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A eukaryotic translation initiation factor 5A from *Tamarix androssowii* (Tamarisk), TaeIF5A1, can form a homodimer and interact with other proteins

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Abstract

Eukaryotic translation initiation factor 5A (eIF5A) proteins, a highly conserved protein family found in all eukaryotic organisms, are involved in translation elongation, mRNA turnover and decay, cell proliferation, programmed cell death, and abiotic stress responses. However, the precise cellular functions of eIF5A proteins are still not fully known, and especially little is known about their interaction partners. In the present study, we report that an eIF5A protein from *Tamarix androssowii*, TaeIF5A1, can form homodimers with itself, but cannot form heterodimers with other eIF5A proteins. In addition, TaeIF5A1 can specifically interact with xyloglucan endotransglucosylase/hydrolase (XTH) and ADP-ribosylation factor GTPase activator (Arf GAP), suggesting that TaeIF5A1 may share similar functions with these proteins. Moreover, expression of the *TaeIF5A1* confers tolerance to oxidative (H_2O_2), salt ($MgCl_2$), heavy metal ($CuSO_4$, $ZnCl_2$, and $CdCl_2$), and alkali ($NaHCO_3$ and Na_2CO_3) stresses to transgenic yeast cells, indicating that it plays a role in abiotic stress tolerance. This study provides information useful in revealing the function of *TaeIF5A1* in depth.

Keywords: eIF5A; Stress response; *Tamarix androssowii*; Yeast two-hybrid assay.
Abbreviations: Arf GAP_ADP-ribosylation factor GTPase activator; CTAB_cetyltrimethylammonium bromide; DHH_deoxyhypusine hydroxylase; DHS_deoxyhypusine synthase; eIF5A_eukaryotic translation initiation factor 5A; XTH_xyloglucan endotransglucosylase/hydrolase; Y2H_yeast two-hybrid.

Introduction

Eukaryotic translation initiation factor 5A (eIF5A) is a small, single polypeptide protein (~18 kDa) that is found in all eukaryotic cells and archaebacteria. It is the only protein known to contain hypusine, which is formed through a two-step post-translational modification involving spermidine and a conserved Lys residue within eIF5A (Cooper et al., 1983; Park et al., 1997; Hu et al., 2005). The first reaction in hypusine formation is catalyzed by the enzyme deoxyhypusine synthase (DHS; EC 1.1.1.249) and involves the transfer of a 4-aminobutyl moiety from spermidine to the ε-amino group of the conserved Lys residue to form deoxyhypusine (Wolff et al., 1997). Subsequently, hydroxylation of deoxyhypusine into hypusine is catalyzed by deoxyhypusine hydroxylase (DHH; EC1.14.99.29) (Abbruzzese et al., 1986; Park et al., 2006). The eIF5A protein was originally identified as a translation initiation factor from rabbit reticulocytes in an in vitro assay (Kemper et al., 1976). However, the proposed function for eIF5A has since been questioned; subsequent studies showed that inactivation of both *eIF5A* genes in yeast only marginally reduces protein synthesis and does not significantly affect polyribosome profiles (Kang and Hershey, 1994; Zuk and Jacobson, 1998). In addition, eIF5A is involved in cellular proliferation and apoptosis (Chatterjee et al., 2006), promotes cell viability and cell growth (Park et al., 2010) and promotes the synthesis of proteins involved in progression of the cell cycle (Clement et al., 2006). Moreover, eIF5A proteins are found to facilitate protein synthesis by participating in the nuclear export of specific mRNAs (Liu et al., 2008). Plant eIF5A, like its mammalian counterpart, is post-translationally modified to hypusine-eIF5A via the aforementioned reaction sequence. So far, a number of eIF5As have been identified and characterized in different plant species, including Arabidopsis (Wang et al., 2003; Feng et al., 2007), tobacco (Chamot and Kuhlemeier, 1992), rice (Chou et al., 2004), and tomato (Wang et al., 2005). Recent studies reported that these eIF5A homologs are involved in multiple biological processes, such as protein synthesis regulation, translation elongation, mRNA turnover and decay, cell proliferation, leaf and root growth, seed yield, leaf, flower and fruit senescence, and programmed cell death (Wang et al., 2003; Ma et al., 2010; Xu et al., 2011). Moreover, increasing evidences have demonstrated that members of the eIF5A family also participate in plant responses to environment stimuli. Overexpression of the *RceIF5A* gene in transgenic *Arabidopsis* plants improved resistance to heat, oxidative and osmotic stresses, while the plants with reduced expression of eIF5A (three *AteIF5A* isoforms in *Arabidopsis* are down-regulated) are more susceptible to these stresses (Xu et al., 2011). The expression of rice *eIF5A* genes, *OseIF5A-1* and *OseIF5A-2*, were induced by salt and heavy metal stresses, suggesting that they are involved in stress tolerance (Chou et al., 2004). Studying the interaction partners of a gene is an important part in characterizing its function. However, few studies have been performed on the interaction partners of eIF5A, and little is known about them. In this study, we found

Table 1. Primers used for QRT-PCR.

Genes	GenBank number	Forward and reverse primers (5'–3')	
TaeIF5A1	AY587771	CACATCGTCATCAAGAACCG	CCACCAGTCTCAGTCAGAAG
ACT1	BK006940	TATATGTTTAGAGGTTGCTGC	CAATTCGTTGTAGAAGGTATG

that an eIF5A from *Tamarix androssowii*, TaeIF5A1, can specifically interact with xyloglucan endotransglucosylase/ hydrolase protein (XTH) and ADP-ribosylation factor GTPase activator (Arf GAP), and further showed that TaeIF5A1 exists as a homodimer. In addition, expression of the *TaeIF5A1* gene in transgenic yeast increased the resistance to oxidative, salt, heavy metal, and alkali stresses. Our study provides helpful insights into the functions of eIF5A and defines the role that eIF5A plays in abiotic stress tolerance in plants.

Results

Expression of TaeIF5A1 gene *in yeast improves the tolerance to oxidative, salt, heavy metal and alkali stresses*

To determine whether *TaeIF5A1* plays a role in tolerance to different abiotic stresses, we employed a yeast strain INVSc1 (*Saccharomyces cerevisiae*) as a fast heterologous model system. The yeast strain INVSc1 was transformed with either pYES2 harboring the coding region of *TaeIF5A1* under the control of the galactose inducible promoter (*GAL1*) or with the empty pYES2 vector (as a control). The expression of *TaeIF5A1* in yeast cells was confirmed by RT-PCR (Fig. 1A). The cultures of two kinds of transformed yeast cells were incubated and adjusted to an equal density for the H_2O_2, $CuSO_4$, $CdCl_2$, $MgCl_2$, $ZnCl_2$, $NaHCO_3$, or Na_2CO_3 treatments. After these treatments for 24 h, the stressed cells were spotted on SC-Ura solid medium (supplying with 2% glucose) with serial dilutions, followed by incubation at 30°C for 60–72 h. There was no difference in growth between the yeast cells transformed with *TaeIF5A1* and empty pYES2 vector (Control) (Fig. 1B). However, the yeast cells expressing *TaeIF5A1* exhibited better growth than control cells (transformed with empty pYES2) under H_2O_2, $CuSO_4$, $CdCl_2$, $MgCl_2$, $ZnCl_2$, $NaHCO_3$, and Na_2CO_3 stress conditions. This result indicated that the abiotic stress tolerance of yeast cells expressing *TaeIF5A1* is improved. Therefore, *TaeIF5A1* plays a role in tolerance to oxidative, salt, heavy metal, and alkali stresses.

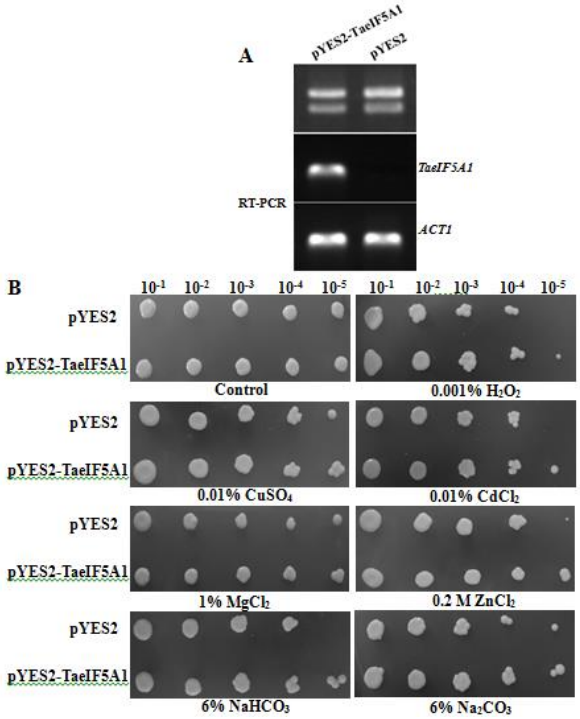

Fig 1. Abiotic stress tolerance of yeast cells expressing the *TaeIF5A1* gene. (A) The expression of *TaeIF5A1* in yeast, grown in liquid SC-Ura-/Gal 2% medium, was confirmed by RT-PCR analysis. *ACT1* was used as an internal control; (B) Comparison of the growth between the transformed yeast cells expressing empty pYES2 (as a control) or pYES2 containing *TaeIF5A1*. The two cultures of transformed cells were incubated at 30°C for 36 h, and adjusted to an equal density for the H_2O_2 (0.001%), $MgCl_2$ (1%), $CuSO_4$ (0.01%), $CdCl_2$ (0.01%), $ZnCl_2$ (0.2 M), $NaHCO_3$ (6%) or Na_2CO_3 (6%) stresses. After these treatments for 24 h, the stressed cells were spotted on SC-Ura solid medium (supplying with 2% glucose) with serial dilution to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} from an initial OD_{600} of 2, and photographed after 60–70 h of incubation at 30°C.

Construction of a cDNA library for Y2H screening

PCR was performed using the Advantage® 2 Polymerase Mix to amplify double-stranded cDNA, and the amplified cDNAs were purified (size-selected) using CHROMA SPIN^{TM} TE-400 columns; peak fractions containing cDNAs larger than 500 bp were pooled (Fig. 2B). The resulting cDNAs were transformed into Y187 yeast cells, incubated at 30°C for 3–4 days on SD/-Leu plates and then pooled together. The titer of this original library was 3.4×10^7 colony-forming units per milliliter. Fifteen colonies from the SD/-Leu plates were randomly selected, and their insert lengths were detected by PCR analysis. As shown in Fig. 2C, the sizes of cDNA inserts are all larger than 500 bp, and the mean size of the inserts is 1.3 Kb, indicating that the quality of the library is suitable for Y2H screening.

Identification of the interaction partners of TaeIF5A1

To further determine the function of TaeIF5A1, we used Y2H screening to identify proteins that interact with TaeIF5A1. The yeast strain Y2H was transformed with either the fusion plasmid pBD-TaeIF5A1 or the control plasmid pGBKT7, and the growth status of these transformants was examined. We found that the yeast cells grew well on SD/-Trp/X-α-Gal without Aureobasidin A (data not shown), while the cells could not grow on the SDO/X/A medium. These results indicated that TaeIF5A1 does not have autoactivation and toxicity and is suitable as bait for screening the cDNA library. After the Y2H screening, 11 positive clones, representing 3 unique genes, have been identified. Among these positive clones, three clones encoded a protein with unknown function (GenBank number: JQ040811), five clones encoded the xyloglucan endotransglucosylase/hydrolase protein (XTH, GenBank number: JQ040812), and another three clones encoded an ADP-ribosylation factor GTPase activator (Arf GAP, GenBank number: JQ040813). Protein interactions between TaeIF5A1 and the putative preys were further confirmed by analyzing growth on selective medium. Growth was observed for positive

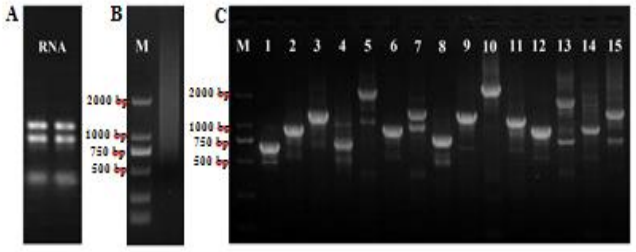

Fig 2. Construction of a cDNA library for yeast two-hybrid screening. (A) Agarose gel (2.0%) showing the total RNA from *T. androssowii* seedlings after salt stress; (B) High-quality cDNA was generated using the SMART cDNA synthesis method. Oligo dT-primed cDNA synthesis was carried out with or without 0.5 μg of *T. androssowii* seedlings poly A+ RNA. PCR was performed using the Advantage® 2 Polymerase Mix and the amplified cDNAs were size-selected using CHROMA SPIN^{TM} TE-400 columns; (C) Detection of the lengths of inserts in the constructed library. Fifteen colonies from the SD/-Leu plates were randomly selected, and the insert length was detected by PCR analysis. M: DL2000 marker; Lane 1–15: the size of cDNA inserts.

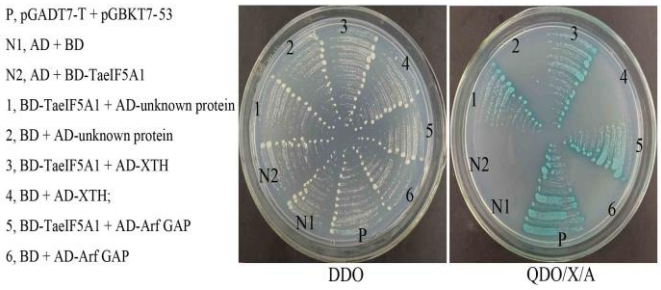

Fig 3. Yeast two-hybrid analysis to identify the interaction partners of TaeIF5A1. The interactions were assayed using the Y2H system to confirm the interactions of TaeIF5A1 with a function unknown protein, xyloglucan endotransglucosylase/ hydrolase (XTH), and ARF GTPase activator (Arf GAP). Growth was monitored on SD/-Leu/-Trp (DDO) and SD/-Ade/-His/-Leu/-Trp/X-α-Gal/Aureobasidin A (QDO/X/A) medium-selective plates. Negative control: N1 (empty pGBKT7 with empty pGADT7); N2 (pGBKT7 harboring TaeIF5A1 with empty pGADT7). Positive control: P (pGBKT7-p53 with pGADT7-T. pGBKT7-53 encodes the Gal4 DNA-BD fused with murine p53, pGADT7-T encodes the Gal4 AD fused with SV40 large T-antigen).

combinations (TaeIF5A1 and the proteins of unknown function, XTH and Arf GAP), but no growth was observed for the control combinations (Fig. 3). These results confirmed the interactions between TaeIF5A1 and these three proteins.

Analysis of the dimerization of TaeIF5A1

To study the interactions between TaeIF5A1 with other eIF proteins (TaeIFs) from *T. androssowii*, five *TaeIF* genes were cloned, including three *eIF5A* genes (named as *TaeIF5A2*, *TaeIF5A3*, and *TaeIF5A4*), one *eIF2A* (*TaeIF2A*), and one *eIF*-like (*TaeIF*-like). Y2H assay was employed to test the ability of TaeIF5A1 to form homo- or heterodimers. When TaeIF5A1 was expressed in yeast cells as both the bait and prey protein, the yeast cells grew on the selective medium and α-galactosidase (MEL1) could be activated, indicating TaeIF5A1 could interact with itself to form a homodimeric complex (Fig. 4). However, when TaeIF5A1 and the other TaeIF proteins were used as bait and prey proteins, respectively, the yeast cells could not grow on the selective medium, indicating that TaeIF5A1 can not interact with these TaeIFs. These results suggested that TaeIF5A1 can only form a homodimeric complex, and cannot form heterodimers with other eIF proteins from *Tamarix androssowii*.

Discussion

Previously, we reported that the *TaeIF5A1* gene is induced in response to multiple abiotic stresses such as high salinity, drought and heavy metal, and transgenic poplar plants overexpressing *TaeIF5A1* exhibited improved salt and drought stress tolerance (Wang et al., 2012). Based on these results, we further investigated whether *TaeIF5A1* confers tolerance to other kinds of abiotic stresses, including oxidant, alkali and heavy metals such as H_2O_2, $CuSO_4$, $CdCl_2$, $MgCl_2$, $ZnCl_2$, $NaHCO_3$, and Na_2CO_3. Our results showed that yeast transformants expressing *TaeIF5A1* displayed increased tolerance to oxidant stress (H_2O_2), salt stress ($MgCl_2$), heavy metal treatments ($CuSO_4$, $CdCl_2$, and $ZnCl_2$), and alkali stresses ($NaHCO_3$ and Na_2CO_3) (Fig. 1). These results suggested that *TaeIF5A1* is involved in eliciting a stress response mechanism that may play a common role in plant tolerance to oxidative, salt, heavy metal, and alkali stresses. Protein–protein interactions are fundamental to understanding biological networks and the functions of proteins (Kortemme and Baker, 2002). Recently, studies showed that eIF5A can interact with ribosomal proteins and phloem proteins involved in protein synthesis (Zanelli et al., 2006; Ma et al., 2010). In this study, our results showed that three proteins can specifically interact with TaeIF5A1, including a protein of unknown function, XTH and Arf GAP (Fig. 3). Among these three proteins, XTHs can catalyze the cleavage and molecular grafting of xyloglucan chains, involved in the reconstruction, rearrangement, breakdown and incorporation of xyloglucan for cell wall rearrangement (Hyodo et al., 2003). Xyloglucan is the predominant hemicellulosic polysaccharide in the primary cell wall of all vascular plants and accounts for up to 20% of the dry weight of the primary wall. TaeIF5A1 specifically interacts with XTH, suggesting that it may play a similar role to XTH. Consistent with this hypothesis, previously studies showed that eIF5A is involved in the WSC/PKC1 signaling pathway (that is involved in cell wall integrity or related processes) and also plays a role in cell wall formation (Valentini et al., 2002), suggesting that TaeIF5A1 may also be involved in cell wall biosynthesis. Different Arf GAP family members localize to various and distinct cellular compartments. However, each Arf GAP prefers a particular Arf and mediates a specific function of this Arf in actin cytoskeleton reorganization and intracellular trafficking (Tanabe et al., 2005). TaeIF5A1 can specifically interact with an Arf GAP, suggesting that TaeIF5A1 may also be involved in intracellular trafficking and actin cytoskeleton reorganization. Structural analyses of some eIF5A proteins from several organisms suggested that these proteins form homodimers. In the eIF5A dimer of *M.jannaschii* (PDB code: 2eif), the two β3 strands interact with each other via six hydrogen bonds to form a continuous six-stranded antiparallel β-sheet, causing the two active site loops to point in opposite directions (Kim et al., 1998). In *Arabidopsis*, the structure of the eIF5A dimer is unique, compared with other plants. Further biochemical and biophysical analyses are needed to prove whether this form of dimer could facilitate RNA binding (Teng et al., 2009). In the human eIF5A dimer (PDB code: 3cpf), the two chains are linked via a disulfide bond. The dimeric structure of eIF5A2 formed between subunits A and B was

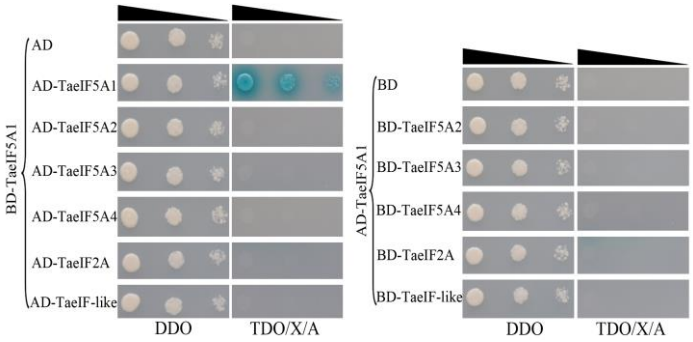

Fig 4. Analysis of the dimerization of TaeIF5A1 using the Y2H assay. The interactions between TaeIF5A1 and other TaeIF proteins used TaeIF5A1 as bait, and the interactions between TaeIF5A1 (as prey) and other TaeIF proteins (as bait). The yeast cells (Y2H) were co-transformed with the indicated plasmids and assayed by spotting serial dilutions (1:1, 1:10, and 1:100) of yeast onto selective dropout media, SD/-Leu/-Trp (DDO) and SD/-His/-Leu/-Trp/X-α-Gal/Aureobasidin A (TDO/X/A), for examination of growth at 30°C for 3–4 days.

significantly different than those observed in other structures (Tong et al., 2009). Gentz et al. have characterized hypusinated yeast eIF5A by size-exclusion chromatography and native PAGE, showing that the protein exists as a homodimer (2009). Consistent with these findings, our data showed that TaeIF5A1 can interact with itself to form a homodimer, but that it fails to form heterodimers with other eIF5A proteins, including TaeIF5A2, TaeIF5A3, and TaeIF5A4, suggesting that TaeIF5A1 may play a functional role distinct from that of TaeIF5A2, TaeIF5A3, and TaeIF5A4.

Materials and Methods

Plant material

Tamarix. androssowii seedlings were grown in pots containing a mixture of turf peat and sand (2:1 v/v) in a growth chamber under controlled conditions (14 h light: 10 h dark; 70–75% relative humidity; 24°C). Uniformly developed 2-month-old seedlings were exposed to 0.4 M NaCl for 0, 6, 12, and 24 h (Wang et al., 2012). After these treatments, whole seedlings were harvested for the construction of a cDNA library for Y2H screening.

RNA isolation and cDNA library construction

Total RNA from *T. androssowii* was extracted by the CTAB method with minor modifications (Chang et al., 1993) and treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), according to the manufacturer's instructions, to ensure no genomic DNA contamination. The first-strand cDNA was reveres transcribed from total RNA from *T. androssowii*, which was then amplified to form double-stranded cDNA by long-distance PCR according to the Make Your Own "Mate & Plate™" Library System User Manual (Clontech, TaKaRa, China). The double-stranded cDNA was purified with a CHROMA SPIN™ TE-400 Column, and peak fractions containing cDNAs larger than 500 base pairs were pooled. The pooled cDNAs were then packaged in vitro, along with *Sma*I-linearized pGADT7-Rec cloning vectors, into Y187 yeast cells using the Yeastmaker™ Yeast Transformation System 2 (Clontech, TaKaRa, China) according to the manufacturer's protocol; the transformed cells were incubated at 30°C for 3–4 days on SD/-Leu plates and pooled together. Finally, the cDNA library was used to transform the yeast bait strains and stored at -80°C.

Construction of the yeast expression vector, yeast transformation and stress tolerance assays

For yeast transformation, the full ORF of *TaeIF5A1* was cloned into the pYES2 vector (Invitrogen) under the control of the inducible *GAL1* promoter, and introduced into the *S. cerevisiae* INVSc1 strain (MATa, his3-1, leu2, trp1-289, ura3-52. His-, Leu-, Trp-, and Ura-) using the EZ-transformation kit (Q-BIOGENE) according to the manufacturer's instructions. The pYES2 empty vector was also transformed as a control. For quantitative RT-PCR, yeast cells were cultivated in SC-Ura liquid medium containing 2% galactose at 30°C. After reaching the exponential growth phase, cells were harvested, and total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed into cDNA with an oligodeoxythymidine as primer using the PrimeScript™ RT reagent Kit (TaKaRa, China) following the manufacturer's instructions. All the primers used are shown in Table 1. For stress tolerance assays, yeast cells harboring pYES2-*TaeIF5A1* or empty pYES2 were cultured in 15 ml of SC-Ura medium containing 2% glucose at 30°C with overnight shaking, adjusted to an OD_{600} of 0.3 in induction medium, and incubated at 30°C for 36 h (RNA gel blot results showed that peak level occurred at this induction time) to induce the expression of the *TaeIF5A1* gene (Wang et al., 2012). After incubation, the two kinds of cultures (harboring *TaeIF5A1* or empty pYES2) were adjusted to an equal density for the following stress experiments: 0.001% H_2O_2 (oxidant stress); 1% $MgCl_2$ (salt stress); 0.01% $CuSO_4$, 0.01% $CdCl_2$, 0.2 M $ZnCl_2$ (heavy metal treatments); 6% $NaHCO_3$ and 6% Na_2CO_3 (alkali stresses). After treatments at 24 h, the stressed cells were spotted on SC-Ura solid medium (supplying with 2% glucose) with serial dilution, and incubated at 30°C for 60–70 h. The growth between yeast cells expressing *TaeIF5A1* and control were compared for stress tolerance assay.

Construction of yeast bait strain

The full-length coding sequence of *TaeIF5A1* was amplified by PCR using the primers 5'-CATGGAGGCCGAATTCATGTCTGACGAGGAGCACCATTTC-3' (forward) and 5'-GCAGGTCGACGGATCCCTTGGGACCAATGTCCTTGAGGGC-3' (reverse); adapter sequences for in vitro recombination are underlined. The PCR fragment was fused to the GAL4 DNA binding domain of the pGBKT7 bait vector (Clontech, Palo Alto, CA, USA) using the In-Fusion™ Advantage® PCR Cloning Kit. The resulting construct and the empty pGBKT7 plasmid (as a control) were respectively transformed into yeast strain Y2H and grown on SD/-Trp/X-α-Gal/Aureobasidin A (SDO/X/A) medium to test for autoactivation and toxicity because it is imperative to confirm that the bait does not autonomously activate the *AbAr* reporter gene in Y2H in the absence of a prey protein.

Yeast two-hybrid assays

Yeast strain Y2H harboring *TaeIF5A1* was used as bait to screen the cDNA library from salt-induced *T. androssowii* using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, TaKaRa, China). Putative positive clones were selected on SD/-Leu/-Trp/X-α-Gal/Aureobasidin A medium (DDO/X/A). To further validate the protein-protein interactions in yeast, the plasmid DNA was retransformed into the yeast bait strain and selected on the high-stringency SD/-Ade/-His/-Leu/-Trp/

X-α-Gal/Aureobasidin A medium (QDO/X/A). Finally, the inserts were sequenced using the specific primers 5'-CTATTCGATGATGAAGATACCCCACCAAACCC-3' and 5'-GTGAACTTGCGGGGTTTTTCAGTATCTACGATT-3', and the results were analyzed via BLAST. At the same time, cells expressing empty pGBKT7 along with the obtained prey constructs, as well as empty pGBKT7 expressed together with empty pGADT7-Rec, were used as negative controls, while pGBKT7-53 expressed with pGADT7-T served as a positive control. To investigate the interactions among TaeIF5A1 and other eIF proteins from *T. androssowii*, five *eIF* genes were cloned from *T. androssowii* (TaeIF5A2, GenBank number: JQ040803; TaeIF5A3, GenBank number: JQ040804; TaeIF5A4, GenBank number: JQ040805; TaeIF2A, GenBank number: JQ040806; TaeIF-like, GenBank number: JQ040807) and used to perform Y2H assays with TaeIF5A1 as either the bait or the prey.

Statistical analysis

Unless otherwise specified, three independent biological replicates were performed in all the experiments.

Conclusions

Our results showed that *TaeIF5A1* is involved in a variety of abiotic stress responses and can form homodimer. In addition, it can specifically interact with XTH and Arf GAP, suggesting that it may also be involved in cell wall biosynthesis and in intracellular trafficking and actin cytoskeleton reorganization.

Acknowledgments

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Competing Interests

The authors have declared that no competing interest exists.

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