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Cullin, a component of the SCF complex, interacts with TaRMD5 during wheat spike development

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Abstract

Cullin, a major component of the SKP1-cullin-F box protein (SCF) complex, is a scaffold protein that binds to both SKP1 and RBX1 for selective protein degradation through the ubiquitin proteasome system. In order to study the role of cullin in common wheat, we isolated *TaCullin* (*Cullin* gene from *Triticum aestivum*) from wheat spike cDNA. *TaCullin* was expressed during all spike/grain developmental stages and in high amounts during early spike/grain development. The *TaCullin* gene is located on the chromosome arm 2DL. Our results suggest that unneddylated *TaCullin* is located in the nucleus. Based on previous proposals of Cullin-SKP1 interactions, we examined the interaction between TaCullin and SKP1-like protein (TaSKP) families by using a yeast two-hybrid approach. Yeast cotransformation demonstrated that the N-terminus of TaCullin physically interacts with TaSKP proteins. Using the yeast two-hybrid screen, we identified potential TaCullin-interacting proteins in a wheat spike library. Among the 9 clones that were identified as potential interacting partners of TaCullin, we identified E3-like ubiquitin ligase, targeting fructose-1,6-bisphosphatase (RMD5) homolog A-like protein. The interaction between TaCullin and the TaRMD5 homolog A-like protein was specifically mediated through the C-terminus of TaCullin. The results of bimolecular fluorescence complementation assay indicated that TaCullin-TaRMD5 is localized in the plasma membrane and cytoplasm. In this study, we present that TaRMD5, such as RING box protein 1 (RBX1), has the potential to interact with TaCullin, depending on the developmental stage and particular organ tissues analyzed.

Additional key words: chromosome location, TaSKP, *Triicum aestivum*, yeast two-hybrid screening.

Introduction

The regulation and degradation of proteins contribute to plant development through the control of processes such as cell cycle progression, DNA repair, hormone response, and diverse developmental pathways (Moon *et al*. 2007). The ubiquitin proteasome system (UPS) is a major mechanism underlying protein degradation in eukaryotes (Pickart *et al*. 2001). Ubiquitination is a post-translational modification in which ubiquitin, a small 8.5-kDa protein, is attached to the target protein. This modification occurs through an ATP-dependent process mediated by the action of 3 sets of enzymes, namely, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases (Scheffner *et al*. 1995). Ubiquitin is activated by E1 and is then passed onto the thiol group of E2. Finally, E3 recognizes and ubiquitinates the target protein for degradation by the 26S proteasome, a multicatalytic protease complex (Smalle *et al*. 2004). E3 ligases confer substrate specificity to the UPS by selecting target substrates (Ciechanover 1998). The UPS plays an important role in the regulation of many cellular processes, including cell cycle transition, differentiation, metabolic regulation, stress response, and

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Abbreviations: APC/C - anaphase-promoting complex or cyclosome; BiFC - bimolecular fluorescence complementation assay; CAND1 - cullin-associated and neddylation-dissociated 1; CRL - cullin-RING ubiquitin ligases; DAF - day after flowering; GFP - green fluorescent protein; HECT - homologous to the E6-AP carboxyl terminus; ONPG - *o*-nitrophenyl-β-galactoside; RBX1 - RING box protein 1; RING - really interesting new gene; RMD5 - E3-like ubiquitin ligase targeting fructose-1,6-bisphosphatase; SCF - SKP1-cullin-F box protein; SKP - S-phase kinase-associated protein 1; UPS - ubiquitin proteasome system; Y2H - yeast two-hybrid.
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signal transduction (Hershko and Ciechanover 1998).

Thousands of E3 ligases exist and have been classified into three groups depending on their subunit composition and ubiquitin transfer mechanisms: the single-subunit RING-finger type, the multisubunit RING-finger type, and the HECT-domain type (Cardozo *et al*. 2004). Skp-Cullin-F-box (SCF) complexes are multi-subunit RING-finger type E3s that contain four protein subunits: the cullin1 (CUL1), the S phase kinase-associated protein 1 (SKP1), the RING-box 1 (RBX1), and the F-box protein. SCF complexes are a major class of E3 ligases in plants and are involved in a variety of biological and developmental processes, including hormone signaling, stress response, flower development, circadian clocks, cell cycle regulation, and photo-morphogenesis (Gray *et al*. 1999, Zhao *et al*. 2001, Moon *et al*. 2004, Hua *et al*. 2011). Cullin, a major component of the SCF complex, is a scaffold protein that binds both SKP1 and RBX1 for selective protein degradation through the UPS (Schwechheimer and Villalobos 2004, Thomann *et al*. 2005).

The N- and C-terminal halves of cullin play distinct roles; the N-terminal domain interacts with SKP1, whereas the C-terminal half forms a globular domain that interacts with RBX1 (Zheng *et al*. 2002). Most eukaryotes contain cullin proteins; in fact, the *Arabidopsis* genome contains at least 11 *CUL-like* genes (Moon *et al*. 2007), but only for a few of them a function is known. CUL1 is involved in the regulation of various pathways, such as those involved in hormone signal transduction, flower formation, senescence, and embryogenesis (Gray *et al*. 2001, Shen *et al*. 2002, Durfee *et al*. 2003, Guo and Ecker 2003, Hellmann *et al*. 2003, Potuschak *et al*. 2003). CUL3 participates in ethylene biosynthesis, embryogenesis, and phytochrome A signal transduction (Wang *et al*. 2004, Dieterle *et al*. 2005, Figueroa *et al*. 2005, Gingerich *et al*. 2005), and CUL4 affects leaf and root development and photo-morphogenesis (Bernhardt *et al*. 2006).

Molecular functions have been suggested for *Cullin* genes in several plant species, but little is known about their function in wheat. To study the role of cullin in common wheat, we isolated *TaCullin* (*Cullin* gene from *Triticum aestivum*) from wheat spike cDNA. The expression of *TaCullin* was investigated in different developmental stages and under various types of stress by RT-PCR. We also used green fluorescent protein (GFP) reporters to examine the subcellular localization of *TaCullin*. Using the yeast two-hybrid (Y2H) system, we identified the TaCullin protein, a TaRMD5 homolog A-like protein, which contains a zinc finger protein domain. TaCullin contains a conserved N-terminal cullin domain as well as a C-terminal neddylation domain. Y2H interaction and bimolecular fluorescence comple-mentation (BiFC) assays revealed that TaCullin specifically interacts with the TaRMD5 homolog A-like protein. Based on previous suggestions that Cullin interacts with SKP1 (Zheng *et al*. 2002), we examined the interaction between TaCullin and the TaSKP protein family by using the Y2H approach (Hong *et al*. 2012). In this study, we have described the isolation, characterization, and expression analysis of *TaCullin*.

Materials and methods

Plants and treatments: The common wheat (*Triticum aestivum* L.) cv. Geumgangmill (IT 213100), developed by the National Institute of Crop Science (RDA, Republic of Korea), was used in this experiment. Imbibed seeds were vernalized at 4 °C for 6 weeks in a dark chamber and then transferred to pots with soil (*Sunshine mix* #1, *Sun Gro Horticulture*, Agawam, MA, USA). Plants were grown in chambers under temperature of 23 - 26 °C, relative humidity of 60 %, a 16-h photoperiod, and irradiance of 600 μmol m^{-2} s^{-1}. Reproductive organs at various developmental stages were sampled following the protocol reported by Hong *et al*. (2012).

In order to obtain wheat seedlings with synchronized growth, seeds were grown in a growth chamber set at 8 °C for 5 d. For phytohormone and abiotic stress treatments, the synchronized wheat seedlings were kept for 14 d in *Magenta* boxes (6.5 × 6.5 × 20 cm, *Greenpia Technology*, Yeoju, Korea) filled with a half-strength Hoagland's nutrient solution (Hong *et al*. 2010).

For plant hormone treatments, the aboveground plant parts of 14-d-old wheat seedlings were submerged in an aqueous solution of abscisic acid (ABA, 100 μM), methyl jasmonate (MeJA, 100 μM), gibberellin (GA_3, 100 μM), and indole-3-acetic acid (IAA, 100 μM). Wheat seedlings were also treated with 25 % polyethylene glycol (PEG 10 000) and sodium chloride (NaCl, 250 mM) for drought and salt stresses, respectively. For a cold treatment, the plants were placed in a cold chamber (4 °C). The leaves were collected at 0, 3, 6, 12, and 24 h after each treatment. All the samples were immediately frozen in liquid nitrogen and stored at -80 °C until analyses.

Cloning of *TaCullin* and *TaRMD5*: In order to obtain the full sequences of *Cullin* and *TaRMD5* from wheat, primer sets were designed from the EST sequences downloaded from *GrainGenes 2.0* (http://wheat.pw.usda.gov/GG2/index.shtml), *Gene Index* (http://compbio.dfci.harvard.edu/index.html), and the *NCBI* databases (http://www.ncbi.nlm.nih.gov/). PCR was performed using specific primers for each gene (Table 1). The PCR cycling conditions were as follows: 95 °C for 10 min followed by 31 cycles at 95 °C for 60 s, annealing at a gene specific temperature for 60 s and 72 °C for 60 s, and a final extension at 72 °C for 4 min. Amplification products were cloned into the *T&A* cloning vector (*Real Biotech Cooperation*, Taipei, Taiwan) and sequenced with the *ABI PRISM* 310 genetic analyzer (*Perkin Elmer*, Waltham, MA, USA). The sequencing results were

analyzed using *GrainGenes 2.0*, *NCBI*, *Gene Index*, and *InterProScan* (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

Genomic DNA extraction and chromosome location: Nulli-tetrasomic and ditelosomic lines of *T. aestivum* cv. Chinese Spring were used for the chromosome localization of the *TaCullin* genes. The nulli-tetrasomic lines were kindly provided by the United States National Plant Germplasm System (www.ars-grin.gov/npgs) and the ditelosomic lines by the National BioResource Project-Wheat (Kyoto University, Kyoto, Japan). The wheat leaves of the synchronized wheat seedlings were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until analyses.

Genomic DNA was extracted from leaves using the *DNeasy* plant mini kit (*Qiagen*, Hilden, USA). The PCR cycling conditions were as follows: 94 °C for 10 min; 29 cycles of 94 °C for 60 s, 56 °C for 60 s, 72 °C for 90 s, and 72 °C for 5 min. Specific primer sets were designed from the cullin repeat-like-containing domain of *TaCullin* (Table 1).

Reverse transcription - polymerase chain reaction (RT-PCR): Total RNA was extracted from each plant tissue [5th and flag leaves, stems, anthers, ovules, and pericarps at the heading and flowering stages (Zadoks scale of Z50 - Z60), the inflorescences at different developmental stages before flowering, and different stages of grain development (1, 3, 6, 10, 16, and 20 d after flowering)] by using a *TRIzol* reagent (*Invitrogen*, Grand Island, NY, USA) (for details see Hong *et al*. 2012).

For RT-PCR analysis, the total RNA samples were first treated with RNase-free DNase I to eliminate any contaminating genomic DNA. First strand cDNA was synthesized from approximately 1 µg of total RNA by using a *DyNamo*^{TM} cDNA synthesis kit (*Finnzymes*, Vanda, Finland) according to the manufacturer's protocol. The semiquantitative RT-PCR was performed with an initial denaturation step at 95 °C for 10 min, followed by 28 cycles at 95 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min, and a final elongation step at 72 °C for 4 min. The quantitative RT-PCR reactions were performed in 96-well blocks with an *iCycler iQ*™ real-time PCR system (*Bio-Rad*, Hercules, USA). *SYBR Green I Master Mix* (*Finnzymes*) was used in 0.025 cm^3 of the reaction mixture that included the first strand cDNA used for PCR amplification along with gene-specific primer pairs (Table 1). The reactions were carried out in biological triplicates with 3 different RNA samples extracted from independent plant materials.

Subcellular localization of *TaCullin* and *TaRMD5*: *Nicotiana tabacum* L. seeds were germinated and seedlings were grown in a controlled greenhouse for 4 to 5 weeks at temperature of 23 °C, relative humidity of 60 %, a 12-h photoperiod, and irradiance of 600 µmol m^{-2} s^{-1}. For the GFP constructs, full-length *TaCullin* and *TaRMD5* gene coding regions were amplified by PCR using specific primer pairs (Table 1) cloned into pCR/GW/TOPO, and then subcloned into gateway-compatible binary GFP vectors (pMDC83, *Invitrogen*) by *LR Clonase* (*Invitrogen*). The constructs were introduced into *Agrobacterium tumefaciens* L. strain GV3101 by using the freeze-thaw method described by Chen *et al*. (1994). A single colony of transformed cells was cultured in 5 cm^3 of a Luria-Bertani (LB) medium containing kanamycin (50 µg cm^{-3}), and grown overnight (at 28 °C and 225 rpm). A 0.1 cm^3 aliquot of *Agrobacterium* cells was used for inoculation of 20 cm^3 of the LB medium supplemented with a 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 5.7, 50 µg cm^{-3} kanamycin, and 0.15 mM acetosyringone (3,5-dime-thoxy-4′-hydroxyacetophenone). *A. tumefaciens* cells were harvested by centrifugation at 5 500 *g* for 20 min. After removal of the supernatant, the pellet was resuspended in infiltration buffer containing 10 mM MES (pH 5.7), 10 mM $MgCl_2$, and 100 µM acetosyringone. The absorbance of the suspension at 600 nm (A_{600}) was adjusted to 0.8 - 1.0 by infiltration buffer and the suspensions were left at room temperature for 3 h. After infiltration of the *Agrobacterium* cells by using a needleless syringe, the tobacco plants were placed under above mentioned growing conditions. Approximately 3 - 4 d after the infiltration, tobacco leaves were cut with a razor blade, the sections were placed on glass slides, and covered with cover glasses. The GFP signal excited at 488 nm was observed using a confocal laser scanning microscope (*LSM 5 Exciter*, *Carl-Zeiss*, Oberkochen, Germany) according to Wydro *et al*. (2006).

Yeast two-hybrid (Y2H) assay: For Y2H screening, total RNA was extracted from wheat spikes (spikes covered by the stem and leaf sheaths just before emergence, and during the initial stage when the spike completely emerged from the stem) with the *TRIzol* reagent. Total RNA (2 µg) was used for the preparation of a cDNA library by using the *Make Your Own Mate & Plate*^{TM} library system (*Clontech*, Palo Alto, CA, USA), according to the manufacturer's instructions. After the colonies were washed with 500 cm^3 of yeast peptone dextrose adenine (YPDA) containing 25 % (v/v) glycerol and 25 mg cm^{-3} kanamycin, they were incubated at 30 °C and 220 rpm for 30 min . Aliquots (1 cm^3) of the cDNA were stored at -80°C until use (Hong *et al*. 2012). Quality analysis of the cDNA showed that the transformation efficiency was 1.6 × 10^6 transformants per 3 µg of pGADT7-Rec DNA.

The pGBKT7:TaCullin construct was transformed into the cells of a yeast strain Y2HGold, and Y2H screening was conducted according to the manufacturer's protocol (*Matchmaker*^{TM} *Gold* Y2H system, *Clontech*). The mating products were plated onto a SD medium lacking leucine and tryptophan (SD/-LW) until colonies appeared (approximately 5 - 7 d later). The resulting diploid yeast was selected on the SD medium lacking histidine, leucine, and tryptophan (SD/-HLW), and was further selected on the SD medium lacking adenine, histidine, leucine, and tryptophan (SD/-AHLW). All the

SD media contained X-α-galactosidase, and an aureobasidin A. *Matchmaker* insert check PCR mix 2 (*Clontech*) was used for a positive clone selection.

The coding regions of *TaCullin*, N-terminal *TaCullin*, and C-terminal *TaCullin* were amplified by PCR with primers containing restriction sites and were cloned into the pGBKT7 vector (bait). The *TaSKP1*, *TaSKP2*, *TaSKP3*, *TaSKP4*, *TaSKP5*, *TaSKP6*, and *TaRMD5* coding regions also amplified by PCR with specific primers containing restriction sites were cloned in a frame into the pGADT7 vector (preys) (Hong *et al*. 2012). For cotransformation, AH109 yeast cells were used with each bait and prey vector by using the lithium acetate method. Transformants were plated onto SD/-LW and SD/-AHLW containing X-α-galactosidase to detect prey-to-bait interactions. AH109 cotransformed with a SV40 large T antigen (pGADT7-T)/p53 (pGBKT7-53) was used as positive control, and AH109 cotransformed with a SV40 large T antigen (pGADT7-T)/lamin-C (pGADT7-Lam) was used as negative control.

β-Galactosidase assay: Y2H β-galactosidase quantifications were performed using *O*-nitrophenyl-β-*D*-galactopyranoside (ONPG) assays as described in the *Clontech* Laboratory Yeast Protocols Handbook. The yeast strains were grown overnight at 30 °C with shaking in 5 cm^3 of SD/-LW. A 2-cm^3 sample of the overnight culture was then transferred to 8 cm^3 of a yeast peptone dextrose medium [1 % (m/v) bacto-yeast extract, 2 % (m/v) bacto-peptone, and 2 % (m/v) dextrose)] and grown at 30 °C with shaking for approximately 2 h. Three replicates were separated from each culture. Yeast cells were pelleted by centrifugation and washed once with a Z buffer consisting of [g dm^{-3}] 16.1 $Na_2HPO_4 . 7H_2O$, 5.5 $NaH_2PO_4 . 7 H_2O$, 0.75 KCl, and 0.24 $MgSO_4 . 7 H_2O$. The yeast cell pellets were then resuspended in the Z buffer and lysed using 5 sequential freeze-thaw cycles, consisting of transfers from liquid nitrogen to a 37 °C water bath. The Z buffer containinga β-mercaptoethanol solution was added along with the Z buffer/ONPG solution. The samples were then incubated in a 30 °C water bath and timed for the rate of yellow colour development. The ONPG reactions were quenched with 1 M Na_2CO_3, after which the samples were centrifuged at 10 000 *g* for 10 min, and A_{420} was measured.

Bimolecular fluorescence complementation (BiFC) assay: To confirm protein-protein interaction *in vivo*, we constructed BiFC vectors (Walter *et al*. 2004). *Nicotiana benthamiana* Domin plants were grown under the above mentioned controlled conditions for 4 - 5 weeks. For the BiFC constructs, *TaCullin* and *TaRMD5* cDNAs were amplified by PCR by using the gene-specific primers (Table 1), cloned into pCR/GW/TOPO (*Invitrogen*) and subcloned into gateway compatible binary BiFC vectors with *LR Clonase.* BiFC vectors (pE-SPYNE-GW and pE-SPYCE-GW) provided by *Wolfgang Dröge-Laser* (Universität Göttingen, Göttingen, Germany). pE-SPYNE:TaCullin was fused with the N-terminal region of YFP, and pESPYCE-TaRMD5 was fused with the C-terminal part of YFP. The BiFC assay was performed using *Agrobacterium*-mediated transient co-expression in *N. benthamiana*. Binary plasmids were transformed into a strain *A. tumefaciens* GV3101 by the

Table 1. Primers used for gene cloning, expression quantification, yeast two-hybrid system, and sub-cellular localization. Sequences underlined in the yeast two-hybrid vectors indicate enzyme restriction sites.

Purpose	List		Forward	Reverse
Full length gene cloning	TaCullin_full		ATGGCGGGCCACGGGCAGGACCGC	TCAAGCCAGATATCTGTATGTGTT
	TaRMD5		ATGGAGCTTGACAGTCTAAGAGA	TTAGAAACGAAGCTGCTTGCACT
RT-PCR	TaCullin_RT		AAGGAGTTCTACGCAACAAGAAC	TAGGCGAAATAGTTCTACCAGCT
	Actin (AB181991)		GCCACACTGTTCCAATCTATGA	TGATGGAATTGTATGTCGCTTC
Chromosome localization	TaCullin_Local		CACACGGAGGTCGCTTACTGC	TGCAAGTAGTGACCAACTCGC
Sub-cellular localization	TaCullin_GFP		ATGGCGGGCCACGGGCAGGACCGC	AGCCAGATATCTGTATGTGTTGGCG
	TaRMD5_GFP		ATGGAGCTTGACAGTCTAAGAGA	GAAACGAAGCTGCTTGCACTGCGA
Yeast two-hybrid system	Bait	TaCullin	GAATTCATGGCGGGCCACGGGCAG	GGATCCCTCAAGCCAGATATCTGTAT
		TaCullin-N terminus	GAATTCATGGCGGGCCACGGGCAG	GGATCCCAGTGCCTTGTGAAAGAG
		TaCullin-C terminus	GAATTCATGGTGAAATGTGTAGAG	GGATCCCTCAAGCCAGATATCTGTAT
	Prey	TaSKP1_Y2H	GGATCCCCATGGCGGCCGCGGGAGAC GCCGG	CTCGAGCTACTCAAAGGCCCACTGG
		TaSKP2_Y2H	GAATTCATGGCGTCCGAGGAAG	GGATCCCCTACTCAAAGGCCCACTG
		TaSKP3,4_Y2H	GAATTCATGGCGGCCGCGGAGGGC	GGATCCCCTACTCAAAGGCCCACTG
		TaSKP5,6_Y2H	GAATTCATGGCGGCCGCGGAGGGC	CTCGAGCCTACTCAAAAGCCCACT
		TaRMD5_Y2H	GAATTCATGGAGCTTGACAGT	GGATCCATTAGAAATGAAGCTGC
		TaRMD5_CRA	GAATTCGGAAGCAGAGATAATGCT	GGATCCTACCGTGGTGAGCTTCAA
		TaRMD5_RING	GAATTCTTCGTATGCCCGGTGCTC	GGATCCAAGCTGCTTGCACTGCGA
		TaRMD5_Partial	GAATTCATGGAGCTTGACAGTCTA	GGATCCTCTGGTGAGTATCTCAAC
BiFC assay	TaCullin_BiFC		ATGGCGGGCCACGGGCAGGACCGC	AGCCAGATATCTGTATGTGTTGGC
	TaRMD5_BiFC		ATGGAGCTTGACAGTCTAAGAGA	GAAACGAAGCTGCTTGCACTGCGA

heat-shock method. All strains of *Agrobacterium* were grown in a LB medium containing a 10 mM MES buffer (pH 5.7), 50 μg cm^{-3} kanamycin, and 0.15 mM acetosyringone (Johansen and Carrington 2001). Transformed cells were pelleted by centrifugation and suspended in an infiltration solution (10 mM $MgCl_2$, 10 mM MES, pH 5.7, and 200 μM acetosyringone) until the A_{600} reached 0.8. The transformed cells containing the pE-SPYNE:TaCullin or pE-SPYCE:TaRMD5 plasmids were mixed in a 1:1 ratio and infiltrated into tobacco leaves. The epidermal cell layers of the tobacco leaves were observed for fluorescence at day 4 after infiltration by using a confocal laser scanning microscope (*LSM 5 Exciter*).

Results

In order to isolate the *Cullin* gene from wheat, data pertaining to the conserved regions of *Cullin* sequences were obtained from the EST sequences downloaded from the *GrainGenes 2.0*, *Gene Index*, and *NCBI* database. Based on the conserved regions of the *Cullin* sequences, primer sets were designed and used for PCR amplification of *TaCullin.* The predicted open reading frame (ORF) of *TaCullin* is 2 238 bp, encoding a peptide of 746 amino acids with a calculated M_r of 86.73 kDa and an isoelectric point of 6.46. Analysis of the predicted TaCullin protein by using the *EBI InterProScan* software showed two domains: one from amino acid 13 to 390 (IPR016159, a cullin repeat like-containing domain) and second from 673 to 740 (IPR019559, a cullin protein neddylation domain). A multiple sequence alignment indicated a considerable conservation of cullin domains across species (Fig. S1).

In order to analyze the expression of *TaCullin* in response to abiotic stresses and the application of phytohormones, RT-PCR was used to detect the expression of *TaCullin* (Fig. 1). *TaCullin* transcript accumulation decreased on the ABA treatment and increased to the highest level at 12 h after the MeJA treatment. During the cold treatment, the transcription of *TaCullin* slightly increased in correlation with the treatment time. In the IAA and NaCl treatments, the expression of *TaCullin* was the lowest at 3 h, after which it increased slightly with time. There was no significant change in the expression of *TaCullin* after either the GA_3 or PEG treatments.

TaCullin expression was also studied during different developmental stages of the spike and grain by qRT-PCR (Table 3). *TaCullin* genes were highly expressed during

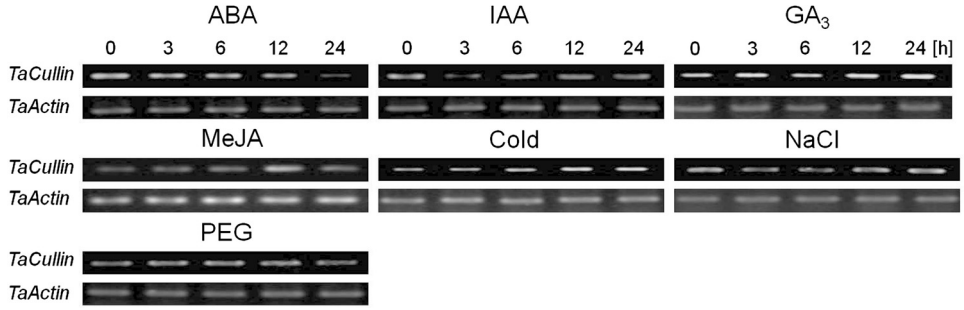

Fig. 1. *TaCullin* gene transcripts of young wheat seedlings harvested at different time points during phytohormone and abiotic stress treatments were analyzed by RT-PCR. The *Actin* gene (GenBank ID: AB181991) was used as reference gene.

Table 2. Transcript accumulation profiles of the *TaCullin* gene expressed in various tissues and at different developmental stages using RT-qPCR analysis. Spikes at different developmental stages: 1 - very young inflorescence, less than 3 mm, 2 - 5 to 10 mm, 3 - 15 to 25 mm, 4 - 50 to 70 mm, 5 - 100 to 120 mm (1 to 4 - spikes covered by stem and leaf sheaths, 5 - the initial stage of spike that is completely emerged from the stem). Different stages of wheat grain development in days after flowering (DAF -1 - 1 day before flowering). *TaCullin* gene expression in various tissues (caryopsis, ovary, pericarp, pollen). Means ± SE, $n = 3$.

Spike development stages	relative expression	Grain development stages	relative expression	Tissues	relative expression
1	22.16 ± 3.07	DAF-1	4.09 ± 1.88	caryopsis	2.64 ± 0.99
2	14.59 ± 1.93	DAF1	16.76 ± 2.43	ovary	9.19 ± 1.08
3	9.40 ± 1.06	DAF3	8.57 ± 1.45	pericarp	1.00 ± 0.42
4	1.62 ± 0.33	DAF6	4.70 ± 1.75	pollen grain	2.52 ± 0.89
5	1.00 ± 0.39	DAF10	1.00 ± 0.53		
		DAF16	2.70 ± 1.11		
		DAF20	2.30 ± 1.16		

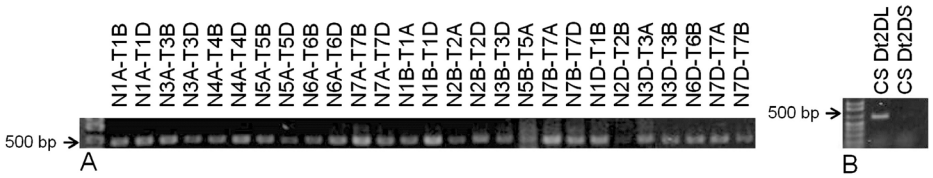

Fig. 2. Chromosomal localizations of the *TaCullin* gene in wheat. *A* - PCR was performed on genomic DNA of the null-tetrasomic (NT) lines derived from *Triticum aestivum* cv. Chinese Spring. *B* - PCR was conducted on the genomic DNA of ditelosomic lines to define the chromosome arm location of the *TaCullin* gene.

Table 3. TaCullin interacting partners identified by yeast two-hybrid screening. Clone used for a β-galactosidase activity analysis and BiFC assay is indicated in bold.

Clone No.	Putative identification	Organisms
1	SNF1-type serine-threonine protein kinase (SnRK2.4)	*Triticum aestivum*
2	SURP and G-patch domain-containing protein 1-like protein-like	*Brachypodium distachyon*
3	zinc finger (C3HC4-type RING finger) protein-like	*Brachypodium distachyon*
4	70 kDa heat shock protein (TaHSP70d) mRNA	*Triticum aestivum*
5	small subunit precursor of RuBPCase	*Triticum aestivum*
6	sufE-like protein, chloroplastic-like	*Brachypodium distachyon*
7	**RMD5 homolog A-like**	*Brachypodium distachyon*
8	ubiquitin-60S ribosomal protein	*Brachypodium distachyon*
9	peroxisomal (S)-2-hydroxy-acid oxidase GLO5-like	*Brachypodium distachyon*

early spike development and the expression decreased as growth and development elapsed (Table 2). During grain development, *TaCullin* was strongly detected at day 1 after flowering (DAF1) and thereafter gradually down-regulated (Table 2). The *TaCullin* is ubiquitously expressed in all tissues, with the highest transcription in the ovary Table 3). These results show that *TaCullin* may play an important role in tissue-specific and develop-ment-related functions.

Chromosome localization of the *TaCullin* gene, based on whether PCR products obtained for the nulli-tetrasomic and ditelosomic lines, showed that the *TaCullin* gene resides on the chromosome arm 2DL (Fig. 2*A*, *B*).

To confirm the interaction between TaCullin and TaSKP proteins, we performed a Y2H assay. Good growth was obtained in the case of the TaCullin-N-terminus, TaSKP1, TaSKP5, and TaSKP6 on SD/-LW/X-α-Gal. However, the TaCullin-C-terminus and all the TaSKP transformed colonies showed comparatively weak interaction in SD/-AHLW/X-α-Gal (Fig. 3). Positive transformants, which appeared blue, were assayed for β-galactosidase activity by using a liquid assay. In the β-galactosidase activity assay, TaCullin and TaSKP1/TaSKP5/TaSKP6 were also found to interact (Table 4), confirming the previous Y2H results.

To identify proteins that interact with TaCullin, we screened TaCullin-interacting proteins by using the Y2H approach. Approximately 1.6×10^6 colonies were screened, and 72 positive clones were identified on the SD/-LW medium. Clones that failed to grow in two consecutive

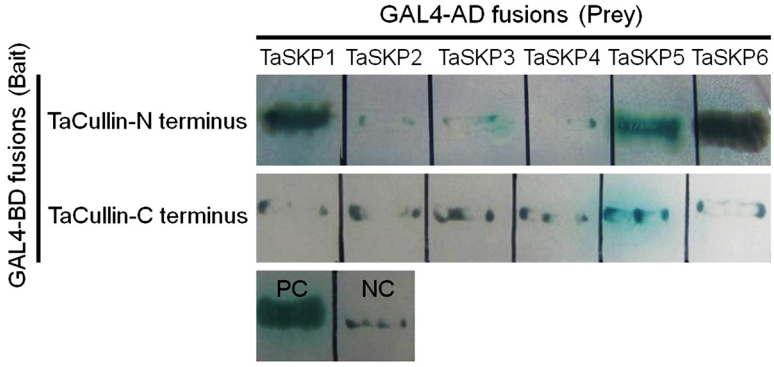

Fig. 3. The yeast two-hybrid interaction between TaCullin proteins and TaSKP proteins. AH109 yeast strain was transformed with pGBKT7-based bait plasmids (TaCullin deletion constructs: TaCullin-N terminus and TaCullin-C terminus) in combination with each one of the following pGADT7-based prey plasmids: TaSKP1, TaSKP2, TaSKP3, TaSKP4, TaSKP5, and TaSKP6. The co-transformants were grown on SD/-LW medium or SD/AHLW (QDO) medium with addition of X-α-galactosidase. SV40 large T antigen (pGADT7-T)/p53 (pGBKT7-53) and SV40 large T antigen (pGADT7-T)/Lamin-C (pGADT7-Lam) indicate positive (PC) and negative (NC) controls, respectively.

Table 4. Interaction of the protein pairs (TaCullin-N terminus and TaSKP proteins, TaCullin and TaRMD5) was tested using the β-galactosidase activity (β-gal) assay. Also, yeast two-hybrid assays were performed with TaCullin-C terminus in the DNA-binding domain vector (pGBKT7) and deletion constructs of TaRMD5 in the activating domain vector (pGADT7): full length of TaRMD5, TaRMD5-RING domain, TaRMD5-CRA domain, and partial length of TaRMD5. The combination of p53 and SV40 large T antigen shows positive interaction (PC), whereas the combination of Lamin-C and SV40 large T antigen shows negative interaction (NC) (Miller unit). Means ± SE, $n = 3$.

	PC	NC	TaSKP1/ TaCullin-N	TaSKP2/ TaCullin-N	TaSKP3/ TaCullin-N	TaSKP4/ TaCullin-N	TaSKP5/ TaCullin-N	TaSKP6/ TaCullin-N
β-gal	318.6 ± 11.2	1.0 ± 0.3	162.8 ± 11.5	1.3 ± 0.1	0.8 ± 0.3	1.1 ± 0.1	80.2 ± 9.5	114.4 ± 10.2

	PC	NC	TaCullin/TaRMD5	TaCullin-N/TaRMD5	TaCullin-C/TaRMD5
β-gal	332.2 ±13.1	1.4 ± 0.1	188.4 ± 9.8	29.2 ± 3.5	244.2 ± 24.1

	PC	NC	TaCullin-C/RMD (full length)	TaCullin-C/RMD (RING domain)	TaCullin-C/RMD (CRA domain)	TaCullin-C/RMD (partial length)
β-gal	418.5 ± 10.3	2.4 ± 0.1	102.5 ± 9.4	52.5 ± 3.4	62.5 ± 4.4	159.1 ± 9.6

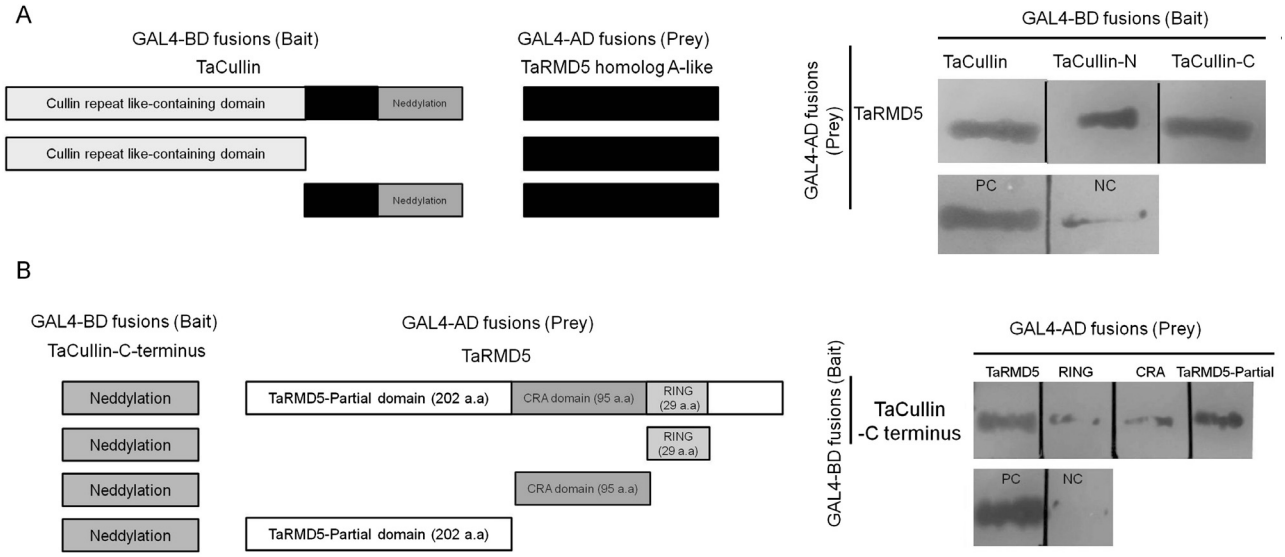

Fig. 4. The interaction between TaCullin and TaRMD5 homolog A-like protein *via* a yeast two-hybrid assay. *A* - The schematic picture of the TaCullin fragments is *on the left*. Deletion analysis of TaCullin-TaRMD5 homolog A-like protein interactions is *on the right*. Yeast two-hybrid assays were performed with full-length TaRMD5 in the activating domain vector (pGADT7) and deletion constructs of TaCullin in the DNA-binding domain vector (pGBKT7): full-length TaCullin, TaCullin-N terminus (Cullin repeat-like containing domain, amino acids 13 - 390), and TaCullin-C terminus (neddylation domain, amino acids 673 - 740). Yeast cells transformed with the plasmid pairs (N- or C-terminal fragments of TaCullin and TaRMD5 homolog A-like protein) were cultured on SD/-LW and transferred to SD/-AHLW + X-α-gal. *B* - The yeast two-hybrid assays were performed with TaCullin-C terminus in the DNA-binding domain vector (pGBKT7) and deletion constructs of TaRMD5 in the activating domain vector (pGADT7): full length of TaRMD5, TaRMD5-RING domain, TaRMD5-CRA domain, and partial length of TaRMD5. The combination of p53 and SV40 large T antigen shows positive interaction (PC), whereas the combination of Lamin-C and SV40 large T antigen shows negative interaction (NC).

rounds of testing were considered false positives (first round, SD/-LW; second round, SD/-AHLW + X-α-gal). Plasmids from these clones were recovered by yeast mini-preparation, sequenced, and subjected to a *BLAST* search in the *NCBI* database. After exclusion of the false positives, nine clones were identified as potential interacting partners of TaCullin (Table 3). To examine whether there is specificity between TaCullin and these

interacting partners, we performed co-transformation of both bait (pGBKT7:TaCullin) and prey (pGADT7: TaCullin interacting proteins) plasmids into the AH109 yeast cells. Yeast colonies were tested for a β-galactosidase activity in 2 additional rounds of selection to test the specificity of interactions (SD/-LW and SD/-AHLW + X-α-gal media). Through yeast cotransformation, the RMD5 homolog A-like protein showed strong interaction with the TaCullin protein and was ultimately identified as TaCullin-interacting protein.

The TaCullin protein is composed of 2 domains, an N-terminal Cullin repeat-like-containing domain and a C-terminal neddylation domain (Fig. 4*A*). We found that full-length TaCullin could interact with TaRMD5. To determine which domain of TaCullin is responsible for its interaction with TaRMD5, we designed two TaCullin deletion mutants carrying deletions of the Cullin repeat-like-containing domain or the neddylation domain. The N-terminal Cullin repeat-like containing domain, the C-terminal neddylation domain, and full-length TaCullin were introduced into the pGBKT7 vector and expressed as a fusion to the GAL4 DNA-binding domain, whereas the *TaRMD5* gene was introduced into the pGADT7 vector and expressed as a fusion to the GAL4 activation domain. The TaRMD5:pGADT7 construct was transformed into yeast AH109 in combination with the TaCullin deletion constructs (TaCullin-N-terminus: pGBKT7, TaCullin-C-terminus:pGBKT7, and TaCullin: pGBKT7). Transformed yeast cells were streaked on the SD/-AHLW + X-α-gal media. All the combinations grew well on the SD/-AHLW + X-α-Gal (Fig. 4*A*).

The β-galactosidase activity assay shows that the full-length TaCullin and C-terminal neddylation domain interacted with the TaRMD5 homolog A-like protein (Table 4). However, a weak interaction was detected between the Cullin repeat-like-containing domain and TaRMD5 homolog A-like protein. We identified the TaRMD5 homolog A-like protein as protein that interacted with both the full-length and the C-terminus of TaCullin. These results indicate that the interaction between TaCullin and TaRMD5 was specifically mediated through the C-terminus of TaCullin which contains the neddylation domain (amino acids 673 - 740).

TaRMD5 contains several predicted functional domains, including a CRA domain (IPR013144) and a zinc finger (RING/FYVE/PHD-type) domain (IPR13083). In order to determine which domain of TaRMD5 is required for its interaction with TaCullin, we tested different TaRMD5 deletion constructs for their ability to interact with TaCullin in the Y2H assay. All the TaRMD5 deletion constructs could interact with the C-terminus of TaCullin in the β-galactosidase activity assay. Amino acids 1 - 202 of TaRMD5 (TaRMD5 partial domain, no distinct domain) showed the strongest interaction with the C-terminus of TaCullin (Fig. 4*B*, Table 4).

In order to investigate the subcellular localization of TaCullin, we prepared a GFP fusion protein construct of the *TaCullin* coding sequence fused with that of GFP driven by the CaMV 35S promoter (35S:TaCullin:GFP). The 35S:TaCullin:GFP construct was transiently expressed in tobacco epidermal cells *via Agrobacterium* infiltration, and the subcellular localization was analyzed by confocal laser scanning microscopy. Fluorescent signals from the TaCullin:GFP fusion protein were detected in nuclei (Fig. 5*B*) and the GFP signal from TaRMD5 was detected in the cytoplasm (Fig. 5*C*).

To confirm the results of the Y2H assay, we examined the protein-protein interactions in plant cells by using BiFC. For the BiFC assay, the full-length ORF of TaCullin was cloned into the pE-SPYNE-GW [split yellow fluorescent protein (YFP) N-terminal fragment expression] vector, and the full-length ORF of TaRMD5 was cloned into the pE-SPYCE-GW (split YFP C-terminal fragment expression) vector by using the *Gateway* system. The transformed *Agrobacterium* strain GV3101 carrying different constructs was co-transformed and infiltrated into tobacco leaves. YFP fluorescence was observed when pE-SPYNE-TaCullin was co-expressed with pE-SPYCE-TaRMD5. We detected a strong reconstituted YFP signal in the plasma membrane when TaCullin and TaRMD5 were co-transformed into tobacco leaves (Fig. 6). This assay confirmed the interaction of TaCullin with TaRMD5 in plants.

Discussion

The ubiquitin proteasome system regulates target protein degradation in the cellular processes of eukaryotes (Ciechanover 1998). E3 ligases provide specificity to the ubiquitination process by recognizing target substrate proteins and mediating transfer of the ubiquitin molecule from an E2 enzyme to the substrate (Deshaies *et al*. 2009). Cullin-RING ubiquitin ligases (CRLs) are well known as group of multisubunit E3 ligases consisting of a Cullin protein that serves as scaffold.

Most eukaryotes contain multiple *Cullin* family genes (Marín 2009). The *Arabidopsis* genome encodes 11 cullin proteins (Sarikas *et al*. 2011). Specific roles for these proteins have been demonstrated in varying stages of plant development, including embryogenesis (Hellmann *et al*. 2003), phytochrome signalling (Dieterle *et al*. 2005), circadian clock (Harmon *et al*. 2008), ethylene biosynthesis (Wang *et al*. 2004), and phytohormone signalling (Gray *et al*. 2001). CUL1 recruits substrate-recognition subunits containing a conserved F-box domain *via* the adaptor protein Skp1 to form Skp1-Cul1-F-box (SCF) E3 ligases. CUL1 interacts with a SKP1-like protein, RING domain protein, and F-box protein with substrate specificity to form an SCF complex (Wang *et al*. 2003, Duda *et al*. 2012). CUL2 and CUL5 are known to interact with the SKP1-like protein elongin C which in turn interacts with F-box protein-like specificity factors

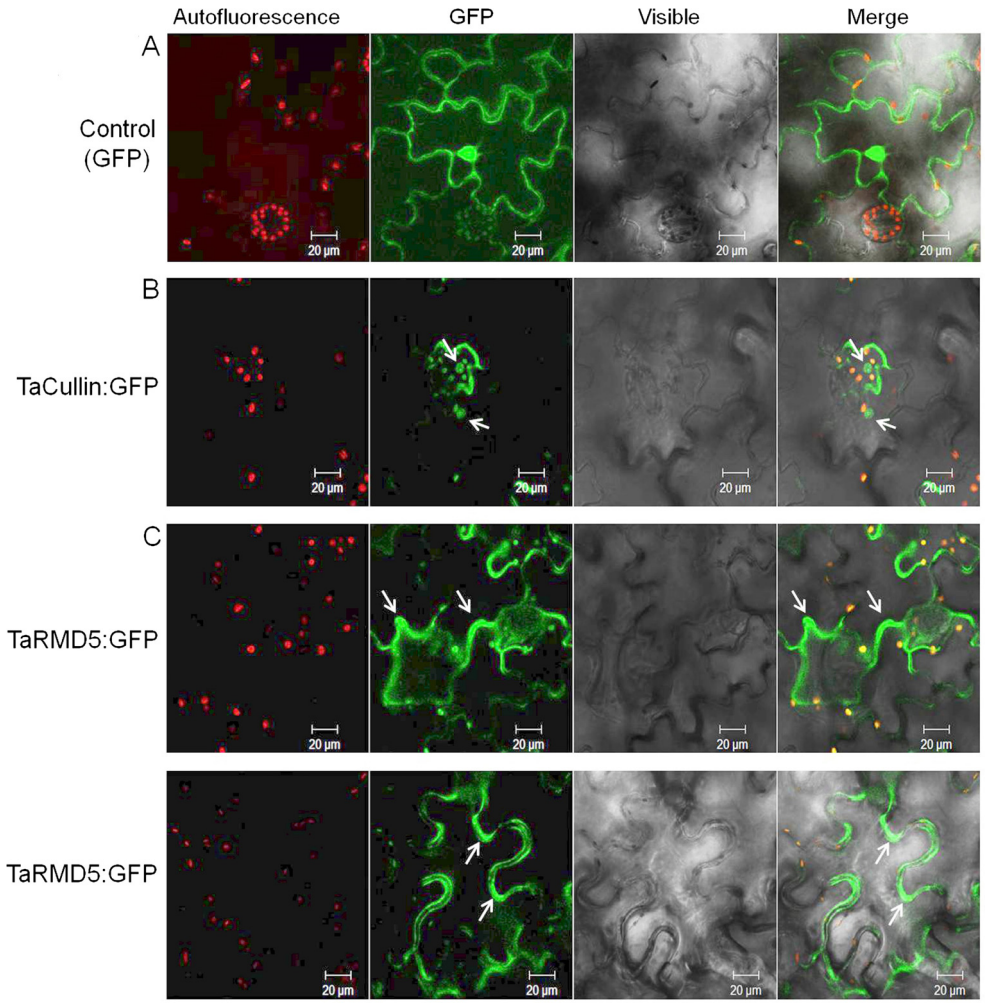

Fig. 5. *A* - Tobacco leaf cells transformed with the vector expressing GFP alone, as control, showed green fluorescence. *B* - Sub-cellular localization of TaCullin-GFP fusion proteins. *C* - Sub-cellular localization of TaRMD5-GFP fusion proteins. Tobacco epidermis was infiltrated with *Agrobacterium tumefaciens* containing a binary vector with the 35S promoter and GFP fusion protein.

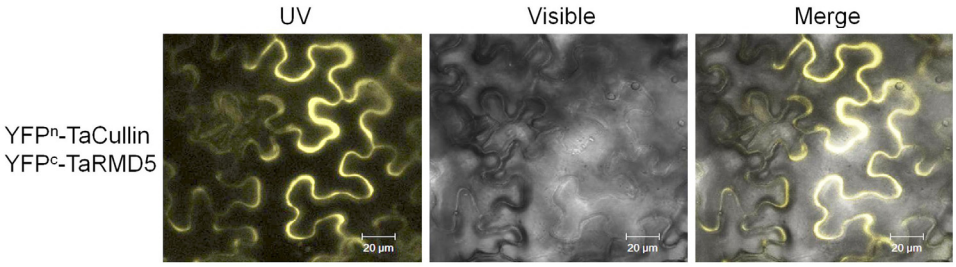

Fig. 6. The interaction between TaCullin protein and TaRMD5 protein using the BiFC assay. Tobacco leaves were co-transformed with confocal images of tobacco epidermal leaf cells expressing YFP fusions of TaCullin and TaRMD5. TaCullin tagged with the N-terminal fragment of YFP (YFP^{n}-TaCullin) was co-expressed in tobacco leaves by *Agrobacterium tumefaciens* transient transformation with the C-terminal fragment of YFP fused to TaRMD5 (YFP^{c}-TaRMD5).

called BC/SOCS-box proteins (Jin *et al*. 2004). CUL3 interacts with BTB/POZ domain proteins (Geyer *et al*. 2003, Weber *et al*. 2005). CUL4-DDB1-CSA-ROC1 complexes appear to function as substrate-specificity modules (Groisman *et al*. 2003, Higa *et al*. 2007). However, the expression and function of cullin proteins in other plant species remain largely unidentified. To our knowledge, this is the first study to examine the *Cullin* gene in hexaploid common wheat. The data presented in this study provide extensive and novel information regarding the function of the *TaCullin* gene in wheat.

Expression of the *TaCullin* gene was detected in all

the tissues examined in this study. The transcription peaked during early spike development and gradually decreased after the spike emerged fully. These results are similar to those of our previous report (Hong *et al*. 2012), where higher expressions of the *TaCFBD* and *TaSKP* genes in developing spikes were observed with peaks during the early stages of spike development. Using the Y2H system, we have demonstrated that the *TaCFBD*, *TaSKP*, and *TaCullin* genes encode core components of SCF complexes. The early stages of spike development are characterized by the initial appearance of floret primordia (Gardner *et al*. 1985) which form various floral organs, such as glumes, lemmas, and anthers. In *Arabidopsis*, many *ASK* (SKP1-like gene) genes are expressed in the inflorescence; this is confirmed by the presence of defects in the regulation of organ identity genes in *Cullin* mutant plants (Zhao *et al*. 2003, Ni *et al*. 2004). In addition, CUL1 is involved in the formation of the apical meristem as organ initiator (Hellmann *et al*. 2003). SCF complexes have often been associated with the development of reproductive organs in *Arabidopsis*, linking the different developmental and physiological processes (Zhao *et al*. 2003). The expression studies described provide an overview of *TaCullin* function during wheat spike development and show that *TaCullin* is important in the early stages of spike formation (Table 2). We believe that, like the *Arabidopsis* SCF complex, the wheat SCF complex may play a role in flower development, although more specific evidence is required to support these results.

SCF complexes, the largest family of cullin-based E3 ligases, recognize specific target proteins. The SCF complex consists of four components: cullin for linking SKP1 and Rbx/Roc/Hrt, the adaptor protein SKP1 for binding cullin and the F-box protein, the RING finger protein Rbx/Roc/Hrt for recruiting the E2 enzyme, and the F-box protein for identifying specific substrates (Deshaies *et al*. 1999, Sun *et al*. 2006). Of these, cullins are the central scaffolding subunits for the assembly of the SCF complex which ubiquitinates a large number of substrate proteins involved in various cellular processes. Cullin proteins contain 2 order modules that are required for the complex: substrate receptor subunits and the RING domain-containing protein Rbx/Roc/Hrt. The N-terminus of cullin binds to a specific adaptor protein, such as SKP1, and *via* this to an F-box protein that binds the substrate (Chew *et al*. 2007, Lijun *et al*. 2009). The CRL complex recognizes protein substrates through F-box proteins and regulates their degradation and ubiquitination (Liu *et al*. 2009).

The Y2H screening results show that TaCullin was able to interact with TaRMD5. The physical interaction of the 2 proteins highlighted by the Y2H assay has been confirmed *in vivo* by the BiFC assay. Multigenic cullin families seem to be widely expressed and they are localized both in nucleus and cytoplasm, however, not much information is available regarding the localization of cullin proteins (Sarikas *et al*. 2011). Some studies reported that the *Arabidopsis* cullin1 (AtCUL1) protein localizes mainly in nucleus and weakly in cytoplasm during interphase and co-localizes with the mitotic spindle during metaphase (Shen *et al*. 2002). Like AtCUL1, the TaCullin protein was also detected in nucleus. The GFP signal was detected in two well-separated nuclei (Fig. 5*B*). The cullin-associated Nedd8-dissociated protein 1 (CAND1) is a cullin-binding protein that only interacts with cullins that are unneddylated and are not connected with adaptor and substrate receptor subunits (Chua *et al*. 2011). CAND1 is an *in vitro* inhibitor of CRLs that stably interacts with unneddylated cullin-RING complexes and co-localizes with cullin mainly in nucleus (Helmstaedt *et al*. 2011). Helmstaedt's report shows that endogenous concentrations of CAND1 and Cullin in cytoplasm are similar, whereas there is considerably less CAND1 than cullin in nucleus. We hypothesize that unneddylated TaCullin is located in nucleus when inhibited by CAND1. The results of the current study can provide insights into the molecular function of TaCullin and the endogenous concentration of CAND1 in nucleus.

Sub-localization results show that the GFP signals of TaCullin and TaRMD5 were present in different areas of cell (nucleus, cytoplasm, and plasma membrane) (Fig. 5). TaCullin-TaRMD5 was detected in plasma membrane and cytoplasm. The GFP signal from either TaCullin or TaRMD5 was difficult to detect in tobacco cells, whereas the TaCullin-TaRMD5 complex was easily detected. These data suggest that the TaCullin-TaRMD5 complex is more stable than TaCullin or TaRMD5 alone in plant cells.

Cullin binds the RING domain-containing protein Rbx1/Rbx2 *via* its C-terminus (Petroski and Deshaies 2005). *InterProScan* analysis showed that TaCullin has a specific N-terminal end, which contains the InterPro IPR016159 cullin repeat-like-containing domain, and a highly conserved C-terminus that includes the IPR019559 cullin neddylation domain. The N-terminus of TaCullin binds to specific recognition subunits, such as TaSKP proteins (SKP1 proteins), and the C-terminus of TaCullin interacts with a TaRMD5 homolog A-like protein (Fig. 4). *InterProScan* analysis showed that the TaRMD5 homolog A-like protein contains a novel functional domain containing a RING-type zinc finger domain (IPR013083) which is required for its role as E3 ubiquitin ligase.

The RING-type E3 ligases can be divided into two subgroups, simple and complex E3 ligases (anaphase-promoting complex or cyclosome and multisubunit SCF complex). The simple RING-type E3 is composed of a substrate-binding domain and an E2 ubiquitin-conjugating enzyme-binding RING domain, as homo-dimer or heterocomplex with different RING proteins (Stone *et al*. 2005). The RING-containing protein of the SCF complexes, Rbx/Roc/Hrt, recruits the E2 enzyme intermediate to the SCF complex (Seol *et al*. 1999). Taken together, our data suggest that TaCullin is a component of the Cullin-based E3 ligase and forms an SCF complex with identical SKP1 (TaSKP protein) and F-box proteins. The Y2H assays indicate that the

TaRMD5 protein seems to recruit the E2 enzyme as RBX1 through the RING-type zinc finger domain.

The activity of cullin-based E3 ligases is controlled by a number of regulatory mechanisms. For instance, cullin neddylation regulates the activity of cullin-based E3 ligase by CAND1. CAND1 is a cullin-binding protein that associates with unneddylated cullin and prevents binding substrate-specific factors, thus inhibiting the formation of an active ligase complex and promoting the dissociation of substrate receptor components from the cullin-based E3 ligases (Chua *et al*. 2011). The ubiquitin-like protein Nedd8 (also called Rub1) is another regulator of cullin-based E3 ligases. The attachment of Nedd8 acts as a positive regulator of cullin-based E3 ligase activity. The combination of cullin and Nedd8 triggers structural changes that enhance the efficient transfer of ubiquitin to the substrate (Duda *et al*. 2008, Siergiejuk *et al*. 2009). Thus, the activity of cullin-based E3 ligases can be predicted. Sequence analysis indicates that TaCullin contains a cullin neddylation domain in its C-terminus. In this study, TaRMD5 (as RBX1) was identified as a protein that interacted with the C-terminus of TaCullin and was required for neddylation. We hypothesize that the activity of the TaCullin-based E3 ligase is regulated by Nedd8 which is attached to the neddylation domain of TaCullin. Our data also suggest that binding CAND1 to cullin prevented the assembly of cullin-based E3 ligases until Nedd8 has been bound to cullin and the adaptor-substrate subunit associated with the cullin-based complex. The TaCullin neddylation and deneddylation mechanisms are still unknown due to the difficulty in determining the intermediate steps of the process.

Using Y2H screening, we showed that TaRMD5 could associate with TaCullin in the wheat spike samples. TaRMD5 is composed of three domains, LisH, CRA, and a RING-type domain. TaRMD5 deduced amino acid sequence has significant homology with yeast Gid2/Rmd5 and *Arabidopsis* AtRanBPM. In yeast cells, Gid2/Rmd5, as component of the GID complex, plays a role in polyubiquitination and the degradation of fructose 1,6-bisphosphatase (Santt *et al*. 2008). In *Arabidopsis*, AtRanBPM, a component of plant homologues of CTLH (C-terminal to the LisH motif) complexes, physically interacts with LiSH-CTLH domain-containing proteins and Yippee proteins (Tomaštíková *et al*. 2012). The wheat *RMD5* gene is structurally conserved, but its biological function is still not fully understood. Although TaRMD5 and AtRanBPM showed high sequence similarity, we predicted the function of TaRMD5 by the Y2H assay results based on the protein interaction data (Table 2 and Fig. 4). We presume that various RBX1 proteins (such as TaRMD5) have the potential to interact with TaCullin depending on the developmental stage and the particular organ tissues analyzed.

Although many factors remained unsolved, the data presented in this study provide new information about the function of wheat *Cullin*. In this study, we have shown molecular evidence for the SCF complex E3 ligase by which TaCullin interacts with TaRMD5 and the substrate-binding subunit, such as SKP1-like adaptor proteins and F-box proteins. F-box proteins confer specificity for the recruitment of the substrate to the SCF complex. TaCullin serves as the primary scaffolding subunit that bridges a substrate-binding subunit to TaRMD5 for the recruitment of E2 ubiquitin-conjugating enzymes. Further studies are required to determine the mechanism by which each cullin specifically recruits a different set of substrates.

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