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Research Paper

Transcriptomic analysis of developing seeds in a wheat (*Triticum aestivum* L.) mutant RSD32 with reduced seed dormancy

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Seed dormancy, a major factor regulating pre-harvest sprouting, can severely hinder wheat cultivation. Reduced Seed Dormancy 32 (RSD32), a wheat (*Triticum aestivum* L.) mutant with reduced seed dormancy, is derived from the pre-harvest sprouting tolerant cultivar, 'Norin61'. RSD32 is regulated by a single recessive gene and mutant phenotype expressed in a seed-specific manner. Gene expressions in embryos of 'Norin61' and RSD32 were compared using RNA sequencing (RNA-seq) analysis at different developmental stages of 20, 30, and 40 days after pollination (DAP). Numbers of up-regulated genes in RSD32 are equivalent in all developmental stages. However, down-regulated genes in RSD32 are more numerous on DAP20 and DAP30 than on DAP40. In central components affecting the circadian clock, homologues to the morning-expressed genes are expressed at lower levels in RSD32. However, higher expressions of homologues acting as evening-expressed genes are observed in RSD32. Homologues of Ca^{2+} signaling pathway related genes are specifically expressed on DAP20 in 'Norin61'. Lower expression is shown in RSD32. These results suggest that RSD32 mutation expresses on DAP20 and earlier seed developmental stages and suggest that circadian clock regulation and Ca^{2+} signaling pathway are involved in the regulation of wheat seed dormancy.

Key Words: mutant, seed development, seed dormancy, transcriptome, wheat.

Introduction

Wheat, similarly to rice and maize, is a major crop worldwide that is crucially important for world food supplies. Pre-harvest sprouting, which is triggered by continuous rainfall during seed development, occurs as seed germination on mother plants. This germination decreases seed quality and causes extensive economic damage to cultivation efforts. Seed dormancy is a major regulating factor affecting pre-harvest sprouting. Seed dormancy, which inhibits seed germination under favorable conditions, particularly of temperature and moisture, occurs after completion of seed maturation (Gubler *et al.* 2005). Therefore, enhancing seed dormancy is an important breeding objective for avoiding pre-harvest sprouting damage.

Seed dormancy begins and develops during seed maturation. The embryo axis and scutellum differentiate and grow extensively at an early developmental stage (5–15 days after pollination: DAP5–DAP15) in wheat (Noda *et al.* 1994). Between DAP15 and DAP20, the embryo dry weight and embryo axis length increase rapidly. The length reaches its maximum at DAP30. The embryo appears to be fully differentiated at DAP30. The endosperm fresh and dry weight reach maximum values at DAP30. Seed development is completed at the middle developmental stage (DAP15–DAP30). At the late developmental stage (DAP30–DAP50), seed moisture contents decrease. Endosperms reach the hard dough stage. Seeds desiccate and change color from yellow to brown. Seed dormancy develops during seed desiccation in the late developmental stage.

A phytohormone, abscisic acid (ABA), plays an important role in controlling seed dormancy. In *Arabidopsis*, many seed-dormancy mutants have been isolated. Results of earlier studies have shown that ABA biosynthesis, catabolism, and sensitivity are involved in regulating seed dormancy (Finkelstein *et al.* 2002, Himmelbach *et al.* 2003, Kushiro *et al.* 2004, Nambara and Marion-Poll 2003, Okamoto *et al.* 2006, Saito *et al.* 2004, Seo *et al.* 2006). *DELAY OF GERMINATION 1* (*DOG1*) has been identified as a quantitative trait locus (QTL) controlling the natural variation of seed dormancy in *Arabidopsis* (Bentsink *et al.* 2006). In fact, *DOG1* interacts with ABA signaling pathway through type 2C protein phosphatases *ABA-HYPERSENSITIVE GERMINATION 1* (*AHG1*) and *AHG2* (Née *et al.* 2017, Nishimura *et al.* 2018). Seed maturation regulators *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *FUSCA3* (*FUS3*) and *ABA INSENSITIVE 3* (*ABI3*) are also involved in the regulation of seed dormancy (Giraudat *et al.* 1992, Kagaya *et al.* 2005, Kroj *et al.* 2003, Lotan *et al.*

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1998, Luerßen *et al.* 1998, Stone *et al.* 2001, To *et al.* 2006). These genes express at the early to late developmental stages of seed in *Arabidopsis*. In monocot species, *MOTHER OF FT AND TFL1* (*MFT*) and *MAP KINASE KINASE* in wheat (Nakamura *et al.* 2011, Torada *et al.* 2016), *SDR4* in rice (Sugimoto *et al.* 2010), and *ALANINE AMINOTRANSFERASE* (*AlaAT*) in barley (Sato *et al.* 2016) have been identified as QTLs regulating seed dormancy. Rikiishi *et al.* (2010) reported that *TaABF1* related with ABA signaling pathway regulates intervarietal variation of seed dormancy in wheat cultivars. Several genes regulating seed dormancy have also been identified in wheat. These genes express at the late seed developmental stage. However, wheat genes homologous to *DOG1* and seed maturation regulators are expressed at the early to middle seed development stage (Rikiishi and Maekawa 2014). The appropriate time for expression differs depending on the regulator gene. These results indicate that different regulatory systems for seed dormancy regulation function at each developmental stage.

Reports of the literature describe that ABA signaling is connected with and integrated with other signaling pathways. Calcium ion acts as a second messenger. In fact, calcium signals in plants are involved in several stress responses to cold, drought, salinity and light (Dodd *et al.* 2010, Zhang *et al.* 2014). The Ca^{2+} signaling pathway is initiated with the acceptance of Ca^{2+} signals by sensor proteins. Plant Ca^{2+} sensors belong to three families (Edel and Kudla 2015, Zhu *et al.* 2015). Calmodulins (CaMs) and CaM-like proteins (CMLs) are grouped in one family. The second family includes calcineurin B-like proteins (CBLs) that specifically activate CBL-interacting protein kinases (CIPKs). The third family includes Ca^{2+}-dependent protein kinases (CDPKs), which have a kinase domain and a Ca^{2+} sensor domain. Sensor proteins accepting Ca^{2+} signals are decoded to downstream responses. Because Ca^{2+} sensor proteins affect ABA sensitivity, Ca^{2+} signaling cooperatively regulates the response to stresses with the ABA signaling pathway (Chen *et al.* 2017, Edel and Kudla 2016, Jiang *et al.* 2013, Midhat *et al.* 2018, Wang *et al.* 2018, Zhao *et al.* 2011). Several reports have described functions of circadian-clock-related genes on ABA sensitivity and dormancy release (Adams *et al.* 2018, Footitt *et al.* 2017, Lee *et al.* 2016, Penfield and Hall 2009, Seung *et al.* 2012). The circadian clock regulates the gene expressions and physiological responses corresponding to a daily cycle of light and darkness. In *Arabidopsis*, *LATE ELONGATED HYPOCOTYL* (*LHY*), *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *TIMING OF CAB EXPRESSION1*/*PSEUDO-RESPONSE REGULATOR* (*TOC1*/*PRR*), *EARLY FLOWERING* (*ELF*) and *LUX ARRHYTHMO* (*LUX*) are involved in the central cores of the circadian clock (Seung *et al.* 2012). These genes encode transcription factors or proteins forming complexes with transcription factor and construct a complex system with feedback loop regulation. Circadian rhythms oscillate based on interactions between morning-expressed *LHY* and *CCA1* and evening-expressed *TOC1*. Circadian clock components interact with various transcription factors such as *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED 1* (*LNK1*), *FAR-RED IMPAIRED RESPONSE 1* (*FAR1*), *PHYTOCHROME INTERACTING FACTOR*s (*PIF*s), *REVEILLE*s (*RVE*s), and *CONSTANS-LIKE* (Gray *et al.* 2017, Ledger *et al.* 2001, Nusinow *et al.* 2011, Ritter *et al.* 2017, Xing *et al.* 2015). Furthermore, circadian clock genes regulate cytosolic Ca^{2+} influx and the signaling pathways of ABA and Ca^{2+}, suggesting that regulatory network integration is necessary for circadian-clock-related fundamental processes in plant growth and development (Dodd *et al.* 2005, 2007, Martí Ruiz *et al.* 2018, Xu *et al.* 2007).

Rikiishi and Maekawa (2010) produced a wheat (*Triticum aestivum* L.) mutant with reduced seed dormancy from 'Norin61', a pre-harvest sprouting tolerant cultivar. Reduced Seed Dormancy 32 (RSD32) was found to be a seed-specific and single-recessive mutation (Kobayashi *et al.* 2006, Rikiishi and Maekawa 2010). Expressions of several transcription factors involved in the regulation of wheat seed dormancy, such as *TaDOG1* and *TaABF1*, were decreased in embryos of RSD32, suggesting RSD32 as an important factor for the regulation of seed dormancy in wheat.

In this study, gene expressions in embryos of 'Norin61' and RSD32 were analyzed using RNA-seq for investigating the regulatory networks of wheat seed dormancy associated with RSD32. Expression profiles were compared at three developmental stages: DAP20, DAP30, and DAP40. Results demonstrate that RSD32 mutation exhibits superior inhibitory effects on gene expression in embryos on DAP20 and DAP30. In embryos of RSD32, homologous genes of circadian clock and Ca^{2+} signaling pathway related genes were expressed differently compared to 'Norin61'. RSD32 is a regulatory factor for wheat seed dormancy expressed at the middle developmental stage. Reduced seed dormancy in RSD32 might result from aberrant signals of the circadian clock and Ca^{2+}.

Materials and Methods

Plant materials and growth conditions

This study used a pre-harvest sprouting tolerant wheat cultivar, 'Norin61', and a mutant RSD32 with reduced seed dormancy selected from M_4 population. They were derived from mutagenized 'Norin61' seeds using NaN_3 treatment (Rikiishi and Maekawa 2010). Seeds were sown in plastic trays for 4 weeks: later, 20 seedlings were transplanted to the field in each line with 20 cm between plants and 90 cm between rows. Plants were grown under a plastic roof to avoid rainfall. Spikes were tagged at anthesis. Seeds were harvested every 10 days from 10 days after pollination (DAP10) to DAP70 and were used in germination tests and RNA-seq analysis. To minimize variation, seeds were collected only from primary and secondary florets of the center spikelets.

Germination test

Ten whole seeds were placed on filter paper in a Petri dish containing 6 ml of distilled water. Seeds were cut transversely into halves. Then ten half seeds with involved embryos were placed in a Petri dish containing 6 ml of distilled water with or without (±) 10 μM of ABA (Sigma Chemical Co.). The Petri dishes were then incubated in the dark at 20°C. All germination tests used three replications. Germinated seeds were counted daily for 14 days. A weighted germination index (GI) was calculated to give maximum weight to seeds that germinated first and to give less weight to those that germinated subsequently, as described by Walker-Simmons and Sesing (1990). The GI values were converted into arcsine-transformed values. They were used for statistical analyses.

RNA isolation, library preparation and sequencing

Embryos of 'Norin61' and RSD32 were collected from 10:00 to 12:00 on the same day. Three embryos from a single panicle were used for total RNA isolation. On DAP20, DAP30, and DAP40 panicles were harvested from three individual plants. Each plant was treated as biological replication. Total RNA was isolated using a commercial kit (FastRNA Pro Green; Qbiogene Inc.). Isolated RNAs were purified (RNA Clean-up Kit; TaKaRa Bio Inc., Tokyo, Japan). All kits were used according to the respective manufacturers' protocols. The concentrations of total RNA samples were quantified using a spectrophotometer (Nano Drop ND-1000; Thermo Fisher Scientific Inc., Waltham, MA, USA). The total RNA sample quality was also verified (Agilent 2100 Bioanalyzer; Agilent Technologies Inc., Santa Clara, CA, USA). The 18 RNA samples (2 lines × 3 stages × 3 biological replications) were sequenced. Library construction and sequencing for the Illumina HiSeq 2500 were provided as a custom service of Eurofins Genomics K.K. (Tokyo, Japan). After the polyA fraction (mRNA) was isolated from total RNA, it was fragmented. Then double-stranded (ds) cDNA was reverse-transcribed from the fragmented mRNA. The ds cDNA fragments were processed for adaptor ligation, size selection (for 200 bp inserts), and amplification to generate strand-specific cDNA libraries. Prepared libraries were subjected to paired-end 2 × 125 bp sequencing on the HiSeq 2500 platform with v4 chemistry.

Bioinformatics analysis

We analyzed RNA-seq read data using RNA analysis tools in Galaxy/NAAC (https://galaxy.dna.affrc.go.jp/). Raw reads were obtained in Fastq format and were assessed for quality using FastQC. Terminal low-quality bases and adaptor sequences were trimmed off (Trimmomatic; Usadel Lab, Aachen, Germany). Clean reads were aligned against wheat survey sequence v3.0 obtained from the International Wheat Genome Sequencing Consortium (IWGSC) using TopHat2 with default parameters (Kim *et al.* 2013a, Trapnell *et al.* 2012). Cufflinks was used to assemble mapped reads. The resulting transcripts were used to quantify the expression of each gene in fragments per kilobase of transcript per million mapped reads (FPKM) units. Cuffdiff was subsequently used to compile a list of differentially expressed genes (DEGs) with fold change ≥3 and *P*-value ≤0.01. BLASTX was used to align genes against the National Center of Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/tools/blast), Wheat Genetic Resources Database (https://shigen.nig.ac.jp/wheat/komugi/blast/blast.jsp), and Barley BioResources Database (http://earth.nig.ac.jp/~dclust/cgi-bin/barley_pub/blast_search.html). The e-value cutoff was set at $1e^{-5}$. Gene names were assigned to each gene based on the top Blastx hit with the highest score. RStudio (https://rstudio.com/) was used for comparing DEGs in three developmental stages.

Results

Growth, seed dormancy and ABA responsibility of RSD32 mutant

Anthesis was observed from 27 April through 16 May in RSD32, similarly to wild type, 'Norin61' (**Supplemental Fig. 1**). Morphological traits of panicles and seeds were not different between 'Norin61' and RSD32 (**Supplemental Fig. 2**). Although heading time and seed development affect the degree of seed dormancy, no disorders of these traits were observed in RSD32.

'Norin61' showed low germination indices (GIs) of whole seeds obtained at DAP50 and earlier stages. Strong seed dormancy was observed (**Fig. 1**). The GIs of half seeds, which were released from dormancy, were significantly lower with ABA treatment until DAP50. 'Norin61' showed sensitivity to ABA on seed germination. Seed dormancy and ABA sensitivity were lost after DAP60 in 'Norin61'. However, RSD32 showed significantly higher GIs of whole seeds on DAP40 (50.0) and on DAP50 (90.5) than those in 'Norin61', although similar GIs of whole seeds were detected on DAP10–DAP30. Results show that RSD32 revealed the reduced seed dormancy phenotype at late developmental stages. The GIs of half seeds were 86.9 and 95.2, respectively, on DAP40 in 'Norin61' and RSD32 and remained at higher levels at later stages. Inhibitory effects of ABA on germination were reduced in RSD32 on DAP20, DAP30, and DAP50. These results indicate that RSD32 showed similar degrees of seed dormancy to those of 'Norin61' on DAP20 and DAP30. However, seed dormancy was found to be markedly lower on DAP40–DAP50 in RSD32. Reduced ABA sensitivity was also detected on DAP50 in RSD32.

Differentially expressed genes (DEGs) during seed development

Reduction of seed dormancy was detected on DAP40 and DAP50 in RSD32. Gene expression was compared using RNA-seq analysis at the middle to late developmental

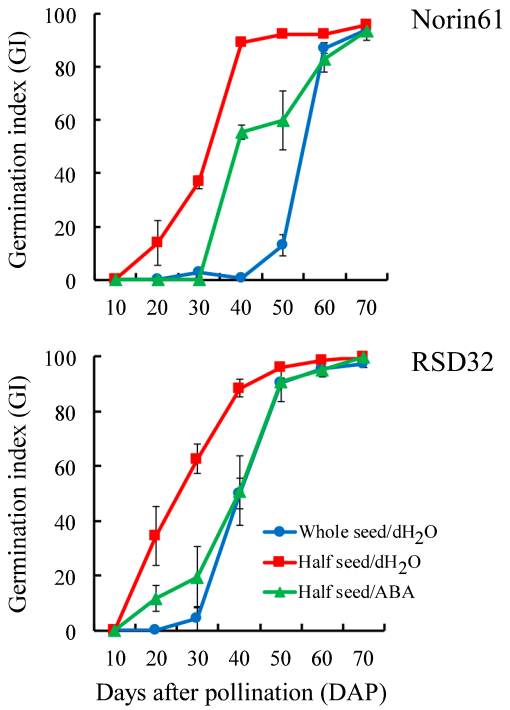

Fig. 1. Germination index (GI) whole seeds in water and half seeds in water with and without 10 μM ABA in 'Norin61' and RSD32 at different developmental stages. Error bars represent SE.

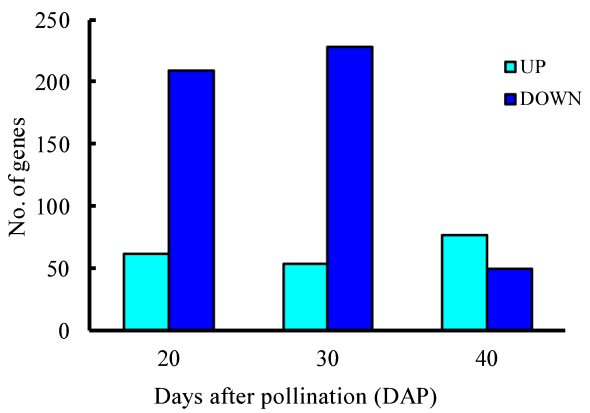

Fig. 2. Numbers of differentially expressed genes (DEGs) between embryos of 'Norin61' and RSD32 at different developmental stages. UP and DOWN respectively denote up-regulated and down-regulated genes in RSD32.

stages: DAP20, DAP30, and DAP40. Numbers of DEGs that were down-regulated in RSD32 were 209, 228, and 49, respectively, on DAP20, DAP30, and DAP40 (**Fig. 2**). Down-regulated genes in RSD32 were detected more on DAP20 and DAP30 than on DAP40. Numbers of DEGs that were up-regulated in RSD32 were similar at all developmental stages. RSD32 mutation preferentially inhibited gene expression. Marked effects were observed at earlier developmental stages than on DAP40 when seed dormancy reduction started.

Comparison of up-regulated genes in RSD32 showed

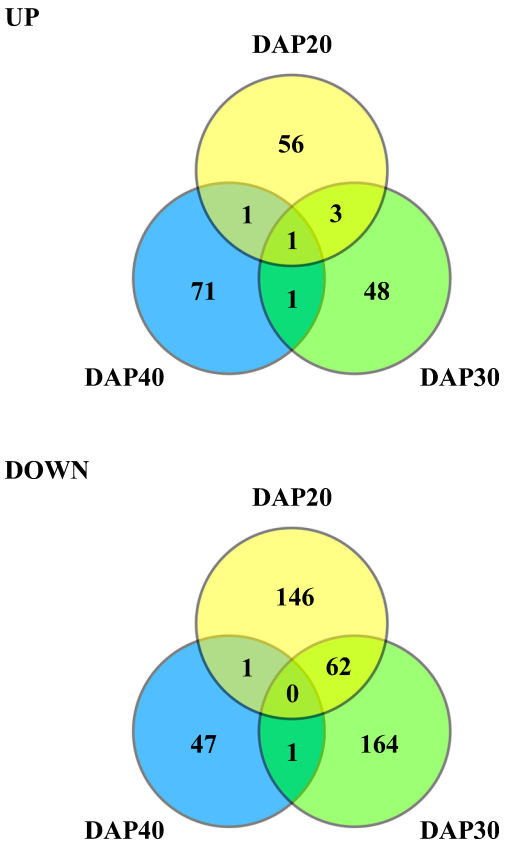

Fig. 3. Venn diagram highlighting the number of differentially expressed genes in the three developmental stages. UP and DOWN respectively denote up-regulated and down-regulated genes in RSD32.

less overlap among developmental stages. Actually, most up-regulated genes were expressed specifically at each developmental stage (**Fig. 3A**). Examination of down-regulated genes in RSD32 among developmental stages revealed that 146 and 164 DEGs, respectively, showed specific expression on DAP20 and DAP30, and that 62 DEGs were down-regulated at DAP20 and DAP30 (**Fig. 3B**). Most down-regulated genes on DAP40 showed stage-specific expression. No overlap with other developmental stages was observed.

At DAP20, down-regulated genes in RSD32 revealed several functions such as gene expression, protein metabolism, oxidation–reduction process, response to stimuli, circadian rhythm, and signal transduction (**Tables 1**, **2**). These genes involved several homologous genes related to the calcium signaling pathway, such as *CALCIUM-BINDING PROTEIN*, *CALMODULIN-BINDING PROTEIN*, *EF-HAND* Ca^{2+}*-BINDING PROTEIN*, *CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE*, *CALCIUM-DEPENDENT PROTEIN KINASE*, and *CALCIUM-TRANSPORTING ATPASE*. Furthermore, down-regulated genes involved circadian-clock-related genes. Genes homologous to *CCA1*, *LHY*, *LNK1*, and *RVE6-LIKE* were expressed lower in RSD32 than in 'Norin61'. Down-regulated genes were classified into Ca^{2+}

Table 1. Down-regulated genes found on DAP20 in RSD32

Putative function	FPKM*		Fold change
	'Norin61'	RSD32	
Cellular component biogenesis			
WAT1-related protein/auxin-induced protein 5NG4	80.4	0	–
Cellular metabolic process			
haloacid dehalogenase-like hydrolase domain-containing protein 3	2235.6	744.7	3.0
pheophorbide a oxygenase	107.9	29.5	3.7
photosystem II reaction center PSB28	62.7	0	–
soluble inorganic pyrophosphatase	312.8	40.6	7.7
Cellular process			
CDGSH iron-sulfur domain-containing protein NEET	160.2	45.6	3.5
CytHSP70	63.1	5.1	12.3
hypoxia-induced gene domain 5	773.8	231.1	3.3
Circadian rhythm			
LNK1	766.5	198.7	3.9
LNK2	125.7	39.9	3.2
LNK4-like	554.4	129.2	4.3
Developmental process			
Senescence associated gene 20 (SAG20)	231.0	45.5	5.1
Gene expression			
antagonist of like heterochromatin protein 1-like (ALP1)	53.9	15.6	3.4
B-box zinc finger protein 25-like	356.3	108.8	3.3
calmodulin-binding protein 60 D-like	213.9	46.0	4.6
lariat debranching enzyme	762.0	154.6	4.9
light-inducible protein CPRF2	436.7	127.3	3.4
multiprotein bridging factor 1 (MBF1)	530.2	81.4	6.5
NAC domain-containing protein 74	574.9	127.1	4.5
pentatricopeptide repeat-containing protein	66.3	16.5	4.0
PsbB mRNA maturation factor Mbb1	721.4	169.4	4.3
ribosomal protein S8	712.9	224.4	3.2
RNA-binding motif protein	278.0	47.2	5.9
WRKY11 transcription factor	97.1	0	–
Localization			
calcium-transporting ATPase 1	199.7	55.3	3.6
glucose-6-phosphate/phosphate-tranlocator-like	192.4	36.7	5.2
P-type ATPase	215.6	68.5	3.1
Molecular function regulator			
kelch repeat-containing protein-like	191.3	38.6	5.0
Organic substance metabolic process			
phosphoglycerate mutase gpmB	112.8	36.1	3.1
Oxidation–reduction process			
3-beta hydroxysteroid dehydrogenase/isomerase	253.2	70.3	3.6
Aldehyde dehydrogenase (ALDH)	186.8	57.7	3.2
cytochrome P450	70.3	21.7	3.2
L-ascorbate oxidase	86.5	20.0	4.3
NAD(P)-binding Rossmann-fold protein	149.6	48.5	3.1
NADH dehydrogenase	855.1	269.2	3.2
nitrate reductase [NAD(P)H]	313.3	44.9	7.0
omega-3 fatty acid desaturase	228.9	71.7	3.2
premnaspirodiene oxygenase-like	162.0	0	–
respiratory burst oxidase B-like	372.0	62.3	6.0
retinal dehydrogenase	72.8	0	–
Protein metabolic process			
ADP-ribosylation factor	74.2	14.6	5.1
ankyrin repeat-containing protein NPR4	305.4	85.5	3.6
Aspartic proteinase Asp1	303.3	61.3	4.9
calcium-dependent protein kinase	1010.3	184.9	5.5
E3 ubiquitin-protein ligase	201.2	65.0	3.1
Leaf rust 10 disease-resistance locus receptor-like protein kinase	98.4	0	–
prolyl 4-hydroxylase 6 precursor	282.7	54.4	5.2
receptor-like protein kinase	165.4	54.5	3.0
RHOMBOID-like protein 2	537.6	132.3	4.1
wall-associated receptor kinase 2-like (WAK2-like)	159.8	44.7	3.6
Response to stimulus			
16.9a kDa heat-shock protein	501.7	152.5	3.3
ATA15	630.6	201.9	3.1
Early responsive to dehydration 15-like	1738.5	461.4	3.8
heat shock cognate 70 kDa protein 2-like	443.6	115.3	3.8
heat-responsive transcription factor	284.0	90.1	3.2
small EDRK-rich factor 2-like (SERF2)	1256.2	376.9	3.3
EXORDIUM-like	184.8	19.9	9.3
hemoglobin Hb2	298.8	78.7	3.8
ethylene-responsive transcription factor ERF071-like	215.8	46.9	4.6
indole-3-acetic acid-amido synthetase (GH3.3)	367.6	110.8	3.3
RVE6-like	241.9	48.5	5.0
Secondary metabolic process			
phenylalanine ammonia-lyase-like	104.1	11.0	9.5
Signal transduction			
calcium-binding protein	93.6	0	–
calmodulin-binding receptor-like cytoplasmic kinase 3	114.2	33.2	3.4
calmodulin-related protein	581.5	133.8	4.3
CBL-interacting protein kinase 31	358.5	103.7	3.5
EF-hand Ca^{2+}-binding protein CCD1	334.6	81.5	4.1
mitogen-activated protein kinase	1358.4	387.6	3.5
SOUL heme-binding domain containing protein	416.0	59.6	7.0
Others			
retrotransposon protein	54.5	0	–
serine-rich protein	179.1	38.2	4.7

* FPKM denotes Fragments Per Kilobase of transcript per Million mapped reads.

Table 2. Down-regulated genes found on DAP20 and DAP30 in RSD32

Putative function	FPKM*		Fold change
	'Norin61'	RSD32	
Cellular component biogenesis			
16.9 kDa class I heat shock protein 1-like	3522.9	815.5	4.3
17.5 kDa class II heat shock protein	990.7	168.8	5.9
17.5 kDa heat-shock protein	312.7	57.3	5.5
17.9 kDa class I heat shock protein	727.0	91.4	8.0
18.6 kDa class III heat shock protein-like	2564.1	693.4	3.7
23.2 kDa heat shock protein-like	189.4	31.3	6.0
heat shock protein 16.9	1006.8	61.4	16.4
small heat shock protein 17.3 kDa	7957.7	1891.0	4.2
small heat shock protein Hsp23.5	1918.5	283.3	6.8
Cellular process			
70 kDa peptidyl-prolyl isomerase	701.2	176.5	4.0
BAG family molecular chaperone regulator 6	99.8	18.5	5.4
peptidyl-prolyl cis-trans isomerase	1101.5	288.2	3.8
regulator of chromosome condensation (RCC1)	376.7	81.8	4.6
Circadian rhythm			
CCA1	1407.7	313.7	4.5
LHY	139.5	27.2	5.1
Gene expression			
lariat debranching enzyme	2892.9	763.9	3.8
zinc finger MYM-type protein 1-like	283.4	62.3	4.5
Oxidation-reduction process			
early nodulin-93-like	421.7	111.5	3.8
Protein metabolic process			
receptor-like protein kinase	158.9	0	–
Response to stimulus			
14.5 kDa heat-shock protein	1341.7	387.5	3.5
23.6 kDa heat shock protein	1410.9	214.1	6.6
heat shock cognate 70 kDa protein	93.9	7.4	12.7
heat shock protein 90	1903.2	407.3	4.7
ultraviolet-B receptor UVR8	266.0	59.7	4.5
universal stress protein PHOS32	739.4	212.8	3.5
RVE6-like	284.6	60.4	4.7
Secondary metabolic process			
4-coumarate--CoA ligase 3	138.8	22.5	6.2

* FPKMs represent values obtained on DAP20.

signal transduction (16 genes) and circadian-clock regulation (14 genes) more than the responses to hormones (9 genes) and light (6 genes). Although many genes of heat shock protein family were observed in down-regulated genes on DAP20 (44 genes) and DAP30 (73 genes), their functions remained unknown. In fact, Ca^{2+} signal transduction and circadian-clock regulation were the leading functional groups in down-regulated genes on DAP20. Circadian-clock-related genes were also identified as up-regulated genes in RSD32. Genes homologous to *FAR1-RELATED SEQUENCE 12-LIKE* and *CONSTANS-LIKE 9* were found to be expressed higher in RSD32 than in 'Norin61' on DAP20 (**Table 3**). A homologous gene to *TOC1* was also expressed 2.4 times higher in RSD32. These results indicate that unusual calcium signaling and circadian-clock regulation occurred in RSD32.

Down-regulated genes found on DAP30 had several functions. Genes homologous to *PIF1-LIKE CCA1* and *LHY* were down-regulated in RSD32 (**Table 2**, **Supplemental Table 1**). However, genes homologous to *PHYTOCLOCK1* and *LUX-B* showed higher expression in RSD32 on DAP30 (**Table 4**). Genes homologous to *CONSTANS-LIKE 9* and *FAR1-RELATED SEQUENCE 5-LIKE* were expressed, respectively, 2.7 times and 2.5 times higher in RSD32. Expressions of genes homologous to *CCA1* and *LHY* were inhibited in RSD32 at both DAP20 and DAP30. Down-regulated genes in RSD32 at both of DAP20 and DAP30 were enriched to *HEAT SHOCK PROTEIN*s (**Table 2**). Although some DEGs were identified as gene expression, oxidation–reduction-process, and protein-

Table 3. Up-regulated genes found on DAP20 in RSD32

Putative function	FPKM		Fold change
	'Norin61'	RSD32	
Cellular component organization			
vasodilator-stimulated phosphoprotein-like	25.9	94.1	3.6
Circadian rhythm			
CONSTANS-LIKE 9	55.1	162.8	3.0
Developmental process			
myb-related protein Zm38-like	50.5	153.8	3.0
Gene expression			
AT hook motif containing protein	0	49.7	–
dehydration-responsive element-binding protein 2C-like	561.8	1696.4	3.0
Eukaryotic translation initiation factor 2 subunit 3	0	414.3	–
heat shock factor C1b	52.7	279.4	5.3
serine/threonine-protein kinase	0	177.8	–
suppressor protein SRP40-like	50.6	224.3	4.4
transcription initiation factor TFIID subunit 4-like	140.2	438.3	3.1
zinc finger MYM-type protein 1	57.8	204.7	3.5
Multi-organism process			
hyphally regulated protein-like	0	63.6	–
Organic substance metabolic process			
3-ketoacyl-CoA synthase 12	115.4	352.0	3.1
glyoxalase family like protein	424.3	1473.3	3.5
thiamine thiazole synthase 2	234.6	750.6	3.2
Oxidation–reduction process			
cytochrome P450 71C1	292.7	1483.4	5.1
divinyl chlorophyllide a 8-vinyl-reductase	0	65.9	–
Protein metabolic process			
glutathione S-transferase GSTU6	738.4	2230.3	3.0
Histone acetyltransferase HAC12	16.1	59.9	3.7
TNP2-like protein	295.6	1157.1	3.9
WD repeat domain phosphoinositide-interacting protein 3	34.8	140.4	4.0
Response to stimulus			
root peroxidase	35.7	198.2	5.6
serine proteinase inhibitor-like allergen	118.3	614.7	5.2
FAR1-related sequence12-like	37.0	121.2	3.3
dormancy-associated protein 1-like/auxin-repressed protein-like protein ARP1	2425.0	7415.5	3.1
Dehydrin DHN3	565.1	1840.4	3.3
Universal stress protein A-like	125.5	382.8	3.0
Signal transduction			
microtubule-associated serine/threonine-protein kinase 4-like	35.1	106.2	3.0
Others			
transposon protein	0	63.2	–

metabolic-process related genes on DAP40, circadian clock related genes were not found in DEGs on DAP40 (**Supplemental Tables 2**, **3**).

Temporal expression of homologues to circadian clock and Ca^{2+} signaling pathway related genes during seed development

Among DEGs, genes homologous to circadian clock and Ca^{2+} signal transduction related genes were abundant. In 'Norin61', genes homologous to *CCA1* and *LHY* showed the highest expression on DAP20. Their expressions were lower following seed development (**Table 5**). RSD32 showed lower expressions of *CCA1* and *LHY* than that of 'Norin61' on DAP20 or on DAP30. However, genes homologous to *TOC1* expressed higher in RSD32 than in 'Norin61' at all developmental stages. An homologous gene to *PHYTOCLOCK1* showed higher expression in RSD32 on DAP20 and DAP30, similarly to *TOC1*, but the difference was less apparent on DAP40. Regarding other circadian clock related genes, genes homologous to *LUX-B* and *CONSTANS-LIKE* showed higher expressions in RSD32. In addition, genes homologous to *LNK1*, *FAR1*, and *RVE6-LIKE* showed lower expression in RSD32. Consequently, RSD32 mutation was inferred to affect the

Table 4. Up-regulated genes found on DAP30 in RSD32

Putative function	FPKM		Fold change
	'Norin61'	RSD32	
Cellular component biogenesis			
WAT1-related protein	0	34.7	–
extracellular glycosidase CRH11-like	0	391.6	–
Cellular metabolic process			
lipid phosphate phosphatase 3	18.8	80.0	4.3
Circadian rhythm			
LUX-B	68.5	246.9	3.6
PHYTOCLOCK1	161.1	536.4	3.3
Developmental process			
myosin-14-like	0	110.3	–
Gene expression			
B3 domain-containing protein	0	218.7	–
trihelix transcription factor GTL1-like	0	369.0	–
zinc finger protein 410	0	8707.7	–
Localization			
sugar transporter ERD6-like 4	39.5	259.6	6.6
Nucleic acid metabolic process			
Superkiller viralicidic activity 2-like 2	259.5	868.1	3.3
Organic substance metabolic process			
plastid alpha-1,4-glucan phosphorylase	10.8	48.9	4.5
Oxidation–reduction process			
premnaspirodiene oxygenase-like	0	1048.3	–
Protein metabolic process			
deSI-like protein sdu1	40.9	185.7	4.5
Lysine-specific demethylase 8	20.9	131.2	6.3
RING-H2 finger protein	0	3545.2	–
Subtilisin-chymotrypsin inhibitor-2A	262.7	913.9	3.5
Response to stimulus			
disease resistance protein RGA2-like	282.2	872.3	3.1
Signal transduction			
Serine/threonine-protein kinase CTR1	91.6	352.7	3.9

expressions of circadian clock related genes in different manners. DEGs related to circadian clock regulation were divided into two groups based on mutant effects on expression for inhibition or enhancement.

In 'Norin61', genes homologous to calcium signaling pathway related genes, *CALMODULIN-BINDING RECEPTOR LIKE CYTOPLASMIC KINASE 3*, *CALCIUM-BINDING PROTEIN*, *CALMODULIN-RELATED PROTEIN*, *CBL-INTERACTING PROTEIN KINASE 31*, *CALMODULIN-BINDING PROTEIN 60D-LIKE*, and *CALCIUM-DEPENDENT PROTEIN KINASE* were found to be expressed specifically on DAP20. Their expressions were found to be diminished on DAP30 and DAP40 (**Table 4**). These genes showed specific expressions at the middle developmental stage. Expressions of genes homologous to calcium signaling pathway related genes were found to be markedly inhibited on DAP20 in RSD32. All Ca signaling pathway related genes were similarly down-regulated.

Discussion

'Norin61' is a pre-harvest sprouting-tolerant cultivar with strong seed dormancy. Although seed dormancy was maintained until DAP50, dormancy release was found on DAP60 and in later developmental stages. By contrast, RSD32 showed reduced seed dormancy on DAP40. Dormancy was found to be completely broken on DAP50. Degrees of seed dormancy in 'Norin61' and RSD32 differed at the late developmental stages (DAP40 and DAP50). Both lines showed low germination ability in whole seeds. No difference was observed at middle developmental stages (DAP20 and DAP30). Because half seeds, which have been released from dormancy, show poor germination, germination ability is not fully developed at this stage. Transcriptome analysis of gene expression in embryos of 'Norin61' and RSD32 at different developmental stages revealed conspicuously different gene expression in these lines at middle developmental stages, but not at

Table 5. FPKMs of circadian rhythm and Ca signaling related genes at different developmental stages in 'Norin61' and RSD32

Putative function	'Norin61'			RSD32			RSD32 Effect
	DAP20	DAP30	DAP40	DAP20	DAP30	DAP40	
Circadian rhythm							
CCA1	1407.7	747.8	435.2	313.7	138.7	686.5	–
LHY	139.5	117.4	39.6	27.2	18.2	43.9	–
TOC1/PPR1	251.2	496.3	532.0	594.9	714.8	694.3	+
PHYTOCLOCK 1	3.0	161.1	557.0	70.6	536.4	448.6	+
LUX-B	0.0	68.5	134.9	30.9	246.9	107.0	+
LNK1	902.4	899.7	414.4	244.4	432.2	480.1	–
FAR1-related sequence 5-like	1.4	617.8	164.2	0.0	176.2	86.6	–
RVE6-like	241.9	110.0	9.2	48.5	38.4	27.3	–
CONSTANS-like	55.1	172.8	282.5	162.8	458.4	317.4	+
Ca signaling							
calcium-dependent protein kinase	1010.3	184.9	93.9	184.9	148.4	122.7	–
calcium-binding protein	93.6	6.1	0.0	0.0	20.2	4.7	–
calmodulin-related protein	581.5	93.8	2.4	133.8	77.7	7.1	–
calmodulin-binding protein 60 D-like	213.9	46.8	5.9	46.0	62.2	8.1	–
CBL-interacting protein kinase 31	358.5	69.4	7.6	103.7	41.4	5.9	–
calmodulin-binding receptor-like cytoplasmic kinase 3	114.2	35.4	11.9	33.2	27.0	11.0	–
EF-hand Ca^{2+}-binding protein CCD1	334.6	47.9	2.3	81.5	71.8	2.3	–

Effects of RSD32 on gene expression represent + (positive) and – (negative).

late developmental stages, which attests to their different degrees of seed dormancy. These results suggest that RSD32 expresses at the middle developmental stage or at an even earlier stage before seed dormancy development. Shallower seed dormancy in RSD32 is associated with genes expressed at the middle developmental stage.

Genes homologous to circadian clock regulation related genes are differentially expressed in embryos of 'Norin61' and RSD32 at the middle developmental stages. For the component of central oscillator, genes homologous to *CCA1* and *LHY* were down-regulated in RSD32. However, *TOC1* and *PHYTOCLOCK1* were up-regulated in RSD32. In *Arabidopsis*, *CCA1* and *LHY* are the morning-expressed type; *TOC1* and *PHYTOCLOCK1* are the evening-expressed type (Seung *et al.* 2012). Apparently, RSD32 affects the expression of circadian clock regulation related genes depending on the circadian clock regulation function. Moreover, genes homologous to *LNK1*, *FAR1-RELATED SEQUENCE5-LIKE*, *RVE6-LIKE*, and *CONSTANS-LIKE*, which interact with clock components, showed modified expression in RSD32. For *Arabidopsis*, Penfield and Hall (2009) reported that circadian clock related genes are involved in dormancy release and that they affect the response to ABA and gibberellic acid (GA). Footitt *et al.* (2017) reported that the balance between the evening and morning phases of the clock contributes to the interpretation of temperature signals, thereby determining cycles of dormancy induction and relief in *Arabidopsis*. Aberrant functioning of central oscillation affects ABA biosynthesis, signal transduction, and several abiotic stress tolerances (Adams *et al.* 2018, Fornara *et al.* 2015, Kim *et al.* 2013b, Kolmos *et al.* 2014, Lee *et al.* 2016, Miyazaki *et al.* 2015, Nakamichi *et al.* 2012, Sanchez-Villarreal *et al.* 2013, Seung *et al.* 2012). The circadian clock might regulate several stress responses through ABA biosynthesis and the signal transduction pathway. Although the relation between circadian clock regulation and seed dormancy remains unknown in wheat, the reduction of seed dormancy in RSD32 might result from aberrant ABA signaling derived from irrelevant regulation of the circadian clock.

Genes homologous to calcium signaling pathway related genes were down-regulated in RSD32 embryos. In actuality, Ca^{2+} signal transduction is involved in several stress responses. In this study, expressions of Ca^{2+} signaling genes were found to be lower in embryos of RSD32. Irregular Ca^{2+} signaling induces inappropriate responses to environmental stresses. However, RSD32 showed no growth defect. RSD32 mutation might limit the effects of irregular Ca^{2+} signaling in the physiological traits of seed in a seed-specific manner.

This study identified Ca^{2+} sensor proteins, calmodulin-related protein, CDPK and CIPK as down-regulated genes in RSD32. In *Arabidopsis*, Ca^{2+} influx, and the expressions of *CALMODULIN-LIKE PROTEIN 39* (*CML39*), *CALCIUM DEPENDENT PROTEIN KINASE* (*CDPK*, *CPK*) and *CBL-INTERACTING PROTEIN KINASE* (*CIPK*) affect ABA signaling (Edel and Kudla 2016, Kong *et al.* 2015, Midhat *et al.* 2018, Sanyal *et al.* 2017, Zhao *et al.* 2011, Zhou *et al.* 2015). In monocot species, *CIPK* and *CPK* also affect sensitivity to ABA (Chen *et al.* 2017, Jiang *et al.* 2013, Wang *et al.* 2018). Furthermore, Somyong *et al.* (2011, 2014) reported that region-located wheat pre-harvest sprouting regulating QTL, QPhs.cnl-2B.1, involved several genes associated with Ca^{2+} signaling pathway, such as

*CDPK*s and *CALMODULIN/Ca^{2+}-DEPENDENT PROTEIN KINASE*. This study found genes homologous to calcium signaling pathway related genes to be temporarily expressed in wheat embryos on DAP20: the middle developmental stage. Temporal induction of these genes was lost in RSD32. Seed dormancy induction might be disturbed by attenuated Ca signaling. Martí Ruiz *et al.* (2018) reported that *CALMODULIN-LIKE 24* (*CML24*) regulates the expression of *TOC1* through Ca^{2+}-dependent pathway in *Arabidopsis*. Calcium signaling might affect the expression of circadian clock related genes in wheat. Relations among seed dormancy, ABA signal transduction, circadian clock regulation and Ca^{2+} signaling remain unknown, but they should be investigated further, especially for wheat. Few reports have described studies of the functions of circadian clock and Ca^{2+} signaling on the regulation of wheat seed dormancy. Results of the present study show that RSD32 is a useful tool for investigating the complex network of these regulatory pathways in wheat. Genes showing diverse functions other than circadian clock and Ca^{2+} signal transduction, such as the gene expression, biological and metabolic processes, are also involved in DEGs. Their effects on seed dormancy are unknown. Relations among these DEGs, circadian clock, Ca^{2+} signals and seed dormancy should be investigated.

Wheat embryos observed at DAP30 appear to be fully differentiated. Furthermore, the fresh and dry weights of endosperm reach their respective maximum values by DAP30 (Noda *et al.* 1994). Seed development is completed at the middle developmental stage (DAP15–DAP30). Moisture contents of seeds remain high at this stage. During the late developmental stage (DAP30–DAP50), moisture contents of seeds decrease; seeds enter the dormant state. Noda *et al.* (1994) reported that reserve accumulation in endosperm and the dry weight increase of embryos between DAP10 and DAP30 as a physiologically distinctive phase. Physiological states differ between seeds in their middle and late developmental stages. Although some DEGs are found to be down-regulated in RSD32 at DAP20 and DAP30, most DEGs identified on DAP40 are specifically expressed. No overlap was observed with other developmental stages. These results also indicate that seeds on DAP20 and DAP30 have similar physiological conditions, but they differ from those found on DAP40. Most studies investigating the regulation of seed dormancy have specifically examined the regulatory pathways of the late developmental stage. In monocot species, *MFT*, *MAP KINASE KINASE* and *AlaAT* have been identified as QTLs for regulating seed dormancy (Nakamura *et al.* 2011, Sato *et al.* 2016, Torada *et al.* 2016). These genes are associated with maintenance and release of seed dormancy. They express at the late developmental stage. Because RSD32 expresses at the middle developmental stage, RSD32 might be an important gene for regulating seed dormancy, acting more upstream in the regulation pathway. In *Arabidopsis*, *DOG1* and seed maturation regulators function to regulate seed dormancy and express at early to middle developmental stages (Bentsink *et al.* 2006, Giraudat *et al.* 1992, Kagaya *et al.* 2005, Kroj *et al.* 2003, Lotan *et al.* 1998, Luerßen *et al.* 1998, Stone *et al.* 2001, To *et al.* 2006). Wheat genes homologous to *DOG1* and seed maturation regulators are also expressed at early to middle developmental stages (Rikiishi and Maekawa 2014). Rikiishi *et al.* (2010) reported decreased expression of *TaDOG1* in the embryos of RSD32. These results suggest that RSD32 acts upstream on *TaDOG1* function. Regulation factors expressed at the middle developmental stage might be associated with seed dormancy initiation. Although many studies have examined the development and maintenance of dormancy, the mechanisms regulating initiation and induction of dormancy remain unknown. Early events of seed dormancy regulation in wheat are elucidated by the identification of RSD32 function. Furthermore, understanding the relations among regulation systems expressed at different developmental stages is necessary to elucidate the overall network regulating seed dormancy.

Author Contribution Statement

Conceived and designed the experiments: KR, MS, MM. Performed the experiments: KR. Analyzed the data: KR, MS, MM. Contributed reagents/materials/analysis tools: KR, MS, MM. Wrote the paper: KR. All authors read the manuscript and agreed to its submission.

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