



# Isolation and characterization of kelch repeat-containing F-box proteins from colored wheat

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## Abstract

F-box proteins play important roles in the regulation of various developmental processes in plants. Approximately 1796 F-box genes have been identified in the wheat genome, but details of their functions remain unknown. Moreover, not much was known about the roles of kelch repeat domain-containing F-box genes (*TaKFBs*) in wheat. In the present study, we isolated five *TaKFBs* to investigate the roles of KFBs at different stages of colored wheat grain development. The cDNAs encoding *TaKFB1*, *TaKFB2*, *TaKFB3*, *TaKFB4*, and *TaKFB5* contained 363, 449, 353, 382, and 456 bp open reading frames, respectively. All deduced TaKFBs contained an F-box domain (IPR001810) and a kelch repeat type 1 domain (IPR006652), except TaKFB2. Expression of *TaKFBs* was elevated during the pigmentation stages of grain development. To clarify how TaKFB and SKP interact in wheat, we investigated whether five TaKFB proteins showed specificity for six SKP proteins using a yeast two-hybrid (Y2H) assay. An Y2H screen was performed to search for proteins capable of binding the TaKFBs and interaction was identified between TaKFB1 and aquaporin PIP1. To examine the subcellular localization of TaKFBs, we transiently expressed TaKFB-green fluorescent protein (GFP) fusions in tobacco leaves; the TaKFB-GFP fusions were detected in the nucleus and the cytoplasm. Y2H and bimolecular fluorescence complementation (BiFC) assays revealed that TaKFB1 specifically interacts with aquaporin PIP1. These results will provide useful information for further functional studies on wheat F-box proteins and their possible roles.

**Keywords** Wheat · F-box protein · Kelch repeat · Grain development · Yeast two-hybrid · TaKFB

## Introduction

Protein degradation via ubiquitin attachment occurs through the following sequential three-step reaction involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes [1]. An E3 ligase recognizes target proteins and transfers ubiquitin from the E2 to a specific substrate via the 26S proteasome pathway. E3 ligases are capable of recognizing and binding specifically to target

proteins to recruit them for protein degradation, thus ensuring that development and growth are timed accurately. The specificity of protein degradation is mainly determined by E3 ligases, so there are many more different kinds of E3 ligase than E1 or E2 ligase [2]. E3 ligases are highly diverse and important enzymes; it is estimated that there are over 1000 in plants [2]. They may be categorized into three subclasses according to their subunit composition and mechanism of action: CRLs (cullin–RING ligases), RING (really interesting new gene)/U-box ligases, and APC/C (anaphase-promoting complex/cyclosome) ligases [3]. RING/U-box E3 ligases are single-subunit complexes that contain both an active site and binding pocket for the E2 intermediate, while CRLs are multi-subunit complexes that consist of a cullin, a RING H2 finger protein that binds to ubiquitin, and variable substrate adaptors [4]. The SCF [S-phase kinase-associated protein 1 (SKP1)/CULLIN1 (CUL1)/F-Box] complex is the largest in the CRL E3 ligase family in plants. The APC/C is a multi-subunit E3 ligase that plays an important role in the progression of mitotic cell cycle [5].

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F-box proteins are one of the components of the SCF complex that function in substrate recognition and substrate recruitment for degradation by the 26S proteasome. The N-terminal of F-box proteins is composed of a well-conserved F-box domain (35–60 amino acids) that directly interacts with SKP1. The C-terminal region contains one or several functional domains such as kelch repeats, leucine-rich repeats, WD40 repeats, F-box-associated domains, and tubby domains, which bind specifically to different substrates [6] via protein–protein interaction to recruit target proteins. F-box proteins serve as specificity determinants due to the extensive variability of these C-terminal target-binding domains. In a previous report, 897, 971, and 425 F-box-coding genes were identified in *Arabidopsis*, rice, and poplar [7], respectively, and categorized into 10 subfamilies based on the type of motif existing at the C-terminal [6].

In plants, F-box proteins are regarded as one of the largest protein superfamilies, which reflect the crucial roles that they play in the modulation of various developmental and physiological processes. Functions of F-box proteins have mainly been reported from *Arabidopsis*; *LKP1/ZTL*, *LKP2/FKL*, and *FKF1* are involved in photomorphogenesis, the circadian clock, and flowering time [8–11], while *EBF1* and *EBF2* regulate ethylene signaling [12] and *SLY1* and *SNE* control gibberellic acid signaling [13]. The role of F-box proteins in jasmonate (*COII*) and auxin (*TIR1*) signaling has also been documented [14, 15]. Additionally, *OsFBK12* reportedly regulates leaf senescence and grain size through the degradation of target substrates in rice [16]. Few of the functions of F-box proteins have been studied in plant species other than the model plant *Arabidopsis*, despite the fact that they play important roles in many aspects of plant growth, development, and other physiological processes.

One region of F-box proteins, the kelch repeat domain, was first identified in *Drosophila melanogaster* [17]. Kelch repeat domain-containing F-box proteins (KFBs) appear more frequently in rice and *Arabidopsis* than in mammals and yeast [6]. In addition to the functions listed above, KFBs are involved in the regulation of pigmentation. The degradation of *Arabidopsis* phenylalanine ammonia-lyase (PAL) isozymes via the 26S proteasome system is regulated by KFBs, which act as repressors in the phenylpropanoid pathway [18, 19]. Shao et al. demonstrated that KFBs regulate pigment synthesis and deposition in rice hulls. However, the molecular functions of KFBs in wheat are largely unknown [20].

Previous studies in *Arabidopsis* reported that up or down-regulation of *Arabidopsis* KFB expression conversely affected the accumulation/deposition of a set of phenylpropanoids, including flavonols, anthocyanins, and condensed tannins [18]. However, not much is known about the roles

of KFB in the development of colored wheat grain. Therefore, we selected and isolated wheat KFB genes that showed high sequence similarity to *Arabidopsis* KFBs. We used colored wheat seeds in the present study; therefore, we tried to search KFB that prompted change in grain color, related to the flavonoid biosynthesis pathway. We isolated kelch repeat domain-containing F-box genes (KFBs) from wheat spike cDNA and examined the expression patterns of *TaKFB* (*Triticum aestivum* kelch repeat domain-containing E-box) genes in grains at different developmental stages by qRT-PCR. Green fluorescent protein (GFP) was used to examine the subcellular localization of TaKFB proteins. We explored proteins that may potentially interact with TaKFBs through yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays. Also, we confirmed that TaKFBs are F-box proteins that can interact with SKPs (a component of the SCF complex). Our results suggest that interaction between TaKFBs and TaSKPs may produce SCF complexes and that TaKFBs have a minor influence on pigmentation in wheat grains.

## Materials and methods

### Plant materials

A line of common hexaploid wheat with a deep purple grain color (accession no. K4191), developed by Korea University (Republic of Korea), was used in this study [21]. Wheat seeds were grown on a radiation breeding research farm located at 35.5699° N and 126.9722° E (Jeongeup-si, Jeollabuk-do, Korea). After flowering, we tagged wheat spikes to confirm the stage of grain development. Then, we collected wheat grain samples according to their developmental stage (days after fertilization: DAF, DAF9, DAF12, DAF16, DAF20, DAF25, DAF29, and DAF35). Samples were immediately frozen in liquid nitrogen and stored at – 80 °C until further analysis.

### Cloning of TaKFBs and respective interacting genes

In order to isolate the full sequences of five *TaKFBs* and respective interacting genes, primer sets were designed based on sequences downloaded from wheat IWGSC Reference Sequence v1.0 and the NCBI database (<https://www.ncbi.nih.gov/Structure/cdd/wrpsb.cgi>) (Table 1). Then, PCR was performed under the following cycling conditions: initial denaturation at 94 °C for 10 min, 31 cycles of denaturation at 94 °C for 1 min, annealing at a gene-specific temperature for 2 min, elongation at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were cloned into a pCR8/GW/TOPO cloning vector (Thermo

**Table 1** List of primer sequences used for gene cloning, qPCR, Y2H, subcellular localization, and BiFC experiments

Objectives	Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Gateway Cloning	<i>TaKFB1</i>	ATGAACCATCACCTTGAAAGTGG	TCAGATGGTGATGACACTGCAC
	<i>TaKFB2</i>	ATGGAAGATTACAAGATTGCAAC	TCAGCACCCCGTTATTGCACAGTT
	<i>TaKFB3</i>	ATGGTTGAGTGCACGATGGTATCG	CTAGATCTTCAGCAGAGCACAGCC
	<i>TaKFB4</i>	ATGGCTTCAATTCGAACAGCTGTC	TCAGATGGTGATGACACTGCACCT
	<i>TaKFB5</i>	ATGGGGGCATCTCTCAGCTTCCCCA	TCATATGCTGAGCGCCTGGCAGCTA
	<i>Aquaporin PIP1</i>	ATGGAGGGCAAGGAGGAGGACGT	CTAGTCGCGGCTCTTGAAGGGGA
qRT-PCR	<i>TaKFB1</i>	ACGGCAGCTCTGAATGACAAG	GTGAATGGTCACCAGCTCATC
	<i>TaKFB2</i>	ATGGAAGATTACAAGATTGC	GCTGAGTGTTCCTTGGCTCCCTC
	<i>TaKFB3</i>	ATGGTTGAGTGCACGATGGTAT	TCCAAGAGCGGCAAACCAGCTGAA
	<i>TaKFB4</i>	ATGGCTTCAATTCGAACAGCTG	ATCTGGAAGACCATGTAGAAGG
	<i>TaKFB5</i>	TTCCTGACCTCCCGGATGAGA	CATCCACACCAAGCTCCTTCC
	<i>Aquaporin PIP1</i>	GGTCACGTTCCGGCTGTT	TCTGGAACCCCTTCACCA
Y2H assay	<i>Actin (AB181991)</i>	GCCACACTGTTCCAATCTATGA	TGATGGAATTGTATGTCGCTTC
	<i>TaKFB1</i>	GAATTCATGAACCATCACCTTGAA	GGATCCCTCAGATGGTGATGACAC
	<i>TaKFB2</i>	GAATTCATGGAAGATTACAAGAT	GGATCCCTCAGCACCCCGTTATTG
	<i>TaKFB3</i>	GAATTCATGGTTGAGTGCACGATG	GGATCCCCTAGATCTTCAGCAGAG
	<i>TaKFB4</i>	GAATTCATGGCTTCAATTCGAACA	GGATCCCTCAGATGGTGATGACAC
	<i>TaKFB5</i>	GAATTCATGGGGCATCTCTCAGC	GGATCCCTCATATGCTGAGCGCCT
BiFC assay	<i>Aquaporin PIP1</i>	GAATTCATGGAGGGCAAGGAGGAG	GGATCCCCTAGTCGCGGCTCTTGA
	<i>TaKFB1</i>	ATGAACCATCACCTTGAAAGTGG	AGATGGTGATGACACTGCACCTTCTC
	<i>Aquaporin PIP1</i>	ATGGAGGGCAAGGAGGAGGACGT	GTCGCGGCTCTTGAAGGGGA

Fisher Scientific, USA) and sequenced. Sequencing results were analyzed using the NCBI database and InterProScan (<https://www.ebi.ac.uk/interpro/search/sequence-search>).

### RNA extraction and qRT-PCR analysis

Total RNA was extracted from wheat seeds according to the protocol described by Meng and Feldman [22]. For qRT-PCR analysis, 1 µg RNA was converted to cDNA using a Power cDNA Synthesis kit (iNtRON Biotechnology, Korea) according to the manufacturer's protocol. Then, qRT-PCR was performed in a 48-well plate (Eco Real-Time PCR System, USA) using 1 µL of reverse-transcribed cDNA and 2×SYBR premix Ex *Taq* II (Tli RNaseH plus, Takara, Japan). The qRT-PCR thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 65 °C for 30 s. Three replicates were performed for all qRT-PCR experiments.

### RNA sequencing analysis

Recombinant inbred lines (RILs) of common wheat used in this study were developed as according to previous reports [23]. Seeds of colored and yellow wheat were grown in controlled environments at 23–26 °C under long-day conditions (16 h/8 h, day/night). We collected plant samples at

different developmental stages, including DAF10, DAF20, and DAF30. Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, Waltham, MA, USA). Total RNA (10 µg) from sample was used for constructing RNA-Seq paired-end libraries by TruSeq RNA Sample Preparation Kit (catalog #RS-122-2001; Illumina, CA, USA), and they were sequenced using Illumina HiSeq2000 platform. For reads mapping to the wheat reference genome sequence, an index of the genome sequence was constructed and all reads were aligned to the wheat genome sequence using HISAT2 with default values [24]. HTSeq v0.6.1 counted the number of reads mapped to the exons of each gene [25]. Read count normalization and calculation of log<sub>2</sub> fold change were conducted using DESeq2 [26]. Read count and log<sub>2</sub> fold changes were used to construct heatmaps of *TaFBX* genes expression profiling at different seed developmental stages using R package.

### Y2H cDNA library construction and screening assay

A wheat (*Triticum aestivum* L.) cDNA library was constructed for Y2H screening from deep purple color wheat seeds (accession no. K4191). First, total RNA was extracted from wheat seeds (DAF20) using Trizol reagent. Next, 2 µg total RNA was used to construct a wheat cDNA library with the Make Your Own Mate & Plant™ Library System (Clontech, Takara, Japan) as per the manufacturer's protocol. All

colonies were pooled with freezing medium (YPDA medium with 25% glycerol), and 1 mL aliquots of the cDNA library were stored at  $-80\text{ }^{\circ}\text{C}$ . For the Y2H screen, full-length TaKFB1, TaKFB2, TaKFB3, TaKFB4, and TaKFB5 coding sequences were transferred into a pGBKT7-GW bait vector using a Gateway LR recombination reaction (Thermo Fisher Scientific, USA) and used as bait protein for screening. The bait constructs (pGBKT7-TaKFBs) were transformed into the yeast strain Y2HGold using the Matchmaker Gold Y2H System (Clontech, Takara, Japan) according to the manufacturer's protocol. Y2HGold strains harboring bait vectors were maintained in minimal SD medium with tryptophan dropout supplement (SD/-Trp). Then, bait strains and cDNA library (prey) were mixed and incubated at  $30\text{ }^{\circ}\text{C}$  with shaking at 30–50 rpm for 20–24 h. The mating cultures were spread onto SD/-Leu/-Trp/X- $\alpha$ -Gal/AbA plates (DDO/X/A) until colonies appeared (after approximately 5–7 days). Colonies on DDO/X/A were transferred to SD/-Ade/-His/-Leu/-Trp/X- $\alpha$ -Gal/AbA (QDO/X/A) plates for further selection.

### Rescue of prey plasmids

Yeast colonies grown on QDO/X/A plates were colonies with potential positive interactions between bait and prey proteins. Yeast colony PCR using Matchmaker Insert Check PCR Mix 2 (Clontech, Takara, Japan) was performed to select positive clones. Prey plasmids were rescued from the yeast using Easy Yeast Plasmid Isolation kits (Clontech, Takara, Japan). After plasmid DNA extraction, the prey inserts were identified by sequencing, the results of which were analyzed using NCBI/GenBank databases.

### Pairwise Y2H assay

cDNAs encoding TaKFBs and PIP1 were amplified by PCR using the gene-specific primers listed in Table 1. Amplification products were cloned into the Gateway-compatible shuttle vector pCR8/GW/Topo (Thermo Fisher Scientific, USA) and subcloned into the Gateway-compatible pGBKT7 (bait) and pGADT7 (prey) vectors to generate bait DNA-binding and activation domain fusion proteins. For pairwise verification between TaKFBs and PIP1, AH109 yeast cells (Clontech, Takara, Japan) were used according to the manufacturer's instructions.

### $\beta$ -galactosidase activity assay

To confirm the strength of a protein interaction, quantitative  $\beta$ -galactosidase activity was assayed with 2-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG, Sigma) using the Clontech yeast protocols handbook (protocol no. PT3024-1). The yeast cells were grown overnight at  $30\text{ }^{\circ}\text{C}$  in SD selective medium; and 2 mL of culture cells were then transferred to

8 mL of YPD media, and the culture was incubated until the  $\text{OD}_{600}$  reached 0.8. Thereafter, yeast cells were centrifuged and resuspended in Z-buffer (60 mM  $\text{Na}_2\text{HPO}_4$ , 40 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM KCl, 1 mM  $\text{MgSO}_4$ , pH 7.0). The yeast cell pellets were lysed via freeze–thaw cycles. All reactions were initiated by the addition of Z-buffer containing  $\beta$ -mercaptoethanol as well as 4 mg/mL ONPG solution to the samples. The reactions were incubated at  $30\text{ }^{\circ}\text{C}$  and stopped after 30 min for the development of yellow color by adding chilled 1 M  $\text{Na}_2\text{CO}_3$ . Absorbance was measured at 420 nm [27], and  $\beta$ -galactosidase activity was calculated according to the following formula:  $\beta$ -galactosidase activity =  $1000 \times A_{420} / (T \times V \times A_{600})$ , where T = time of reaction (min), and V = volume of culture used in the assay (mL).

### Subcellular localization analysis and BiFC assay

For subcellular localization analysis, the open reading frames (ORFs) of TaKFBs and PIP1 were cloned into a pMDC43 vector [28] to be fused to the N-terminus of an enhanced GFP gene with LR clonase (Thermo Fisher Scientific, USA). The plasmids, 35S:GFP-TaKFBs and 35S:GFP-PIP1, were introduced into *Agrobacterium tumefaciens* GV3101 using the freeze–thaw method. *Agrobacterium* GV3101 harboring 35S:GFP-TaKFBs and 35S:GFP-PIP1 was grown in LB medium until the optical density of the culture reached 1.0 at 600 nm. The *Agrobacterium* cells were pelleted at 5000 g for 10 min at room temperature and the pellets were resuspended in infiltration buffer (pH 5.6; 10 mM MES, 10 mM  $\text{MgCl}_2$ , 200 mM acetosyringone). Then, tobacco leaves were injected with *Agrobacterium* solution using a syringe. For the BiFC assay, GFP fusion proteins were produced by the recombination (LR clonase reaction) of the entry vectors pCR/GW/Topo-TaKFBs, and PIP1 with the destination vectors pGTQL1211-YN (GFP-N) or pGTQL1221-YC (GFP-C) using the Gateway system [29]. All constructs were then introduced into *Agrobacterium* GV3101 and infiltrated into tobacco leaves based on the methods detailed in a previous report [30]. Tobacco leaves were assayed for fluorescence 72 h after infiltration for both analyses of subcellular localization and the BiFC assay using a confocal laser-scanning microscope (Zeiss LSM800, Germany).

### MG132 treatment and western blot

Four-week-old *N. benthamiana* (tobacco) plants were used for infiltration. *Agrobacterium tumefaciens* GV3101 carrying the 35S-GFP-PIP1 constructs were infiltrated into tobacco leaves and incubated for 48 h at  $22\text{ }^{\circ}\text{C}$ . For proteasome inhibitor treatments, tobacco leaves were injected with 50  $\mu\text{M}$  MG132 24 h after *Agrobacterium* infiltration, then collected after a further 12 h. Total protein was extracted from the tissues in protein extraction buffer (Elpis Biotech,

Korea). The concentration of total protein was determined using a Bradford assay kit (Takara, Japan). For western blotting, total proteins were separated on 12% SDS-PAGE gel and transferred to nitrocellulose membranes using the iBlot 2 Dry Blotting system. Membranes were incubated with primary antibody (rabbit anti-GFP antibody, abcam, UK). Then, HRP-conjugated goat anti-rabbit (Thermo Fisher Scientific, USA) was used as secondary antibody to generate the light signal. The membrane was visualized using Amersham ECL western blotting detection reagents (GE Healthcare, USA) and the chemiluminescence was recorded with the iBright CL1000 imaging system (Invitrogen, USA).

## Results

### Isolation and sequence analysis of KFBs in wheat

Previous reports demonstrate that a group of KFBs interacts with flavonoid biosynthesis proteins to regulate phenylpropanoid biosynthesis in *Arabidopsis* [18, 19, 31]. In the present study, four *Arabidopsis* KFBs were used to isolate wheat KFBs: *AtKFB1* (AT1G15670.1), *AtKFB20* (AT1G80440.1), *AtKFB39* (AT2G44130.1), and *AtKFB50* (AT3G59940.1). These were BLAST searched against a wheat reference genome sequence (wheat IWGSC Reference Sequence v1.0) provided by URGI (<https://urgi.versailles.inra.fr/>) with an E-value threshold  $< 1e^{-5}$ . We investigated the F-box proteins that regulate anthocyanin biosynthesis during wheat grain development using colored wheat, the seed coat of which starts to change color from green to red on DAF20 [21]. We collected KFBs from a wheat database (*Triticum aestivum* IWGSC1.0), and putative wheat KFBs were confirmed by the presence of F-box domains in the N-terminus and the kelch repeat domain of the C-terminus using the InterProScan database (<https://www.ebi.ac.uk/interpro/search/sequence-search>). We performed BLAST-search of the protein sequences of TaKFBs and only selected the sequences with maximum number of hits with quality score (Supplementary Table 1). Finally, KFB1 (TraesCS6A02G037900.1), KFB2 (TraesCS2B02G484200.1), KFB3 (TraesCS6D02G195800.1), KFB4 (TraesCS6D02G043400.1), and KFB5 (TraesCS7D02G016400.1) were selected five complete TaKFB ORFs (*TaKFB1*, *TaKFB2*, *TaKFB3*, *TaKFB4*, and *TaKFB5*) were isolated from wheat spike cDNA. These ORFs were 1092, 1350, 1062, 1149, and 1371 bp in size, respectively, and encoded 363, 449, 353, 382, and 456 amino acids, respectively (Table 2). The molecular weights of the TaKFBs had a pI range between 6.10 and 7.53 and a mass range between 38.80 and 49.98 kDa (Table 2). According to the blast results, TaKFB were likely to be located on chromosomes 2, 6, and 7. TaKFB1, TaKFB3, and TaKFB4

produced significant hits to sequences located on wheat chromosome 6A, 6B, and 6D (Supplementary Table 1). Especially, TaKFB1 and TaKFB4 represented hits of same region in wheat chromosome 6. Moreover, TaKFB1 and TaKFB4 shared higher levels of amino acid sequence similarity (Supplementary Fig. 1). The domain annotation of *TaKFB* genes was conducted using DoMosaics software based on a local installation of HMMscan against Pfam-defined models (Table 1). Sequence analysis indicated that all deduced TaKFBs contain an F-box domain (IPR001810), except TaKFB2 (Fig. 1). TaKFB2 encoded a truncated form of F-box protein in wheat (Supplementary Fig. 2). The C-terminus region of the TaKFBs consists of approximately 1–3 kelch repeat type 1 domains (IPR006652), as predicted (Fig. 1).

### TaKFBs interact with TaSKPs

F-box protein, a component of the E3 ubiquitin ligase SCF complex, interacts with SKP1 [32]. To confirm the interaction between TaKFBs and TaSKPs, we investigated whether five TaKFB proteins exhibited specificity for six TaSKP proteins using Y2H assay. The TaKFB sequences were fused to the DNA-binding domain of GAL4 in a pGBKT7 vector to serve as bait, and six TaSKP sequences were fused to the DNA activation domain of GAL4 in a pGADT7 vector [33]. After mating, all combinations grew well on DDO/X/A (Fig. 2a), whereas several combinations showed no interaction on QDO/X/A. We found that TaKFB1, TaKFB3, and TaKFB4 could interact with TaSKP1 and TaSKP6 on QDO/X/A (Fig. 2b). In addition, TaKFB4 interacted with TaSKP3. However, there was no interaction between TaKFB2 and TaKFB5 and any of the TaSKPs examined. Results of the Y2H screening for the quantification of the relative interaction strength between TaKFBs and TaSKPs were measured by performing  $\beta$ -galactosidase assay (Fig. 2c).  $\beta$ -galactosidase activities for the interaction between TaKFBs and TaSKPs ranged from 18.6 to 33.9 units. Although all constructs showed low activities compared with the positive control, the relative strength of interaction between TaKFB1, TaKFB3, and TaKFB4 as well as between TaSKP1, TaSKP4, and TaSKP6 was at remarkable levels. As expected,  $\beta$ -galactosidase activity was very low in the negative control.

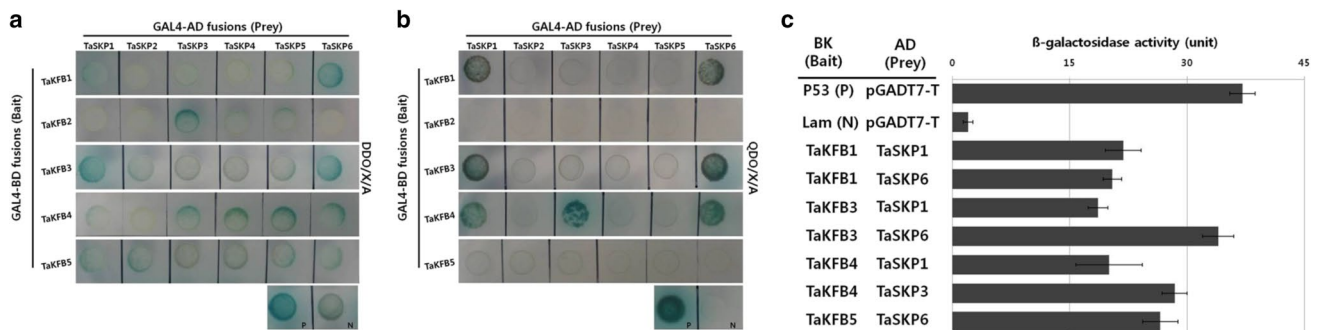
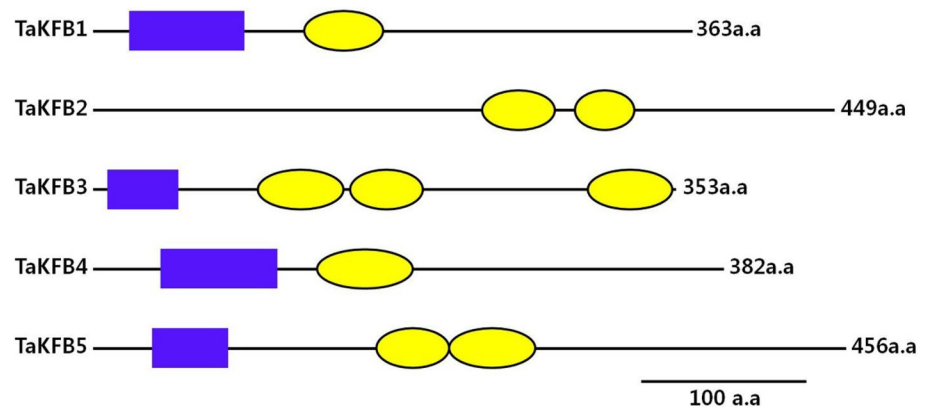
### Screening of proteins interacting with TaKFBs

To identify potential TaKFB-binding partners, each TaKFB fragment fused with the GAL4 DNA-binding domain was used as bait to screen the wheat grain development cDNA library fused to a synthetic activation domain in yeast. From a total of approximately  $2 \times 10^8$  transformants, the first screening, involving blue/white selection on DDO/X/A, identified 61 (TaKFB1-interacting partners) and 74

**Table 2** Sequence analysis of five kelch domain-containing F-box proteins in wheat

CDS (bp)	Total length (aa)	MW (kDa)	pI	InterProScan		HMMScan results	Pfam ID	Position	E-value
				F-box domain (IPR001810)	Kelch repeats (IPR006652)				
TaFBX1	363	41.10	6.51	22–92	128–175	F-box Kelch_1	PF00646 PF01344	28–68 117–162	1.60E-07 6.60E-08
TaFBX2	449	49.98	6.10	–	234–280, 294–327	Kelch_4 Kelch_1 Kelch_4	PF13418 PF01344 PF13418	125–163 234–280 234–279	9.30E-07 6.20E-09 2.30E-04
TaFBX3	353	38.80	6.39	9–52	109–166, 167–215, 297–352	F-box Kelch_1 Kelch_1	PF00646 PF01344 PF01344	11–46 99–152 155–201	2.00E-06 1.40E-07 2.70E-12
TaFBX4	382	43.25	6.51	41–112	136–194	Kelch_4 Kelch_6 Kelch_2	PF13418 PF13964 PF07646	155–200 156–201 157–199	2.40E-04 3.60E-06 2.50E-05
TaFBX5	456	49.72	7.53	36–82	171–217, 218–268	F-box Kelch_1 Kelch_4	PF00646 PF01344 PF13418	47–89 136–181 144–182	3.80E-07 7.10E-08 1.00E-06
						F-box Kelch_1	PF00646 PF01344	39–80 159–204	4.20E-05 3.60E-12
						Kelch_1	PF01344	212–255	1.70E-08

**Fig. 1** Predicted F-box and kelch repeat domains of the TaKFBs analyzed in the present study. The conserved domains were first BLAST searched using the NCBI online domain search engine, and the schema was drawn using the online software InterProScan. F-box (blue) and kelch repeat domains (yellow) are indicated. (Color figure online)



**Fig. 2** Interaction between TaKFBs and various SKP proteins as investigated by Y2H assay. Co-transformants were grown on **a** SD/-LW or **b** SD/-AHLW with X-α-Gal. SV40 large T antigen (pGADT7-T)/p53 (pGBKT7-53) and SV40 large T antigen (pGADT7-T)/

Lamin-C (pGBKT7-Lam) were used as positive (P) and negative controls (N), respectively. **c** The strength of interactions of the TaKFBs and SKPs was measured by performing β-galactosidase assay using ONPG as a substrate

(TaKFB3-interacting partners) clones that consistently activated LacZ expression. TaKFB2, TaKFB4, and TaKFB5 showed no interaction with any transformants. To avoid false positive clones, we performed a secondary screening on QDO/X/A plates, which identified six and five blue colony clones from TaKFB1 and TaKFB3, respectively. After the exclusion of false positive and out-of-frame clones, we obtained four and three clones that potentially interacted with TaKFB1 and TaKFB3, respectively (Table 3). Finally, aquaporin1 (PIP1) was identified as a TaKFB1-interacting protein.

### Subcellular localization of TaKFB proteins

The subcellular localization of TaKFBs was predicted using the WoLF PSORT II program, the results of which are shown in Table 4. Subcellular localization of TaKFBs was different according to the amino acid composition. To confirm the results of the bioinformatic predictions, we identified the subcellular localization of TaKFB proteins in plant cells. We constructed a vector expressing translational GFP-TaKFB fusion proteins and then infiltrated tobacco leaves with *Agrobacterium tumefaciens* strain

**Table 3** TaKFB interaction partners identified by Y2H screening

Bait	Putative identification	Organism
TaKFB1	Chlorophyll a-b binding protein CP26	<i>Aegilops tauschii</i>
	NAD(P)H-quinone oxidoreductase subunit 1	<i>Aegilops tauschii</i>
	<b>Aquaporin PIP1 (PIP1)</b>	<i>Triticum aestivum</i>
TaKFB3	Nonspecific lipid transfer protein 5 (LTP5)	<i>Triticum aestivum</i>
	Alpha-gliadin storage protein	<i>Triticum aestivum</i>
	Serine carboxypeptidase-like 18	<i>Brachypodium distachyon</i>
	Heat shock protein 90	<i>Triticum aestivum</i>

Clones used for pairwise Y2H and BiFC assays are indicated in bold

**Table 4** Prediction of the subcellular localization of TaKFBs using WoLF PSORT II

Name	PSORT predictions	Localization in plant cell
TaKFB1	cyto: 10, mito: 2, chlo: 1	Nucleus
TaKFB2	nucl: 9, cyto: 2, mito: 1, chlo:1	Plasma membrane or cytoplasm
TaKFB3	nucl: 3, cyto: 5, chlo:3, cysk: 2	Nucleus
TaKFB4	chlo: 9, extr: 2, ER: 2	Nucleus
TaKFB5	nucl: 1.5, nucl_plas: 2, cyto: 6, chlo: 3, plas: 1.5, pero: 1	Nucleus
PIP1	plas: 7, cyto: 3, cysk: 2.5, cysk_nucl: 2	Plasma membrane or plasmodesmata

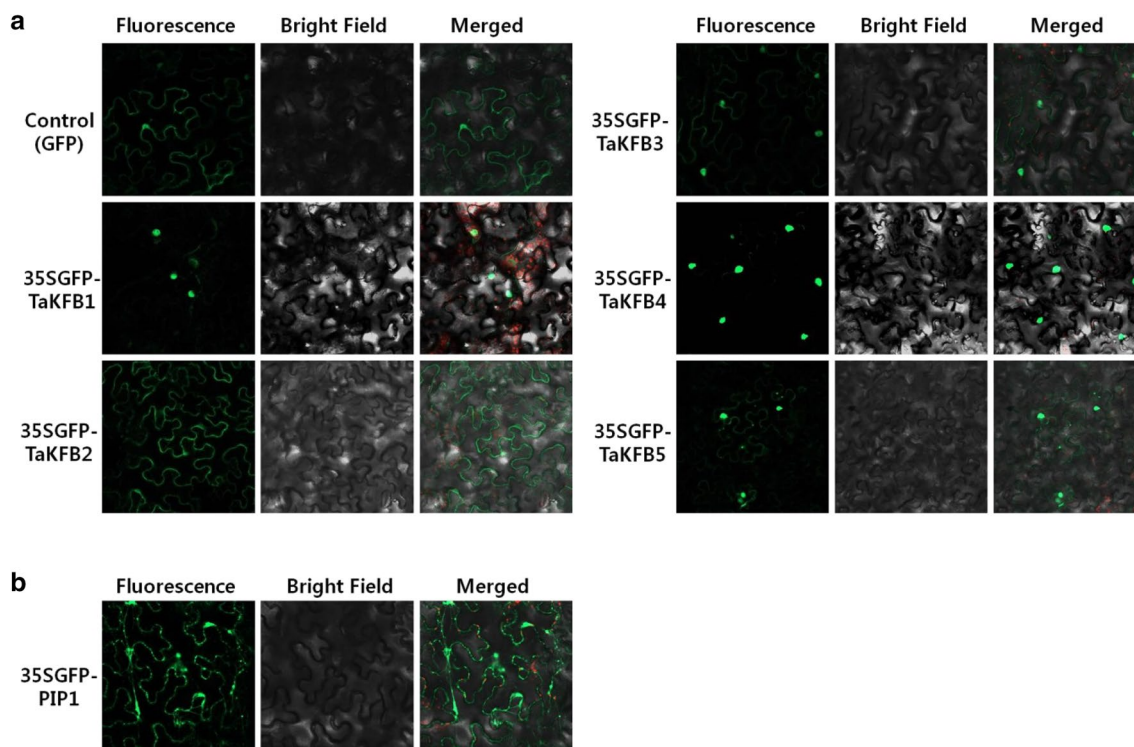
*nucl* nuclear, *cyto* cytosol, *mito* mitochondria, *chlo* chloroplast, *plas* plasma membrane, *cysk* cytoskeleton, *extr* extracellular, *pero* peroxisome

GV3101. pMDC43 (GFP alone) was used as a control. Tobacco leaves transformed with pMDC43 showed bright fluorescence localized to the nucleus and the cytoplasm. The result of subcellular localization of TaKFBs by GFP in vivo assay in tobacco cell showed different results from the prediction by WoLF PSORT II program. The GFP signals of TaKFB1, TaKFB3, TaKFB4, and TaKFB5 were detected in the nucleus (Fig. 3a), but the fluorescence signal of the GFP-TaKFB2 fusion protein was detected in the plasma membrane or the cytoplasm (Fig. 3a). PIP1, a TaKFB1-interacting partner, was mainly observed in the plasma membrane and plasmodesmata, and PIP1 GFP signals were also detected in the cytoplasm or cytoskeleton

(Fig. 3b). The bioinformatic prediction for the PIP1 protein was therefore similar to the actual experimental results. However, the experimental results and bioinformatic predictions conflicted for TaKFBs (Table 4).

### TaKFB1 protein interacts with aquaporin PIP1

To confirm the specificity of the interaction between TaKFBs and candidate proteins, we amplified the full-length cDNAs of the TaKFBs and their respective candidate interacting genes and revalidated their pairwise interactions by yeast co-transformation. The experiment showed that all five TaKFB proteins interact with the PIP1 protein, as

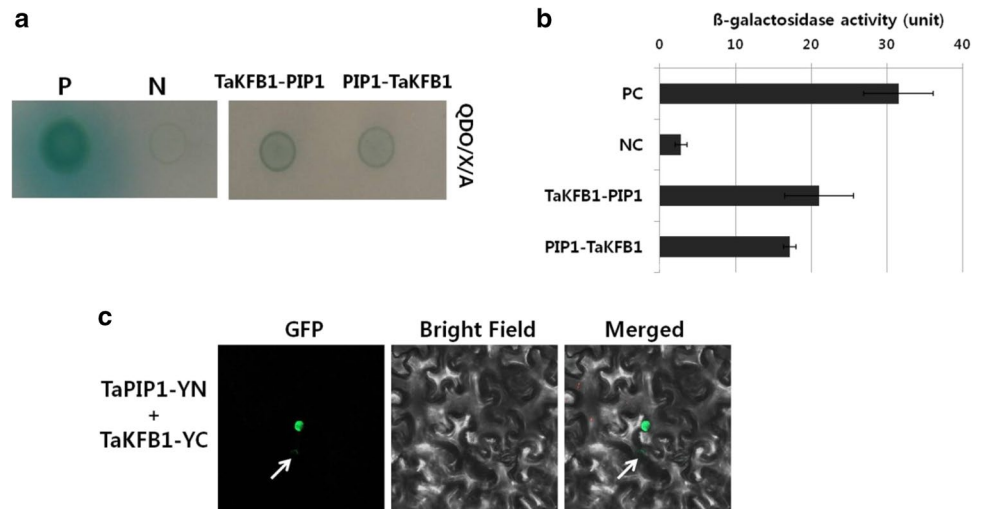


**Fig. 3** Subcellular localization of GFP-TaKFBs and PIP1-fusion proteins visualized by confocal microscopy. Tobacco epidermis was infiltrated with *Agrobacterium tumefaciens* containing a binary vector

harboring a GFP-TaKFB fusion construct driven by the 35S promoter. **a** GFP signals of control and five TaKFB proteins. **b** GFP signal of the TaKFB1 interacting partner PIP1



**Fig. 4** Interaction between TaKFB1 and PIP1 protein as determined by Y2H and BiFC assays. **a** Results of Y2H assay showing the interaction between TaKFB1 and PIP1 proteins. **b** Quantification of the relative interaction strength between TaKFB1 and PIP1 was assayed by performing  $\beta$ -galactosidase assay. **c** Results of BiFC assay for the interaction between TaKFB1 and PIP1 in *Agrobacterium*-infiltrated tobacco leaves. White arrows indicate nuclei. *DIC* differential interference contrast, *YN* N-terminal domain of GFP, *YC* C-terminal domain of GFP

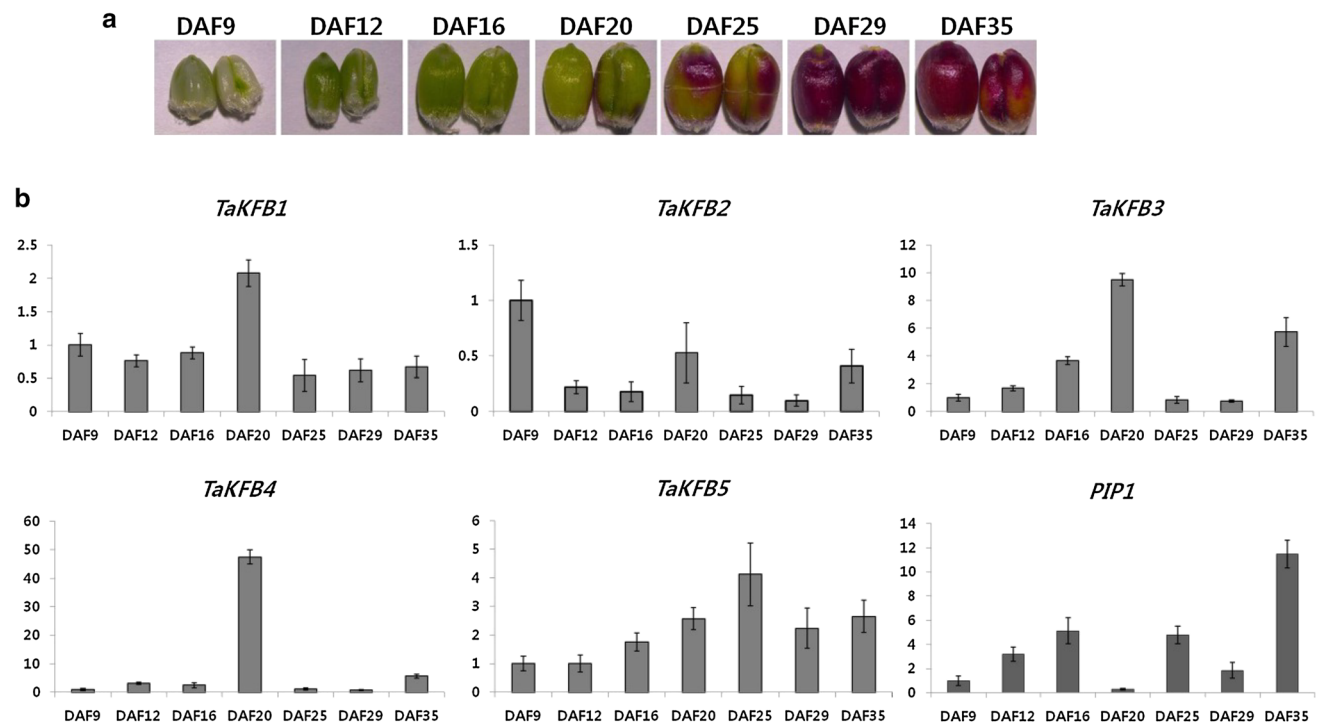


evaluated by growth on QDO/X/A plates. However, the strength of these interactions was lower than the positive control, but the  $\beta$ -galactosidase activity between TaKFB1 and PIP1 was strong enough (Fig. 4b). To examine the interactions between TaKFB1 and PIP1 protein *in planta*, we performed a BiFC assay using tobacco leaves. TaKFB1 was fused to the N-terminal fragment of GFP (EGFP-N) to generate pGTQL1211-TaKFB1, and PIP1 was fused to the C-terminal fragment of GFP (EGFP-C), generating

pGTQL1221-PIP1. GFP signals of pairwise expression of TaKFB1 and PIP1 were detected in the nucleus (Fig. 4c).

### Expression of *TaKFB* genes and their respective interacting partners during grain development

To explore the functions of the *TaKFB* genes and their respective interacting genes, we examined gene expression patterns by qRT-PCR. The wheat grain sample used in this



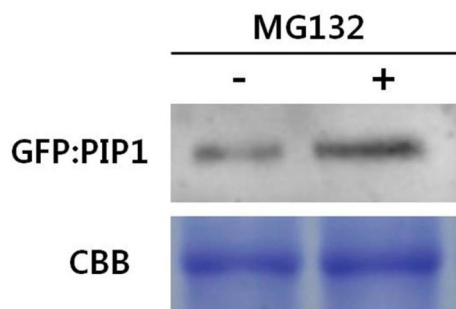
**Fig. 5** Relative expression of *TaKFB* genes at different time points during grain development. **a** Different stages of wheat grain development. Wheat seed coats started to change color from green to purple at DAF20. *DAF* day after fertilization. **b** *TaKFBs* and *PIP1*

transcripts quantified by qRT-PCR in wheat grains at various developmental stages. Each bar represents the mean  $\pm$  SD of three independent experiments. (Color figure online)

study started to change color from green to purple at DAF20 (Fig. 5a). The pattern of expression of *TaKFB1* and *TaKFB4* was similar. *TaKFB1* and *TaKFB4* transcripts were detected in high numbers at DAF20, but their expression levels were similarly low at all other developmental stages (Fig. 5b). *TaKFB2* expression was highest at DAF9, then decreased at DAF12 and DAF16, and slightly increased at DAF20. *TaKFB3* showed the highest expression at DAF20, followed by a sharp decline at DAF24 and DAF29 before increasing again at DAF35. The *TaKFB5* transcripts were continuously induced up to DAF24, then decreased thereafter. The expression of the TaKFB1-interacting protein aquaporin PIP1 (*PIP1*) was continuously induced up to DAF16, then sharply decreased at DAF20, and increased again at DAF25 (Fig. 5b). After that, a strong induction of *PIP1* transcripts was observed at DAF35. In addition, we used RNA-seq analysis to compare the expression of *TaKFB* genes between colored and yellow wheat during different developmental stages to determine whether their expressions are specific to colored seed pericarp. Corresponding with the results of qRT-PCR, heatmap analysis of *TaKFB3* showed a high expression pattern at DAF20 and DAF30, and the expression levels of other *TaKFB* genes were increased at DAF20, with the pericarp color turning purple because of the accumulation of pigments, such as anthocyanin (Supplementary Fig. 3).

### TaKFB1-interacting protein is degraded by the 26S proteasome

In order to investigate PIP1 protein degradation by the 26S proteasome, we analyzed a cell-free extract in either the presence or absence of the proteasome inhibitor MG132 (Sigma, USA) using the transient expression in tobacco. Western blotting with GFP antibodies was used to validate the expression of the transgene. As shown in Fig. 6,



**Fig. 6** Inhibition of protein degradation by 26S proteasome *in planta*. Western blot analysis using protein extracts prepared from transiently transformed tobacco leaves expressing GFP-PIP1 treated with (+) or without (–) 50  $\mu$ m MG132. The GFP-PIP1 was monitored by western blot using anti-GFP antibody. CBB staining served as the control for the amount of protein loading

the GFP-PIP1 signal was increased in extracts treated with MG132 at the same point (Fig. 6). These results indicate that the TaKFB1 target substrate protein (aquaporin PIP1) is regulated by 26S proteasome-mediated proteolysis.

## Discussion

F-box proteins are involved in controlling various processes in plants, including photomorphogenesis, the circadian clock, flower development, hormone signaling, self-incompatibility, and senescence [34]. F-box proteins, a component of the SCF complex and a large protein superfamily in plants, contain an F-box motif in the N-terminal region and various types of protein–protein interaction domains in the C-terminal region, such as leucine-rich repeats, kelch repeats, tetratricopeptide repeats, and WD40 repeats [6] that bind target substrates and can be used to categorize the F-box protein. The diversity of the C-terminal domain confers specificity to the F-box proteins, which bind specific interacting substrates. In plants, the kelch domain is one of the most frequently occurring subfamilies among the many C-terminal domains of F-box proteins [35]. Previous reports indicate that a few characterized wheat KFBs are involved in inflorescence development or tolerance to heat stress [36, 37]. However, little information is available about KFBs in wheat. The present study was carried out to elucidate the functions of KFBs in wheat grain color changes during grain development.

The conserved F-box domain interacts with SKP1-related proteins to form the SCF complex and F-box proteins recruit specific substrates via a protein–protein interaction domain in the C-terminal region to degrade target substrates [7]. Previous reports demonstrate that *Arabidopsis* KFBs function as regulators controlling phenylpropanoid biosynthesis via the mediation of PAL ubiquitination and subsequent degradation, affecting seed coloration [18, 19]. Furthermore, Yu et al. demonstrated that *Arabidopsis* *SMALL AND GLOSSY LEAVES1* (*SAGL1*), a Kelch repeat F-box protein, regulates phenylpropanoid biosynthesis post-translationally at PAL1 and transcriptionally at *anthocyanin synthase* (*ANS*) [38]. However, the functions of wheat KFBs in grain development are not known. To investigate this topic, we isolated five *TaKFBs* from colored wheat grains by PCR-based cloning. Although the sequence compositions of the five respective TaKFB proteins are different, we confirmed that all contained a conserved F-box domain at their N-terminal and one to three kelch repeats at the C-terminal (Fig. 1). Except for TaKFB2, all TaKFBs had one F-box domain in their N-terminal region. The F-box motif is a well-conserved N-terminally located sequence of about 70 amino acids that mediates protein–protein interaction [39]. F-box proteins can interact with SKP1-related proteins via an N-terminal F-box

domain to form the SCF complex for ubiquitination by the 26S proteasome [6]. In the present study, we confirmed the interactions between TaKFBs and TaSKPs (Fig. 2). Our data showed that TaKFB1, TaKFB3, and TaKFB4 can interact with TaSKP1 and TaSKP6, and that TaKFB4 can interact with TaSKP3, whereas TaKFB2 and TaKFB5 did not show any significant interaction with any of the tested TaSKPs. No interactions between TaKFB2, which lacks an F-box domain, and TaSKPs were found. These results indicate that F-box domains at the N-terminus end of F-box proteins are essential for interaction with SKP1 proteins in wheat. Also, the GFP signals of TaKFB2 were detected in the plasma membrane, but all other TaKFB-GFP signals were localized to the nucleus (Fig. 3). These data indicate that the F-box domain may be involved in the translocation of TaKFBs to the nucleus. Y2H assay showed that TaKFB5 did not interact with any TaSKPs, even though TaKFB5 contains an F-box domain. It is possible that not all F-box domains participate in the formation of an SCF complex, and some F-box proteins seem to function without interacting with SKP1.

TaKFB1 and TaKFB4 contain one conserved domain, kelch repeat type 1 (IPR006652), at the C-terminal. TaKFB2 and TaKFB5 have two kelch repeats and TaKFB3 has three kelch repeats in the C-terminal region. The kelch motif is composed of about 44–56 amino acid residues, which constitute five to seven repeats that form a  $\beta$ -propeller tertiary structure [40]. Kelch  $\beta$ -propellers have the ability to serve as scaffolds for protein–protein interaction [41]. F-box and kelch domains are the most commonly discovered protein domains in the living organism; however, the co-existence of F-box and kelch domains is an interesting feature of plant F-box proteins [35]. TaKFBs contain F-box domain and one to three kelch repeats like plant feature. We hypothesized that TaKFBs recognize specific proteins via the kelch repeat domain at C-terminus. Also, sequence differences in the kelch repeat suggest that TaKFBs may be able to interact with multiple substrates. In this study, we carried out a yeast library screening analysis in order to elucidate the roles of TaKFBs and their interacting proteins in wheat grain development. The Y2H assay showed that TaKFB1 physically interacts with aquaporin PIP1 (Table 3, Fig. 4).

Aquaporins are thought to play an important role in increasing membrane permeability to facilitate the mobilization of water [42]. The roles of *Arabidopsis* aquaporin (TIP1;1, TIP1;2) in anthocyanin content changes and catalase activity reduction have been reported [43]. Also, aquaporin is known as a regulator, which is involved in various environmental stress responses and is generally located in the plasma membrane [44, 45]. As in previous reports, aquaporin PIP1-GFP signals were mainly detected in the plasma membrane and plasmodesmata in the present study (Fig. 3b). The BiFC assay revealed that an interaction occurs between TaKFB1 and PIP1 in the nucleus (Fig. 4c); it is,

therefore, possible that PIP1 protein is regulated by TaKFB1 in the nucleus. Using a Y2H assay and BiFC analysis, we confirmed that TaKFB1 could interact with aquaporin PIP1 (Fig. 4). Our results suggest that TaKFB proteins are involved in the formation of the SCF complex and act as a connector between the SCF complex and target substrates.

We used qRT-PCR analysis to investigate the functions of TaKFBs and their respective interacting proteins (Fig. 5) and confirmed that *TaKFB* genes were upregulated during the pigmentation stage of grain development in wheat (Fig. 5b). The result of phylogenetic tree showed that TaKFB1 and TaKFB4 belong to same clade (Supplementary Fig. 1). Moreover, high levels of sequence similarity between TaKFB1 and TaKFB4 showed similar expression patterns. These results indicated that sequence similarity between TaKFB1 and TaKFB4 may influence similar functions of gene expression at grain development stages. Although we only performed expression studies of *TaKFB*s at the grain development stage, the expression of *TaKFB* genes might be associated with the characteristics of organ- or tissue-specific expression partitioning in different tissues and under various environmental conditions. For example, the expression profiles of *TaGFs*, one of the wheat gene families, were diverse in different tissues and grain developmental periods, which could be inferred from the analysis of WheatExp in public transcriptome data [46]. Therefore, further work is still needed to demonstrate expression patterns of TaKFBs in different tissues under diverse conditions.

*PIP1* transcripts showed the lowest level at the seed pigmentation stage (Fig. 5b). The *Arabidopsis* double mutant *tip1;1-1 tip1;2-1* exhibited a slightly increased anthocyanin content when compared with that in wild type [43]. Similar to TIP1;1-1 TIP1;2-1, the change in anthocyanin content at grain development in colored wheat might be associated with the action of PIP1. According to our analysis of gene expression and co-localization, we hypothesized that an increase in anthocyanin content due to increased *TaKFB* gene expression might be associated with decreased PIP1 owing to the functions of F-box proteins. Using the Y2H assay and BiFC analysis, we confirmed that TaKFB1 is an F-box protein and could interact with SKP1 in an F-box domain-dependent manner, and it acts as a connector between aquaporin PIP1 and SKP1. Moreover, we confirmed that PIP1 protein is regulated by the 26S proteasome by using tobacco leaves treated with MG132, a proteasome inhibitor that prevents protein degradation by the 26S proteasome. GFP-PIP1 was highly detectable in MG132-treated samples (Fig. 6), indicating that MG132 treatment greatly blocked the degradation of PIP1 protein.

According to the present study, it is possible that TaKFB1 acts as a connector between PIP1 and the SCF complex and provides SCF with substrate specificity for ubiquitin ligase complexes. Also, we found that PIP1 protein is targeted by

TaKFB1 for degradation by the 26S proteasome. Here, we report that TaKFB1, a kelch repeat F-box protein, interacts with PIP1 protein for proteasome-mediated degradation to regulate anthocyanin content in colored wheat. Although further studies are needed to reveal the functions of TaKFBs and their respective interacting proteins, identification and characterization of KFBs in the present study will help to facilitate future research into the molecular basis of F-box proteins in wheat.

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## Compliance with ethical standards

**Conflict of interest** All authors declare that they have no conflict of interest.

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