Interactions between wheat Tubby-like and SKP1-like proteins

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Tubby-like genes were first verified in obese mice. In plants, 11 Tubby genes have been identified in Arabidopsis, 14 in rice, and 11 in poplar. However, there is very little information about Tubby-like proteins in wheat. In this study, we identified four Tubby-like protein genes (TtULP1-TtULP4) in wheat. A comparison of the gene structure showed a conserved exon number pattern in TaTULPs, although the length of the introns differed. With the exception of TaTULP2, TaTULPs had four exons. To identify the chromosome localization of TaTULPs, BLASTn analyses were performed using the URG database to predict the chromosomal location of TaTULP genes. TaTULP1, 2, 3 and 4 genes were localized on chromosomes 4, 5, 7 and 2. All TaTULPs harbor a Tubby domain in their C-terminal region and an F-box domain in the N terminus. We investigated protein–protein interactions between the F-box domain of TaTULPs and various wheat SKP1-like proteins (TaSKPs) using the yeast two-hybrid system. TaTULP1, TaTULP3 and TaTULP4 were found to interact with TaSKP1, TaSKP3 and TaSKP6, whereas TaTULP2 showed no interaction with TaSKP proteins. TaTULP proteins tagged with green fluorescent protein were targeted to the Golgi apparatus in plant cells. Our analysis of TaTULPs will aid in understanding the functions of TaTULPs in plants.

Key words: F-box protein, SKP protein, Tubby protein, TaTULP, yeast two-hybrid system

INTRODUCTION

Tubby-like genes were first found in mammalian cells (Coleman and Eicher, 1990), and Tubby-like proteins (TULPs), including Tub, Tulp1, Tulp2 and Tulp3, have been identified in humans and mice (Kleyn et al., 1996; Noben-Trauth et al., 1996; North et al., 1997). Tubby and Tubby-like proteins, which were initially linked to the spontaneous maturity-onset obesity syndrome in mice, were later shown to be involved in the development and functions of neuronal cells during post-differentiation in mice (Coleman and Eicher, 1990; Kleyn et al., 1996; Li et al., 2001; Ikeda et al., 2002; Yang et al., 2008). TULPs play crucial roles in photoreceptor degradation (Ikeda et al., 1999), embryonic lethality (Nishina et al., 1998) and membrane receptor trafficking (Mukhopadhyay et al., 2010). Tubby proteins are highly conserved among different species of mammals, while TULPs are found in a wide range of multicellular organisms (Boggon et al., 1999; Lai et al., 2004). Tubby and TULPs are characterized by a conserved Tubby domain (approximately 270 amino acids) located at the C terminus (North et al., 1997; Yang et al., 2008; Lai et al., 2012). The N-terminal region of Tubby proteins in humans is less conserved than the C-terminal region, suggesting that the C-terminal region is functionally redundant among the Tubby gene family members (Ikeda et al., 1999; Ikeda et al., 2001). Unlike human Tubby proteins, most tubby-like proteins in plants contain both a highly conserved F-box domain at the N terminus and a Tubby domain at the C terminus (Yang et al., 2008). The F-box domain mediates binding between specific substrates and components of the SCF E3 ubiquitin ligase complexes (Kipreos and Pagano, 2000; Gagne et al., 2002; Jin et al., 2004; Jain et al., 2007). The N-terminal F-box domain interacts with SKP1-like proteins (Bai et al., 1996), whereas the C terminus contains several highly variable protein–protein interaction domains, such
as LRR, Kelch repeats, FBD, WD40, PAS/PAC, ring finger, Tubby and PPR, that bind to specific targets (Patton et al., 1998; Gagne et al., 2002; Kuroda et al., 2002; Jain et al., 2007).

Gene duplication is a prominent characteristic of angiosperm evolution (Lockton and Gaut, 2005). Duplication has played an important role in the evolution and expansion of new gene functions and novel developmental and physiological pathways (Hanada et al., 2008). In plants, tandem and segmental duplication events generally lead to the generation of new members of existing nuclear gene families (Cannon et al., 2004). Plants have multigene Tubby families. For example, there are 11 members of the Tubby gene family in Arabidopsis (AtTLP1–AtTLP11) (Lai et al., 2004), 14 in rice and 11 in poplar (Yang et al., 2008). Further, in rice and poplar, the TULP family has expanded, mainly through segmental duplication events (Yang et al., 2008). However, Tubby genes in wheat remain unknown.

Bread wheat has a large genome (~17 Gb) that consists of three (A, B, and D) homoeologous chromosome sets. Because the wheat genome is hexaploid, most genes are present in three homoeologous forms ([The International Wheat Genome Sequencing Consortium (IWGSC), 2014]). Wheat is a well-known example of genome evolution through allopolyploidization. Hexaploid wheat is generated by the hybridization of a tetraploid (AABB) genome with a diploid (DD) genome (Kihara, 1944; McFadden and Sears, 1946). The wheat genome consists of highly repetitive DNA elements (> 80%), and three potential homoeologous copies of each gene may be present on more than one chromosomal group as a result of gene duplication ([The International Wheat Genome Sequencing Consortium (IWGSC), 2014]). In this study, we cloned and characterized Tubby-like protein genes in wheat. We named them Triticum aestivum Tubby-like protein genes (TaTULPs). To study the role of TaTULPs in wheat, we isolated TaTULPs from the cDNA of wheat spike. We used green fluorescent protein (GFP)-tagged reporters to examine the subcellular localization of TaTULPs. We used a yeast two-hybrid (Y2H) system to identify partners that interact with TaTULPs and confirm that TaTULPs are F-box proteins that can interact with TaSKPs.

MATERIALS AND METHODS

Plant materials and treatments

Seeds of the wheat cultivar ‘Geumgangmill’ (accession no. IT 213100), provided by the National Institute of Crop Science (RDA, Republic of Korea), were vernalized at 4 °C for 6 weeks in a dark chamber and then transferred to soil (Sunshine Mix 1) in pots. Plants were grown at 23–26 °C under a 16-h light/8-h dark photoperiod, and spike tissues were sampled between the stage when the spikes were covered by the stem and leaf sheath just before emergence (Feekes Scale 10) and the initial stage when the spike completely emerged from the stem (Feekes Scale 10.1) (Hong et al., 2012). Three independent replicates of the spike samples were collected for the construction of a yeast cDNA library.

To obtain wheat seedlings with synchronized growth, the seeds were grown in a growth chamber maintained at 8 °C for 5 days. The synchronized wheat seedlings were transferred to a magenta box (6.5 × 6.5 × 20 cm; Greenpia Technology, Yeoju, Korea) filled with half-strength Hoagland’s nutrient solution for 14 days. To examine the response of TaTULP genes to plant growth hormones, the above-ground plant parts of 14-day-old wheat seedlings were sprayed with an aqueous solution of abscisic acid (ABA, 100 μM) and methyl jasmonate (MeJA, 100 μM). Wheat seedlings were treated with NaCl (250 mM) for salt stress. For cold treatment, the plants were placed in a cold chamber (4 °C). Treated wheat seedlings were collected at various time intervals after the treatments and then immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Molecular cloning

Total RNA was extracted from wheat seedlings using the Trizol reagent (Invitrogen, New York, USA), according to the manufacturer’s protocol. RNA samples were pretreated with RNase-free DNase I to eliminate any contaminating genomic DNA. First-strand cDNA was synthesized from total RNA (~1 μg) using the Power cDNA Synthesis Kit (iNtRON, Seongnam-si, Gyeonggi-do, Korea). Gene-specific primer pairs were designed using the information on the ORF sequences (Table 1). The cDNA was used as a template for PCR with gene-specific primers. The PCR cycling conditions were as follows: 94 °C for 10 min, followed by 32 cycles of 94 °C for 60 s, 56 °C for 60 s and 72 °C for 90 s, and a final incubation at 72 °C for 5 min. The PCR products were cloned in a pCR/GW/TOPO TA vector (Invitrogen), transformed into Escherichia coli, and sequenced.

To isolate the genomic sequence of TaTULPs, genomic DNA was extracted from fully expanded leaves of 3- to 4-week-old plants using the DNeasy Plant Mini Kit (QIAGEN, Germany), according to the manufacturer’s instructions. PCR was performed using a 25-μl solution containing 50 ng of the genomic DNA template. The PCR products were separated by agarose gel electrophoresis to confirm successful amplification.

Primers for the isolation of genomic TaTULP genes were identical to those used for the isolation of cDNA for TaTULP genes (Table 1, Supplementary Fig. S1).

Sequence analysis

A BLAST search based on the rice Tubby (XM_006662698) and Brachypodium Tubby (XM_003577765) sequences identified a matching sequence in Triticum aestivum. To isolate Tubby genes
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from wheat, conserved regions of Tubby sequences were collected from the EST sequence from Gene Index (TC404716, TC399190, TC390333), GrainGenes (BM138619, BG608313) and NCBI databases (AK333166). Six EST sequences were scanned for open reading frames (ORFs) using the NCBI ORF finder tool.

F-box and Tubby-like domain positions were predicted using an InterProScan sequence search (http://www.ebi.ac.uk/Tools/pfa/iprscan/). In addition, the common wheat chromosome sequence in EnsemblPlants (http://plants.ensembl.org/Triticum_aestivum/Info/Index) is the Chromosome Survey Sequence (CSS) for *T. aestivum* cv. Chinese Spring, combined with the reference sequence of chromosome 3B, both generated by the IWGSC. The CSS assemblies have been refined into chromosomal pseudomolecules using POPSEQ data generated by Chapman et al. (2015). BLASTn analysis was performed using the IWGSC 1.0 + popseq DB of *T. aestivum* in EnsemblPlants to predict the chromosomal locations and gene structures of TaTULPs and their homoeologous genes.

**qRT-PCR analysis** qRT-PCR with gene-specific primers was performed to obtain cDNA clones for each TaTULP gene (Table 1). qRT-PCR was performed in 96-well blocks using the iCycler iQ (Bio-Rad) real-time PCR system. SYBR Green Master Mix (TaKaRa, Otsu, Shiga, Japan), including the first-strand cDNA, was used for PCR amplification along with gene-specific primer pairs. The two-step thermal cycling profile included incubations for 5 s at 95 °C and 30 s at 60 °C. qRT-PCR was performed in triplicate using RNA extracted from independent tissue samples. The primer sequences are listed in Table 1. For the quantification of gene expression, we used the Actin (AB18199) gene as an endogenous control for relative quantification (RQ) of the expression of each TaTULP. RQ is fold change in each sample compared to the calibrator. For quantification normalized to an endogenous control, standard curves were prepared for both the target and endogenous reference. For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard curve. The target amount was then divided by the endogenous reference amount to obtain a normalized target value. Each normalized target value was divided by that of the calibrator to generate the relative expression levels.

**Y2H assay** A wheat spike two-hybrid cDNA library was generated using the pGADT7-Rec vector with the Make Your Own Mate & Plate Library system (Clontech, Palo Alto, CA, USA), according to the manufacturer’s instructions. Two micrograms of total RNA was used for cDNA synthesis (Hong et al., 2012). The coding regions of TaTULPs were amplified using PCR with specific primer pairs containing restriction sites and were cloned into the

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**Table 1. Primers used for gene cloning, expression quantification, Y2H and subcellular localization**

<table>
<thead>
<tr>
<th>Purpose</th>
<th>List</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
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<tr>
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<td>PIP2</td>
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<td>GGATCCCGAACGCGCAATCTGTGGTTC</td>
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<td></td>
<td>TaActin</td>
<td>GCCACACGTTCCTCAATCTGTA</td>
<td>AGCGTTGTGTTGAGGAGTA</td>
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</table>

Sequences underlined in the Y2H primers indicate restriction enzyme sites.
pGBK7 vector (bait; Table 1). Approximately $1.6 \times 10^6$ transformants of the amplified library were subjected to screening by yeast mating. The Matchmaker Gold Yeast Two-Hybrid System (Clontech #630489) was used for the isolation of interacting proteins. The mating products were grown on SD medium without leucine (Leu) or tryptophan (Trp) until the appearance of colonies (5–7 days). The resulting diploid yeasts were selected on SD medium without adenine (Ade), histidine (His), Leu or Trp. All growth media were supplemented with X-alpha-Gal and Aureobasidin A.

Interactions of the hybrid proteins were examined by co-transformation of yeast cells with pGBK7:TaTULPs as the bait and pGADT7:TaSKPs as the prey (Hong et al., 2013). Full-length TaSKP, phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and N-(phosphoribosyl)anthranilate isomerase-like (PRAI) genes were also amplified using PCR with specific primers containing restriction sites and cloned in-frame into the pGADT7 vector (Table 1). AH109 yeast cells were co-transformed with each bait and cloned in-frame into the pGADT7 vector (Table 1). PCR with specific primers containing restriction sites and cloned in-frame into the pGADT7 vector (Table 1). AH109 yeast cells were co-transformed with each bait and cloned in-frame into the pGADT7 vector (Table 1). AH109 yeast cells were co-transformed with each bait and cloned in-frame into the pGADT7 vector (Table 1). AH109 yeast cells were co-transformed with each bait and cloned in-frame into the pGADT7 vector (Table 1).

Subcellular localization of TaTULPs To generate TaTULP-GFP fusion constructs, the coding sequence of each full-length TaTULP cDNA without the stop codon was amplified using specific primers (Table 1) and cloned into the pCR8/GW/TOPO TA vector (Invitrogen). The constructs were cloned into Gateway-compatible binary BiFC vectors using LR clonase II (Invitrogen). The BiFC assay was performed using Agrobacterium-mediated transient co-expression in tobacco leaves (Walter et al., 2004). The epidermal cell layers of tobacco leaves were observed for evidence of fluorescence 3 days after infiltration, using a confocal laser scanning microscope (Carl Zeiss).

RESULTS

Identification and isolation of TaTULP genes from wheat Four genes that encode a Tubby domain were identified and named TaTULP1, TaTULP2, TaTULP3 and TaTULP4. Sequence analysis indicated that these genes encode 1317–1413-bp transcripts, which translate into putative proteins comprising 439–471 amino acid residues (Fig. 1). Wheat Tubby proteins contain two motifs (F-box domain and Tubby-like domain) that we found to be highly conserved among Tubby proteins in other plant species (Fig. 2, A and C). A phylogenetic tree was constructed using ClustalW software on the basis of the amino acid sequences of Tubby proteins (Fig. 2B). ClustalW analysis revealed a 60% sequence identity between any two TaTULP protein sequences (Fig. 2C). TaTULP1/TaTULP2 and TaTULP3/TaTULP4 proteins were in the same clade of the phylogenetic tree, with a high degree of protein sequence identity. The phylogenetic tree showed that TaTULP proteins belonged to several clades. Analysis of the sequence of the TaTULPs by

<table>
<thead>
<tr>
<th>TaTULP</th>
<th>Genomic DNA length (PF00646)</th>
<th>Amino acid length</th>
<th>F-box domain (PF01167)</th>
<th>Tubby C-terminal domain (PS01201, TUB_1)</th>
<th>Tubby, C-terminal conserved site (PS01201, TUB_2)</th>
<th>Gene structure</th>
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<td>TaTULP1</td>
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<td>12-100</td>
<td>117-434</td>
<td>354-372</td>
<td>**</td>
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<tr>
<td>TaTULP2</td>
<td>2629</td>
<td>439</td>
<td>12-100</td>
<td>117-435</td>
<td>424-439</td>
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<tr>
<td>TaTULP3</td>
<td>1914</td>
<td>436</td>
<td>62-112</td>
<td>124-459</td>
<td>362-375</td>
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<td>TaTULP4</td>
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<td>471</td>
<td>18-111</td>
<td>124-465</td>
<td>359-375</td>
<td>**</td>
</tr>
</tbody>
</table>

Fig. 1. List of TaTULP genes, Tubby-like family members in wheat. *Black and white boxes indicate exons and introns, respectively.
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InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) predicted the presence of an F-box domain (PF00646) in the N-terminal region of all TaTULPs. In addition, two PROSITE signature motifs, termed TUB1 (PS01200) and TUB2 (PS01201), were identified in the C-terminal region of both TaTULP3 and TaTULP4. Interestingly, only a single TUB1 domain was observed at the C terminus of both TaTULP1 and TaTULP2 (Fig. 1).

Exon-intron structures were examined by comparing genomic DNA sequences against cDNA sequences (Supplementary Fig. S1). Although the lengths of the introns were different, three of the four TaTULPs (TaTULP1, TaTULP3 and TaTULP4) had four exons; in contrast, TaTULP2 had five exons (Fig. 1). The sequences of the TaTULPs have been added to GenBank. The accession numbers for TaTULP1, TaTULP2, TaTULP3 and TaTULP4 are KP966378, KP966379, KP966380 and KP966381, respectively.

Chromosomal locations of the TaTULP genes  The wheat chromosome draft sequence was used to identify the chromosome arm locations of TaTULPs by BLASTn analysis. TaTULP1 was localized to chromosome arms 4AL, 4BS and 4DS (Supplementary Fig. S2). TaTULP2 was located in chromosome arms 5AL, 5BL and 5DL. TaTULP3 was localized to chromosome arms 7AL, 7BL and 7DL. TaTULP4 was located on chromosome arms 2AL, 2BL and 2DL. Supplementary Fig. S2 shows detailed BLAST results for searches using TaTULPs against the wheat chromosome survey sequence. The information obtained for predicted genes, e-values, sequence identities and gene structures for each TaTULP is presented in Fig. 1 and Supplementary Fig. S2.

Expression of TaTULP genes  Changes in the expression of TaTULPs in response to abiotic stresses and application of phytohormones were analyzed using qRT-PCR with gene-specific primers (Fig. 3, Table 1 and Supplementary Fig. S2).
In general, stress gradually increased the expression of TaTULP1, TaTULP3, and TaTULP4. Transcripts of TaTULP1, TaTULP3, and TaTULP4 were induced over time by salt and cold stress, whereas TaTULP2 transcripts were repressed by salt and cold stress (Fig. 3, A and B). Interestingly, transcripts of TaTULP1, TaTULP3, and TaTULP4 were induced by MeJA within 6 h after the treatment, and later returned to control levels at 12 h after the treatment (Fig. 3C). However, TaTULP2 was repressed by MeJA treatment, but the number of transcripts was lowest at 6 h. With the exception of TaTULP3, transcripts of all TaTULPs were also induced 6 h after ABA treatment (Fig. 3D). These data therefore demonstrate that TaTULPs are differentially regulated by different stress treatments.

Subcellular localization of TaTULPs

To examine the subcellular localization of TaTULPs, GFP-tagged TaTULP constructs driven by the 35S promoter (35S:TaTULP:GFP) were transiently expressed in tobacco leaves using Agrobacterium-mediated transformation. The destination vector pMDC83 contained the mGFP reporter gene and the hygromycin phosphotransferase (hpt II) gene, both regulated by the CaMV 35S promoter with a double enhancer (Curtis and Grossniklaus, 2003; Nair et al., 2014). All four TaTULP-GFP fusion proteins were localized to the Golgi apparatus. To confirm the localization of TaTULP proteins, mCherry (red fluorescent protein: RFP) was used as a specific organelle marker for the Golgi apparatus (Nelson et al., 2007). Fluorescence imaging results showed that TaTULP-GFP fusion proteins partially overlapped with the Golgi organelle marker (Fig. 4). These data indicate that TaTULP fusion proteins localize to the Golgi apparatus.

Screening for partners that interact with TaTULPs

Previous studies have shown that the Tubby domain (C terminus of the Tubby protein) has a notable dual binding role as it is capable of interacting with both DNA and phosphatidylinositol (Boggon et al., 1999; Santagata et al., 2001). The amino acid sequence of the putative PIP2 gene from T. aestivum is homologous to that of the Arabidopsis PIP2 gene. To obtain the full sequence of TaPIP2, primers were designed from contig sequences downloaded from GrainGenes. The cDNA sequence of TaPIP2 comprises an 873-bp ORF. To confirm the interaction between TaTULPs and TaPIP2 in the Y2H assay, we co-transformed AH109 yeast cells with bait (pGBK7:TaTULPs) and prey (pGADT7:PIP2) plasmids (Fig. 5A). TaPIP2 protein showed strong interactions with TaTULP1, TaTULP3, and TaTULP4 and a relatively weak interaction with TaTULP2, as confirmed by the growth of the colonies on SD/-AHLW + X-a-Gal.

To identify other proteins that interact with TaTULPs, we used full-length TaTULPs as bait in the Y2H assay and screened a wheat spike cDNA library constructed
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We did not find any protein candidates that interacted with TaTULP1, TaTULP2 or TaTULP3 through the Y2H assay; however, PRAI was identified as a protein candidate that interacted with TaTULP4. To examine the interaction of all TaTULPs with PRAI, co-transformation was performed using the PRAI protein as the substrate. Through yeast co-transformation, PRAI showed a strong interaction with TaTULP1, TaTULP2 and TaTULP4 (Fig. 5B).

TaTULPs interact with TaSKPs

Previous studies have demonstrated that F-box proteins interact with SKP1 to form an SCF complex. To confirm the interaction between TaTULPs and TaSKP proteins (Hong et al., 2013), we performed a Y2H assay. The four TaTULPs were fused to the DNA-binding domain of the GAL-4 transcription factor to serve as bait constructs and were tested in combination with six TaSKPs fused to the activation domain GAL-4. We co-transformed AH109 yeast cells with bait and prey plasmids. Our sequence analysis showed that the TaTULPs contain a cyclin-like F-box domain (IPR001810) (Fig. 2C). The F-box proteins directly interact with SKP1 through the F-box motif (Schulman et al., 2000). After mating, all combinations grew well on SD-LW, except TaTULP1-TaSKP1, TaTULP1-TaSKP2 and TaTULP3-TaSKP1 (Fig. 6A). The growth of TaTULP3-TaSKP6- and TaTULP4-TaSKP1-transformed colonies on SD-AHLW + X-a-Gal suggests a relatively strong interaction between the partners, whereas the growth of TaTULP1-TaSKP3- and TaTULP1-TaSKP6-transformed colonies suggests a relatively weak interaction between them (Fig. 6B).

BiFC assay confirms the interaction of TaTULPs with TaSKPs

To confirm the results of the Y2H assay, we examined the protein–protein interactions in plant cells using a BiFC assay. TaTULP1, TaTULP3, TaTULP4,
TaskP1, TaskP3 and TaskP6 genes were used in this experiment. For BiFC analysis, we generated Gateway vectors that expressed TaTULP1, TaTULP3 or TaTULP4 fused to the N terminus of the yellow fluorescent protein (YFP) and TaskP1, TaskP3 or TaskP6 fused to the C terminus of YFP (YFP*), respectively. The Agrobacterium strain GV3101 co-transformed with specific constructs (YFP*-TaTULP1 and YFP*-TaskP3, YFP*-TaTULP1 and...
YFPn-TaTULP3 and YFPc-TaSKP6 (Fig. 7A). The YFP signal of TaTULP4-TaSKP1 was detected in both the Golgi apparatus and the plasma membrane (Fig. 7B). However, no fluorescence signal was observed when YFPn-TaTULP1 was co-expressed with YFPc-TaSKP3 or TaSKP6 (Fig. 7, C and D).

**DISCUSSION**

The F-box domain may function as a binding motif for specific E3 ubiquitin ligases and interact with the SKP1-like protein. F-box proteins have the potential to determine the specificity of SCF (SKP1-Cullin-F-box protein) complexes via several functional domains in the C-terminal
region. Tubby-like proteins represent a novel type of F-box protein (Gagne et al., 2002), and are highly conserved in multicellular organisms, including plants. In mammals, Tubby proteins play an important role in fundamental physiological processes, as shown by neuronal cells during development and post-differentiation (Mukhopadhyay and Jackson, 2011). In plants, Tubby-like proteins participate in plant stress signaling (Lai et al., 2004; Reitz et al., 2013). In Arabidopsis, most Tubby proteins are ubiquitously expressed, although some have a more restricted expression on the basis of the plant tissue or organ or abiotic/biotic conditions. In particular, Lai et al. (2004) showed that AtTLP9 interacts with ASK1 and is involved in the ABA signaling pathway during seed germination and early seedling development. Results of the expression analysis showed that exogenous ABA treatment rapidly induced the transcription of all TaTULPs except TaTULP3. Transcripts of TaTULP1, TaTULP3 and TaTULP4 gradually increased after exposure to salt treatment, but the expression of TaTULP2 decreased (Fig. 3). Likewise, transcripts of TaTULPs were also induced by cold treatment. Overall, transcript abundance indicates that TaTULP1, TaTULP2, TaTULP3 and TaTULP4 may act in salt and abiotic stress signaling during wheat development. However, we do not currently have mutant lines for TaTULPs to test this proposal.

When TaTULP sequences were compared with the Arabidopsis Tubby protein sequences, TaTULP genes were found to encode proteins that were most similar to the AtTLP amino acid sequence, with an overall 51–77% amino acid sequence identity. Generally, similar sequences tend to have similar expression patterns. Like AtTLP gene expression, this study indicated that TaTULPs may be involved in ABA or abiotic stress signaling and interact with SKP1 protein. However, although TaTULP3 and TaTULP4 were in the same clade of the phylogenetic tree, the expression of TaTULP3 showed a different response to stress. Orthologs usually have the same functions in different species, whereas paralogs may have different functions in the same genome as a result of gene duplication events (Tatusov et al., 1997). The different expression behavior of TaTULP genes was likely generated by gene duplication events, and differentially expressed TaTULPs may have subfunctionalization in hormone signaling. Gene duplication, small-scale segmental losses, and additional motif/domain acquisition events have probably contributed to the diversity of the Tubby gene family (Lai et al., 2012). Wheat (2n = 6x = 42) is an allohexaploid with three relatively collinear genomes (AA, BB and DD). There are 11 Tubby genes in Arabidopsis, 14 in rice, and 11 in poplar (Lai et al., 2004; Yang et al., 2008). In this study, we isolated four Tubby genes in wheat. TaTULP1, TaTULP2 and TaTULP4 have the same gene structure, although the intron sizes differ (4 exons, 3 introns) (Fig. 1). The N-terminal F-box domain and C-terminal Tubby domain are highly conserved among these TaTULP proteins. TaTULP3 and TaTULP4 contain two conserved domains, TUB1 and TUB2, at the C terminus. Chromosomal localization experiments demonstrated that all four genes were localized to different chromosome arms (Supplementary Fig. S2).

F-box proteins interact with the SKP1-related protein through the N-terminal F-box domain and bind to multiple specific substrates via a protein–protein interaction domain in the C-terminal region (Jain et al., 2007). Arabidopsis Tubby-like proteins interact with the ASK protein via an N-terminal F-box domain and with a specific substrate via a protein–protein interaction domain in the C-terminal region (Lai and Shaw, 2012). Our data show that three TaTULPs (TaTULP1, TaTULP3 and TaTULP4) can interact with TaSKPs (TaSKP1, TaSKP3 and TaSKP6) and two (TaTULP3 and TaTULP4) can interact with two TaSKPs (TaSKP3 and TaSKP6), whereas TaTULP2 did not show any significant interaction with any of the tested TaSKPs. These results suggest that the TaTULPs proteins can form SCF complexes and have the ability to target specific protein degradation through the C-terminal Tubby domain. F-box proteins contain several protein–protein interaction domains, including Kelch repeats, leucine-rich repeats, armadillo repeats, WD40 repeats, RING finger and the F-box associated domain (Kuroda et al., 2002). The C-terminal domain of several F-box proteins has a specific domain for recruiting interacting proteins. The Y2H assay indicated that TaTULP4 interacts with the PRAI protein. We demonstrated for the first time that the Tubby domain in TaTULP proteins interacts with the PRAI protein. PRAI is an enzyme that catalyzes the third step of sequential reactions in the biosynthesis of tryptophan (Priestle et al., 1987).

In animal cells, Tubby proteins localize to the plasma membrane by specific interactions with the phospholipid phosphatidylinositol 4,5-bisphosphate (Santagata et al., 2001; Bateman et al., 2009). Tubby is transported from the plasma membrane to the nucleus through G protein-coupled receptor activation (Mukhopadhyay et al., 2010). Tubby proteins have a conserved PIP2 binding site in the C-terminal Tubby domain for targeting to the plasma membrane. However, specific substrates for the Tubby domain have not yet been identified in plant systems. In the present study, we used the Y2H assay to identify substrates of the TaTULP proteins (Fig. 5). We found that TaTULP proteins could interact with PIP2-like protein. This result suggests that TaTULP proteins have binding sites for PIP2 interaction, although TaTULP and PIP2 interactions were not detected in vivo. In plants, the C-terminal part of Arabidopsis Tubby protein 3 (AtTLP3) has been reported to localize to the plasma membrane. However, TaTULP-GFPs localized to the
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Golgi apparatus (Fig. 4). Using the BiFC assay, we showed that TaTULP3-TaSKP6 and TaTULP4-TaSKP1 YFP signals were detected in both the plasma membrane and the Golgi apparatus (Fig. 7). The expression and functions of the Tubby proteins in wheat have not yet been identified. Furthermore, the localization of wheat Tubby proteins is unknown. These results suggest that, although Tubby domains are conserved across species and kingdoms, subcellular localization of Tubby proteins may change because of interactions with specific proteins.

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