Characterization of a eukaryotic translation initiation factor 5A homolog from *Tamarix androssowii* involved in plant abiotic stress tolerance

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**Abstract**

**Background:** The eukaryotic translation initiation factor 5A (eIF5A) promotes formation of the first peptide bond at the onset of protein synthesis. However, the function of eIF5A in plants is not well understood.

**Results:** In this study, we characterized the function of eIF5A (*TaeIF5A1*) from *Tamarix androssowii*. The promoter of *TaeIF5A1* with 1,486 bp in length was isolated, and the cis-elements in the promoter were identified. A WRKY (TaWRKY) and RAV (TaRAV) protein can specifically bind to a W-box motif in the promoter of *TaeIF5A1* and activate the expression of *TaeIF5A1*. Furthermore, *TaeIF5A1*, TaWRKY and TaRAV share very similar expression pattern and are all stress-responsive gene that functions in the abscisic acid (ABA) signaling pathway, indicating that they are components of a single regulatory pathway. Transgenic yeast and poplar expressing *TaeIF5A1* showed elevated protein levels combined with improved abiotic stresses tolerance. Furthermore, *TaeIF5A1*-transformed plants exhibited enhanced superoxide dismutase (SOD) and peroxidase (POD) activities, lower electrolyte leakage and higher chlorophyll content under salt stress.

**Conclusions:** These results suggested that *TaeIF5A1* is involved in abiotic stress tolerance, and is likely regulated by transcription factors TaWRKY and TaRAV both of which can bind to the W-box motif. In addition, *TaeIF5A1* may mediate stress tolerance by increasing protein synthesis, enhancing ROS scavenging by improving SOD and POD activities, and preventing chlorophyll loss and membrane damage. Therefore, eIF5A may play an important role in plant adaptation to changing environmental conditions.

**Background**

Eukaryotic initiation factor 5A (eIF5A) is a small protein ubiquitously present throughout the eukaryotic kingdom. The protein was initially identified in rabbit reticulocytes as a factor involved in formation of the first peptide bond [1,2]. eIF5A is a highly conserved protein and contains the post-translationally synthesized amino acid hypusine [3]. Molecular and biochemical studies in yeast and mammalian cells demonstrated that eIF5A is synthesized as an inactive precursor that is activated by a post-translational hypusine modification that is only detected in the eIF5A protein, and consists of a two-step sequential reaction catalyzed by deoxyhypusine synthase (DHS, EC:2.5.1.46) and deoxyhypusine hydroxylase (DHH, EC1.14.99.29) [4,5].

The precise cellular function of eIF5A is not fully understood. It was originally considered to be a translation initiation factor as it can stimulate methionyl-puromycin synthesis *in vitro* and transiently attach to ribosomes to begin eukaryotic cellular protein synthesis. In addition, eIF5A promotes the formation of the first peptide bond at the initiation of protein synthesis [1]. Recent studies have demonstrated that eIF5A dysfunction significantly decreases protein synthesis in yeast, and that eIF5A promotes translation elongation in *Saccharomyces cerevisiae* [6-8]. Henderson and Hershey found that although eIF5A is not required for protein synthesis, eIF5A can stimulate the process by about 2-
to 3-fold. They further draw a conclusion that the poly-
some profiles observe during and after elf5A depletion
are diagnostic for a role in initiation [9]. In addition,
elf5A is involved in cellular proliferation and apoptosis
[10], promotes cell viability and cell growth [11] and the
synthesis of proteins involved in progression of the cell
cycle [12]. Moreover, elf5A proteins are found to facilit-
tate protein synthesis by participating in the nuclear ex-
port of specific mRNAs [5]. Furthermore, elf5A proteins
also play a role in RNA binding, and contain a C-
terminal domain with a structure that resembles an
oligonucleotide-binding fold [13].

Plant elf5A proteins are also highly conserved that are
involved in multiple biological processes, including pro-
tein 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 [14-16]. Ma et al. showed that
elf5A plays roles in supporting plant growth and in
regulating responses to sub-lethal osmotic and nutrient
stress [17]. Valentini et al. showed that elf5A is involved
in the WSC/PKC1 signaling pathway that controls cell
wall integrity or related processes, and plays a role in cell
wall formation [18]. Hopkins et al. reported that elf5A
plays a vital role in signal transduction pathways
involved in pathogen-induced cell death and in the
development of plant disease symptoms. Plant elf5A genes
are also involved in abiotic stress responses [3]. For in-
fstance, Xu et al. showed that transgenic Arabidopsis
plants overexpressing RceIF5A show improved resistance
to heat, oxidative and osmotic stresses, while the plants
with reduced elf5A expression (three AtelIF5A isoforms
in Arabidopsis are down-regulated) are more susceptible
to these stresses [16]. Chou et al. reported that salt and
heavy metal stresses induce the expression of rice elf5A
genmes, OseIF5A-1 and OseIF5A-2, suggesting that they
are involved in stress tolerance [19].

However, little is known of the upstream regulators or
its regulatory network, and its role in stress tolerance. In
addition, if elf5A does in fact confer stress tolerance in
plants, the physiological changes mediated by elf5A de-
serve further study.

Tamarix (Tamaricaceae) species, which include small
trees or shrubs, are widely distributed in the saline soils
of drought-stricken areas of Central Asia and China. Tamarix androssowii Litvinov is highly tolerant to abiotic
stresses, such as salinity, drought and high tempera-
tures. These characteristics make the species a suitable
source of stress tolerance genes and for investigating en-
dogenous stress resistance mechanisms.

In the present study, we cloned and functionally charac-
terized an elf5A from T. androssowii. We showed that
Taelf5A1 is a stress-responsive gene involved in the ABA
signal transduction pathway. TaWRKY and TaRAV can
active the expression of Taelf5A1. In addition, Taelf5A1
facilitates protein synthesis and regulates several physio-
logical pathways to improve stress tolerance. This study
reveals a physiological role for elf5A and defines a possible
mechanism for elf5A-mediated stress tolerance in plants.

**Results**

**Cloning and analysis of Taelf5A1 and its promoter**

The Taelf5A1 gene (GenBank number: AY587771),
801 bp in length and encoding a 159 aa protein with a
predicted molecular weight of 17.33 kDa, was cloned
from a T. androssowii. To investigate the homology of
known elf5A proteins, a phylogenetic tree was con-
structed (Figure 1), which showed that Taelf5A1 is most
similar to the elf5A from Manihot esculenta and has a
long evolutionary distance from elf5As of yeast and
mammalian. However, there is little published informa-
tion about the biological functions of these elf5A pro-
zeins and their molecular functions await further study.

The promoter of Taelf5A1 was cloned using TAIL-
PCR, and a promoter fragment with 1486 bp (from –1
to −1486) in length was obtained. We identified diverse
cis-elements in the promoter, including AARRIAT, DOF-
COREZ, MYB1AT, MYBCORE and W-box (Additional
file 1) using PLACE (http://www.dna.afrcc.go.jp) [20].
The W-box sequence “CTGACT” was identified in
Taelf5A1 promoter that shows high binding affinity
to WRKY [21].

**Expression of the Taelf5A1 gene**

Real-time RT-PCR showed that Taelf5A1 can be
detected in roots, stem and leaves, and is differentially
regulated by different abiotic stresses. The expression of
Taelf5A1 was induced in roots by salt stress at 6 or
24 h, but not at other time points. In stems, Taelf5A1
was down-regulated after 24 and 72 h of NaCl stress.
Moreover, Taelf5A1 was strongly down-regulated in
leaves after 6, 24 and 72 h of NaCl stress, but its expres-
sion was unaffected at other time points (Figure 2A).
The Taelf5A1 was down-regulated in roots, stems and
leaves following exposure to PEG stress for 24–72 h
(Figure 2B). Interestingly, Taelf5A1 exhibited the same
expression pattern in roots, stems, and leaves under
NaHCO3 stress, being up-regulated after 12, 24 and 72 h
of stress and down-regulated at all other time points
(Figure 2C). Following CdCl2 stress, Taelf5A1 was gen-
erally down-regulated in roots, stems and leaves
(Figure 2D). ABA treatment induced a marked inhibition
of Taelf5A1 expression in roots, stems and leaves by
6 h, followed by recovery thereafter (Figure 2E).

To further investigate the promoter activity of
Taelf5A1, the transgenic Arabidopsis plants expressing
GUS under the control of Taelf5A1 promoter were ana-
lyzed using GUS staining (Figure 3B). In young
Arabidopsis (less than 3-week old), GUS activity was mainly confined to the cotyledons, the main root, the leaf tips, the tips of leaf teeth, veins and hydathodes (Figure 3Ba−e). GUS activity was present throughout the whole plant in five-week-old plants, and showed a step-wise reduction in both expression area and level in three-week-old and four-week-old plants (Figure 3Be−g). Further, changes in GUS expression pattern were observed during the development of the reproductive organs (Figure 3Bj−o). In flowers, high GUS expression was predominantly observed in the pistils, stigma, stamens, anther and petals, but not in the sepals (Figure 3Bj−l). In the siliques, GUS activity was present in the adhesion zones (Figure 3Bn). Consistent with real-time PCR results (Figure 2), these results indicated that TaIF5A1 is expressed in all the tissues including leaves, roots, stems at all growth stages and reproductive organs.

Targeting TaIF5A1 to nucleus and cytoplasm
The subcellular localization of TaIF5A1 was determined using the TaIF5A1::GFP fusion gene under the control of the CaMV 35S promoter. The TaIF5A1::GFP fusion gene and GFP control were transformed into onion epidermal cells by particle bombardment. We detected the green fluorescent signal of TaIF5A1−GFP in the nucleus and uniformly distributed throughout transformed cells (Figure 4), suggesting that the TaIF5A1 protein showed nuclear and cytoplasm localization.

Analysis of the upstream regulator of TaIF5A1
A W-box element was found in the TaIF5A1 promoter, suggesting that TaIF5A1 may be regulated by transcription factors that interact with W-box motif. To investigate the upstream regulator of TaIF5A1, yeast
A one-hybrid assay was performed using the pHIS2-cis (containing triple tandem repeats of the W-box) reporter vector as bait to screen a *Tamarix* cDNA library. In total, two genes were found to specifically bind the W-box motif (Figure 5B): WRKY transcription factor (TaWRKY, GenBank number: JQ040808) and AP2/ERF and B3 domain-containing transcription factor (TaRAV, GenBank number: JQ040809).

To characterize the specific interaction between the W-box and TaWRKY and TaRAV, we mutated the core W-box motif “TGAC” to “TGCC,” “TAAC” and “TTTT”. Both TaWRKY and TaRAV could bind the W-box motif, but they failed to bind to each of the mutants (Figure 5B), indicating that both “G” and “A” in “TGAC” are necessary for W-box recognition. These results indicate that TaWRKY and TaRAV can specifically bind to the W-box.
To further investigate whether these two genes can activate the expression of TaeIF5A1, promoter fragments of 461 and 165 bp in length containing the W-box motif, and promoter fragment of 165 bp in length containing mutated core sequence “TTTT” were respectively inserted into pHIS2, and the interactions between these promoter fragments and the two genes were determined using the yeast one-hybrid system. We found that both TaWRKY and TaRAV can specifically bind to the two promoter fragments containing the W-box, but failed in binding to the promoter fragment containing the mutated core sequence “TTTT” and the control (N1, N2) (Figure 5C), indicating that they may regulate the expression of TaeIF5A1 through binding to the W-box motif in the promoter of TaeIF5A1.

To further confirm the above interactions, we co-transformed the effector constructs (pROKII-TaRVA or pROKII-TaWRKY) in which TaRVA or TaWRKY is driven by 35S promoter and their corresponding reporter plasmids (pCAM-W-box, pCAM-W165, pCAM-mW165) into tobacco leaves. Histochemical staining and GUS activity assay showed that the GUS gene was activated in tobacco cells when co-transformation of pROKII-TaRVA or pROKII-TaWRKY with pCAM-W-box and pCAM-W165; however co-transformation of pROKII-TaRVA or pROKII-TaWRKY with pCAM-mW165 failed in GUS activation (Figure 5D). These data clearly indicated that both TaRVA and TaWRKY can activate expression of TaeIF5A1 by binding to W-box motif in its promoter.

The expression patterns of TaRAV, TaWRKY and TaeIF5A1 were investigated using real-time RT-PCR. We found that both the expression of TaRAV and TaWRKY are induced by osmotic stress and negatively regulated
by ABA treatment, suggesting that TaRAV and TaWRKY are stress response genes and involved in ABA signaling pathway. Moreover, TaRAV, TaWRKY and TaeIF5A1 all share very similar expression patterns under different stress conditions (Figure 6A, B).

Given the facts that TaRAV and TaWRKY can activate the expression of TaeIF5A1, we next studied if this mechanism of transcriptional regulation is also maintained in the model plant Arabidopsis. Using BLASTX program in Tair, we identified the homologs of TaeIF5A1, TaRAV and TaWRKY in Arabidopsis are AT1G13950, AT1G68840 and AT1G13960, respectively. Real time RT-PCR showed that as in T. androssowii, AT1G13950, AT1G68840 and AT1G13960 also shared a very similar expression patterns in Arabidopsis when exposed to ABA and osmotic stress (Figure 6C, D).

Functional analysis of the TaeIF5A1 gene using a S. cerevisiae expression system

Yeast transformants harboring TaeIF5A1 or the empty pYES2 vector were generated to investigate the role of TaeIF5A1 in stress tolerance. RNA gel blot showed that TaeIF5A1 can be induced in yeast cells, and reached a peak of expression after induction for 36 h (Figure 7A). Therefore, 36 h was selected as a suitable induction time. Yeast transformants harboring TaeIF5A1 or empty vector were grown on induction medium for 36 h, and then treated with different stress-inducing agents. Yeast cells expressing TaeIF5A1 exhibited better growth than control did under NaCl, KCl, LiCl and sorbitol stress conditions (Figure 7B), suggesting that expression of TaeIF5A1 in yeast increases tolerance to abiotic stresses.

We next compared the soluble protein content in three yeast transformants harboring TaeIF5A1, TaPrx1 (as control) or empty pYES2 (as control for investigating if the exogenous gene expressed). The protein content of yeast transformants harboring TaeIF5A1 was the highest, followed by transformants harboring TaPrx1 then empty vector (Figure 7C). Transformants harboring empty pYES2 failed to produce an exogenous gene and exhibited the lowest overall protein expression; the two other transformed strains expressed greater levels of protein, suggesting that the exogenous genes had been expressed. Yeast transformants harboring TaeIF5A1 displayed significantly (P < 0.05) higher overall protein levels than those harboring TaPrx1, indicating that the TaeIF5A1 expression increases protein level in yeast cells.

Overexpression of TaeIF5A1 improves salt tolerance in transgenic plants

To investigate whether overexpression of TaeIF5A1 in plants enhances stress tolerance, transgenic poplar plants expressing the TaeIF5A1 were generated. DNA and RNA gel blot analyses were conducted to confirm the integration and expression of exogenous TaeIF5A1 in transgenic plants (Additional file 2B, C). Salt tolerance test on tube seedlings showed that there was no difference in height growth under normal growth conditions. However, under salt stress conditions, many transgenic lines exhibited significantly increased height growth relative to WT plants (Figure 8A, B). In addition, salt tolerance test on plantlets in soil indicated that all the transgenic lines except line 7 displayed significantly improved height and basal diameter growth than control did (Figure 8C). All these results suggested that salt tolerance of the transgenic lines was improved due to the overexpression of TaeIF5A1.

Additional abiotic stress tolerance assays

Transgenic and WT plantlets were treated with CuSO4, CdCl2 and ZnCl2 or sorbitol for 16 d, and then their
Figure 5 Yeast one-hybrid analyses of the factors binding to W-box elements. A. Scheme of reporter and effector vectors. Fragments of the TaeIF5A1 promoter were inserted into the upstream of the His3 reporter gene. Promoter fragments of 461 bp and 165 bp containing the W-box motif (construct R1, R2) and promoter fragment of 165 bp containing the mutated sequence (construct mR2) were tested. The W-box and mutated W-boxes were respectively cloned into the upstream of the His3 reporter gene: W-box (construct R3); the mutants (constructs R4–R6). The effector vectors, E1: pGADT7-Rec2 harboring TaRAV; E2: pGADT7-Rec2 harboring TaWRKY. B. TaRAV and TaWRKY interaction with W-box (R3) or mutants (R4–R6) sequences. C. Determination of TaRAV and TaWRKY binding to the promoter fragments containing W-box or mutated motif (R1, R2, mR2). The effector and the reporter constructs were co-transformed into yeast strain Y187. Positive transformants were determined by spotting serial dilutions (1:1, 1:10, 1:100, 1:1000) of yeast onto SD/-His/-Leu/-Trp plates with 3-AT. Negative controls: N1, p53HIS2 + E1 (AD-TaRAV); N2, p53HIS2 + E2 (AD-TaWRKY); Positive control: P, p53HIS2 + pGAD-Rec2-53. D. TaRAV and TaWRKY binding to the W-box and the promoter fragment containing the W-box motif in tobacco leaves. (a) Construction of reporter and effector plasmids for transient trans-activation assays; (b) GUS staining of tobacco leaves co-transformed with reporter and effector plasmids; (c) GUS activity assay of the co-expression of effector and reporter plasmids. The data represent mean values of three independent experiments.
growth was compared. We found no difference in growth height between WT and transgenic plants under normal growing condition. However, relative to the WT, the transgenic plants exhibited greatly increased height growth under the various stress conditions (Figure 8D, E), indicating that plants overexpressing TaeIF5A1 possess increased tolerance to these forms of abiotic stresses.

Measurement of soluble protein content
Prior to salt stress, soluble protein levels in transgenic plants did not significantly differ from those of WT plants. However, after 4 d and 7 d of salt stress, all transgenic lines contained significantly (P < 0.05) higher levels of soluble protein than WT plants (Figure 9A), indicating that overexpression of TaeIF5A1 greatly increases soluble protein levels in transgenic plants under salt stress conditions compared to WT plants.

POD activity assay
Transgenic and WT plants had similar POD activity prior to salt stress. Under salt stress conditions, POD activity in WT plants was transiently elevated after 1 d of stress, and subsequently recovered. However, in all transgenic plants POD activity was markedly improved and was significantly (P < 0.05) higher than that in the WT during the entire 4–7 d period of exposure to stress conditions (Figure 9B).

SOD activity comparison
SOD activity in the transgenic lines was either higher or lower than that of WT plants prior to salt stress (Figure 9C). Under salt stress conditions, SOD activity in WT plants did not change; however SOD activity was increased in transgenic lines, being significantly (P < 0.05) higher after 4 and 7 d of exposure to salt stress (Figure 9C), indicating that TaeIF5A1 overexpression specifically increases SOD activity under salt stress condition.

Electrolyte leakage assay
Electrolyte leakage was not significantly different (P > 0.05) between transgenic and WT plants prior to stress. Electrolyte leakage increased in both transgenic and WT plants under stress conditions, with maximal electrolyte leakage levels after 7 d of salt stress. However, the increase of
electrolyte leakage in transgenic lines was significantly lower than in WT plants (Figure 9D). These results indicated that transgenic plants suffer less membrane damage under salt stress conditions compared to WT plants.

Relative chlorophyll content (RCC) comparison

The RCC of WT plants did not notably vary following exposure to salt stress for 1–7 d, but markedly decreased after 14 d of exposure to stress. However, the RCC in transgenic plants increased over 4–7 d, and reached significantly (P < 0.05) higher levels than that of WT control plants under stress for 7–14 d (Figure 9E), indicating that TaeIF5A1 overexpression may enhance chlorophyll levels and prevent chlorophyll loss under salt stress condition.

Discussion

TaeIF5A1 is expressed in all tissues of plants, and tolerant to different abiotic stresses

In the present study, we found that the TaeIF5A1 gene was significantly differentially regulated by NaCl, NaHCO₃,
Figure 8 Effects of the overexpression of TaeIF5A1 on abiotic stress tolerance in transgenic poplar plants. A–B. The seedlings of WT and transgenic lines grown on 1/2MS medium supplemented with 0 and 0.6% NaCl for 20 d, the phenotypes of plantlets were photographed and the height was measured. C. Comparison of the relative rates of growth in height and basal diameter in WT and transgenic poplar lines. The plantlets with similar height from each line and control plants (sample size of 10 plantlets) growing in soil were treated with 0.8% NaCl solution for 30 d then watered normally, the height and basal diameter were measured after 90 d of treatment. RGH or RDG were calculated as: (final value - baseline value) / baseline value × 100 and presented as a percentage. D–E. The plantlets of WT and transgenic plants (2 and 3) with similar size were grown on 1/2MS medium supplied with 300 μM of CuSO₄, CdCl₂, 1 mM of ZnCl₂, and 200 mM of sorbitol. After 16 d of stress, the phenotypes of plantlets were photographed and the height was measured. The error bars represent standard deviations of the mean measurements. * means significant difference (P < 0.05) between transgenic lines and WT plants. WT, wild type poplar plants; 1–10, ten lines of transgenic poplar plants.
PEG and CdCl$_2$ treatments (Figure 2A–D), suggesting roles in the abiotic stress response. In addition, the expression of *TaeIF5A1* and its upstream regulators, *TaRAV* and *TaWRKY*, were strongly inhibited by ABA (Figure 6A), suggesting that they are involved in ABA-dependent signaling pathway. Moreover, both yeast transformants and transgenic plants expressing *TaeIF5A1* displayed increased tolerance to NaCl, KCl, ZnCl$_2$, CuSO$_4$, CdCl$_2$ and sorbitol stresses. Furthermore, *TaeIF5A1* gene is expressed in all the tissues including leaves, roots and stem at different growth stages, and also in reproductive organs (Figure 3B); therefore they may play role of stress tolerance in all of these tissues of plants.

The W-box motif “CTGACT” can be specifically recognized by TaRAV and TaWRKY. RAV transcription factors contain an AP2/ERF domain in their N-terminal regions and a B3 domain in their C-termini. Some RAV genes can interact with the sequences “CAACA”, “CACCTG” and the GCC-box “AGCCGCC” [22], which are involved in plant defense pathways [23] and diverse bioprocesses such as flowering, germination and the early events of leaf senescence [24,25]. In the present study, a RVA gene, *TaRVA*, was found to bind to the W-box motif “CTGACT” (Figure 5B, D), and the binding is lost following “G” to “A” or “A” to “G” mutation of core “TGAC” motif (Figure 5B), suggesting a specific interaction.

Figure 9 Physiological analyses of WT plants and four transgenic *TaeIF5A1* poplar lines. A–E. Comparison of soluble protein levels, POD activity, SOD activity, electrolyte leakage and relative chlorophyll content (RCC) between WT and transgenic poplar plants. Plantlets (60–100 cm in height, each sample contains at least ten plantlets) from wild-type (WT) and transgenic plants (1, 2, 3 and 5) grown in soil were watered with 0.8% (w/v) NaCl for 0, 1, 4, 7 and 14 d, respectively. The error bars represent standard deviations of the mean measurements. * means significant difference (P < 0.05) between WT and transgenic lines plants.
WRKY genes play a variety of roles in plant developmental and physiological processes. Previous studies showed that most WRKY proteins can bind to the cognate cis-acting element “CTTGACT/C” in the promoter or the 5’ untranslated regions of target genes [21]. Our results showed that a WRKY homolog (TaWRKY) can specifically bind to the W-box motif “CTGACT” and that the core “TGAC” motif is sufficient for binding (Figure 5B).

The expression of Taelf5A1 is likely regulated by TaWRKY and TaRAV both of which can bind to the W-box

A W-box motif is present in the Taelf5A1 promoter, indicating that it may be regulated by W-box-binding transcription factors. Both yeast one-hybrid analysis and co-expression of reporter and effector genes demonstrated that two proteins, TaWRKY and TaRAV, which can specifically bind to Taelf5A1 promoter fragments containing the W-box, but failed in binding to the same fragments containing the mutated core sequence “TTTT” (Figure 5C, D). These results clearly suggested that both TaWRKY and TaRAV can activate the expression of Taelf5A1 by binding to the W-box motif present in the promoter of Taelf5A1. Moreover, the expression of Taelf5A1, TaWRKY and TaRAV share very similar expression patterns, are all inhibited following ABA treatment and induced by osmotic stress (Figure 6A, B), suggesting that Taelf5A1, TaWRKY and TaRAV may be components of a single regulatory pathway. Therefore, these combined results strongly suggest that TaWRKY and TaRAV are the upstream regulators of Taelf5A1 that can regulate the expression of Taelf5A1 through binding to the W-box motif in the Taelf5A1 promoter. In Arabidopsis, the homologs of Taelf5A1, AT1G13950, also has three W-box motifs in its promoter (from −10 to −670), suggesting that it may also be regulated by RAV and WRKY. Interestingly, real time RT-PCR results showed that the AT1G13950, AT1G68840 and AT1G13960 all shared very similar expression profiles in response to ABA and osmotic stress (Figure 6C, D), implying that these genes may also be components of a single regulatory pathway. These results indicated that the RAV and WRKY activate the expression of elf5A1 may be a conserved mechanism of transcriptional regulation, which is also maintained in the model plant Arabidopsis.

Taelf5A1 facilitates protein synthesis to improve stress tolerance

In the present study, we introduced Taelf5A1 into yeast S. cerevisiae and poplar to investigate the role of Taelf5A1 in protein synthesis. We found that overall protein levels in S. cerevisiae and poplar expressing exogenous Taelf5A1 significantly improved compared with controls (Figure 7C and Figure 9A). These results suggest that Taelf5A1 facilitates protein synthesis. In plants, protein synthesis is highly sensitive to salt stress and overexpression of elf1A can improve protein translation under stress conditions [1,26]. Increased salt tolerance is observed in plants transformed with elf1A, indicating that protein synthesis positively correlates with stress tolerance [26]. In addition, protein accumulation provides a stored form of nitrogen that can be utilized to adjust osmotic potential [27], suggesting that a vital component of stress tolerance is the maintenance and enhancement of protein synthesis under stress conditions. Therefore, soluble protein levels may be closely associated with plant abiotic stress tolerance. These facts suggested that overexpression of Taelf5A1 improves both protein levels and stress tolerance supports this hypothesis.

Taelf5A1 regulates some physiological pathways to improve stress tolerance

Abiotic stresses, including salt, drought and extreme temperatures, can induce the rapid generation and accumulation of reactive oxygen species (ROS) that cause secondary oxidative stress to plants. Therefore, improving the ROS scavenging capacity is vital for plants to resist abiotic stress conditions. Both POD and SOD are important ROS scavenging enzymes integral to plant stress tolerance. In the present study, two results must be considered together: transgenic Taelf5A1 lines had significantly higher POD and SOD activity than WT plants (Figure 9B, C); and transgenic Taelf5A1 lines exhibited elevated protein synthesis (Figure 9A) under stress conditions. Consequently, we hypothesize that Taelf5A1 increases SOD and POD activity by elevating POD and SOD enzyme synthesis and/or the synthesis of related proteins. In addition, elevated POD and SOD activity may enhance the ROS scavenging capacity of plants under salt stress. Moreover, abiotic stresses in plants also typically result in cell membrane damage, leading to electrolyte leakage. Therefore, electrolyte leakage is a common indicator of membrane damage and leakage is closely related to a loss in water potential. Our results indicate that the level of electrolyte leakage in WT plants is significantly higher than in transgenic plants (Figure 9D). These results suggested that Taelf5A1 may also serve a role in membrane protection under stress conditions.

Chlorophyll is the green plant pigment that absorbs light energy vital for photosynthesis. Salinity causes a reduction in chlorophyll levels and inhibits the net photosynthetic rate. Thus, chlorophyll content is a good indicator of the photosynthetic function of plants under adverse environmental conditions. Previous studies have shown that RCC in trees is reduced by aggravated salt stress due to the degradation of enzymatic chlorophyll
We found that the RCC in WT and TaeIF5A1-transformed plants was similar under normal conditions. However, TaeIF5A1-transformed plants displayed an increased RCC following stress, and RCC was significantly higher in transgenic relative to WT plants following 4–14 days of stress (Figure 9E). These results suggest that eIF5A also has a role in preventing chlorophyll loss under salt stress. The increased RCC indicates that TaeIF5A1-transformed plants should increase or maintain a stable photosynthetic rate under salt stress compared to WT plants. A previous study showed that RceIF5A confers tolerance to heat, oxidative and osmotic stress, and overexpression of RceIF5A can enhance SOD activity and proline level, and decrease electrolyte leakage [16]. In the present study, we further showed that TaeIF5A1 is also tolerant to salt and heavy metal stresses, and overexpression of TaeIF5A1 can not only enhance SOD activity and decrease electrolyte leakage, but also facilitate protein synthesis, increase POD activities and maintain higher chlorophyll content under salt stress. These results suggested that eIF5A is involved in eliciting a stress response mechanism that may play a common role in plant tolerance to salt, heat, oxidative, osmotic and heavy metal stresses.

Conclusion
In summary, TaeIF5A1 is a stress responsive gene that forms part of the ABA signal transduction pathway. The expression of TaeIF5A1 is likely regulated by the transcription factor TaWRKY and TaRAV both of which can bind to W-box “CTGACT”. Furthermore, TaeIF5A1 can facilitate protein synthesis and confer abiotic stress tolerance. We propose that TaeIF5A1 increases plant salt tolerance via several physiological pathways, including enhancement of protein synthesis, elevation of SOD and POD activity, increase or maintenance of photosynthetic rates and the protection of cell membranes. Therefore, eIF5A serves essential and multiple roles in the reduction and elimination of stress imposed on plants by various abiotic factors.

Methods
Plant materials, growth conditions, and treatments
T. androssowii seedlings were grown in pots containing a mixture of turf peat and sand (2:1 v/v). Thoroughly watered 2-month-old seedlings were each exposed to the following treatments: 0.4 M NaCl, 20% (w/v) PEG6000, 0.3 M NaHCO₃, 150 μM CdCl₂, and 150 μM ABA for 0, 6, 12, 24, 48, and 72 h, respectively. Following these treatments, leaves, stems and roots of seedlings from each sample (sample size of 10 seedlings) were harvested at the indicated times after initiation of each treatment and pooled for real-time RT-PCR analyses.

Seedlings of Arabidopsis were grown into pots filled with perlite/soil mixture in a growth chamber under the controlled conditions (16 h light: 8 h dark; 70-75 % relative humidity; 22°C). Three week-old seedlings were each exposed to the following treatments: 20% (w/v) PEG6000, or 100 μM ABA for 0, 3, 6 and 12 h, respectively. After these treatments, the leaves and roots of seedlings were respectively harvested and pooled (sample size of 10 seedlings) for real-time RT-PCR analyses.

Cloning and expression analysis of TaeIF5A1
A TaeIF5A1 (AY587771) gene was cloned from T. androssowii cDNA library [29]. The sequence alignments of the eIF5A proteins from different species, including plants, yeast, mammalian and other eukaryotes was conducted using CLUSTALX1.81, and a phylogenetic tree was constructed using the Neighbor-joining method provided by the computer program MEGA5. The promoter of TaeIF5A1 was PCR-amplified from genomic DNA of T. androssowii using the Genome Walking Kit (TaKaRa, China). To analyze the activity of promoter of TaeIF5A1, the 35S promoter in pCAMBIA1301 was replaced with the TaeIF5A1 promoter (1,486 bp in length) to drive the β-glucuronidase (GUS) gene (Figure 3A). The TaeIF5A1 promoter::GUS construct was transferred into Arabidopsis plants by floral dip method. The T₃ seedlings were employed for spatial expression analysis of TaeIF5A1 using GUS staining.

Real-time RT-PCR was performed in Opticon 2 System (Bio-Rad, Hercules, CA) with α-tubulin, β-tubulin and β-actin genes as internal references. Primers used for RT-PCR are listed in Additional file 3: Table S1. The amplification was performed using the following cycling parameters: 94°C for 30 s followed by 45 cycles at 94°C for 12 s, 60°C for 30 s, 72°C for 40 s and 82°C for 1 s for plate reading. A melting curve was generated for each sample at the end of each run to assess the purity of the amplified products. Each reaction was conducted in triplicate to ensure reproducibility of results. Expression levels were calculated from the cycle threshold according to the delta delta Ct method [30].

Subcellular localization of the TaeIF5A1 protein
The TaIF5A1 coding region without the termination codon was ligated in frame to N-terminal of the green fluorescent protein (GFP) to generate the TaeIF5A1::GFP fusion gene. A CaMV 35S promoter was employed to drive TaeIF5A1::GFP, and the GFP gene under the control of the CaMV 35S promoter (35S::GFP) was used as a control. The constructs were introduced into the onion epidermis cells by particle bombardment (Bio-Rad). The transformed cells were analyzed using confocal laser scanning microscopy LSM410 (Zeiss, Jena, Germany).
Identification of the upstream regulator of *TaeIF5A1*

One W-box motif ("CTGACT") [21] was found to exist in the promoter of *TaeIF5A1* (Additional file 1). To study which gene can recognize this W-box and regulate the expression of *TaeIF5A1*, the three tandem copies of promoter sequence fragment ("AGGCTGACT") containing W-box motif sequence were cloned into a pHIS2 vector (construct R3, Figure 5A), and were screened with *Tamarix* cDNA library for a one-hybrid assay (Clontech, Palo Alto, CA, USA). To investigate the interactions between the W-box and positive clones, we mutated the W-box core motif “TGAC” [31,32] with “TGCC”, “TAAC” or “TTTT” (constructs R4, R5, R6, Figure 5A), and the interactions between the mutant W-box sequences and the positive clones were performed using yeast one hybrid. To further confirm the upstream regulator of *TaeIF5A1*, a 461 bp fragment of *TaeIF5A1* promoter (from −456 to −916) containing the W-box motif (construct R1, Figure 5A), and a 165 bp fragment of *TaeIF5A1* promoter (from −591 to −755) containing the W-box motif and a 165 bp fragment of *TaeIF5A1* promoter (from −591 to −755) containing the mutated W-box core motif “TTTT” (construct R2, mR2, Figure 5A), were cloned into pHIS2, respectively. The interactions between putative upstream regulators and the promoter fragments containing W-box or mutated W-box were performed using a yeast one-hybrid assay. In the above experiments, the p53HIS2 plasmid (pHIS2 contains three copies of p53 DNA element) was used as a negative control vector. All primers are shown in Additional file 3: Table S2.

For further verification of these interactions, the three tandem copies of the W-box and the 165 bp promoter fragments containing W-box motif or mutated core motif “TTTT” were fused with 35S CaMV −46 minimal promoter and respectively cloned into pCAMBIA1301 to replace with its 35S promoter for driving the GUS gene (constructs containing three tandem copies of the W-box named as pCAM-W-box, containing promoter fragment with W-box and mutated W-box named as pCAM-W165 and pCAM-mW165). The effector vectors were constructed by cloning the full ORF of *TaRVa* or *TaWRKY* into pROKII under the control of 35S promoter (named as pROKII-TaRVa and pROKII-TaWRKY) (Figure 5 Da).

All primers are shown in Additional file 3: Table S3. Both the reporter and effector vectors were co-transformed into tobacco leaves using the particle bombardment. GUS staining assay was performed as described by Jefferson [33], and GUS activity was determined according to the method of Jefferson [34].

To investigate the expression patterns of the upstream regulators of *TaeIF5A1*, real-time PCR was performed to determine the expression of *TaeIF5A1* and the upstream regulators in *Tamarix* under ABA and osmotic stress conditions. For investigation of the expression of the homologs of *TaeIF5A1*, *TaRAV* and *TaWRKY* in *Arabidopsis* in response to ABA and osmotic stimulus, BLASTX research on Tair (http://www.arabidopsis.org/Blast/) was performed, we identified the homologs of *TaeIF5A1*, *TaRAV* and *TaWRKY* in *Arabidopsis* are AT1G13950, AT1G68840 and AT1G13960, respectively. An *actin* gene (AT3G18780) was used as internal reference to normalize the amount of total RNA present in each reaction. The primers used are listed in Additional file 3: Table S1, and the real-time PCR conditions were the same as above.

Expression of *TaeIF5A1* in *S. cerevisiae* and stress-tolerance assays

The full ORF of *TaeIF5A1* was cloned into pHYES2 vector (Invitrogen), and was introduced into *S. cerevisiae* strain, INVSc1 (MATa, his3-1, leu2, trp1-289, ura3-52, His-, Leu-, Trp-, and Ura-). To determine the expression peak of *TaeIF5A1* in yeast, yeast transformants harboring the *TaeIF5A1* were cultivated in induction medium (SC-U medium containing 2% galactose) at 30°C for 0, 12, 24, 36, 48 and 60 h, and harvested for RNA gel blot analysis.

For stress tolerance assays, clones harboring *TaeIF5A1* and empty pHYES2 (control) were cultured into SC-U medium containing 2% glucose at 30°C with overnight shaking, adjusted to OD600 of 0.4 in induction medium, and incubated at 30°C for 36 h (RNA gel blot result showed that peak level of exogenous gene induced at this time). After incubation, cell densities were adjusted to equal and incubated in different concentrations of NaCl, KCl, LiCl or sorbitol, then they were incubated at 30°C with overnight shaking. The growth rates were evaluated by measuring the OD600 for liquid medium in each sample.

To analyze protein content, yeast transformants harboring Peroxiredoxin gene (*TaPrx1*, GenBank number: JQ082512) from *Tamarix* were used as control (it can remove the protein synthesis differences between the yeast transformants harboring *TaeIF5A1* and empty pHYES2; since transformants harboring empty pHYES2 failed in producing an exogenous gene-Elf5A compared with that harboring *TaeIF5A1*). The yeast transformants harboring *TaeIF5A1*, *TaPrx1* and empty pHYES2 were cultured in induction medium at 30°C for 0, 12, 24, 36 and 48 h, adjusted to equal quantity, and harvested for soluble protein content analysis. The experiment was repeated at least three times. The protein extraction followed the procedure described by Kushnirov [35] and protein content analyses were performed following the Bradford method [36].

Construction of plant expression vector and poplar transformation

The *TaeIF5A1* was cloned into pROKII (Additional file 2A), in which *TaeIF5A1* under the control of CaMV
35S promoter, and transferred into poplar plants (Populus davidiana Dode × P. bollena Lauche) using the Agrobacterium-mediated transformation. Kanamycin-resistant lines were detected by DNA gel blot and RNA gel blot. DNA probe for RNA and DNA gel blot were prepared by PCR amplification of the coding region of the TaeIF5A1 using digoxigenin (DIG) - PCR labeling mix (Roche). Total DNA (30 μg) from samples was digested with BamHI and SacI and separated by electrophoresis on a 0.8% agarose gel. The DNA was denatured with NaOH and then transferred to Hybond N+ membranes (Amersham). Hybridization and detection was performed following the manual instruction (DIG High Prime DNA Labeling and Detection Starter Kit II; Roche). To detect the expression of exogenous TaeIF5A1, total RNA (20 μg) was fractionated on formaldehyde agarose gels, blotted on Hybond N+ membranes and fixed by UV cross-linking (254 nm, 8 min). Hybridization and detection were conducted following the manufacturer’s instructions (Dig Northern starter kit, Roche).

Physiological analysis of transgenic and nontransgenic poplar

The wild-type and the transgenic plants exhibiting similar height (about 1 cm in length) were grown on 1/2MS medium supplemented with 0.6% NaCl (16 h light: 8 h dark, 25°C in tube). The phenotypes of plantlets were photographed, and the heights of plantlets were measured after 20 d of growth.

For growth comparison of plants in soil, plantlets from WT and transgenic plants with similar height (about 70 cm in height) were employed. The height and basal diameter of each sample (sample size of 10) were measured before stress as baseline values. The plantlets were treated with 0.8% NaCl solution for 30 d then watered normally. Following 90 days of growth, the height and basal diameter (final values) were measured, and the relative growth rates of growth in height or basal diameter were calculated.

For physiological analysis, plantlets (60–100 cm in height) from WT and transgenic plants grown in soil were watered with 0.8% (w/v) NaCl solution for 0, 1, 4 and 7 d, and leaves were harvested for analyses. For measurement of concentration of soluble protein, a standard curve for protein level with known concentrations of bovine serum albumin (0–100 μg, at 20 mg intervals) was generated. Phosphate buffer (1.5 mL, 0.01 M, pH 7.0) was added with sample leaf powder (0.1 g), extracted for 3 min, and centrifuged. One mL of supernatant was added with 2 mL of coomassie brilliant blue G250 regent, and the light absorbance was determined at 595 nm. Water was used instead of supernatant as control, and the protein concentrations were calculated using the standard curve. For POD activity measurement, each sample powder (0.05-0.1 g) was incubated with 1.5 mL of 0.01 M phosphate buffer (pH 7.2) at 4°C for 30 min. After centrifugation, 20 μL of supernatant was diluted to 500 μL with water, then added with 0.5 mL of 0.8% H2O2, 0.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.1 M Guaiacol buffer, and incubated at 30°C for 8 min. Light absorbance (ΔA470) of the reaction solution was measured at 470 nm. Water was used instead of H2O2 as a control. POD activity (Apod) was calculated as follows: Apod = (ΔA470 V) / WTv × 100. Where V: total enzyme volume, v: the volume of enzyme used in reaction, W: the material weight, T: reaction time (min). For SOD activity assay, phosphate buffer (1.5 mL) was added with the leaf powder and incubated at 4°C for 30 min. After centrifugation, 30 mL of the supernatant was diluted to 500 mL with water and added with 1.5 mL of reaction buffer (0.013 M Met, 6.3 × 10^-6 M NBT, 6.5 × 10^-6 M riboflavin, 1 × 10^-4 M EDTA, 0.05 M phosphate buffer, pH 7.8), and incubated at 50°C for 10 min under 6000 LX. The solution was measured at 560 nm. SOD activity was calculated as ASOD[Ug^-1 min^-1 (FW)]= (ΔA560 × N) / (50% WT); where ΔA560 is the decrease absorbance at 560 nm (%), N: the dilution folds, W: the weight and T: the reaction time (min). Electrolyte leakage was determined according to Wang et al. [37]. Soluble protein contents were measured following Bradford method [36]. A chlorophyll analyzer (Konica Minolta, Japan) was used to determine relative chlorophyll content (RCC) in plants stressed for 1–14 d. Each sample contained at least ten plantlets and each experiment was performed in triplicate to ensure the accuracy of analyses.

For other abiotic stress tolerance tests, the plantlets with similar size were grown on 1/2MS medium supplied with 300 μM of CuSO4, CdCl2, 1 mM of ZnCl2, and 200 mM of sorbitol. Plantlets growing in normal 1/2MS medium were used as the control. After 16 d of stress, the height between WT and transgenic lines plants were compared.

Statistical analysis

Data analyses were carried out using SPSS 16.0 (SPSSInc, Chicago, Ill, USA) software. For all the analyses, the significance level was set at P < 0.05. Sample variability is given as the standard deviation (S.D.) of the mean.

Additional files

Additional file 1: The promoter sequence of TaeIF5A1 and the cis-elements within the promoter. The cis-elements are shown in different colors and the PCR primers used for the amplification of promoter fragments used in the yeast one-hybrid assay are indicated by a solid line. The primers Pro-af and Pro-ar were used amplifying 461 bp
promoter fragment, and Pro-bf and Pro-br were used amplifying 165 bp promoter fragment. The putative transcription start site is underlined and the start codon (ATG) is labeled with a rectangle.

Additional file 2: DNA and RNA gel blot analyses of TaeIF5A1-transformed poplars. A. Diagram of the T-DNA region of the pROKII-TaeIF5A1 vector used for transformation. B. DNA gel blot analysis of transformed plants. DNA (30 μg) from each sample was digested with BanHI and SacI, separated on agarose gels, denatured and transferred to Hybond N+ membranes. C. RNA gel blot analysis of WT and the transgenic poplar plants. Total RNA (20 μg) from each sample was fractionated on formaldehyde agarose gel and blotted on Hybond N+ membranes. P. pROKII-TaeIF5A1 vector using as positive control; WT, wild type poplar plants; 1–10, ten lines of transgenic poplar plants.

Additional file 3: Primers used in the study.

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Authors’ contributions
YW conceived and designed the experiments. LW, CX and CW performed the experiments. LW, CX and CW analyzed the data. YW wrote the manuscript. All authors read and approved the final manuscript.

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