2), 4A (3), 5A (2), 5B (3), 5D (1), and 6A, whereas the candidate genes included *FAR1-related sequence 6-like protein*, *delta-1-pyrroline-5-carboxylate synthase (P5CS)*, and *MICOS complex subunit mic60 protein*, which is essential for the integrity of cellular membranes and genomes [129].

The most recent report addressing seed longevity in wheat from a genetic perspective appeared in 2020 when an analysis of 150 DH lines [130] was reported after six aging treatments, in which 49 additive QTLs for seed vigor-related traits were mapped onto chromosomes 1B, 2D, 4A, 4D, 6D, and 7A and all group 3 and group 5 chromosomes. In

addition, 25 pairs of epistatic QTLs were reported on all chromosomes except chromosomes 5D, 6A, and 7D. This study pioneered the mapping of epistatic loci of seed longevity in wheat. The same approach has been adopted to map epistatic loci in ITMI/MP to explain further variation in this trait [117].

4.5. Maize (Zea mays L.)

Natural variability of maize seed viability at the beginning of 21st century using RILs has been examined [131,132]. The investigators found that viability decreased linearly with age for most inbred lines, with a few exceptions. They also discovered that the same enduring seeds of most RILs, when sown, produced new seeds with superior viability and vigor compared to average values, suggesting natural selection for viability and vigor during storage. This prompted another study by the same group [132] and they identified genes of seed longevity in maize employing sweet corn inbred line 'P39' and the field corn inbred line 'EP44'. Simple sequence repeats (SSRs) in the bulk of living and dead seeds after 20 and 22 years of storage were compared, and the differences between dead and living seeds could be explained by residual variability, spontaneous mutation, or aging. Chromosome 7 exhibited more variability than the other chromosomes. Likewise, variability was more pronounced for distal SSRs. Six known genes, including *pathogen-related protein 2*, *superoxide dismutase 4*, *catalase 3*, *opaque endosperm 2*, *metallothionein 1*, and *golden plant 2* have been reported as candidate genes for longevity. In addition, five novel candidate genes (three of which could be involved in resistance to diseases, one in detoxification of electrophilic compounds, and another in transcription regulation) were reported.

Early harvesting is preferred in maize, but it can result in poor quality and low seed vigor [133]. To tackle this, the genetics of seed vigor at different stages of maturity were determined using a set of maize RILs harvested 32, 40, and 45 days after pollination, coupled with a genetic linkage map covering a distance of 2438.2 cM through 217 SSRs. In total, there were sixteen different QTLs for seed vigor at three sampling times, five for germination energy (number of seedlings on day 3 divided by total seeds sown), three for germination percentage, four for germination index, and four for vigor index. The four QTLs for seed vigor, which were detected at all three sampling times, were located in a comparable region on chromosome 7, confirming the findings of the authors of [132] who also reported chromosome 7 to be an important carrier of longevity genes in maize.

The first comprehensive use of SNPs for studying seed longevity also appeared in maize in 2014 [134] in two unrelated RIL populations subjected to four different treatments. Sixty-five QTLs distributed between the two populations were identified, from which sixty-one were integrated into eighteen meta-QTLs (mQTLs). Finally, twenty-three candidate genes coincided with thirteen mQTLs. Eight of these genes were associated with the glycolytic pathway. The functions of two of the candidate genes were associated with stress responses, including a *stress-responsive glyoxalase family* gene and an *aldehyde dehydrogenase* gene. The functions of the remaining six candidate genes were associated with energy processes, including an *ATP synthase F1 subunit alpha gene*, *glyceraldehyde 3-P dehydrogenase* gene, *V-type (H+)-ATPase domain* gene, *phosphoglucomutase* gene, *3-phosphoglycerate kinase* gene, and *isocitrate lyase* gene. Eight candidate genes were functionally associated with protein metabolism. Four candidate genes had functions related to general protein metabolism or translation, including an *elongation factor 1-g2* gene, an *elongation factor 1B-g* gene, a *calreticulin 1* gene, and an *Asp aminotransferase* gene. The other four candidate genes encoded HSPs, including a hypothetical *ACD ScHsp26-like* gene, *HSP16.9*, *HSP17.2*, and *hsp20/alpha crystallin* family protein gene. In addition, five candidate genes were associated with protein modification and signal transduction. These included a *ubiquitin E2* gene, *calcium-dependent protein kinase,* a gene containing a predicted *RING-finger domain*, a *CAAX prenyl protease 1* gene, and a *cucumisin-like serine protease*. Two other candidate genes had functions related to cell growth and division, including a predicted *cyclin-dependent kinase A* gene and an *MEK homolog1* gene.

The RIL population used in [133] and an immortalized F_2 (IF_2) population were convened in 2015-16 to examine genetic differences between homo-and heterozygous maize lines after AA and subsequent evaluation in field experiments [135]. Twenty-eight and twenty-one QTLs were detected in the IF_2 and RIL populations, respectively, with only one QTL (*qGP5*) common between both. In the IF_2 population, a QTL (*qGI10b*) was detected on chromosome 10 in the same region as that reported in the same population [133], which demonstrated that this locus corresponds to major genes associated with seed germination or seed aging. Likewise, a QTL on chromosome 3 (*qGE3a*) in the RIL population in this study was the same QTL previously reported [133]. In addition, candidate genes for QTLs *qVI4b* and *qGE3a* detected in the RIL population were suggested to be *ZmLOX1* and *ZmPLD1.*

Another report on QTL mapping for seed longevity in maize after AA appeared in 2018 in an $F_{2:3}$ population and RIL population [136]. The authors reported 13 QTLs on chromosomes 1, 3, 4, 5, and 7, where 2–4 QTLs were co-located in one region. In each region, three to eight previously identified aging-related QTLs were located, confirming the importance of these regions in controlling seed longevity in different maize populations. The parents (I178 and X178) of the same population used in this study [136] were also subjected to transcriptome sequencing before and after five days of AA treatment [137], which resulted in the detection of 286 and 220 differentially expressed genes (DEGs) in I178 and X178, respectively. Of these DEGs, 98 were detected in both I178 and X178, which were enriched in Gene Ontology (GO) terms of the cellular component of the nuclear part, intracellular part, organelles, and membrane. Only 86 commonly downregulated genes were enriched in GO terms of the carbohydrate derivative catabolic process. Additionally, transcriptome analysis of alternative splicing (AS) events in I178 and X178 showed that 63.6% of transcript isoforms occurred in AS in all samples, and only 1.6% of transcript isoforms contained 169 genes that exhibited aging-specific AS after aging treatment. Combined with the reported QTL mapping result, seven DEGs exhibited AS after aging treatment, and thirteen DEGs in the mapping interval were potential candidates that were directly or indirectly related to seed longevity. The authors also reported very low expression of *ZmPIMT* and *ZmLOX11* genes in seeds, which implies that they are highly tissue-specific or even differentially expressed in mono- and dicots. In addition, several novel candidate genes have been suggested for longevity.

In 2020, the use of maize mutants provided evidence that *ZmDREB2A* regulates the longevity of maize seeds following AA by stimulating the production of raffinose while simultaneously limiting auxin-mediated cell expansion [138]. The authors detected that unaged seeds of two independent maize *DREB2A* mutant (*zmdreb2a*) lines, with decreased expression of *GRETCHEN HAGEN3.2* (*ZmGH3.2*, encoding indole-3-acetic acid (IAA) deactivating enzyme) and increased IAA in their embryos, produced longer seedling shoots and roots than the null segregant (NS) controls. In contrast, *zmdreb2a* seeds with decreased expression of *RAFFINOSE SYNTHASE* (*ZmRAFS*) and less raffinose in their embryos, exhibited decreased seed aging tolerance compared to the NS controls. When they overexpressed *ZmDREB2A* in maize protoplasts, there was an increased expression of *ZmGH3.2*, *ZmRAFS* genes and that of a *Rennila* LUCIFERASE reporter (*Rluc*) gene, which was controlled by either the *ZmGH3.2* or *ZmRAFS* promoter. They also provided evidence that *ZmDREB2A* directly binds to the DRE motif of the promoters of both *ZmGH3.2* and *ZmRAFS*.

Another attempt to map QTLs for seed longevity in maize was made in 2021 by utilizing an $F_{2:3}$ population and a population of RILs constructed from a cross between "Dong156" and "Dong237" [139]. Genotyping was performed using SSR markers and the seeds were subjected to AA. Two consistent regions, *cQTL-7* on chromosome 7 (also in line with [132]), and *cQTL*-10 on chromosome 10, were identified by comparing QTL analysis results of the two populations. The four SSR markers (*umc1671*, *phi328175*, *umc1648*, and *phi050*) linked to *cQTL-7* and *cQTL*-10 could be used to select maize germplasms with a high degree of seed storability.

4.6. Soybean (Glycine max (L.) Merr.)

The first study to resolve the genetics of seed longevity after experimental ageing in soybean was initiated in 2008 [140] in an $F_{2:3}$ population composed of 153 lines from a cross of 'Birsa soya-1' × 'JS 71-05'. Four (*Satt538*, *Satt600*, *Satt434* and *Satt285*) independent SSR markers were significantly associated with seed longevity at a distance of 158.63 cM, 75.4 cM, 105 cM and 25.51 cM on chromosomes A2, D1b, H and J, respectively [141]. The same population was also subjected to seed coat permeability and electrolyte leaching by the same group [142], where four SSRs (*Satt434*, *Satt538*, *Satt281*, and *Satt598* on chromosomes H, A2, C2, and E, respectively) were associated with seed coat permeability. In addition, SSR *Satt281* has been linked to electrolyte leaching on chromosome C2. The SSR *Satt281* was also reported in a subsequent longevity analysis of 21 soybean genotypes [143].

To link seed coat color with longevity, Hosamani et al. used 33 genotypes of soybean that differed in storability and seed coat color with 53 SSR and 51 randomly amplified polymorphic DNA (RAPD) markers [144]. Black seed coat genotypes were more stable under both natural and AA conditions. SSRs grouped the genotypes into two major clusters, representing black- and yellow-seeded genotypes. SSR markers *Satt371*, *Satt453*, and *Satt618* were identified as candidate markers for linkage with seed storability and testa color. In the same year, a QTL mapping study of soybean for seed longevity in an $F_{2:3}$ population derived from a soybean line (MJ0004-6; characterized by poor longevity) and a landrace cultivar from Myanmar (R18500; characterized by superior longevity) was published [145]. The $F_{2:4}$ seeds after AA, as well as under ambient conditions, revealed 13 markers from six linkage groups (C1, D2, E, F, J, and L) in association with seed storability. QTL mapping detected three QTLs on linkage groups C1, F, and L, and the SSRs involved were *Satt476* and *Satt399*, *Satt269*, *Satt423*, *Satt523*, and *Satt143*, respectively.

Another investigation to elucidate the genetic mechanism behind seed longevity was undertaken during detailed physiological and molecular characterization of late seed maturation [146]. A two-fold increase in longevity was observed by the time seeds were dry. This increase in longevity was linked to the expression of genes encoding protective chaperones, including HSPs, and the repression of nuclear and chloroplast genes involved in a range of chloroplast activities, including photosynthesis. An increase in the RFO/sucrose ratio, together with changes in RFO metabolic genes, was also associated with longevity. Twenty-seven transcription factors were identified, whose expression profiles were highly correlated with longevity, including homologues of *ERF110*, *HSF6AB*, *NFXL1*, and members of the *DREB2* family, as well as transcription factors associated with auxin and developmental cell fate during flowering, organ growth, and differentiation. Transcriptional transition occurred concomitantly with seed chlorophyll loss and detachment from the mother plant, which was enriched with AP2/EREBP and WRKY transcription factors and genes associated with growth, germination, and post-transcriptional processes that prepared the seed for the dry quiescent state and subsequent germination.

A comprehensive investigation to identify QTLs employing high-density genetic linkage maps of soybean for seed longevity in two RIL populations derived from "Zhengyanghuangdou" × "Meng 8206" and "Linhefenqingdou" × "Meng 8206" using both natural and AA protocols identified 34 QTLs on 11 chromosomes, all of which were novel [147]. Twenty-one of these QTLs were clustered in five QTL-rich regions on four chromosomes: Chr3, Chr5, Chr17, and Chr18. In addition, "QTL hotspot A" on Chr17 and "QTL hotspot B" on Chr5 carried seven and six QTLs, respectively. Hence, stable genomic regions governing the inheritance of seed storability in soybeans have been reported.

Biochemical and molecular analyses of the parents exhibiting black (Birsa soya-1) and yellow seed coat color (EC 241780) and the 11 F_3 progenies of the cross exhibiting brown, yellow, and black seed coat color have been undertaken [148] to understand the physicochemical attributes related to soybean seed longevity. Vitamin E, lignin, calcium content, and antioxidant enzyme activity were higher in black and brown seed coat color progenies, whereas lower lipid peroxidation rates were recorded in black and brown seed coat color parents and progenies with better seed longevity. The SSR primers *Satt162*,

Satt523, and *Satt453*, which are linked to seed coat color and seed permeability, exhibited a specific-size allelic fragment in soybean genotypes and crosses with better seed longevity, confirming the results of a previous report [142].

The latest report on seed longevity in soybean appeared in 2021, where the investigators analyzed the QTLs for seed longevity in an RIL population under −20 °C conservation and AA conditions [149]. Two major QTLs and eight QTL hotspots localized on chromosomes 3, 6, 9, 11, 15, 16, 17, and 19 were detected for a variety of seed vigor-related traits across the two treatments. No common QTLs were detected in the RIL populations. Furthermore, 15 promising candidate genes were reported that could possibly determine seed vigor in soybeans, which would help to explore the mechanisms responsible for maintaining high seed vigor .

4.7. Lettuce (Lactuca sativa L.)

Seed longevity has been thoroughly explored at the genetic level in one report [150]. QTL mapping for seed longevity after conventional storage (30% RH and various temperatures for various durations) and CDT on RILs produced from a cross between "*Lactuca sativa* cv. Salinas" × "*Lactuca serriola* accession UC96US23" identified multiple QTLs under CD and conventional storage conditions. However, they did not find a correlation between the results of CD and conventional storage, which led to the conclusion that CD conditions are not predictive of aging in conventional storage environments. QTLs for seed longevity were reported on chromosomes 1, 2, 3, 4, 6, 7, and 8 after various treatments, where chromosomes 1 and 4 were involved in multi-treatment QTLs. Four genes (*AtHSF4*, *AtIRX14*, *AtMAT3*, and *AtUBC16*) were suggested as candidate genes on chromosome 4, including *heat shock transcription factor 4*, *phosphoglycerate/biphosphoglycerate mutase* protein family, *CC-NBS-LRR* and *TIS-NBS-LRR* class disease resistance protein, *glycosyl transferase* family 43, *methionine adenosyltransferase 2*, *ubiquitin-conjugating enzyme 16*, and *pfkB-type carohydrate kinase* protein family. The other locus on chromosome 4 carried *AtPAP2* as a candidate gene, which produces phytochrome-associated protein 2 (AUX/IAA family). There were other possible candidate genes, including *AMP-dependent synthetase* and *ligase*, a *zinc finger* (*GATA* type) family protein, an *ABC transporter* family protein, and *LOX*. The two regions on chromosome 1 were reported to carry approximately nine candidate genes, viz. *AtCPN60A*, *AtCKS1*, *AtMTN2*, *AtABA2*, *AtCYSA*, *AtEIN2*, *AtBGL2*, *RPT1A*, and *AtHSD5*, which include *RuBisCO subunit binding protein*, *cyclin-dependent kinase*, *methyladenosine nucleosidase*, *ABA2*, *cysteine-type endopeptiase inhibitor*, *EIN2*, *glucosidase*, *26S proteasome AAA-ATPase subunit RPT1a*, and *hydroxysteroid dehydrogenase 5*.

4.8. Tobacco (Nicotiana tabacum L.)

One of the first systematic studies to approach seed longevity loci under ambient conditions in tobacco was initiated on a collection of 122 RILs derived from a cross between the cultivars "Florida 301" and "Hicks" [151]. They were able to detect four genomic regions located in four different linkage groups (LGs): 6, 7, 8/18 and 23. The loci on LGs 6 and 7 carried loci specifically detected under treated and controlled conditions, respectively, whereas LGs 8/18 and 23 carried loci detected under both controlled and treated conditions at almost the same locations. The LG 8/18 locus was reported in the same region where a disease resistance locus against black shank disease caused by *Phytophthora nicotianae* was detected in a previous study [152]. A subsequent study examined 118 DHLs developed from a cross between cultivars, "Beinhart-1000" and "Hicks" [153] where another 24 loci were detected on 11 linkage groups. Additional variation was captured by performing epistasis analysis, which resulted in the detection of five pairs (four in the control group after treatment) of epistasis QTLs. Thus, epistatic QTL exploration for seed longevity has become an active topic, and it is expected that with the development of more sophisticated QTL analysis tools, more epistatic QTLs will be identified.

4.9. Tomato (Solanum lycoperscum L.)

To address the problem of reduced seed longevity after AA in tomato, QTL mapping in 50 RILs of tomato (from a cross of "*Solanum lycopersicum* cv Moneymaker" × *Solanum pimpinellifolium*) was performed [56]. Two QTLs were detected on chromosome 2, and one on chromosome 6. Two additional QTLs were detected for galactinol content on chromosomes 2 and 4. One of the QTLs for longevity and galactinol content on chromosome 2A falls into the same interval as the *galactinol synthase* gene (*Solyc02g084980.2.1*), which is a key enzyme of the RFO pathway. A reverse genetics approach using T-DNA knockout lines in genes encoding enzymes of the RFO pathway (*galactinol synthase 1, galactinol synthase 2, raffinose synthase, stachyose synthase, and alpha-galactosidase*) and overexpression of the cucumber *galactinol synthase 2* gene in *Arabidopsis* was undertaken. The analysis demonstrated that the galactinol synthase 2 mutant and galactinol synthase 1 galactinol synthase 2 double mutant contained the lowest seed galactinol content, which coincided with lower seed longevity. It was thus concluded that galactinol content of mature dry seeds can be used as a biomarker for seed longevity in Brassicaceae and tomatoes.

Seed priming is a way to achieve synchronized and rapid germination in tomatoes. However, it comes at the cost of reduced longevity. This can be ameliorated by heat shock to primed seeds. The genetic determinants of this improvement in longevity were investigated using primed and dried tomato seeds [154]. RNA-seq and subsequent transcriptome analysis of the tomato seeds after priming and after heat shock treatment post-priming demonstrated that from a total of 368 differentially expressed genes, 298 genes were up-regulated and 70 were down-regulated. An increase in messenger RNA levels of *heat shock factor-like* and *HSP-like* chaperone genes demonstrated their pivotal role in the enhancement of longevity in primed tomato seeds.

To further understand the acquisition of desiccation tolerance and seed vigor, including longevity in tomato, Bizouerne et al. [155] used temporal RNA-seq analyses of the different seed tissues during maturation. Gene networks and trait-based correlations were used to explore the transcriptome signatures associated with longevity. A total of 15,173 differentially expressed genes were detected, forming a gene network representing 21 expression modules, with three being specific to the seed coat and embryo and five to the endosperm. A gene–trait significance measure identified a common gene module between the endosperm and embryo associated with longevity, which included antioxidant and repair genes, in addition to LEA-, HSP-, and ABA-responsive genes. Longevity enhancement was correlated negatively with dormancy emancipation which was released concomitantly with the increase in longevity throughout fruit ripening, until 14 days after the red fruit stage, which also equated with an increment in SlDOG1–2 and PROCERA transcripts. The step-by-step increase in seed vigor was captured by two tissue-specific and one common gene module (between embryo and endosperm). Each module carried specific genes. For example, the common module carried genes responsible for mRNA processing, flowering time, and post-transcriptional regulation. ABI4 and CHOTTO1 were associated with seed vigor in the embryo-specific module, whereas the endosperm-specific module revealed diverse processes involved in genome stability, defense against pathogens, and ABA/GA response genes.

5. Future Directions

Biological and agricultural research is at a crossroads in the third decade of the 21st century, where boundaries between various disciplines are rapidly diminishing. Multiple approaches are being adopted to understand the mechanisms underlying biological processes such as seed longevity. Thus, it is imperative that researchers from different disciplines (e.g., plant breeders, seed technologists, plant physiologists, and molecular biologists) collaborate to further our understanding of the processes of seed aging. Although the quest for seed immortality is a mission impossible [156], researchers have achieved substantial success in understanding seed deterioration at the genetic, molecular, and physiological levels. However, much remains to be done.

Genetic research, in particular, involves two versatile methods: linkage analysis and association mapping to dissect quantitative traits [157] including seed longevity [111]. The former relies on trait segregation in a population derived from a bi-parental cross, whereas the latter is a population-based method that relies on the detection of linkage disequilibrium between a trait and a genetic marker in unrelated accessions [158]. Both the bi-parental population and the genetic map need to be created before phenotypic traits can be associated with the linkage groups of the plant species under consideration. For example, in the case of wheat, an SSR map was initially developed for ITMI/MP [159]. SSR maps were developed using manual pipetting in the laboratory, which took years to complete. However, with the development of genotyping technology, large-scale genotyping has been outsourced to invest the saved time in intensive phenotyping. One example is the development of DArT markers in wheat, which have been used to map important loci of dormancy and pre-harvest sprouting [160] and longevity [111]. Further developments have led to the discovery of SNPs, and readily available new populations have been genotyped using this new set of markers [116]. Hence, SNPs represent the ultimate form of molecular markers, the plant populations of any species previously mapped with primitive marker systems can be genotyped with SNPs, and the data can be used to understand the genetics of any trait.

Experimental examination of seed longevity is a time-consuming and labor-intensive process as it involves growing a population of plants for one complete cycle, harvesting the seeds and post-harvest treatments, and subsequent seed storage. The harvested/stored seeds are then treated experimentally, followed by germination tests (where germinated seeds were visually counted) to obtain an estimate of longevity in that population. With the arrival of high throughput scoring of seed germination in at least model plants (*Arabidopsis*) [161], new methods for phenotype germination on a large-scale in limited time in other plant species could soon be undertaken in future to accelerate research on the seed longevity of important plants.

Sophisticated methods of QTL detection have been developed and incorporated in various programs to facilitate breeders to capture hidden or otherwise uncaptured trait variation. Initially, in wheat, a single marker analysis was performed to map longevity loci in pioneer longevity studies [111]. Similarly, interval mapping, composite interval mapping, and inclusive composite interval mapping methods have been developed and incorporated into important QTL detection programs, including *Qgene* [162], *QTL Cartographer* [163] and *ICIM mapping* [118]. More recently, whole-genome composite interval mapping (*QTL.gCIMapping*) [164] has been proposed to detect small-effect QTLs. As longevity QTLs also have small effects, especially in polyploid species [3,111], *QTL. gCIMapping* [164] might be a useful option to explore seed longevity QTLs in future studies. All of the above-mentioned studies, with the exception of [117,130,153], have reported additive QTLs. However, further variation can be detected by exploiting epistatic interactions among loci to explain the genetic determinants of seed longevity. The detection of epistatic interactions is possible using *ICIM mapping* software (version 4.2.53) [118] in constructed populations, including RILs or DHLs. No direct software is available to detect such interactions in natural populations, including germplasm collections. Hence, in-house scripts were developed to look for loci interactions for various traits, as has been reported for yield and Karnal bunt resistance in wheat [165,166]. Hence, in the future, epistatic QTLs should be examined to capture hidden variance and define missing links in seed longevity research.

The discussion above indicates that the most important steps to discover the genetic determinants of seed longevity are population development, genotyping, intensive phenotyping, and computational analysis. All of these steps are expensive. Consequently, scientists, breeders, seed technologists, physiologists, molecular biologists, and genebank curators must exchange the germplasm developed by them in their respective projects to accelerate genetic research on seed longevity. Table 1 provides some examples of germplasm resources of different plant species that can be exploited to hasten research on seed longevity.

Table 1. Germplasm collections with genotype data for future research on seed longevity of various crops.

Sr. No.	Plant Species	Number of Accessions	Genotyping Platform	References
1	Hexaploid wheat (*Triticum aestivum* L.)	>2500 accession in SeeDs of Discovery project	7180 genotyping by sequencing (GBS) SNPs	[167,168]
2	Durum wheat (*Triticum durum* Desf.)	(i) 6280 RILs (50 interconnected families constituting a nested association mapping population (NAM)) (ii) 1336 genotypes (from 25 families constituting a NAM)	(i) 13,000 SNPs from Infinium 15K Ultra HD chip (ii) 5398 SNPs from Illumina Infinium iSelect HD 9k chip	(i) [169] (ii) [170]
3	Sorghum (*Sorghum bicolor* (L.) Moench.)	971 world wide accessions	GBS SNPs	[171]
4	Rice (*Oryza sativa* L.)	1568 inbred varieties	700,000 high density rice array SNPs	[172]
5	Tomato (*Solanum lycopersicum* L.)	163 accesions, 291 accesions and 402 accesions	5995 SNPs, 9013 SNPs and 2014,488 SNPs, respectively	[173]
6	Pepper (*Capsicum* spp.)	10,038 genebank accession	GBS SNPs	[174]
7	Soybean (*Glycine max* L.)	(i) 421 accesions (ii) 305 accessions	(i) 1536 SNPs (ii) 37,573 SNPs	(i) [175] (ii) [176]

With the arrival of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein-9 (Cas9) and its increasing popularity in the plant research community [177], substantial improvements in our understanding of the genetic mechanisms of important biological functions in model and crop plants are imminent. Much detailed information is already available, as previously reviewed for horticultural plants [177]. However, there are no reports where seed longevity was targeted by this tool in any plant species. Thus, we also propose that the seed longevity genes identified and discussed in the preceding paragraphs in a range of plant species can be a useful target for CRISPR/Cas9 scientists for future research. Next-generation sequencing platforms provide new insights into the genome, transcriptome, and epigenome of plants, providing an advanced understanding of functional genes [178]. We propose that a time-course transcriptomic analysis can be used to identify transcriptional changes during aging treatments at key intervals in response to stress, as recently demonstrated. Reference [179] studied salt stress in wheat via Massive Analysis of cDNA 3′-ends (MACE). MACE sequencing can be used as a substitute for regular RNA sequencing, in which a single fragment represents one transcript [180]. Digital and strand-specific outputs of MACE with increased accuracy of expression data have provided high-resolution mapping to detect genes with moderate expression levels and short transcripts [180,181]. Similar to CRISPR/Cas9, MACE sequencing to detect the genetic control of seed longevity has yet to be undertaken.

In conclusion, genetic advances have been successful in establishing genetic determinants of seed longevity in wheat, barley, rice, maize, and other crop plants, in addition to horticultural plants, including tomato and lettuce. New techniques, including CRISPR/Cas9, RNA sequencing, and MACE sequencing, remain to be applied in the seed science research.

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Abbreviations

1-Cys Prx	1-cys peroxiredoxin and/or PER1
AA	artificial ageing
ABA	abscisic acid
AKR1	aldo-ketoreductases
Ale	Aleurain
AM	association mapping
AS	alternative splicing
ASPG1	ASPARTIC PROTEASE IN GUARD CELL 1
ATM	ATAXIA TELANGIECTASIA MUTATED (ATM)
ATR	RAD3-RELATED
BIL	backcross inbred lines
BR	brassinosteroid
BSA-seq	bulked segregant analysis through whole genome sequencing
Cas-9	CRISPR-associated protein-9
CDT	controlled deterioration test
CRISPR	clustered regularly interspaced short palindromic repeats
CSSL	chromosome segment substitution lines
DArT	diversity array technology
DEG	differentially expressed genes
DH	doubled haploid
DREB	dehydration responsive element binding protein
EIN2	ETHYLENE-INSENSITIVE 2-like
GA	gibberellins
ERE	ethylene-responsive element
GAAS	germination ability after storage
GBS	genotyping by sequencing (GBS
GO	gene ontology
GWAS	genome wide association study
HSP	heat shock proteins
IF_2	immortalized F_2
IAA	indole-3-acetic acid [IAA]
ITMI/MP	International Triticeae Mapping Initiative" mapping population
LACS2	long-chain acyl-CoA synthetase 2
LEA	late embryogenesis abundant
LG	linkage groups
LOX	lipoxygenase
MACE	massive analysis of cDNA 3′-ends
miRNA	microRNA
mQTL	meta-QTL
NADP-ME	NADP-dependent malic enzyme
NAM	nested association mapping
NIL	near isogenic line
NS	null segregant
OS	oligosaccharides
OsALDH7	rice aldehyde dehydrogenase
Per2	peroxidase
PIMT1	protein L-isoaspartyle methyltransferase
PPR	pentatricopeptide repeat
QTL	quantitative trait loci
RAB18	responsive to abscisic acid 18
RFO	raffinose family oligosaccharide
Rluc	Rennila LUCIFERASE reporter
RFO	raffinose series oligosaccharides
RIL	recombinant inbred population

RNA-seq	RNA sequence
SNP	single nucleotide polymorphism
SSP	seed storage proteins
SSR	simple sequence repeats
TIP	tonoplast intrinsic proteins
TPP	trehalose-6-phosphate phosphatase
Vps29	vacuolar protein sorting 29
Wip	wound-induced protein

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Seed-to-Seedling Transition in *Pisum sativum* L.: A Transcriptomic Approach

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Abstract: The seed-to-seedling transition is a crucial step in the plant life cycle. The transition occurs at the end of seed germination and corresponds to the initiation of embryonic root growth. To improve our understanding of how a seed transforms into a seedling, we germinated the *Pisum sativum* L. seeds for 72 h and divided them into samples before and after radicle protrusion. Before radicle protrusion, seeds survived after drying and formed normally developed seedlings upon rehydration. Radicle protrusion increased the moisture content level in seed axes, and the accumulation of ROS first generated in the embryonic root and plumule. The water and oxidative status shift correlated with the desiccation tolerance loss. Then, we compared RNA sequencing-based transcriptomics in the embryonic axes isolated from pea seeds before and after radicle protrusion. We identified 24,184 differentially expressed genes during the transition to the post-germination stage. Among them, 2101 genes showed more prominent expression. They were related to primary and secondary metabolism, photosynthesis, biosynthesis of cell wall components, redox status, and responses to biotic stress. On the other hand, 415 genes showed significantly decreased expression, including the groups related to water deprivation (eight genes) and response to the ABA stimulus (fifteen genes). We assume that the water deprivation group, especially three genes also belonging to ABA stimulus (LTI65, LTP4, and HVA22E), may be crucial for the desiccation tolerance loss during a metabolic switch from seed to seedling. The latter is also accompanied by the suppression of ABA-related transcription factors ABI3, ABI4, and ABI5. Among them, HVA22E, ABI4, and ABI5 were highly conservative in functional domains and showed homologous sequences in different drought-tolerant species. These findings elaborate on the critical biochemical pathways and genes regulating seed-to-seedling transition.

Keywords: desiccation tolerance; gene expression; germination; post-germination; RNA-seq; *Pisum sativum* L.; seeds; transcriptome

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1. Introduction

The transition from a growth-arrested seed to a germinating seed and then to a seedling is a crucial step in the plant life history [1–4]. During and after germination, early seedling growth is supported by the catabolism of stored reserves of protein, oil, or starch accumulated during seed maturation [5,6]. These reserves support cell expansion and seedling

development. Successful seed germination and establishing a normal seedling are determining features in plant propagation, having both economic and environmental importance.

During late maturation, orthodox seeds lose up to 95% of their water and successfully enter dormancy. This feature allows them to sustain unfavorable environmental conditions, such as extremely high or low temperatures and drought [7–10]. Desiccation-tolerant seeds survive drying to 0.1 g H_2O/g dry weight or less [2]. The mechanisms behind desiccation tolerance are activated at the late seed-maturation stage and associated with the accumulating LEA proteins, small heat shock proteins, non-reducing oligosaccharides, and antioxidants [9,11–14]. Regulation of seed maturation and the onset of desiccation tolerance is based on the ABA/GA ratio along with the network of four master regulators, usually referred to as LAFL, i.e., LEAFY COTYLEDON1 (LEC1), ABSCISIC ACID INSENSITIVE3 (ABI3), FUSCA3 (FUS3), and LEC2. Namely, LAFL is directly involved in the coordination of seed maturation and inhibition of seed germination [15,16]. Epigenetic modifications, such as changes in DNA methylation and histone post-translational modifications, are also crucial for seed desiccation tolerance and longevity [17–20]. The switch of the developmental program from maturation to germination is accompanied by the suppression of LAFL genes and activation of those involved in vegetative growth [16].

The process of seed germination *sensu stricto* can be divided into two phases [2,21]. Phase I is characterized by rapid water uptake, enhanced hydration of macromolecules, activation of respiration, and repair of membranes, mitochondria, and DNA. Phase II is characterized by mobilization of reserves, translation of stored mRNAs, transcription and translation of newly synthesized mRNAs, and activation of protein biosynthesis [22].

Protrusion of a radicle is regarded as the beginning of phase III, which is a post-germination stage. At this moment, germination ends, and the post-germination phase III begins [2]. The transition from germination to the post-germination stage corresponds to the loss of desiccation tolerance [23–26]. Up to this point, the seeds can lose water without any viability loss, and their metabolic processes can resume upon subsequent rehydration [27–29]. This stage, when reversible dehydration/rehydration cycles are still possible without losses of germination efficiency, is often referred to as the "window of desiccation tolerance" [28]. A loss of desiccation tolerance in seeds during germination supposedly occurs upon the resumption of DNA synthesis in the radicle cells with the entry of radicle cells into the G_2 phase of the cell cycle, during which the cell prepares itself for mitosis [30]. Cells in the G_2 phase of the cell cycle, with duplicated DNA, were more sensitive to stresses (desiccation in particular) than cells that were still in the pre-synthetic phase G_1 [31]. Whether seeds maintain dormancy or germinate depends on the balance of phytohormones and environmental factors, such as temperature, moisture, and light [32–37]. Seed germination also includes such critical processes as silencing of seed development genes and activation of vegetative growth genes [16,38,39]. Key modulators of the transition from seeds to seedlings are epigenetic modifications such as DNA methylation, demethylation, histone modifications, and sRNA pathways [40–42]. Apparently, at this moment, the program controlled by the LAFL network is blocked [43,44]. For example, the LAFL genes are repressed during seed germination by the Polycomb machinery via the histone H3K27 trimethyltransferase activity of the PRC2 and the histone H2A E3 monoubiquitin ligase activity of the PRC1 [38,45–47].

To provide novel insights into the mechanisms of the seed-to-seedling transition, we performed transcriptome profiling of embryonic axes isolated from germinated pea (*Pisum sativum* L.) seeds before and after the beginning of radicle growth. Pea is a widespread crop plant representing one of Europe's global food protein sources. Pea genome was fully annotated only in 2019 [48]. This reference sequence is a valuable tool for high-quality transcriptomic analysis to understand the molecular basis of agronomically important traits and support crop improvement.

2. Results

2.1. Physiological Status of Germinated Pea Seeds

We incubated the pea seeds between moist filter paper layers at 22 °C for 72 h, isolated the cotyledons and embryonic, axes and measured their moisture content. The start of root cell elongation and division, accompanied by the so-called "radicle protrusion," was observed in 72 h of seed germination. Since that process was not synchronous, we divided the 72-hour-germinated seeds into two batches: (a) before the start of embryonic root growth (before radicle protrusion) and (b) after the start of embryonic root growth (after radicle protrusion). The appearance of axes is shown in Figure 1.

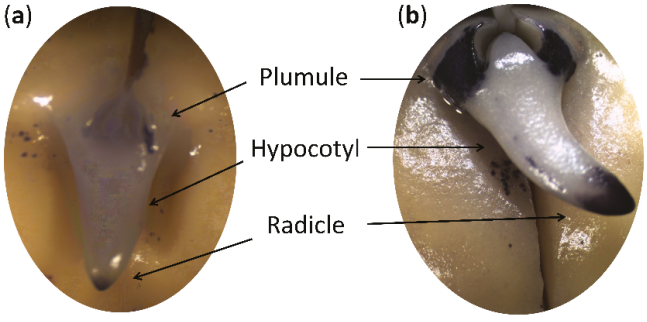

Figure 1. Axes of germinating pea seeds before radicle protrusion (**a**) and after radicle protrusion (**b**). Seeds incubated for germination for 72 h were stained with nitroblue tetrazolium. Dark-blue staining shows the superoxide anion radical localization.

Moisture content in the isolated axes before and after radicle protrusion was 72% and 87%, respectively (Figure 2a). Meanwhile, the moisture content in the isolated cotyledons was 65% in both batches. Radicle protrusion was accompanied by ROS generation in seed axes. We observed the accumulation of superoxide anion radical in the embryonic root and plumule (Figure 1). On the biochemical level, the radicle protrusion manifested in an approx. 2-fold increase ($p \leq 0.05$) in the thiobarbituric acid-reactive substances, expressed as malondialdehyde equivalents (Figure 2b). It was accompanied by a 3-fold increase ($p \leq 0.001$) in hydrogen peroxide (Figure 2c).

To measure the level of desiccation tolerance in seeds imbibed for 24, 48, and 72 h, they were dried at 22 °C for 24 h to the initial moisture content. It is essential that drying was done only for the seed batch before radicle protrusion. After drying, the seeds were returned to moist filter paper to germinate. Radicle protrusion started after two days of germination and reached 50–60% after three days, as in control and previously dried seeds (Figure 2d). However, in nine days, the total amount of seeds did not statistically differ in all versions. We evaluated the germination after nine days; the assessment covered normally developed seedlings, abnormally developed seedlings, and non-germinated seeds (Figure 2e). Normally developed seedlings had a well-formed embryonic axis, including the root, hypocotyl, epicotyl, and plumule with true leaves (Figure S1). Abnormally developed seedlings had an underdeveloped epicotyl or a too short main root. No statistically significant differences existed between the shoot and root lengths in seedlings grown from the control seeds or previously dried seeds (Figure 2f and Figure S2). It is important to note that drying at the different germination stages up to the visible radicle protrusion did not entail seed damage. However, when we dried seeds after the radicle protrusion, they lost their viability (Figure 2e).

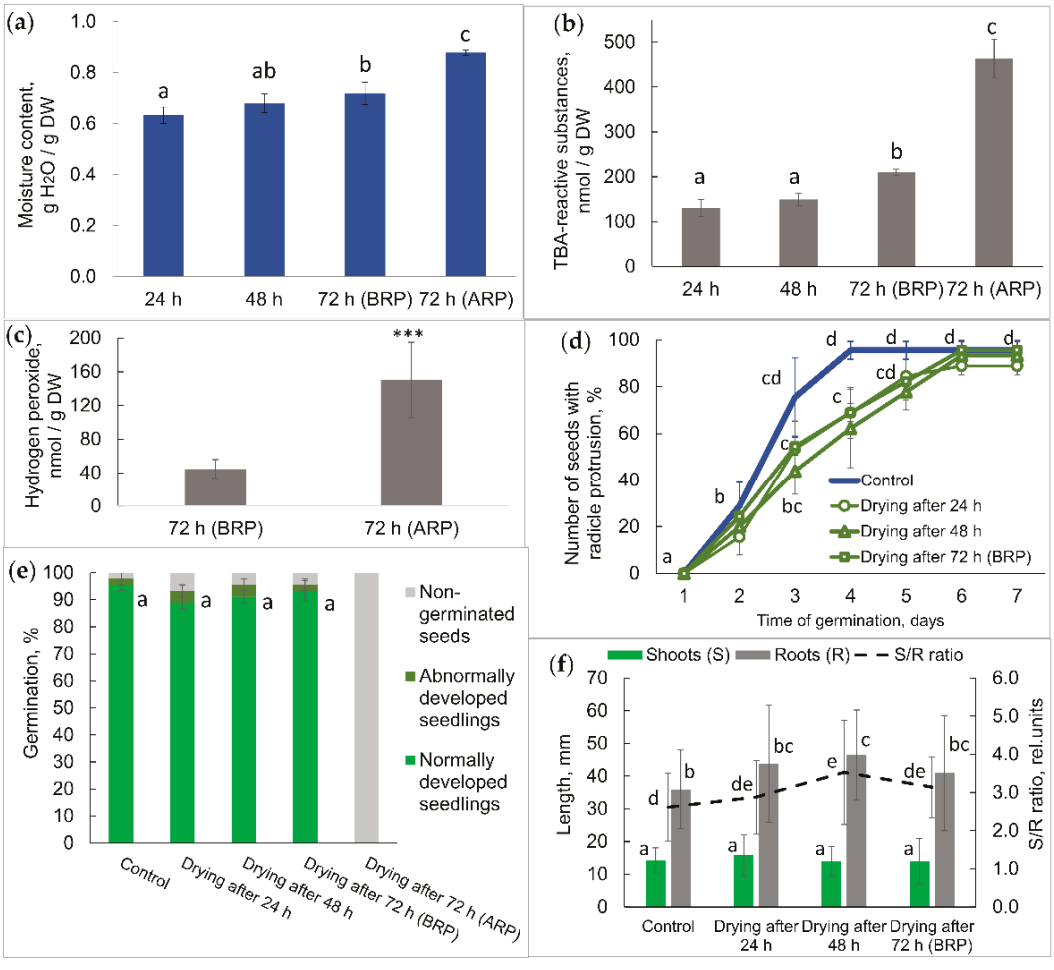

Figure 2. Physiological characterization of pea seeds and seedlings: Control, untreated seeds; BRP, before radicle protrusion; ARP, after radicle protrusion. (**a**) Moisture content in embryonic axes isolated from seeds after 24, 48, and 72 h of germination. The data represent the mean ± standard error of five biological replicates. The statistical significance was determined by the Kruskal–Wallis test. (**b**) Germination rate was recorded as the number of seeds with visible radicle protrusion. The data represent the mean ± standard error of three biological replicates. The statistical significance was determined by the *t*-test. (**c**) Content of thiobarbituric acid (TBA)-reactive substances in seed axes. The data represent the mean ± standard deviation of five biological replicates. The statistical significance was determined by the Kruskal–Wallis test. (**d**) Numbers of normally developed seedlings, abnormally developed seedlings, and non-germinated seeds after nine days of germination. The data represent the mean ± standard error of three biological replicates. The statistical significance was determined by the *t*-test. (**e**) Content of hydrogen peroxide in seed axes. The data represent the mean ± standard deviation of nine biological replicates. The statistical significance was determined by the *U*-test. (**f**) Length of the shoot and root of seedlings after nine days of germination. The data represent the mean ± standard deviation of 14–19 biological replicates. The statistical significance was determined by the Kruskal–Wallis test. Letters above the bars indicate significant differences between the mean values for (**a**,**b**,**d**–**f**) ($p \leq 0.05$). Asterisks indicate significant differences between the mean values for (**c**) ($p \leq 0.001$).

2.2. Comparative Transcriptome Profiling of Pea Seed Axes during Seed-to-Seedling Transition

To dissect the molecular basis for massive changes during the seed-to-seedling transition, we performed RNA sequencing of isolated embryonic axes before and after radicle protrusion with three biological replicates. The analysis was performed using the Illumina NovaSeq 6000 SP high-throughput whole-genome sequencing system. After removing the low-quality sequencing reads, a total of 511,182,576 reads were obtained (length of reads: 100 bp on one side of the fragments), with an average of 85 million filtered reads for each replicate.

Quality control of the sequencing results showed the high quality of the reads, with a small number of adapter sequences. After filtering the reads by length and quality and upon removing the adapter sequences, the reads were mapped to the reference Pea Genome International Consortium, version 1a (*Pisum sativum* v1a) [48]. Differentially expressed genes (DEG) with a false-discovery rate (FDR) < 0.05 and log base 2-transformed fold change (|logFC|) > 2 were acknowledged as differentially expressed.

The principal component analysis (PCA) based on the expression of 24,184 genes demonstrated a high level of differences between the seed axes before and after radicle protrusion (Figure 3). The percentage of the explained dispersion for the PCA model was 99% (98% for PC1 and 1% for PC2).

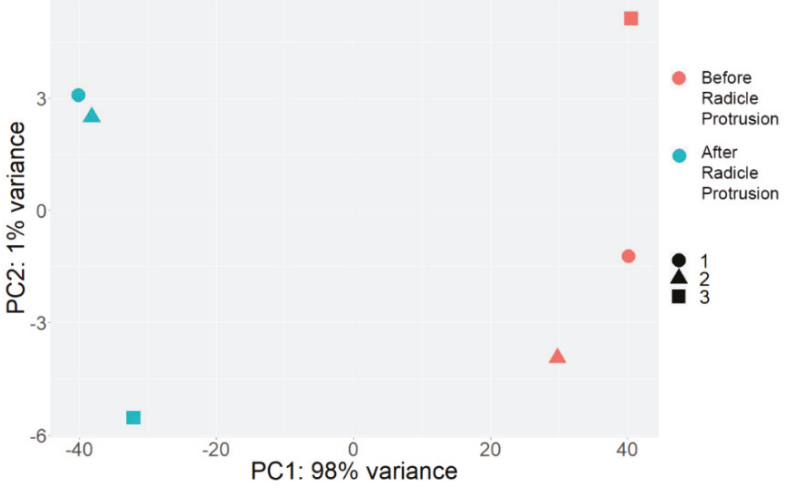

Figure 3. Principal component analysis (PCA) plot of all expressed genes in the RNA−seq data. The *X*−axis indicates the first principal component; the *Y*−axis indicates the second principal component. The percentage of variance explained by each PC is shown in each case. 1–3, biological replicates.

A volcano plot shows the distribution of differentially expressed genes (DEGs) by FDR and *p*-value. Among them, we observed a 4-fold increase in the expression of 2101 genes and a 4-fold decrease in the expression of 415 genes (|logFC| > 2). Genes with significant expression changes are marked in the diagram with red dots (Figure 4).

2.3. Categorization and Functional Annotation of the DEGs in Pea Seed Axes during Seed-to-Seedling Transition

2.3.1. GO Functional Enrichment Analysis

The DEGs specifically identified in seed axes after 72 h of germination were used to map the gene ontology (GO) database to explore significantly enriched terms compared with the genome background using the AgriGO v2.0 toolkit with false-discovery rate (FDR values < 0.05) as the threshold. The number of significant annotated genes whose expression decreased was 250, while the expression of 1361 genes increased. Treemap

visualization of the GO terms significantly enriched in downregulated and upregulated genes is shown in Figure S3a,b. We identified the 50 significant GO terms among the downregulated DEGs and 205 significant GO terms among the upregulated ones.

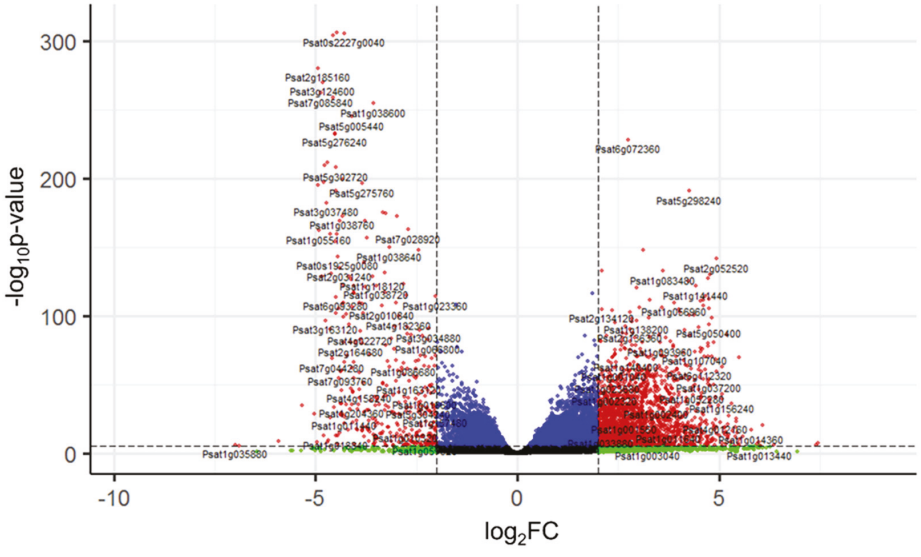

Figure 4. Volcano plot representing 24,184 differentially expressed genes. The $X-$axis indicates the log2$-$transformed gene expression fold changes between seed axes before and after radicle protrusion. The $Y-$axis indicates the log10$-$transformed $p-$value. Dashed lines indicate logFC and $p-$value thresholds. The scattered points represent each gene. Significant differentially expressed genes are highlighted in red. Genes with a significant logFC value but nonsignificant p-value are highlighted in green. Genes with a significant $p-$value but nonsignificant logFC value are highlighted in blue. The absence of significant differences is highlighted in black.

In the downregulated gene group, we identified the main categories contributing to the seed development process ("seed development", "developmental process", "reproduction", "multicellular organismal process", and "regulation of transcription, DNA template") and seed tolerance ("response to heat" and "response to stimuli") (Figure S3a). Among them, "response to heat" was the largest group, containing 14 GO terms, including "response to abiotic stimulus", "response to temperature stimulus", "response to ROS", "response to water", and "response to water deprivation" (Figure 5).

In the upregulated gene group, we identified the large categories containing multiple GO terms: "regulation of biological activity", "photosynthesis", "post-embryonic morphogenesis", and "lipid localization" contributing to the post-germination seedling development (Figure S3b). Four categories belonged to stress tolerance: "response to chitin", "response to stimuli", "immune system process", and "immune effector process". Three categories belonged to secondary metabolism: "secondary metabolic process", "plant-type secondary cell wall biogenesis", and "flavonoid metabolic process".

2.3.2. Overview of Metabolism

We annotated metabolic pathways using the MapMan software and visualized the changes in the metabolism of pea seed axes after radicle protrusion (Figure 6). As shown in Figure 6a, the seed-to-seedling transition was related to the biosynthesis of cell wall components; secondary metabolism; photosynthesis; metabolism of lipids, sugars, and amino acids; and redox status. Among the cell wall, lipids, and amino acids, some genes were upregulated and some downregulated. Starch and sucrose metabolism, light reaction,

and photorespiration were enhanced. Secondary metabolism contained many upregulated genes participating in synthesizing the terpenes, flavonoids, phenylpropanoids, and phenolics.

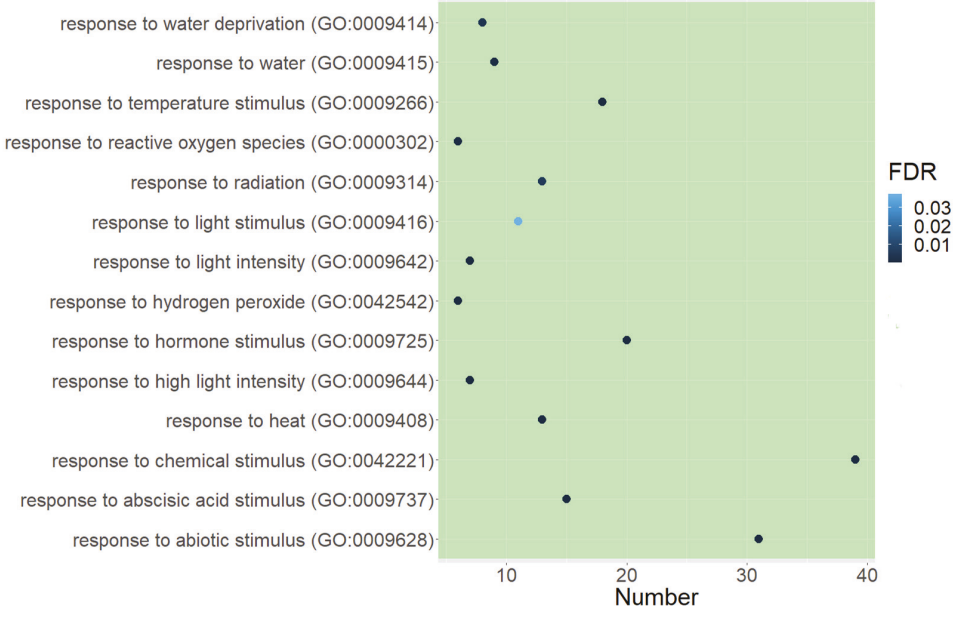

Figure 5. Bubble plot of GO terms associated with the response to stimuli and significantly enriched in genes downregulated in pea seed axes after seed radicle protrusion. The *X*-axis corresponds to the number of genes related to the GO term. The *Y*-axis corresponds to the GO term. The point color indicates FDR: the darker the color, the more significantly enriched the GO term.

DEGs were also mapped to genes associated with response to stressors (Figure 6b). Genes related to hormone signaling changed differently. Some genes associated with abscisic acid (ABA) were upregulated, and some were downregulated. At the same time, we observed the increased expression of most of the genes associated with auxin, ethylene, salicylic acid (SA), and jasmonic acid (JA). We divided the abiotic stress genes into equal upregulated and downregulated groups. However, the expression of heat shock proteins was mainly downregulated. The changes were associated with the expression of genes responsible for the cell redox status (ascorbate, glutathione, thioredoxin, and peroxidases). We also identified the upregulation of secondary metabolism genes and genes related to pathogen attack (PR-proteins).

2.4. Quantitative PCR Analysis of Selected Genes Related to Water Deprivation

qRT-PCR was performed for sixteen genes to validate the differential expression of genes selected by RNA-Seq. Among them, the seven upregulated DEGs and nine downregulated DEGs were detected in the pea seed axes before and after radicle protrusion (Figure S4a). The results showed that the expression pattern obtained by qRT-PCR corroborated the one obtained from RNASeq data for all sixteen genes. Spearman's coefficient attested to a significant correlation (R = 0.978), supporting the reliability of sequencing results (Figure S4b).

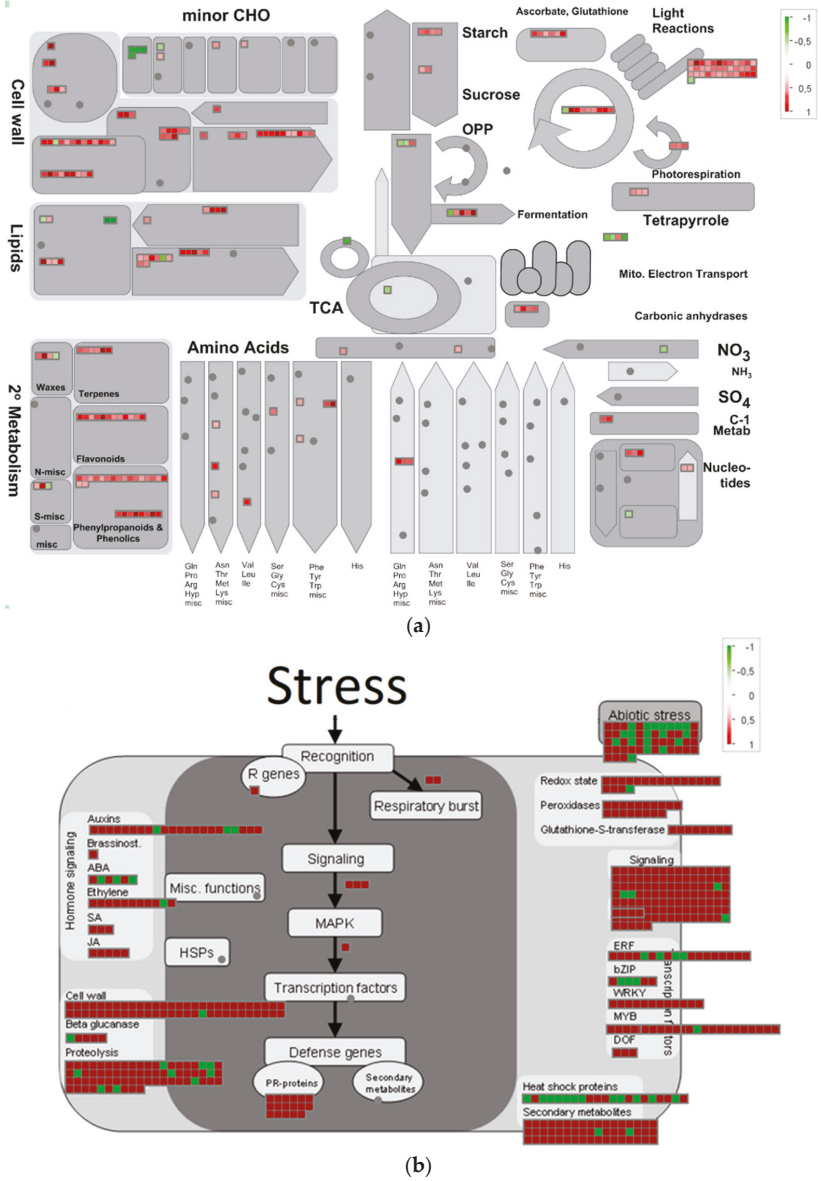

Figure 6. Overview of the changes in the metabolism of pea seed axes after radicle protrusion, obtained using the MapMan software. All squares represent genes with a significant differential expression assigned to the various metabolic pathways. The red color indicates an increase in gene expression; the green color indicates a decrease in gene expression. (**a**) Snapshot of modulated genes over the central metabolic pathways. (**b**) Snapshot of modulated genes over the stress metabolism.

The eight and fifteen identified DEGs downregulated in seed axes after radicle protrusion were related to GO terms "response to water deprivation" and "response to abscisic acid stimulus", respectively (Figure S3, Table S6). The DEGs were first annotated with BLASTX search against *A. thaliana* and then mapped to the Pea Genome Assembly v1a.

In the "response to water deprivation" group presumably associated with the loss of drought tolerance by seeds there were the *ABI4 (ABA INSENSITIVE 4*, Psat2g031240), *ABI5 (ABA INSENSITIVE 5*, Psat3g033680), three copies of *LEA14 (LATE EMBRYOGENESIS ABUNDANT 14*, Psat4g157880, Psat4g157760, Psat3g105280), *HVA22 (HORDEUM VULGARE ABUNDANT 22E*, Psat5g052360), *LTI65 (LOW-TEMPERATURE-INDUCED 65*, Psat0s2227g0040), three copies of *RD22 (RESPONSIVE TO DESSICATION 22*, Psat6g033920, Psat6g033960, Psat0s4403g0040), *LTP4 (LIPID TRANSFER PROTEIN 4*, Psat7g227120), and *PER1 (1-CYS PEROXIREDOXIN*, Psat7g085840).

Genes from the first group were partially overlapped with "response to abscisic acid stimulus", i.e., the five genes *HVA22E*, *ABI4*, *ABI5*, *LTI65,* and *LTP4.* These genes were represented well among mono- and dicotyledonous plant species (Figures S5–S9). Among these genes, *ABI4*, *ABI5*, and *HVA22E* are highly conservative in functional domains (Tables S10 and S11). *LTI65* and *LTP4* are less conservative; their homologs have been identified only in the genomes of higher plants (Figures S8 and S9). Therefore, the essential gene *ABI3 (ABA INSENSITIVE 3)*-encoded AP2/B3-like transcriptional factor family protein (Psat3g142040) was selected from the second group for the qRT-PCR analysis. According to the analysis results, the expression of all genes corresponded to the predicted and significant decrease during the seed-to-seedling transition (Figure 7).

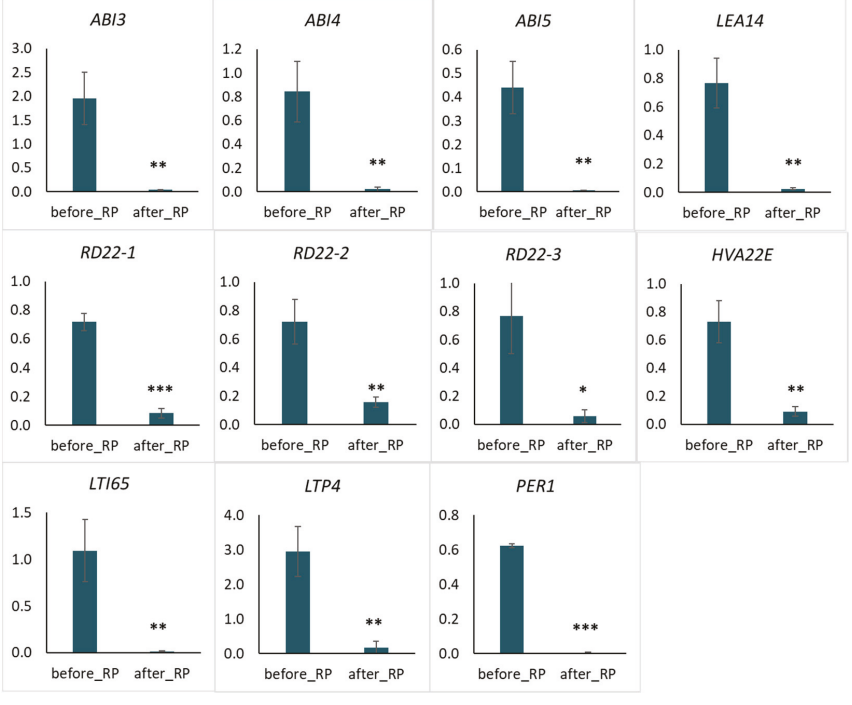

Figure 7. Relative expression levels of the protein-encoding genes ABA INSENSITIVE 3/4/5 (ABI3, ABI4 and ABI5), LATE EMBRYOGENESIS ABUNDANT 14 (LEA14), RESPONSIVE TO DEHYDRATION 22 (RD22), HORDEUM VULGARE ABUNDANT 22E (HVA22E), LOW-TEMPERATURE INDUCED 65 (LTI65), LIPID TRANSFER PROTEIN 4 (LTP4), and 1-CYSTEINE PEROXIREDOXIN (PER1) in pea seed axes before and after radicle protrusion (RP). Y-axis corresponds to relative expression levels analyzed by qRT-PCR. Data were normalized to the expression of the Psat1g039040 (GenBank Z25888) gene encoding the phosphoprotein phosphatase 2A. The data represent the mean ± standard error of three biological replicates. Significant differences between the mean values are indicated (*** $p \leq 0.001$, ** $p \leq 0.005$, * $p \leq 0.05$) (*U*-test).

We obtained exciting results for three copies of genes represented in the *P. sativum* genome. The expression of Psat4g157880, Psat4g157760, and Psat3g105280 annotated as *LEA14* has differed between copies. The decrease in expression during the switching of the developmental stage was detected only for the gene Psat4g157880 (Figure 7). We did not make the qRT-PCR analysis of other genes. According to the RNA-seq results, the Psat4g157760 expression was close to zero, and Psat3g105280 expression was equally high before and after radicle protrusion. However, we obtained the opposite results for the *RD22* genes Psat6g033920, Psat6g033960, and Psat0s4403g0040. The expression level of all copies significantly decreased after radicle protrusion. These genes were designated as *RD22-1*, *RD22-1*, and *RD22-1*, respectively (Figure 7).

3. Discussion

Seed germination is considered the first developmental phase in higher plants' life cycle, followed by post-germination growth and seedling development [2,49,50]. Until now, most studies focused on understanding the mechanisms of *sensu stricto* germination, but little is known about the molecular events taking place during the proper transition to the post-germination stage. Silva et al. analyzed the dynamics of the primary metabolites [50] and transcript profiles [51] in *A. thaliana* from the seed germination to seedling establishment. They revealed the two major metabolic shifts with changes in amino acids, organic acids, carbohydrates, and their derivatives. The first one occurred from the imbibition of dry seed to the early-seedling stages, including testa rupture, radicle protrusion, and root hair formation. The second occurred between radicle protrusion and seedling establishment when cotyledons fully opened. Correlation analysis between metabolite and transcript profile enabled the identification of the genes that influence the fluxes of metabolites during the seed-to-seedling transition.

In the present study, we focused on the seed-to-seedling transition in *Pisum sativum* L. Our experimental model included pea seeds after 72 h of germination sorted into two seed batches—just before and after the start of embryonic root growth (Figure 1). Radicle protrusion was accompanied by ROS generation in seed axes. The process of ROS accumulation during seed germination and the beneficial role of ROS in the regulation of cell elongation and division is well established [52]. We observed the increase in TBA-reactive products and hydrogen peroxide with localization of ROS in embryonic root and plumule (Figures 1 and 2b,c). Since the germination process is accompanied by an increase in water content from the dry state at the beginning to complete tissue hydration at the end, we compared the moisture content in isolated seed axes. It was 72% and 87% before and after radicle protrusion, respectively (Figure 2a). This shift in the water status of the analyzed embryonic axes correlated with their desiccation tolerance. The seeds sorted before radicle protrusion survived after drying and developed normal seedlings, but those selected after radicle protrusion lost their desiccation tolerance and died after drying (Figure 2e).

The phenomenon of desiccation-tolerance acquisition in orthodox seeds is well-known in literature [11,27–29]. Its molecular basis is grounded on the accumulation during late maturation of such compounds as the LEA proteins, small heat shock proteins, non-reducing oligosaccharides of the raffinose group [11,53], glutathione [54], tocochomanols [55], and carotenoids [56].

Progressive water loss in matured seeds induces the so-called cellular "glassy state" and a slowdown of chemical reactions [57,58]. Such glassy matrix consists of soluble sugars, which immobilize macromolecules offering protection to membranes and proteins [10,53]. The viscosity of cytoplasm also increases, while water and oxygen diffusion are suppressed, and the rates of all chemical reactions become dramatically reduced [57–59]. Due to these shifts in the physicochemical properties of multiple cellular structures, orthodox seeds can maintain viability for decades [60]. However, there is little information about the molecular mechanism of desiccation-tolerance loss at the post-germination stage.

3.1. Key Genes of the Response to Drought Tolerance of Pea Seeds

We compared RNA-sequencing-based transcriptomics in the isolated embryonic axes before and after radicle protrusion and identified 24,184 DEGs with FDR < 0.05 and (|logFC|) > 2. The PCA analysis demonstrated a high level of differences between versions, with 99% of the explained information (Figure 3). After radicle protrusion, the expression of 2101 genes increased, and that of 415 genes decreased more than four times (Figure 4). Among the downregulated DEGs, the largest group included the GO terms "response to heat", "response to abiotic stimulus", "response to temperature stimulus", "response to ROS", "response to water", and "response to water deprivation" (Figure S3a and Figure 5). At the same time, we observed the upregulation of the DEGs from the GO terms "response to stimuli", "immune system processes", and "immune effector process" (Figure S3b).

Annotation of the metabolic pathways using the MapMan software confirmed the significant genetic rearrangement of the general metabolic processes. Transition to the post-germination stage was accompanied by the gene expression related to photosynthesis, metabolism of lipids, sugars and amino acids, biosynthesis of cell wall components, redox status, and secondary metabolism (Figure 6a). The expression of the genes encoding heat shock proteins was mainly downregulated (Figure 6a). However, the expression of genes responsible for the ascorbate, glutathione, thioredoxin, and peroxidases level was increased (Figure 6b). Remarkably, we observed strong activation of the genes involved in defense responses to the biotic stress caused by fungi, bacteria, and viruses. These included the genes associated with pathogenesis-related (PR) proteins, salicylate- and JA-dependent defense pathways, and synthesis of terpenes, flavonoids, and phenylpropanoids.

The bioinformatic processing emphasized the genes from GO terms "response to water deprivation" and "response to abscisic acid stimulus" downregulated in seed axes after radicle protrusion. As is known, ABA is a phytohormone regulating the maintenance of seed dormancy and seed response to stress, particularly water deprivation [61]. Three genes (*LTI65*, *LTP4*, and *HVA22E*) were related to "response to water deprivation" and "response to abscisic acid stimulus" (Figure 7).

LTI65/RD29B (LOW-TEMPERATURE-INDUCED 65/RESPONSIVE TO DESICCATION 29B) encodes a protein expressed in response to such water deprivation conditions as cold, high-salt, and desiccation [62–64]. In the *Arabidopsis thaliana* genome, the promoter region of these gene contains two ABA-responsive elements (ABREs) [65]. In the genome of *P. sativum*, this gene encodes a protein with a total length of 1036 amino acids (aa) (Table S9). Analysis of phylogenetic similarity of *LTI65* based on full protein sequences showed no annotated homologues in the genomes of *Hordeum vulgare* L., some mosses, and drought-tolerant plants, such as the model resurrection specie *Selaginella moellendorffii* Hieron (Table S9, Figure S8).

LTP4 encodes the lipid transfer protein, which is strongly up-regulated by ABA [66]. LPTs proteins can bind and transfer fatty acids, acylCoA esters, and several phospholipids [67]. *LTP4* acts redundantly with its closest homologue LTP3 by modulating the ABA pathway [66]. In *P. sativum*, *LTP4* encodes the probable lipid transfer protein (88aa). However, we did not find the *LTP4* homologues in the genomes of mosses and resurrection plants (Table S10, Figure S9). This family's proteins are a members of PR-14 pathogenesis-related protein family [68]. Several plant LTPs act positively in plant disease resistance. *LTP3*, a pathogen- and ABA-induced gene, negatively regulates plant immunity in *A. thaliana*. *LTP3/LTP4* overexpression enhanced the susceptibility to virulent bacteria and compromised the resistance to avirulent bacteria [66]. LTP3 and its closest paralogue, *LTP4*, are highly induced during wilt disease development caused by the soil-borne pathogen *Ralstonia solanacearum* [69].

HVA22 encodes one more of the ABA-induced LEA proteins isolated from the aleurone tissue of barley [70]. This protein is short (147aa) and highly conservative (Table S10, Figure S7). Previously the bioinformatic analysis has revealed that 354 HVA22 homologs are present in diverse eukaryotic organisms. These homologs share high amino acid sequence similarity in a conserved TB2/DP1 domain. HVA22E encodes one of five HVA22 homologs

in *A. thaliana* and is upregulated in response to cold and salt stress, ABA treatment, or dehydration [71,72].

The three genes included only in the "response to water deprivation" group were *LEA14*, *RD22*, and *PER1*. Most *LEA* genes are known to have ABA response elements (ABRE) in their promoters, and their expression can be induced by ABA, cold, or drought [65,73]. The desiccation-related protein LEA14 belongs to Group II LEA proteins, also known as dehydrins [73–75]. *LEA14* was induced in response to salt and light stress in *A. thaliana* [76,77] and low temperatures in *Pyrus communis* L. [78].

RD22 encodes proteins of the BURP family, members of which share a highly conserved BURP domain [79,80]. AtRD22 is up-regulated by high-salt, desiccation, and exogenously supplied ABA [81–83]. It is possible that RD22 also regulates cell wall peroxidases under stress conditions [84].

Peroxiredoxins (Prx) are thiol-dependent antioxidants containing one (1-Cys) or two (2-Cys) conserved Cys residues [85]. *PER1* encodes a protein 1-Cys peroxiredoxin. 1-Cys Prx (also named PER1) is expressed in developing seeds but disappears rapidly after germination [86,87]. PER1 has a vital function during the stages accompanied by dramatic ROS generation by different processes such as desiccation during late maturation [88], "imbibing stress", and germination under abiotic stress [87,89]. 1-Cys Prxs function not only to relieve mild oxidative stresses but also as molecular chaperones under severe conditions during seed germination and plant development [90]. Overoxidation controls the switch in function of 1-Cys-Prxs from peroxidases to molecular chaperones [90]. Chen et al. (2020) demonstrated that AtPER1 eliminates ROS to suppress ABA catabolism and GA biosynthesis, thus improving the primary seed dormancy and making the seeds less sensitive to adverse environmental conditions [91].

Altogether, our data showed that after radicle protrusion in pea seed axes, there was a significant change in the pattern of genes responsible for adaptation to abiotic and biotic stresses. The seed and seedling demonstrated different pictures of gene expression in response to abiotic stresses. Seedlings lost their capability to survive after drying but acquired a system of defense against biotic stresses caused by fungi, bacteria, and viruses. Such massive rearrangements of gene expression programs should be under hormone control. We observed that genes related to hormone signaling changed differently (Figure 6b). Some of the ABA-related genes were upregulated, and some were downregulated. However, most genes encoding other hormones, such as auxin, ethylene, SA, and JA, were expressed.

3.2. The LAFL Network and the Developmental Program

As known, the master negative regulators of seed germination include the transcription factors LEC1, ABI3, FUS3, LEC2 (so-called LAFL), and DOG1 [16,41,92]. Switching the developmental program from maturation to germination is accompanied by gene suppression of the LAFL network and activation of the genes involved in vegetative growth [15]. Our results evidenced that among the *LAFL* genes, only *ABI3* was expressed in pea seed axes before radicle protrusion (Figure 7). The expression of *LEC1*, *LEC2*, *FUS3*, and *DOG1* was apparently suppressed at the early germination stage. Together with *ABI3*, we observed the expression of the ABA-dependent genes *ABI4* and *ABI5*.

ABI3, *ABI4*, and *ABI5* are the core transcriptional factors containing B3, AP2, and bZIP domains [93,94]. They control the expression of the ABA-responsible genes involved in the seed-specific events, such as maturation, dormancy, longevity, germination, and post-germination growth [61,95–100]. Interestingly, these genes are tightly co-expressed during all steps of plant ontogenesis [101]. *ABI3* and *ABI5* are involved in the regulation of chlorophyll catabolism, raffinose family oligosaccharides synthesis, and *LEA* genes expression at the late maturation stage [16,92,102–105]. *ABI5* can also act as a signaling integrator of ABA and other hormones [103,106,107]. Disruption of *ABI5* decreases the expression of many ABA-responsive genes, whereas *ABI5*-overexpressing plants were hypersensitive to ABA during seed germination and early seedling development [108]. *ABI4* is involved in hormone synthesis and signaling, seedling establishment, plastid-

to-nucleus signaling, synthesis and breakdown of lipids, and disease resistance [109]. It was suggested that *ABI4* could be activated in response to redox signals involved in developmental processes and response to oxidative stress [110]. In seeds, *ABI4* is mainly expressed during late maturation and early germination, mediating the balance between ABA and GA and thereby regulating the dormancy [11,98,101,111].

It is interesting that *ABI4* and *ABI5* are the highly conservative genes in the diverse drought-tolerant species. *ABI5* is the only gene represented in genomes of resurrection plants *Xerophyta humilis* (Baker) T. Durand & Schinz and *S. moellendorffii* (Table S10, Figures S5 and S6). In *P. sativum* genome, they encode the proteins with the length of 240aa and 422aa, respectively.

In our study, the *ABI3*, *ABI4*, and *ABI5* genes were expressed before radicle protrusion but reduced to zero after the protrusion. Thus, the seed-to-seedling transition was associated with blocking the expression of the ABA-related genes *ABI3*, *ABI4*, *ABI5*, and *LEA14*, of which only *ABI3* belongs to the LAFL regulatory network. Together, we observed a sharp decrease in the expression of *HVA22E*, *LTI65/RD29B*, and *LTP4* involved in responses to water deprivation and *PER1* involved in the suppression of ABA catabolism and GA biosynthesis via ROS elimination.

4. Conclusions

Thus, this study provided a primary global view of transcriptomic changes during the seed-to-seedling transition in *P. sativum*. Seedling establishment was accompanied by growth initiation of embryonic roots, increased water content, and ROS accumulation. Importantly, seeds were tolerant to desiccation before radicle protrusion and survived after drying to initial moisture content. However, radicle protrusion resulted in the loss of desiccation tolerance, and seedlings died after drying. Through the transcriptomic comparison of embryonic axes isolated from 72-hour-germinated pea seeds before and after radicle protrusion, we deciphered the significant genetic rearrangement of metabolic processes. This rearrangement includes the upregulation of gene expression related to the photosynthesis; metabolism of lipids, sugars, and amino acids; and cell wall biosynthesis. It was accompanied by (i) a desiccation tolerance loss and (ii) the initiation of secondary metabolism and activation of genes involved in defense responses to biotic stress. Most genes associated with auxin, ethylene, salicylic, and jasmonic acids were upregulated. However, ABA-related genes demonstrated a different behavior. Several of them were upregulated, while others were downregulated. Among them, *ABI3*, *ABI4*, *ABI5*, and *LEA14* were expressed in embryonic axes before radicle protrusion but downregulated at the post-germination stage. Surprisingly, among the *LAFL* genes, only *ABI3* was expressed in pea seed axes before radicle protrusion. In summary, we observed the downregulation of ABA-related genes *HVA22E*, *LTI65/RD29B*, and *LTP4* were involved in responses to water deprivation and *PER1* involved in the suppression of ABA catabolism and GA biosynthesis via ROS elimination. These results provide the next step toward identifying the master regulators controlling seed-to-seedling transition in plants and open new perspectives for understanding the complex regulatory mechanisms underlying the seedling establishment. Further exploration of genes controlling the switch of stress-tolerance programs can facilitate the breeding of plants more tolerant to drought.

5. Materials and Methods

5.1. Preparation of Plant Material

Pea seeds of the commercial cultivar "Prima" were obtained from the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (St. Petersburg, Russia). Seeds were incubated between layers of moist filter paper. Seeds imbibed for 72 h were visually divided into two batches: (a) before the start of embryonic root growth (before radicle protrusion) and (b) after the start of root growth (after protrusion). Seed axes from both batches were isolated, frozen in liquid nitrogen, and stored at −80 °C before DNA and RNA extraction.

5.2. Physiological Evaluation

In situ localization of the superoxide anion. Pea seeds germinated for 72 h, before and after radicle protrusion, were incubated in 6 mM nitroblue tetrazolium (NBT) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 30 min [112]. The superoxide anion was visualized as deposits of dark-blue insoluble formazan compounds [113].

TBA-reactive substances. Content of thiobarbituric acid (TBA)-reactive substances were quantified as malondialdehyde equivalents (MDA) as described by [114]. TBA content was measured separately using five replicates. The differences among variants were tested with Past software (https://www.nhm.uio.no/english/research/infrastructure/past accessed on 18 May 2022), taking $p \leq 0.05$ as significant.

Hydrogen peroxide content. The hydroperoxide content was calculated as 13S-hydroperoxy-9*Z*, 11*E*-octadecanoic acid equivalents [115] with some modifications described by [116]. Hydrogen peroxide content was measured separately using nine replicates. The differences among variants were tested with Past software (https://www.nhm.uio.no/english/research/infrastructure/past, accessed on 18 May 2022), taking $p \leq 0.001$ as significant.

Seed moisture content. Moisture content was measured separately in seed axes and cotyledons using three replicates of 9 seeds each by oven method at 103 °C for 17 h. The calculation was done on a dry weight basis, with the degree of moisture expressed as a percentage. The differences among variants were tested with Past software (https://www.nhm.uio.no/english/research/infrastructure/past, accessed on 18 May 2022), taking $p \leq 0.05$ as significant.

Desiccation tolerance. Seed desiccation tolerance was assessed based on the embryonic axes survival after drying. We incubated seeds between layers of moist filter paper at 22 °C. After 24, 48, and 72 h of germination, we transferred the seeds to dry paper and dried them to the initial moisture for three days at 22 °C. Dried seeds were returned to moist filter paper to continue the germination process.

Seed germination. Twenty-five seeds in 4 replicates were incubated between layers of moist filter paper at 22 °C for nine days. The number of seeds with visible radicle protrusion was counted for each day. After nine days, the numbers of normally developed seedlings, abnormally developed seedlings, and non-germinated seeds were calculated [117]. The differences among variants were tested with Past software (https://www.nhm.uio.no/english/research/infrastructure/past, accessed on 18 May 2022), taking $p \leq 0.05$ as significant.

5.3. Isolation of Total DNA and RNA

The seed axes were frozen in liquid nitrogen and homogenized with a pestle in a mortar. According to the manufacturer's instructions, total DNA was isolated using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) (www.qiagen.com, accessed on 18 May 2022). Total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions (www.qiagen.com, accessed on 18 May 2022), followed by DNase treatment with the RNase-free DNase set (QIAGEN). The quality of the isolated DNA and RNA was evaluated in 1% agarose gel prepared based on the TAE buffer with the addition of ethidium bromide as an intercalating dye. The Sky-High 250 b–10 kb marker (BioLabMix, Novosibirsk, Russia) was used as a DNA molecular weight marker. The resulting isolation products were visualized using the BioRad ChemiDoc MP gel-documenting system (Bio-Rad Laboratories, Moscow, Russia). Concentrations of the isolated DNA and RNA were measured using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

5.4. RNA Library Preparation

The TruSeq mRNA-stranded reagent kit (Illumina, San Diego, CA, USA) was employed to enrich total RNA samples with the poly(A+) fraction, followed by cDNA synthesis using Superscript II Reverse Transcriptase, followed by the second strand cDNA synthesis. cDNA was used to prepare libraries compatible with Illumina sequencing technology. The quality

of the obtained libraries was checked using the Fragment Analyzer (Agilent, Moscow, Russia). Quantitative analysis was performed by real-time polymerase chain reaction (qPCR). After quality control and DNA quantity evaluation, the library pool was sequenced using the Illumina NovaSeq 6000 SP high-throughput whole-genome sequencing system (www.illumina.com, accessed on 18 May 2022).

5.5. Reads Preprocessing, Mapping and Differential Expression Analysis

The quality of raw reads was checked using FASTQC v. 0.11.9 [118]. Filtering of the libraries was performed using the bbmap software (https://sourceforge.net/projects/bbmap/, accessed on 12 March 2021). It resulted in removing unidentified bases (N) or bases with a Phred quality score below 20 from both 3′- and 5′-ends of the read and removing the reads with a length less than 60. The filtered reads were then mapped to the Pea Genome Assembly v1a from the UGRI server (https://urgi.versailles.inra.fr/Species/Pisum, accessed on 12 March 2021). The Hisat2 tool was applied to map the short-read libraries to the reference genome [119]. The number of reads aligned to each gene was counted with the Subread software package [120]. The PCA analysis using DESeq2 was conducted to assess library quality and search for outliers [121]. The differential gene expression analysis was performed using the edgeR package for R [122]. Genes with low expression were eliminated. Genes with a false-discovery rate (FDR) < 0.05 and log base 2-transformed fold change ($|\log FC|$) > 2 were considered differentially expressed. A volcano plot showing the distribution of differentially expressed genes (DEGs) was made using the EnhancedVolcano package [123].

5.6. Differentially Expressed Genes (DEGs) Annotation

DEGs were annotated with the blastx search against the *A. thaliana* (TAIR 10) protein database (with a threshold e-value < 10×10^{-9}). The GO and MapMan annotations were assigned based on the *A. thaliana* homologous proteins. The AgriGO v.2 toolkit was used to perform singular enrichment analysis of DEG lists [124]. The GO terms with an adjusted p-value < 0.05 were considered significantly enriched. Reduction of redundancy and TreeMap visualization of GO terms were performed using the ReviGO tool [125]. The diversity and abundance of differentially expressed genes were investigated with MapMan using the *A. thaliana* mapping database (Ath_AGI_ISOFORM_MODEL_TAIR10_Aug2012) downloaded from the MapMan server [126]. The full list of DEGs (upregulated and downregulated) was used as a query experiment and mapped on the metabolism overview and stress metabolism pathways.

5.7. Quantitative Reverse-Transcription PCR (qRT-PCR) and Sequence Analysis

A single-stranded cDNA was synthesized from the RNA template using the M-MuLV–RH First Strand cDNA Synthesis Kit (BioLabMix, Novosibirsk). Quantitative PCR was performed using the SYNTOL SYBR Green I+ROX kit (Syntol) on the CFX96 Touch Real-Time PCR Detection System (BioRad). PCR was made in 15 µL of the reaction mixture under the following conditions: 1 cycle at 50 °C for 10 min; 1 cycle at 95 °C for 5 min; 40 cycles at 95 °C for 10 s and 60 °C for 30 s. PCR product melting curves were constructed under the following conditions: 10 s at 95 °C; 5 s at 65 °C; and 5 s at 95 °C. To standardize the amount of cDNA template, qPCR was performed with the primers for the housekeeping gene *Phosphoprotein Phosphatase 2a* (GenBank: Z25888): 5′-ccacattacctgtatcggatgaca-3′ and 5′-gagcccagaacaggagctaaca-3′ [127]. Each sample was amplified in three technical replicates. Plots of the dependence of the threshold cycle on the initial concentration of matrices were built based on three consecutive 3-fold dilutions. The differences among genotypes were tested by *U*-test in Past software (https://www.nhm.uio.no/english/research/infrastructure/past, accessed on 18 May 2022), taking $p \leq 0.001$, $p \leq 0.005$, and $p \leq 0.05$ as significant. The primer design for determining the relative level of gene expression was made using the IDT PrimerQuest software (http://eu.idtdna.com/PrimerQuest/Home, accessed on 19 June 2021) and Oligo

Primer Analysis Software v.7 (https://www.oligo.net accessed on 19 June 2021) (Table S9). The annotation of the functional domains was carried out using MOTIF Search (https://www.genome.jp/tools/motif/, accessed on 19 June 2021). The evolutionary analysis was performed in MEGA X software (http://www.megasoftware.net, accessed on 19 June 2021) [128] by maximum likelihood method and JTT matrix-based model [129] with 1000 bootstrap replicates. LALIGN/PLALIGN has been used to identify percent sequence identity (https://fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=lalign&pgm=lal, accessed on 19 June 2021).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11131686/s1, Figure S1: Appearance of the embryonic axes of normally and abnormally developed 9-day-old seedlings; Figure S2: Appearance of 14-day-old; Figure S3: Treemap visualization of GO terms significantly enriched in genes downregulated and upregulated in pea seed axes after seed radicle protrusion; Figure S4: qRT-PCR validation of RNA-seq results; Figure S5: Analysis of phylogenetic similarity of ABI4 genes based on full protein sequences; Figure S6: Analysis of phylogenetic similarity of ABI5 genes based on full protein sequences; Figure S7: Analysis of phylogenetic similarity of HVA22E genes based on full protein sequences; Figure S8. Analysis of phylogenetic similarity of LTI65 genes based on full protein sequences; Figure S9: Analysis of phylogenetic similarity of LTP4 genes based on full protein sequences; Table S1: Statistics of the obtained RNA-seq libraries; Table S2: Raw counts; Table S3: RPKMs; Table S4: Differential expression of *Pisum* genes in seed axes after radicle protrusion; Table S5: Result of the BLASTX search of *P. sativum*. DEGs against *A. thaliana* protein database (TAIR10); Table S6: GO term; Table S7: Genes mapped to the overview of the metabolic pathway using MapMan; Table S8: Genes mapped to the stress metabolic pathway using MapMan; Table S9: Primers used in this work; Table S10: The percentage of identity of proteins of *P. sativum* and other plant species; Table S11: Functional domains of *P. sativum* proteins.

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Transcripts Expressed during Germination *Sensu Stricto* Are Associated with Vigor in Soybean Seeds

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Abstract: The rapid and uniform establishment of crop plants in the field underpins food security through uniform mechanical crop harvesting. In order to achieve this, seeds with greater vigor should be used. Vigor is a component of physiological quality related to seed resilience. Despite this importance, there is little knowledge of the association between events at the molecular level and seed vigor. In this study, we investigated the relationship between gene expression during germination and seed vigor in soybean. The expression level of twenty genes related to growth at the beginning of the germination process was correlated with vigor. In this paper, vigor was evaluated by different tests. Then we reported the identification of the genes *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *N-glycosylase/DNA lyase OGG1* and *Cellulose synthase A catalytic subunit 2*, which are expressed during germination, that correlated with several vigor tests commonly used in routine analysis of soybean seed quality. The identification of these transcripts provides tools to study vigor in soybean seeds at the molecular level.

Keywords: germinative process; seedling establishment; molecular tools

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1. Introduction

Soybean (*Glycine max* (L.) Merrill.) ($2n = 40$ chromosomes) is a herbaceous and autogamous plant belonging to the Fabaceae family. Soybean production accounts for 56% of the world oilseed production [1]. It is considered a global commodity and is highly affected by biotic and abiotic factors [2]. Thus, obtaining soybean seeds with high physiological quality which germinate uniformly and rapidly is paramount for the success of the production of this oilseed. Seeds with superior physiological quality are the input for agriculture [3] because they are responsible for the successful establishment of the desired stand, which is essential to achieve high productivity [4].

Vigor is an important component of the physiological quality [5] and is responsible for the seed's resilience [6]. Thus, vigorous seeds present better physiological performance under non-ideal conditions for their germination (capacity for the new individual to grow from the embryo), and/or germinate faster according to Fonseca de Oliveira et al. [3] and Ebone et al. [4], which leaves the seed exposed to adverse conditions for a shorter time. The most robust and accepted definition of vigor is 'the sum total of those properties of the seed that determine the potential level of activity and performance of the seed during germination and seedling emergence' [7,8]. In this sense, vigorous seeds have a faster and more uniform seedling establishment [9], which contributes to the initial growth of the

plants. It is important to highlight that, seeds with similar germination may have variation in their vigor. This makes the germination analysis (mandatory for its commercialization) insufficient to determine the seed lots with the highest level of vigor. This is due to the germination test only evaluating the number of individuals with normal characteristics in an isolated way, at a certain period after sowing. Therefore, the screening of this property is commonly performed using measurements of germination speed, seedling growth and tolerance to stress, since these characteristics are more pronounced in seeds with a higher level of vigor. In parallel, to help in the diagnosis of more vigorous seeds, other tests can be used, such as membrane integrity, lipid peroxidation level and more recently the use of spectral images that map seed constituents [3,10].

In soybean, vigor is known as a physiological quality attribute that contributes to the increase in crop yield [4]. In this species, the acquisition of this property occurs progressively after the acquisition of desiccation tolerance at stage R7.1.3 in the maturation phase [11]. Despite the knowledge of its importance and of the time when vigor is acquired, research has been incipient in elucidating the molecular mechanisms underlying its expression in soybean seeds. Apparently, this is due to an overlap of physiological and molecular events in the temporal pattern of acquisition of physiological quality in soybean seeds during the maturation and late maturation phases [11,12]. This makes it difficult to separate and characterize the events that contribute to the acquisition of vigor at the molecular level.

Faced with the difficulty of studying vigor at the molecular level when it is acquired during maturation, an alternative is to study it during the germination process, i.e., when this property manifests itself and provides the establishment of seedlings quickly or under adverse conditions. This would be possible since the germination process has been well explored in relation to the association of its physiological and molecular mechanisms. For example, several groups of genes have been related to germination *sensu stricto* (the first stage of the germination process), mainly those involved with DNA repair, such as oxidative stress defense, cell respiration and hydrolysis of reserves, and cell elongation and expansion [13–17]. At the same time, the synchrony between the processes promotes a chain reaction that culminates in the growth of the embryonic axis, which leads to the occurrence of visible germination, i.e., growth of the new individual from the embryo, through the protrusion of the radicle due to the elongation of the transition zone between the hypocotyl and the radicle [18] as occurring in soybean embryos [19].

Recently, Ribeiro-Oliveira et al., [6] showed that the process that occurs during germination *sensu stricto* has a positive linear influence on vigor in soybean seeds. In other words, vigor is the key for the efficiency of the germination process, and the consequence of this is observed in the establishment of the seedling (quickly and under adverse conditions). Thus, theoretically, the study of the expression of genes related to growth acting during germination may reveal transcripts associated with seed vigor. One way to explore this would be using seeds with similar germination rates, but with differences in their vigor. Therefore, to test our hypothesis, we used seeds with differences in their vigor levels, previously screened from two commercial cultivars (cv1 and cv2) divided two lots (lot 1 and lot 2). Our aim was to verify if there was a correlation between the genes expressed during germination *sensu stricto* with seed vigor, evaluated by different properties related to this, i.e., speed germination, stress tolerance, organ elongation of seedlings, membrane integrity and tissue deterioration.

2. Results

2.1. Characterization of Seed Properties

Initially, we screened the physiological quality of our samples through several tests related with germination and vigor for commonly used soybean seeds. We noticed that normal seedlings and emergence did not differ between the lots of cultivars analyzed (Figure 1A,B). There was no difference regarding the length of seedlings of cv1 (Figure 1C). As for cv2, seedlings from lot 2 were longer in relation to those of lot 1 (Figure 1C). The

seedling dry mass data were not influenced (Figure 1D). After accelerated aging there was a reduction in the ability to generate normal seedlings in seeds from lot 2 in all the cultivars analyzed (Figure 1E). After aging, the water content ranged from 13.2 to 13.5% between our seed samples, indicating that the moisture content did not influence the results obtained, since it did not change more than 0.5% between the samples.

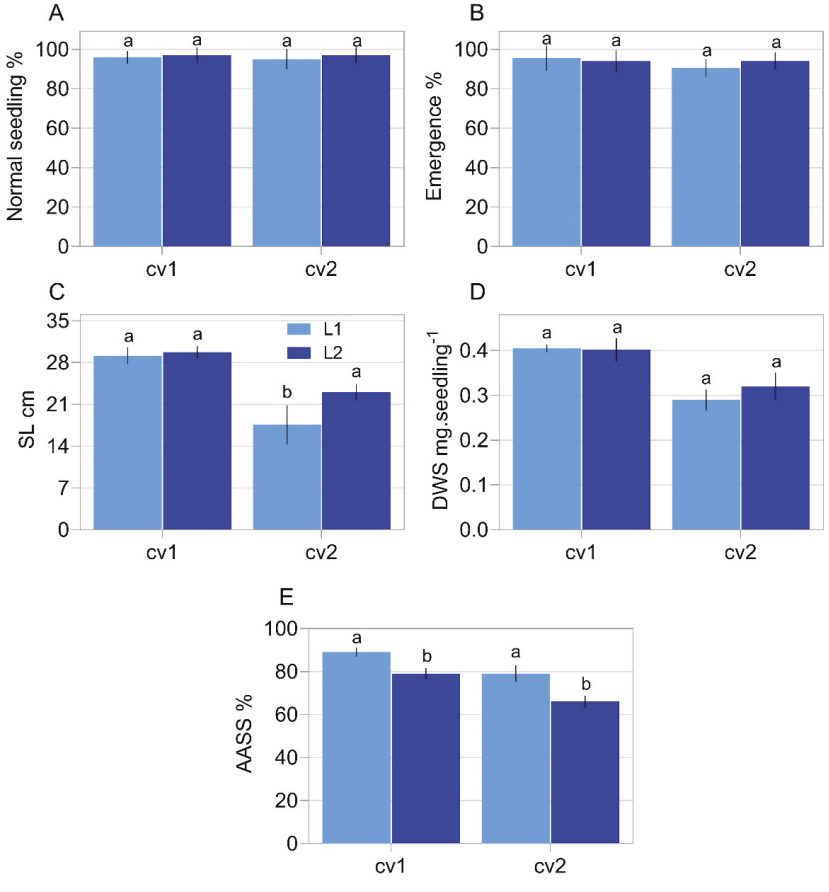

Figure 1. Physiological properties in two commercial cultivars (cv1-BRS133 and cv2-MG/BR 46 (Conquista)) and their respective seed lots (L1: lot 1 and L2: lot 2). Normal seedlings (**A**), seedling emergence (**B**), seedling length (**C**), seedling dry weight (**D**) and accelerated ageing using saline solution–AASS (**E**). Different letters indicate a significant difference at the 5% probability level ($p < 0.05$). Error bars show standard deviation ($n = 8$).

Excluding the results of time to reach 50% of germination (t50) at 25 °C with and without the use of saline solution in the germination substrate for cv1, in all other conditions the seeds of lot 2 took longer to reach 50% of germination than the seeds of lot 1 (Figure 2). In general, as the test conditions became more adverse for germination, the differences between the lots became more evident, and at 10 °C and 15 °C, the t50 results showed significant differences between all the samples analyzed (Figure 2 B,C).

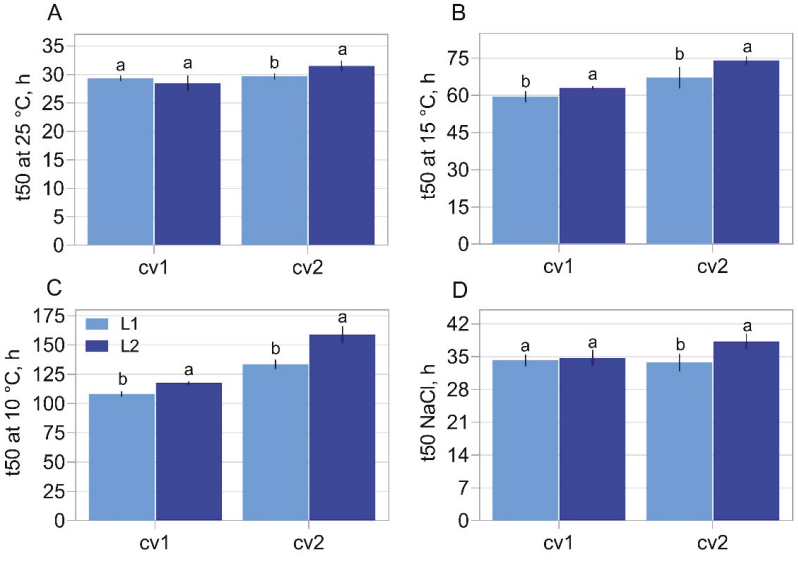

Figure 2. Time to reach 50% of root protrusion (t50) in two commercial cultivars (cv1-BRS133 and cv2-MG/BR 46 (Conquista)) and their respective seed lots (L1: lot 1 and L2: lot 2). t50 at 25 °C (**A**), 15 °C (**B**) and 10 °C (**C**), and t50 in saline substrate at 100 mmol.L^{-1} of NaCl (**D**). Different letters indicate a significant difference at the 5% probability level ($p < 0.05$). Error bars show standard deviation ($n = 8$).

Electrical conductivity (Figure 3A) showed higher amounts of leachate in the seeds of lot 2 of cv1 and cv2. Finally, malonaldehyde analysis showed that seeds from lot 2 had a higher estimated level of lipid peroxidation (Figure 3B).

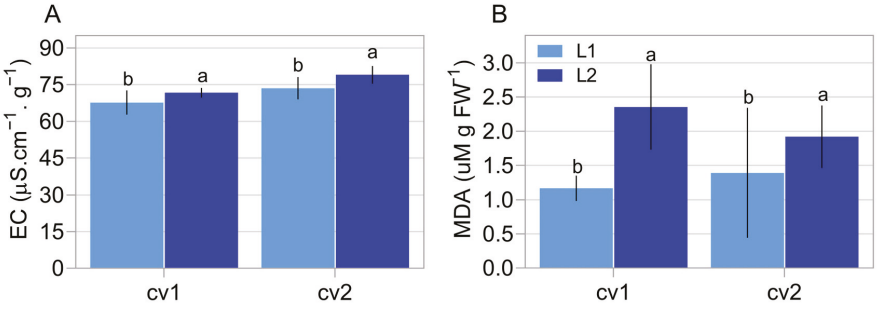

Figure 3. Biochemical properties in two commercial cultivars (cv1-BRS133 and cv2-MG/BR 46 (Conquista)) and their respective seed lots (L1: lot 1 and L2: lot 2). Electric conductivity (**A**) and Estimation of lipid peroxidation (MDA) (**B**). Distinct letters indicate a significant difference at the 5% probability level ($p < 0.05$). Error bars show standard deviation ($n = 8$).

There was an increase of 25% in the average length of the embryonic axis during germination *sensu stricto*, and the dynamics of growth did not change depending on the cultivar and its respective lots (Figure 4).

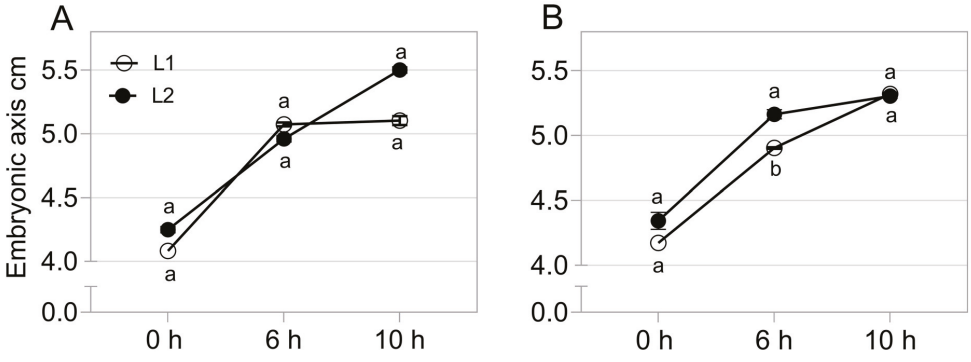

Figure 4. Embryonic axis length during germination in two commercial cultivars (A: cv1-BRS133 and B: cv2-MG/BR 46 (Conquista)) and their respective seed lots (L1: lot 1 and L2: lot 2) during germination *sensu stricto*. cv1 (**A**) and cv2 (**B**) at 0 h, 6 h and 10 h of germination. Distinct letters in each point of evaluation indicate a significant difference at the 5% probability level ($p < 0.05$). Error bars show standard deviation ($n = 6$).

2.2. Gene Expression Related to Growth in the Embryonic Axis during Germination

In sequence, to further test our idea we explored gene expression during germination *sensu stricto* in previously characterized seeds with distinct physiological potential of each cultivar (Figure 5). Among the genes involved in DNA repair, the relative expression of the gene encoding *N-glycosylase/DNA lyase OGG1* stands out, which was twice as high at 6 h and 10 h in the germination of seeds from lots 1 and 2 of cv1. The seeds of lot 2 from cv2 showed a decrease in expression after 6 h of imbibition, with a greater expression at 10 h. In seeds from cv1 the expression of the *DNA apurinic lyase* expression was more accentuated at 10 h in the seeds of L1 and at 6 h in the seeds of L2 (Figure 5).

The relative expression of *Metallothionein-like protein 1* (*MT1*) was high along germination for seed lots 1 and 2 of cv1. The seeds of lot 1 from cv2 showed a decrease in three times on *MT1* expression in the beginning of germination (Figure 5). The expression of *enolase*, an enzyme involved in the glycolytic pathway, was observed in the embryonic axis of soybean seeds and was not significant for any of the cultivars and their respective lots (Figure 5).

Regarding the reserve mobilization process, it was observed in seeds from lot 1 of cv1 that the relative expression of *Thiol protease* in the embryonic axis increased by 3 × and that *Cysteine proteinase* increased by 2 × 10 h after beginning germination. For seeds from lot 2 of cv1, the level of *Thiol protease* was highest at 6 h. In cv2 seeds, there was no variation in the relative expression of these transcripts during germination *sensu stricto* and among seeds of the studied lots (Figure 5).

The relative expression of *expansin-A10-like* (*EXPA10*), *expansin-A6* (*EXPA6*) and expansin-like A1 (*EXPA1*) progressively increased in seeds from lot 1 of cv1. In the seeds of lot 2, however, there was a similar pattern of the increase profile only for *EXPA10*, which was 18 × greater than from seeds of lot 1. Reductions were observed along germination for *EXPA6* and *EXPA1*. In cv2 seeds, there was an increase for *EXPA10* and *EXPA6* in lot 1 seeds, while in lot 2 the tendency was for a reduction in the expression with advance of the germination process (Figure 5).

As observed for expansins, the relative expression of transcripts *α-xylosidase 1-like* (*XYL1*), *Tubulin α-4 chain-like* (*TUA*), *Xyloglucan endotransglucosylase/hydrolase 2* (*XTH2*), *Endoglucanase 8-like* (*EGase*), *Pectinesterase 3* (*PME*) and *LRR receptor-like serine/threonine-protein kinase FEI* increased throughout germination *sensu stricto* in seed lot 1 of cv1. For cv2 seeds, the variations in the relative expression of transcripts were more accentuated during germination, in which, in general, lot 1 seeds showed greater expression at 6 h, while lot 2

seeds at 10 h after the beginning of germination (Figure 5), which suggest a delay in the expression of the genes mentioned earlier in these seeds.

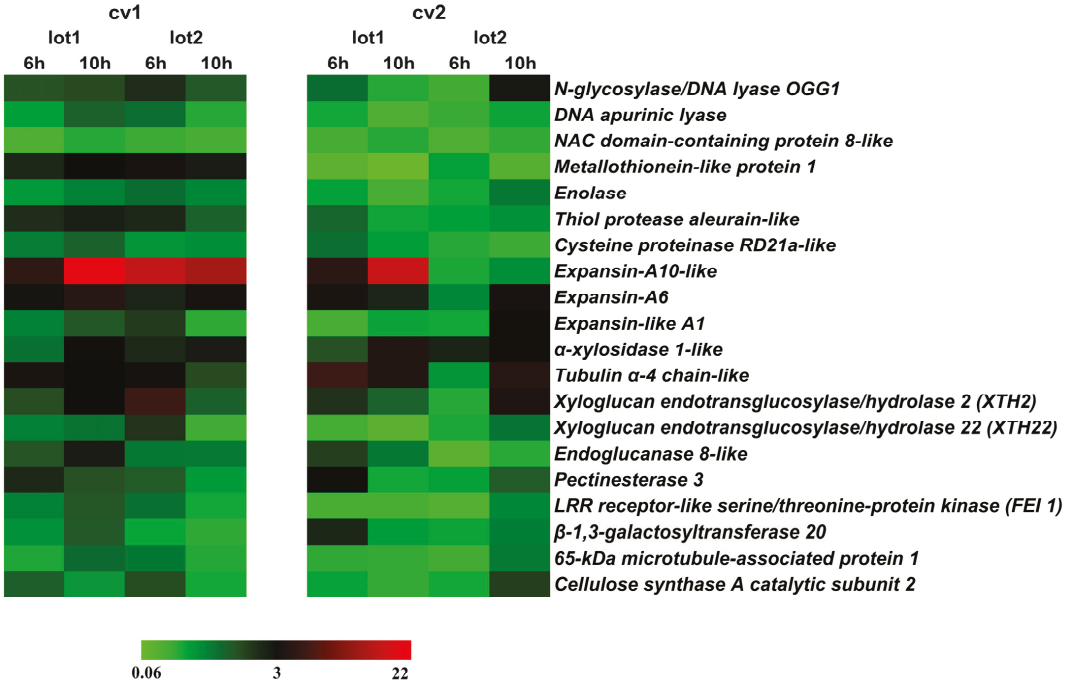

Figure 5. Heatmap of relative expression ($2^{-\Delta\Delta CT}$) during germination *sensu stricto* in the embryonic axis in two commercial cultivars (cv1-BRS133 and cv2-MG/BR 46 (Conquista)) and their respective seed lots (L1: lot 1 and L2: lot 2). The relative expression was obtained using the Pair-Wise Fixed Reallocation Randomization Test ($n = 6$) in relation to the expression of dry embryonic axis.

The expression of *β-1,3-galactosyltransferase 20* increased strongly at 6 h of germination in lot 1 of cv2 and reduced afterwards. For cv2, although apparently showing the same trend, this increase was not observed in the same proportion of cv1 seeds (Figure 5).

In general, for *65-kDa microtubule-associated protein 1 gene*, an increase of two times in the expression during germination in our samples was verified. (Figure 5). An increase in the expression of *Cellulose synthase A catalytic subunit 2* occurred at 6 h in seeds from lot 1 and lo2 of cv1. For cv2 the expression was more accentuated only at 10 h in seeds of lot 2 (Figure 5).

Regarding the gene expression divergence between the cultivars used in this study, our expression analysis demonstrated that, in general, cv1 seeds have superior expression of the genes studied during the germination process, especially those belonging to lot 1 (Figure 5).

2.3. Association between Gene Expressions during Germination Sensu Stricto with Seed Vigor

Finally, considering the natural divergence in vigor of our samples and the changes in the expression profile of transcripts during germination as previously screened, we sought to find the genes most associated with the expression of seed vigor, here evaluated by different tests. For this, the cultivars and their respective lot parameters were analyzed jointly. Initially, we explored whether there were differences in the association of gene expression analyzed at 6 h and 10 h during germination *sensu stricto* with aspects of vigor. To this end, the bootstrap method was performed with 10,000 resamplings, using the gene expression of each sample obtained at 6 h and 10 h during germination in relation to the

aspects of seed vigor (Figures 1–3); and for these two bootstrap resamplings (6 h and 10 h) the analysis of regulatory canonical correlation (RCC) was applied. Thus, a mean correlation >0.7 was verified for the two process (Supplementary Figure S1), corroborating that the association of gene expression with seed vigor is equal for the different germination times analyzed. Thus, for subsequent analyses, the expression samples at 6 h and 10 h were used jointly to find associations with seed vigor.

Correlation coefficients indicated the existence of associations between the genes analyzed in the study, and notably certain genes analyzed were related to one or more distinct genes through high and positive correlation ($r > 0.70$). As an example, there is the *N-glycosylase/DNA lyase OGG1* transcript with a DNA repair function, which positively interacts with cell wall relaxation genes (*65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *Cellulose synthase A catalytic subunit 2* and *LRR receptor-like serine/threonine-protein kinase FEI*) (Figure 6).

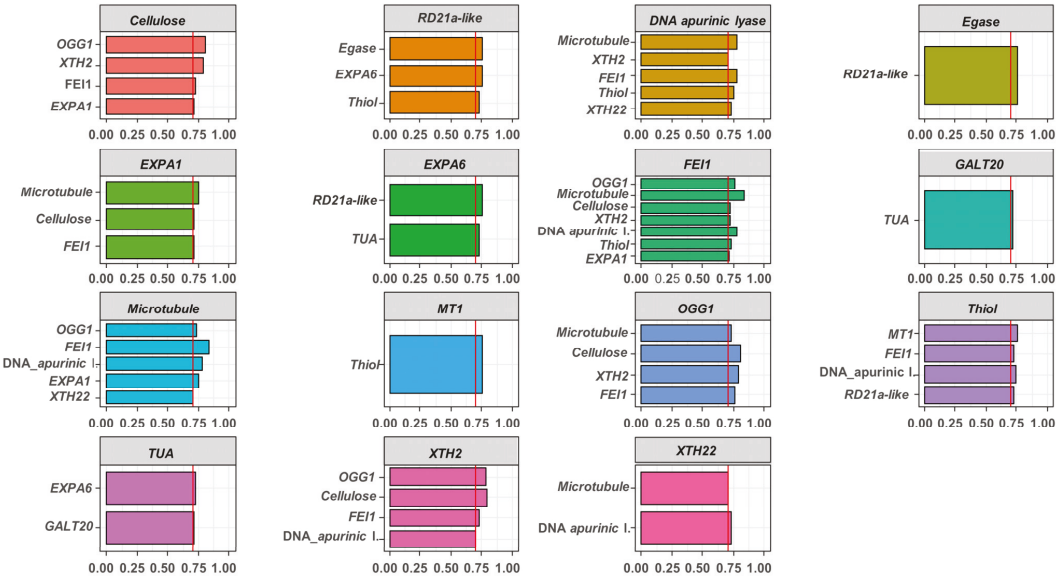

Figure 6. Correlation between genes analyzed during germination *sensu stricto* in seed embryos of two cultivars and their respective lots. Spearman method was used to obtain correlation coefficient (r) ($p \leq 0.05$). Only values of $r > 0.70$ were presented. *N-glycosylase/DNA lyase OGG1* (*OGG1*), *DNA apurinic lyase*, *Metallothionein-like protein 1* (*MT1*), *Thiol protease aleurain-like* (*Thiol*), *Cysteine proteinase RD21a-like* (*RD21a-like*), *Tubulin alpha-4 chain-like* (*TUA*), *Expansin-like A1* (*EXPA1*), *Expansin-A10-like* (*EXPA10*), *Expansin-like A6* (*EXPA6*), *Xyloglucan endotransglucosylase/hydrolase 22* (*XTH22*), *Xyloglucan endotransglucosylase/hydrolase 2* (*XTH2*), *Endoglucanase 8-like* (*Egnase*), *LRR receptor-like serine/threonine-protein kinase FEI* (*FEI1*), *β-1,3-galactosyltransferase 20* (*GALT20*), *65-kDa microtubule-associated protein* (*microtubule*) and *Cellulose synthase A catalytic subunit 2* (*cellulose*).

Faced with the undeniable correlation between the genes analyzed (Figure 6), we sought to group them according to an expression pattern. For this, a clustering of these was performed, applying a homogeneity criterion in the decomposition of the singular values of the first principal component (data not shown). Thus, the relationships between genes were maximized [20] enabling us to separate them into five different groups (Figure 7).

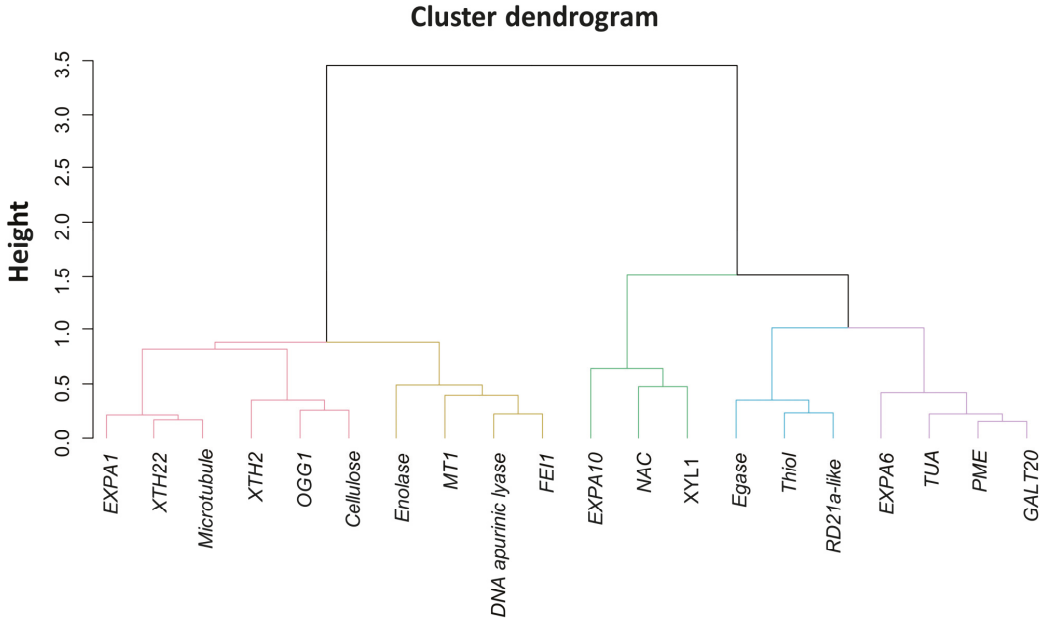

Figure 7. Gene clustering analyzed in soybean seed embryos during germination *sensu stricto* of two cultivars and their respective lots. *N-glycosylase/DNA lyase OGG1* (*OGG1*), *DNA apurinic lyase*, *NAC domain-containing protein 8-like* (*NAC*), *Metallothionein-like protein 1* (*MT1*), *Enolase*, *Thiol protease aleurain-like* (*Thiol*), *Cysteine proteinase RD21a-like* (*RD21a-like*), *Alpha-xylosidase 1-like* (*XYL1*), *Tubulin alpha-4 chain-like* (*TUA*), *Expansin-like A1* (*EXPA1*), *Expansin-A10-like* (*EXPA10*), *Expansin-like A6* (*EXPA6*), *Xyloglucan endotransglucosylase/hydrolase 22* (*XTH22*), *Xyloglucan endotransglucosylase/hydrolase 2* (*XTH2*), *Endoglucanase 8-like* (*Egnase*), *Pectinesterase 3* (*PME*), *LRR receptor-like serine/threonine-protein kinase FEI* (*FEI1*), *Beta-1,3-galactosyltransferase 20* (*GALT20*), *65-kDa microtubule-associated protein* (*microtubule*), *Cellulose synthase A catalytic subunit 2* (*cellulose*).

The bootstrap re-sampling process was performed for each clustered group (Figure 7) with the aspects of vigor expression (Figures 1–4), and the canonical correlation were estimated in each process generated by this re-sampling and expressed in Boxplots (Figure 8). Thus, it was possible to verify that the genes *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *N-glycosylase/DNA lyase OGG1* and *Cellulose synthase A catalytic subunit 2* (group 1) have a greater correlation with the expression of seed vigor (Figure 8). This demonstrated that of the twenty genes studied, only six were strongly associated with the vigor properties of soybean seeds (Figures 7 and 8).

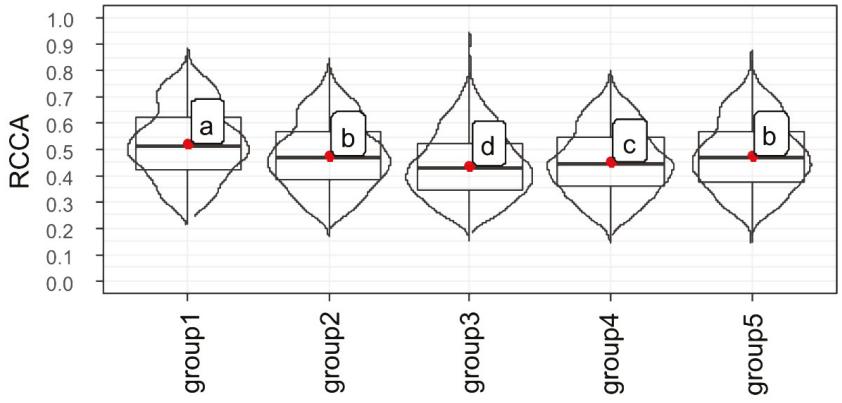

Figure 8. First eigenvalue of the Regulatory canonical correlation (RCCA) from the computational process Bootstrap 10,000 generated from each gene group (1, 2, 3, 4 and 5) with aspects of soybean seed vigor expression. Distinct letters indicate a significant difference at the 5% probability level ($p < 0.05$). Group 1: *N-glycosylase/DNA lyase OGG1* (*OGG1*), *Expansin-like A1* (*EXPA1*), *Xyloglucan endotransglucosylase/hydrolase 22* (*XTH22*), *Xyloglucan endotransglucosylase/hydrolase 2* (*XTH2*), *65-kDa microtubule-associated protein* (*microtubule*), *Cellulose synthase A catalytic subunit 2* (*cellulose*). Group 2: *DNA apurinic, Metallothionein-like protein 1* (*MT1*), *Enolase*, *LRR receptor-like serine/threonine-protein kinase FEI* (*FEI1*). Group 3: *NAC domain-containing protein 8-like* (*SOG1*), *Expansin-A10-like* (*EXPA10*), *Alpha-xylosidase 1-like* (*XYL1*). Group 4: *Endoglucanase 8-like* (*Egnase*), *Cysteine proteinase RD21a-like* (*RD21a-like*), *Thiol protease aleurain-like* (*Thiol*). Group 5: *Tubulin alpha-4 chain-like* (*TUA*), *Expansin-like A6* (*EXPA6*), *Pectinesterase 3* (*PME*), *Beta-1,3-galactosyltransferase 20* (*GALT20*).

3. Discussion

Here we studied the expression of genes associated with growth during germination *sensu stricto* and traced their association with the expression of the seed vigor evaluated. We wanted to take a step towards understanding the physiological expression of vigor in soybean seeds and, in parallel, propose the use of certain transcripts, among those studied here, as a tool to assess seed vigor at a molecular level.

It was noted that the germination and emergence rate had not changed depending on the lot (Figure 1A,B). However, the vigor tests demonstrated a clear distinction in the vigor level between the lots of each cultivar, regularly marked by the accelerated aging test and the germination speed (t50) (Figures 1E and 2). The difference in the vigor levels of the analyzed lots was confirmed by the divergence regarding their lipid peroxidation (Figure 3B), which can be considered an indication of the "health status" of the seeds, where a higher concentration of peroxides indicates more advanced tissue deterioration. In addition, the seeds of cv1 demonstrated superior vigor compared to the seeds of cv2 (Figures 1E, 2 and 3). Thus, the seeds used in this study have different ways of performing the germination process. This characterizes divergence in their vigor, our target phenomena.

An increase of 25% of the embryonic axis length during germination was observed (Figure 4). This occurred as a result of events involved in germination *sensu stricto*. Among these, is the beginning of cell elongation, responsible for boosting radicle protrusion. This process depends on the expression of genes involved in cell wall modification and relaxation, such as expansins (*EXP*), *xyloglucan endotransglycosylase/hydrolases* (*XTH*), *pectin methyl esterases* (*PME*) and *β-1,4-glucanases* [13], which jointly with genes associated to DNA repair, were targeted in our study. The expression of these transcripts changed throughout the germinative process (Figure 5) due to the signaling necessary for the growth of the embryonic axis. According Smolikova et al. [21], this phase is marked by rearrangements of signaling pathways and a switching of gene expression programs. This explains the intercorrelations between the genes here evaluated, by demonstrating the interactivity

between processes necessary for the occurrence of germination (Figure 6), i.e., different signals are required for its success.

Among the target genes analyzed in our study, *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *N-glycosylase/DNA lyase OGG1* and *Cellulose synthase A catalytic subunit 2* transcripts have a greater association with vigor in soybean seeds (Figures 7 and 8). To explain these results, it is necessary to consider that the germination process has several initial events and checkpoints that need to be completed for germination to occur normally [22]. In this context, a vigorous seed completes these stages more quickly, completing the program sooner than a less vigorous seed [7] as showed by our t50 data (Figure 2B,C). Based on that, the increase in the expression of *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *N-glycosylase/DNA lyase OGG1* and *Cellulose synthase A catalytic subunit 2* transcripts allowed the seeds to complete the necessary steps for germination in a more efficient way, which mainly contributes to an increase in the germination speed and tolerance to artificial aging, important characteristics of seed vigor, which had contrasts in our biological material (Figures 1E and 2). Thus, as the expression of the genes mentioned earlier was enhanced in more vigorous seeds, the association of these genes with vigor was evident (Figure 8).

To explain the reason for the correlation of these genes with seed vigor (Figures 7 and 8), it is necessary to consider the role played by these genes during the germination process. In this sense, among the steps for efficient germination mentioned earlier, DNA repair is one the first process activated and represents an important limitation for seed vigor [23], so that the time spent for metabolic repair directly influences the seed germination rate [24]. These reports, in parallel with our results, highlights the idea that increased expression of the *N-glycosylase/DNA lyase OGG1* transcript in soybean seeds with high vigor allows the embryo to acquire germination competence earlier in this species since metabolism does not deal with the effects of DNA damage. This is supported by the rapid germination, even under adverse conditions, which occurs in vigorous seeds (Figure 2), which exhibited an increase in *N-glycosylase/DNA lyase OGG1* expression during germination. These findings explain the association of this gene expression during germination with the vigor of soybean seeds as shown by our analyses (Figure 8). Here we must emphasize that this association goes beyond rapid germination, including the ability to establish seedlings from aged seeds (Figure 1E), another face of the seed vigor. This may be linked to the role of the *N-glycosylase/DNA lyase OGG1* gene in combating DNA damage caused by reactive oxygen species as demonstrated by Chen et al., [14]. These molecules have devastating effects on seed metabolism since they are associated with lipid peroxidation, protein oxidation and damage to nucleic acids according to Li et al. [25]. These molecules are abundantly present in aged seeds. Here, we emphasize that the lipid peroxidation was high in seeds with a lower vigor level (Figure 3B). In this sense, if a possible role of this gene in stress tolerance exists, it deserves to be further studied.

In relation to the expression of genes that result in growth, here associated with seed vigor (Figures 7 and 8), it is necessary to consider that the activity of proteins involved in cell wall relaxation, such as expansins, XTHs, PMEs, endo-β-mannanases and other hydrolases, results in the sliding and moving away of the cellulose microfibrils. Taken together, the synchronous actions of these processes allow cell elongation, which determines radicle protrusion [18]. In soybean embryos, a radicle-derived growth pattern occurs, although the hypocotyl represents most of the cell elongation [19] and our embryonic length measurement showed that the growth occurred in a similar way (see the growth behavior of the embryonic axis on Figure 4), regardless of the different vigor of our samples (Figures 1E, 2 and 3). However, there is an undeniable association between *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2* and *Cellulose synthase A catalytic subunit 2* expression with the embryonic axis growth, jointly with other vigor parameters here evaluated (Figures 7 and 8).

The explanation for this is that the advanced molecular stage through the increase in the level of transcription of the genes mentioned earlier was decisive for the manifestation of vigor in soybean seeds even if not noticeable in the embryonic axis growth in the evaluated points. Even so, it can be inferred that an increase in the *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2* and *Cellulose synthase A catalytic subunit 2* expression ensures the fast germination under suboptimal conditions (Figure 2) and the ability to form normal seedlings after exposure to accelerated deterioration imposed by accelerated ageing test in high vigor seeds (Figure 1E) as a reflection of their contribution on vigor. [26] verified in *Arabidopsis* seeds that the overexpression of the *AtEXP2* gene leads to higher germination speed. It was demonstrated that submodules related to genes of the expansin group have a greater contribution in the hypocotyl elongation in soybean seeds [27], an important step in the construction of young plants in post germination events. These results permeate our findings and support us accordingly.

Our results demonstrated the assertiveness of our hypothesis of associating genes related to the growth of the embryonic axis with the manifestation of vigor, here evaluated by germination speed, stress tolerance, organ elongation of seedlings, membrane integrity and tissue deterioration. These associations have a significant contribution since seed scientists have sought to associate functional characteristics of germination to the events following this. In previous studies, we demonstrated that using biophysics signals it is possible to predict superior seedling formation [28] and that the process that occurred during germination *sensu stricto* has a positive influence in greater seedling establishment [6]. However, up to now, the contribution of gene expression on vigor has not been explored in soybean seeds, which highlights the contribution of this work to seed science and technology.

Thus, here we increase previous knowledge by demonstrating that *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *N-glycosylase/DNA lyase OGG1* and *Cellulose synthase A catalytic subunit 2* expression during germination contributes significantly to vigor in soybean (our study measured not only seedling performance but also germination speed, artificial aging, and biochemical properties). We noticed that this link occurred through an increase in the level of these transcripts in seeds with high vigor (Figure 5). We would like to mention that other genes related to the germination process can be analyzed to increase the range of targets in future research. Taken together, our results allow us to suggest that the expression of the genes mentioned earlier can be used to study vigor at the molecular level.

4. Conclusions

The gene expression of the *Expansin-like A1*, *Xyloglucan endotransglucosylase/hydrolase 22*, *65-kDa microtubule-associated protein*, *Xyloglucan endotransglucosylase/hydrolase 2*, *N-glycosylase/DNA lyase OGG1* and *Cellulose synthase A catalytic subunit 2* is associated with seed vigor in soybean seeds. Besides the use to study the vigor at the molecular level, our findings open the possibility of using the expression of the genes reported here to analyze seed vigor in soybean commercial seed lots, which should be validated for a greater number of genotypes and seed lots in future research. This use can be a strong tool for the diagnosis of vigor by the seed industry.

5. Materials and Methods

5.1. Seed Samples

Seeds of two commercial cultivars (BRS133-cv1 and MG/BR 46 Conquista-cv2) were used in this study. These cultivars have different tolerance to the production environment and are constantly propagated by our seed laboratory team. The distinct tolerance mentioned earlier allows obtaining different levels of natural vigor throughout the seasons and years. In each production season the seeds were collected manually at the mature stage (R9) according to criteria described in Basso et al. [11] and the immature, green and unformed

seeds were removed from the seed lots. We screened and separated two lots (lot 1 and 2) of each cultivar produced in separate areas in Botucatu, São Paulo State, Brazil, during the 2014/15 crop season (data on environmental conditions of each area are not presented here). The screening was based on a natural difference in their seed vigor according to the results of different vigor tests. The divergence in the seed vigor in these samples would allow us to test our hypothesis. The seeds were stored at 12 °C and 50% relative humidity (RH) until the beginning of the experiments, which was not longer than fifteen days.

5.2. Physiological Assays

Seeds were evaluated for their physiological and biochemical properties as described below.

Water content: determined using two replicates of 20 seeds each by oven method at 105 °C for 24 h. The calculation was done on a wet basis, with the level of moisture expressed as a percentage according to the Rules for Seed Testing [29].

Normal seedlings: four replicates of 25 seeds were germinated on paper rolls moistened with water at 2.5 times the paper dry weight at 25 °C in the dark. The count of normal seedlings (here considered those with aerial part and main root $\geq$5 cm) was carried out after eight days according to the Rules for Seed Testing [29] criteria. The results were expressed in percentage of normal seedlings.

Time to 50% of root protrusion (t50): four replicates of 25 seeds were germinated on a paper roll as described earlier, but with the following variations. One was germinated at 25 °C, one at 15 °C, one at 10 °C, and one at 25 °C using a saline solution of NaCl at a concentration of 100 mmol.L^{-1}. Every six hours, the seeds that presented root protrusion $\geq$ 2 mm were computed as germinated, and the t50 was calculated using the curve fitting module of the Germinator software [30].

Embryonic axis length: three replicates of 10 seeds were germinated as described by the normal seedling-test, and their embryonic axis were collected at six and 10 h. In parallel, the embryonic axis of the dry seeds were collected. The embryonic axis were photographed under a blue background and their length was measured using ImageJ software [31].

Length and dry mass of seedlings: four replicates of 10 seeds were placed longitudinally in the upper third of paper roll, which was moistened 2.5 times the paper dry weight and kept at 25 °C [32]. Eight days after germination, the seedlings were measured (cm), placed at 60 °C for 72 h hours and the dry mass results expressed in milligrams [33].

Seedling emergence: four replicates of 50 seeds were sown in sand at a depth of 3 cm and irrigated whenever necessary. The seedling count was carried out fifteen days after sowing and the results expressed in percentage [32].

Accelerated aging with NaCl saline solution (SSAA): seeds were placed in a single layer on a metallic screen suspended inside plastic boxes (11 cm × 11 cm × 3 cm) over a saturated saline solution of NaCl (75% RH) and kept at 41 °C for 72 h. After this period, the seeds were germinated as described by the normal seedling-test, and the percentage of normal seedlings was counted on the fifth day after the installation of the test [34].

Electrical conductivity: this test was performed with four replicates of 50 seeds. Seeds were placed in plastic containers with 75 mL of distilled water and kept at 25 °C for 24 h. After this period, the electrical conductivity of the solutions was performed using a conductivity meter (Digimed D31) and the results expressed in $\mu S.cm^{-1}$. g^{-1} [35].

Malondialdehyde (MDA): this estimate was determined from four 100 mg seed samples macerated and homogenized in 1 mL of 50 mM phosphate buffer (pH 7.0) containing 0.67% TCA, followed by centrifugation at 15,000 *g* for 15 min. To 1.0 mL of the supernatant, 2.0 mL of 0.5% thiobarbituric acid (TBA) was added in 20% TCA. The mixture was heated at 95 °C for 30 min in a water bath and then cooled in ice. Afterwards, centrifugation was carried out at 15,000 *g* for 20 min, followed by the absorbance of the supernatant at 532 nm. The value for the nonspecific absorption of each sample at 600 nm was also recorded and subtracted from the absorbance recorded at 532 nm.

5.3. Gene Expression during Germination Sensu Stricto

Three biological replicates of 50 embryonic axis were isolated from dry seeds and from seeds germinated at six and 10 h, for each cultivar and lot. For total RNA extraction, the kit-NucleoSpin RNA Plant® (Macherey-Nagel, Düren, Germany was used. For cDNA synthesis, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Victoria, Australia) was used. The recommendations of each manufacturer were followed to perform these steps.

The target genes were identified by literature searches for genes associated with germination, such as DNA repair, oxidative stress defense, cell respiration, reserve mobilization, cell stretching and expansion. The list of genes, their functions and the sequence of primers used are listed in Supplementary Table S1.

The genes were amplified in real time-qRT-PCR-using the KiCqStart® SYBR® Green qPCR ReadyMix kit (Sigma-Aldrich, St. Louis, MO, USA), in an Eco Real-Time optical thermal cycler (Illumina, San Diego, CA, USA), and the data was analyzed using Illumina's EcoStudy v5.0 software. Relative quantification (RQ) was determined by the $2^{\wedge-\Delta\Delta Ct}$ method [36], using two reference genes, Importin beta-2 subunit family protein (Glyma.20G106300) and 20S proteasome subunit beta (Glyma.06G078500) [37].

5.4. Statistical Design

The physiological properties were subjected to analysis of variance, and when significant, the means were compared by the Tukey test at 0.05 confidence level. The relative gene expression data were obtained using the REST® program, which performs the comparative quantification by the method of "Pair-Wise Fixed Reallocation Randomization Test" [38]. The dry seeds from lot 1 of each cultivar were adopted as a control group to perform this calculation.

The subsequently analyses were performed using the R programming language v4.1.0 through the RStudio integrated development environment platform v1.4.1717. For the analysis of correlations between gene expression and physiological assays, two techniques were used jointly. The non-parametric bootstrap resampling method, which computationally generated 10,000 new combinations of data from random iterative selections from the original dataset. Then, for each resampling, the correlation between the 2 groups of variables (gene expression and physiological properties) was measured. This measurement was found by means of the canonical regulatory correlation (RCCA) analysis of the CCA::rcc (...) library and function. As described by Leurgans et al. [39] and Vinod [40], this is a process similar to ridge regression, as the RCCA uses an estimated constant to penalize the covariance matrices that are used for the calculation, avoiding problems of high linear dependence. Therefore, 10,000 measurements of the first (most expressive) eigenvalue of the RCCA were obtained and characterized by means of a Boxplot graph. For the analysis of clusters of variables, the ClustOfVar library was used with the hclustvar (...) function. This is due to the objective of finding similarity between variables, different from the usual method of clustering between observations that uses distances (e.g., Euclidean). The similarity measurement used in the work is based on the first principal components of the decomposition of singular values of the PCAmix method (Main Components Method for mixing quantitative and qualitative variables), and thus through a homogeneity criterion measured by the square of the measure of Pearson's correlation, it was possible to characterize the greatest relationship between groups of variables [20].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants11101310/s1. Table S1: Genes (mRNAs) studied in the embryonic axis of soybean seeds during germination *sensu stricto* and primers used for real-time PCR reactions. Figure S1: First eigenvalue of the regulatory canonical correlation (RCCA) from the Bootstrap 10,000 of gene expression at 6 h and 10 h during germination *sensu stricto* and vigor properties.

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T.B.B. and E.A.A.d.S.; writing—original draft preparation, K.R.D., C.C.G.; writing—review and editing, K.R.D., T.B.B., G.W.B.; E.A.A.d.S.; supervision, G.W.B. and E.A.A.d.S. Funding acquisition, E.A.A.d.S. All authors have read and agreed to the published version of the manuscript.

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Acceleration in Germination *Sensu stricto* Plays a Central Role on Seedling Vigor in Post-Germination

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Abstract: An obvious relationship between germination *sensu stricto* and seedling development during post-germination has been considered, but not explained concerning vigor. Taking this into account, we used measurements of water dynamics in germinating seeds and seedling development to clarify that relationship. The biological model was soybean seeds, since it is the most relevant 'true seed' produced around world. Our findings suggest that the way energy is used (acceleration) and not its input (velocity) is the main aspect relating seed germination and seedling development, especially when considering vigor. However, velocity and acceleration can be complementary in analyses of seed physiology. Other measurements proposed here also have potential uses for testing vigor in seed lots, such as seedling vigor index and biological activity in the lot. Therefore, water dynamics in germinating seeds can be an interesting way for testing seed lots, because it is an easier, faster and cheaper method in relation to other non-destructive procedures.

Keywords: acceleration; imbibition; biological activity; indexes; germination measurements; seed physiology; soybean; velocity

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1. Introduction

Vigor is a functional trait acquired in the last stages of seed maturation and is responsible for seed resilience. In this sense, it is expected that vigorous seeds should possess more capacity to produce early seedlings [1]. This in turn reduces the possibility of their suffering with inter- and intra-specific competition during early plant development. Because of that, this idea has been explored by basic and technical science to predict the behavior of seed germination and plant establishment in field conditions.

Even though the impact on cultivation caused by seed vigor differs among species, depending on specific production practices [2,3], considerable reductions in yield might be expected when non-viguorous seeds are sown. As an example, over the past few decades, there have been reports indicating reductions of up to 50% in yield components of soybean due to the use of non-vigorous seeds [4,5]. This justifies continuous research to provide practical, rapid and simple ways of predicting seed-lot vigor. By the way, not surprisingly, most information on seed-lot vigor is based on early plant development, especially on seedling development, which is the basis of national and international seed trade [6,7]. However, details about interactions between germination *sensu stricto* [1 sense] and seedling vigor (immediate post-germination) still have to be clarified. Thus, we asked: what is the relationship between germination *sensu stricto* and seedling development of commercial lots? The answer can not only be used to promote a new and rapid test of seed vigor, but can also be a bridge between seed physiology and seed technology. Recent studies consider this bridge to be water dynamics in germinating seeds [2,8,9].

Water dynamics in germinating seeds has been used as a trivial laboratory test, which is incorrectly called imbibition test [9]. However, studies focusing on this relation dating back to the early 1980s demonstrate that the entrance and exit of water in a live cell is an important physiological trait of germination *sensu stricto* because of the integration with biophysical and/or biochemical processes [10–14]. In this occasion, although some authors explored the kinetic idea for water influx in germinating seeds, vigor in germination *sensu stricto* was poorly explored [12]. In previous years, a physiological sense was offered to define seed vigor, by considering it a characteristic related to the embryo's ability to perform the germination process in a coordinated and sequential way [2]. In this sense, new tests of water dynamics in germinating seeds were based on infrared and/or magnetic resonance [15–19]. These tests are useful, but still expensive and therefore not always possible to be used routinely in seed testing. To make use of the phenomena in an easy and practical way, water dynamics measurements were proposed [9,20]. More specifically, velocity and acceleration were proposed as a tool to investigate germination *sensu stricto* in the seed-seedling transition [21]. Velocity is a functional trait for metabolism since it describes the variation of mass over germination time, whereas acceleration measures the variation of velocity. However, no study has been done to understand the relationship between measurements of water dynamics in germinating seeds and seedling development in immediate post-germination. Thus, our hypothesis is that velocity and/or acceleration of water dynamics in germinating seeds can be a biomarker of vigor, improving the knowledge about how this influences the seedling development of a commercial lot. We also expand on how measurements of water dynamics can be used in a physiological sense.

2. Material and Methods

2.1. Biological Model

Soybean was used as a biological model in this paper, since (i) the species is the world's largest source of animal protein feed and the second largest source of vegetable oil [22], being the number one 'true seed' in cultivated areas of the world (only maize, wheat and rice, which have caryopses, are more cultivated); (ii) it is the agricultural product with the highest commercial growth rate in world [23]; and (iii) it has classical reports related to water dynamics in germinating seeds [13,14]. Soybean is also one of the most important genetically engineered (GE) crops, the main type of cultivation in traditional grain producers as the United States, Argentina and Brazil [24]. Taking this into account, seeds of NA5909RR cultivar transgenic soybean (*Glycine max* L.) were used, with an initial moisture content of 11%. The cultivar is one of the most cultivated in Brazil because of its high adaptability in diverse edafic-climatic conditions.

A seed private company provided the seed lots (produced according to cultivation and harvesting procedures defined by the protocol of the donor) without any chemical treatment. These seeds were produced in the 2013–2014 Brazilian crop season. At first, we made a screening of 15 lots. The physiological quality of the lots was evaluated in pre-testing, quantifying germinability, viability and germination initial time. From this, we defined three samples (with low, intermediate and high quality). The designations low, intermediate and high follow the ones proposed by Ribeiro–OliveiraandRanal [9,25], who defined them based on viability and germination measurements. Samples composed by seeds with viability (V) and germinability (G) higher than 90% were considered high-quality, whereas $V \leq 60\%$ and $G \leq 50\%$ were considered low-quality. The seeds with intermediate quality had intermediate values for both characteristics. This also allowed us to comply with the International Seed Testing Association (ISTA) methods for seed-testing validation [26]. The ISTA, based on ISO5725-2 [27], determines that any method of germination analysis can only be validated when using samples with different physiological standards. We highlight that these inferior boundaries are not considered a seed sample in a seed technology context, but they are important for plant breeders and seed physiologists who study samples of lineages and/or other plant species with germinability and viability below the soybean-seed trade standards.

2.2. Water Dynamics in Germinating Seeds

Seeds were sowed in germination boxes (plastic boxes) over paper soaked with distilled water (volume in mL equivalent to six times the mass of the paper in g) and then placed on a laboratory bench at 25.3 ± 1.5 °C under white fluorescent light (11.29 ± 2.84 μmol m^{-2} s^{-1} Photosynthetic Photon Flux Density—PPFD). The initial water volume (lost either by experimental manipulation or possible evaporation) was maintained by adding 1 mL (defined in pre-testing) of distilled water in the germination box after each mass recording. These seeds (n = 50; more than sufficient sample size for this test according to 9) were individually weighed on a digital scale (at 0.0001 g precision) every hour until 2 h after embryo protrusion (root or cotyledon emergence). The germination boxes were opened just for the mass recording of the seed individual, assuring the germination boxes would have characteristics similar to a humidity chamber (high vapor pressure). The water dynamics in germinating seeds was measured by means of this recording of mass over time. As this is associated with germination *sensu stricto*, measurements of water dynamics in germinating seeds are functional traits related to the embryo.

Modelling the Water Dynamics in Germinating Seeds

For modelling the water dynamics in germinating soybean seeds, we used the algorithm proposed by Ribeiro-Oliveira et al. [21]. Therefore, the weighted mass of the water dynamics in germinating seeds was obtained from mass data collected over time and weighted by initial mass (hydroscopic equilibrium). Raw data of mass were used to calculate the coefficient of initial diffusion, which is a function of the water diffusion ratio by the seed radius (D/ρ^2; n = 50). This ratio was used focusing on a practical sense, since we used seeds with similar dimensions (Φ = 6.9 ± 0.3; mean ± standard error) for each sample. We parameterized D/ρ^2 since errors from approximations from seed shapes are partially compensated [28]. We used the diffusion coefficient per unit area as an initial diffusion coefficient because it makes the parameter comparable for species or samples with different seed sizes. We also used the bootstrap method with 1000 re-samplings, since values generated above this number are similar according to the convergence test, as an assumption to calculate weighted mass and, then, means of velocity and acceleration. To calculate these confidence intervals, the Algorithm AS 214 for Fortran was used [29], which is recommended to perform the Monte Carlo Confidence Intervals. The central point of these measurements and the base of the quantitative treatment is m, the water mass over time. The tabulated values of the normalized mass $m(t)$ were obtained from total mass $M(t)$ divided by initial (dry) seed mass (M_0).

The first step in the numerical calculation is the interpolation of $m(t)$ by cubic splines [30], and as the main characteristic of this method is the interpolated function, its first and second derivatives are continuous. The result is a smooth function that can be used to study metabolic changes as a function of time. For details see Ribeiro-Oliveira et al. [21].

Velocity is expressed as

$$v = \frac{dm}{dt} \tag{1}$$

and acceleration in our analyses will be

$$a = \frac{d^2m}{dt^2} \tag{2}$$

Within the interpolation scheme, it is simple to integrate the data from Equations (1) and (2), and calculate the average values v_m and a_m for velocity and acceleration, respectively. It is worthwhile mentioning that velocity is associated to the flux of water from the outside to the inside of the seed. The time average a_m of a parameter $a(t)$ is defined by

$$a_m = \frac{1}{\tau}\int_0^\tau a(t)dt \tag{3}$$

where τ is the last time tabulated for each sample. The definition (3) is also used to calculate v_m.

The diffusion ratio coefficient defined by D/ρ^2 and the saturation mass M_∞ were fitted for a series solution of the Fick differential equation for a spherical model of the seed through the expression (4)

$$\frac{M(t)}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}exp\left(-Dn^2\pi^2t/\rho^2\right) \quad (4)$$

where $M(t)$ is the total mass of the diaspore at a time t, D is the diffusion coefficient, ρ is the radius of the seed, t is the time, n is the number of terms in the series. The best fit in a sequence with different values defines the choice of n. The values of D/ρ^2 and M_∞ were obtained by a nonlinear fitting, using the Levenberg–Marquardt method [31]. These parameters were fitted using only data from the first hours of the experiment, limiting them to the purely imbibition phase, just before the presence of water trigger biochemical processes in the seed. $M(t)$ in the Equation (4) is the total mass, to account for the boundary conditions imposed by the Equation correctly (4).

2.3. Seed Germination Assays

We maintained the seeds used in the water dynamics assessment for 48 h in the same experimental conditions to analyze them in relation to germinability (*G*; percentage of germination) and viability (*V*). Viability was calculated using the proportion of viable seeds (evaluated by means of 2,3,5-triphenyl-2H-tetrazolium chloride solution—TTC) in relation to the total seeds analyzed [32].

From the data of germinability and viability, we calculated Biological Activity (*A*) in the sample. *A* is a measurement classically used as an analogous to the equation relating the thermodynamic activity of a solute to its concentration via an activity coefficient [33]. The activity is related to the capacity of a biological system to manage chemical processes (endogen and exogen), which is similar to the idea of seed germination in a sample. For that, the expression considers the amount-of-substance concentration or, in this case, proportion of germinated seeds (*c*) and a parameter designated as inherent activity, i.e., here, the proportion of viable seeds in the sample (*f*). We are transposing this concept to seed science by using an adaptation in the algebraic expression (5):

$$A = G\ V\ 100 \quad (5)$$

2.4. Seedling Development Assays

The seedling development assays were performed in germination chamber from a completely randomized design (CRD). The chamber was configured under continuous white fluorescent light (15.79 ± 3.70 μmol m^{-2} s^{-1} PPFD) at 25.0 ± 2.7 °C. We performed the sowing in layers of germination paper moistened with distilled water in the proportion of 2.5 times the dry mass of the paper in milliliters [32]. We used a sample (*n*) with 200 seeds (*n* = 200). The seedlings were observed and classified on the eighth day after sowing [32]. They were classified as normal seedlings, damaged and deteriorated abnormal seedlings, and dead or dormant seeds [32].

The seedling development protocol was also the basis for the experimental design of the accelerated ageing test (*AA*), one of the most used seed vigor tests in the world for several species [34], which is recommended for soybean seed testing [35]. We used this test to determine seedling vigor in place of other classical measurements, such as relative growth rate, because our interest is to connect seed physiology to seed technology. Thus, for the AA test, the seeds were exposed to non-optimum relative humidity and temperature {41 °C ± 0.3 °C and ~95% humidity for 72 h ± 15 min, according to [35]} before sowing in germination paper, to generate a stress condition for the embryo. On the fifth day after sowing, the number of normal seedlings was determined, and their percentage in relation to the number of seeds sowed was expressed as the test result.

In addition to the *AA* test, seedling vigor was examined by means of two other characteristics, seedling vigor index (*SVI*) and seed-seedling transition yield index (*SSYI*). Both indexes are based on the ability of a seed to produce a normal seedling. The *SVI* is based on the vigor index proposed by [36] and modified by [37]. The authors also used the *AA* and seedling emergence results to define a measurement for seedling vigor of soybean seeds. However, as our purpose was to analyze how germination *sensu stricto* is correlated to seedling vigor, we performed an adaptation, since it is only possible to correlate characteristics obtained in similar experimental conditions [38]. Taking this into account, we adapted the vigor index to seedling vigor index by using the algebraic expression (6):

$$SVI = \frac{AA}{NS}100 \quad (6)$$

where: *SVI* is the seedling vigor index, expressed in percentage, *NS* is the proportion of normal seedlings developed from non-stressed seeds, and *AA* is the proportion of normal seedlings developed from stressed seeds exposed to the accelerated ageing test. By taking only normal seedlings from each condition into account [39], the measurement can be more interesting to predict the seedling vigor in a *sensu stricto* view. Apart from that, we used a simple index based on the yield idea to observe how many seeds in the sample were able to develop a normal seedling. For that, we used the following algebraic expression (7):

$$SSYI = \frac{NS}{G}100 \quad (7)$$

where *SSYI* is the seed-seedling transition yield index, expressed in percentage, *NS* is the percentage of normal seedlings developed from non-stressed seeds and in an optimum experimental condition, and *G* is the germinability of the sample in an optimum experimental condition.

2.5. Statistical Analysis

For water dynamics in germinating seeds, confidence intervals at 0.05 significance were calculated for the mean values by means of Algorithm AS 214 for Fortran [30], which is recommended to perform the Monte Carlo Confidence Intervals. Overlapping confidence intervals indicate non-significant differences [32]. For seed germination and seedling measurements, we adopted the Binomial distribution with logit function (from glm2 package in R project; https://cran.r-project.org/, accessed on 7 September 2021); every zero value was observed. Means comparisons were performed using the Tukey test and by using confidence intervals calculated by the Šidák correction to reduce the familywise error rate–FWER [40]. In these cases, we also used at 0.05 significance ($\alpha = 0.05$). In addition, we calculated the Pearson linear correlation between the water dynamics in germinating seeds, seed germination (embryo protrusion), seed viability and post-germination (seedling development) measurements by using the residuals to standardize the effect of samples, as was recommended by [38]. The *r* values were tested by the Student *t* test at 0.01 significance ($\alpha = 0.01$), and only characteristics with differences were used to build a heatmap from script defined by [41]. The adjectives to describe the magnitude of the correlations were proposed by [42], where the values from $r = 0.01$ to 0.09 are negligible correlations, $r = 0.10$ to 0.29 are low, $r = 0.30$ to 0.49 are moderate, $r = 0.50$ to 0.69 are substantial, $r = 0.70$ to 0.99 are very high, and $r = 1.0$ is the perfect correlation.

3. Results

The water dynamics in germinating seeds of the three samples based on weighted mass over time (germination *sensu stricto*) were shown to be similar (Figure 1). The seed samples had three behaviors in relation to germinability and viability (Table 1), ratifying the pre-testing. These behaviors were also observed regarding the capacity of seed-seedling transition (Table 1), being the sample previously categorized as high physiological quality with a high ability to produce normal seedlings (*NS* = 98.00%). All seeds germinated in this

sample developed a normal seedling, reaching 100% in the seed-seedling transition yield index (*SSYI*). In addition, the high-quality seed sample, with 99.94% of biological activity (*A*; Table 1), developed lower abnormal seedlings and dead seeds in the sample, both at about 1% (Table 1). These values were coherent with the high resilience of seeds of the sample, developing 94% normal seedlings after accelerated ageing (Table 1). Besides seed resilience, the sample also demonstrated a high capacity to develop vigorous seedlings (*SVI* = 95.92%).

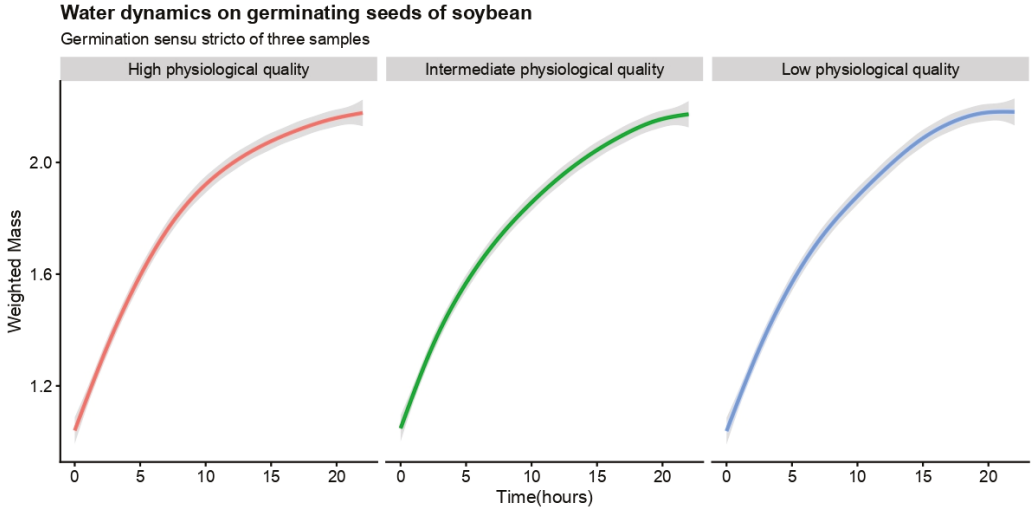

Figure 1. Water dynamics in germinating seeds of soybean based on weighted mass over time. Physiological quality describes viability (*V*) and germinability (*G*) in the sample from pre-testing: High physiological quality is related to a seed sample with viability (*V*) and germinability (*G*) higher than 90%, intermediate physiological quality is a seed sample with $61\% \leq V \leq 89\%9$ and $51\% \leq G \leq 89\%$, and low physiological quality is a seed sample with $V \leq 60\%$ and $G \leq 50\%$. The solid line (red, green and blue) shows the mean value, and the gray area delimits the lower and upper confidence intervals from 1000 Monte Carlo simulations at 0.05. The embryo protrusion in at least one sample occurred 2 h before the last recording. $n = 50$ diaspores.

The lower the physiological seed quality in a sample, the lower the biological activity (Table 1). This led to 24% and 48% normal seedlings developed from seeds of low- and intermediate-quality samples, respectively (Table 1). Seeds that failed to germinate were more relevant to physiological designations than seedling growth, since although the number of abnormal seedlings was similar between low (*AS* = 25%) and intermediate (*AS* = 27%) physiological samples, the number of dead seeds in a low-quality sample was approximately twice as large when compared to an intermediate quality sample (Table 1). Although seed resilience to non-optimum relative humidity and temperature is lower in low-physiological seeds (*AA* = 16%) than in intermediate-physiological seeds (*AA* = 34%), the seedlings developed from these seeds had a similar vigor to those originated from seeds of intermediate quality ($77.27\% \leq$ seedling vigor index $\leq 80.00\%$).

Table 1. Seed-seedling transition measurements in soybean (*Glycine max* L.).

Seed-Seedling Transition Step	Seed Sample	D/ρ^2 (h^{-1})	v_m (g H_2O h^{-1})	a_m (g H_2O h^{-2})			
Germination *sensu stricto*	Low physiological quality	0.0014	0.055 (0.0279; 0.0822)	−0.0184 (−0.0391; 0.0298)			
	Intermediate physiological quality	0.0018	0.058 (0.0278; 0.0882)	−0.0182 (−0.0468; 0.0105)			
	High physiological quality	0.0016	0.055 (0.0355; 0.0074)	−0.0164 (−0.0310; −0.0019)			
		G (%)	*V* (%)	*A* (%)			
Embryo protrusion	Low physiological quality	28.00 ± 3.8 c	48.00 ± 4.2 c	26.36 ± 0.36 c			
	Intermediate physiological quality	54.00 ± 4.2 b	62.00 ± 4.1 b	65.66 ± 0.39 b			
	High physiological quality	98.00 ± 1.2 a	98.00 ± 1.2 a	99.94 ± 0.02 a			
		NS (%)	*AS* (%)	*DS* (%)	*AA* (%)	*SVI* (%)	*SSYI* (%)
Post-germination (seedling development)	Low physiological quality	24.00 ± 3.4 c	25.00 ± 0.008 b	51.00 ± 0.008 c	16.00 ± 3.7 c	80.00 ± 3.6 b	71.43 ± 3.81 c
	Intermediate physiological quality	48.00 ± 4.0 b	27.00 ± 3.61 b	23.00 ± 3.66 b	34.00 ± 4.0 b	77.27 ± 3.4 b	81.48 ± 3.29 b
	High physiological quality	98.00 ± 1.0 a	1.00 ± 3.7 a	1.00 ± 4.21 a	94.00 ± 2.0 a	95.92 ± 1.7 a	100.00 ± 0.01 a

Physiological quality describes viability (*V*) and germinability (*G*) in the sample from pre-testing: High physiological quality is related to a seed sample with viability (*V*) and germinability (*G*) higher than 90%, intermediate physiological quality is a seed sample with $61\% \leq V \leq 89\%$ and $51\% \leq G \leq 89\%$, and low physiological quality is a seed sample with $V \leq 60\%$ and $G \leq 50\%$. *A:* biological activity in the sample; D/ρ^2: Initial diffusion coefficient of water dynamics in germinating seeds; v_m: mean velocity of water dynamics in germinating seeds; a_m: mean acceleration of water dynamics in germinating seeds; *NS*: percentage of normal seedlings; *AS*: percentage of abnormal seedlings; *DS*: dead seeds; *AA*: percentage of normal seedlings after accelerated ageing test; *SVI*: Seedling vigor index; *SSYI*: seed-seedling transition yield index. D/ρ^2 is a parameter of Fick's Second Law from a data set to perfect fitting and, hence, without statistical dispersion measurements; only absolute values. The values of germination *sensu stricto* measurements represent the mean (lower confidence interval; upper confidence interval) obtained from 1000 Monte Carlo simulations at 0.05. For each measurement of embryo protrusion and/or post-germination measurements, means and confidence intervals of the Šidák method followed by different letters are significantly different ($p < 0.05$) as compared using a Tukey test.

No sample had seeds with physical dormancy [42] (0% of hard seeds), enabling practical inferences about germination *sensu stricto* from measurements of water dynamics in germinating seeds (Table 1). As detected by the accelerated ageing test, the initial diffusion coefficient also demonstrated three distinct behaviors of seeds from the samples (Table 1). The higher initial diffusion coefficient ($D/\rho^2 = 0.0014\ h^{-1}$) of water in germinating seeds of soybean was observed in those from the intermediate physiological quality sample ($D/\rho^2 = 0.0018\ h^{-1}$). These seeds also had the highest values of mean velocity ($v_m = 0.058\ g\ H_2O\ h^{-1}$) of water dynamics during germination *sensu stricto* (Table 1). On the other hand, the highest values of mean acceleration of water dynamics were obtained for seeds from the high-quality sample ($a_m = -0.0164\ g\ H_2O\ g^{-2}$; Table 1).

The early steps of germination *sensu stricto* affected the velocity positively and linearly as the germination occurred, but they did not impact acceleration (Figure 2). This was proved by the perfect [43] linear correlation to the initial diffusion coefficient and mean velocity of water dynamics in germinating seeds, and moderate linear correlation to former measurements and mean acceleration (Figure 2). From these measurements, only mean acceleration had a positive, linear and substantial correlation to seedling development (post-germination), observed by correlations among seed germinability, viability and normal seedlings developed. In addition, mean acceleration of water dynamics in germinating seeds had a positive and substantial linear correlation to the seedling vigor index, *SSYI*, and *A*; whereas, mean acceleration was substantially negatively correlated to abnormal seedlings developed and the number of dead seeds in a sample (Figure 2). This demonstrates that mean acceleration is associated to both embryo and seedling vigor. By the way, germinability, viability, biological activity, normal seedlings developed; seedling vigor index and *SSYI* had substantial positive linear correlation among themselves; and there was a substantial negative correlation to abnormal seedlings development and dead seeds (Figure 2). What draws our attention, on the one hand, is the low and negative linear correlation between mean velocity and the seedling vigor index as well as between initial diffusion coefficient and dead seeds; on the other hand, it is also interesting to note the low and positive linear correlation between mean velocity and abnormal seedlings developed. The seedling vigor index also had a negligible negative linear correlation to dead seeds, whereas the initial diffusion coefficient had a negligible positive linear correlation to all measurements related to protrusion (germinability, viability and biological activity), normal seedlings (from seeds exposed or not to stress) and *SSYI* (Figure 2). These results not only demonstrate that the initial diffusion coefficient is an interesting measurement to infer germination, but they also mean that velocity and mean acceleration of water dynamics in germinating seeds are complementary to analyses about seed physiology and immediate post-germination.

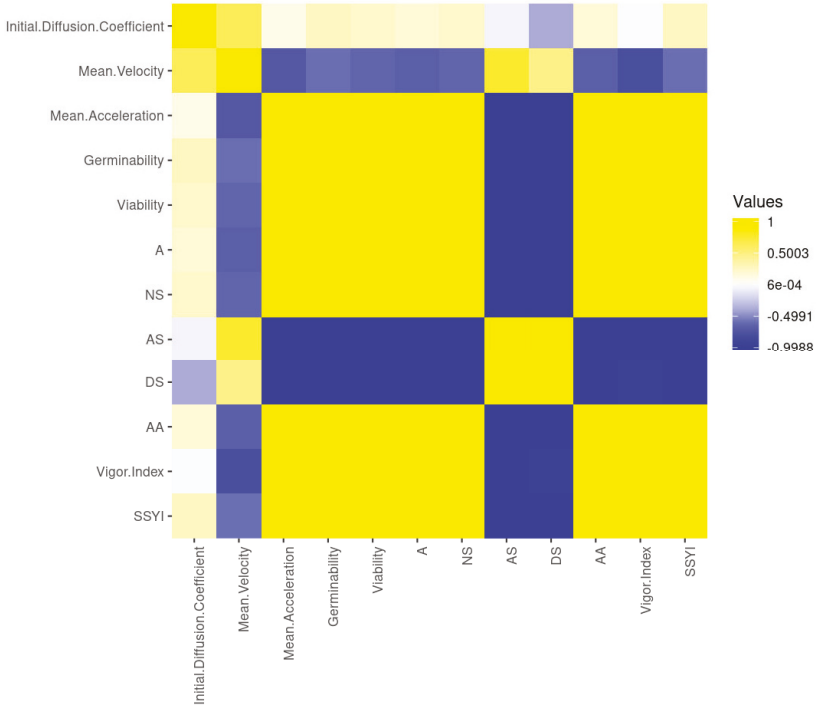

Figure 2. Correlation matrix heatmap based on pair-wise Pearson correlation coefficients (rank correlation; $p \leq 0.01$) for germination *sensu stricto* and post-germination (seedling development) measurements of soybean seeds. Initial diffusion coefficient: D/ρ^2, h^{-1}; mean velocity: v_m, g H_2O h^{-1}; mean acceleration: a_m, g H_2O h^{-2}; *G*: germinability (%); *V*: viability (%); *A*: Biological Activity in the sample (%); *NS*: Normal seedlings (%); *AS*: Abnormal seedlings (%); *DS*: dead seeds (%); *AA*: percentage of normal seedlings after accelerate ageing test; Vigor index: *SVI* = Seedling vigor index (%); *SSYI*: seed-seedling transition yield index (%).

4. Discussion

Our findings demonstrate that although germination *sensu stricto* and seedling vigor related to post-germination do not always have the same contribution to seedling development, there are traits of water dynamics in germinating seeds capable of building a bridge between them. Specifically, the mean acceleration of water dynamics in germinating seeds is a measurement related to both germination *sensu stricto* and post-germination, including the development of vigorous normal seedlings. For the first time, we prove the relevance of acceleration in seed germination for seed-seedling transition. Up to now, only velocity has been considered as an important aspect for this transition. However, with a new model for the germination process, based on biophysics measurements and with robustness to predict metabolism, we amplify the concept around the seed-seedling transition in a context of energy management by means of acceleration.

Other methods have been established as a bridge between germination *sensu stricto* and seedling development, such as biospeckle in coffee seeds [44]. The fact is: as the transition zone hypocotyl-radicle elongates during germination *sensu stricto* [45,46], correlations between a biophysical signal and cell elongation demonstrate that it is possible to make inferences of seedling development from a germinating seed [44]. What we offer here is a safer and cheaper way to do it, by using parameters from water dynamics in germinating seeds from a simple mass measurement over time. These measurements were recently

proposed by our group to demonstrate phases of germination and post-germination, as well as to prove that the 'three-phase germination model' is not a standard, but an exception [21].

Mean acceleration and mean velocity were applied here by means of knowledge from biophysics. According to our assumption, better functioning and metabolism during germination is accompanied by greater velocity and acceleration. This relationship is expected because velocity is directly associated with water flux and, consequently, is a measurement of the variation of both diffusion (especially during imbibition per se, respecting Fick's law) and catabolism and anabolism [21]. In addition, acceleration is a rate of velocity change and, therefore, could reflect metabolism more accurately. Here, our assumption is validated for the seed-seedling transition since the mean acceleration was highly and positively correlated to seedling vigor.

Biological activity is a principle of pharmacology in which the greater the presence of an active principle in a living system, the more biological activity it has [33,47,48]. This activity is mainly dependent on the uptake, distribution and metabolism of energy by the living system. It is important to note that metabolism is defined by velocity and acceleration. Velocity is considered an energy input measurement, whereas acceleration is an energy use measurement [21]. This is naturally related to chemical kinetics, but needs to be considered in more complex models such as that defined by Onsager [49]. In seed science, only mean velocity has been used [50], and it was recently considered a species-specific trait [51]. This is coherent with our findings, since only intermediate-quality seeds demonstrated a different mean velocity. This sample was obtained by applying thermal stress to seeds in anhydrobiosis. Stress conditions have the potential to repress species-specific traits because of selective pressure, leading to a possible segregation in the population [52–54]. In addition, it is expected that extreme differences in physiological quality of seeds of the same sample will promote, on the one hand, faster and non-controlled water dynamics, and, on the other hand, slower and controlled water dynamics in germinating seeds [2,4,55]. This can be considered a limitation for using mean velocity. However, when we overlapped it with acceleration, this limitation was overcome. By inferring energy use, mean acceleration is expected to be more related to embryo vigor than to mean velocity. Because of that, low- and intermediate-physiological quality seeds possess similar mean acceleration, which is lower than in high-quality seeds. The low- and intermediate-physiological quality samples had a similar seedling vigor index since the seedlings from the sample had similar development. However, the intermediate-physiological quality seeds had a greater ability to transit from seed to seedling than low-quality ones because they possessed more biological activity. This was corroborated by the correlation analysis, and it also explains why the intermediate-physiological quality sample had more resilience to the accelerated ageing test than the low-quality sample.

As might be expected [56], low- and intermediate-physiological quality seeds stand out in imbibition per se due to high intraspecific variability (see parameters and confidence intervals in Table 1). That occurred with soybean seeds, which have a slightly thick tegument and a protein reserve, due to peculiarities in membrane rehydration and high affinity of protein to water. As low-quality seeds have high cellular unviability (low viability), there is a failure in membrane selectivity, which controls water influx and inner cell maintenance, especially in the imbibition phase [55,57], making the initial diffusion in low-quality seeds faster than in high-quality seeds. In intermediate-quality seeds, membrane control occurs, but not as efficiently as in high-quality seeds, increasing intraspecific differences observed by CI and velocity of imbibition. Due to that, the initial diffusion coefficient is correlated to metabolic (mean velocity and mean acceleration) and physiological (germinability, viability and biological activity) measurements of embryo protrusion and, therefore, can infer seedling vigor. This also demonstrates that the initial diffusion coefficient must be able to infer physical and physiological quality in a seed sample, especially soybean. After all, the more damaged a tegument is, the lower the membrane control is in the imbibition process [9,10,14,19]. However, this can be studied in the future by using a more adequate experimental design.

The explanation about the supposed incoherence between *SSYI* and the seedling vigor index is algebraic and physiological. In the algebraic sense, *SSYI* is a simple index of yield, based only on the quantity of germinated seeds that grow and get normal seedling status, whereas the seedling vigor index is based on the number of normal seedlings developed from stressed and non-stressed seeds, in this case simulated by the ageing acceleration test. In all cases, *SSYI* and the seedling vigor index are measurements defined from viable seeds, whereas the results of *AA* consider both viable and non-viable seeds in its calculation. Therefore, *SSYI* and seedling vigor index are weighted characteristics, which enable inferences on seedling vigor *sensu stricto*. Contrary to this, *AA* offers an estimative on seed sample vigor based on the resilience behavior of seeds in non-optimum humidity and temperature [6,28,54]. This is more objective by considering the physiological sense from the General Adaptation Syndrome of Hans (János) Selye [52,58]. From that, stressed seeds have three stages before death. First, they recognize the environmental cues as 'priming' (alarm phase). If the stress persists, the seeds trigger molecular defenses based on species-specific adjustments, which can lead to resilience (resistance phase) or a degradation of biological functions (exhaustion phase). A consequence is an increase of abnormal seedlings and/or dead seeds, which is expected as one of the last events in seed deterioration (both natural and artificial) [52]. This occurred here and made seedlings that developed from the low-quality sample have similar vigor to the ones developed from the intermediate-quality sample. This led to a similar ability in germinated seeds to transition to seedling in both samples, but with differences in quality of the samples when considering the number of seeds in each one.

Several authors have been trying to establish a relationship between functional traits of germination *sensu stricto* and post-germination [8,18,59], but few got representative results about seedling vigor [2,44,45]. Nowadays, magnetic resonance has built a bridge between those two phenomena [15–17,19] but this technique is both expensive and specialized, requiring an advanced laboratory. Here, we are presenting not only relations between these steps, but also demonstrating that water dynamics is associated with both embryo vigor and seedling vigor. This can be useful to predict vigor in seed samples in routine and academic laboratories in an easier, cheaper and more practical way in the near future. For example, image analyses can be useful (such as those based on ImageJ®; https://imagej.nih.gov/ij/, accessed on 7 September 2021). Several software applications have been presented to the seed industry that can be updated for analysis of germination *sensu stricto* to predict seedling vigor. Taking this into account, this paper can serve as a guideline. In any case, other studies must be designed, especially for other species with dormant seeds. In the end, the findings suggest that embryo vigor can influence seedling vigor and, therefore, seed sample vigor, because the energy used by the embryo during germination *sensu stricto* is fundamental to early plant development. This directly affects the resilience of a commercial sample to stresses and, consequently, its ability to produce normal seedlings. It is important to note that the kinetics of germination *sensu stricto* is not very representative of vigor, which may come as a surprise to seed technologists. However, again, an addendum is that in seed technology, the germination *sensu stricto* could be considerate secondary; for this science, the kinetics observed are about seedling development, which can justify velocity as an important aspect of vigor. Here, we are presenting a way of speeding the testing of vigor seed for both seed physiologists and seed technologists, by associating both processes of early plant development. In this sense, mean acceleration of the water dynamics in germinating seeds can be an important measurement to demonstrate how germination *sensu stricto* affects the seedling vigor positively.

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Characteristics of the Seed Germination and Seedlings of Six Grape Varieties (*V. vinifera*)

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Abstract: Intraspecific recurrent selection in *V. vinifera* is an effective method for breeding of high quality, disease-, cold-, and drought-resistance grapes. Exploring the optimal treatment methods for grape (*V. vinifera*) seeds can help to accelerate the process of intraspecific recurrent selection and improve breeding efficiency. In this study, seeds of six *V. vinifera* varieties were used as experimental materials, and the germination and seedling formation characteristics were studied by single factor treatment and orthogonal compound treatment, respectively. To do this, stratification, chemical substances, beak cutting, and pre-germination treatments were tested, and the optimal treatment combination was determined for each variety. The results indicated that the optimal conditions obtained in the orthogonal experiments were not completely consistent with those in the single-factor experiments. Single factor experiment results demonstrated that two stratification methods (chilling gauze-storage and chilling sand-storage) and two pre-germination methods (pre-germination in petri dishes and pre-germination in a bean sprouter) vary in effectiveness for different varieties. gibberellin acid (GA_3) soaking and beak-cutting promote the germination and seedling rate of the tested varieties. Orthogonal test results demonstrate that, for Dunkelfelder and Cabernet Sauvignon, the optimal treatment combination was chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in petri dishes. For Meili, the optimal treatment combination was chilling sand-storage + acetic acid (HAc) soaking seed + beak cutting + pre-germination in petri dishes. For Ecolly, the optimal treatment combination was chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter. For Garanior, the optimal treatment combination was chilling sand-storage + HAc soaking seed + no beak cutting + pre-germination in petri dishes. For Marselan, the optimal treatment combination was chilling gauze-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter. This study identified the optimal conditions for seed germination and seedling formation of six grape varieties, which will facilitate future work to characterize the seed germination and seedling formation of seeds obtained by intraspecific hybridization of these varieties. This work also provides a reference for addressing problems of low seed germination rate and suboptimal seedling formation for better utilization of grape germplasms.

Keywords: *V. vinifera*; grape seeds; seed germination; seedling formation

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1. Introduction

Grapes have been cultivated and bred for more than 200 years. The successful breeding of different grape varieties for different purposes requires high-quality grape genetic

resources and typically a variety of breeding methods and techniques have been used. Breeding is done to improve the quality of grapes, including enhanced resistance of plants to biological and abiotic stress or an extended or shortened maturity period to meet market needs [1]. Hybrid breeding and seed selection are important aspects of the breeding process [2]. As the most widely cultivated grape with the highest economic value in the world, *V. vinifera* is widely used as the main material for breeding new grape varieties [3,4]. Intraspecific recurrent selection in *V. vinifera* is an effective method for breeding of high quality, disease-, cold-, and drought-resistance grapes [4]. However, more effective breeding strategies require improved germination and seedling formation rates of intraspecific hybrid seeds in *V. vinifera*, to obtain larger seedling populations in a short time and enable more rapid grape breeding.

The cultivation of seeds into seedlings is one of the most important determinants of breeding efficiency. The germination rate of grape seeds is typically low, 30–50%, with a final seedling rate that is even lower [5–7]. Low germination rates can be due to the selection of parents, an incompletely developed seed embryo of the female parent, or a hard seed coat that is not easy to crack [8]. During seed collection and storage, seeds that are not fully mature or subjected to improper temperature and humidity during storage can result in seed decay or premature germination [9]. If the stratification time is too long or too short, the phenolic compounds in seeds can inhibit the germination rate [10]. Humidity can be difficult to control when trying to germinate seeds. If the humidity is too low, the germination rate will be low due to a lack of water, but if the humidity is too high, seeds are prone to mold and rot [5]. After sowing, the seedling stage may suffer blight or be inhibited by improper cultivation conditions [11]. Previous studies on grape seed germination and seedling formation examined differences of germination rates among different populations and varieties, differences of seedling formation rates among different cultivation and transplanting methods or characterized the physiology of germination and the optimal way to release dormancy [12,13]. Chai et al. compared the germination and seedling rates of cultivars *V. vinifera*, *V. labrusca*, and Franco-American, with *V. vinifera* and Franco-American varieties displaying higher and lower germination rates, respectively [2,5]. Grape seeds experience physiological dormancy, and the dormancy degree and method of dormancy release differ among different varieties [14–16]. Pan et al. studied the effects of gibberellin acid (GA_3) on the seed germination characteristics of *V. adenoclada* Hand. -Mazz, *V. davidi*, wine grape, and table grape, finding that GA_3 significantly improved the germination rate, germination potential, and germination index of grape seeds, and shortened the length of germination [9,17–19]. In addition, effects of 6-benzyl aminopurine (6-BA), Forchior fennron (CPUU), acetic acid (HAc), polyethylene glycol, indole acetic acid, 2,4-dichlorophenoxyacetic acid, lime nitrogen, ammonium nitrate, and other chemicals on seed germination rate were described [6,8,20–22]. Zhang et al. investigated the effects of direct sowing seeds in field or in a greenhouse and evaluated the use of film mulching of seedlings in the field and in a greenhouse. The highest formation rate of hybrid seedlings was obtained by sowing seeds in a greenhouse with the hole disc method and then transplanting the seedlings to the field in mid to late May after the growth of 4–5 true leaves [7].

An understanding of dormancy, germination, and seedling formation in grape seeds remains lacking, and more comprehensive studies are needed. In this study, six grape varieties were used as intraspecific hybrid parents. Single factor and orthogonal test systems were used to analyze the influences of different stratification methods of chilling gauze-storage and chilling sand-storage on germination and seedling rates, the effects of chemical treatments on seed dormancy before germination, the influence of cutting beak on germination and seedling rates, and the efficacy of pre-germination methods using petri dishes or a bean sprouter to improve germination and seedling rates. The aim of this study was to provide technical reference to improve the germination and seedling formation of the intraspecific hybrid progenies of six grape varieties of *V. vinifera* and increase the efficiency of recurrent selection.

2. Results

2.1. Effects of Single Factor Treatment on Seed Germination Characteristics

As can be observed in Figure 1, compared with chilling gauze-storage, chilling sand-storage significantly improved the seed germination rate and potential of Dunkelfelder, but had no significant effect on the seed germination rate, potential, or index of Meili, Ecolly, Garanior, Marselan, and Cabernet Sauvignon.

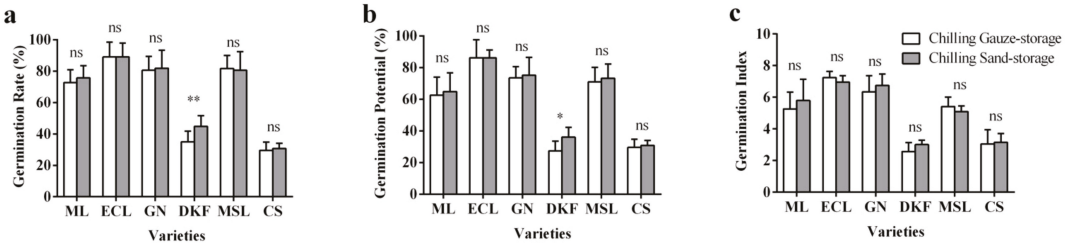

Figure 1. Effect of stratification treatments on seed germination characteristics of six tested varieties: (**a**) influence of stratification treatments on seed germination rate of tested varieties, (**b**) influence of stratification treatments on seed germination potential of tested varieties, and (**c**) influence of stratification treatments on seed germination index of tested varieties. Data in this figure were tested by Student's t test; * $p < 0.05$ and ** $p < 0.01$ represent significant differences between treatments, and ns indicates not significant. ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

It can be observed in Figure 2 that GA_3, 6-BA, CPUU, and HAc improved seed germination rate, potential, and index of the six tested varieties. The effects of GA_3 were the highest, especially for Ecolly, with seed germination rate, potential, and index of 95.72%, 94.29%, and 9.64% respectively.

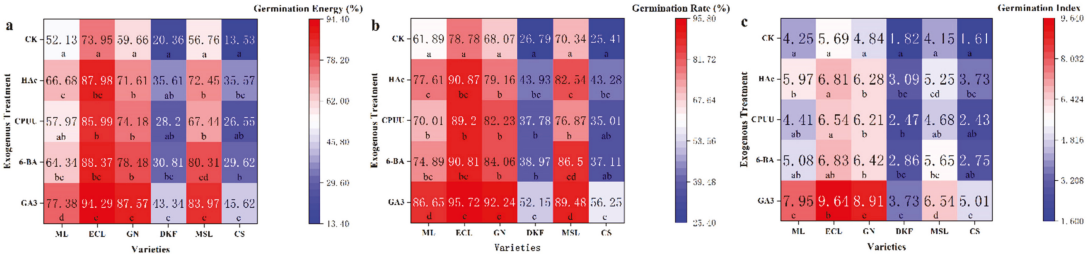

Figure 2. Effect of chemical treatments on seed germination characteristics of six tested varieties: (**a**) influence of chemical treatments on seed germination rate of tested varieties, (**b**) influence of chemical treatments on seed germination potential of tested varieties, and (**c**) influence of chemical treatments on seed germination index of tested varieties. Data in this figure were tested by One-way ANOVA; means followed by the same letter in a column do not differ according to Tuker's test ($p \leq 0.05$). ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

As displayed in Figure 3, beak cutting treatment improved seed germination rate, potential, and index of the six tested varieties, with very significant effects for Meili, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon, and significant effects for Ecolly.

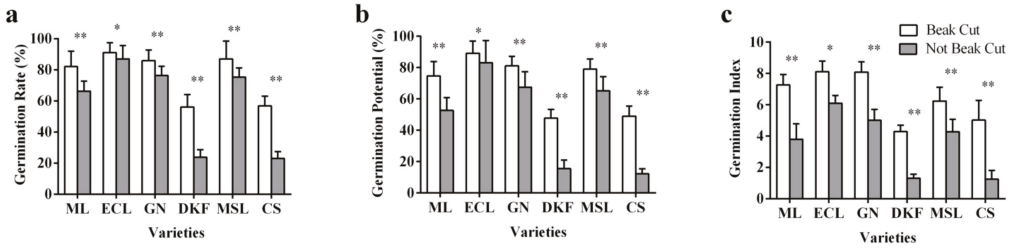

Figure 3. Effect of beak cutting treatment on seed germination characteristics of six tested varieties: (**a**) influence of beak cutting on seed germination rate of tested varieties, (**b**) influence of beak cutting on the germination potential of the tested varieties, and (**c**) influence of beak cutting on the germination index of the tested varieties. Data in this figure were tested by Student's t test; * $p < 0.05$ and ** $p < 0.01$ represent significant differences between treatments, and ns indicates not significant. ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

The effects of pre-germination treatments on seed germination rate, potential and index varied for the six tested different varieties. As illustrated in Figure 4a, the two pre-germination methods had no significant effect on the seed germination rate of Dunkelfelderand and Marselan but resulted in extremely significant differences in the seed germination rates of Meili and Ecolly, and a significant difference in the seed germination rate of Garanior and Cabernet Sauvignon. As illustrated in Figure 4b, there was no difference in the effects of two pre-germination methods on the seed germination potential of Meili, Garanior, Dunkelfelder, and Cabernet Sauvignon, but very significant differences were found for Ecolly and Marselan. As displayed in Figure 4c, there was no difference for the two pre-germination methods on the seed germination index of Garanior, Dunkelfelderand, and Marselan, but a very significant difference for Meili, Ecolly, and Cabernet Sauvignon.

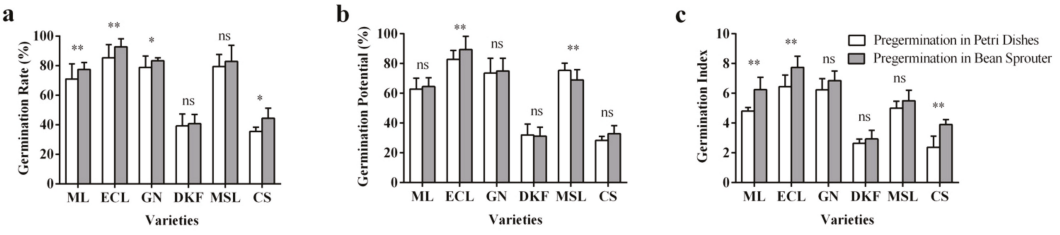

Figure 4. Effect of pre-germination treatments on seed germination characteristics of six tested varieties: (**a**) influence of pre-germination treatments on seed germination rate of tested varieties, (**b**) influence of seed germination treatments on seed germination potential of tested varieties, and (**c**) influence of pre-germination treatments on the germination index of tested varieties. Data in this figure were tested by Student's t test; * $p < 0.05$ and ** $p < 0.01$ represent significant differences between treatments, and ns indicates not significant. ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

2.2. Effects of Single Factor Treatment on Seed Seedling Characteristics of 6 Tested Varieties

As illustrated in Figure 5, there was no significant difference in the effects of the two stratification treatments on the emergence rate and seedling rate of Meili, Ecolly, Garanior, Marselan, and Cabernet Sauvignon, but there were very significant effects for Dunkelfelder.

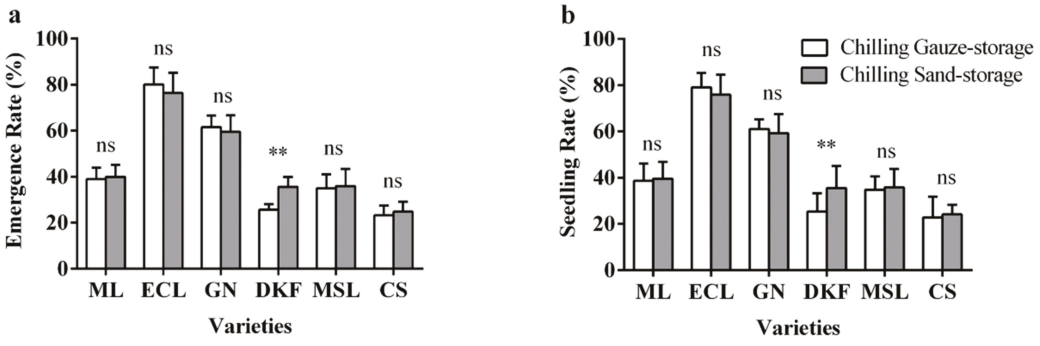

Figure 5. Effect of stratification treatments on seed seedling characteristics of six tested varieties: (**a**) influence of stratification treatments on seed seedling rate of tested varieties, and (**b**) influence of stratification treatments on seed seedling rate of tested varieties. Data in this figure were tested by Student's t test; ** $p < 0.01$ represent significant differences between treatments, and ns indicates not significant. ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

As demonstrated in Figure 6, different chemical treatments improved the seed emergence and seedling rates of Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon seeds. Figure 6a illustrates that there was no difference in the effects of different chemical treatments on the seed emergence rate of Meili, Dunkelfelder, and Cabernet Sauvignon. For all but Meili, the seed emergence rate was highest for the GA_3 treated seeds, and for Ecolly, the seed emergence rate was highest, 93.76%. The same effects were observed for the seedling rate. As displayed in Figure 6b, there was no difference in the effects of different chemical treatments on the seed seedling rate for Meili, Dunkelfelder, and Cabernet Sauvignon. For all varieties but Meili, the seed seedling rate of GA_3 treated seeds was the highest, especially for Ecolly, seed seedling rates reached 93.76%.

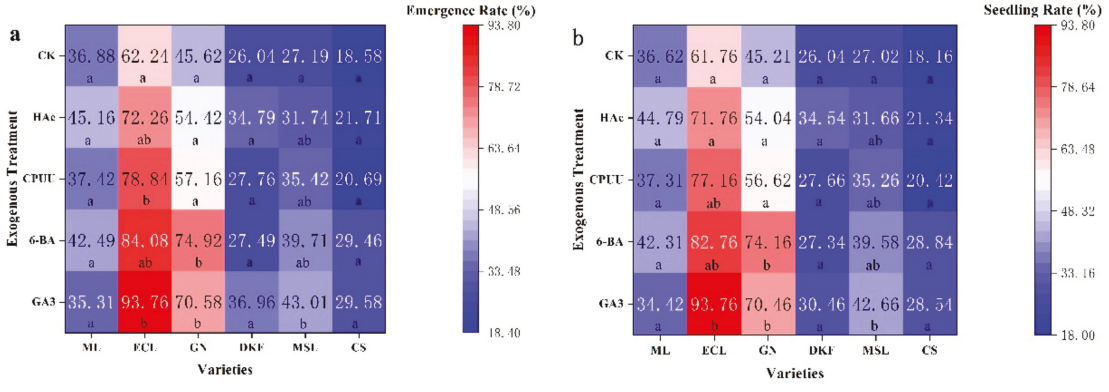

Figure 6. Effect of chemical treatments on seed seedling characteristics of six tested varieties: (**a**) effect of chemical treatments on seedling rate of tested varieties, and (**b**) effect of chemical treatments on seed seedling rate in tested varieties. Data in this figure were tested by One-way ANOVA; means followed by the same letter in column do not differ according to Tuker's test ($p \leq 0.05$). ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

It can be observed in Figure 7 that beak cutting treatment increased the seed emergence rate and seedling rate for the six tested varieties. As presented in Figure 7a, there was no difference in the effect of beak cutting treatment on the seed emergence rate of Meili, Ecolly, and Garanior, but a very significant effect was found for Dunkelfelder and Cabernet Sauvignon, and a significant effect was observed for Marselan. As displayed in Figure 7b, there was no difference in the effect of beak cutting treatment on the seedling rate of Meili and Ecolly, a significant effect for Garanior and Marselan, and a very significant effect for Dunkelfelder and Cabernet Sauvignon.

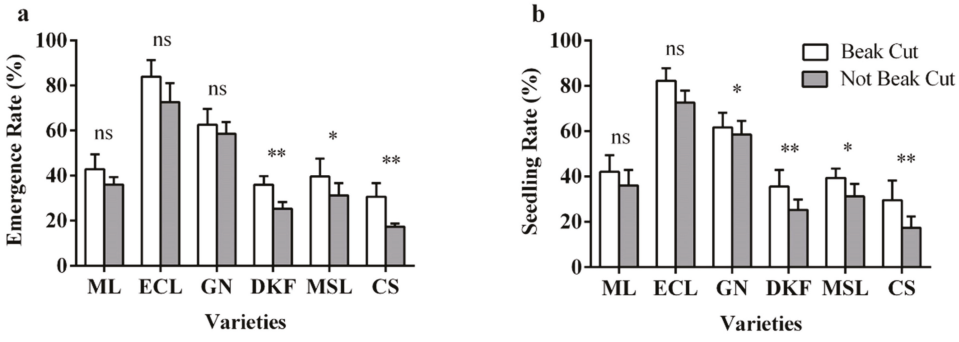

Figure 7. Effect of beak cutting treatment on seedling characteristics of six tested varieties: (**a**) influence of beak cutting treatment on emergence rate of tested varieties, and (**b**) influence of beak cutting treatment on seedling rate of tested varieties. Data in this figure were tested by Student's t test; * $p < 0.05$ and ** $p < 0.01$ represent significant differences between treatments, and ns indicates not significant. ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

It can be observed in Figure 8 that pre-germination treatments had varied effects on seed emergence and seedling rates for the six tested varieties. As displayed in Figure 8a, there was no difference between the two pre-germination methods on the seed emergence rates of Meili, Ecolly, Garanior, and Dunkelfelder, but a significant difference for Marselan and Cabernet Sauvignon. As illustrated in Figure 8b, there was no difference between the two pre-germination methods on the seedling rate for Meili, Ecolly, Garanior, Dunkelfelder, and Cabernet Sauvignon, but a significant difference was found for Marselan.

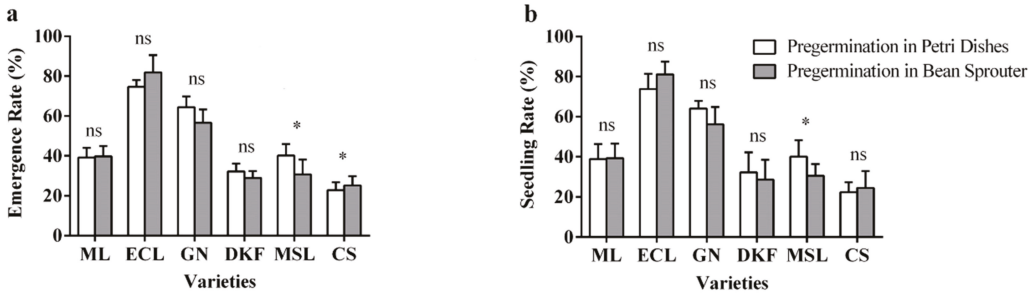

Figure 8. Effect of pre-germination treatments on seed seedling characteristics of tested varieties: (**a**) influence of pre-germination treatments on seed emergence rate of tested varieties, and (**b**) influence of pre-germination treatments on seed seedling rate of tested varieties. Data in this figure were tested by Student's t test; * $p < 0.05$ represent significant differences between treatments, and ns indicates not significant. ML, ECL, GN, DKF, MSL, and CS represent varieties of Meili, Ecolly, Garanior, Dunkelfelder, Marselan, and Cabernet Sauvignon.

2.3. Effects of Composite Treatments on Seed Germination and Seedling Characteristics

Using the seed germination and emergence rates as evaluation indexes, the orthogonal test was performed. The results for Meili seeds are presented in Table 1. Ki represents the average number of germination or emergence rates corresponding to any column with level number i (i = 1, 2, 3, 4, 5), and range R represents the difference between the maximum and minimum values of Ki in any column. The best treatment combination of seed germination and seedling formation of each variety can be obtained by range analysis.

Table 1. Orthogonal experimental design and optimum results of Meili grape seeds.

Experiment Number		Experimental Factor				Experimental Result	
		A	B	C	D	Germination Rate (%)	Emergence Rate (%)
	1	1	3	1	2	84.33	47.52
	2	1	1	1	1	98.67	48.00
	3	1	3	1	1	78.61	38.90
	4	2	3	2	1	68.18	44.43
	5	2	5	1	2	85.41	63.89
	6	2	2	1	1	91.33	49.55
	7	2	1	2	1	88.08	42.36
	8	2	1	2	2	89.75	50.00
	9	1	2	2	2	74.00	38.18
	10	2	3	2	2	72.95	34.00
	11	1	1	1	2	99.00	59.33
	12	1	5	2	1	56.33	34.67
	13	1	4	2	1	80.27	73.03
	14	2	4	1	2	96.00	51.67
Germination rate (%)	k1	81.6	93.87	90.48	80.21		
	k2	84.53	82.67	75.65	85.92		
	k3		76.02				
	k4		88.13				
	k5		70.87				
	Range (R)	2.93	23	14.83	5.71		
	Primary and secondary order	B > C > D > A					
	Superior level	A2	B1	C1	D2		
	Excellent combination	A2B1C1D2					
Emergence rate (%)	k1	48.52	49.92	51.27	47.28		
	k2	47.99	43.86	45.24	49.23		
	k3		41.21				
	k4		62.35				
	k5		49.28				
	Range (R)	0.53	21.14	6.03	1.95		
	Primary and secondary order	B > C > D > A					
	Superior level	A1	B4	C1	D2		
	Excellent combination	A1B4C1D2					
Comprehensive optimum combination		A2B4C1D2					

A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

For Meili seeds, factor A's influence on the germination and emergence rates ranks fourth, but for the germination rate, A2 was taken and for the emergence rate, A1 was taken. For A1, the germination rate was reduced by 3.46% and the emergence rate was increased by 1.10%, so factor A is A2. For factor B, the influence on the germination and emergence rates ranked first, but for the germination rate, B1 was taken and for the emergence rate, B4 was taken. For B1, the germination rate was increased by 6.11% and the emergence rate was reduced by 19.92%, so factor B is B4. Factor C's influence on the germination and emergence rates ranks second, and both take C1, so factor C takes C1. For factor D, the influence on the germination and emergence rates ranked third, and D2 was taken, so factor D was taken as

D2. Therefore, for Meili seeds, this results in an optimal combination of A2B4C1D2, that is, chilling sand-storage + HAc soaking seed + beak cutting + pre-germination in petri dishes.

For Ecolly seeds (Table 2), factor A's influence on the germination and emergence rates ranked third, and A2 was taken, so factor A is A2. For factor B, the influence on the germination and emergence rates ranked first, and both take B1, so factor B takes B1. Factor C's influence on the germination and emergence rates ranked fourth, but for the germination rate, C1 was taken and for the emergence rate, C2 was taken. For C1, the germination rate was increased by 2.90% and the emergence rate was reduced by 2.39%, so factor C is C1. For factor D, the influence on the germination and emergence rates ranked second, but for the germination rate, D2 was taken and for the emergence rate, D1 was taken. For D1, the germination rate was reduced by 6.23% and the emergence rate was increased by 24.86%, so factor D is D1. Therefore, for Ecolly seeds, this results in an optimal combination of A2B1C1D1, that is, chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter.

Table 2. Orthogonal experimental design and optimum results of Ecolly grape seeds.

Experiment Number		Experimental Factor				Experimental Result	
		A	B	C	D	Germination Rate (%)	Emergence Rate (%)
	1	1	3	1	2	91.33	21.00
	2	1	1	1	1	95.33	48.03
	3	1	3	1	1	84.00	43.00
	4	2	3	2	1	87.88	40.50
	5	2	5	1	2	88.51	38.11
	6	2	2	1	1	90.82	35.60
	7	2	1	2	1	92.49	65.93
	8	2	1	2	2	97.67	37.33
	9	1	2	2	2	91.67	34.83
	10	2	3	2	2	91.87	20.33
	11	1	1	1	2	97.00	39.00
	12	1	5	2	1	76.81	30.71
	13	1	4	2	1	86.26	36.48
	14	2	4	1	2	96.33	35.01
Germination rate (%)	k1	88.91	95.62	91.9	87.66		
	k2	92.22	91.25	89.24	93.48		
	k3		88.77				
	k4		91.3				
	k5		82.66				
	Range (R)	3.31	12.96	2.67	5.83		
	Primary and secondary order	B > D > A > C					
	Superior level	A2	B1	C1	D2		
	Excellent combination	A2B1C1D2					
Emergence rate (%)	k1	36.15	47.57	37.11	42.89		
	k2	38.97	35.22	38.02	32.23		
	k3		31.21				
	k4		35.75				
	k5		34.41				
	Range (R)	2.82	16.37	0.91	10.66		
	Primary and secondary order	B > D > A > C					
	Superior level	A2	B1	C2	D1		
	Excellent combination	A2B1C2D1					
Comprehensive optimum combination		A2B1C1D1					

A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

For Garanior seeds (Table 3), factor A's influence on the germination and emergence rates ranks fourth and third respectively, and A2 was taken, so factor A is A2. For factor B, the influence on the germination and emergence rates ranks first, but for germination rate, B1 was taken and for emergence rate, B4 was taken. For B1, the germination rate was increased by 4.80% and the emergence rate was reduced by 6.15%; B4 was taken. Factor C's influence on the germination and emergence rates ranks second, but for the germination rate, C1 was taken and for emergence rate, C2 was taken. For C1, the germination rate was increased by 12.62% and the emergence rate was reduced by 14.48%, so factor C is C2. Factor D's influence on germination and emergence rates ranks third and fourth respectively, but for the germination rate, D2 was taken and for the emergence rate, D1 was taken. For D1, the germination rate was reduced by 6.22% and the emergence rate was increased by 3.19%, so factor D is D2. Therefore, for Garanior seeds, this results in an optimal combination of A2B4C2D2, that is, chilling sand-storage + HAc soaking seed + no beak cutting + pre-germination in petri dishes.

Table 3. Orthogonal experimental design and optimum results of Garanior grape seeds.

	Experiment Number	Experimental Factor				Experimental Result	
		A	B	C	D	Germination Rate (%)	Emergence Rate (%)
	1	1	3	1	2	85.24	66.74
	2	1	1	1	1	96.49	48.20
	3	1	3	1	1	84.71	51.33
	4	2	3	2	1	83.08	59.77
	5	2	5	1	2	87.92	54.27
	6	2	2	1	1	90.77	51.59
	7	2	1	2	1	86.33	72.00
	8	2	1	2	2	91.19	72.00
	9	1	2	2	2	81.00	52.15
	10	2	3	2	2	78.69	51.69
	11	1	1	1	2	97.62	49.33
	12	1	5	2	1	55.46	41.37
	13	1	4	2	1	81.68	81.78
	14	2	4	1	2	95.22	46.90
Germination rate (%)	k1	83.17	92.91	91.14	82.65		
	k2	87.6	85.89	79.63	88.13		
	k3		82.93				
	k4		88.45				
	k5		71.69				
	Range (R)	4.43	21.22	11.51	5.48		
	Primary and secondary order	B > C > D > A					
	Superior level	A2	B1	C1	D2		
	Excellent combination	A2B1C1D2					
Emergence rate (%)	k1	55.84	60.38	52.62	58.01		
	k2	58.32	51.87	61.54	56.15		
	k3		57.38				
	k4		64.34				
	k5		47.82				
	Range (R)	2.47	16.52	8.91	1.85		
	Primary and secondary order	B > C > A > D					
	Superior level	A2	B4	C2	D1		
	Excellent combination	A2B4C2D1					
Comprehensive optimum combination		A2B4C2D2					

A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

For Dunkelfelder seeds (Table 4), factor A's influence on the germination and emergence rates ranks fourth and second respectively, and A2 was taken, so factor A is A2. For factor B, the influence on the germination and emergence rates ranks first, and both take B1, so factor B is B1. For factor C, the influence on the germination and emergence rates ranks second and fourth respectively, and both take C1, so factor C is C1. For factor D, the influence on the germination and emergence rates ranks third, but the germination rate, D2 was taken and for emergence rate, D1 was taken. For D1, the germination rate was reduced by 10.54% and the emergence rate was increased by 10.46%, so factor D is D2. Therefore, for Dunkelfelder seeds, this results in an optimal combination of A2B1C1D2, that is, chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in petri dishes.

Table 4. Orthogonal experimental design and optimum results of Dunkelfelder grape seeds.

Experiment Number		Experimental Factor				Experimental Result	
		A	B	C	D	Germination Rate (%)	Emergence Rate (%)
	1	1	3	1	2	53.59	35.02
	2	1	1	1	1	81.22	55.52
	3	1	3	1	1	54.02	28.00
	4	2	3	2	1	59.68	50.20
	5	2	5	1	2	52.85	78.62
	6	2	2	1	1	73.77	45.33
	7	2	1	2	1	46.85	81.25
	8	2	1	2	2	55.70	44.67
	9	1	2	2	2	40.00	23.33
	10	2	3	2	2	52.00	27.33
	11	1	1	1	2	92.89	50.00
	12	1	5	2	1	24.06	18.00
	13	1	4	2	1	39.73	51.11
	14	2	4	1	2	77.00	36.00
Germination rate (%)	k1	55.07	69.16	69.33	54.19		
	k2	59.69	56.88	45.43	60.58		
	k3		54.82				
	k4		58.37				
	k5		38.45				
	Range (R)	4.62	30.71	23.9	6.39		
	Primary and secondary order	B > C > D > A					
	Superior level	A2	B1	C1	D2		
	Excellent combination	A2B1C1D2					
Emergence rate (%)	k1	37.28	57.86	46.93	47.06		
	k2	51.91	34.33	42.27	42.14		
	k3		35.14				
	k4		43.56				
	k5		48.31				
	Range (R)	14.63	23.52	4.66	4.92		
	Primary and secondary order	B > A > D > C					
	Superior level	A2	B1	C1	D1		
	Excellent combination	A2B1C1D1					
Comprehensive optimum combination		A2B1C1D2					

A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

For Marselan seeds (Table 5), factor A's influence on the germination and emergence rates ranks second and fourth respectively, but for the influence on the germination rate, A1 was taken and for the emergence rate, A2 was taken. For A1, the germination rate was increased by 5.83% and the emergence rate was reduced by 2.50%, so factor A is A1. For factor B, the influence on the germination and emergence rates ranks first, but for the

germination rate, B1 was taken and for the emergence rate, B2 was taken. For B1, the germination rate was increased by 4.97% and the emergence rate was reduced by 0.37%, so factor B is B1. Factor C's influence on the germination and emergence rates ranks third, and both take C1, so factor C is C1. For factor D, its influence on the germination and emergence rates ranks fourth and second respectively, and both take D1, so factor D is D1; Therefore, for Marselan seeds, this results in an optimal combination of A1B1C1D1, that is, chilling gauze-storage + GA_3 soaking seed + beak cutting + pre-germination in bean sprouter.

Table 5. Orthogonal experimental design and optimum results of Marselan grape seeds.

Experiment Number		Experimental Factor				Experimental Result	
		A	B	C	D	Germination Rate (%)	Emergence Rate (%)
	1	1	3	1	2	91.41	48.50
	2	1	1	1	1	98.67	72.00
	3	1	3	1	1	90.61	48.00
	4	2	3	2	1	84.17	56.02
	5	2	5	1	2	66.49	22.00
	6	2	2	1	1	94.00	79.43
	7	2	1	2	1	94.33	49.50
	8	2	1	2	2	98.00	72.00
	9	1	2	2	2	91.47	43.44
	10	2	3	2	2	69.14	33.33
	11	1	1	1	2	99.33	51.33
	12	1	5	2	1	74.39	48.70
	13	1	4	2	1	84.09	45.62
	14	2	4	1	2	87.09	54.48
Germination rate (%)	k1	90	97.58	89.66	88.61		
	k2	84.75	92.74	85.09	86.13		
	k3		83.83				
	k4		85.59				
	k5		70.44				
	Range (R)	5.25	27.14	4.57	2.47		
	Primary and secondary order	B > A > C > D					
	Superior level	A1	B1	C1	D1		
	Excellent combination	A1B1C1D1					
Emergence rate (%)	k1	51.09	61.21	53.68	57.04		
	k2	52.39	61.44	49.8	46.44		
	k3		46.46				
	k4		50.05				
	k5		35.35				
	Range (R)	1.31	26.09	3.88	10.59		
	Primary and secondary order	B > D > C > A					
	Superior level	A2	B2	C1	D1		
	Excellent combination	A1B1C1D1					
Comprehensive optimum combination		A1B1C1D1					

A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

For Cabernet Sauvignon seeds (Table 6), factor A's influence on the germination and emergence rates ranks fourth and third respectively, and A2 was taken, so factor A is A2. For factor B, the influence on the germination and emergence rates ranks second, but for the germination rate, B1 was taken and for emergence rate, B2 was taken. For B1, the germination rate was increased by 30.73% and the emergence rate was reduced by 25.32%, so factor B is B1. Factor C's influence on the germination and emergence rates ranks first, and both take C1, so factor C is C1. For factor D, the influence on the germination and emergence rates ranks third and fourth respectively, and both take D2, so factor D is D2. Therefore, for

Cabernet Sauvignon seeds, this results in an optimal combination of A2B1C1D2, that is, chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in petri dishes.

Table 6. Orthogonal experimental design and optimum results of Cabernet Sauvignon grape seeds.

Experiment Number		Experimental Factor				Experimental Result	
		A	B	C	D	Germination Rate (%)	Emergence Rate (%)
	1	1	3	1	2	89.67	56.56
	2	1	1	1	1	97.33	54.33
	3	1	3	1	1	91.35	40.67
	4	2	3	2	1	41.18	28.60
	5	2	5	1	2	81.18	47.55
	6	2	2	1	1	85.28	86.44
	7	2	1	2	1	66.01	32.00
	8	2	1	2	2	72.67	34.00
	9	1	2	2	2	30.41	28.89
	10	2	3	2	2	48.89	32.06
	11	1	1	1	2	98.00	51.93
	12	1	5	2	1	9.63	13.33
	13	1	4	2	1	38.17	22.67
	14	2	4	1	2	93.11	46.00
Germination rate (%)	k1	64.94	83.5	90.85	61.28		
	k2	69.76	57.84	43.85	73.42		
	k3		67.77				
	k4		65.64				
	k5		45.41				
	Range (R)	4.82	38.09	47	12.14		
	Primary and secondary order	C > B > D > A					
	Superior level	A2	B1	C1	D2		
	Excellent combination	A2B1C1D2					
Emergence rate (%)	k1	38.34	43.06	54.78	39.72		
	k2	43.81	57.67	27.36	42.43		
	k3		39.47				
	k4		34.33				
	k5		30.44				
	Range (R)	5.47	27.23	27.42	2.71		
	Primary and secondary order	C > B > A > D					
	Superior level	A2	B2	C1	D2		
	Excellent combination	A2B2C1D2					
Comprehensive optimum combination		A2B1C1D2					

A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

2.4. Verification Experiment

Since the optimal compound treatment predicted by the orthogonal experiment of Ecolly, Garanior, Dunkelfelder, and Cabernet Sauvignon grape seeds does not appear in the orthogonal experiment table, the germination and emergence rates of the seeds under this optimal treatment combination need to be verified, to ensure the authenticity of the experimental results. Therefore, it was verified by three sets of parallel experiments under the optimal compound treatment conditions, which are listed in Table 7. It can be observed in the table that the results of the verification experiment are representative, indicating that the optimal compound treatment is reasonable and feasible, and has good reproducibility.

Table 7. Results of repeated experiment.

Varieties	Optimal Combination	Germination Rate	Emergence Rate
Ecolly	A2B1C1D1	94.21 ± 4.33	53.24 ± 4.19
Garanior	A2B4C2D2	92.18 ± 4.56	67.65 ± 5.34
Dunkelfelder	A2B1C1D2	79.99 ± 5.72	51.52 ± 4.24
Cabernet Sauvignon	A2B1C1D2	82.49 ± 7.01	56.17 ± 3.55

The data based on three replicates was represented as M ± SD. A, B, C, and D represent experimental factor of stratification method, chemical substance, beat cutting, and pre-germination method respectively. 1, 2, 3, 4, and 5 correspond to the level factors of each experimental factor.

3. Discussion

3.1. Effects of Exogenous Treatment on Seed Germination Characteristics

Temperature and humidity are key factors for seed germination and also important environmental conditions for seed germination effect [23,24]. Different treatments may be more effective for different varieties based on differences in morphological structure and growth characteristics [25]. The results demonstrated significant effects on the germinating rate and potential of Dunkelfelder by chilling sand-storage. The seed coat of Dunkelfelder is hard, and humidity is low during stratification. This could limit seed expansion, resulting in slow germination and low germination rate [26,27]. Chemical treatments can change the internal metabolic mechanism of seeds, relieve seed dormancy, and promote seed germination [28,29]. The results of this study demonstrated that GA_3, 6-BA, CPUU, and HAc improved the germination rate, potential, and index of grape seeds of the tested varieties, with GA_3 providing the largest treatment effect. This is consistent with the results of previous studies [8,9,20,21,30]. This may be because GA_3, as a plant growth regulator, participates in the signal transmission during seed germination, and improves the activity of various enzymes to accelerate the decomposition and synthesis of internal substances in seeds, to improve the germination rate, potential, and index of the seeds [31–33]. Beak-cutting treatments also improved the germination rate, potential, and index of the tested varieties, which was consistent with the results of previous studies [21]. During seed germination, the control of humidity is particularly important. When the humidity is too low, the seed germination rate will be affected because of water limitation, but high humidity can make seeds become moldy and rotten [5]. Pre-germination in a bean sprouter also improved germination, with different effects observed for different varieties. This effect may be related to the constant humidity during pre-germination in a bean sprouter [30].

3.2. Effects of Exogenous Treatments on Seed Seedling Characteristics

Seed germination is the first stage of life in which plants can perceive the external environment, and this is also the most sensitive stage to environmental changes [34]. Germination directly affects seedling emergence and subsequent growth [35]. There were very significant differences in the influence of the two stratification methods on the emergence and seedling rates of Dunkelfelder. This may be due to the better relative swelling effect of seeds in the sand storage process, which improved germination and seedling formation [26]. Varieties with high germination efficiency and rate can more easily produce seedlings under appropriate environmental conditions [36]. There was no difference in the effects of different chemical treatments on the emergence and seedling rates of Meili, Dunkelfelder, and Cabernet Sauvignon, but there were different effects on the emergence and seedling rates of Ecolly, Garanior, and Marselan, potentially related to the relatively higher germination rate and potential of these varieties. Seed germination and seedling growth are the beginning stage of the plant life cycle, and exogenous treatment affects seed vigor [37,38]. Beak-cutting treatments improved the germination rate, potential, and index of the six tested varieties, but also improved the emergence and seedling rates, consistent with the results of previous studies [21,39]. Increasing the seedling rate can conserve resources [40]. The emergence and seedling rates of Ecolly and Cabernet Sauvignon were increased by pre-

germination in a bean sprouter, but these rates were decreased for Garanior, Dunkelfelder, and Marselan. This may be related to differences between varieties, such as seed maturity, endogenous hormone content, seed coat thickness, thousand-grain weight, size, water content, dormancy type, and the amount of cold needed to break dormancy [7,15,16,41,42]. In addition, we found that for all exogenous treatments, the seedling and emergence rates of the six tested varieties had a similar ratio, which may be related to the use of a seed coating agent before germination. Previous studies have found that a seed coating agent exhibits a good prevention and control effect on seedling stage wilt disease, which greatly increases the seedling rate [8].

3.3. Effects of Compound Treatments on Seed Germination and Seedling Characteristics

Seed germination and seedling growth are key stages of plant growth and development but are also the most vulnerable. Germination rate, potential, and index, and emergence and seedling rate are important indicators of seed germination and seedling growth, which together determine the seedling efficiency [43]. In conventional hybrid breeding and seed selection, the phenotypic traits of seeds drive the selection of high-quality strong seedlings, and the germination and seedling characteristics of seeds allow the selection of the best female parent for hybrid breeding [2]. The results of this study demonstrated that the optimal combination of treatments for Dunkelfelder and Cabernet Sauvignon was chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in petri dishes. The optimal treatment combination for Meili was chilling sand-storage + HAc soaking seed + beak cutting + pre-germination in petri dishes. The optimal treatment combination for Ecolly was chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter. The optimal treatment combination for Garanior was chilling sand-storage + HAc soaking seed + no beak cutting + pre-germination in petri dishes. The optimal treatment combination for Marselan was chilling gauze-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter. Overall, for Meili, Ecolly, Garanior, Dunkelfelder, and Cabernet Sauvignon seeds, chilling sand-storage was more effective than chilling gauze-storage, which may be related to the more constant humidity of sand storage [7]. For chemical treatments, GA_3 had larger effects for Ecolly, Dunkelfelder, Marselan, and Cabernet Sauvignon, and HAc had greater effects on Meili and Garanior. As an efficient and broad-spectrum plant growth regulator, GA_3 can increase the gibberellin content in seeds after soaking, regulate the proportion balance between inhibitor and promoter in advance, and enhance germination and seedling formation [33,44]. Soaking seeds with acetic acid can reduce the barrier of the seed coat, with enhanced permeability for improved seedling germination rates [45]. Beak-cutting is better than no-beak-cutting for the seeds of Melly, Ecolly, Dunkelfelder, Marselan, and Cabernet Sauvignon, and no-beak-cutting is better than beak-cutting for Garanior seeds. This may be related to the thickness of the seed coat. Beak-cutting is performed to release the binding of the hard peel to the embryo. If the seed coat of the seed is thin, beak-cutting may reduce the germination and emergence rates of the seed by damaging the embryo [21]. For Ecolly and Cabernet Sauvignon seeds, pre-germination in a bean sprouter is better than pre-germination in petri dishes, and for Meili, Garanior, Dunkelfelder, and Marselanseeds, pre-germination in petri dishes is better than in a bean sprouter. This may be related to the characteristics of seed germination. In our research, the germination potential of Ecolly and Marselan seeds is higher. This is helpful for the seeds to complete the germination process as soon as possible, effectively avoiding moldy and rotten seeds under the condition of prolonged moist germination and reducing the germination rate of the seeds. In addition, the characteristics of seed germination and seedling formation are also related to the growth cycle of the variety and the hardness of the seed coat. Early-maturing varieties have a short growth cycle, and the embryo development is not complete. The seed coat is hard, and the seed embryo is easily confined by the hard outer shell. These are not conducive to the germination of seeds [9].

The optimal combination treatments for the six grape varieties differed due to differences in the seeds of the different varieties. Dunkelfelder and Cabernet Sauvignon are red

medium or late ripening wine grape varieties, suggesting that the conditions identified here would be suitable for the treatment of other red medium and late ripening varieties. The germination and seedling characteristics of hybrid seeds are very similar to the characteristics of the female parent [8], so the optimal treatment for a female parent can provide reference for the germination and seedling formation of seeds produced by intraspecific hybrid combination, using the six tested varieties as parents. Further, the results of this study reveal higher germination and seedling rates for Ecolly, Garanior, and Marselan, with better consistency at the seedling stage, suggesting the suitability of these varieties for use as the female parent of grape hybridization.

4. Materials and Methods

4.1. Materials

With the primary research, the seeds of six grape varieties of *V. vinifera* were used as experimental materials: Ecolly, Meili, Garanior, Cabernet Sauvignon, Marselan, and Dunkelfelder (Table 8). The seeds were collected from an experimental vineyard of the Northwest Agriculture and Forestry University (NWAFU) located in Yangling of Shanxi Province (lat. 34° N, long. 108° E), China. This area has a semiarid continental monsoon climate, and the soil type is bauxite. Self-rooted vines of *V. vinifera*. were planted in 2013. Vine rows were oriented west-east, with vines spaced in 1.0 × 2.5 m rows. The vines were cordon-trained and pruned to two buds per spur. All viticultural practices were performed according to local standards. The characteristics of seeds of the six varieties are presented in Table 1.

Table 8. Characteristics of the six test *V. vinifera* varieties.

Variety	Breeding Method	Seed-Parent	Variety Type	Seed Characteristics
Meili [46]	Intraspecific current selection	[Muscat Hamburg × (Merlot × Riesling)] × (Muscat Hamburg, Merlot, Riesling)	Red mid-variety	Large seed, seed coat is medium thickness.
Ecolly [47]	Intraspecific current selection	[Chenin Blanc × (Chardonnay × Riesling)] × (Chenin Blanc, Chardonnay, Riesling)	White mid-variety	Small seed, seed coat is relatively thin.
Garanior [48]	Intraspecific hybridization	Gamay × Reichensteiner	Red early-maturing variety	Small seed, seed coat is relatively thin.
Dunkelfelder [47]	Intraspecific hybridization	Madeleine Angevine × Teinturier	Red mid-variety	Medium-sized seed, seed coat is relatively thin.
Marselan [47]	Intraspecific hybridization	Cabernet Sauvignon × Grenache	Red mid-late maturity variety	Small seed, seed coat is relatively thin.
Cabernet Sauvignon [47]	Intraspecific hybridization	Cabernet Franc × Sauvignon Blanc	Red late-maturing variety	Medium-sized seed, seed coat is medium thickness.

Meili and Ecolly selected the mixed pollen of the three parents in the process of intraspecific current selection. All tested varieties were obtained through intraspecific hybridization in *V. vinifera*.

4.2. Treatment Methods of Seeds

4.2.1. Collection and Storage

Grapes were collected randomly from different vines and different parts of clusters after reaching full maturity. One cluster was randomly selected for each vine, and six grapes (two each from the upper, middle, and lower parts) were sampled from each chosen cluster. The pulp and peel of the collected grapes were removed, and the mature seeds

with full shapes were rinsed several times with water. The washed seeds were sterilized with 1% sodium hypochlorite for 15 min, and then rinsed four times with sterile water. After natural drying in the shade, the seeds were put into nylon bags, hung in a ventilated place for air drying, and then stored for later use [9,16,49].

4.2.2. Stratification Treatment

Two stratification treatments were tested. In the first, the seeds were mixed with 3–4 times volumes of wet sand and then chilled in a refrigerator. The sand humidity was 60–80%, the refrigerator temperature was about 4 °C, and the chilling sand-storage is carried out for three months [50]. In the other stratification treatment, the seeds were wrapped with wet gauze, sub-packed in plastic bags with air holes, and then stored in a 4 °C refrigerator for three months [30]. The temperature and humidity were frequently checked during storage to prevent seed decay and premature germination.

4.2.3. Chemical Treatments

Different chemicals were tested using previously determined amounts and 8 h of soaking time: 150 mg/L GA_3, 100 mg/L 6-BA, 200 mg/L CPUU, HAc at 1:70, or pure water as a control [8,9,20,21,30].

4.2.4. Beak-Cutting Treatments

Before pre-germination, the seeds were sterilized, soaked in 1% sodium hypochlorite for 15 min, and then fully stirred with 25 g/L Syngenta fludioxonil seed coating agent (slurry: seeds of 1:50) [15,18]. Then the beak-cutting and non-beak-cutting treatments were performed for the pre-germination test.

4.2.5. Pre-Germination Treatment

Two kinds of pre-germination treatment were tested. In the traditional method, the treated seeds were placed in petri dishes containing double-layer filter paper in the upper and lower parts and then placed in a light incubator to accelerate germination (the temperature of the light incubator is 25 °C, the filter paper is timely moisturizing, 12 h of light and 12 h of darkness) [17]. In the other method, the sterilized seeds were uniformly sown in a bean sprouter, and distilled water was added to accelerate germination (the temperature of the bean sprouter is 25 °C, intermittent spray moisturizing, 12 h of light and 12 h of darkness) [30].

4.2.6. Sowing and Planting

Germination was measured by evaluating seed cracking and white exposure or long root. The number of sprouting seeds per day was determined for each treatment of different varieties. Germinated seeds were sown in the greenhouse using burrow plate seedling cultivation [2,7]. After seed emergence, wheat bran mixed with insecticides was sprinkled around the burrow tray to prevent insect pests. Carbendazim solution (1 g/L) was applied one or two times to prevent blight. Emergence was determined as the time when the seedlings had grown two pieces of cotyledon, and then the seedlings were moved into a 10 cm gallon pot (The growing medium is nutrient soil). When the seedlings had four true leaves and one heart seedling, the seedlings were counted [7].

4.3. Experimental Design

The study utilized a single factor experiment and an orthogonal experiment. The two experiments were conducted simultaneously, with three technical replicates for each treatment setting.

4.3.1. Single Factor Experiment

The experiment was carried out to investigate the key steps of seed germination and seedling formation. The experiment included two stratification methods (chilling

gauze-storage and chilling sand-storage), five chemicals (GA_3, 6-BA, CPUU, HAc, and CK), beak-cutting or no beak-cutting before germination, and two pre-germination methods (pre-germination in petri dishes and pre-germination in a bean sprouter). The germination and seedling status were measured after sowing. Three biological replicates were performed, and 100 seeds were taken from each replicate.

4.3.2. Orthogonal Experiment

The stratification, chemical substance, beak cutting, and pre-germination treatments were used as the four research factors, and the orthogonal experiment of composite treatments was designed using SPSS, as listed in Table 9. For each treatment, 100 seeds were collected, with three repeats. The germination and seedling formation were measured after sowing. Analysis was performed to determine the optimal compound treatment conditions.

Table 9. Level factors.

Level	Experimental Factor			
	Stratification Method (A)	**Chemical Substance (B)**	**Beak Cutting (C)**	**Pre-Germination Method (D)**
1	chilling gauze-storage	GA_3	beak cutting	pre-germination in bean sprouter
2	chilling sand-storage	6-BA	no beak cutting	pre-germination in petri dishes
3		CPUU		
4		HAc		
5		CK		

4.3.3. Verification Experiment

The experiment was repeated three times under the optimal compound treatment conditions of the orthogonal experiment to verify the results.

4.4. Assay Method of Primary Indicators

The germination rate (%) equals the total number of germinated seeds divided by the total number of seeds tested × 100%. The germination potential (%) is the number of seeds germinated within eight days of germination divided by the total number of seeds tested × 100%. The germination index = Σ (Gt/Dt), where, Gt is the germination at different times, Dt is the number of germination days (in this study, the first eight days are calculated), and Σ is the sum. The emergence rate (%) equals the number of seedlings (two pieces of cotyledon) divided by the number of seeds sown × 100%. The seedling rate (%) equals the number of seedlings (number of four true leaves and one heart seedlings) divided by the number of seeds sown × 100%.

4.5. Data Processing

Microsoft Excel 2013 was used to record and process the original data and calculate the mean and range. An analysis of variance and multiple comparisons were performed in SPSS 22.0 statistical software. Data were averaged by treatment, and treatments (stratification treatment, beak-cutting treatments, and pre-germination treatments) were compared using the Student's t test. The multiple comparisons tests (chemical treatments) were performed between treatments with Tukey's test (* $p < 0.05$, ** $p < 0.01$). Origin 9.0 and GraphPad Prism 6 were used to present the results.

5. Conclusions

In this study, single factor and composite orthogonal experiments were used to determine the optimal pre-treatment methods for seed germination and emergence of six *V. vinifera* varieties. The single factor experiment demonstrated that two stratification methods (chilling gauze-storage and chilling sand-storage) and two pre-germination methods

(pre-germination in petri dishes and pre-germination in a bean sprouter) vary in effectiveness for different varieties, so a simple and economical hierarchical or pre-germination method could be selected as needed. GA_3 soaking and beak-cutting promote the germination and seedling rates of the tested varieties. The optimal way to improve the germination and emergence rates of the tested varieties was determined by a compound orthogonal experiment comparing several methods. The optimal treatment combination of Dunkelfelder and Cabernet Sauvignon were chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in petri dishes. The optimal treatment combination of Meili was chilling sand-storage + HAc soaking seed + beak cutting + pre-germination in petri dishes. The optimal treatment combination for Ecolly was chilling sand-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter. The optimal treatment combination of Garanior was chilling sand-storage + HAc soaking seed + no beak cutting + pre-germination in petri dishes. The optimal treatment combination for Marselan was chilling gauze-storage + GA_3 soaking seed + beak cutting + pre-germination in a bean sprouter.

This study identified the optimal conditions for seed germination and seedling formation of six grape varieties, which will facilitate future work to characterize the seed germination and seedling formation of seeds, obtained by intraspecific hybridization of these varieties. This work also provides a reference for addressing problems of low seed germination rate and suboptimal seedling formation for better utilization of grape germplasms. However, this study only evaluated the effects of exogenous treatments on the germination, potential, index, emergence and seedling rates of the six tested varieties. The physiological changes inside seeds, the effect of seed embryo vigor, and the quality of seedlings after exogenous treatment were not studied in detail. Future work should determine the effects of physiological and biochemical reactions on seed germination and seedling formation characteristics of grapes.

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