Tamyb10-D1 restores red grain color and increases grain dormancy via suppressing expression of *TaLTP2.128*, *non-specific lipid transfer protein* in wheat

Eiko Himi · Shiho Kurihara-Yonemoto · Fumitaka Abe · Hidekazu Takahashi · Keisuke Tanaka · Takakazu Matsuura · Masahiko Maekawa · Takuji Sasaki · Kazuhide Rikiishi

Abstract Grain dormancy of wheat is closely associated with grain color: red-grained lines show higher dormancy than white-grained lines. The production of red pigments is regulated by *R-1*, *Tamyb10* gene. However, the relation between grain color and dormancy remains unknown. For this study, we generated transgenic lines which were introduced a DNA fragment containing *Tamyb10-D1* gene and its a 2 kb promoter including the 5′ untranslated region into white-grained wheat. Transgenic lines showed red-grained and higher dormant traits. Contents of plant hormones and gene expression of embryos at 30 days after pollination were examined in a wild type and a transgenic line. No differences were observed in the contents of plant hormones, but several genes are differentially expressed between these lines. One differentially expressed gene, *TaLTP2.128*, is a member of non-specific lipid transfer proteins. It was expressed higher in white grains than in red grains. A putative amino acid sequence showed similarity to that of *OsHyPRP5*, which is identified as QTL controlling low-temperature germinability in rice. Expression of *TaLTP2.128* was increased by grain imbibition. The

Eiko Himi and Shiho Kurihara-Yonemoto have equal contributors to this work and should be designated as co-first authors.

E. Himi
Kibi International University, 370-1 Shichi-Sareo, Minamiawaji, Hyogo 656-0484, Japan

S. Kurihara-Yonemoto
Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-8555, Japan

F. Abe
Institute of Crop Science, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8518, Japan

H. Takahashi
Fukushima University, 1, Kanayagawa, Fukushima, Fukushima 960-1296, Japan

K. Tanaka
NODAI Genome Research Center, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

T. Matsuura · M. Maekawa · K. Rikiishi (✉)
Institute of Plant Science and Resources, Okayama University, 2-20-1 Chuo, Kurashiki, Okayama 710-0046, Japan
e-mail: riki@okayama-u.ac.jp

T. Sasaki
NODAI Research Institute, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

increasing levels were higher not only in other white-grained lines, but also in non-dormant red-grained lines. *TaLTP2.128* was expressed at a quite early stage of germination. These study findings indicate that *Tamyb10* regulates dormancy release by the modification of *TaLTP2.128* acting as trigger of germination.

Introduction

Wheat (*Triticum aestivum* L.) is an important crop consumed as bread and noodles all over the world. Pre-harvest sprouting, seed germination before harvesting, causes severe damage to crop production because of starch degradation. Therefore, dormancy-related genes have been studied for a long time to breed higher dormant lines. Grain color is one factor that is closely linked to seed dormancy not only of wheat but also other plant species: darker seeds are more dormant than those lighter colored seeds (Debeaujon et al. 2000; Warner et al. 2000). In wheat, an MYB-type transcription factor, *Tamyb10*, is reportedly a strong candidate as a regulator of grain color, called *R-1* gene (Himi et al. 2011; Himi and Noda 2005). Because wheat is hexaploid, the *R-1* genes are triplicated, located on chromosomes 3A, 3B, and 3D, and respectively designated as *R-A1*, *R-B1*, and *R-D1*. White-grained lines have all recessive alleles (*R-A1a/R-B1a/R-D1a*), whereas red-grained lines have at least one of the dominant alleles (*R-A1b/R-B1b/R-D1b*). Nevertheless, the relation between grain pigmentation and dormancy remains unknown.

A lipid transfer protein (LTP) was first isolated from an active fraction that stimulates the exchange of phospholipids by gel filtration from potatoes (Kader 1975). Later, it was revealed that plant LTPs consist of a large protein family and have various functions (Carvalho and Gomes 2007). They are called non-specific lipid transfer protein (nsLTPs), which have a characteristic conserved motif, eight-cysteine motif (8CM) generally formed by C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C (Salminen et al. 2016). Kouidri et al. (2018) reported 461 putative nsLTPs (TaLTPs) identified in wheat and classified into five types:

Types 1, 2, C, D, and G. Nevertheless, the functions of these TaLTPs are poorly understood.

Some reports have described that members of *nsLTPs* are involved in seed maturation and germination. Lee and Suh (2018) reported that *Arabidopsis LTPG15,* a member of glycosylphosphatidylinositol-anchored LTP, is involved in suberin monomer export in seed coats and consequently seed coat permeability. *OsLTPL36* of rice, predominantly expressed in developing seed, might be involved in seed development and germination (Wang et al. 2015). Additionally, rice *qLTG3-1*, which was identified as QTL for low-temperature germinability on chromosome 3 and which has the conserved motif, 8CM, was reported as involved in seed germination (Fujino et al. 2008).

For this study, we generated transgenic red-grained wheat lines into which *Tamyb10-D1* had been introduced into white-grained line, Fielder using its native promoter. Transgenic lines showed higher dormancy than wild type. Gene expressions of embryos were compared between them. A member of *nsLTPs*, *TaLTP2.128*, was found in differentially expressed genes. Expressions of *TaLPT2.128* were strongly enhanced in white-grained lines of different genetic backgrounds during imbibition and also in the reduced seed dormancy mutant. We infer that TaLTP2.128 might be a novel candidate for the regulation of dormancy release.

Materials and methods

Production of transgenic wheat lines

For the generation of transgenic wheat, *Tamyb10-D1* was selected from three orthologous *Tamyb10* genes located in each chromosome of 3A, 3B and 3D. This was done because the longest upstream region sequence data (GenBank acc. no. AB191460) was available from the database at the time of cloning. PCR amplification was performed with Prime STAR*GXL DNA Polymerase (Takara Bio Inc., Japan) using primer set 1 (Table S1) from genomic DNA of Zenkojikomugi (red-grained); a 4119-bp DNA fragment was obtained. The resultant amplicon was first cloned into the vector pCR-Blunt II-TOPO (Thermo Fisher Scientific Inc., USA) and sequenced.

A fragment of the nos terminator with *Sac* I and *EcoR* I restriction enzyme sites at the ends was

prepared because we were not sure whether the terminator contained the full length in the short 3′ untranslated region of the 131 bp of *Tamyb10-D1*. To the 4,119-bp length of *Tamyb10-D1* genomic fragment *Hin*dIII and *Bam*HI recognition sequences were added primer set 2 (Fig. S1 and Table S1). The nos terminator fragment and the genomic *Tamyb10-D1* fragment were fused into pZH2B (Kuroda et al. 2010), a binary vector derived from pPZP202 (Hajdukiewicz et al. 1994) that contained hygromycin phosphotransferase as a selectable marker.

Agrobacterium-mediated transformation of wheat (*Triticum aestivum* L.) Fielder was performed using immature embryos as described in an earlier report (Abe et al. 2019; Ishida et al. 2015). The transformation efficiency was calculated as the percentage of wheat embryos that regenerated transformed seedlings relative to the number of embryos infected. Rooted regenerated plants were transplanted to soil in a growth chamber 20/15 °C (day/night) under a 12 h light/ 12 h dark photoperiod.

Molecular analysis of transgenic lines

Genomic DNAs of the regenerated plants were isolated from the seedling tips using a DNeasy Plant Kit (Qiagen Inc.) according to the manufacturer's instructions. To confirm the presence of introduced genes of *hygromycin phosphotransferase* (*htp*) and *Tamyb10-D1*, primer sets 3 and 4 were used, respectively (Fig. S1 and Table S1).

To detect the junction between T-DNA and the host plant, inverse PCR was performed. After 10 μg of DNA was digested with 10 U of *Alw*44I (Toyobo Co. Ltd.) for 16 h at 37 °C or 10 U of *Taq*I (Toyobo) for 16 h at 65 °C, the digested DNA was purified using an UltraClean GelSpin DNA Extraction Kit (MO BIO). It was then ligated with 1000 U of T4 ligase (Nippon Gene Co. Ltd.) for 23 h at 16 °C. Self-ligated DNA was cleaned using the UltraClean GelSpin DNA Extraction Kit and was used as a template for inverse PCR. Genomic DNA (200 ng) of T_3 lines was used as a template in a 10 μl reaction solution ($1 \times$ GC buffer I (Takara), 0.2 mM dNTP mix (Takara), 0.3 μM of primer set 5, and 0.5 U Ex Taq (Takara). PCR conditions were the following: 2 min denaturation at 94 °C, then 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C. Nested PCR was subsequently performed with primer set 6, using the same conditions as those for the first PCR. Amplified fragments were cloned into a pGEM-T Easy vector (Promega Corp.). The DNA sequence was determined using a sequencer (ABI 3100; Applied Biosystems) and was analyzed with GENETYX ver.13.0.3 (Genetyx Corp.). The T-DNA insertions were verified with primers based on the vector and the adjacent sequences (primer sets 7–9).

Total RNA was extracted from whole grains of 5 DAP and leaves of shoots of T_2 plants and their original line, Fielder, and red-grained Zenkojikomugi and white-grained Tamaizumi as controls using TRIzol Reagent (Invitrogen Corp.). After 10 μg RNA for each sample were treated with DNase I (Roche Diagnostics GmbH, Germany), random hexamers were used as primers for reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany). For each reverse transcription reaction, 5 μg of RNA treated with DNase I was used. Then the remaining 5 μg of RNA was used as a negative control using distilled water instead of reverse transcriptase. Expression of the transfected *Tamyb10-D1* gene and flavonoid biosynthetic gene of *CHS*, *CHI*, *F3H*, *DFR* were examined using primer sets 10–13 according to Himi et al. (2005) and primer set 15. A set of primer for *actin* (primer set 14) was used as an internal control. These are listed in Table S1.

Growth conditions and germination test for transgenic lines and fielder

Each seed was sown in a 5 L pot of soil. Ten plants each of T_2 generation and Fielder were grown in a transgenic plant greenhouse at 14–29 °C (average temperature about 21 °C). The daylength was the natural daylength of Sapporo, Japan, located at 43°N, during March–July 2016. Each pot was given approximately the same amount of water daily. The position of each was rotated weekly to ensure that the climatic conditions of the 30 pots in the greenhouse were as equal as possible.

Spikes were tagged at pollination day and were harvested at 50 and 60 days after pollination (DAP) of Fielder and G8 and G12 lines of T_2 generation derived from homozygous T_1 plants. T_3 grains of primary and secondary florets of the central spikes were hand-threshed. After-ripened (AR) grains were obtained by storing 60 DAP grains at room temperature for 55 days. Two 8.5 cm diameter filter papers

(No.2 qualitative filter paper; Advantec) were placed in a 9 cm diameter petri dish. Then 25 seeds were placed in each dish; 6 ml of distilled deionized water was poured. The dishes were placed inside a plastic box with a lid. Germination tests were conducted with the fresh grains and AR grains at 15 and 20 °C in the dark for 10–14 days. Each replication petri dish was replaced in turn from top to bottom daily. The position in the box was rotated daily. The box was rotated in the incubator daily. Results are presented as the means of four replicates for 50 DAP and of five replicates for 60 DAP and AR. The germination Index (GI) for estimating germinability was calculated as $GI = (14n_1 + 13 \times n_2 + 12 \times n_3 + 11 \times n_4 + 10 \times n_5 + 9 \times n_6 + 8 \times n_7 + 7 \times n_8 + 6 \times n_9 + 5 \times n_{10} + 4 \times n_{11} + 3 \times n_{12} + 2 \times n_{13} + 1 \times n_{14}) \times 100/ (14 \text{ (days)} \times 25 \text{ (total number of grains)})$, where n_1, n_2, n_3 … n_{14} respectively represent the numbers of grains that germinated on the first, second, third and subsequent days until the 14th day.

Other plant materials, growth conditions, and germination tests

Other wheat lines used for this study are portrayed in Table 4: EMS-AUS is a white-grained mutant derived from EMS treatment with red-grained AUS1490 (Himi et al. 2011; Mares et al. 2005). CS-W1 and -W2 are also white-grained mutants generated by sodium azide treatment with red-grained Chinese Spring (Warner et al. 2000). An F_2 population was generated by crossing between Chinese Spring and Novosibirskaya 67, a white-grained line. RSD32 is a non-dormant mutant derived from sodium azide treatment with Norin 61 (Rikiishi et al. 2021); both are red-grained lines. These lines were grown in a field of the Institute of Plant Science and Resources, Okayama University, under plastic roof to avoid rain.

Spikes were tagged at pollination day and were harvested at 30 DAP of Chinese Spring, CS-W1, -W2 and F_2 individuals, 40 DAP of Norin 61 and RSD32, and 20, 30, and 40 DAP of AUS1490 and EMS-AUS. Grains of primary and secondary florets of the central spikes were hand-threshed. For germination tests, fresh grains were used immediately according to our earlier report (Himi et al. 2002).

Determination of grain color

Grain colors of transgenic lines, control cultivars, and individuals of the F_2 population were determined by incubating grains in 5% sodium hydroxide (NaOH) solution according to an earlier report (Chmelar and Mostovoj 1938). The incubation time was 1 h, longer than that used for that earlier study. The NaOH treatment emphasized the reddish color of the grains. The white grains became a distinct cream color.

Tamyb10-D1 genotyping

Tamyb10-D1 genotyping was performed with each of the F_2 population according to our earlier report (Himi et al. 2011, 2015).

RNA sequencing and analysis

Fielder and T_3 generation of G8 were grown in a biosafety greenhouse under natural lighting conditions with 15–20 °C air temperature. Grains of 30 DAP of Fielder and G8 were used for RNA sequencing. Collected grains from a spike were divided into two groups. Whole grains of the first group were imbibed with distilled water. Grains of the second group were cut into embryo-half grains and were imbibed with 10 μM ABA solution. Grains of both groups were imbibed at 20 °C for 24 h in dark conditions. Three biological replicates were prepared with three spikes. Total RNAs of embryos were extracted with an RNA extraction kit following the manufacturer's procedures (NucleoSpin RNA; Machery-Nagel). Each cDNA library was prepared using a NEBNext Ultra RNA Library Prep Kit for Illumina following the manufacturer's procedures (New England Biolabs). High-throughput sequencing was run by a paired-end read 2×100-bp on a HiSeq 2500 (Illumina Inc.). The raw reads of RNA-seq datasets were filtered with FastQC (ver. 0.11.5) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low-quality reads were removed using the FASTX Toolkit (ver. 0.0.13) (http://hannonlab.cshl.edu/fastx_toolkit/). The clean RNA-seq data were aligned to the reference genome (Triticum_aestivum.TGACv1.dna.toplevel.fa) using HISAT2 (ver. 2.1.0) (Kim et al. 2019). Fragments per kilobase per million fragments mapped (FPKM) values were calculated using

Table 1 Up-regulated genes found on DAP30 in G8

Putative gene	Gene ID	Fielder		G8		log$_2$FC	FDR
		H$_2$O	ABA	H$_2$O	ABA		
Protein kinase	TRIAE_CS42_2DL_TGACv1_157886_ AA0500190	0.0	0.0	61.3	62.3	–	2.E-22
Ras-related protein RABC1	TRIAE_CS42_2DL_TGACv1_158540_ AA0521440	0.0	0.0	51.3	43.3	–	1.E-18
NB-ARC	TRIAE_CS42_2BL_TGACv1_129522_ AA0387020	0.0	0.0	26.0	45.0	–	7.E-09
NB-ARC	TRIAE_CS42_2AL_TGACv1_095805_ AA0314680	0.0	0.0	10.0	15.3	–	1.E-02
Histone-lysine N-methyltransferase activity	TRIAE_CS42_2DL_TGACv1_162257_ AA0562260	1.0	0.0	167.3	141.0	8.3	7.E-56
Ras-related protein RABC1	TRIAE_CS42_2BL_TGACv1_129841_ AA0397790	0.0	3.0	393.3	318.3	7.9	1.E-31
Serine/threonine protein kinase	TRIAE_CS42_2AL_TGACv1_094920_ AA0304840	1.7	2.0	158.3	161.0	6.4	9.E-23
Znf_TTF	TRIAE_CS42_5AL_TGACv1_374645_ AA1205410	0.7	1.0	66.0	61.7	6.3	6.E-21
WAMP-1, antimicrobial peptide	TRIAE_CS42_2DL_TGACv1_159104_ AA0532470	4.3	4.3	312.7	329.3	6.2	1.E-51
Metalloendopeptidase activity	TRIAE_CS42_7AL_TGACv1_560652_ AA1802500	2.0	2.3	87.0	130.0	5.6	9.E-30
NB-ARC	TRIAE_CS42_2AL_TGACv1_093745_ AA0285890	18.7	11.7	597.0	672.3	5.4	2.E-22

StringTie (ver. 1.3.3) (Pertea et al. 2016). Differential gene expression analysis was performed using the package DESeq2 (ver. 1.16) in R (v3.4.0).

DESeqDataSetFromMatrix(countData

= countdata, colData = coldata, design

=~ genotype + condition).

Quantitative reverse-transcription PCR analysis

After total RNA was extracted from embryos with NucleoSpin RNA (Machery-Nagel), it was reverse transcribed with PrimeScript Reagent Kit Perfect Real Time (TaKaRa) according to the manufacturer's instructions. Quantitative reverse-transcription PCR was performed with TB Green Premix Ex Taq II (TaKaRa) using LightCycler 2.0 (Roche) under the following conditions: 30 s denaturation at 95 °C, then 40 cycles of 5 s at 95 °C, 20 s at 60 °C. Primer sets15–17 used for qRT-PCR are listed in Supplemental Table 1. Each qRT-PCR analysis was performed in triplicate. Cell division control protein (CDCP;

primer set 18) was used as a reference gene for data normalization (Paolacci et al. 2009). Reverse transcription PCR (RT-PCR) was also performed with CS, CS-W1 and -W2 using Quick Taq (Takara) under the following conditions: 2 m denaturation at 94 °C, then 30 or 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 68 °C. Primer sets 19 and 20 used for RT-PCR are listed in Table S1.

Quantification of plant hormones in embryos

Grains of 30 DAP collected from a spike were divided into three groups for fresh grains, imbibed whole grains, and imbibed embryo-half grains. The imbibed grains were prepared by imbibition with distilled water at 20 °C for 24 h in a dark condition. Three biological replicates were prepared with three spikes of Fielder and G8. Plant hormones, ABA, DHZ, GA$_1$, GA$_4$, IAA, iP, JA, SA and tZ, of the embryos were extracted, purified and quantified with LC–ESI–MS/MS as described in an earlier report (Matsuura et al. 2019).

Results

Characteristics of transgenic lines with introduced *Tamyb10-D1*

Genomic *Tamyb10-D1* fragment isolated from Zenkojikomugi (a red grained and dormant cultivar) contained a 2 kb promoter including the 5' untranslated region (5' UTR), three exons, two introns, and short 3′ untranslated region 131 bp. Comparison of the isolated sequences with data from IWGSC Chinese Spring RefSeq_v2.1_chromosome 3D (https://wheat-urgi.versailles.inra.fr/Seq-RepositoryBLAST) showed 99.6% identity in the 2 kb promoter including 5' UTR and 100% identity from the start codon to 131 bases downstream of the stop codon. Results show that the fragment *Tamyb10-D1* cloned from Zenkojikomugi has no polymorphism with Chinese Spring in amino acid sequence. A white-grain cultivar, Fielder, was transformed with the genomic *Tamyb10-D1* fragment (Fig. 1a). The transformation efficiency was 2.9%. We obtained 24 hygromycin-resistant T_0 plants, G1 to G25. These regenerated plants were confirmed to harbor *hygromycin phosphotransferase* (*htp*) gene

(Fig. S2a). The inserted *Tamyb10-D1* gene was found in these regenerated plants except for 1 plant (G13); the grains of the G13 appeared white (Fig. 1b, Fig. S2b and c). From the 24 regenerated plants, 2 plants (G8 and G12) that were assumed to have single T-DNA insertion were selected. For T-DNA insertion, T_1 progenies of these T_0 plants were segregated at a ratio of 3:1. We decided to use G8 and G12 progeny for germination tests.

The T-DNA/plant DNA junction sequence was found using inverse PCR with two restriction enzymes: *Alw*44I and *Taq*I. In G8, flanking sequences of the amplified fragments of both digested and self-ligated DNA were found to be identical, which suggests that the inserted T-DNA is a single copy onto the distal region of chromosome 7D (Fig. 1a). The insertion site was verified by PCR using primers based on T-DNA and the flanking sequence (Fig. 1c). The T-DNA insertion site was found between predicted genes, TraesCS7D02G490100 and TraesCS7D02G490200, respectively separate from about 160 kb and 50 kb. Therefore, the insertion is not expected to have affected the functions of other genes. The right-border of T-DNA was truncated

Fig. 1 Characteristics of transgenic lines with *Tamyb10-D1*. **a**. Diagrams of an induced vector (upper), a T-DNA inserted site on chromosome 7D (lower) and partial sequences of the insertion site (middle). **b**. Grain color of Fielder and G8 before NaOH treatment (upper) and after treatment (lower). Bar represents 1 cm. **c**. PCR products of Fielder and transgenic line G8. Three individuals were used: G8-1, 2, and 3
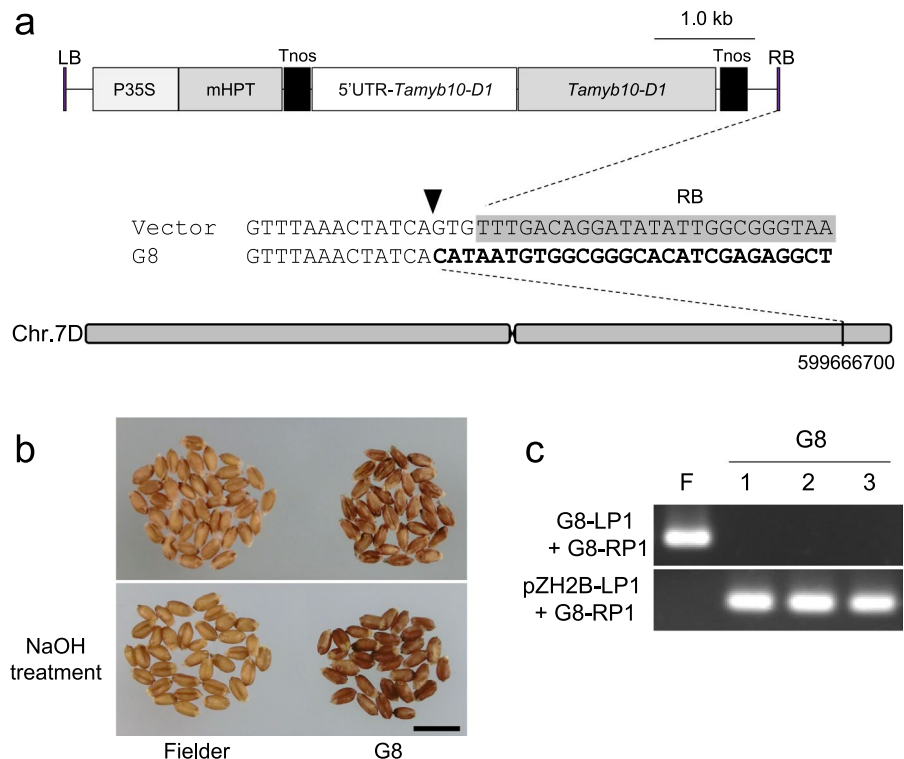
(Fig. 1a), but the loss of this region might not affect transgene expression as described in reports of earlier studies (Bartlett et al. 2014). Similarly, the insertion site of G12 was surveyed. The T-DNA was inserted onto an upper region of a putative gene, TraesC-S5A02G166400 on chromosome 5A (Fig. S3a–c). We selected the G8 line and used it for additional experiments to avoid unanticipated effects of insertion.

Flavonoid biosynthetic genes, *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*F3H*), and *dihydroflavonol 4-reductase* (*DFR*), are essential genes for proanthocyanidin synthesis. These genes are known to be expressed in immature grains of red-grained lines (Himi et al. 2005). Expressions of these genes were strongly upregulated in immature T_3 grains of G8 and G12 as well as the red-grained cultivar, Zenkojikomugi (Fig. S4a, b). Expression of the inserted *Tamyb10-D1* gene driven by its own promoter, probably present in the 2 kb-5′UTR of the inserted genomic fragment, was observed in G8 and G12 in immature grains but not in leaves (Fig. S4c). Roots of hydroponically grown Fielder and G12 were also subjected to testing, but the expression was not observed (data not shown). These data demonstrated that the promoter of this gene engenders testa tissue specific expression in immature grains.

Grain dormancy of red-grained transgenic lines

The phenotype and fertility of transgenic lines G8 and G12 (T_2 plants) were not clearly different from those of Fielder except for grain testa color. Germination tests were conducted at 15 and 20 °C using 50 and 60 DAP and after-ripened. Although after-ripened grains of all lines which had broken dormancy showed higher germination indices, the germination index of G8 was lower than those of wild type (Fig. 2a, b). G12 also showed lower germination index at 20 °C treatment, but no significant difference was found at 15 °C (Fig. 2b). These results suggest that *Tamyb10-D1* gene regulated both grain color and grain dormancy.

Quantification of plant hormones in embryos

Plant hormones of both lines were quantified using liquid chromatography – electrospray tandem mass spectrometry (LC-ESI-MS/MS) to ascertain whether grain color influenced the synthesis and/or metabolism of plant hormones. Embryos of 30 DAP were collected from freshly harvested grains (FH), 24-h imbibed whole grains (IW), and 24-h imbibed embryo-half grains (IEH) from the same spike and were used for quantification as depicted in Fig. 3a. Contents of indole acetic acid (IAA) tended to increase by imbibition, but no significant difference was found (Fig. 3b). ABA levels of both lines were rapidly decreased by imbibition. Significant differences ($p < 0.05$) were found between non-imbibed whole grain and imbibed embryo-half grain in both lines, but no significant difference were found between the two lines (Fig. 3c). Amounts of jasmonic acid (JA) in non-imbibed whole grains of Fielder were significantly higher than in the others (Fig. 3d). No significant differences were found in the amounts of salicylic acid (SA), *trans*-zeatin (tZ), or isopentenyladenine (iP) (Table S2). It is particularly interesting that gibberellin A_1 (GA_1), gibberellin A_4 (GA_4), and dihydrozeatin (DHZ) were not detected even in imbibed grains.

Differentially expressed genes between fielder and G8

Gene expressions in embryos of Fielder and G8 (T_4 grains) were compared by RNA-seq analysis using the grains of 30 DAP. Embryos were collected from imbibed whole grain in water and imbibed embryo-half grain in ABA solution (Fig. 4a). RNA-seq analysis showed that 79 and 45 differential expressed genes (DEGs) were up-regulated, respectively, in water absorption and ABA absorption of G8. Moreover, 30 genes were common (Fig. 4b). Table 1 presents a list of the common up-regulated genes, except for uncharacterized protein genes and non-coding RNA. Protein kinase and Ras-related protein RABC1, which were located on homeologous chromosomes, were involved.

Numbers of down-regulated genes in G8 were 79 and 14, respectively, in water and ABA; 6 genes were common (Fig. 4b). Table 2 shows the down-regulated genes in G8 except for an uncharacterized protein gene. Lipid transfer proteins (LTPs) located on homeologous chromosomes were involved in down-regulated DEGs. These genes were strong candidates as novel dormancy-related genes. Expression analysis was performed in other pairs of wheat lines that had different dormancy levels.

Fig. 2 Germination indices of Fielder, G8, and G12. Grains of 50 and 60 DAP and after-ripened (AR) were used. Germination assays were performed at 15 °C. (**a**) and 20 °C (**b**). White, black and gray bars respectively denote Fielder, G8 and G12. Bars represent standard errors. Different letters above the bars were found to be significantly different according to Tukey's test ($p < 0.05$)
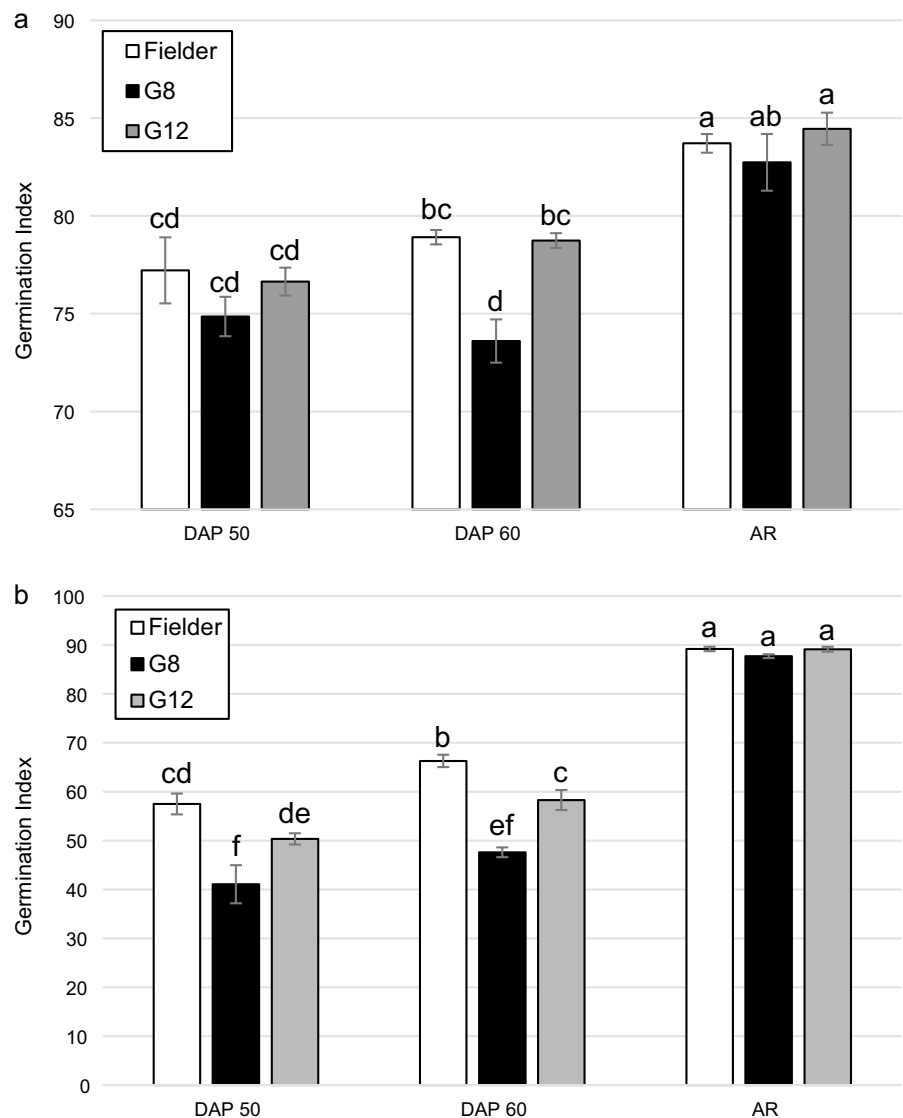

Results of the plant hormone quantification suggest that the expression of genes related to hormone synthesis/metabolism might be of equal levels in both lines. Actually, the expressions of ABA 8′-hydroxylase (which catalyzes the first step in the oxidative degradation of ABA), Gibberellin 3-beta-dioxygenase 2–2 (GA3ox2-2, which converts the inactive gibberellin precursors GA_9 and GA_{20} in the bioactives gibberellins GA_4 and GA_1), Jasmonic acid-amido synthetase (JAR1, which catalyzes the synthesis of jasmonates-amino), and Allene oxide cyclase 1 (AOC1, which is involved in the production of 12-oxo-phytodienoic acid (OPDA), a precursor of jasmonic acid) were not different between G8 and Fielder (Table 3). These results support that the contents of plant hormones between the lines were not different.

Additionally, no significant differences were found in the expression levels of already known dormancy-related genes, *MOTHER OF FT AND TFL1* (*MFT-3A*), *Viviparous-1* (*Vp1*), *mitogen-activated protein kinase kinase 3* (*TaMKK3*), *TaDOG1L4*, *Protein phosphatase 2C* (*PP2C*), and *dehydration-responsive element-binding protein 1* (*DREB1*). However, G8 showed higher grain dormancy (Table 3). *Tamyb10-D1* showed no effects on the expression of known dormancy-related genes.

Fig. 3 Plant hormone quantification of Fielder and G8. **a**. Grains of a same 30 DAP spike were divided into three groups: (1) freshly harvested grains (FH), (2) 24-h imbibed grains with water (IW), and (3) 24-h imbibed embryo-half grains with water (IEH) and embryos of each group were used for plant hormone quantification. **b**. Contents of indole-3-acetic acid (IAA). **c**. Contents of abscisic acid (ABA). **d**. Contents of jasmonic acid (JA). Different letters above error bars (i.e., $\pm$ SE) denote significant differences ($p < 0.05$) among groups (Tukey's test)
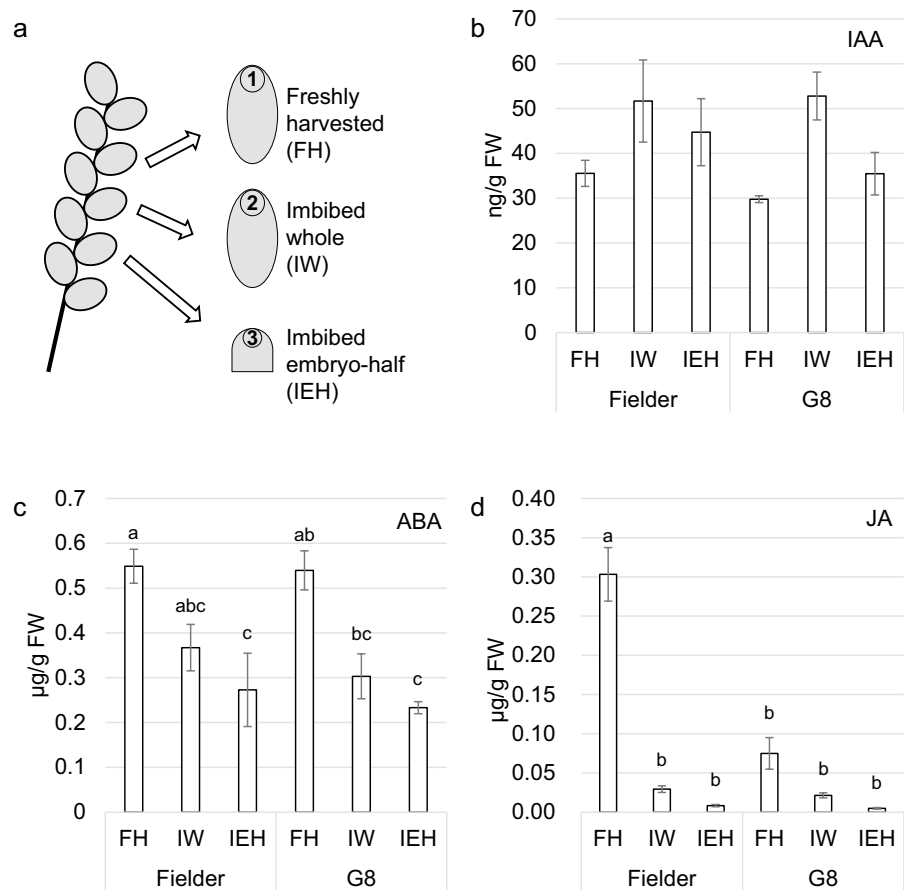
Expression patterns of DEGs during imbibition

Protein kinase (*PK*), *ras-related protein* (*Ras*), and *lipid transfer protein* (*LTP*) were regarded as strong candidates for novel dormancy-related genes because these genes were found in duplicate, derived from homeologous genes (Tables 1 and 2). By contrast, *F-box* genes consisted of multifamilies; the sequences of the two genes were quite dissimilar, suggesting that these F-box genes were not homeologous. For this study, we specifically examined *LTP, PK*, and *Ras* by reliability of multiple detection. We designed sets of primers for expression analysis (Table S1).

Expressions of candidate genes were examined in other lines showing different genetic backgrounds. Chinese Spring and its white-grained mutant, CS-W1, were used for expression analysis (Fig. 5a). *Tamyb10-D1* was disrupted in CS-W1 (Table 4). This mutant also showed less dormancy than wild type (Fig. 5b), as reported from an earlier study (Warner et al. 2000).

Figure 5C–E show the expression levels of *LTP* on chromosome 4B (*LTP-B*), *PK* on chromosome 2A (*PK-A*), and *Ras* on chromosome 2D (*Ras-D*) of 30 DAP embryos from freshly harvested grains and 24-h imbibed grains. Significant difference ($P < 0.05$) was found for the expressions of LTP-B between imbibed Chinese Spring and CS-W1 (Fig. 5c). However, *PK-A, -D* and *Ras-B, -D* showed no significant differences between these lines (Figs. 5d, e, S5).

The F_2 population derived from a cross between Chinese Spring and Novosibirskaya 67, white-grained line, was used for another set for verifying expression analysis. Dormancy levels of 16 red-grained lines and 5 white-grained lines are shown in Fig. S6a. Lower dormancy of white-grained lines was observed. Expression levels were analyzed using selected 5 red-grained and 4 white-grained individuals. Higher expression levels were observed in white-grained lines than in red-grained lines (Fig. S6b). Moreover, the expression of *LTP-B* gene was accelerated by
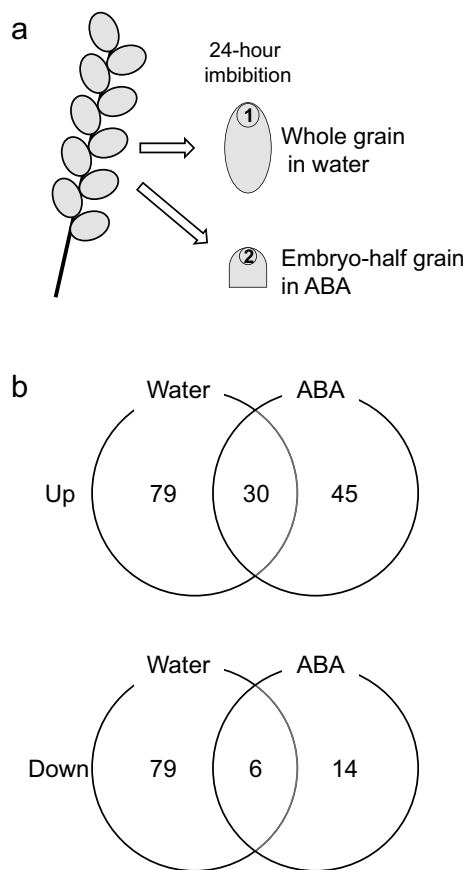
Fig. 4 RNA-seq analysis of Fielder and G8. **a**. Grains of a same 30 DAP spike were divided into two groups: (1) 24-h imbibed grains with water and (2) 24-h imbibed embryo-half grains with ABA and embryos of each group were used for RNA-seq analysis. **b**. Venn diagram of the number of up-regulated differentially expressed genes of G8 (upper) and down-regulated genes of G8 (lower)

imbibition in both lines. These results indicate that *LTP-B* was acting on germination processes.

Characteristics of LTP-B

LTP-B located on the distal region of the long arm of chromosome 4B (Fig. 6a). The putative amino acid sequences had characteristic eight cysteine motif (8-CM) as do other plant nsLTPs (Fig. 6b). Kouidri et al. (2018) reported 461 putative wheat nsLTP identified in Chinese Spring and classified them into five types (Types 1, 2, C, D, and G). The *LTP-B* in this study is a member of Type 2, the largest group, consisting of 274 genes. It was designated as *TaLTP2.128* according to Kouidri et al. (2018). It is particularly interesting that Type 2 of *nsLTP* in rice comprises only 13 genes. The C-term inal region including 8-CM of TaLTP2.128 showed high similarity to OsHyPRP3. However, several amino acid residues of the N-terminal region were shared with OsHyPRP5 (Fig. 6b).

Expression patterns of *TaLTP2.128* in developing grains of wheat

TaLTP2.128 gene was found by RNA-seq with 30 DAP grains (Table 2) and was verified with other lines at the same developmental stage (Figs. 5c and S6b). Then, expression levels of *TaLTP2.128* during grain development were examined in AUS1490 and EMS-AUS. The former, AUS1490, is a red-grained and dormant line; EMS-AUS is a white-grained and non-dormant mutant of AUS1490 (Himi et al. 2002; Mares and Himi 2021). *Tamyb10-A1* was disrupted in EMS-AUS (Table 4). In immature 20 DAP grains, *TaLTP2.128* was expressed in EMS-AUS. The expression level increased by imbibition, whereas low expression levels in AUS1490 were stable during imbibition (Fig. 7a). In 30 DAP grains, *TaLTP2.128* expression was identified in imbibed AUS1490 grains, but the expression level was significantly

Table 2 Down-regulated genes found on DAP30 in G8

Putative gene		Fielder		G8		log$_2$FC	FDR
		H$_2$O	ABA	H$_2$O	ABA		
Lipid transfer protein	TRIAE_CS42_4BL_TGACv1_321925_AA1066890	29.0	24.7	1.0	4.7	− 3.2	3.E-02
F-box	TRIAE_CS42_7BS_TGACv1_592370_AA1936780	15.7	13.0	1.0	1.0	− 3.8	3.E-03
F-box	TRIAE_CS42_6AS_TGACv1_485781_AA1552080	27.0	13.0	0.0	2.0	− 4.3	2.E-02
Lipid transfer protein	TRIAE_CS42_4DL_TGACv1_343478_AA1135040	72.7	17.7	1.0	2.0	− 4.9	4.E-03
dUTPase-like	TRIAE_CS42_3B_TGACv1_220816_AA0720070	39.0	45.0	1.0	1.7	− 5.0	5.E-10

Table 3 Expression of phytohormone and dormancy-related genes

Genes	Fielder		G8		log$_2$FC	FDR
	H$_2$O	ABA	H$_2$O	ABA		
ABA 8′ hydroxylase	1220.7	2048.3	1120.3	1502.7	− 0.3	1.0
GA3ox2-2	51.0	6.3	17.0	6.0	− 1.3	1.0
JAR1	56.0	44.0	29.3	34.7	− 0.6	1.0
AOC1	782.3	483.3	1221.0	673.3	0.6	1.0
Tamyb10-D1	0.0	0.0	0.0	0.0	–	1.0
MFT-3A	1874.0	1834.7	1818.7	2021.7	0.1	1.0
Vp1	1284.7	1579.3	1396.7	1616.0	0.1	1.0
TaMKK3	522.3	503.3	488.7	487.0	− 0.1	1.0
TaDOG1L4	67.0	41.3	92.0	95.0	0.8	0.5
PP2C	1912.3	3784.7	2426.7	2485.0	− 0.2	1.0
DREB1	1602.0	1356.0	1679.3	1594.3	0.1	1.0

higher in EMS-AUS (Fig. 7b). Figure 7c presents the expression levels in 40 DAP. The figure suggests that extremely high expression was observed in 48 h imbibition of EMS-AUS. Two-day (48 h) imbibition caused some grains of EMS-AUS to germinate (Fig. S7). Low and stable expression levels were also found with 40 DAP grains of AUS1490. These results indicate that the expression of *TaLTP2.128* is closely linked with germination.

TaLTP2.128 expression of red-grained non-dormant mutant

These results presented up to this point were compared between red-grained and white-grained lines. To investigate the effects of the dormancy level on *TaLTP2.128*, expression patterns were determined in non-dormant mutant RSD32: a single recessive mutant that showed reduced grain dormancy derived from the Norin 61 by NaN$_3$ treatment (Rikiishi et al. 2021). Norin 61 and RSD32 were a red-grained dormant line that possesses all dominant *R-1b* genes (Kaneko et al. 1994). Then, the effects of dormancy level on *TaLTP2.128* were evaluated independently of the grain color. *TaLTP2.128* expression was strongly induced by imbibition with RSD32 (Fig. 8). The reduced dormancy level of RSD32 affected the expression of *TaLTP2.128* similarly to grain color.

Discussion

Tamyb10 regulates grain color and dormancy

It has long been known that a relation exists between grain color and grain dormancy in wheat. Although the reason for this relation remains unclear, it has been suggested that the *R-1* gene, which determines the grain color, might regulate not only pigment synthesis but also genes related to grain dormancy. Our earlier studies (Himi et al. 2011; Himi and Noda 2005) have indicated *Tamyb10-1* gene as a strong candidate of the grain color regulator, *R-1* gene. In this study, grain color and dormancy were modified in transgenic lines into which *Tamyb10-D1* had been introduced. These results demonstrated directly that the *Tamyb10-1* gene is the *R-1* gene which is expressed predominantly in grains and which regulates dormancy.

Because ABA sensitivities differ among red-grained lines and white-grained lines (Himi et al. 2002), it has been predicted that the *R-1* gene interacts with genes related to ABA sensitivity and biosynthesis. Matsuura et al. (2019) have already reported that no difference exists in plant hormone contents among different grain colors. Our results are consistent with their results. By contrast, Lang et al. (2021) reported that the function of *Tamyb10-D1* confers pre-harvest sprouting resistance by enhancing ABA biosynthesis to delay germination. They introduced the same gene into the same host cultivar and produced transgenic red grain lines. However, their transgenic wheat
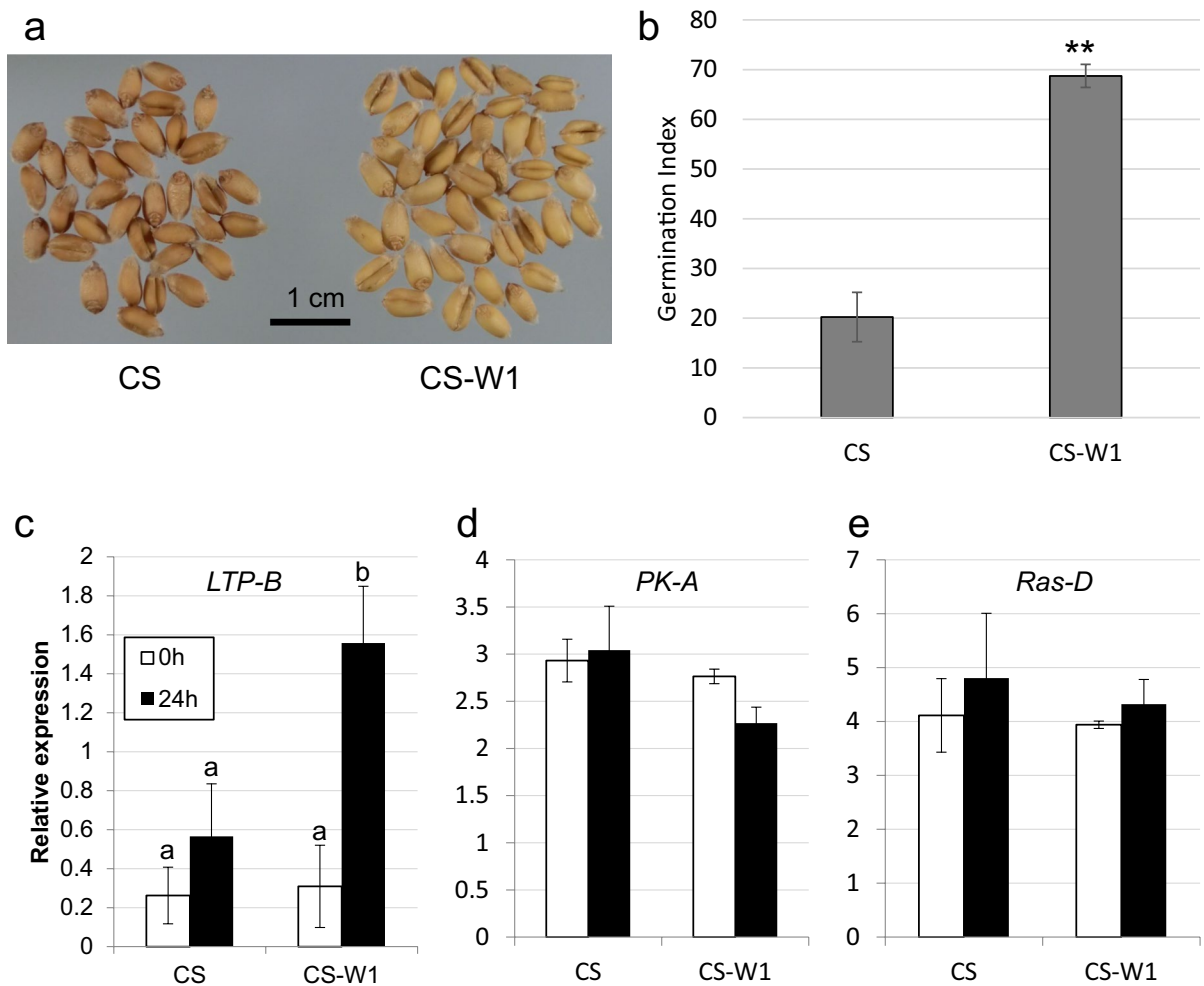
Fig. 5 Germination indices and gene expression in Chinese Spring and white-grained mutant. **a**. Chinese Spring (CS), red-grained line, and its white-grained mutant, CS-W1. **b**. Germination Index of 40 DAP grains with CS and CS-W1. Error bars represent the standard error (SE). ** denotes significant differ-

ence (*P* < 0.01). **c-e**. Relative expression of LTP-B (**c**), PK-A (**d**), and Ras-D (**e**) per CDCP gene of embryos from freshly harvested grain (0 h; white bar) and 24 h-imbibed grain (24 h; black bar). Different letters above error bars (i.e., ± SE) denote significant differences (*p* < 0.05) among groups (Tukey's test)

was a high-expression system in which the cDNA is driven by a ubiquitin promoter, whereas our material is driven by its own promoter of the genomic fragment. The difference of promoter might affect ABA biosynthesis in transgenic lines. Our results indicate that sensitivity might be important rather than endogenous content in *R-1* regulation pathway in an ABA-dependent manner of *R-1* regulation for grain dormancy. As shown in Fig. 3, the amount of jasmonic acid is higher in white-grained grains in freshly harvested grains. The reason for this is unknown, but Matsuura et al. (2019) also reported no correlation

between jasmonic acid content and grain dormancy, suggesting that jasmonic acid content does not cause the germinability of white-grained grains.

TaLTP2.128 is an ortholog of qLTG3-1

LTP has a common motif, 8CM, on the C-terminus, whereas the N-terminal side is more varied. The N-terminal region of the putative TaLTP2.128 amino acid sequence has similarity to that of rice *OsHyPRP5* gene, which is a synonym of *qLTG3-1*, known as a factor that controls low-temperature germinability in

Table 4 *Tamyb10* genotype of the wheat lines used for this study

	Tamyb10-			Grain color	References
	A1	*B1*	*D1*		
Fielder	*a*	*a*	*a*	White	Kurihara-Yonemoto and Matsunaka (2019)
G8	*a*	*a*	*b*	Red	This study
G12	*a*	*a*	*b*	Red	This study
AUS1490	*b*	*a*	*a*	Red	Mares et al. (2005)
EMS-AUS	*a*	*a*	*a*	White	Himi et al. (2011)
Chinese Spring	*a*	*a*	*b*	Red	Heyne and Livers (1953)
CS-W	*a*	*a*	*a*	White	Warner et al. (2000)
Norin 61	*b*	*b*	*b*	Red	Kaneko et al. (1994)
RSD32	*b*	*b*	*b*	Red	This study
Novosibirskaya 67	*a*	*a*	*a*	White	Koval (1997)
Tamaizumi	*a*	*a*	*a*	White	Himi et al. (2011)
Zenkojikomugi	*a*	*b*	*b*	Red	Miura et al. (2002)

rice (Fujino et al. 2008). *OsHyPRP3, 4*, and *5* were tandemly located on chromosome 3 spanning about 2 and 8 kb intervals. That finding might indicate that these genes were triplicated, but that the temporal and special gene expressions of these genes varied, i.e. root (*OsHyPRP3*), root and germinating seed (*OsHyPRP4*), and specifically expression in germinating seed (*OsHyPRP5*) (Fujino et al. 2014). These findings indicate that the *LTP-B*, *TaLTP2.128*, might also play a role in controlling seed germination in wheat similar to *OsHyPRP5*. The function might be determined by the N-terminal motif (Fig. 6b).

qLTG3-1 locates on rice chromosome 3, which has synteny with wheat chromosome 4, supporting the possibility that these genes are orthologous. Because *qLTG3-1* promotes grain germination at low temperatures (Fujino et al. 2008), the expression of wheat *TaLTP2.128* might also be temperature-dependent. It is particularly interesting that Yazdanpanah et al. (2017) reported that *Arabidopsis AT2G45180*, a member of *nsLTP*, was found by microarray analysis using freshly harvested (dormant) and after-ripened (non-dormant) seeds and the *AT2G45180* expressed higher in non-dormant seeds. Figure S8 presents a comparison of the putative amino acid sequence of *AT2G45180* with that of *TaLTP2.128*, revealing that some residues at the N-terminal regions were common, which are also found in *qLTG3-1* of rice. The findings suggest that this gene of *Arabidopsis* might have a similar function in rice and wheat.

Both rice and *Arabidopsis* have 13 genes of type-2 *nsLTP* genes, but there are 274 genes of type-2 *nsLTPs* in wheat (Kouidri et al. 2018). This redundancy suggests that other *nsLTPs* might also be associated with grain dormancy. Fujino et al. (2014) reported collinearity of chromosomal regions around *qLTG3-1* and its orthologous genes as conserved among rice, *Brachypodium*, sorghum, and maize. They also discussed that structural changes i.e., deletion, insertion and duplication in this area were thought to be useful for analyzing evolution and diversity. The redundancy of wheat *nsLTPs* is expected to be useful in this study as well.

Interconnections between grain color, *TaLTP2.128*, and dormancy

As mentioned above, ABA sensitivity is thought to vary with grain color. We used grains of 30 DAP and treated them with water or ABA solution to search for differentially expressed genes. Although no differences were found between lines, several peroxidase genes were down-regulated by ABA treatment in both lines (data not shown). This result may suggest that ROS is occurred in imbibed grains. The grain pigment accumulated in wheat testa is proanthocyanidins which are composed of (+)-catechin mainly, and both (+)-catechin and proanthocyanidins have been known as antioxidant. Miyamoto and Everson (1958) also showed that catechins have an inhibitory effect on wheat germination. Additionally, it was
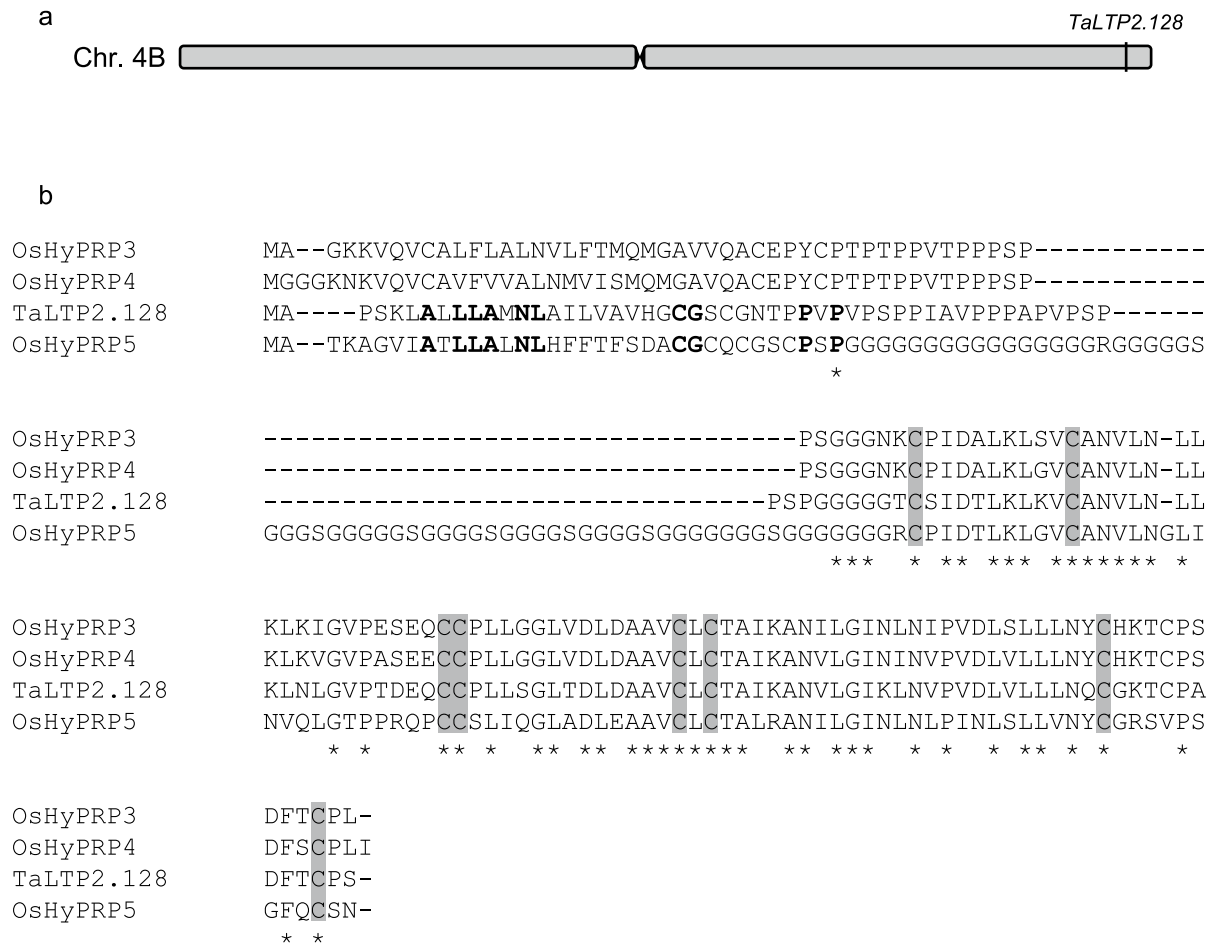
a

b

```
OsHyPRP3    MA--GKKVQVCALFLALNVLFTMQMGAVVQACEPYCPTPTPPVTPPPSP-----------
OsHyPRP4    MGGGKNKVQVCAVFVVALNMVISMQMGAVQACEPYCPTPTPPVTPPPSP-----------
TaLTP2.128  MA----PSKLALLLAMNLAILVAVHGCGSCGNTPPVPVPSPPIAVPPPAPVPSP------
OsHyPRP5    MA--TKAGVIATLLALNLHFFTFSDACGCQCGSCPSPGGGGGGGGGGGGGGGGGRGGGGGS
                                                   *

OsHyPRP3    ----------------------------------PSGGGNKCPIDALKLSVCANVLN-LL
OsHyPRP4    ----------------------------------PSGGGNKCPIDALKLGVCANVLN-LL
TaLTP2.128  -----------------------------PSPGGGGGTCSIDTLKLKVCANVLN-LL
OsHyPRP5    GGGSGGGGGSGGGGSGGGGSGGGGSGGGGGGGGSGGGGGGGRCPIDTLKLGVCANVLNGLI
                                                    ***   *  ** ***  ******* *

OsHyPRP3    KLKIGVPESEQCCPLLGGLVDLDAAVCLCTAIKANILGINLNIPVDLSLLLNYCHKTCPS
OsHyPRP4    KLKVGVPASEECCPLLGGLVDLDAAVCLCTAIKANVLGININVPVDLVLLLNYCHKTCPS
TaLTP2.128  KLNLGVPTDEQCCPLLSGLTDLDAAVCLCTAIKANVLGIKLNVPVDLVLLLNQCGKTCPA
OsHyPRP5    NVQLGTPPRQPCCSLIQGLADLEAAVCLCTALRANILGINLNLPINLSLLVNYCGRSVPS
                  *  *    ** *   ** **  *******  **  ***   *  *   *  **  *  *        *

OsHyPRP3    DFTCPL-
OsHyPRP4    DFSCPLI
TaLTP2.128  DFTCPS-
OsHyPRP5    GFQCSN-
                 *  *
```

Fig. 6 *LTP-B* gene as *TaLTP2.128*. **a**. Location of *TaLTP2.128* gene on chromosome 4B. **b**. Alignment of putative amino acid sequences of TaLTP2.128 and, OsHyPRP3, OsHyPRP4, and OsHyPRP5 of rice. Cysteine residues shown in grey are components of the eight cysteine motif (8-CM). Bold letters signify common residues among TaLTP2.128 and OsHyPRP5

reported that reactive oxygen species (ROS) act as dormancy-breaking substances in grain germination in wheat and barley (Ishibashi et al. 2012, 2008), and it is possible that grain pigments may have an effect on removing ROS leading to inhibit germination. ABA might promote to delete ROS for inhibition of germination.

A lipid transfer protein gene was found to be an up-regulated gene in white-grained line in both ABA and water treatments, and identified as *TaLTP2.128* according to an earlier report (Kouidri et al. 2018). *R-1* not only regulated grain color but also modified the expression of *TaLTP2.128* in imbibed grains. Furthermore, *TaLTP2.128* showed high expression levels in other white-grained mutant lines, increasing with the duration of water absorption (Fig. 7). A similar expression pattern was also observed in non-dormant mutant RSD32, irrespective of grain color. *TaLTP2.128* might be indirectly regulated by the *R-1* gene through the modification of dormancy level. In
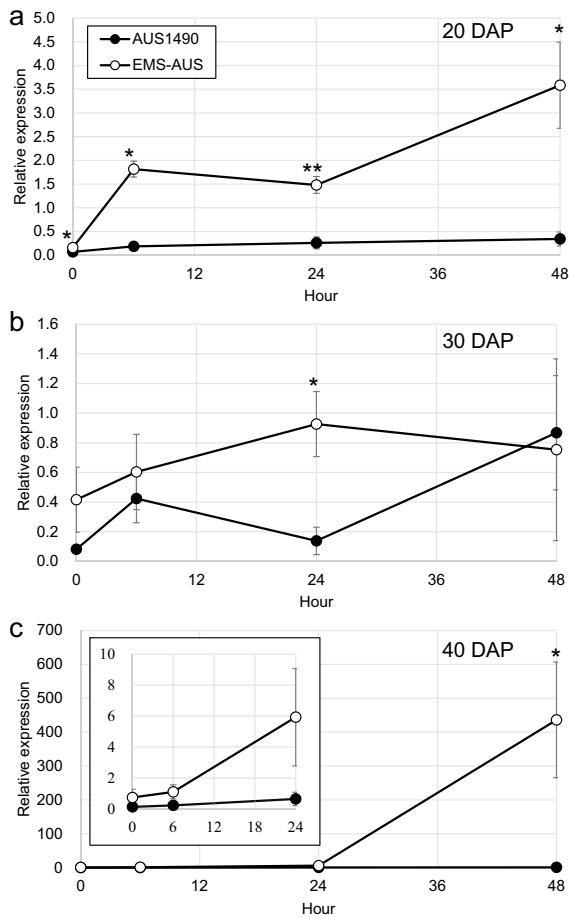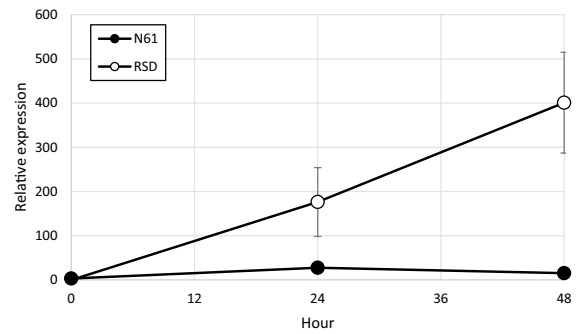
Fig. 7 Relative expressions of *LTP-B* gene in AUS1490 and EMS-AUS. Embryos were extracted from freshly harvested whole grains (0 h) and 6, 24, and 48 h-imbibed grains. **a**. 20 DAP. **b**. 30 DAP. **c**. 40 DAP. Black and white circles respectively denote AUS1490 and EMS-AUS. Expression levels of 0, 6, and 24 h-imbibed grains are presented in the inset

Fig. 8 Relative expressions of *LTP-B* gene in Norin 61 and RSD32. Embryos were extracted from freshly harvested whole grains (0 h) and 6, 24, and 48 h-imbibed grains of 40 DAP. Black and white circles respectively stand for Norin 61 and RSD32

addition, the expression of *TaLTP2.128* was detected before germination, suggesting that *TaLTP2.128* might act as a trigger of a transition from the dormancy stage to the germination stage.

Data availability The datasets analyzed during the study are available in the DDBJ Sequenced Read Archive under accession numbers DRX411818 – DRX411829.

Declarations

Conflict of interest. The authors report no conflict of interest.

References

Abe F, Haque E, Hisano H et al (2019) Genome-edited triple-recessive mutation alters seed dormancy in wheat. Cell Rep 28(1362–1369):e1364

Bartlett JG, Smedley MA, Harwood WA (2014) Analysis of T-DNA/host-plant DNA junction sequences in single-copy transgenic barley lines. Biology 3:39–55

Carvalho AO, Gomes VM (2007) Role of plant lipid transfer proteins in plant cell physiology—a concise review. Peptides 28:1144–1153

Chmelar F, Mostovoj K (1938) On the application of some old and the introduction of new methods for testing genuineness of variety in the laboratory. Proc Int Seed Test Assoc 10:67–74

Debeaujon I, Leon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. Plant Physiol 122:403–414

Fujino K, Sekiguchi H, Matsuda Y, Sugimoto K, Ono K, Yano M (2008) Molecular identification of a major quantitative trait locus, qLTG3-1, controlling low-temperature germinability in rice. Proc Natl Acad Sci USA 105:12623–12628

Fujino K, Obara M, Sato K (2014) Diversification of the plant-specific hybrid glycine-rich protein (HyGRP) genes in cereals. Front Plant Sci 5:489

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 25:989–994

Heyne EG, Livers RW (1953) Monosomic analysis of leaf rust reaction, awnedness, winter injury and seed color in Pawnee wheat. Agron J 45:54–58

Himi E, Noda K (2005) Red grain colour gene (R) of wheat is a Myb-type transcription factor. Euphytica 143:239–242

Himi E, Mares DJ, Yanagisawa A, Noda K (2002) Effect of grain colour gene (R) on grain dormancy and sensitivity of the embryo to abscisic acid (ABA) in wheat. J Exp Bot 53:1569–1574

Himi E, Nisar A, Noda K (2005) Colour genes (R and Rc) for grain and coleoptile upregulate flavonoid biosynthesis genes in wheat. Genome 48:747–754

Himi E, Maekawa M, Miura H, Noda K (2011) Development of PCR markers for Tamyb10 related to R-1, red grain color gene in wheat. Theor Appl Genet 122:1561–1576

Himi E, Maekawa M, Matsuura T, Taketa S (2015) Real-time PCR-mediated diagnosis of hemizygosity at the Tamyb10-D1 locus controlling grain color in wheat. Mol Breed 35:90

Ishibashi Y, Yamamoto K, Tawaratsumida T, Yuasa T, Iwaya-Inoue M (2008) Hydrogen peroxide scavenging regulates germination ability during wheat (Triticum aestivum L.) seed maturation. Plant Signal Behav 3:183–188

Ishibashi Y, Tawaratsumida T, Kondo K, Kasa S, Sakamoto M, Aoki N, Zheng SH, Yuasa T, Iwaya-Inoue M (2012) Reactive oxygen species are involved in gibberellin/abscisic acid signaling in barley aleurone cells. Plant Physiol 158:1705–1714

Ishida Y, Tsunashima M, Hiei Y, Komari T (2015) Wheat (Triticum aestivum L.) transformation using immature embryos. Methods Mol Biol 1223:189–198

Kader JC (1975) Proteins and the intracellular exchange of lipids: I. stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber. Biochim Biophys Acta Lipids Lipid Metab 380:31–44

Kaneko S, Komae K, Nagamine T, Yamada T (1994) Development of an extraction procedure for wheat red coat pigments and determination of varietal differences for this trait. Breed Sci 44:263–266

Kim D, Paggi JM, Park C, Bennett C, Salzberg SL (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol 37:907–915

Kouidri A, Whitford R, Suchecki R, Kalashyan E, Baumann U (2018) Genome-wide identification and analysis of non-specific Lipid Transfer Proteins in hexaploid wheat. Sci Rep 8:17087

Koval SF (1997) The catalog of near-isogenic lines of Novosibirskaya-67 common wheat and principles of their use in experiments. Russ J Genet 33:995–1000

Kuroda M, Kimizu M, Mikami C (2010) A simple set of plasmids for the production of transgenic plants. Biosci Biotechnol Biochem 74:2348–2351

Kurihara-Yonemoto S, Matsunaka H (2019) A simple method for genotyping of wheat seed coat color genes by multiplex PCRs [in Japanese with English summary, tables, and figures]. Bull NARO Agric Res Hokkaido Reg 207:35–49. https://doi.org/10.24514/00002276

Lang J, Fu Y, Zhou Y et al (2021) Myb10-D confers PHS-3D resistance to pre-harvest sprouting by regulating NCED in ABA biosynthesis pathway of wheat. New Phytol 230:1940–1952

Lee SB, Suh MC (2018) Disruption of glycosylphosphatidylinositol-anchored lipid transfer protein 15 affects seed coat permeability in Arabidopsis. Plant J 96:1206–1217

Mares D, Himi E (2021) The role of TaMYB10-A1 of wheat (Triticum aestivum L.) in determining grain coat colour and dormancy phenotype. Euphytica 217:89

Mares D, Mrva K, Cheong J, Williams K, Watson B, Storlie E, Sutherland M, Zou Y (2005) A QTL located on chromosome 4A associated with dormancy in white- and red-grained wheats of diverse origin. Theor Appl Genet 111:1357–1364

Matsuura T, Mori IC, Himi E, Hirayama T (2019) Plant hormone profiling in developing seeds of common wheat (Triticum aestivum L.). Breed Sci 69:601–610

Miura H, Sato N, Kato K, Amano Y (2002) Detection of chromosomes carrying genes for seed dormancy of wheat using the backcross reciprocal monosomic method. Plant Breed 121:394–399

Miyamoto T, Everson EH (1958) Biochemical and physiological studies of wheat seed pigmentation. Agron J 50:733–734

Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10:11

Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL (2016) Transcript-level expression analysis of RNA-seq experiments

with HISAT, StringTie and Ballgown. Nat Protoc 11:1650–1667

Rikiishi K, Sugimoto M, Maekawa M (2021) Transcriptomic analysis of developing seeds in a wheat (*Triticum aestivum* L.) mutant RSD32 with reduced seed dormancy. Breed Sci 71:155–166

Salminen TA, Blomqvist K, Edqvist J (2016) Lipid transfer proteins: classification, nomenclature, structure, and function. Planta 244:971–997

Wang X et al (2015) A lipid transfer protein, OsLTPL36, is essential for seed development and seed quality in rice. Plant Sci 239:200–208

Warner RL, Kudrna DA, Spaeth SC, Jones SS (2000) Dormancy in white-grain mutants of Chinese Spring wheat (*Triticum aestivum* L.). Seed Sci Res 10:51–60

Yazdanpanah F, Hanson J, Hilhorst HWM, Bentsink L (2017) Differentially expressed genes during the imbibition of dormant and after-ripened seeds—a reverse genetics approach. BMC Plant Biol 17:151