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ORIGINAL PAPER

Genome-Wide Identification, Biochemical Characterization, and Expression Analyses of the YTH Domain-Containing RNA-Binding Protein Family in *Arabidopsis* and Rice

Dayong Li · Huijuan Zhang · Yongbo Hong · Lei Huang · Xiaohui Li · Yafen Zhang · Zhigang Ouyang · Fengming Song

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Abstract RNA-binding proteins are critical to RNA metabolism in cells and, thus, play important roles in diverse biological processes. In the present study, we identified the YTH domain-containing RNA-binding protein (RBP) family in *Arabidopsis thaliana* and rice at the molecular and biochemical levels. A total of 13 and 12 genes were found to encode YTH domain-containing RBPs in *Arabidopsis* and rice and named as *AtYTH01–13* and *OsYTH01–12*, respectively. The phylogeny, chromosomal location, and structures of genes and proteins were analyzed. Electrophoretic mobility shift assays demonstrated that recombinant AtYTH05 protein could bind to single-stranded RNA in vitro, demonstrating that the YTH proteins have RNA-binding activity. Analyses of publicly available microarray data, gene expression by qRT-PCR, and *AtYTH05* promoter activity indicate that the *Arabidopsis AtYTHs* and rice *OsYTHs* genes have distinct and diverse expression patterns in different tissues and developmental stages, showing tissue- and developmental-specific expression patterns. Furthermore, analyses of publicly available microarray data also indicate that many of the *Arabidopsis AtYTHs* and rice *OsYTHs* genes might be involved in responses to various abiotic and biotic stresses as well as in response to hormones. Our data demonstrate that the YTH family proteins are a novel group of RBPs and provide useful clues to define their biological functions of this RBP family in plants.

D. Li · H. Zhang · Y. Hong · L. Huang · X. Li · Y. Zhang · Z. Ouyang · F. Song (✉)
State Key Laboratory for Rice Biology, Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China
e-mail: fmsong@zju.edu.cn

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Introduction

The regulation of gene expression at the posttranscriptional level is important for plant growth, development, and stress responses. Posttranscriptional regulation includes pre-messenger RNA (mRNA) splicing, capping, polyadenylation, mRNA stability, and mRNA transport (Glisovic et al. 2008; Morris et al. 2010; Mittal et al. 2011; Stower 2014). In these processes, regulation is mainly achieved either directly by RNA-binding proteins (RBPs) or indirectly, whereby RBPs modulate the function of other regulatory factors (Janga and Mittal 2011). In eukaryotes, RBPs participate in synthesizing, processing, editing, modifying, transporting, and exporting RNA molecules from the nucleus (Ambrosone et al. 2012; Huh and Paek 2013; Pallas and Gomez 2013) and, thus, are involved in all aspects of translating mRNAs, as well as in storing them while they are not being translated (Johnstone and Lasko 2001; Zhong et al. 2001). RBPs also regulate and maintain the stability of mRNA in cells (Glisovic et al. 2008; Ambrosone et al. 2012). In addition to their involvement in these very central aspects of decoding the information stored in DNA, RBPs are also involved in certain aspects of chromosome structuring and coarse regulation such as telomere maintenance (Chen and Lingner 2012).

All aspects of RNA metabolism are accompanied by the activities of a myriad of RBPs (Glisovic et al. 2008). Most of the RBPs are built-in versatile modular structures with multiple repeats of a few conserved

domains arranged in a variety of ways to fulfil their diverse functional requirements (Lunde et al. 2007). RBPs are characterized by the presence of one or more conserved RNA-binding domains such as RNA recognition motif (RRM), heterogeneous nuclear ribonucleoprotein K homology domain, Piwi Argonaut and Zwille domain, and the double-stranded RNA-binding domains (Ambrosone et al. 2012). It was estimated that there are more than 200 putative RBP genes in *Arabidopsis thaliana* genome (Lorkovic 2009), whereas about 250 RBP genes have been identified in *Oryza sativa* (Cook et al. 2011). Many RBPs seem to be unique to plants, suggesting that they might serve specific functions in plants (Ambrosone et al. 2012).

The YTH (YT521-B homology) domain, comprising 100–150 amino acids, is highly conserved and was identified by comparing some known protein sequences with the splicing factor YT521-B (Stoilov et al. 2002). The first member of the YTH-containing proteins is the YT521-B, which is a nuclear protein interacting with other proteins (e.g., Sam68/p62, rSLM-1, rSLM-2, hnRNPG, rSAF-B, and emerin) and is implicated in RNA metabolism (Imai et al. 1998; Hartmann et al. 1999; Stoss et al. 2004). The human splicing factor YT521 (YTHDC1) is characterized by alternatively spliced isoforms with regulatory impact on cancer-associated gene expression (Zhang et al. 2010a; Hirschfeld et al. 2014). Recent studies also found that the human YTH family members YTHDF1/2/3 selectively bind to N^6-methyladenosine and thus regulate the stability of mRNA (Dominissini et al. 2012; Wang et al. 2014). In yeast, Mmi1 (meiotic mRNA interception), a YTH protein, was found to be a part of the mRNA-destruction system that eliminates meiotic-specific mRNAs (Harigaya et al. 2006; McPheeters et al. 2009), whereas Ydr374c (Pho92), the homologue of human YTHDF2, was shown to participate in cellular phosphate metabolism through the regulation of *PHO4* mRNA stability (Kang et al. 2014).

It was speculated that the YTH domain is especially abundant in plants (Stoilov et al. 2002). However, information on the YTH genes or proteins in plants is very limited. In the present study, we performed genome-wide identification, biochemical characterization, and expression patterns of the YTH genes in *Arabidopsis* and rice. Gene expression analyses based on publicly available microarray data, qRT-PCR, and *AtYTH05* promoter activity indicate that members of the YTH family in *Arabidopsis* and rice should have yet-unknown functions in growth and development as well as in stress responses. Our studies demonstrate that the YTH family proteins are a novel group of RBPs and provide clues for studying their biological functions in the future.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis ecotype Columbia-0 was used in all experiments. Plants were grown in soil in a growth room under a 10-h light (100 μmol s^{-1} m^{-2} photons m^{-2} s^{-1} of intensity) and 14-h dark cycle at 22±2 °C with 60 % relative humidity. Samples for the analysis of gene expression patterns were collected from 6-week-old soil-grown plants and stored at −80 °C until use. *Nicotiana benthamiana* plants used for subcellular localization study were grown in the same growth room under same conditions.

Identification and Analysis of the YTH Genes in *Arabidopsis* and Rice

Two rounds of multiple database searches were performed to identify YTH genes in *Arabidopsis* and rice. In the first round, the rice (http://rice.plantbiology.msu.edu/, version 6.1) and *Arabidopsis* (http://www.arabidopsis.org/, TAIR10) genome databases were searched using the default settings with the YTH domain (Pfam PF04146, http://pfam.sanger.ac.uk/family/PF04146) sequence as a query. The protein and predicted complementary DNA (cDNA) and genomic sequences for putative YTH genes were downloaded from http://rice.plantbiology.msu.edu and http://www.arabidopsis.org websites, respectively. In the second round, the predicted protein and cDNA sequences of the YTH genes were used as queries for blastp or blastn searches against several nucleic acid and protein databases including TIGR (http://www.tigr.org/tdb/agi/), DDBJ database (http://www.ddbj.nig.ac.jp/E-mail/homology.html), and GenBank (http://www.ncbi.nlm.nih.gov/) to identify other possible YTH genes and retrieve full-length cDNA sequences. Intron/exon organization for each YTH gene was analyzed at Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) by comparing the predicted cDNA, available full-length cDNA, and genomic DNA sequences. Searches for putative conserved domains in the YTH proteins were performed using Scanprosite tool at http://prosite.expasy.org/scanprosite/. Molecular weight calculations and p*I* prediction were carried out using Compute pI/Mw tool at http://us.expasy.org/tools/pi_tool.html (Bjellqvist et al. 1994). The putative secondary structure of the YTH domains was determined using the PHD program (Rost 1996). Further domains or motifs in the YTH proteins were searched using SMART (Letunic et al. 2012; Schultz et al. 1998).

Phylogenetic Analysis

The conserved YTH domain sequences for the identified *Arabidopsis* and rice YTH proteins were aligned using

ClustalX (version 2.0.8) followed by manual adjustment. A phylogenetic tree was generated by the neighbor-joining (NJ) algorithm with *p*-distance method and pairwise deletion of gaps using MEGA version 6.05 with default parameters (Tamura et al. 2013). A bootstrap statistical analysis was performed with 1,000 replicates to test the phylogeny.

Subcellular Localization of AtYTH05 and AtYTH07 Proteins

Total RNA was extracted from the leaves of 2-week-old *Arabidopsis* seedlings, and first-strand cDNAs were synthesized using AMV reverse transcriptase following the manufacturer's recommendation (TaKaRa, Dalian, China). Coding sequences of *AtYTH05* and *AtYTH07* were amplified using specific primers AtYTH05-GFP-F (5′-GGA AGA TCT ATG GAT TCG AAT GGT CAA GTT C-3′, a *Bgl*II site underlined)/AtYTH05-GFP-R (5′-TCC CCC GGG TTA CAT GGT AGA ATC AAC AGT-3′; a *Sma*I site underlined) and AtYTH07-GFP-F (5′-CGC GGA TCC ATG GCT GGA GCC GCT TCT-3′, a *Bam*HI site underlined)/AtYTH07-GFP-R (5′-TGC TCT AGA TCA ACT CAA GTT AAT TGT TTC-3′, a *Xba*I site underlined) and cloned into pFGC-Egfp. The resulting pFGC-Egfp-AtYTH05 and pFGC-Egfp-AtYTH07 were transformed into *Agrobacterium tumefaciens* strain GV3101 by the electrotransformation method. *Agrobacterium* cells were grown at 28 °C overnight, collected by centrifugation and resuspended in MES buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 150 mM acetosyringone) to OD_{600}=0.6. The *Agrobacterium* suspension was infiltrated into fully expanded leaves of *N. benthamiana* plants (Goodin et al. 2002). GFP-expressing cells were detected with a confocal laser scanning microscope (Zeiss LSM 510 META; argon laser exCitation wavelength, 488 nm).

Purification of the Recombinant AtYTH05 Protein and RNA-Binding Assay

The coding region of *AtYTH05* was amplified with primers AtYTH05-pRSET-1F (5′-CC CTC GAG ATG GAT TCG AAT GGT CAA GTT C-3′, a *Xho*I site underlined) and AtYTH05-pRSET-1R (5′-GG GGT ACC TTA CAT GGT AGA ATC AAC AGT-3′, a *Kpn*I site underlined). The amplified fragment was digested with *Xho*I/*Kpn*I and cloned into pRSET-A (Invitrogen, Carlsbad, CA, USA). The resulting plasmid, pRSET(A)-AtYTH05, was confirmed by sequencing and introduced into *Escherichia coli* BL21(DE3) plus cells. Purification of the recombinant protein from induced and uninduced bacteria pRSET(A)-AtYTH05 was carried out with the His-Bind kit (NovaGen, Madison, WI, USA) following the manufacturer's recommendations. Protein concentration was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

RNA-binding activity of the recombinant AtYTH05 protein was analyzed by electrophoretic mobility shift assay. All reactions were performed on ice in 20 μl binding buffer (20 mM HEPES, pH 8.0, 50 mM KCl, 1 mM DTT, and 5 % glycerol). RNA substrate used for RNA-binding activity assays was prepared as described (Iost et al. 1999). A 58-nt single-strand RNA substrate (5′-UGC AUG CCU GCA GGU CGA CUC UAG AGG AUC CCC GGG UAC CGA GCU CGA AUU CGU AUU C-3′), labeled with [α-^{32}P]-dCTP, was generated by in vitro transcription from pGEM-4Z (Promega, Madison, WI, USA) using the MAXIscript system according to the manufacturer's instructions (Ambion, Austin, TX, USA). The synthesized transcript was purified by native polyacrylamide gel electrophoresis. Binding reactions contained 1 nM labeled RNA and 1 or 2 μg of protein, with or without cold competitor RNA, incubated on ice for 1 h, and run on a 6 % nondenaturing acrylamide gel (Mini Protean II, Bio-Rad, Hercules, CA, USA) at 100 V for 45 min at 4 °C. Gels were dried, exposed to a storage phosphor screen for 6–12 h, and autoradiographed by exposure to storage phosphor screens (GE Healthcare, Pittsburgh, PA, USA) for 4 h.

Generation of $AtYTH05_{pro}$::GUS Transgenic Lines and GUS Staining

A 1.5-kb fragment upstream of the *AtYTH05* gene was amplified from genomic DNA using specific primers AtYTH05p-1F (5′-GC GTC GAC CAA AGA CAG ACA AAA TTC AGC AC-3′, a *Sal*I site underlined) and AtYTH05p-1R (5′-AT GAA TTC AGC TCC ATA CTG GTA AAC ACC-3′, a *Eco*RI site underlined). The PCR product was cloned into pMD19-T vector (Takara, Dalian, China) and sequenced for confirmation. The entire 1.5-kb promoter fragment was cloned into the pCAMBIA1301 to create AtYTH05p-GUS fusion construct and transformed into *A. tumefaciens* strain GV3101. The floral dip method was used for *Arabidopsis* transformation (Clough and Bent 1998). Independent AtYTH05p-GUS transgenic lines were carried to T3 homozygous and stained for GUS activity as described previously (Jefferson et al. 1987).

Expression Analyses of the *AtYTHs* and *OsYTHs* Genes

Microarray expression data from various datasets were obtained using GENEVESTIGATOR (https://www.genevestigator.com/gv/) with the *Arabidopsis* and rice Gene Chip platform. The web site provides a web-based search interface to search for probes of genes on the *Arabidopsis* and rice GeneChip by using keywords as well as probe ID numbers and GO terms as query terms. The expression data for each gene in different development stages, organs, and under different abiotic and biotic stress conditions were mined. Results are given as heatmaps in different color coding that reflects absolute signal

values. Only the expression data from *Arabidopsis* ecotype Columbia-0 were mined, while the expression data from different rice cultivars were extracted for analyses. The *Arabidopsis* microarray expression analyses were obtained from samples infected by *Botrytis cinerea* (*Bc*), *Blumeria graminis* f. sp. *hordei* (*Bgh*), *cabbage leaf curl virus* (*CaLCuV*), *Golovinomyces cichoracearum* (*Gc*), *Golovinomyces orontii* (*Go*), *Hyaloperonospora arabidopsidis* (*Ha*), *Meloidogyne incognita* (*Mi*), *Phytophthora infestans* (*Pi*), *Phytophthora parasitica* (*Pp*), *Pseudomonas syringae* pv. *maculicola* (*Psm*), *P. syringae* pv. *phaseolicola* (*Psp*), *P. syringae* pv. *tomato* (*Pst*), or *turnip mosaic virus* (*TuMV*). The rice microarray expression analyses were performed on samples infected with *Magnaporthe grisea* (*Mg*), *Magnaporthe oryzae* (*Mo*), *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), or *X. oryzae* pv. *oryzicola* (*Xoc*). Elicitors and their concentrations used for microarray expression analysis in *Arabidopsis* are as follows: elongation factor Tu (EF-Tu, 1 μM), Flg22 (10 μM for seedlings, 100 nM for leaves), necrosis-inducing Phytophthora Protein 1 (NPP, 1 μM), HarpinZ (HrpZ, 10 μM), LPS (1 μM), oligogalacturonides (OGs, degree of polymerization 9–16; 50 μg/ml), or Pep2 (1 μM). Abiotic stress treatments in *Arabidopsis* microarray expression analyses include cold (4 °C), drought (withholding water), heat (38 °C for seedlings, 37 °C for leaf and whole plants), hypoxia (0.1 % oxygen), osmotic (300 mM mannitol), oxidative (10 μM methyl viologen), salt (150 mM NaCl for leaf and 140 mM for root), or submergence (placing plants into water-filled tanks in darkness). Rice abiotic stress treatments include anaerobic (seeds submerged at 30 °C in darkness and continuously bubbled with nitrogen), cold (4 °C), drought (slowly draining to relative water content of 65–75 % in leaves), heat (42 °C), salt (200 mM NaCl), or submergence (submerged in deionized water). Hormones and their concentrations used in *Arabidopsis* microarray expression analyses are as follows: abscisic acid (ABA, 10 μM for seedlings, 100 μM for leaf and whole plants, or 50 μM for guard cells) , 1-aminocyclopropane-1-carboxylic acid (ACC, 10 μM), brassinolide (BL, 10 μM), ethylene (5 ppm), gibberellic acid (GA3, 1 μM), methyl jasmonate (MeJA, 10 μM), 1-naphthalene acetic acid (NAA, 1 μg/ml), salicylic acid (SA, 2 mM for seedlings and 0.3 mM for leaf), 1-*N*-naphthylphthalamic acid (NPA, 10 μM), or Zeatin (20 μM). Hormones and their concentrations used in rice microarray expression analyses include 6-benzylaminopurine (BAP, 50 μM), GA3 (100 μM), indole-3-acetic acid (IAA, 50 μM), kinetin (KT, 100 μM), NAA (100 μM), or *trans*-zeatin (5 μM).

For qRT-PCR analysis of gene expression, total RNA was extracted from different plant tissues with TRIzol (Invitrogen, Carlsbad, CA, USA) and then treated with RNase-free DNase (TaKaRa, Dalian, China). First-strand cDNA was synthesized from 0.6 μg total RNA using AMV reverse transcriptase (TaKaRa, Dalian, China) as instructed. Each qPCR reaction contained 12.5 μl 2× SYBR Premix Ex Taq (TaKaRa, Dalian, China), 0.1 μg cDNA, and 7.5 pmol of each gene-specific primer (Table 1) in a final volume of 25 μl and had three independent biological replicates. *AtActin2* expression was used as an internal control. The qPCR was performed in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ or $2^{-\Delta CT}$ method as described (Livak and Schmittgen 2001).

Results

Identification of the *Arabidopsis* and Rice YTH Gene Family

Considering that most of the plant YTH genes or proteins have not been studied for their biological functions, we therefore performed genome-wide identification, biochemical

Table 1 List of qRT-PCR primers used in this study

Genes	Primers (5′–3′)	Size (bp)
AtYTH01	AtYTH01-F: ACTTTGCGTGGAGATCGACCAA AtYTH01-R: ATTGGGAGGAAGTCTACCAGCACC	108
AtYTH02	AtYTH02-F: GCAGCATTATGGCGACCTCTA AtYTH02-R: CTATCAAAATGTCTAGAGTTCG CCT	144
AtYTH03	AtYTH03-F: ACCTCAGGGGCAAGTTCCAAT AtYTH03-R: GCAAAGGATGAGATGTTCTGTTCG	155
AtYTH04	AtYTH04-F: TTGTTGTTGACCAGGGCATGT AtYTH04-R: GAGAATTTTCATTCTGCCCACC	156
AtYTH05	AtYTH05-F: GCTGCCTTTCCTTACTCACCAG AtYTH05-R: GCAGGAACAGACGAAGCGAAA	137
AtYTH06	AtYTH06-F: TGATGCCAGGGTTTCAGTCTTATG AtYTH06-R: AACCCTGAGCAGCATACATCG	108
AtYTH07	AtYTH07-F: GCTGGCAATCTGCTAAATGGTG AtYTH07-R: GTTGCGGTCATAACCAAACCTC	114
AtYTH08	AtYTH08-F: CTTACTGGACCTTATGGTTTAGCCG AtYTH08-R: ATTCGCCTGTGGGGTTTTCAT	115
AtYTH09	AtYTH09-F: AGGACCCCAGGTATGCCTATGA AtYTH09-R:CGTGTAGTGAGAATTTGAGCGG TAG	176
AtYTH10	AtYTH10-F: CTCAGACCCTCCGTATGAAAGTG AtYTH10-R: GACCACCATTCGCTACCAAAGA	113
AtYTH11	AtYTH11-F: AGTTGAGAAAGGGATTTGGGCTAC AtYTH11-R: GAATCCGCTCATGTTCACGCT	112
AtYTH12	AtYTH12-F: GTCCAACAGCAGGTTTCTCACG AtYTH12-R: ATTGGTAGGGGTTTGAAGAACG	120
AtYTH13	AtYTH13-F: GGCAAATCCTGACCATGTTTCA AtYTH13-R: AGTCATTACCGTTTCCGCCAT	122
AtActin	AtActin-1F: GTCGTACAACCGGTATTGTGCTG AtActin-1R:CTCTCTCTGTAAGGATCTTCATGA GGT	153

characterization, and comparative expression analysis of the YTH genes in *Arabidopsis* and rice. Using the YTH domain sequence as a query, database searches against the *Arabidopsis* and rice genomic sequences identified totals of 13 and 12 genes encoding putative YTH domain-containing proteins in *Arabidopsis* and rice, respectively (Table 2). No more putative YTH genes in *Arabidopsis* and rice were identified in further searches against other databases using the obtained YTH genes as queries, suggesting that there are 13 and 12 genes encoding for the YTH domain-containing proteins in *Arabidopsis* and rice genomes, respectively. For convenience, we assigned names to these YTH genes as *AtYTH01–13* (*Arabidopsis thaliana YTHs*) and *OsYTH01–12* (*Oryza sativa YTHs*) according to the chromosomal positions. Information of the gene loci, molecular weights, p*I*s, and chromosomal locations for *AtYTH01–13* and *OsYTH01–12* are listed in Table 2. Full-length cDNAs were identified in the GenBank database for most of the *Arabidopsis* and rice YTH genes except for *AtYTH03*, *AtYTH11*, and *OsYTH09*, indicating that the *YTH* genes are normally expressed in *Arabidopsis* and rice plants. The YTH family members show an uneven distribution on the rice and *Arabidopsis* chromosomes. The *AtYTHs* genes are distributed on chromosomes I, III, IV, and V, and three pairs *AtYTH02*/*AtYTH03*, *AtYTH08*/*AtYTH09*, and *AtYTH12*/*AtYTH13* are located closely (interval distance ~1,000 kB) on chromosomes I, III, and V, respectively (Table 2). By contrast, the *OsYTHs* genes separately distribute on chromosomes I, III, IV, V, VI, VII, and VIII (Table 2). According to their exon and intron organizations, the *Arabidopsis* and rice YTH genes can be divided into different groups with similar exon and intron arrangements within each group (Fig. 1). Interestingly, none of the members in each group shows identical gene structure with respect to the size and arrangement of the exons and introns (Fig. 1). The

Table 2 Genes encoding YTH domain-containing proteins in *Arabidopsis* and rice

Genes	Loci	cDNA	Size (aa)	Genomic positions	MW (Da)	p*I*	Note
Arabidopsis thaliana							
AtYTH01	At1g09810	AK226991	470	chr1: 3181138-3183354	53,525.44	8.79	ECT11[a]
AtYTH02	At1g27960	AK226628	539	chr1: 9742359-9745648	61,277.05	5.90	ECT9[a]
AtYTH03	At1g30460	NM_102782	631	chr1: 10771469-10775323	70,014.73	5.85	CPSF30[b]
AtYTH04	At1g48110	AK229281	639	chr1: 17770339-17772806	70,862.51	6.50	ECT7[a]
AtYTH05	At1g55500	AY050425	599	chr1: 20719747-20722416	65,968.51	7.53	ECT4[a]
AtYTH06	At1g79270	AF326910	528	chr1: 29816157-29818811	59,549.33	6.26	ECT8[a]
AtYTH07	At3g03950	AY097342	428	chr3: 1021502-1023767	48,231.63	6.70	ECT1[a]
AtYTH08	At3g13060	AK228498	634	chr3: 4180625-4183632	69,481.39	5.36	ECT5[a]
AtYTH09	At3g13460	AY123992	667	chr3: 4385274-4388220	72,475.38	5.68	ECT2[a]
AtYTH10	At3g17330	AK229019	595	chr3: 5917253-5919458	65,649.73	8.02	ECT6[a]
AtYTH11	At4g11970	NM_117267	444	chr4: 7181223-7182953	50,491.07	5.41	
AtYTH12	At5g58190	AK227630	528	chr5: 23546434-23549363	58,470.30	5.54	ECT10[a]
AtYTH13	At5g61020	AK226952	495	chr5: 24557485-24559780	55,225.68	8.22	ECT3[a]
Rice (*Oryza sativa*)							
OsYTH01	Os01g22630	AK073536	708	chr1: 12725141-12731526	78,204.96	8.68	
OsYTH02	Os01g48790	AK120262 AK121361	609	chr1: 27983688-27990383	67,071.45	5.65	
OsYTH03	Os03g06240	AK111578 AK111489	708	chr3: 3130483-3125933	76,574.54	8.10	
OsYTH04	Os03g20180	AK099818	709	chr3: 11402649-11408106	77,046.19	5.50	
OsYTH05	Os03g53670	AK101299	662	chr3: 30777720-30781832	71,749.88	8.35	
OsYTH06	Os04g04000	AK109511	675	chr4: 1843006-1848258	76,438.79	6.08	
OsYTH07	Os04g51940	AK065298	574	chr4: 30821130-30825520	63,792.73	5.58	
OsYTH08	Os05g01520	AK069692	638	chr5: 311132-304131	73,169.31	6.90	
OsYTH09	Os06g46400	NM_001064894	665	chr6: 28151360-28156784	72,597.53	6.32	
OsYTH10	Os07g07490	AK072392	602	chr7: 3731170-3726574	66,242.11	7.61	
OsYTH11	Os08g12760	AK101331	577	chr8: 7563188-7559098	63,866.56	5.41	
OsYTH12	Os08g44200	AK068463	624	chr8: 27830316-27825032	68,266.32	5.13	

[a] Ok et al. 2005

[b] Thomas et al. 2012

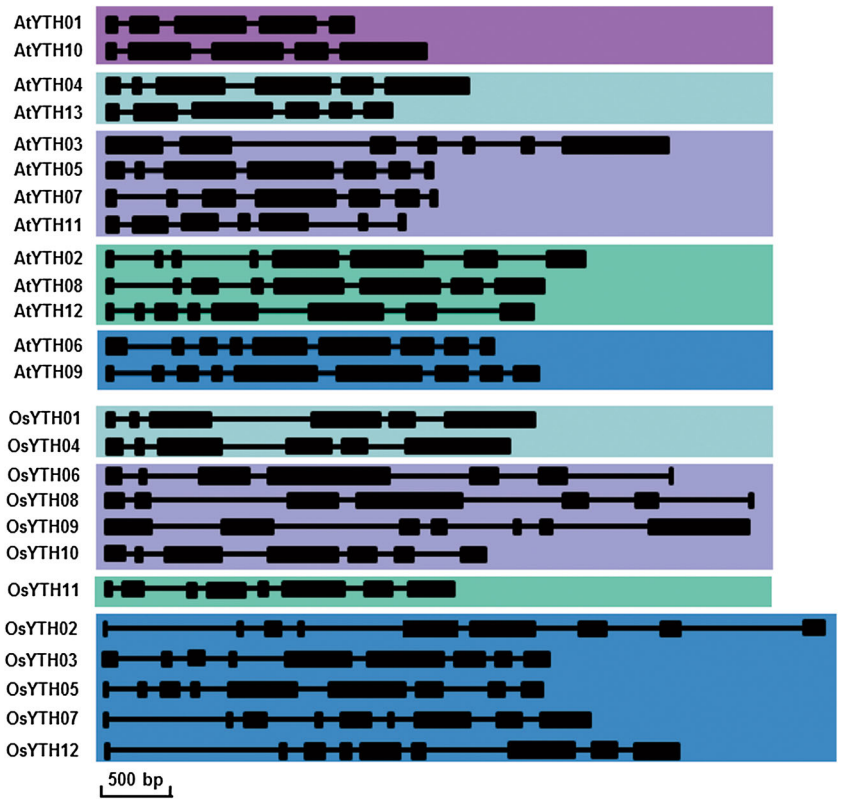

Fig. 1 Exon/intron organization of the *AtYTHs* and *OsYTHs* genes. *Filled box*, exon; *solid line*, intron

features in gene structure and exon/intron organization might indicate that the members of the *Arabidopsis* and rice YTH family might evolve independently.

Characterization of the *Arabidopsis* and Rice YTH Proteins and Phylogenetic Analysis

The *Arabidopsis* and rice YTH proteins comprise 360~717 amino acids (Table 2). Each of the predicted AtYTHs and OsYTHs protein contains an YTH domain as confirmed by blastp searches at GenBank and the YTH domain is usually located near the C-terminal except the AtYTH03, AtYTH11, OsYTH04, and OsYTH09, whose YTH domains are located at the middle regions in the proteins (Fig. 2). The YTH domains in the AtYTHs and OsYTHs proteins are highly conserved with 14 invariant and 19 highly conserved residues and are predicted to have a mixed αβ-fold secondary structure with four α-helices and six β-strands (Fig. 3). A notable feature of the YTH domain is the presence of highly conserved aromatic residues located in the β-sheet (Fig. 3). Overall, the sequences outside the conserved YTH domain in the AtYTHs and OsYTHs proteins vary dramatically without significant similarity. The SMART and PFAM databases were searched for additional known domains probably existing in the AtYTHs and OsYTHs proteins, and these searches identified a conserved CCCH-type zinc finger domain in AtYTH03 and OsYTH09 proteins (Fig. 2). No other conserved domain and motifs were found in all the rest of the AtYTHs and OsYTHs proteins (Fig. 2).

To investigate the molecular evolution and phylogenetic relationships among the YTH proteins in *Arabidopsis* and rice, a phylogenetic tree was constructed by alignment of the YTH domain sequences because the sequences outside the YTH domain in the OsYTHs and AtYTHs proteins showed very low levels of similarity (Fig. 4). This phylogenetic tree analysis identified roughly four main groups and most of the AtYTHs and OsYTHs fall into one of these four groups except that OsYTH01 could cluster into any group (Fig. 4). Members of groups I, II, and III only contain one conserved YTH domain without any other conserved domain or motif. AtYTH03 and OsYTH09 in group IV have two conserved domains: one YTH domain and one CCCH zinc finger domain. By contrast, AtYTH11 in this group, most similar to AtYTH03 in blast searching, contains only one YTH domain and does not have other conserved domain or motif. These data indicate that the *Arabidopsis* and rice YTH proteins might be structurally divergent for their different biological and biochemical functions.

Subcellular Localization of AtYTH05 and AtYTH07 Proteins

The AtYTH05 and AtYTH07 were clustered in the same group III with the rice OsYTH03 (Fig. 4), whose gene was found to be differentially expressed gene in rice response to

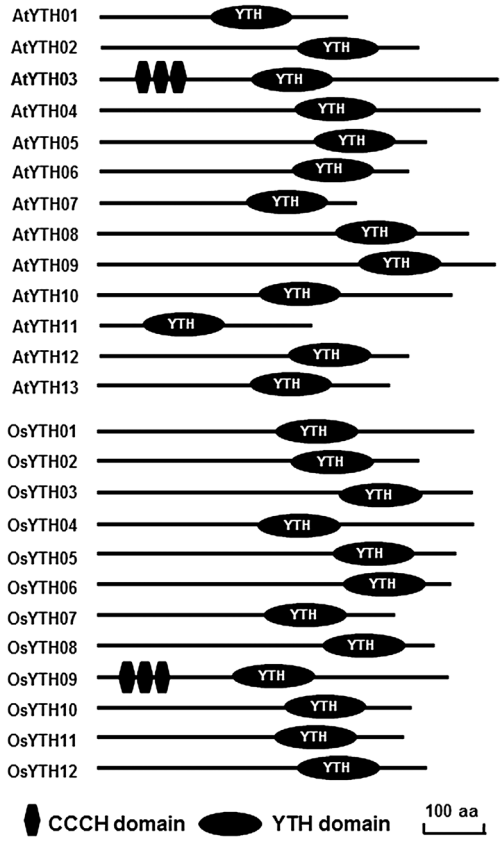

Fig. 2 Conserved domains in the AtYTHs and OsYTHs proteins

M. grisea in another study. Therefore, we chose these two AtYTHs proteins for further biochemical analysis. To examine the subcellular localization of the YTH proteins, we fused the coding sequences of AtYTH05 and AtYTH05 to GFP at the N-terminals under the control of CaMV 35S promoter and transiently expressed in *N. benthamiana* leaves. Transient expression assays reveal that GFP signals in the GFP-AtYTH05- and GFP-AtYTH07-expressed *N. benthamiana* leaves were observed in the cytoplasm, membrane, and nucleus of the cells, similar to the pattern as seen for GFP alone (Fig. 5a). These results indicate that the AtYTH05 and AtYTH07 proteins do not target specific compartments and are likely to distribute ubiquitously in cells.

Recombinant AtYTH05 Protein Showed RNA-Binding Activity

Recent studies showed that splicing factor YT521-B, a typical YTH domain-containing protein, can bind to a short, degenerated, single-stranded RNA sequence (Zhang et al. 2010b). To determine whether the plant YTH proteins have RNA-binding activity, we expressed the *AtYTH05* in *E. coli*, purified the recombinant AtYTH05 protein, and examined its RNA-binding activity in vitro. In SDS-PAGE gel, the recombinant AtYTH05 protein from induced bacteria carrying pRSET(A)-AtYTH05 showed a single band with the calculated molecular weight similar to the predicted amino acid sequence (Fig. 5b). In gel mobility shift assays, the negative controls, in the presence of BSA only or absence of the AtYTH05 protein in the binding reactions containing the ssRNA probe, did not show any signal (Fig. 5c, lanes 1, 2, and 3). The recombinant AtYTH05 protein bound to the ssRNA probe, and this binding activity was in competition with an unlabeled wild-type probe (Fig. 5c, lanes 4 and 5). These results demonstrate that the AtYTH05 protein can bind to single-strand RNA molecules.

Development-Specific Expression Patterns of the YTH Genes

To gain insights into the possible functions of the YTH genes in *Arabidopsis* and rice growth and development, we took advantage of the publicly available microarray data and analyzed the expression patterns using the GENEVESTIGATOR tool. Expression patterns of the *AtYTHs* and *OsYTHs* genes over different developmental stages of *Arabidopsis* and rice plants are shown in Fig. 5. The data showed that expression of the *AtYTHs* and *OsYTHs* genes was highly developmentally regulated in *Arabidopsis* and rice plants. Most of the *AtYTHs* genes except *AtYTH08* and *AtYTH10* have high expression potentials during senescence stage. *AtYTH09* and *AtYTH13* showed relatively high levels of expression potentials in vegetative and senescence stages and relatively low potentials in the flowering stage (Fig. 6a). Relatively high levels of expression potentials were detected for *AtYTH02* and *AtYTH04* in mature siliques and for *AtYTH06* in rosettes (Fig. 6a). Most of the *AtYTHs* genes show expression potentials during different developmental stages, but the expression potential of *AtYTH12* was primarily limited to the late stages of silique development and senescence process (Fig. 6a). *AtYTH01*, *AtYTH03*, *AtYTH08*, and *AtYTH10* showed low expression potentials in developmental stages including germinated seed, seedlings, and rosettes (Fig. 6a). In rice, *OsYTH01*, *OsYTH06*, and *OsYTH08* showed high expression potentials in the flowering stage, while *OsYTH02*, *OsYTH03*, *OsYTH04*, *OsYTH05*, *OsYTH07*, *OsYTH10*, and *OsYTH12* had low potentials of expression in the flowering stage (Fig. 6b). *OsYTH02*, *OsYTH03*, *OsYTH05*, *OsYTH07*, and *OsYTH10* exhibited relatively high expression potentials in the early stages of rice development (Fig. 6b). Notably, expression of *OsYTH01* was largely restricted to the late stages such as flowering, milking, and dough stages, whereas only *OsYTH10* showed an upregulated expression during seed germination (Fig. 6b).

To confirm the expression patterns established by analysis of the public microarray data, we examined by qRT-PCR analysis the expression patterns of the *AtYTHs* genes using total RNA extracted from the roots, leaves, stems, flowers, and siliques of soil-grown 6-week-old plants (Fig. 7a). Distinct expression patterns of the *AtYTHs* genes were

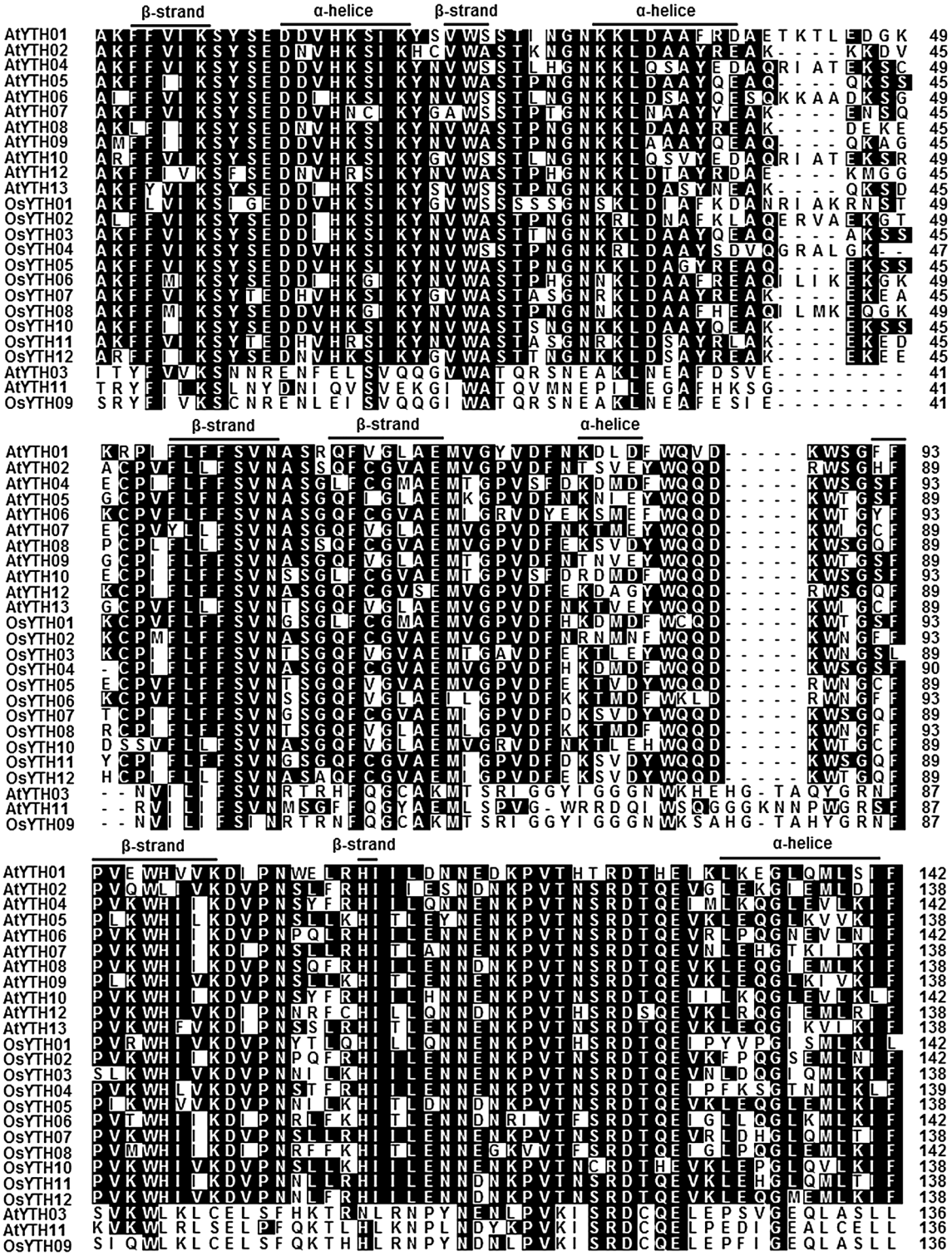

Fig. 3 Sequence alignment of the YTH domains in AtYTHs and OsYTHs proteins. Identical amino acids are marked in *black*. Putative α-helices and β-strands are indicated on the top of the sequences

observed. In the roots, most of the *AtYTHs* genes showed high levels of expression except *AtYTH11* and *AtYTH12*. Higher levels of expression were detected in the stems for *AtYTH01~04*, *AtYTH08~11*, and *AtYTH13*, especially for *AtYTH04* and *AtYTH13*, whereas in the same samples, the expression levels of the rest of the *AtYTHs* genes were low (Fig. 7a). *AtYTH04~06*, *AtYTH08*, *AtYTH09*, and *AtYTH13* showed high levels of expression in the leaves. In contrast, high levels of expression

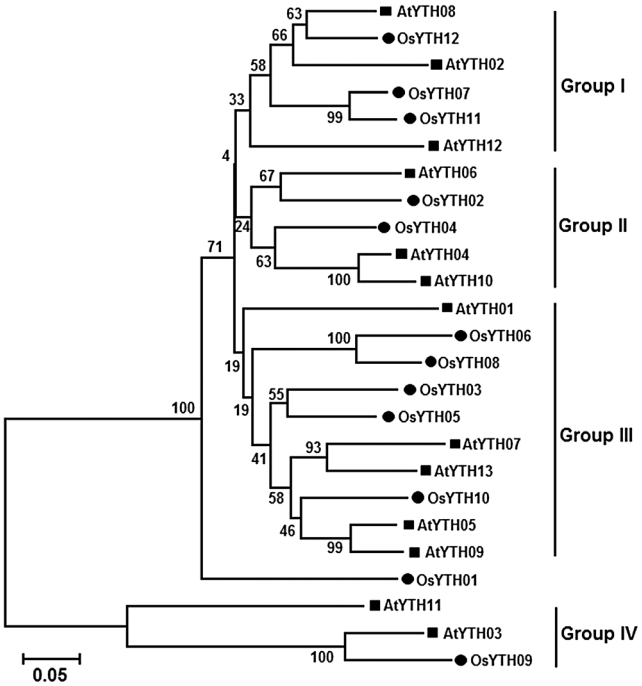

Fig. 4 Phylogenetic tree of the AtYTHs and OsYTHs proteins. An unrooted neighbor-joining tree was generated from multiple sequence alignment of the YTH domains in AtYTHs and OsYTHs proteins. Sequence alignment was done using ClustalX 2.0.8 and the tree was generated using MEGA6. The AtYTHs and OsYTHs proteins are grouped based on the bootstrap supporting value ≧50 %. *Scale bar* represents 0.05 amino acid substitutions per site

were detected for *AtYTH05*, *AtYTH06*, and *AtYTH07* in the flowers and siliques (Fig. 7a). *AtYTH06* showed high levels of expression in the leaves, flowers, and siliques but low levels in the roots. However, expression of *AtYTH13* was detected in all organs examined with relatively high levels of expression in the roots, stems, leaves, and siliques and modest levels in the flowers. On the contrary, *AtYTH12* showed relatively low expression level in all the organs examined. Overall, the expression patterns established by qRT-PCR analysis of the *AtYTH01–13* genes in different developmental stages were similar or comparable to the patterns obtained from the microarray data.

The expression pattern of *AtYTH05* was further confirmed by analysis of its promoter activity in transgenic plants. A 1.5-kb promoter sequence upstream of the *AtYTH05* coding region was fused to the GUS reporter gene, and stable transgenic $AtYTH05_{pro}$::GUS lines were generated. In 2-week-old $AtYTH05_{pro}$::GUS seedlings, no significant GUS staining could be detected in the entire cotyledons including petioles, distal part of the first leaves, vascular tissues of the hypocotyls, and maturation and elongation zones of the roots (data not shown). GUS staining could not be detected until the $AtYTH05_{pro}$::GUS plants started into the flowering stage. When the $AtYTH05_{pro}$::GUS plants began flowering, GUS staining was detected in the margin of mature leaves, stem, style of the mature flowers, and developing siliques (Fig. 7b). These data further confirm that the *AtYTH01–13* genes, at least for *AtYTH05*, are regulated developmentally.

Tissue-Specific Expression Patterns of the *YTH* Genes

To gain further information on the possible involvement of the *YTHs* genes in growth and development, we analyzed the tissue-specific expression patterns of *AtYTHs* and *OsYTHs* genes in *Arabidopsis* and rice plants. Generally, no identical expression pattern for each of the *AtYTHs* or *OsYTHs* gene in different tissues examined was observed (Fig. 8). However, most of the *AtYTHs* or *OsYTHs* genes showed overlapping expression patterns at least in two or more different tissues (Fig. 8). The *AtYTHs* genes except *AtYTH06* showed relatively high expression potentials in seeds such as the embryo, endosperm, and testa and in the shoot, especially for *AtYTH03*, *AtYTH07*, *AtYTH08*, *AtYTH10*, and *AtYTH12* in seeds and for *AtYTH13* in axillary shoot (Fig. 8a). The *AtYTHs* genes except *AtYTH01*, *AtYTH02*, and *AtYTH11* showed high expression potentials in the root tip (Fig. 8a). *AtYTH01–04*, *AtYTH08*, *AtYTH09*, and *AtYTH11-13* but not *AtYTH05-07* and *AtYTH10* exhibited moderate expression potentials in raceme such as flowers and pedicel (Fig. 8a). However, the *AtYTHs* genes except *AtYTH09* and *AtYTH13* showed low expression potentials in inflorescence, rosettes (stem, leaf, and bud), and pericarp (Fig. 8a). Notably, *AtYTH12* in pedicel, *AtYTH06* in embryo and pericarp, and *AtYTH01* and *AtYTH03* in stem exhibited very low expression potentials (Fig. 8a). In rice, more diverse patterns of expression potentials for *OsYTHs* genes in different tissues of rice plants were observed as compared with those for *AtYTHs* in *Arabidopsis* plants. Most of the *OsYTHs* genes showed differential expression potentials in almost all the examined tissues in rice plants (Fig. 8b). Surprisingly, *OsYTH02* and *OsYTH06* exhibited high expression potentials in the stele of internodes (Fig. 8b). *OsYTH09* in coleptile, *OsYTH05* in node, and *OsYTH04* in collar and crown tissues exhibited very low expression potentials (Fig. 8b). Taken together, these data demonstrate that the *AtYTHs* and *OsYTHs* genes have tissue-specific expression potentials in *Arabidopsis* and rice plants.

Expression Patterns of the *YTH* Genes in Response to Pathogen Infection and Elicitor Treatment

To gain insights into the possible involvement of the *Arabidopsis* and rice YTH genes in response to various stresses, we analyzed the expression patterns of *AtYTHs* and *OsYTHs* in *Arabidopsis* and rice plants, respectively, after infections with different types of pathogens. Differential expression patterns of *AtYTHs* were detected in *Arabidopsis* plants after pathogen infections (Fig. 8a). Expression of *AtYTH06* and *AtYTH10* in the leaf and root tissues was

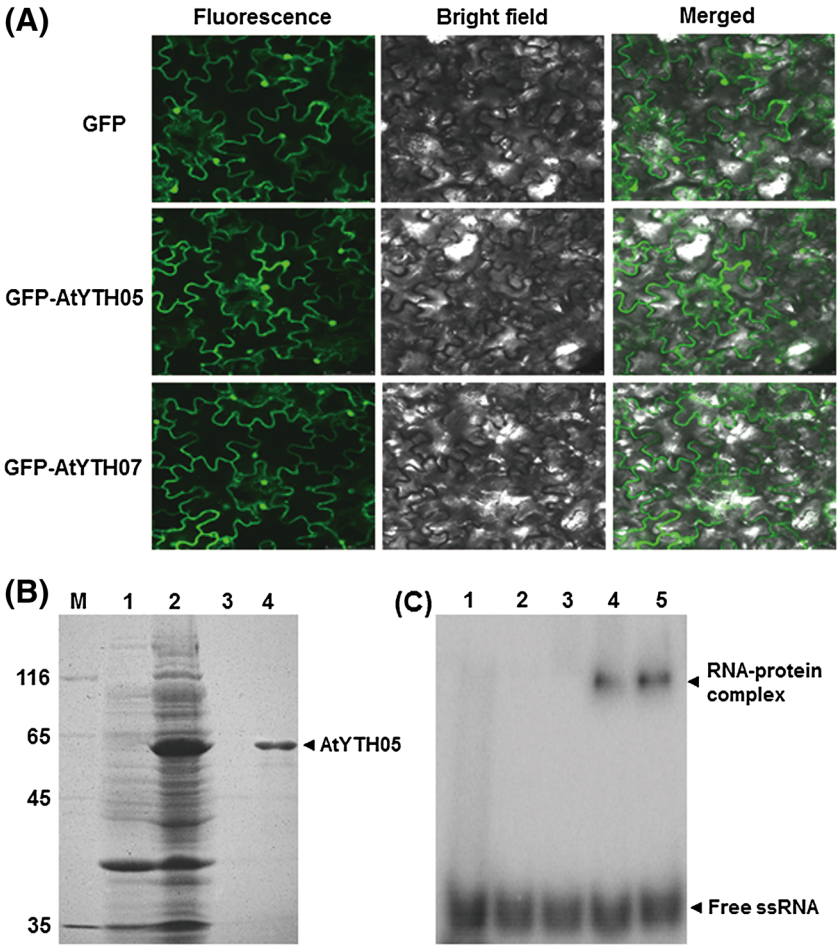

Fig. 5 Subcellular localization of AtYTH05 and AtYTH07 and RNA-binding activity of AtYTH05. **a** Subcellular localization analysis. The AtYTH05::GFP, AtYTH07::GFP, and GFP alone constructs were transiently expressed through agroinfiltration in *Nicotiana benthamiana* leaves and green fluorescence of the GFP was viewed using confocal laser microscopy (*left row*). The same cells were also viewed by transmission microscopy (*middle row*) and both images were merged (*right row*). **b** Purification of recombinant AtYTH05 protein from *E. coli*. *M*, marker for protein molecular weight; *lane 1*, total cell proteins from uninduced bacteria carrying pRSET(A)-AtYTH05; *lane 2*, total cell protein from induced bacteria carrying pRSET(A)-AtYTH05; *lane 3*, purified protein from uninduced bacteria carrying pRSET(A)-AtYTH05; *lane 4*, purified AtYTH05 protein. **c** RNA-binding activity of the recombinant AtYTH05 protein. *Lane 1*, BSA alone as the negative control; *lane 2*, [α-^{32}P]-labeled ssRNA probe; *lane 3*, competition assay with unlabeled ssRNA probe; *lanes 4* and *5*, 2 ng [α-^{32}P]-labeled ssRNA probe and 1 μg (*lane 4*) or 2 μg (*lane 5*) AtYTH05 protein, respectively

reduced in response to infection by *G. cichoracearum* and *G. orontii* (powdery mildew), *H. arabidopsidis* (downy mildew), *P. parasitica* (phytophthora blight), *P. syringae* pv. *maculicola* (leaf spot), or *M. incognita* (root knot) (Fig. 9a). Notably, expression of *AtYTH06* in the roots of plants infected with *P. parasitica* or *M. incognita* and expression of *AtYTH10* in the leaves of plants infected with *G. cichoracearum* or *P. syringae* pv. *maculicola* were significantly reduced (Fig. 9a). Infection by *M. incognita* could upregulate the expression of *AtYTH01*, *AtYTH05*, and *AtYTH11* in the roots of the plants grown on MS medium. Slight increases in expression levels for *AtYTH05* and *AtYTH02* in the leaves infected by a nonadapted pathogen *B. graminis* f. sp. *hordei*, *AtYTH02* and *AtYTH12* in the leaves infected with *P. infestans* (late blight), and *AtYTH06* in the leaves infected by *P. syringae* pv. *maculicola*, *P. syringae* pv. *tomato*, or *B. cinerea* were detected (Fig. 9a). Infection by cabbage leaf curl virus or turnip mosaic virus also resulted in changes of expression levels for some of the *AtYTH* genes. These results imply that the *Arabidopsis* YTH genes show differentially expression patterns upon infection by different types of pathogens including fungi, oomycetes, bacteria, viruses, and nematodes.

We also analyzed the expression patterns of *OsYTHs* genes in the leaves or roots of different rice cultivar plants after infection by *M. oryzae* (*M. grisea*) (rice blast), *X. oryzae* pv. *oryzae* (leaf blight) or *X. oryzae* pv. *oryzicola* (leaf streak). *OsYTH12* showed decreased expression levels in the leaves of cv. Nipponbare and cv. IR24 plants infected by *M. grisea* strain FR13 or *X. oryzae* pv. *oryzae* strain PXO99A but exhibited an increased expression level in the leaves of cv. Nipponbare plants infected by *X. oryzae* pv. *oryzae* strain PXO99A (Fig. 9b). Increased expression levels for

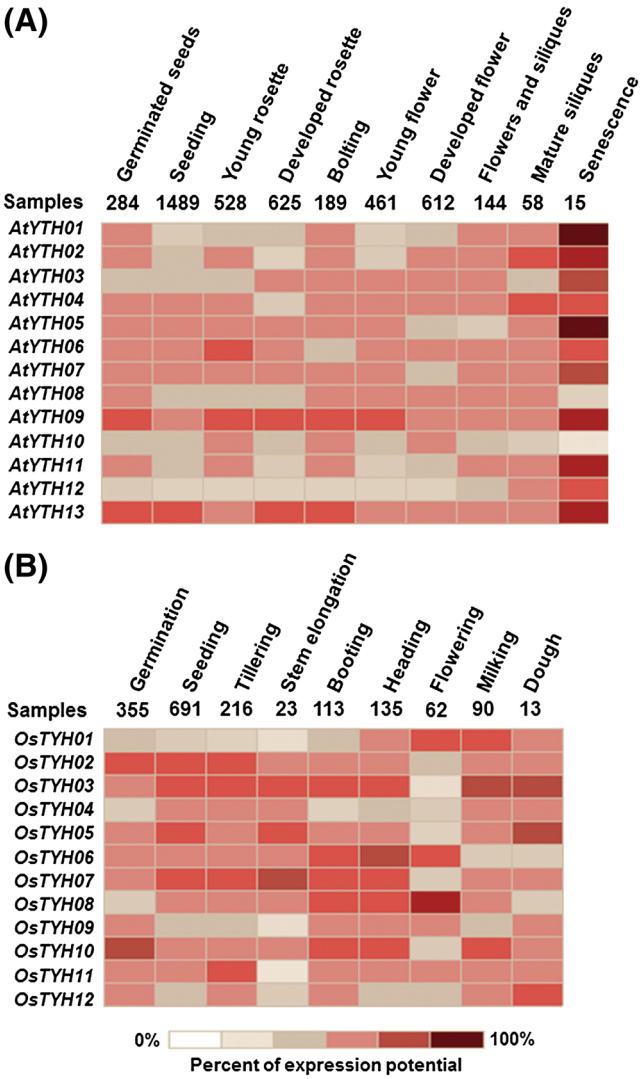

Fig. 6 Expression patterns of *AtYTHs* (**a**) and *OsYTHs* (**b**) in different developmental stages. The color scheme represents $\log_2$ ratio of expression levels with the color *deep red* indicating relatively high expression and the scale representing the relative signal values is shown. The developmental stages and numbers of samples used for analysis are shown on the top

OsYTH01, *OsYTH03*, and *OsYTH10* were also detected in the leaves of cv. Nipponbare and cv. Taipei 309 plants infected with *M. oryzae* or in the leaves of cv. IR24 plants infected by *X. oryzae* pv. *oryzae* strain PXO99A or *X. oryzae* pv. *oryzicola* strain BLS303 (Fig. 9b). These data indicate that the members of the rice YTH genes respond differentially to different fungal and bacterial pathogens.

We further examined whether the *AtYTHs* genes could be induced by some known effectors or elicitors from pathogenic fungi and bacteria. Expression of *AtYTH06* was reduced in the seedlings after treatments with effectors including EF-Tu (Zipfel et al. 2006), Flg22 (Felix et al. 1999), or Pep2 (Albert 2013) (Fig. 9c). Treatments with EF-Tu or Pep2 also affected the expression of *AtYTH01*, *AtYTH02*, and *AtYTH10* in seedlings (Fig. 9c). However, treatments with other elicitors including NPP1 (Fellbrich et al. 2002), HrpZ (Alfano et al. 1996), LPS (Newman et al. 2007), or OGs (Denoux et al. 2008) did not significantly affect the expression of the *AtYTH* genes (Fig. 9c). These data indicate that some of the *Arabidopsis* YTH genes can also respond to pathogen-derived effectors or elicitors.

Expression Patterns of the *YTH* Genes in Response to Abiotic Stresses

We next analyzed the expression patterns of the *AtYTHs* and *OsYTHs* genes in *Arabidopsis* and rice plants after treatments with different abiotic stresses. In *Arabidopsis*, expression of *AtYTH06* in the leaves, rosettes, and seedlings was significantly upregulated during cold, drought, osmotic, salt, and heat stress conditions but was markedly downregulated in bolting tissues and rosettes under hypoxia and submergence stress (Fig. 10a). Expression of *AtYTH07* and *AtYTH10* in seedlings and flowers was also dramatically induced at early stages after heat treatment (Fig. 10a). Increased expression levels for *AtYTH05* under cold stress, *AtYTH02* under hypoxia stress, and *AtYTH01* and *AtYTH02* under submergence stress were also detected (Fig. 10a). In contrast to its upregulated expression during heat stress, expression levels of *AtYTH10* were generally decreased under cold, drought, salt, and osmotic stress, especially in rosettes during the late stage of osmotic stress (Fig. 10a). *AtYTH08* also showed decreased expression at 30 h after treatment with heat stress (Fig. 10a). Expression of other *AtYTHs* genes was affected by most of the stresses examined leading to upregulated or downregulated levels; however, oxidative stress did not affect the expression of almost all *AtYTHs* genes (Fig. 10a).

In rice, different expression patterns of *OsYTHs* genes in response to abiotic stress were detected. Expression levels of *OsYTH10* were decreased significantly in the shoots of cv. C418 and cv. K354 plants during cold stress and in panicle leaves of cv. DK151 plants under drought stress, but increased significantly in seedlings by a temperature shift from cold (4 °C) to normal condition (29 °C) (Fig. 10b). Expression of *OsYTH05*, *OsYTH06*, *OsYTH07*, and *OsYTH09* was also downregulated in the shoot of rice plants under cold stress (Fig. 10b). Rice plants under anaerobic stress showed decreased expression levels of *OsYTH01*, *OsYTH02*, *OsYTH05*, *OsYTH09*, and *OsYTH12* but exhibited an increased expression of *OsYTH11* (Fig. 10b). Decreased expressions of *OsYTH08* in seedlings by a temperature shift of 4 to 29 °C, *OsYTH11* and *OsYTH12* in seedlings under heat stress, and *OsYTH11* in the roots of tillering plants under drought stress were also observed (Fig. 10b). Meanwhile, expression of *OsYTH03* in rice plants under submergence stress and expression of *OsYTH08* in seedlings under heat stress was upregulated markedly (Fig. 10b). Drought stress treatment

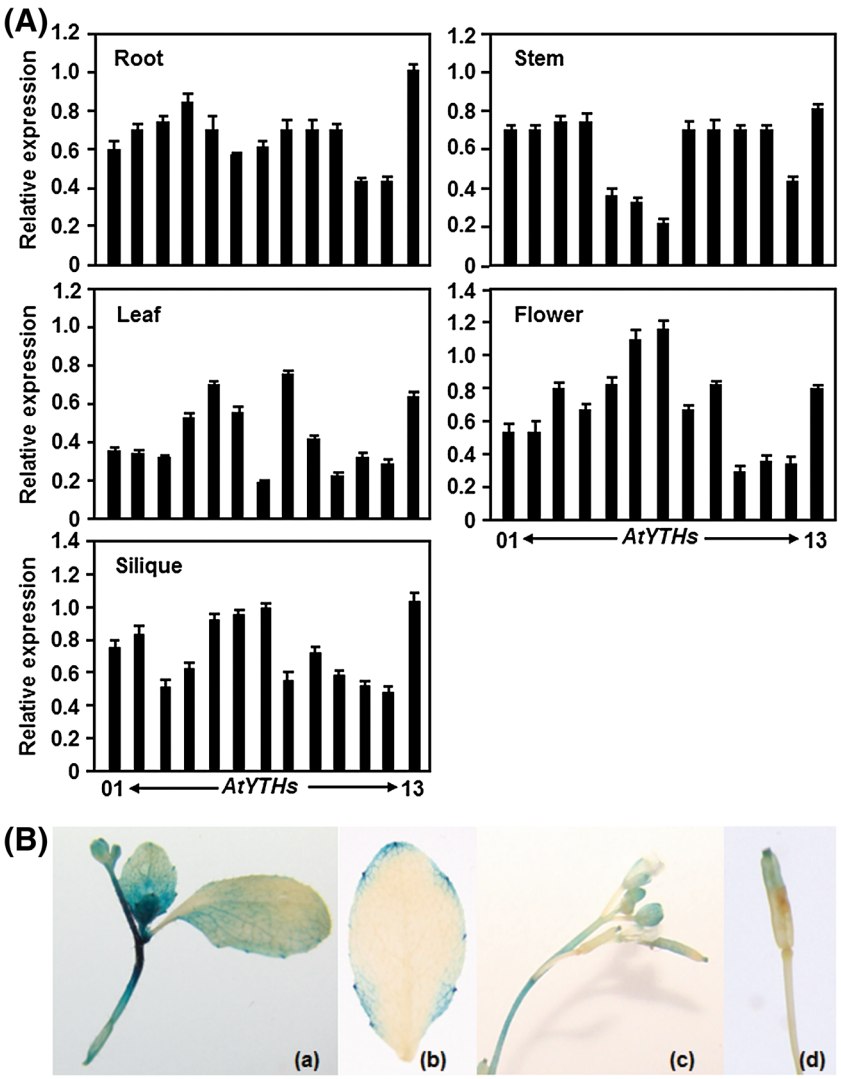

Fig. 7 Expression patterns of the *AtYTHs* genes in different tissues of *Arabidopsis* plants. **a** qRT-PCR analyses of *AtYTHs* expressions in different tissues. **b** GUS staining-based analysis of *AtYTH05* expression in $AtYTH05_{pro}$::GUS transgenic plants. *a* Emerging leaves and shoot apex in seedlings; *b* leaf from 4-week-old transgenic plant; *c* flower buds, a mature flower, and stem; *d* a developing silique

increased the expression of *OsYTH01*, *OsYTH02*, *OsYTH03*, *OsYTH05*, and *OsYTH12* in the root and leaf tissues, while a temperature shift of 4 to 29 °C also increased the expression of *OsYTH12* in seedlings (Fig. 10b). Notably, none of the *OsYTHs* genes showed an altered expression pattern in rice plants under salt stress, whereas *OsYTH01*, *OsYTH02*, *OsYTH03*, *OsYTH04*, and *OsYTH08* also did not exhibit altered expression patterns in response to cold stress (Fig. 10b).

Expression Patterns of the YTH Genes in Response to Hormones

Considering that the *AtYTHs* and *OsYTHs* genes were differentially regulated in responses to various abiotic and biotic stresses and that different hormones are involved in the regulation of growth/development and the responses to abiotic and biotic stresses, we therefore analyzed the expression patterns of the *AtYTHs* and *OsYTHs* genes after treatments with different hormones. In *Arabidopsis*, expression of *AtYTH06* was significantly upregulated in seedlings, suspension cells, leaves, and guard cells after treatments with abscisic acid (ABA) or brassinolide (BL); however, *AtYTH01*, *AtYTH02*, *AtYTH10*, and *AtYTH13* showed decreased expression in ABA-treated leaves (Fig. 11a). Expression of *AtYTH05* and *AtYTH13* in the root and seedlings after treatment with 1-naphthalene acetic acid (NAA) or zeatin and expression of *AtYTH08* in suspension cells after treatment with BL were upregulated (Fig. 11a). Interestingly, treatments with 1-aminocyclopropane-1-carboxylic acid (ACC, precursor of ET), ethylene (ET), methyl jasmonic acid (MeJA), or salicylic acid (SA), well-known defense hormones involved in plant defense response, and treatments with indole-3-acetic acid (IAA) and gibberellic acid (GA3) did not affect the expression patterns of all *AtYTHs* genes in *Arabidopsis* plants (Fig. 11a). In rice, decreased expression of *OsYTH08* in seedlings or leaves after treatments with GA3, kinetin (KT), or zeatin was detected (Fig. 11b). However, expression of *OsYTH01*

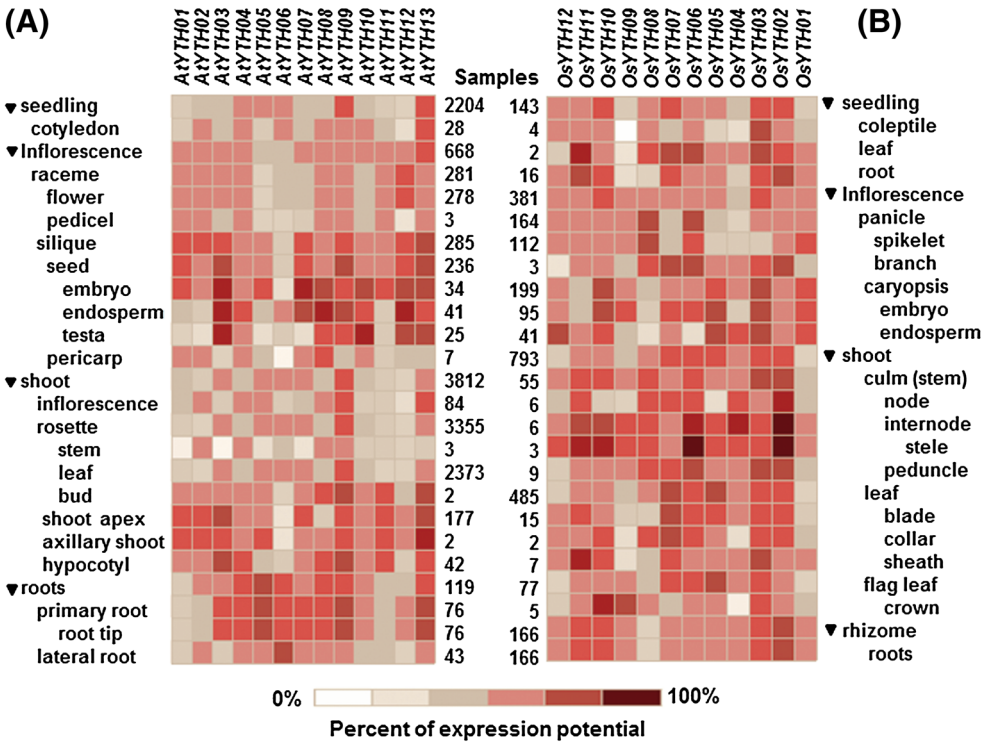

Fig. 8 Expression patterns of *AtYTHs* (**a**) and *OsYTHs* (**b**) in different tissues. The color scheme represents $\log_2$ ratio of expression levels with the color *deep red* indicating relatively high expression and the scale representing the relative signal values is shown. Different tissues used for analysis are shown and numbers of samples used are listed in the *middle*

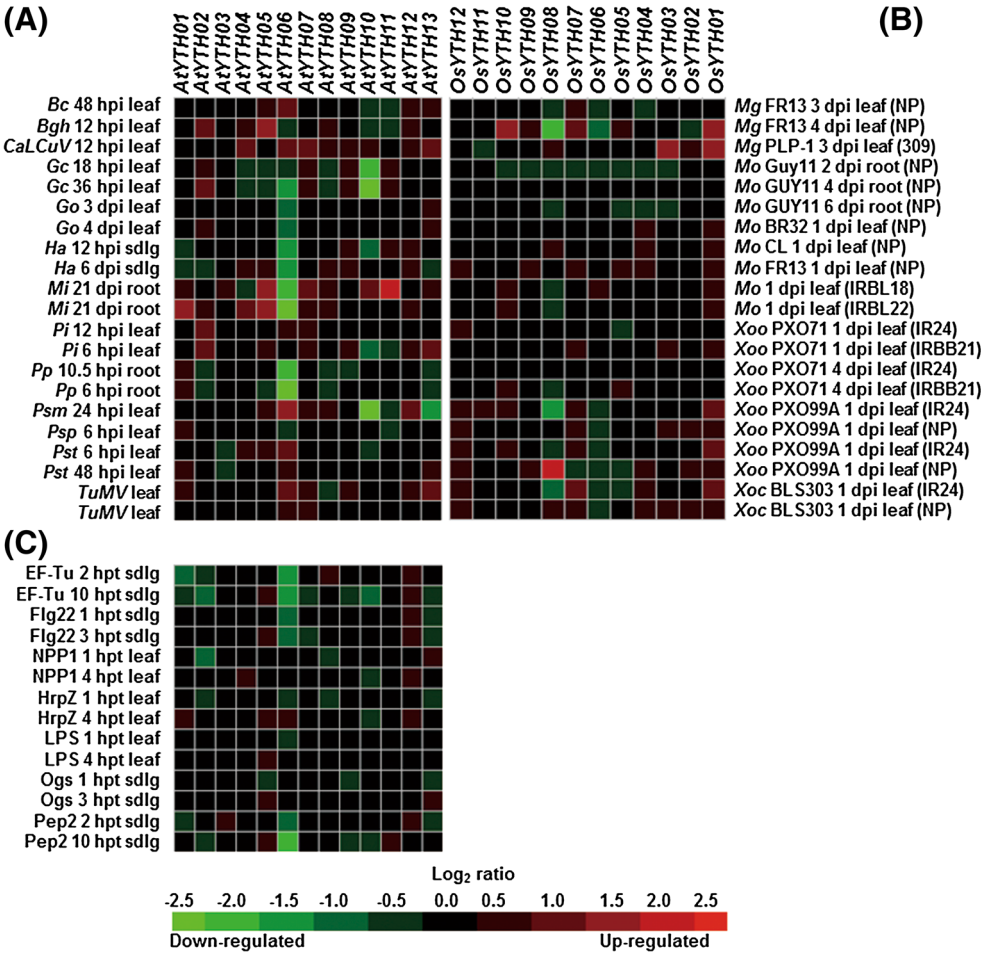

Fig. 9 Expression patterns of the *AtYTHs* (**a**) and *OsYTHs* (**b**) genes in response to pathogen infection and elicitor treatment. The *red* or *green shading* represents an upregulated or downregulated expression level, respectively. The color scale with heatmap is given in $\log_2$ ratio values. **a** Expression patterns of the *AtYTHs* genes in response to pathogen infection. **b** Expression patterns of the *OsYTHs* genes in response to pathogen infection. *NP* Nipponbare, *309* Taipei 309. **c** Expression patterns of the *AtYTHs* genes in response to elicitor treatment. Time points of sampling are indicated as days post-inoculation (dpi) or hours post-inoculation (hpi), and tissues sampled for analyses are also indicated as root, leaf, or seedlings (sdlg)

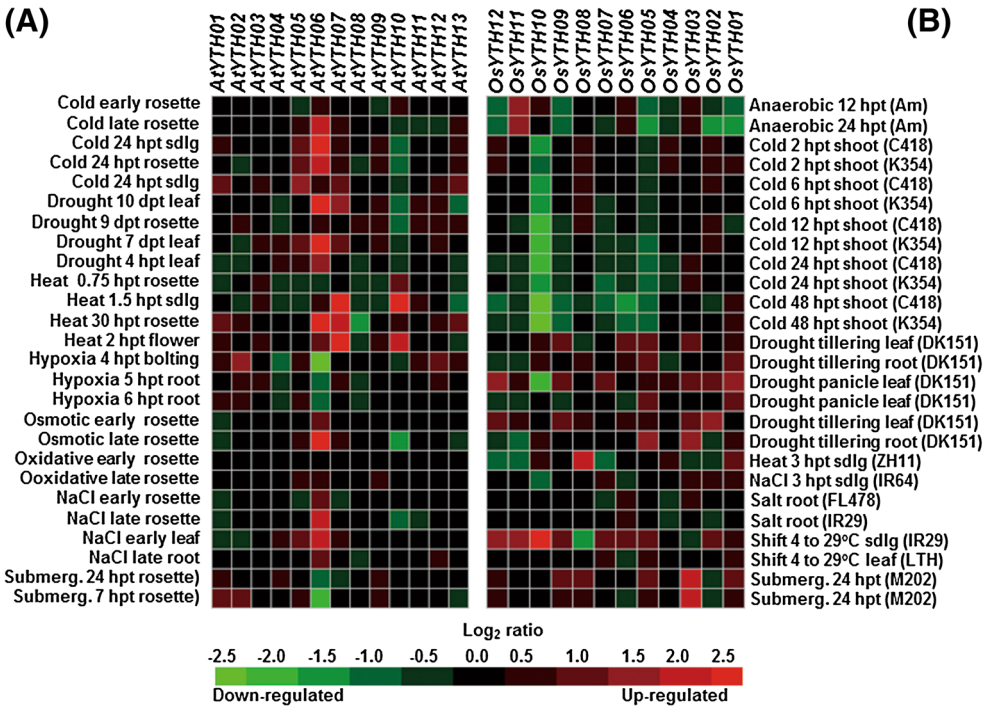

Fig. 10 Expression patterns of the *AtYTHs* (**a**) and *OsYTHs* (**b**) genes in response to abiotic stress treatment. The *red* or *green shading* represents an upregulated or downregulated expression level, respectively. The color scale with heatmap is given in $\log_2$ ratio values. **a** Expression patterns of the *AtYTHs* genes in response to abiotic stress. **b** Expression patterns of the *OsYTHs* genes in response to abiotic stress. *Am* cv. Amaroo, *ZH11* cv. Zhonghua 11, *LTH* Lijiangxintuanheigu, *Submerg.* submergence stress. Time points of sampling are indicated as days post-treatment (dpt) or hours post-treatment (hpt) and tissues sampled for analyses are also indicated as root, leaf, or seedlings (sdlg)

and *OsYTH09* was upregulated in the leaves or seedlings after treatments with KT, NAA, or trans-zeatin (Fig. 11b). These data indicate that the *AtYTHs* and *OsYTHs* genes respond differentially to hormones in different tissues or developmental stages of *Arabidopsis* and rice plants.

Discussion

The YTH domain in diverse proteins was recognized as a structurally conserved domain involved in the binding of RNA molecules (Glisovic et al. 2008; Janga and Mittal 2011). Information on the YTH proteins and their biological functions in plants is limited. In the present study, we performed a comprehensive genome-wide identification, biochemical characterization, and comparative expression analyses of the YTH gene family in *Arabidopsis* and rice. Our results demonstrated that the YTH proteins in plants have RNA-binding activity in vitro, and expression of the YTH genes in *Arabidopsis* and rice is regulated in response to developmental cues as well as in response to abiotic and biotic stresses. Therefore, our study not only characterizes systematically the YTH gene family in *Arabidopsis* and rice at genomic and biochemical levels but also provides new information on the possible biological functions of the YTH genes in plant development and stress response.

Our genome-wide searches using the conserved YTH domain sequence as a query identified a total of 13 and 12 genes encoding YTH domain-containing proteins in *Arabidopsis* and rice genomes, respectively. Ok et al. (2005) identified 11 ECTs for their interacting with Calcineurin B-Like-Interacting Protein Kinase1 (CIPK1) through yeast two-hybrid screening, and all these ECTs contain the conserved YTH domains in their C-terminals. The genes encoding for these 11 ECTs are the same with *AtYTH01*, *AtYTH02*, *AtYTH04-10*, *AtYTH12*, and *AtYTH13* (Table 2). In this study, we identified two more *Arabidopsis* genes, *AtYTH03* and *AtYTH11*, which encode YTH domain-containing proteins (Table 2), and thus, we considered that they should be members of the YTH gene family in *Arabidopsis*. *AtYTH03* was also identified as a gene encoding AtCPSF30 (cleavage and polyadenylation specificity factor) that is involved in alternative polyadenylation of mRNA (Delaney et al. 2006; Thomas et al. 2012). Structures such as the feature of exon/intron organization of the *AtYTHs* and *OsYTHs* genes are quite different between each other (Fig. 1), implying that the YTH gene family in *Arabidopsis* and rice may evolve independently. Although most of the *AtYTHs* genes separately distribute on different chromosomal locations, one pair of the *AtYTHs* genes, *AtYTH08*/*AtYTH09*, locates within 25-kb distance on chromosome III (Table 2), indicating that some members of the *Arabidopsis* YTH gene family may result from tandem duplication. Thus, it is likely that both tandem duplication and segmental duplication events are involved in the evolution of the *AtYTHs* gene family in *Arabidopsis*. By contrast, the rice *OsYTHs* genes do not form clusters and are separately distributed on different chromosomes, indicating that tandem duplication is not involved in the evolution of *OsYTHs* gene family in rice. Indeed, the rice *OsYTHs* genes show scattered distribution on the chromosomes and any two of the *OsYTHs* genes have quite large

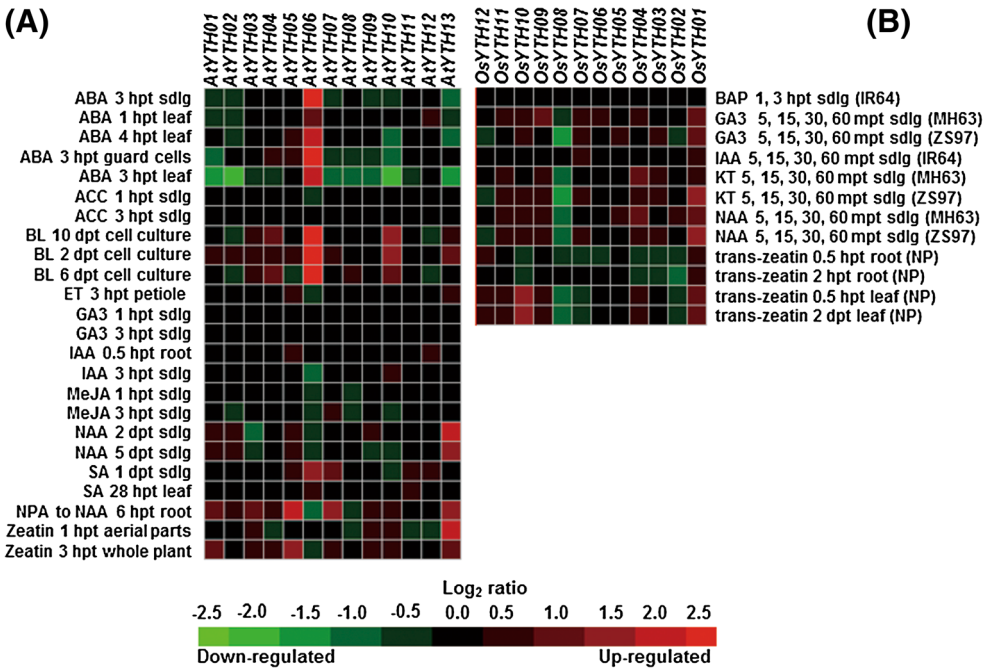

Fig. 11 Expression patterns of the *Arabidopsis* (**a**) and rice (**b**) YTH genes in response to hormones. The *red* or *green shading* represents an upregulated or downregulated expression level, respectively. The color scale with heatmap is given in $\log_2$ ratio values. **a** Expression patterns of the *AtYTHs* genes in response to different hormones. **b** Expression patterns of the *OsYTHs* genes in response to different hormones. *MH63* cv. Minghui 63, *ZS97* cv. Zhenshan 97. Time points of sampling are indicated as days post treatment (dpt), hours post-treatment (hpt), or minutes post-treatment (mpt), and tissues sampled for analyses are also indicated as root, leaf, or seedlings (sdlg)

physical distances on the chromosomes. It is thus speculated that segmental duplication events may be the main source for the evolution of the *OsYTHs* gene family in rice. This is similar to the observations on the evolution of the maize *ZmMPKs* gene family (Liu et al. 2013).

The biochemical characteristics have been studied in some of the YTH proteins. In our study, we found that the recombinant AtYTH05 could bind to single-stranded RNA molecules, demonstrating that the AtYTH05 protein has RNA-binding activity in vitro (Fig. 5c). This is similar to YT521-B that was previously shown to bind to single-strand RNA sequences (Zhang et al. 2010b). The YTH proteins generally only have conserved YTH domains but do not contain any other well-known RNA-binding domains or motifs such as RRM, pseudouridine synthase and archaeosine transglycosylase (PUA), and oligonucleotide/oligosaccharide binding fold (OB-fold) (Stoilov et al. 2002). It was recently found that the YTH domain is required for RNA-binding activity as deletion of the YTH domain abolished the RNA-binding activity in YT521-B (Zhang et al. 2010b). Structurally, the β-strands in the YTH domain contain highly conserved aromatic residues, which are often present in RRM, PUA, and OB-fold (Stoilov et al. 2002) and are crucial for RNA binding (Hoffman et al. 1991). These structural features and experimental evidence demonstrate that the YTH domain is a novel RNA-binding domain (Zhang et al. 2010b). However, AtYTH03 (AtCPSF30), showing less sequence similarity to other AtYTHs proteins, contains an additional CCCH zinc finger domain except a YTH domain and was shown that the CCCH domain is necessary for its RNA-binding activity (Addepalli and Hunt 2007). On the other hand, it was shown that the yeast Mmi1 and the human YT521-B are localized in dynamic nuclear foci (Rafalska et al. 2004; Harigaya et al. 2006), especially that the YT521-B is localized in a specific nuclear compartment, the YT body (Rafalska et al. 2004). By contrast, results from our experiments revealed that, when transiently expressed in the leaves of *N. benthamiana*, AtYTH05 and AtYTH07 were not localized in a specific compartment of the cells (Fig. 5a). This is in agreement with the observation that the human YTH protein family members reside both in the nucleus and cytosol (Zhang et al. 2010b). It is thus likely that the YTH domain is not a cellular targeting signal and the YTH proteins may function in different aspects of cellular RNA metabolism (Zhang et al. 2010b).

Generally speaking, a gene expressed abundantly or increasingly in a tissue, a developmental stage, or a stress condition may indicate its function related to developmental and stress response. Different approaches including analyses of publicly available microarray data, gene expression by qRT-PCR, and promoter activity in transgenic plants were taken to explore possible involvement of the *AtYTHs* and *OsYTHs* genes in development and stress response. The facts that the expression patterns of the *AtYTHs* and *OsYTHs* genes are quite divergent and no two *AtYTHs* or *OsYTHs* genes show similar or same expression patterns in different tissues and developmental stages (Figs. 6 and 8) suggest that each member of the *Arabidopsis* and rice YTH gene family may have specific functions in growth and development. This is partially supported by the observations that expression of some of the

AtYTHs and *OsYTHs* genes could be affected by exogenous treatments with growth/development-related hormones such as NAA, BL, KT, and zeatin (Fig. 11). The existence of homozygous lines for each of the *AtYTHs* gene (the SALK T-DNA insertion population at http://signal.salk.edu/cgi-bin/tdnaexpress?; Zhang et al. 2008 for *AtYTH03*) indicates that mutations in the *AtYTHs* genes do not result in a lethal effect. Surprisingly, most of the *AtYTHs* genes, especially for *AtYTH01* and *AtYTH05*, showed higher expression potentials during senescence stage (Fig. 6a), indicating a relationship of the *AtYTHs* genes to senescence, probably having specific and most important functions. Notably, *AtYTH05*, *AtYTH06*, and *AtYTH07* were expressed at relatively higher levels in flowers and siliques than those in other tissues (Fig. 7a), and the high level of *AtYTH05* expression in flowers and siliques was further confirmed by the *AtYTH05* promoter activity in $AtYTH05_{Pro}$::GUS transgenic plants (Fig. 7b), indicating that these three *AtYTHs* genes might be associated with their functions in the reproduction process. This is supported by the tissue-specific expression patterns of the *AtYTHs* genes, which show relatively high levels of expression potentials in seeds such as embryo, endosperm, and testa than those in inflorescence and rosettes (Fig. 8a). Interestingly, the *AtYTHs* genes expressed abundantly in the root, stem, or leaves showed relatively low expression in the flowers and siliques, and vice versa (Fig. 7a). Such opposite expression patterns indicate that the *AtYTHs* genes have specific functions during different developmental stages. For the rice *OsYTHs* genes, it is interesting that two genes, *OsYTH02* and *OsYTH06*, have the highest levels of expression potentials in internode and stele tissues, suggesting that both of the *OsYTH02* and *OsYTH06* have functions in the regulation of stem development in rice.

Several lines of evidence supporting the involvement of the YTH family in response to abiotic and biotic stresses came from our comparative analyses of the expression patterns of the *AtYTHs* and *OsYTHs* genes under stress conditions. Expression of *AtYTH06* and *AtYTH10* was reduced significantly after infection by different types of pathogens including fungi, oomycetes, bacteria, and nematode and by treatment with well-known pathogen-associated molecular patterns (PAMPs) such as EF-Tu and Pep2 (Zipfel 2008) (Figs. 9a and 11a), indicating that *AtYTH06* and *AtYTH10* should have a function in response to pathogen infection. In rice, expression of the *OsYTH08* gene showed different patterns in response to infection by *M. oryzae* or *X. oryzae* pv. *oryzae* (Fig. 9b), indicating that *OsYTH08* is involved in the interactions between rice and its pathogens. Surprisingly, however, exogenous treatments with SA, JA, ET, or ACC, which are well-known defense signaling hormones (Pieterse et al. 2012), did not significantly affect the expression of the *AtYTHs* genes in *Arabidopsis* plants (Fig. 11a). Thus, the involvement and function (if any) of the YTH family in response to biotic stress need to be further examined experimentally. On the other hand, the involvement of the YTH family in response to various abiotic stress is supported by the altered expression of *AtYTH06*, *AtYTH07*, *AtYTH10*, *OsYTH04*, and *OsYTH10* (Fig. 10) under abiotic stress conditions and by the interactions of some AtYTHs proteins (also known as ECTs) with CIPK1 that is involved in the regulation of abiotic stress response (Ok et al. 2005). ABA is well documented to play important roles in signaling pathways involved in the regulation of abiotic and biotic stress responses (Chinnusamy et al. 2008; Cao et al. 2011; Kim 2012; Lee and Luan 2012; Nakashima and Yamaguchi-Shinozaki 2013; Roychoudhury et al. 2013). Similar expression patterns of *AtYTH06* in *Arabidopsis* after treatments with cold, drought, heat, and osmotic stress and by treatment with ABA (Figs. 10a and 11a) indicate that *AtYTH06* has diverse functions in responses to multiple abiotic stresses. Surprisingly, none of the *AtYTHs* genes including *AtYTH03* responded with altered expression levels at early and late stages after treatment with oxidative stress (Fig. 10a). However, it was found that mutation in *AtYTH03* (also known as *AtCPSF30*) led to enhanced tolerance against oxidative stress (Zhang et al. 2008; Addepalli et al. 2010). Interestingly, the *AtYTHs* and *OsYTHs* genes exhibited different expression patterns in response to same abiotic stress, e.g., induced expression of *AtYTH06* vs. reduced expression of *OsYTH10* in cold stress and reduced expression of *AtYTH06* vs. induced expression of *OsYTH03* in submergence stress (Fig. 10). Therefore, it is likely that members of the YTH family function with different modes of action in response to various abiotic stresses in *Arabidopsis* and rice plants.

In summary, this study presents a comprehensive characterization of the YTH gene/protein family in *Arabidopsis* and rice and comparative analyses of expression of the *AtYTHs* and *OsYTHs* genes in different tissues or developmental stages as well as in response to abiotic and biotic stresses. Further studies are absolutely required to elucidate the biological function for each member of the YTH family in *Arabidopsis* and rice using functional genomics approaches by phenotypic analyses of transgenic lines with altered expression (knockout/knockdown and overexpression).

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Conflict of Interest The authors declare that they have no conflict of interest.

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