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Article

Transcriptome Profiling of Cold-stored Potato Tubers Revealed Similarities in the Regulation of Bud Dormancy Release, Tuberization, and Flowering Initiation

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Abstract

Potato tubers can be dormant for 1–15 weeks after harvest, and the germination of tuber eye-buds is suppressed by cold. Genetic mechanisms regulating bud dormancy at low temperatures are not completely understood. We performed RNA-seq to compare gene expression in tubers after 0.5, 3.5, and 6.5 months of cold storage. Differentially expressed genes (DEGs) in non-dormant (3.5/6.5 months) vs dormant (0.5 months) tubers were associated with transcriptional and translational activation, cell growth, metabolism, hormonal signaling, meristem development, dormancy break, and reproduction, confirming the non-dormant state of the meristem in the middle of storage. DEGs encoding transcription factors (TFs) (*CBF1*, *FLC*, *SVP*, *HY5*, *GI*, *CO*, *FT*, *SOC1*, *CDF1*, *POTM1*) were associated with the regulation of dormancy, flowering, and tuberization. TF DEGs upregulated (78) or downregulated (224) over four times in non-dormant vs dormant tubers are considered potential coordinators of the endo- to non-dormancy transition and upcoming tuber sprouting during cold storage. RT-qPCR analysis of non-dormant and germinating buds revealed the upregulation of tuberigenes (*StSP3D*, *StFTL1-1*, *StFTL1-2*), anti-tuberigenes (*StSP5G*-like), and TF genes positively associated with tuberization or flowering/germination (*StFDL1*, *StFDL*, *StCDF1*, *StCO*-like). Our results should further investigation of the mechanisms underlying tuber meristem dormancy release and sprouting during long-term cold storage.

Keywords: potato; *Solanum tuberosum* L.; tuber dormancy; long-term cold storage; transcriptome; gene expression; transcription factor

1. Introduction

Potato (*Solanum tuberosum* L.) is the most important non-cereal food crop in the world. Potato tubers, which are formed at the ends of modified stem shoots (stolons), are rich in starch, proteins, and vitamins [1]. Commercial cultivars are propagated vegetatively through tuberous reproduction, whereas in breeding practice seeds are used to contribute to the morphophysiological variability of the species [2].

Potato flowering and tuberization occur simultaneously depending on endogenous cues and seasonal changes in temperature and photoperiod. Thus, in response to day length phloem-mobile signals are transmitted from the leaves to the shoot apical meristem and the tips of stolons, where flower and tuber formation is stimulated through similar regulatory mechanisms called florigen- and tuberigen-activating complexes (FAC and TAC, respectively) [3].

Both FAC and TAC include transcription factors (TFs). The *FLOWERING LOCUS T (FT)* gene is a component of FAC; its mRNA and protein (TF of the PEBP family) are transported from the leaves to the shoot meristem and trigger flowering [3]. Tuber formation in potato is induced in a similar

manner through the activity of *FT*-like genes. The potato genome contains seven *FT*-like genes: florigen *SELF-PRUNING 3D* (*StSP3D*), main tuberigen *StSP6A*, additional tuberigen *StSP3D* and *StFTL1*, and anti-tuberigen *StSP5G-A/B* and *StSP5G-like* which suppress tuber formation [4].

Besides FT factors, FAC and TAC include bZIP-TF FLOWERING LOCUS D (FD; StFDL1 in potato), which is bound to FT through a 14-3-3 protein [3].

Tuberization is negatively regulated by BBX-TF CONSTANS (CO) and CO-like TFs StCOL1 and StCOL2, which activate the transcription of anti-tuberigen *StSP5G* [4–6]. Under inductive conditions, *StCOL* expression is suppressed by CYCLIC DOF FACTOR 1 (CDF1), which otherwise is degraded by the joint activity of TF GIGANTEA (GI) and blue light receptor FLAVIN-BINDING KELCH-REPEAT F-BOX 1 (FKF1) [5]. The stability of StCOL is positively regulated by phytochromes (PHYs) StPHYB and StPHYF [4]. The expression of *StCDF1*, *StSP6A*, and some *StSP6A* target genes is induced by BELL-TF BELLRINGER-1 LIKE 5 (StBEL5) and KNOX-TF POTH1 [4,6]. In the stolon, TAC activates the *AGL8*-like MADS-box TF gene, *StMADS1* (*POTM1*), which stimulates *StSP6A* transcription in a feedback manner [7].

Tuber formation is negatively regulated by competitive replacement of the FT factor in TAC by FT homologue PEBP-TF TERMINAL FLOWER 1 (TFL1 or CENTRORADIALIS [StCEN]) [8]. Besides, tuberization depends upon the activity of microRNAs, which can stimulate (miR172) or suppress (miR156) the process [9].

Under inductive conditions, stolons swell and turn into tubers. Mature tubers enter a state of dormancy, which can be of three possible types: endodormancy or true dormancy regulated by endogenous signals, ecodormancy determined by environmental signals, and paradormancy or apical dominance (suppression of lateral bud outgrowth) when the bud meristem is in a non-dormant state but can grow only at favorable conditions [1,10]. The duration of endodormancy depends on the cultivar and can range from 1 to 15 weeks under growth-promoting conditions (15–20°C and humidity of about 90%). Harvested tubers are usually stored at low temperatures (2–4°C) for the maintenance of the dormant state to prevent germination until seasonal sowing as well as desiccation and disease development [11,12].

The onset and maintenance of tuber dormancy is accompanied by the accumulation and activation of phytohormones such as abscisic acid (ABA), jasmonic acid (JA), strigolactones (SLs), and salicylic acid (SA), whereas the exit from dormancy is promoted by cytokinins (CKs), gibberellins (GAs), and auxins. Ethylene (ETH) signaling has been shown to be associated with both tuber states, and brassinosteroid (BR) signaling modulate tuber sprouting [12–15].

The morphological sign of the tuber dormancy release is the germination of buds [12]. The process is accompanied by an increase in the content of amino acids and their derivatives, alkaloids, flavonoids, reducing sugars, a decrease in sucrose and starch, and significant changes in zeatin and phenylpropanoid biosynthesis, glutathione metabolism, and pentose phosphate pathway [15,16].

The regulation of potato tuber dormancy depends on multiple genes. Dormancy release is associated with the activation of genes involved in the cell cycle, metabolism of phytohormones and carbohydrates, stress response, DNA methylation, and histone acetylation [11,13]. Visible tuber germination is preceded by changes in the expression of genes encoding deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) [17] and ADP-ribosylation factor (ARF1) [18] and those regulating the cell cycle, carbohydrate metabolism, phytohormone synthesis and signaling, and vesicular transport [11]. RNA-seq analysis of dormant and germinating tubers has identified 7,728 differentially expressed genes (DEGs) and revealed changes in the expression of *stu-miR319c*, *miRNA8047*, and *miRNA482a* genes [19,20], indicating the complexity of the dormant state control in potato.

Previous studies have addressed molecular mechanisms of tuber dormancy and dormancy release focusing mainly on the cell cycle and metabolic pathways; however, the intricate regulatory network behind dormancy exit is still not understood. In our study, we performed comparative transcriptomics of mature potato tubers at the endodormancy and ecodormancy states (0.5–6.5

months at 3°C) and after induction of sprouting (20°C), focusing on potential molecular triggers of dormancy break.

2. Materials and Methods

2.1. Plant Material

In this study, we used potato (*S. tuberosum* L.) cultivar 'Lady Clair'. In September 2023, one-plant tubers of a similar size (55–60 mm in diameter) were harvested, defined as fully matured tubers, and stored at 3°C in the dark for 0.5 month (putative dormant tubers) and 3.5 and 6.5 months (putative non-dormant tubers). At each of three time points, the tubers were sampled by cutting around the apical eye-bud to include periderm, cortex, vascular ring, and perimedulla; two biological replicates were used. Samples were frozen in liquid nitrogen and stored at -80°C until analysis.

Tubers stored in cold for 6.5 months were also used to study the expression of genes putatively associated with dormancy release. For this, apical eye-buds were cut from tubers at 0 time (just taken from cold) and after exposure to room temperature (20°C) for 1, 7, and 9 days; the two latter time points corresponded to sprouted (0.2–1 and 1–2 mm, respectively) eye-buds.

2.2. mRNA Library Sequencing

Total RNA was extracted from tuber samples using the RNeasy Plant Mini Kit and RNase free DNase set (QIAGEN, Hilden, Germany). RNA was quantified (Qubit® RNA Assay Kit and Qubit® 2.0 Fluorometer, Life Technologies, Carlsbad, CA, USA) and qualified using agarose gel electrophoresis and the RNA integrity number (RIN) determination (Agilent 2100 Bioanalyzer, Agilent Technologies Inc., Santa Clara, CA, USA). According to the analyses, all six RNA preparations were of good quality for RNA-seq (RIN ~ 8.5–9.6).

A NEBNext® mRNA Library Prep Reagent Set for Illumina® (New England BioLabs Inc., Ipswich, MA, USA) was used to prepare mRNA libraries. Six libraries were obtained and sequenced by MyGene Co. (Moscow, Russia) using the Illumina HiSeq 2500 (Illumina, San Diego, CA, USA).

2.3. Mapping Reads to the Reference Genome

The *S. tuberosum* cv. DM 1-3 516 R44 genome (SolTub_3.0; GCF_000226075.1) was used for mapping of the obtained transcript reads. After removing adapter-containing (cutadapt v.1.17 [21]) and low quality (sickle v.1.33 [22]) sequences, paired overlapping reads were merged using FLASH v.1.2.11 (<https://ccb.jhu.edu/software/FLASH/>; accessed on 10 November 2024) and aligned to the annotated genes in the potato genome. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed with ShinyGO 0.82 ([23,24]; <https://bioinformatics.sdstate.edu/go/>; accessed on 18 April 2025).

2.4. Differentially Expressed Genes

Relative transcription levels of the identified genes were calculated as numbers of reads per kilobase of gene exon region per million mapped reads (RPKM). Differentially expressed genes (DEGs) were identified using the DESeq2 software; DEGs were considered if the expression change was not less than two-fold (absolute log₂FoldChange [FC] ≥ 1) and padj (P-value adjusted, or false discovery rate) was < 0.05. To visualize DEGs, Venn diagrams, Heatmaps, Volcano plots, and PCR vs RNA-seq plots were used ([25]).

2.5. Gene Expression Analysis by Quantitative Real-Time PCR

Based on the RNA-seq data, a number of DEGs were selected and analyzed for expression by quantitative (q) real-time (RT) PCR. Total RNA extracted from tuber samples (section 2.2) was used for cDNA synthesis (Reverse Transcription System; Promega, Madison, WI, USA). RT-qPCR was performed with SYBR Green and ROX RT-qPCR mixture (Syntol, Moscow, Russia), 3 ng cDNA, and

10 μ M gene-specific primers (Supplementary Table S1) in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at the following conditions: pre-denaturation at 95°C for 5 min and 40 cycles at 95°C for 15 s and 62°C for 50 s. The data were normalized to the expression of two *S. tuberosum* genes, *elongation factor 1-alpha (ef1)*, LOC102600998) and *exocyst complex component (SEC3A)*, LOC102599118), which are stably expressed under abiotic stress conditions and can be used in pairs to improve the efficiency of normalization of target gene expression [26]. One-way ANOVA (multiple comparisons corrected with Bonferroni test) in GraphPad Prism v.9 software (<https://www.graphpad.com>, San Diego, CA, USA) was used to test for significant differences ($P < 0.05$) between mean gene expression values in each sample (two biological and three technical replicates were used).

3. Results

3.1. Transcriptome Sequencing, Mapping, and Primary Analysis

Eye-buds of mature potato tubers (0.5 months of cold storage) were considered dormant. The time point of 3.5 months corresponded to the maximum known period for endodormancy release of the tuber bud meristem (about 15 weeks [10]) and that of 6.5 months corresponded to the approaching end of storage.

To analyze changes in gene expression between tuber dormancy and dormancy release states, we performed RNA-seq of 0.5-, 3.5-, and 6.5-month tuber transcriptomes. Reads were aligned to the potato reference genome. Sequencing yielded 19,436,490 (0.5 months), 21,677,357 (3.5 months), and 17,269,387 (6.5 months) good quality reads, representing approximately 4 GB of sequence data for each time point and approximately 4.7 times the size of the potato genome (0.84 Gb).

A total of 23,425 (0.5 months), 22,601 (3.5 months), and 22,576 (6.5 months) expressed genes were identified, which covered 24,957 of 32,873 annotated potato genes (Supplementary Table S2). The distribution of the expressed genes in the three stages is summarized in Venn diagrams (Figure 1). In total, 20,962 genes were expressed across all three stages and approximately 500–600 genes were co-expressed between stage pairs. The highest number of genes (1,333) were specifically expressed in mature dormant tubers (0.5 months) (Figure 1a).

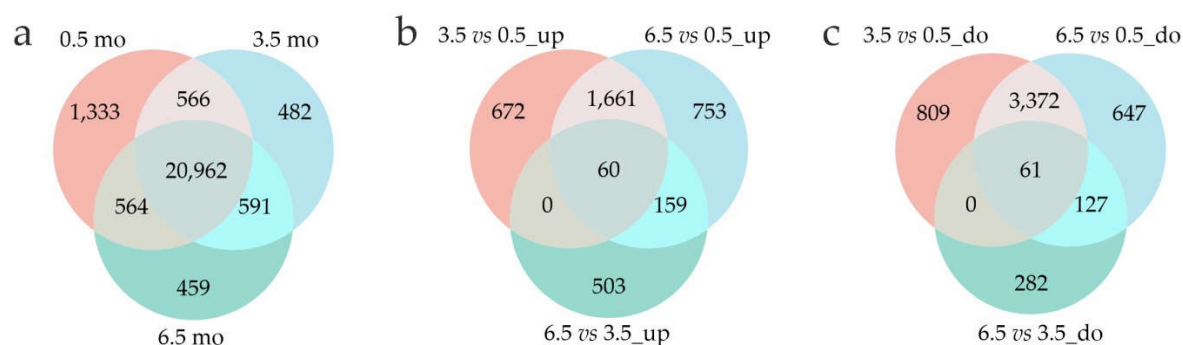


Figure 1. Venn diagrams of the expressed genes (a) and DEGs upregulated (b) and downregulated (c) between the 0.5-, 3.5-, and 6.5- month transcriptomes.

Among the expressed genes, 26.6%, 27.6%, and 4.8% were identified as DEGs (at least a two-fold change) at 3.5 vs 0.5 months (2,393/4,242 up/downregulated), 6.5 vs 0.5 months (2,633/4,207), and 6.5 vs 3.5 months (722/470), respectively (Figure 1b, c, Supplementary Tables S3-5). Compared with 0.5-mo, 3.5- and 6.5-mo tubers had 1,721 upregulated and 3,433 downregulated common DEGs (Figure 1b, c). The changes were quantitatively similar between 3.5- vs 0.5-mo and 6.5- vs 0.5-mo samples and were notably higher than those for 6.5- vs 3.5-mo tubers, indicating that the most significant transcriptional shifts occurred in tubers during the 0.5–3.5-month period.

DEGs with the most significant expression changes (Volcano plots in Figure 2) may serve as key biomarkers of the transition between tuber dormancy and dormancy release without visually

detectable morphological changes. In particular, the DEGs significantly upregulated in 3.5- and 6.5- vs 0.5-mo tubers included *dUTPase* (LOC102589306) (Supplementary Table S3-5) considered as a marker of tuber dormancy release [17]. Another such marker is ADP-ribosylation factor (*ARF1*) [18], and we found that one of the *ARF1*-like genes (LOC102577686) was significantly downregulated in 3.5- vs 0.5-mo tubers (Supplementary Table S3-5).

3.2. Enriched GO and KEGG Terms

To reveal the functions of the overrepresented DEGs, we characterized them in GO and KEGG terms. It was found that in 3.5- and 6.5- vs 0.5-mo samples, the most enriched genes were associated with transcriptional and translational activation, post-translational modifications, cell cycle/differentiation, phytohormone metabolism and signaling, and other metabolic pathways (carbohydrate, lipid, etc.), as well as with the suppression of photosynthesis and biosynthesis of secondary metabolites (carotenoids, phenolic compounds, and porphyrins). In 6.5- vs 3.5-mo tubers, the most enriched genes were related to photosynthesis and secondary metabolism (Figure 3, Supplementary Tables S6-11), indicating the upcoming germination of eye-bud meristems.

As expected, storage of tubers at low temperature affected the expression of stress response- and lipid metabolism-related genes. In both 3.5- and 6.5- vs 0.5-mo tubers, the activity of ~15% genes in the categories “response to cold”(GO:0009409) and “response to temperature stimulus”(GO:0009266) was increased, whereas that of 15% was decreased. Also, 15% of the genes belonging to the categories “response to water deprivation”(GO:0009414) and “response to osmotic stress”(GO:0006970) were upregulated and 15% were downregulated (Supplementary Tables S6-9), which is consistent with the tendency of tubers to desiccate during long-term storage.

At 3.5 vs 0.5 months, many DEGs were found to be associated with main phytohormone signaling pathways. The activity of genes involved in SA-, JA-, and BR-related processes was rather suppressed (corresponding categories were enriched only in the downregulated DEGs), whereas some genes associated with ETH, CK, and ABA pathways were stimulated and the other such genes – inhibited. The auxin-mediated pathway was activated, but no DEGs related to the GA pathway were found (Supplementary Tables S6, 7). These data are somewhat inconsistent with the results of previous studies showing that dormancy release is associated with the upregulation of CK, GA, auxin, and BR signaling and downregulation of ABA, ETH, JA, SL, and SA pathways [12–15]. Thus, the status of phytohormones after 3.5 months of cold storage could indicate that the tubers differed in terms of the time of exit from endodormancy and ecodormancy.

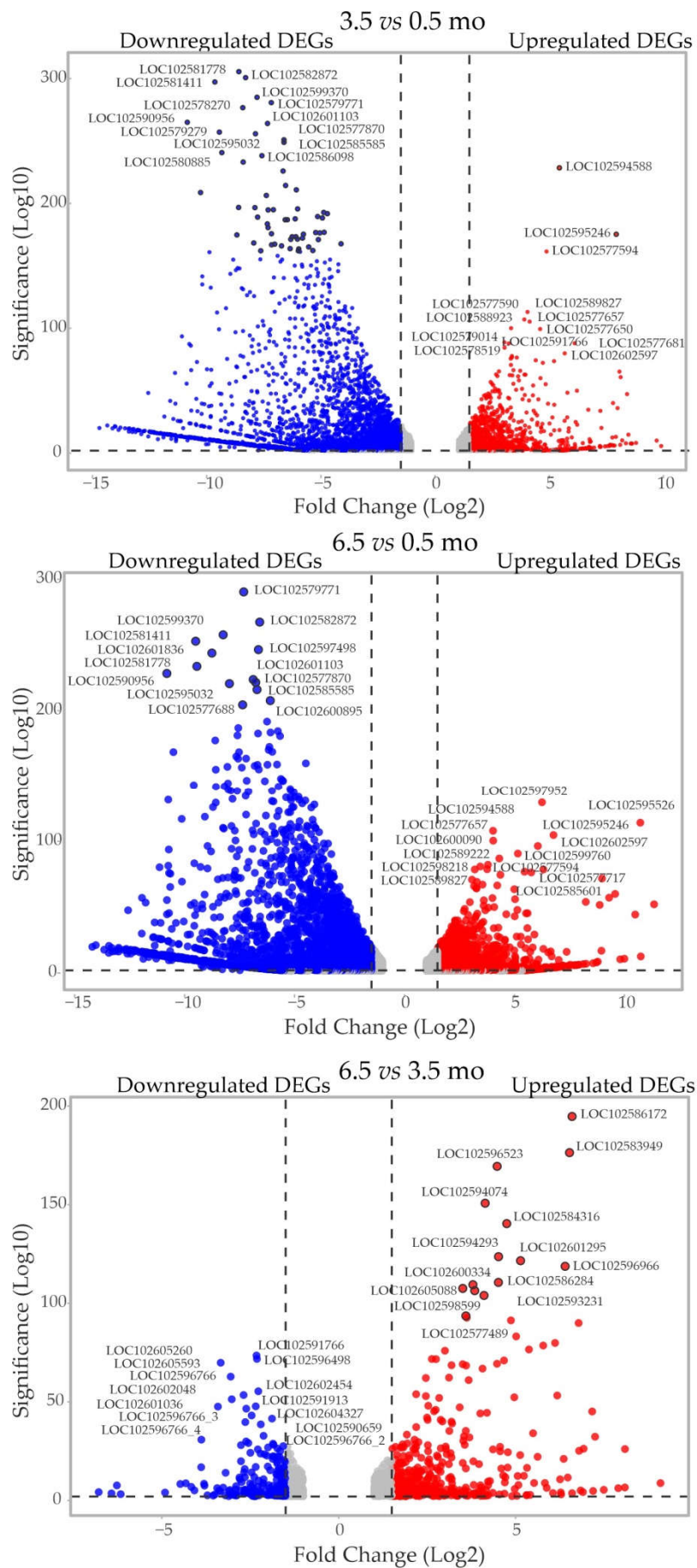


Figure 2. DEGs with the most significant expression changes between the 0.5-, 3.5-, and 6.5-mo tuber transcriptomes.

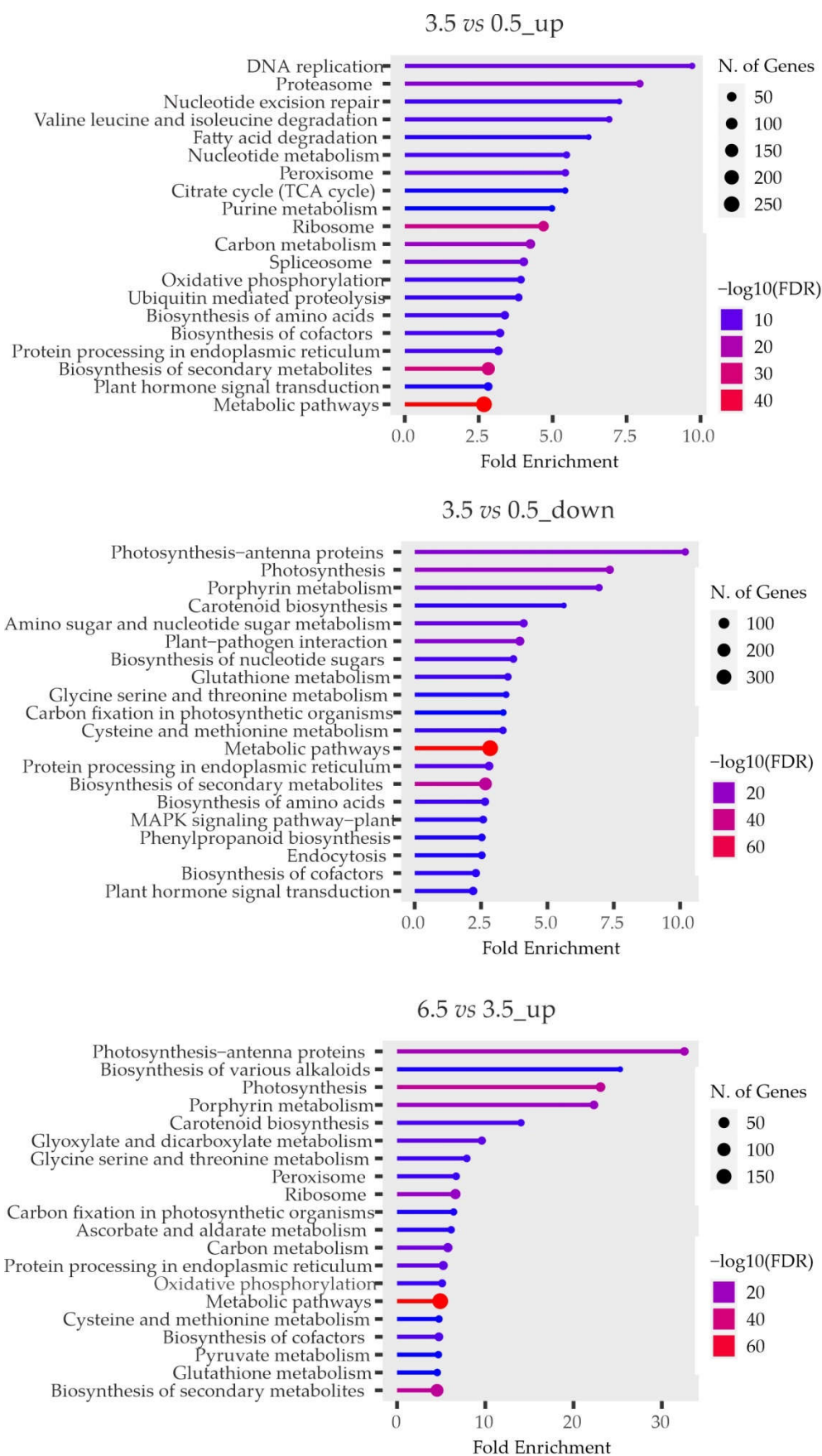


Figure 3. The most enriched functional categories (KEGG terms) of DEGs between the 0.5-, 3.5-, and 6.5-mo transcriptomes.

Both 3.5- and 6.5- vs 0.5-mo tubers were characterized by the enrichment of DEGs related to the development of tuber embryonic meristems (e.g. “embryo development” GO:0009790, “embryo development ending in seed dormancy” GO:0009793, and “meristem development” GO:0048507) (Table 1, Supplementary Tables S6–9). The enrichment in DEGs belonging to the category “postembryonic development” (GO:0009791) and “plant organ development” (GO:0099402) was even more pronounced in 6.5-mo vs 3.5-mo samples. Taken together, these results may indicate the activation of the eye-bud meristems in potato tubers at 3.5 months and their progressive development and upcoming sprouting at 6.5 months of cold storage.

Analysis of DEGs associated with plant reproductive development produced interesting results. Thus, genes belonging to the category “vegetative to reproductive phase transition of meristem” (GO:0010228) were upregulated at 3.5 months, which may indicate the involvement of flowering initiation-related genes in the determination of the non-dormant state of the bud. This result was supported by the finding that DEGs (both up- and downregulated) associated with the initiation of flowering and flower development were enriched in the corresponding categories (“flower development” GO:0009908, “regulation of photoperiodism flowering” GO:2000028, “inflorescence development” GO:0010229, and “reproductive shoot system development” GO:0090567) (Table 1, Supplementary Tables S6,7). These genes may be related to the regulation of tuber dormancy release, which causes transcriptomic/metabolomic changes previously detected by comparative analysis of dormant and non-dormant tubers ([12,13]).

Table 1. Enriched GO categories related to biological processes (BPs) and associated with the meristem and reproductive development.

GO: BP term	Pathway (number of genes in the category)	Upregulated DEGs		Downregulated DEGs			
		3.5 vs	6.5 vs	6.5	3.5	6.5 vs	6.5
		0.5	0.5	vs	vs	0.5	vs
				3.5	0.5		3.5
GO:0009790	Embryo development (103)	20	17	0	0	0	0
GO:0009793	Embryo development ending in seed dormancy (99)	19	17	0	0	0	0
GO:0009791	Post-embryonic development (431)	63	59	22	68	62	25
GO:0048507	Meristem development (85)	15	0	0	0	0	0
GO:0010073	Meristem maintenance (54)	10	8	0	0	0	0
GO:0099402	Plant organ development (267)	29	30	0	41	40	11
GO:0010228	Vegetative to reproductive phase transition of meristem (54)	7	0	0	0	9	6
GO:2000028	Regulation of photoperiodism flowering (22)	4	0	0	5	6	3
GO:0003006	Developmental process involved in reproduction (370)	55	48	0	56	55	18

GO:00224	Reproductive process (571)	68	58	0	73	73	22
14							
GO:00099	Flower development (130)	17	16	0	26	23	0
08							
GO:00101	Fruit development (154)	23	21	0	0	0	7
54							

3.3. Detailed Analysis of the DEGs

3.3.1. Storage Proteins

Patatins are the major potato tuber storage proteins, which are degraded during tuber sprouting due to suppression of protease inhibitors; accordingly, the amino acids necessary for the initial sprouting are released [27]. Our results indicate that DEGs of most protease inhibitors were upregulated in 3.5- vs 0.5-mo tubers (dormancy release) and then downregulated (many of them sharply) in 6.5- vs 3.5-mo samples (upcoming sprouting state). Under stimulating conditions (20°C), the expression of patatin genes decreases when tubers exit from dormancy and increases during their germination [12]. In our study, the profile of patatin genes was different, probably because of cold storage: five patatin DEGs were upregulated at 3.5 vs 0.5 months and downregulated at 6.5 vs 3.5 months, whereas seven such DEGs were downregulated at both 3.5 and 6.5 vs 0.5 months (Figure 4a, Supplementary Table S12).

Analysis of lipoxygenase genes showed that 2 and 3 of them were upregulated and 9 and 6 – downregulated in 3.5- and 6.5- vs 0.5-mo samples, respectively (Figure 4a, Supplementary Table S12). These data are consistent with the finding that lipoxygenases are involved in the formation of secondary metabolites that regulate shoot development [27].

Thus, under low-temperature conditions, when bud endodormancy breaks (presumably at 3.5 months or earlier), the expression of most protease inhibitor genes increased, whereas that of about half of the patatin genes and most lipoxygenase genes decreased, suggesting maintenance of the ecodormancy state with the suppression of eye-bud germination. These tendencies continued in 6.5-mo tubers (upcoming sprouting), in which the expression of most genes encoding patatins, protease inhibitors, and lipoxygenases was decreased.

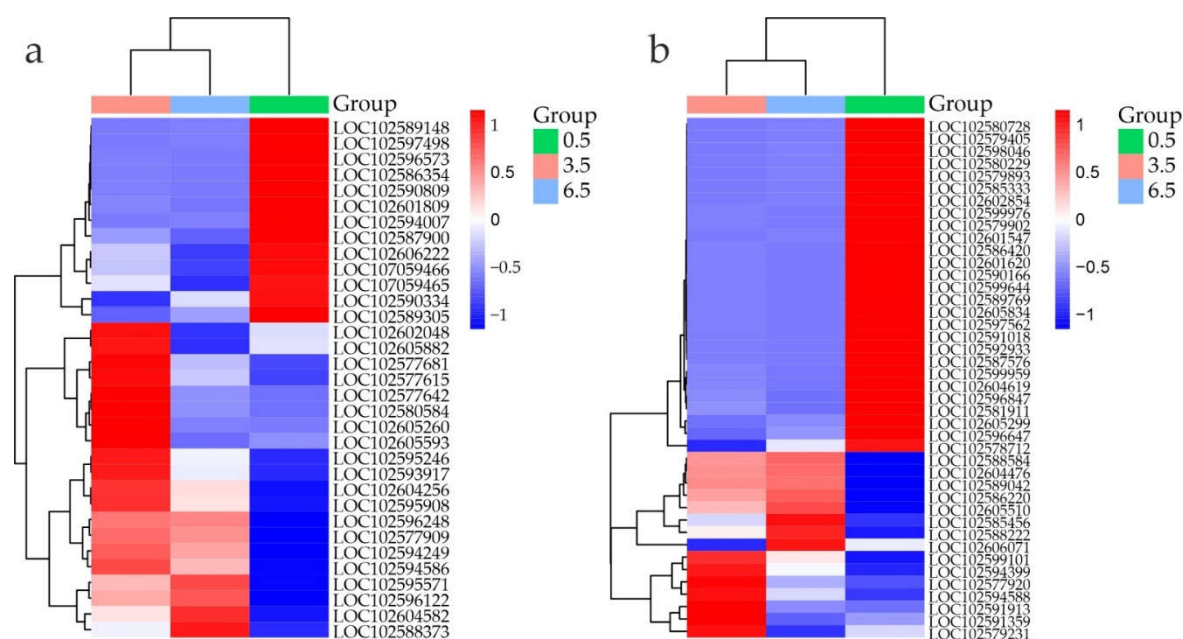


Figure 4. Heatmap of DEGs related to storage proteins (a) and ETH signaling (b) in 0.5-mo (green), 3.5-mo (pink), and 6.5-mo (blue) *S. tuberosum* tubers based on the transcriptome data (RPKM). The color gradient indicates

changes in gene expression from low (blue) to high (red); the names of the proteins corresponding to the LOC numbers are given in the Supplementary Table S12.

3.3.2. Phytohormones

Potato tuber dormancy is maintained by the ABA, JA, SL, and SA pathways and is broken by the upregulation of auxin, CK, and GA signaling, whereas tuber sprouting is regulated by BRs [14]. ETH is positively associated with both tuber states but more with dormancy maintenance; in dormant tubers, ETH accumulation is increased, which supports the dormancy state through effects on carbohydrate and phenylpropanoid metabolism [14] and on the inhibition of cell division, DNA synthesis, meristem activation, and sprouting [28]. In contrast, short-term treatment with exogenous ETH breaks dormancy by stimulating the expression of the ABA 8'-hydroxylase gene *StCYP707A2* associated with ABA catabolism, which leads to a decrease in ABA levels [14]. Dormancy-to-dormancy release transition in tubers is accompanied by an increase in the expression of ETH signaling-related genes [15].

Table 2 and Figures 4–6 summarize the data on the expression change of some genes associated with phytohormone signaling in tubers at 3.5 vs 0.5 months; more detailed information on the relevant DEGs can be found in Supplementary Table S12.

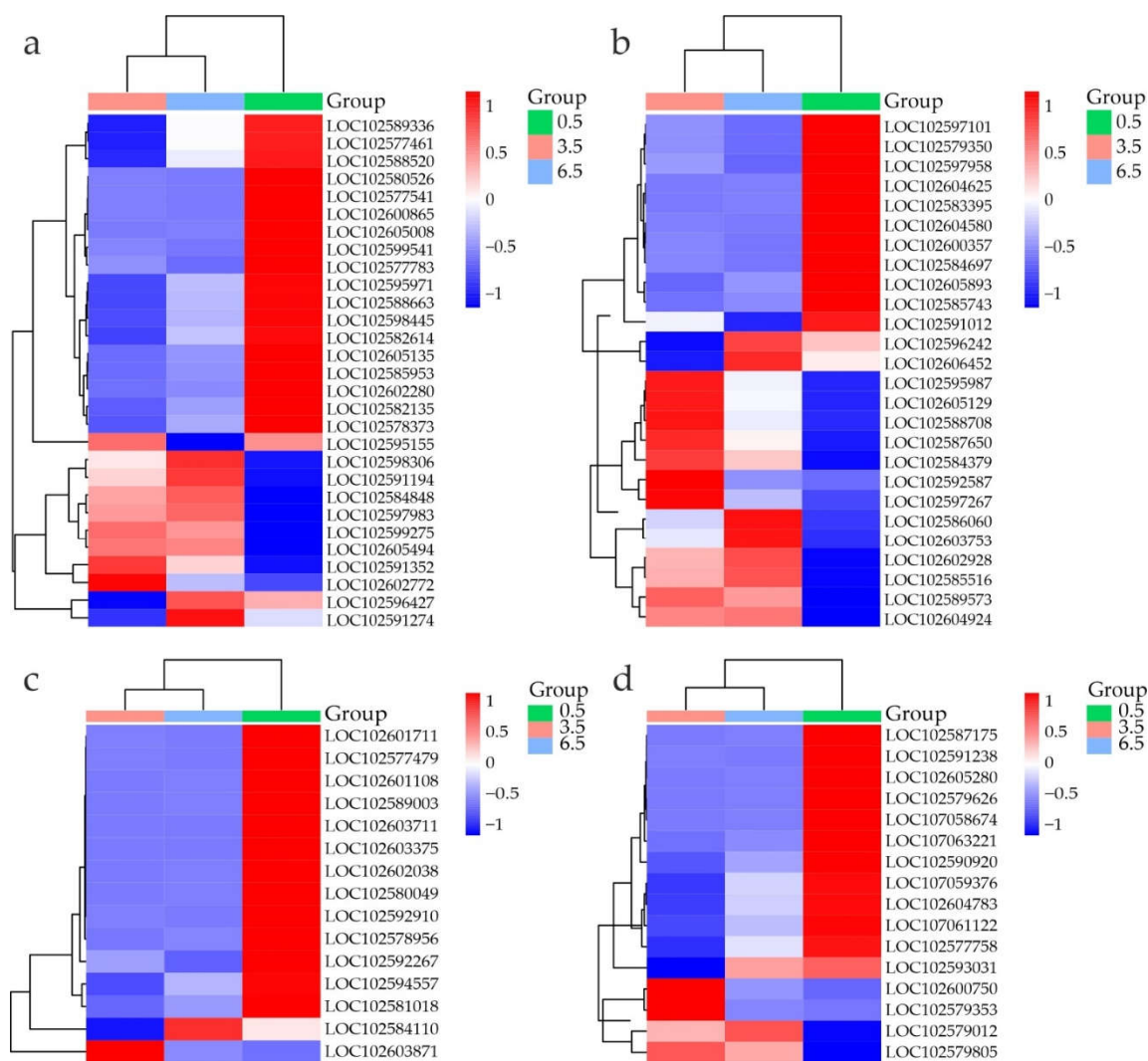


Figure 5. Heatmap of DEGs related to the carotenoid/ABA/SL pathway (a) and GA (b), JA (c), and CK (d) signaling in *S. tuberosum* tubers at 0.5 (green), 3.5 (pink), and 6.5 (blue) months based on transcriptome data (RPKM). The color gradient indicates changes in gene expression from low (blue) to high (red); the names of the proteins corresponding to the LOC numbers are given in the Supplementary Table S12.

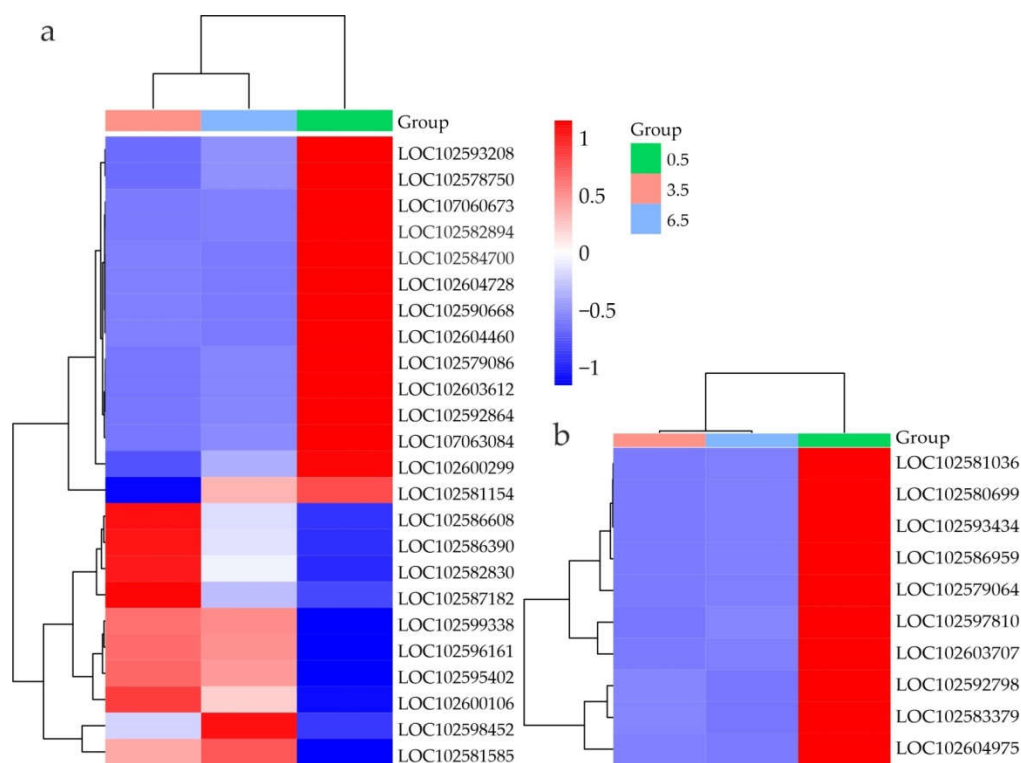


Figure 6. Heatmap of DEGs related to auxin (a) and BR (b) signaling, in *S. tuberosum* tubers at 0.5 (green), 3.5 (pink), and 6.5 (blue) months based on transcriptome data (RPKM). The color gradient indicates gene expression changes from low (blue) to high (red); the names of the proteins corresponding to the LOC numbers are given in the Supplementary Table S12.

Table 2. Phytohormone signaling-related DEGs at 3.5 vs 0.5 months.

Phytohormone	DEGs and their functions
ETH	43 DEGs associated with ETH signaling were identified. The upregulated DEGs encode ETHYLENE-OVERPRODUCTION PROTEIN 1 (ETO1), ETHYLENE INSENSITIVE 3-like (EIN3), 13 ETH-responsive TFs (ERFs), and three ETH receptors homologous to <i>Arabidopsis thaliana</i> ETHYLENE RESPONSE 1 (ETR1), ETR2, and ETHYLENE RESPONSE SENSOR 1 (ERS1); the most upregulated genes were ETH-responsive <i>ERF114-like</i> and <i>RAP2-3</i> (Figure 4b).
ABA, SLs	30 DEGs associated with carotenoid/ABA/SL synthesis/signaling were identified. All DEGs related to carotenoid metabolism, such as <i>NCED1</i> and 3 (9- <i>cis</i> -epoxycarotenoid dioxygenase), <i>AAO</i> (abscisic-aldehyde oxidase), and ABA 8'-hydroxylase genes were downregulated (Figure 5a). <i>CCD8</i> (carotenoid cleavage dioxygenase) associated with SL synthesis [14] was upregulated (Figure 5a), supporting the notion about ABA content decrease. DEGs encoding ABA signaling-related proteins, including ABA receptors (<i>PYL4-like</i> LOC102602772 and LOC102580526, <i>PYL9-like</i> , and <i>PYL8-like</i>), ABA-responsive element-binding factors (<i>ABF</i>), ABA-INSENSITIVE 5-like protein 4 (<i>ABI5-like 4</i>), and sucrose non-fermenting-1-related protein kinases 2 (<i>SnRK2</i>) [29] were either upregulated (<i>PYL4-like</i> LOC102602772, <i>PYL9-like</i> , <i>ABI5-like 4</i> , four <i>SnRK2</i> genes) or downregulated (<i>PYL4-like</i> LOC102580526, <i>PYL8-like</i> , <i>ABF</i> -binding bZIP-TFs, one <i>SnRK2</i>).

JAs	16 DEGs associated with JA synthesis/signaling were identified. DEGs related to JA signaling, such as <i>TIFY</i> (JA ZIM-domain protein), <i>TPL</i> (TOPLESS), and <i>AOS</i> (allene oxide synthase, the first enzyme in JA synthesis), were downregulated, except for one of the nine <i>TIFY</i> genes, <i>TIFY8</i> , which was activated by 2.6-fold (Figure 5c). In response to JA, the F-box protein CORONATINE-INSENSITIVE 1 (<i>COI1</i>) enters the SCF ^{COI1} E3 ubiquitin ligase complex involved in the degradation of JA signaling negative regulators <i>TIFY</i> and represses the JA cascade through interaction with corepressor <i>TPL</i> [30]. The expression of the <i>COI1</i> gene, the only positive regulator of JA signaling among the listed, did not change at 3.5 vs 0.5 months (although it was increased by 1.5 times at 6.5 vs 0.5/3.5 months (Figure 5c), which may be related to the inhibition of non-dormant bud sprouting). Thus, JA signaling was likely downregulated in 3.5-mo tubers because of <i>TIFY8</i> activation and JA synthesis suppression.
GAs	27 DEGs associated with GA synthesis/signaling were identified. The expression of the DEGs encoding GA synthesis enzymes copalyl pyrophosphate synthase (<i>CPS</i>), Ent-kaurene synthase (<i>KS</i>), Ent-kaurene oxidase (<i>KO</i>), GA20 oxidase (<i>GA20ox</i>), GA3 oxidase (<i>GA3ox</i>), and GA2 oxidase (<i>GA2ox</i>) changed in both directions: some (<i>CPS</i> , two <i>GA20ox</i> , one <i>GA3ox</i> , and three <i>GA2ox</i>) were repressed, whereas the other (<i>KS</i> , <i>KO</i> , one <i>GA20ox</i> , and one <i>GA3ox</i>) – activated. The <i>GAI</i> - and <i>RGL1</i> -like genes of DELLA TFs, which negatively regulate GA signaling but activate GA receptor (<i>GID1</i>) [31,32], were downregulated as well as one of the <i>GID1B</i> -like genes, whereas the other gene was upregulated. The expression of most genes that belong to the nuclear factor Y (NF-Y) TF complex and positively regulate GA-mediated dormancy [14,31], including <i>StNF-YA8</i> and <i>StNF-YB20</i> , was increased and that of <i>StNF-YC5</i> – decreased (Figure 5b).
CKs	17 DEGs associated with CK synthesis/signaling were identified. The expression of CK metabolism/signaling genes [14,33] changed insignificantly or was repressed, with the exception of <i>IPT</i> (isopentenyl transferase regulating CK accumulation), two <i>ABCG14-like</i> (regulating CK efflux across the plasma membrane), and <i>ARR17-like</i> (type-A response regulator negatively affecting CK signaling) genes which were activated. One of the CK-responsive genes <i>WUS</i> (<i>WUSCHEL</i> TF maintaining the stem cell niche in the shoot meristem) was strongly upregulated, whereas <i>WUS-related homeobox 8-like</i> (<i>WOX8</i>) was downregulated (Figure 5d).
Auxins	25 DEGs associated with Auxin signaling were identified. Auxin target genes [34] such as two <i>SAUR</i> (<i>SMALL AUXIN UP-REGULATED RNA</i>) and <i>GH3</i> (<i>GRETCHEN HAGEN 3 indole-3-acetic acid-amido synthetase</i>) DEGs were upregulated, whereas four <i>SAUR</i> and five <i>GH3</i> DEGs were downregulated (Figure 6a). <i>ARF5</i> (auxin response factor) and four <i>AUX/IAA</i> (<i>AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE repressor</i>) genes were upregulated, and two <i>IAA</i> genes were downregulated (Figure 6a). Two <i>ARF6</i> -like genes were slightly upregulated (Figure 6a); the upregulation of the <i>ARF6</i> gene in tuber buds during dormancy release has also been shown earlier [35].
BRs	12 DEGs associated with BR synthesis/signaling were identified. Of the five genes encoding important enzymes in the GA biosynthetic pathway (Ent-kaurenoic acid oxidase 2 (<i>KAO2</i>), deetiolated2 (<i>det2</i>), dwarf1 (<i>DWF1</i>), and <i>DWF5</i> [36]), only <i>DWF5</i> expression was significantly changed (upregulated) at 3.5 vs 0.5 months. <i>BIN2</i> (BR receptor BRASSINOSTEROID-

INSENSITIVE 2 kinase [34]), three *WRKY53* and two *BZR1* (BRASSINAZOLE-RESISTANT 1) TF-encoding genes (positive regulators of BR-related processes [37]), and five *EXO* (*EXORDIUM* mediator of BR-promoted growth [38]) genes were downregulated at 3.5 and 6.5 vs 0.5 months (Figure 6b).

Comparison of DEG profiles at different time points (Figures 4–6) revealed that the expression of hormonal signaling-related genes had a turning point at 3.5 months of tuber cold storage. The patterns of DEGs at 3.5/6.5 vs 0.5 months may indicate a decrease in the content of storage proteins and carotenoids and a turn to the opposite mode of phytohormone signaling pathways.

3.3.3. Stress Response

To prevent diseases and sprouting, field-grown tubers are stored at low temperature, which, however, also has negative effects such as cold stress, desiccation, and cold-induced sweetening (CIS) [39].

Master regulators of cold acclimation Dehydration-Response Element-Binding Protein 1/C-Repeat Binding Factors (*DREB1/CBF1-3*) are induced by cold stress and upregulate *COLD-REGULATED* (*COR*) and *RESPONSIVE TO DEHYDRATION* (*RD*) genes. *DREB1* expression is activated by Inducer of CBF Expression 1 (*ICE1*), Calmodulin-Binding Transcription Activator 3 (*CAMTA3*), *CAMTA5*, *REVEILLE 4* (*RVE4*), and *RVE8*, and negatively regulated by Late Elongated Hypocotyl (*LHY*) and Phytochrome-Interacting Factors (*PIFs*). Basic Transcription Factor 3 (*BTF3*) and *BTF3*-like (*BTF3L*) promote *DREB1* stability. *BZR* and Zinc Finger of Arabidopsis thaliana 12 (*ZAT12*) TFs also positively regulate cold-stress tolerance. Low temperature promotes the gene expression and protein stability of Elongated Hypocotyl 5 (*HY5*) TF, causing upregulation of its downstream targets such as anthocyanin biosynthesis enzymes [40]. Prolonged low temperature storage induces genes encoding heat shock proteins (*Hsps*), which may prevent tuber cold damage [41].

At 3.5 and 6.5 vs 0.5 months, the expression of four *DREB1*-like, *RVE8*, *LHY*, *CAMTA3*, *CAMTA4*, *PIF1*-like, and two *BZR*-like genes was downregulated and that of two *COR*-like, *HY5*, and *EARLY RESPONSIVE TO DEHYDRATION 15* (*ERD15*) genes was upregulated. Among 23 *Hsp* DEGs, two (*Hsp21* LOC102604571 and *Hsp70* LOC102579387) were upregulated at 3.5 vs 0.5 months and then downregulated at 6.5 vs 3.5 months, whereas 14 other *Hsp* genes showed an upregulation trend (Supplementary Table S12, Figure 7a).

The *HY5* gene and some genes encoding flavonoid/anthocyanin biosynthesis enzymes, such as *CHS* (chalcone synthase), *CHI* (chalcone isomerase), *F3H* (flavanone 3-hydroxylase), *FLS* (flavonol synthase), *IFR* (isoflavone reductase), *DFR* (dihydroflavonol-4-reductase), *ANR* (anthocyanidin reductase) were upregulated, whereas the other genes such as *F3'5'H* (flavonoid 3',5'-hydroxylase-like), *DFR*, *UFGT2* (anthocyanidin 3-O-glucosyltransferase 2), *UFGT5*, and *UFGT6* were downregulated. DEGs encoding TFs that target the anthocyanin pathway (*ANTHOCYANINLESS 2*, *GLABRA 2*, *GLABRA 1*, *MYB113*, *MYBA1*, *StAN2*, and *StAN1*) were downregulated (Supplementary Table S12, Figure 7b).

CIS is characterized by the accumulation of soluble sugars, which is mostly regulated by the combined activity of granule-bound starch synthase 1 (*GBSS1*), beta-amylases (*BAM*), invertase inhibitors (*INH*), and fructokinases stimulating disaccharide and polyglucan breakdown [41]. At 3.5 vs 0.5 months, *BAM7*, inhibitor of fructosidase 1 (LOC102586828), and fructokinase (LOC102577816) DEGs were upregulated and *BAM1* and *BAM3* – downregulated, whereas two *GBSS*-like and two soluble starch synthase-encoding genes were slightly upregulated (by 1.3–1.6-fold) (Supplementary Table S12, Figure 7c).

Some DEGs related to disaccharide hydrolysis were downregulated (sucrose-phosphate synthase *SPS4*, alkaline/neutral invertase A, beta-fructosidase *PAIN-1*, cell wall invertase *CWINV1*, beta-galactosidases 3 and 4, alpha-galactosidase) and some – upregulated (sucrose synthase *SS16*,

alkaline/neutral invertase D, beta-galactosidase 16, and alpha-galactosidase 3). The following DEGs encoding enzymes involved in polyglucan breakdown (including that of starch) were downregulated (nine glucan endo-1,3-beta-D-glucosidases, beta-glucosidases 11 and 40, alpha-amylase, and alpha-1,4 glucan phosphorylase L-1 isozyme) and the other such genes were upregulated (beta-glucosidases 18, 42 and 46, phosphoglucan phosphatase DSP4, and alpha-glucan phosphorylase type H isozyme) (Supplementary Table S12, Figure 7c).

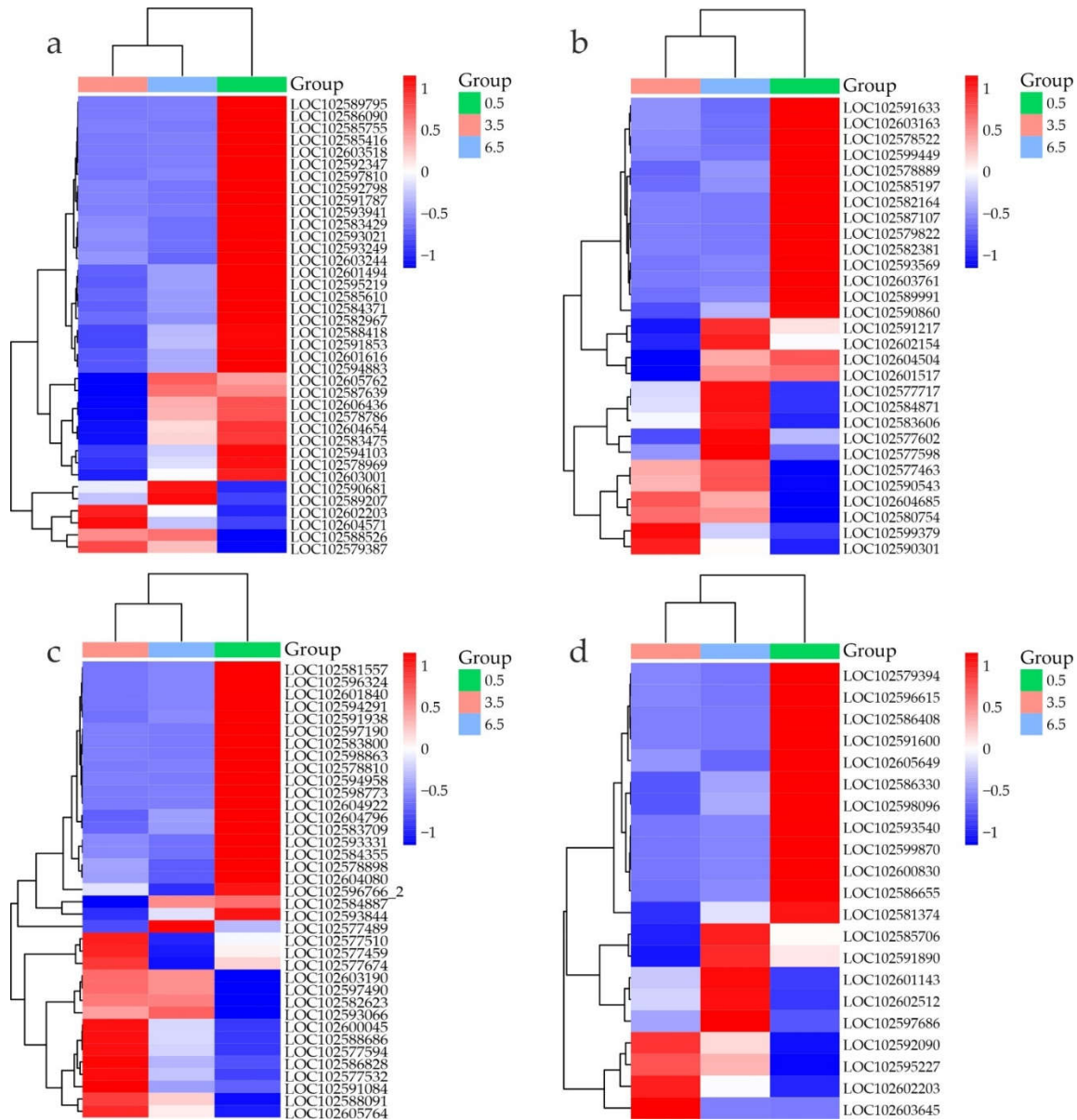


Figure 7. Heatmap of DEGs related to cold response (a), flavonoid/anthocyanin synthesis (b), carbohydrate metabolism (c), and desiccation response (d) in *S. tuberosum* tubers at 0.5 (green), 3.5 (pink), and 6.5 (blue) months based on transcriptome data (RPKM). The color gradient indicates gene expression changes from low (blue) to high (red); the names of the proteins corresponding to the LOC numbers are given in the Supplementary Table S12.

The DEGs strongly upregulated at 6.5 vs 3.5 months encoded BAM1, PAIN-1, beta-galactosidase 8, and beta-glucosidase 11 and those downregulated encoded two GBSS-like, soluble starch synthase LOC102577674, fructokinase, inhibitor of fructosidase 1, alpha-glucan phosphorylase type H isozyme, alpha-1,4 glucan phosphorylase L-1 isozyme, and sucrose synthase (Supplementary Table S12, Figure 7c).

The resistance of long-term stored tubers to desiccation depends on the formation of hydrophobic barriers at the periderm level to reduce water loss, including suberization which directly depends on ABA concentration. The enzymes of the suberin biosynthesis pathway in potato are β -ketoacyl-coenzyme A (CoA) synthase *StKCS6*, cytochrome *CYP86A33*, and feruloyl transferase (*FHT*) [42]. In addition, late embryogenesis abundant proteins (*LEA*) play an important role in the adaptation of tubers to dehydration [43]. *ERD15* negatively affects ABA sensitivity [44], and aquaporin membrane channels maintain water balance [45].

At 3.5 vs 0.5 months, three *LEA*-like DEGs were upregulated and two – downregulated. The expression of the *FHT* suberization gene was upregulated and that of *StKCS6*, *StKCS4*, and *StKCS11* was downregulated. *ERD15* and three aquaporin genes (*TIP2-1* and two *TIP3-2*) were upregulated, whereas eight aquaporin DEGs were downregulated. At 6.5 vs 3.5 months, five aquaporin DEGs (*PIP1-1*, *PIP2-1*, *TIP1-1*, and two *TIP3-2* genes) were strongly upregulated (Supplementary Table S12, Figure 7d). It could be suggested that these DEGs are involved in the regulation of water balance during long-term cold storage and dormancy release of potato tubers.

It should also be noted that at 3.5 vs 0.5 months, most DEGs encoding pathogenesis-related proteins (chitinases, aspartic proteases, glucosidases, thaumatin-like proteins, peroxidases, etc. [46]), as well as most DEGs coding for WRKY-TFs important for plant defense responses [47] were downregulated, except for *WRKY3*, *WRKY5*, *WRKY12*, *WRKY41*, and *WRKY65* related to cold and/or osmotic stresses (drought, salt), which were upregulated (Supplementary Table S3). Three of four detected DEGs for universal stress proteins A-like (USPAs), which are vital for plant survival under stressful conditions [48], were upregulated by 3–5-fold (Supplementary Table S3).

Overall, these results suggest that the detected changes in the expression of genes associated with adaptive responses to low temperature (including *CIS*) and desiccation are associated not only with stress conditions but also with the stage of tuber development – dormancy release (3.5 months) and upcoming sprouting (6.5 months).

3.3.4. Transcription Factors

In search of possible regulators initiating tuber exit from dormancy, we analyzed the DEGs encoding TFs.

At 3.5 vs 0.5 months, some TF families (*BZIP*, *ARF*, *NF-YA*, *GRF*, *C3H*, *TALE*, *LSD*, *Dof*, *HB-other*, *HD-ZIP*, *TCP*, and *WOX*) were enriched with the upregulated DEGs and the other (*ERF*, *GRAS*, *WRKY*, *C2H2*, *AP2*, *CO-like*, *DBB*, *SBP*, *BES1*, *MYB*, *MYB-related*, *HB-other*, *Nin-like*, *TCP*, *BZIP*, *BHLH*, *Trihelix*, and *ZF-HD*) with the downregulated DEGs (Supplementary Table S13).

At 6.5 vs 0.5 months, the *ARF*, *C3H*, *G2-like*, and *HSF* families contained downregulated DEGs. At 6.5 vs 3.5 mo, the *CO-like* and *DBB* TF families were enriched with the upregulated DEGs and families such as *C2H2*, *Trihelix*, *BHLH*, *BZIP*, *HB-other*, *ARF*, *TALE*, *DBB*, *C3H*, *GRAS*, *HD-ZIP*, and *WOX* – with the downregulated DEGs (Supplementary Table S13).

Heatmap analysis revealed distinct TF DEG profiles in the samples, pointing on the differences in the regulation of gene expression between the dormant (0.5 months) and dormancy release (3.5 and 6.5 months) states (Supplementary Table S2, Figure 8).

At 3.5 vs 0.5 months, the expression of 78 TF genes was activated and that of 224 was inhibited by more than 4 times. Both DEG groups were mainly related to abiotic (cold, drought, desiccation) and/or biotic stress responses, flavonoid/anthocyanin biosynthesis, phytohormone signaling, shoot/root development, and meristem/leaf patterning. More detailed information on the functional activity of the identified TF DEGs is presented in Supplementary Tables S14 and S15. These genes can be the subject of future studies on the regulation of dormancy release and sprouting suppression during long-term cold storage of potato tubers.

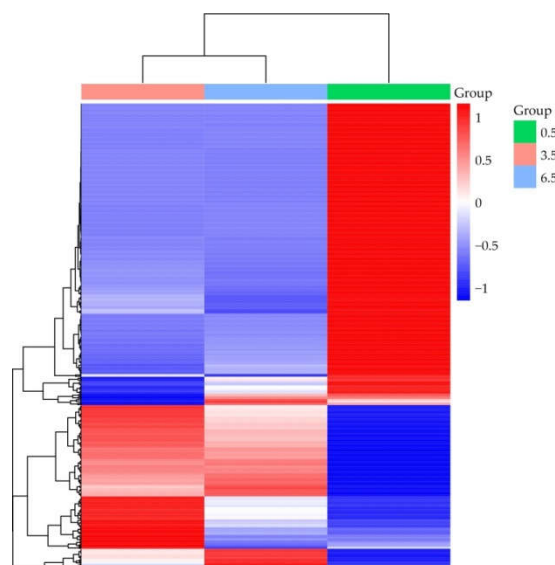


Figure 8. Heatmap of TF DEG expression in *S. tuberosum* tubers at 0.5 (green), 3.5 (pink), and 6.5 (blue) months based on transcriptome data (RPKM). The color gradient indicates gene expression changes from low (blue) to high (red).

Next, we focused on TF DEGs associated with the processes related to dormancy, germination of seeds/buds, flowering, tuber sprouting, and meristem development.

The pool of the meristem-related upregulated (> 4-fold) DEGs included *WUS* homologs which maintain stem cell niche in the shoot apical meristem [49], *ZPR2* which regulates shoot meristem activity [50], *TSJT1* involved in shoot/stem development [51], and *LET6* (*POTH15*) and *HAT3* important for shoot meristem maintenance and leaf development [52].

Examples of the upregulated TF DEGs that may be related to dormancy/release/sprouting include *MFT* homologs playing a role in seed dormancy regulation [53], *NAC25* and *bZIP53* involved in the seed-to-seedling transition regulation [54,55], *AGL21*-like *StMADS23* controlling seed germination and early post-germination growth [56], *StGRF1* involved in potato tuber dormancy release and sprouting, and *StGRF4* and *StGRF9* playing a role in tuber dormancy maintenance [57].

Among the downregulated (> 4-fold) DEGs associated with dormancy/germination, there are *WRKY2* homologs which mediate seed germination [58], *WRKY71* which may be involved in dormancy release in lily bulbs [59], *bHLH92* which reduces seed dormancy [60], and *bZIP53*, *ERF2*, *ERF4*, and *ERF12* which regulate dormancy release and seed germination [55,61–63].

In addition, the genes upregulated more than 4-fold included *COL2* which, together with *COL1*, is one of the most important factors in potato tuberization process, *GI* involved in tuber initiation [6], and an *AGL27* homolog which belongs to the *FLOWERING LOCUS C* (*FLC*) clade of cold-sensitive floral repressors [64]. Considering the similarity of pathways regulating flowering initiation and tuberization in potato [3], we hypothesized that the eye-bud dormancy release may involve related mechanisms.

We made a list of flowering/tuberization-associated TF DEGs which demonstrated significant expression changes (by more than 2-fold or at least ≥ 1.4 -fold for particularly important genes) at 3.5 and/or 6.5 vs 0.5 months (Table 3). Some DEGs in this list were associated with the *FLC*-mediated plant response to vernalization (*FLC*-clade *StAGL27*) and *FLC* expression regulation (*FRI*, *FRL3*, *FRI-ESSENTIAL 1*, and *FLK*), whereas the other DEGs were related to *FLC*-independent flowering/tuberization and regulation of *FT* expression (*AGL19*, *NF-YB3*, *APL*, *BBX19*, and *COL*). The expression of TAC components *StBEL5* and *FD* did not change significantly during tuber storage. The list also included dormancy-associated DEGs (*StMADS11*, *StMADS16*, *StMADS23*, *StSOC1*, *MFT*, *GI*, *LHY*, *HY5*, *AGL62*, *ELF3*, and *ELF4*) (Table 3).

Table 3. Selected TF-encoding DEGs related to flowering/tuberization.

Gene ID	Protein	3.5 vs 0.5	6.5 vs 0.5	Comments
LOC1025850 59	Agamous-like MADS-box protein AGL27	5.3 up	3.1 up	Belongs to the FLC subfamily consisting of FLC, MADS AFFECTING FLOWERING 1 (MAF1, AGL27, or FLOWERING LOCUS M (FLM)), MAF2, MAF3, MAF4, and MAF5; some interact with SHORT VEGETATIVE PHASE (SVP) to inhibit flowering (cold dependent) by directly repressing <i>FT</i> and <i>SOC1</i> transcription [64].
LOC1026039 65	Flowering locus K homology domain-like (FLK)	3.0 up	3.5 up	FLK and FPA promote flowering by suppressing <i>FLC</i> . [65].
LOC1025936 82	Flowering time control protein (FPA)	1.6 up	2.6 up	
LOC1025981 10	FRIGIDA-like 3	2.6 up	3.7 up	
LOC1025882 99	FRIGIDA-like 4a	2.2 up	2.3 up	FRI induces <i>FLC</i> transcription, which is epigenetically repressed by vernalization [64]; FRI-like protein 3
LOC1025975 00	FRIGIDA- ESSENTIAL 1- like	2.1 do	3.7 do	(FRL3) functions in flowering suppression [66]; FRI-ESSENTIAL 1 genetically interacts with FRI and FRI-LIKE 1 to promote <i>FLC</i> expression [67].
LOC1025895 37	FRIGIDA-like (FRI)	2.1 up	2.3 up	
LOC1025933 87	VIN3-like protein 1 (VIL1)	2.3 do	2.0 do	VIL1 participates in both photoperiod and vernalization pathways by regulating <i>FLC</i> and <i>FLM</i> expression. In the vernalization pathway, VIL1, along with VIN3, is necessary for the modifications of <i>FLC</i> and <i>FLM</i> chromatin associated with the epigenetically silenced state and acquisition of competence to flower [68].
LOC1025996 42	Agamous-like MADS-box protein AGL19	4.9 do	4.3 do	AGL19 acts in the FLC-independent vernalization pathway; elevated AGL19 levels result in the activation of <i>LEAFY</i> and <i>APETALA1</i> and flowering [69].
LOC10260194 9	SENSITIVITY TO RED LIGHT	4.8 do	4.3 do	
LOC1026038 64	REDUCED 1-like	4.2 do	-	SRR1 delays flowering by controlling <i>FT</i> repression [70].

LOC1025892 40	Transcription factor TCP8-like	7.9 do	7.2 do	TCP8 upregulation leads to <i>FLC</i> increase [71].
LOC1025887 08	Nuclear transcription factor Y subunit B-3-like	8.2 do	4.1 do	NF-YB3 is essential for flowering induction through regulation of <i>FT</i> expression [72].
LOC1025973 23	MYB family transcription factor APL	136 do	91.7 do	APL promotes flowering through <i>FT</i> activation [73].
LOC1025935 69	TRANSPARENT TESTA GLABRA 1-like	22.7 do	13.8 do	TTG1 can repress the transcription of floral integrators <i>FT</i> and <i>SOC1</i> and activate that of <i>FLC</i> [74].
LOC1026031 63		9.7 do	30.1 do	
LOC1026063 59	B-box zinc finger protein 19-like	4.5 do	-	BBX19 suppresses seed germination via ABI5 induction [75] and interacts with CO to repress FT transcription, thus defining flowering time [76].
LOC1025804 67	MOTHER of FT and TFL1	50 up	15 up	MOTHER-OF-FT-AND-TFL1 (MFT) is seed dormancy-promoting factor [53].
LOC1026062 92	FLOWERING LOCUS T-like (StSP5GB)	2.4 up	-	StSP5G genes are repressors of tuber formation [4]; FT is involved in temperature responses and has an uncharacterized role in the promotion of seed germination [77].
LOC1025791 94	FLOWERING LOCUS T-like (StSP5GA)	1.7 up	-	
LOC1025834 57	GIGANTEA-like	11 up	5.8 up	<i>A. thaliana</i> accessions with <i>gigantea</i> (<i>gi</i>) mutations have significantly increased seed dormancy; the phenotype is shared by <i>constans</i> (<i>co</i>) and <i>flowering locus t</i> (<i>ft</i>) mutants [77]. In potato, <i>GI</i> is involved in flowering and tuber initiation [6].
LOC1025787 19	GIGANTEA-like	2.2 up	-	
LOC1025777 95	Agamous-like MADS-box protein AGL8 homolog (POTM1)	2.4 up	3.8 up	POTM1 (StMADS1) stimulates the transcription of tuberigen <i>StSP6A</i> in a feedback manner [7].
LOC1025913 75	Agamous-like MADS-box protein AGL62	3.4 do	4.2 do	<i>AGL62</i> regulates the transport of auxin (positively associated with dormancy release) from endosperm [78].
LOC1025967 67	Floral homeotic protein AGAMOUS-like	3.7 do	9.2 do	A homolog of AG-clade gene <i>SHATTERPROOF 1</i> (<i>SHP1</i> , <i>AGL1</i>) is upregulated during flower bud endodormancy to ecodormancy transition in <i>Prunus</i> species during chilling [79].

LOC1025776 64	MADS transcriptional factor STMADS11	6.5 do	12.6 do	<i>STMADS11</i> and its homolog <i>STMADS16</i> belong to the <i>SVP/AGL24</i> -clade MADS-box genes, which include <i>DORMANCY-ASSOCIATED MADS-box (DAM)</i> ; DAM/SVP represses the bud dormancy break; <i>DAM</i> expression is decreased during dormancy release in response to prolonged chilling [80].
LOC1025778 49	MADS16 protein	1.4 up	-	expression is decreased during dormancy release in response to prolonged chilling [80].
LOC1025937 15	MADS-box TF 23-like	2.0 do	2.7 do	<i>AGL16</i> homolog directly interacting with <i>SVP</i> and indirectly with <i>FLC</i> ; reduces flowering time [81].
LOC1026016 50	MADS-box protein SOC1- like	3.8 do	1.8 do	<i>SOC1</i> is linked to flowering regulation and plays a key role in bud dormancy transition [82].
LOC1025860 19	MADS-box protein SOC1- like	2.1 do	-	role in bud dormancy transition [82].
LOC1025828 84	MADS-box TF 23-like	8.6 up	3.9 up	<i>AGL21</i> homolog involved in seed germination and early post-germination growth [56].
LOC1025970 46	EARLY FLOWERING 3 (<i>ELF3</i>)	2.1 do	2.1 do	
LOC1025866 41	EARLY FLOWERING 3- like	15.6 do	5.9 do	<i>ELF3</i> and <i>ELF4</i> are negative regulators of flowering time [83] and may have a role in seed dormancy control [84].
LOC1025877 75	<i>ELF4-LIKE 3</i>	1.9 up	2.2 up	
LOC1025776 71	CONSTANS	12.4 do	3.6 do	
LOC1025873 52	zinc finger protein CONSTANS-like	4.7 do	5.9 do	In potato, <i>CO</i> , <i>COL2</i> , and <i>COL1</i> activate anti-tuberigen expression and function as regulators of the tuberization pathway [6].
LOC1025879 05	CONSTANS- LIKE 2	14.0 do	10.1 do	
LOC1025980 89	CONSTANS- LIKE 2-like	2.9 do	6.4 do	
LOC1025850 80	CONSTANS- LIKE 4-like	3.9 do	1.5 do	
LOC1025784 95	CONSTANS- LIKE 4-like	3.1 do	1.4 do	<i>COL4</i> is a flowering repressor [85].
LOC1025828 32	CONSTANS- LIKE 5-like	3.5 do	3.2 do	<i>COL5</i> acts as a flowering inductor [86].
LOC1026000 93	CONSTANS- LIKE 3-like	3.9 do	-	

LOC1025823 39	CONSTANS- LIKE 13	3.2 do	3.7 do	LONG HYPOCOTYL 5 (HY5)-COL3-COL13 regulatory chain functions in hypocotyl elongation [87].
LOC1025885 26	transcription factor HY5	5.1 up	5.4 up	HY5 is positively associated with dormancy release [88].
LOC1026033 07	cyclic dof factor 1	-	1.9 up	StCDF1 represses CO expression, stimulating tuberization [5].
LOC1025976 15	cyclic dof factor 1-like	13–15 RPKM*		
LOC1025774 60	BEL1-related homeotic protein 5	29–65 RPKM*		StBEL5 together with POTH1 positively regulates the expression of <i>StCDF1</i> , <i>StSP6A</i> , and some <i>StSP6A</i> target genes [4,6].
LOC1026025 63	FLOWERING LOCUS D (FD)	7–8 RPKM*	13–20 RPKM*	FD is included in both FAC and TAC [3].
LOC1025935 35	Agamous-like MADS-box protein AGL27			
LOC1026058 12	Truncated TF CAULIFLOWER A-like (MAF4 homolog)	4–6 RPKM*		Flowering repressors [64].
LOC1026052 45	MADS AFFECTING FLOWERING 5- like	14–21 RPKM*		

Note: * indicates insignificant gene expression changes between 0.5-, 3.5-, and 6.5-mo tubers; “up” or “do” – upregulated or downregulated, respectively.

3.3.4. MiRNAs

Four microRNA genes were found to be differentially expressed at 3.5 vs 0.5 months: *MIR172B*, *MIR6023*, and *MIR8047* were upregulated and *MIR482A* – downregulated (Supplementary Table S13).

MiRNA482a belongs to the miRNA482/2118 superfamily; it is specifically involved in GA signaling via phasiRNA-mediated targeting of *GID1* genes and, as such, in seed development [20]. The expression of two DEGs encoding GA receptor *GID1B*-like proteins was upregulated by 5-fold (LOC102604924) or downregulated by 2.2-fold (LOC102579350) (Supplementary Table S13), suggesting them as possible targets of miRNA482a during tuber dormancy maintenance or release.

MiRNA172 targets AP2/ERF-TF genes. In tree peony, miRNA172 targeting of the ETH-responsive *TOE3* gene was shown to suppress bud dormancy release [89]. Among potato DEGs, the expression of the *TOE3*-like gene (LOC102582479) was increased by 2.5-fold at 3.5 vs 0.5 months (indirectly confirming tuber dormancy release), whereas the expression of other AP2/ERF family genes mostly decreased (29 of 39 genes), suggesting their involvement in dormancy maintenance (Supplementary Table S13).

MiRNA6023 mediates potato cold resistance by targeting pectinesterases [90]. In our transcriptomes, the expression of pectinesterase DEGs was either downregulated by 1.4–37.5-fold (or even to zero) (LOC102588428, LOC102584217, LOC102583978, and LOC102605006) or upregulated by 6–7-fold (LOC102598671 and LOC102598327) at 3.5 vs 0.5 months (their expression level at 0.5 months was very low [~ 0.2 RPKM]) (Supplementary Table S13).

MiRNA8047 targets *ARF* genes and is associated with tuber dormancy release [19] which should be accompanied by the activation of auxin signaling [14]. The upregulation of this microRNA at 3.5 vs 0.5 months was accompanied by a 2.4- to 9.7-fold increase in the expression of four *ARF* DEGs (*ARF5* LOC102590158, *ARF17* LOC102588218, *ARF18* LOC102586871, and *ARF18-like* LOC102599257) which are likely responsible for the induction of auxin signaling, and by a 1.4–1.8-fold decrease in the expression of the other four *ARF* genes (*ARF1* LOC102604408, *ARF2* LOC102582348, *ARF7* LOC102594021, and *ARF18-like* LOC102592252) (Supplementary Table S13).

3.4. RT-qPCR Analysis of Selected DEGs

3.4.1. Validation of Transcriptome Data on Selected DEGs

To validate the RNA-seq results, the expression of eight genes in tubers was analyzed by RT-qPCR. Candidate genes were randomly selected among those putatively associated with dormancy release. As shown in Figure 9, the expression dynamics of these genes generally corresponded to the transcriptome data.

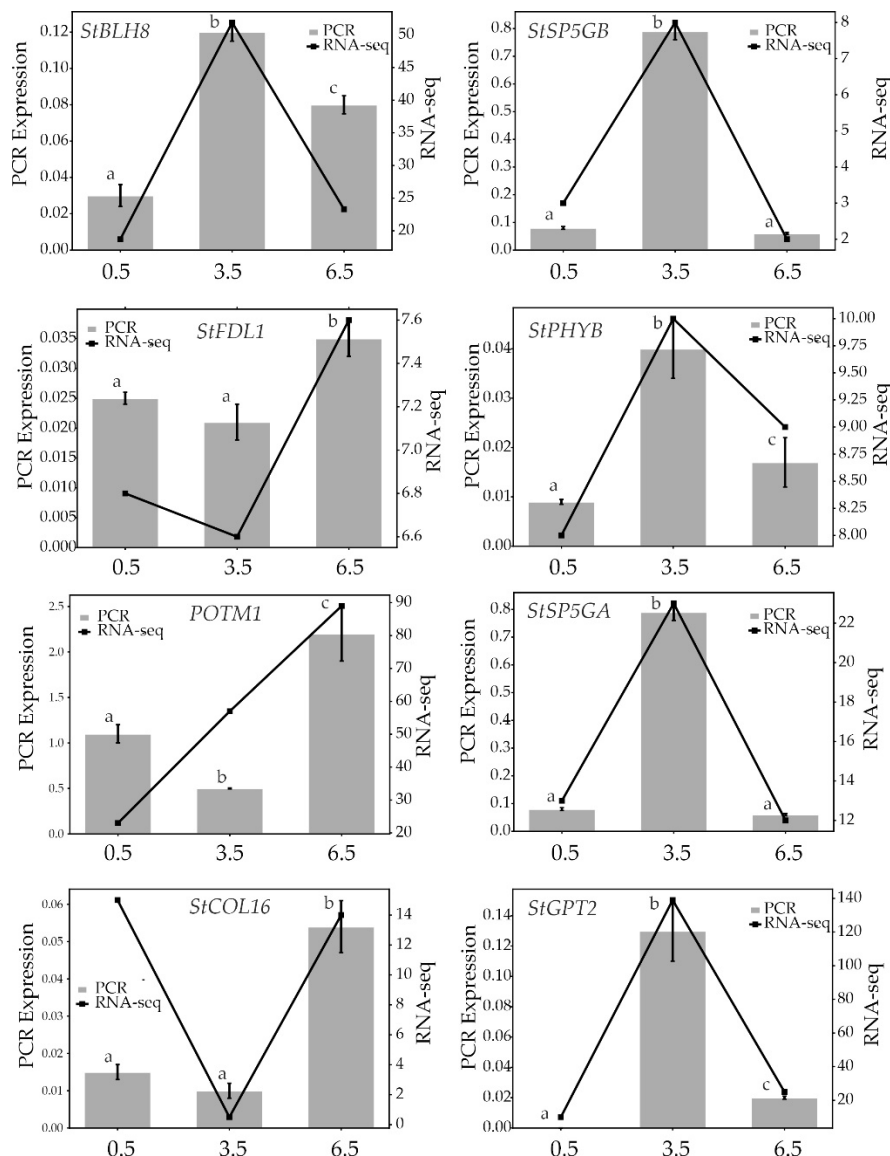


Figure 9. Comparison of RT-qPCR and RNA-seq results on the expression of *StBLH8*, *StSP5GB*, *POTM1*, *StSP5GA*, *StFDL1*, *StPHYB*, *StCOL16*, and *StGPT2* presented as RT-qPCR vs RNA-seq dual Y axis plots. ^{a-c}*P* < 0.05 indicates significant differences in RT-qPCR-data between 0.5-, 3.5-, and 6.5-mo samples.

3.4.2. Expression of Genes Presumably Related to Dormancy Release in Tuber Buds

The expression of 17 selected genes (*StCO*, *StCOL*, *StCOL2*, *StCOL16*, *StPHYB*, *StCDF1*, *POTM1*, *StSP3D*, *StFTL1-1*, *StFTL1-2*, *StSP5GA*, *StSP5GB*, *StGPT2*, *StBLH8*, *FDL1*, and *StFDL*) putatively associated with dormancy release or tuberization was compared in the eye-buds of 6.5-mo tubers (non-dormant, 6.5) and 6.5-mo tubers kept at 20°C for 1 day (6.5-0), 7 days (0.2–1 mm shoots, 6.5-1), and 9 days (1–2 mm shoots, 6.5-2).

All analyzed *FT*-like genes, including tuberigenes (*StSP3D*, *StFTL1-1*, and *StFTL1-2*) and anti-tuberigenes (*StSP5GA* and *StSP5GB*) [4] were upregulated in 6.5-0 vs 6.5 samples. The expression of the main tuberigen *StSP6A* [4] was not analyzed, since the RPKM of the gene in the transcriptomes was less than 1 for all three time points and did not change significantly between them. The two other possible TAC components [3] were either upregulated (*StFDL*) or did not change (*StFDL1*). In 6.5-1 vs 6.5-0 samples, the expression of tuberigenes and anti-tuberigenes continued to increase along with that of *StFDL1* and *StFDL* but then all these genes were downregulated in 6.5-2 vs 6.5-1 samples (Figure 10).

In 6.5-0 vs 6.5 samples, one of potential positive regulators of tuberigenes [4,5,7] (*StBLH8*) was activated, whereas the other were either repressed (*StCDF1* and *POTM1*) or did not change (*StPHYB*). At later points, *POTM1* continued to be repressed, *StBLH8* did not change, and *StCDF1* and *StPHYB* were slightly activated and then repressed (Figure 10).

The expression of possible *CO*-like regulators of tuberization/flowering [4–6] in 6.5-0 vs 6.5 samples was increased (*StCO* and *StCOL2*) or did not change (*StCOL* and *StCOL16*). In 6.5-1 vs 6.5-0 samples, the expression of *StCO* continued to increase but then decreased in 6.5-2 samples, whereas that of *StCOL2* decreased in 6.5-2 vs 6.5-1 samples, and that of *StCOL* and *StCOL16* did not change (Figure 10).

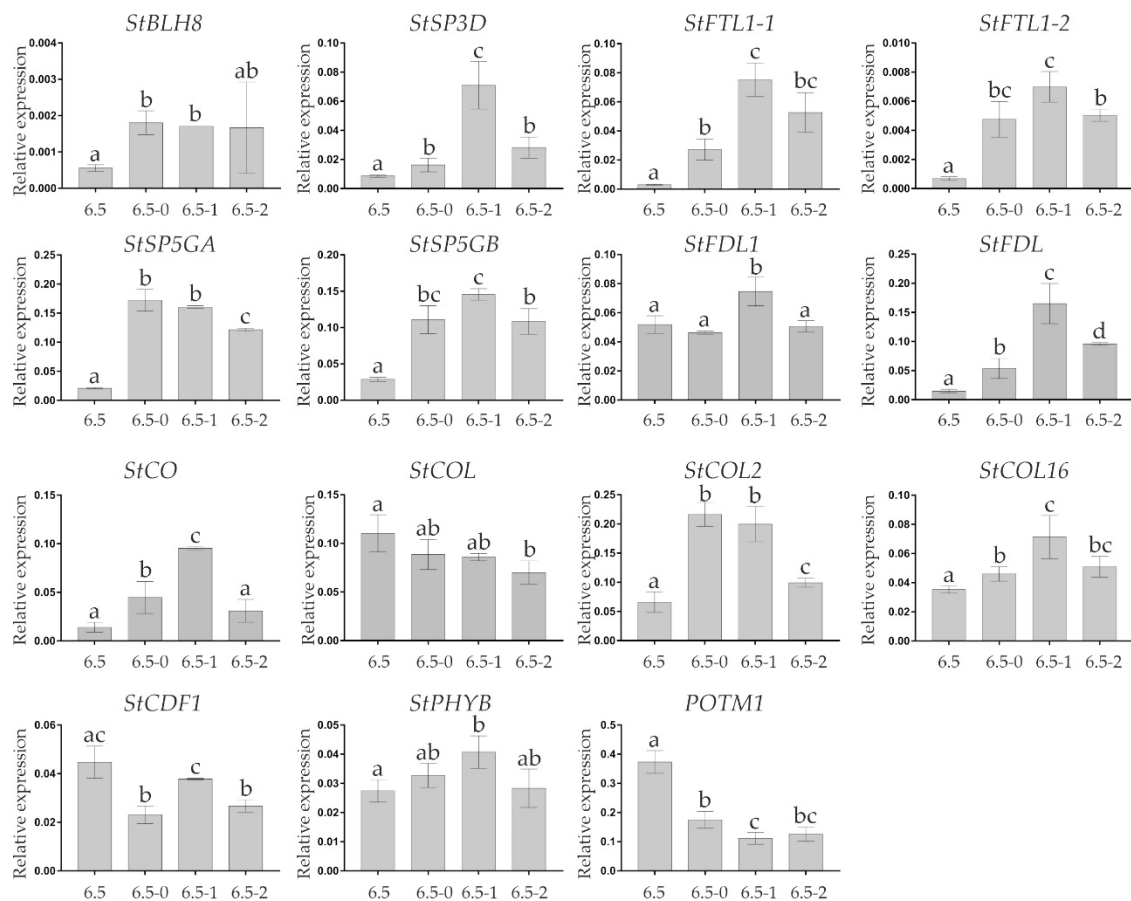


Figure 10. Expression of the *StBLH8*, *StSP3D*, *StFTL1-1*, *StFTL1-2*, *StSP5GA*, *StSP5GB*, *FDL1*, *StFDL*, *StCO*, *StCOL*, *StCOL2*, *StCOL16*, *StCDF1*, *StPHYB*, and *POTM1* genes in *S. tuberosum* non-dormant (6.5) tuber eye-buds and buds in stimulating conditions (6.5-mo tubers after 1 d (6.5-0), 7 d (6.5-1), and 9 d (6.5-2) at 20°C); the letters above the columns indicate the difference in gene expression between the corresponding eye-bud states ($a-dP < 0.05$).

4. Discussion

In potato tubers, the endodormancy state of eye-bud meristems persists from 1 to 15 weeks after harvesting, depending on the genotype and environmental conditions, and is regulated by endogenous signals [10]. Long-term storage of tubers at 2–4°C prevents bud sprouting, disease development, and weight loss but leads to sweetening [12,18,28,41]. An analogue of such storage in the wild is seasonal cold, when tuber endodormancy is maintained, among other things, through molecular pathways triggered by low temperatures [10]. Moreover, prolonged vernalization is necessary for the efficient exit of buds, seeds, or storage organs from dormancy under favorable conditions, as demonstrated for many plant species [10,67,79,80].

If the duration of cold exposure exceeds the tuber genotype-specific dormancy breaking time, endodormancy is likely to be lost during storage, but germination should nevertheless be inhibited by low temperature and darkness. Although the dormancy break is not visually detectable, it can still be monitored at the molecular level owing to significant changes in gene transcription and metabolic indicators [12,15,18,57]. Despite of that, RNA-seq analysis of cold-stored tubers has only been used to study the CIS phenomenon [41], whereas the dormancy release mechanism has been mainly investigated under dormancy exit-promoting conditions, i.e., at the temperature about 20°C [12,15,18,57] or after treatment of tubers with chemical agents [13,15].

In the present study, we attempted to analyze the transcriptomes of potato tubers during storage in the dark at low temperature for 6.5 months in order to clarify the regulatory mechanism(s) of eye-bud dormancy release, especially considering vernalization-mediated pathways. Gene expression patterns were compared at three time points: 0.5 months (endodormancy), 3.5 months (mid-storage), and 6.5 months (end of storage). The mid-point was chosen as the longest possible endodormancy release period (15 weeks [10]).

The considerable changes in gene expression, which we found at 3.5- vs 0.5 months (Figures 1–8), including a significant increase in the expression of *StdUTPase* considered as a marker of tuber bud exit from dormancy [17], confirmed that bud endodormancy had already been broken by the middle of the cold storage period (or even earlier), whereas ecodormancy (suppression of sprouting) had been maintained.

These findings were supported by the results of GO/KEGG analysis, which showed that in 3.5- vs 0.5-mo tubers, the most enriched functional categories of the DEGs were related to transcriptional and translational activation, cell cycle/differentiation, post-translational modifications, phytohormone signaling, and metabolic pathways. (Figure 3, Supplementary Tables S6–11). In 6.5- vs 3.5-mo samples, the DEGs in the enriched GO/KEGG categories were associated with the activation of photosynthesis (Figure 3), which may indicate the upcoming germination of the buds exposed to cold for 6.5 months. This notion is supported by previous results demonstrating the induction of photosynthetic activity in germinating buds [12]. Therefore, the 6.5-mo tubers were used to obtain eye-buds at different stages of sprouting induced at 20°C (6.5-0, 6.5-1 and 6.5-2 samples) for further expression analysis of individual regulatory genes.

The activation of eye-buds after 3.5 months of cold storage was also confirmed by the enrichment of DEGs in functional categories associated with seed dormancy termination and embryo/meristem and reproductive system development (Table 1, Supplementary Tables S6–9). Multiple changes at 3.5 vs 0.5 months in the expression of genes related to hormonal signaling, cell division and cell cycle activity, storage proteins, carbohydrate, carotenoid/flavonoid metabolism, and stress response (Figures 4–7, Table 2, Supplementary Table S12) were consistent with previous studies on dormancy-released potato tubers [12,14,15,18,57].

Analysis of ETH-associated genes revealed transcriptional repression at 3.5 vs 0.5 months (Figure 4b, Table 2, Supplementary Table S12), indicating a decrease in ETH-mediated signaling, which is characteristic for non-dormant tuber buds [14].

Most DEGs related to the biosynthesis of carotenoids and their by-products ABA/SLs [14] were downregulated, suggesting a decrease in the ABA content. At the same time, only some genes of the ABA signaling pathways were downregulated, whereas the others were upregulated (Figure 4a, Table 2, Supplementary Table S12). Nevertheless, given that carotenoid biosynthesis is positively correlated with the ABA content [28], there could be a decrease of the ABA level in 3.5-mo tubers, suggesting broken dormancy. In this case, the decrease in the expression of genes involved in JA synthesis and signaling (Figure 5c, Table 2, Supplementary Table S12) suggests negative association between JA content and dormancy release, which is consistent with the observation that JAs inhibit potato tuber sprouting and MeJA maintains dormancy [14].

The reduction of ABA levels activates hormones that promote dormancy break, such as GA; thus, a high ABA/GA ratio corresponds to dormancy and low – to sprouting [14]. Given that transcriptional changes in the genes encoding enzymes of GA biosynthesis and its positive regulators were both positive and negative (Figure 5b, Table 2, Supplementary Table S12), it is unclear whether GA production was activated in tubers at 3.5 vs 0.5 months. However, the suggested decrease in the ABA content should in turn decrease the ABA/GA ratio and promote dormancy break.

The expression of genes involved in the metabolic and signaling pathways of CK, another dormancy-breaking hormone [14,33], changed insignificantly or was repressed (Figure 5d, Table 2, Supplementary Table S12). One of the few exceptions was the upregulation of the CK-responsive *WUS* gene at 3.5 vs 0.5 months (Table 2), which may correspond to the activation of meristem development in tuber buds.

Among the DEGs of auxin-mediated signaling, which is positively associated with tuber dormancy release [14], two *ARF6*-like genes were slightly upregulated (Figure 6a, Table 2, Supplementary Table S12). This finding is consistent with a previous study showing *ARF6* activation in tuber buds during dormancy release [35].

Considering the role of BRs as key modulators of potato tuber sprouting [14], the decreased expression of BR-related DEGs at both 3.5 and 6.5 months vs 0.5 months (Figure 6b, Table 2, Supplementary Table S12) suggests suppression of BR signaling, probably until bud germination. The repression of BR pathway-related genes during tuber dormancy release has also been reported earlier [12,36].

The proximity of the 3.5-month time point to the moment of tuber transition from the dormant to non-dormant state was also confirmed by the sharply changed dynamics of the expression of regulatory TF genes (Figure 8). Given the similarity of the mechanisms controlling flowering initiation and tuber induction [3], we hypothesized that the tuber exit from dormancy could be regulated in a similar way but with the adjustment for the vernalization effect.

Previous transcriptomic studies of tuber dormancy break under break-promoting conditions have identified several TF genes possibly involved in the regulation of this process: *GRF3*, *GRF4*, *GRF10* (*GRF4*-like), and *StMADS16* [12], *POTM1* (*AGL8*-like MADS-box gene PGSC0003DMT400010451) [13], and *SVP*, *ASYMMETRIC LEAVES 1 (AS1)*, *SHOOT MERISTEMLESS (STM, POTH15)*, and *MONOPTEROS (ARF5)* [36]. We found that at 3.5/6.5 vs 0.5 months, the expression of *POTM1*, *POTH15*, *ARF5*, *GRF4*, and *GRF10* was upregulated and that of *AS1* – downregulated, whereas that of *GRF3*, *SVP*-like *StMADS11*, and *StMADS16* did not change (Supplementary Table S13). Our results confirm possible involvement of these TFs in the regulation of dormancy release in potato tubers.

In addition to the TF genes mentioned above, *HY5* and *AGL21*-like *StMADS23* were upregulated at 3.5 vs 0.5 months. These genes are positively associated with dormancy breaking and sprouting in several plant species through transcriptional control of the corresponding targets [56,88]. Other significant genes found included upregulated *MFT* and downregulated two *SOC1*-like genes which promote dormancy [53,82], upregulated *GRF1* and *GRF9* involved in dormancy break and

maintenance [57], induced *GI* genes positively associated with potato flowering/tuber initiation [4,6] and *A. thaliana* seed dormancy release [77], downregulated *StCO*-like genes, which repress potato tuberization [4,6] and promote *A. thaliana* seed dormancy break [77], upregulated *NAC25*, downregulated *bZIP53*-like, and three differentially regulated miRNA genes (*MIR482A*, *MIR172*, and *MIR8047*) regulating bud dormancy/sprouting [19,20,54,55,89]. Considering that these genes are associated (positively or negatively) with dormancy break, their expression dynamics at 3.5 vs 0.5 months (Table 3, Supplementary Table S13) provide evidence of the tuber exit from endodormancy.

The effect of vernalization on the regulation of the tuber eye-bud state may be associated with *CBF1* and *DAM/SVP* genes, whose expression is rapidly and sequentially activated by short-term chilling, thus promoting dormancy. After prolonged exposure to cold, *DAM/SVP* expression is suppressed, leading to the upregulation of *CO* and *FT* genes and, consequently, dormancy exit. This effect has been demonstrated in perennial tree buds in response to seasonal cold [10].

Another group of TF genes, which constitute the *FLC* clade, downregulate the transcription of *FT* and *SOC1* genes at low temperatures and may play a role in inhibiting the germination of non-dormant meristems [64,91].

In view of the data described above, our finding that *CBF1* and *SVP*-like genes (*StMADS11* and *StMADS16*) were downregulated at 3.5 vs 0.5 months may indicate a non-dormant state of the eye-buds, whereas the simultaneous increase in the expression of *FLC*-like gene *StAGL27* may be consistent with the lack of sprouting of non-dormant meristems (Table 3, Supplementary Tables S13–S15).

Among the *SVP* and *FLC* target genes, most *StCO*-like and two *StSOC1* genes were downregulated, *FT*-like anti-tuberigenes upregulated, and *FT*-like tuberigenes remained unchanged at 3.5 vs 0.5 month (Table 3, Supplementary Tables S13–S15). This transcriptional profile suggests that the changes in the expression of potato *CO* and *SOC1* genes and *FT* tuberigenes are associated not with the exit of buds from dormancy but rather with the induction of germination in non-dormant buds. At the same time, the increase in the expression of *FT*-like anti-tuberigenes *StSP5GA* and *StSP5GB* may suggest their participation in the suppression of premature germination of meristems in cold conditions.

Given that in *Arabidopsis*, *FT* plays a role in temperature responses and promotes seed germination [77], it is reasonable to suggest that in potato, which has several *FT* homologs [4], the eye-bud germination may be finely regulated through joint activity of tuberigenes (stimulators) and anti-tuberigenes (repressors). This notion is supported by the RT-qPCR analysis showing that under favorable conditions (20°C), not only anti-tuberigenes but also other *FT*-like potato genes were activated in non-dormant buds (Figure 10).

More TF genes involved in the control of flowering/tuberization timing [3,4,6] were also differentially expressed at 3.5 vs 0.5 months (Table 3, Supplementary Tables S13–S15); they can be considered to play a role in the initiation of tuber bud dormancy release as secondary regulators directed by CBF-SVP-FLC.

Other two important genes, including *StBEL5* which together with *POTH1* upregulates *StCDF1* expression, and *StFDL1* which is a component of FAC and TAC [3–6], were expressed at a relatively constant level throughout storage (0.5–6.5 months) (Table 3, Supplementary Tables S13–S15). Thus, despite the supposed importance of these genes in the control of tuber dormancy release, they may not exert a decisive effect but may coordinate eye-bud germination. For example, in 6.5-0 and 6.5-1 vs 6.5-mo samples, the expression of *StCDF1* (*CO* suppressor [5]), and *POTM1* (*StCDF1* activator [7]) was decreased, whereas that of *StFDL1*, *StFDL*, *StCO*, *StCOL2*, and *StCOL16*, potential positive regulators of germination, was increased (Figure 10). Furthermore, the upregulated expression of *StBLH8* (Figure 10), a *POUND-FOOLISH* homolog involved in the maintenance of the shoot apical meristem [92], may indicate progression of the eye-bud meristem development during germination.

5. Conclusions

RNA-seq of potato tubers during long-term cold storage (0.5–6.5 months) revealed that the gene expression profiles at 3.5/6.5 months differed significantly from that at 0.5 months, suggesting possible transcriptional and translational activation and stimulation of cell growth and development, metabolism, and hormonal signaling. These expression changes may reflect the release from dormancy and meristem activation and at the same time, the suppression of bud sprouting due to low temperature. Among the DEGs, a number of TF genes associated with dormancy were identified, including those regulating the vernalization pathway (*StCBF1*, FLC-clade *StAGL27*, and SVP-like *StMADS11* and *StMADS16*), initiation of flowering and/or tuber formation (CO-like *StCO* and *StCOLs*, and FT-like *StSP3D*, *StFTL1-1*, *StFTL1-2*, *StSP5GA*, and *StSP5GB*), flowering, and/or dormancy (two *SOC1*-like genes, AGL8-like *POTM1*, *StMFT*, and STM-like *POTH15*). Four miRNA genes were also differentially expressed at 3.5 vs 0.5 months: *miRNA172b*, *miRNA482a*, and *miRNA8047* associated with dormancy and *miRNA6023* associated with cold stress response. Changes in the expression of *StCO*, *StCOL*, *StCOL2*, *StCOL16*, *StPHYB*, *StCDF1*, *POTM1*, *StSP3D*, *StFTL1-1*, *StFTL1-2*, *StSP5GA*, *StSP5GB*, *StGPT2*, *StBLH8*, *FDL1*, and *StFDL* genes in sprouting versus non-dormant tuber eye-buds suggest that these genes, jointly with those of the vernalization pathway, may play an important role in the finely tuned regulation of bud dormancy exit and sprouting during long-term cold storage. The set of differentially expressed TF genes (78 activated and 224 repressed by more than 4 times) which were identified in non-dormant vs dormant buds can be considered as regulators of the dormancy break and upcoming germination of potato tuber eye-buds during long-term cold storage and could form a basis for future research.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Table S1: List of primers for RT-qPCR analysis of *Solanum tuberosum* gene expression. Table S2: List of genes expressed in 0.5, 3.5 and 6.5 mo potato tubers. Table S3: List of genes differentially expressed at 3.5 vs 0.5 mo. Table S4: List of genes differentially expressed at 6.5 vs 0.5 mo. Table S5: List of genes differentially expressed at 6.5 vs 3.5 mo. Table S6: GO categories enriched in upregulated DEGs at 3.5 vs 0.5 mo. Table S7: GO categories enriched in downregulated DEGs at 3.5 vs 0.5 mo. Table S8: GO categories enriched in upregulated DEGs at 6.5 vs 0.5 mo. Table S9: GO categories enriched in downregulated DEGs at 6.5 vs 0.5 mo. Table S10: GO categories enriched in upregulated DEGs at 6.5 vs 3.5 mo. Table S11: GO categories enriched in downregulated DEGs at 6.5 vs 3.5 mo. Table S12: Selected DEGs related to the pathways of storage proteins, phytohormones, cold response, flavonoids, carbohydrates and desiccation response. Table S13: List of DEGs of transcription factors. Table S14: DEGs of transcription factors upregulated more than 4 times. Table S15: DEGs of transcription factors downregulated more than 4 times.

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