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A novel eukaryotic translation initiation factor 5A gene (*eIF5A*) from *Populus simonii* × *P. nigra* confers $CuSO_4$ and NaCl stress tolerance in *Arabidopsis thaliana*

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Abstract

The role of plant eIF5A proteins in multiple biological processes such as protein synthesis regulation, translation elongation, mRNA turnover, programmed cell death and stress tolerance is well-known. In the present study, we cloned and characterized a *PsneIF5A2* from poplar (*Populus simonii* × *P. nigra*) leaves. With the support of bioinformatics prediction, the results showed the domain structures of PsneIF5A2, which was predicted by multiple alignment analysis. It has a S1_eIF5A motif, which is an ortholog of eIF5A1 in *Arabidopsis*. The plant expression vector of *PsneIF5A2* was transformed into *Arabidopsis* using *Agrobacterium tumefaciens* strain EHA105. Ectopic expression of *PsneIF5A2* in *Arabidopsis* significantly conferred $CuSO_4$ and NaCl stress tolerance. Furthermore, *PsneIF5A2*-transgenic *Arabidopsis* exhibited enhanced SOD and POD activities, lower MDA content and electrolyte leakage under $CuSO_4$ and NaCl stress, compared with control plants. Our results suggested that the *PsneIF5A2* gene (GenBank No. KC521463) is an excellent candidate for genetic engineering to improve salt and heavy metal tolerance in agricultural plants.

Keywords *Populus simonii* × *P. nigra*, Eukaryotic translation initiation factor eIF5A, Abiotic tolerance, *Arabidopsis thaliana.*
Abbreviations: CaMV 35S_cauliflower mosaic virus 35S promoter; CAT_catalase transcripts; DAB_3,30-diaminobenzidine; EC_electrical conductivity; eIF5A_eukaryotic translation initiation factor eIF5A; MDA_malondialdehyde; NBT_nitroblue tetrazolium; OE_over expression; ORF_open reading frame; Pnos_nopaline synthase gene promoter; POD_peroxidase; qRT-PCR_quantitative real time polymerase chain reaction; ROS_reactive oxygen species; RT-PCR_reverse transcriptase polymerase chain reaction; SD_standard deviation; SOD_superoxide dismutas; Tnos_nopaline synthase gene terminator; WT_wild-type

Introduction

Eukaryotic translation initiation factor eIF5A has been found in all eukaryotic organisms as well as in archaebacteria being one of the most highly conserved proteins. The eIF5A protein was originally isolated from polyribosomes of rabbit reticulocyte lysates (Kemper et al., 1976; Benne et al., 1978). Protein structural analyses have shown that eIF5A is phosphorylated at the N-terminal serine residue (Kang et al., 1993; Klier et al., 1993) and contains a C-terminal domain with a structure that resembles an oligonucleotide binding fold (Yao et al., 2003; Teng et al., 2009). This suggests a role in RNA binding as a candidate of the translation initiation factor. In earlier studies, the depletion of yeast eIF5A was reported to cause only a 30% reduction in the rate of protein synthesis (Kang and Hershey, 1994; Zuk and Jacobson, 1998), suggesting that translational initiation can be processed in the absence or at very low concentrations of eIF5A. The precise cellular function of eIF5A is not fully understood; therefore, the translation initiation function of eIF5A has since been questioned and proved (Kyrpides and Woese, 1998; Hanawa-Suetsugu et al., 2004; Zanelli et al., 2006; Gregio et al., 2009). Saini et al. (2009) have demonstrated that eIF5A promotes translation elongation in *Saccharomyces cerevisiae*. Henderson and Hershey (2011) found that eIF5A can stimulate the process of protein synthesis by about 2-to 3-fold, and further draw a conclusion that the polysome profiles observe during and after eIF5A depletion are diagnostic for a role in initiation. In addition, eIF5A proteins are found to facilitate protein synthesis by participating in the nuclear export of specific mRNAs, and play a role in RNA binding (Liu et al., 2008; Teng et al., 2009).

Plant eIF5A proteins are involved in multiple biological processes, including the regulation of protein synthesis, translation elongation, mRNA turnover and programmed cell death (Wang et al., 2003; Ma et al., 2010; Xu et al., 2011). Plant *eIF5A* genes are also involved in abiotic stress responses (Hopkins et al., 2008). Transgenic *Arabidopsis* plants over-expressing *RceIF5A* from *Rosa chinensis* showed improved tolerance to heat, oxidative and osmotic stresses (Xu et al., 2011). *OseIF5A1* and *OseIF5A2* from *Oryza sativa* can be induced by salt and heavy metals, suggesting that they are involved in stress tolerance (Chou et al., 2004). In recent centuries, human activities are the primary sources of heavy metal contamination (Moreira 1996; Schell et al., 2006). Moreover, with the land desertification and salinization, the arable lands on earth are becoming less and less (Foster and Chilton 2003; Araújo et al., 2015). Bioremediation has emerged as a potential tool to clean up the contaminated/broken environment. Especially derived processes by phytoremediation are environment protection (Ma et al., 2011).

Populus simonii x *P. nigra* hybrid was obtained from *Populus simonii* and *Populus nigra* by the Chinese academy of forestry in 1959. Many years practice proves that *P. simonii* x *P. nigra* is a good tree species, with the excellent characteristics of growth speed, cold tolerance, drought tolerance, disease resistance, and others. Especially in the drought and sandy wasteland of the north China, *P. simonii* x

P. nigra as an excellent fast-growing afforestation tree species can become building materials in 10 years. So far, there is no report about the function of poplar *eIF5A* gene. Based on the *P. simonii* x *P. nigra* as experimental material, a *eIF5A* homologous gene was cloned. To investigate the function of poplar *eIF5A* genes, phenotypes of transgenic *A. thaliana* under salt and heavy metal stress were determined.

Results

Cloning and bioinformatics analysis of PsneIF5A2

A cDNA sequence of the homologous gene *eIF5A*, i.e., *PsneIF5A2* (GenBank No. KC521463), was obtained from *P. simonii* × *P. nigra* using RT-PCR. The gene contains a 483 bp ORF corresponding to a deduced protein of 160 amino acids. The estimated molecular weight and isoelectric point of the putative protein was approximately 17.5 kD and 5.60, respectively. The protein sequence exhibited significant Pfam matches with the S1_eIF5A motif (85–154 aa; PF01287) (http://pfam.sanger.ac.uk/), suggesting that this gene belongs to an eukaryotic translation initiation factor gene family (Yao et al., 2003). A comparison of the deduced amino acid residues indicated that PsneIF5A2 shared 100% identity with *P. trichocarpa* PtreIF5A2, and 89% identity with *A. thaliana* eIF5A1 (Fig. 1). A homologous sequence analysis of the deduced protein sequences of PsneIF5A indicated considerable similarities with the corresponding eIF5A, which previously characterized in other plants (Fig. 1). To investigate the homology of known eIF5A proteins, a phylogenetic tree was constructed (Fig. 2), showing that PsneIF5A2 is most similar to the eIF5A from dicotyledonous plants and has a long evolutional distance from eIF5As of yeast and mammalian.

Over expression of PsneIF5A in transgenic Arabidopsis

The physiological function of *PsneIF5A2* was performed by plant transformation to obtain transgenic *Arabidopsis* (*35S::PsneIF5A2*) under the control of a CaMV 35S promoter (Fig. 3a). In total, 10 independent lines were generated. Northern blotting and qRT-PCR confirmed that the exogenous *PsneIF5A2* gene was successfully expressed at the mRNA level in *Arabidopsis* (Fig. 3b and 3c). The qRT-PCR results also showed that *PsneIF5A2* was expressed in all 10 lines, and OE6 was expressed at the lowest level. These lines exhibiting high-level expression (*i.e.*, OE1, 2, 4, 5, 9, and 10) were selected for further experiments (Fig. 3c).

Physiological characterization of 35S::PsneIF5A2 in transgenic Arabidopsis

Under normal conditions, there was no difference in growth between the transgenic and wild-type *Arabidopsis* plants (Fig. 4a and d). However, the transgenic lines showed significantly improved germination and root growth under $CuSO_4$ or NaCl stress compared with the wild-type (Fig. 4b, c, e, f, and g). In addition, the *PsneIF5A2* transgenic seedlings had a higher fresh weight under $CuSO_4$ or NaCl stress in comparison with the wild-type plants (Fig. 4h). Moreover, the transgenic plants had a higher number of lateral roots under $CuSO_4$ or NaCl stress, compared with the wild-type plants (Fig. 4e and f). There was no significant difference in the aboveground components between the transgenic and wild-type plants before the stress treatments. However, the *PsneIF5A2* transgenic lines had considerable differences in leaf color and higher chlorophyll contents than the wild-type under $CuSO_4$ or NaCl-stress conditions (Fig. 4i). These results suggest that *PsneIF5A2* conferred an enhanced tolerance to $CuSO_4$ and NaCl stresses in *35S::PsneIF5A2* transgenic plants.

In situ histochemical examination of 35S::PsneIF5A2 transgenic Arabidopsis

The H_2O_2 and O_2^- levels in the transgenic lines were examined using DAB and NBT staining, respectively (Fig. 5a and b). The H_2O_2 and O_2^- levels in the transgenic plants were almost identical to the wild-type plants under normal growth conditions. However, these differences increased after $CuSO_4$ or NaCl stress treatments. The level of staining in the transgenic lines was considerably lower than in the wild-type (Fig. 5a and b). The SOD and POD activity levels did not differ significantly between the transgenic and wild-type plants under normal conditions (Fig. 5d and e). Under $CuSO_4$ or NaCl stress conditions, the SOD and POD activities increased in both the transgenic and wild-type plants. However, the SOD and POD activities in both transgenic lines were significantly higher than in the wild-type plants. The Evans blue staining assay (Fig. 5c) was used to measure cell death, and the MDA content and relative electrical conductivity were also examined to compare the transgenic and wild-type plants before and after stress treatments (Fig. 5f and g). No differences were observed in the cell death, MDA content or relative electrical conductivity between the transgenic and wild-type plants before treatments; however, the wild-type plants had higher rates of cell death or damage than the transgenic lines under the imposed stress treatments. Under $CuSO_4$ and NaCl stresses, the MDA content averaged 1.39-fold and 1.45-fold higher, respectively, in wild-type plants than in *35S::PsneIF5A2* transgenic plants, while the relative electrical conductivity was 1.53-fold and 1.34-fold higher, respectively, in the wild-type plants compared with the *35S::PsneIF5A2* transgenic plants.

Discussion

The eIF5A protein has been implicated in many vital functions in eukaryotic cells. It is a highly conserved protein containing a nucleotide binding motif (Kim et al., 1998; Yao et al., 2003) and the RNA binding ability appears to be hypusine dependent (Xu and Chen, 2001). However, it is interesting that in contrast with the existence of two genes for eIF5A in animals (Jenkins et al., 2001), plant genomes often contain more than two eIF5A isoforms (Wang et al., 2001; Chou et al., 2004; Zhou et al., 2006). There are three isoforms of eIF5A in *Arabidopsis thaliana*, i.e., *AteIF5A1*, *AteIF5A2*, and *AteIF5A3* (Wang et al., 2003; Thompson et al., 2004). *AteIF5A1* plays an important role in xylogenesis of *Arabidopsis* (Liu et al., 2008), *AteIF5A2* is a key element of the signal transduction pathway that results in both plant development and programmed cell death (Feng et al., 2007; Hopkins et al., 2008), and *AteIF5A3* influences plant growth and plays a regulatory role in the response of plants to sub-lethal osmotic and nutrient stress (Ma et al., 2010). With the prediction of bioinformatics, there are four isoforms of eIF5A in *P. trichocarpa* (Fig. 1). Homology analysis of amino acids indicated that eIF5A family was highly conserved. Four PtreIF5As differ from each other, merely based on several amino acid positions (Fig. 1). The discovery of isoforms of the *eIF5A* genes illustrates the high similarity. However, different isoforms may contribute to the several special physiological functions. Our earlier data showed that *PsneIF5A2* is a stress responsive gene expressed under abiotic stress, and increases abiotic stress tolerance in yeast (In press). In this study, we present evidences that *PsneIF5A2* is involved in the increased abiotic stress tolerance in

Table 1. Primers used in this study

Name	Sequences (5'...3')	Usage
PsneIF5A2-prokII-F	ATCTCTAGAGAGATGTCGGACGAGGAGCACCACTTTG	Plant expression vector
PsneIF5A2-prokII-R	ATCGAGCTCTCAATTTTTTGGGCCAATGTCCTTAAGGG	
PsneIF5A2-probe-F	ATGTCGGACGAGGAGCACCACTTTG	Probe of *eIFA5A2* cDNA labeled with DIG-dUTP
PsneIF5A2-probe-R	TCAATTTTTTGGGCCAATGTCCTTAAGGG	
PsneIF5A2-RT-F	GTCATCGTCCTCTTTCACCTG	Primers used in qRT-PCR
PsneIF5A2-RT-R	CTTGCTGAGGGTAAGTCTTGG	
Atactin-RT-F	AGAGATTCAGATGCCCAGAAGTCTTGTTCC	
Atactin-RT-R	AACGATTCCTGGACCTGCCTCATCATACTC	

Note：The sites of *Xba* I or *Sac* I are underlined.

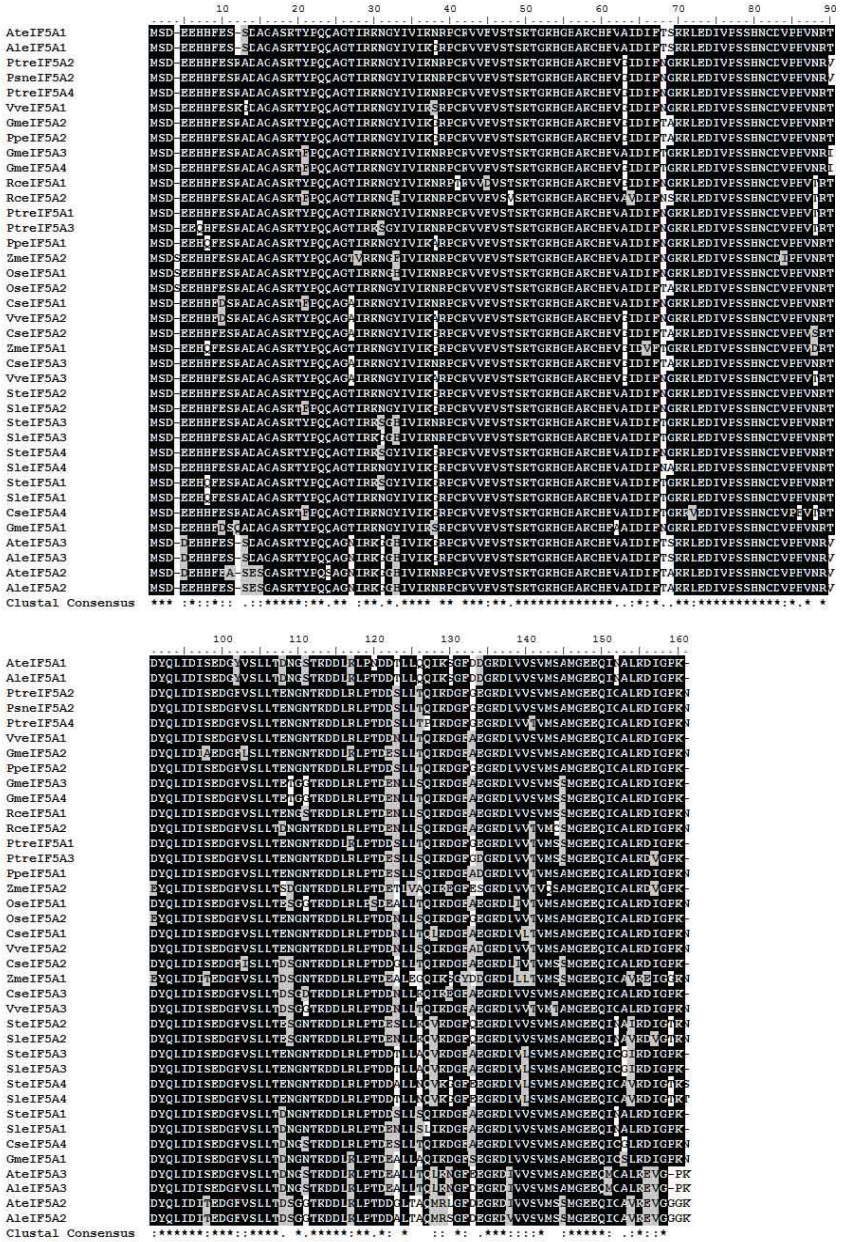

Fig 1. Alignment of the deduced amino acid sequences of eIF5A homologs including PsneIF5A2. RceIF5A1/2 from *Ricinus communis* (EEF32970, EEF47818), PtreIF5A1/2/3/4 from *Populus trichocarpa* (ABK93726, ABK93569, ABK95708, ABK96021), GmeIF5A1/2/3/4 from *Glycine max* (ACU13213, ACJ76773, ACJ76772, ACU13235), CseIF5A1/2/3/4 from *Cucumis sativus* (XP_004146682, XP_004135157, XP_004145502, XP_004138702), PpeIF5A1/2 from *Prunus persica* (EMJ03891, EMJ24836), AteIF5A1/2/3 from *Arabidopsis thaliana* (AAG53646, AAM61392, AAG60110), AleIF5A1/2/3 from *Arabidopsis lyrata* (EFH66290, EFH69650, EFH63493), VveIF5A1/2/3 from *Vitis vinifera* (XP_003634303, XP_002273265, XP_002285505), SteIF5A1/2/3/4 from *Solanum tuberosum* (BAA20878, BAA20877, BAA20879, BAA20876), SleIF5A1/2/3/4 from *Solanum lycopersicum* (AAG53649, AAG53648, AAG53647, AAG53650), ZmeIF5A1/2 from *Zea mays* (ACG32474, ACG30843), and OseIF5A1/2 from *Oryza sativa* Japonica Group (ABF98985, ABA98689). Black shadows indicate identical amino acids; dashed lines indicate gaps to optimize the alignment.

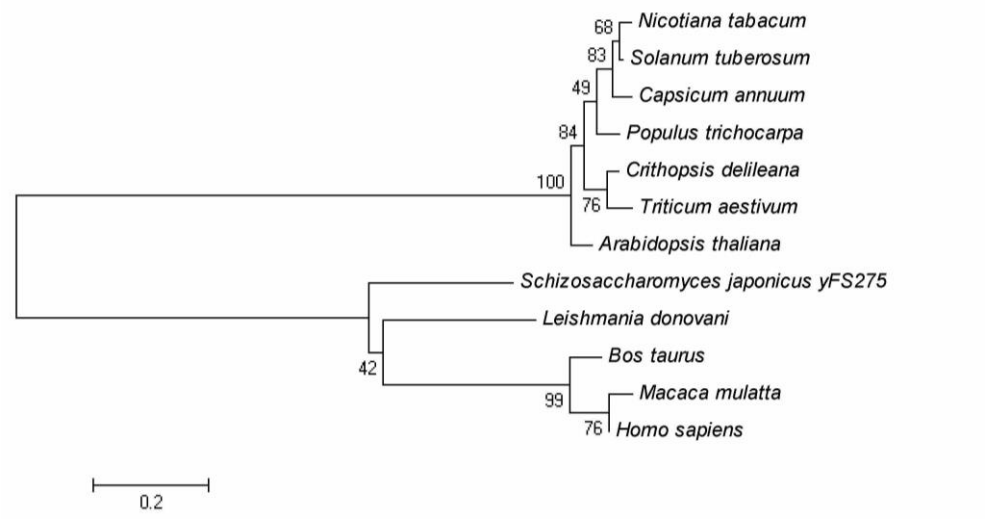

Fig 2 . Phylogenetic relationships among eIF5A homolog proteins. All protein sequences used in the phylogenetic analysis were retrieved from GenBank and their GenBank accession numbers are as follows: *Populus trichocarpa* (ABK93726), *Nicotiana tabacum* (CAA45105), *Capsicum annuum* (AAS48586), *Solanum tuberosum* (ABB16995), *Arabidopsis thaliana* (AAG53646), *Triticum aestivum* (AAZ95172), *Crithopsis delileana* (ABB90163), *Homo sapiens* (NP_112594), *Leishmania donovani* (ADJ39999), *Schizosaccharomyces japonicus* (XP_002173495), *Macaca mulatta* (AFH28009), and *Bos taurus* (NP_001069354). Branch length values are indicated.

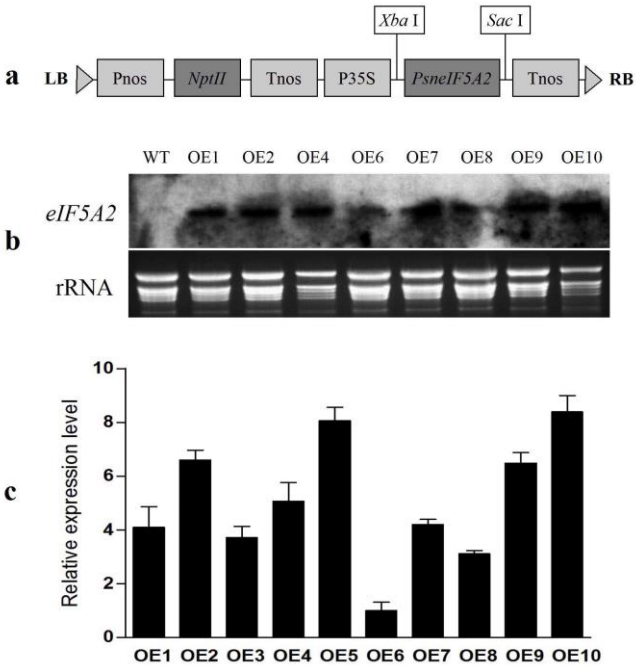

Fig 3 . Northern blot and qRT-PCR analysis of *PsneIF5A2* expression in transgenic *Arabidopsis*. (a) Schematic diagram of the T-DNA region of the binary vector pROKII-*PsneIF5A2*. LB, left border; Pnos, nopaline synthase promoter; *NptII*, kanamycin resistance gene; Tnos, nopaline synthase terminator; P35S, CaMV35S promoter; RB, right border. (b) Northern blot analysis of *35S::PsneIF5A2* in transgenic *Arabidopsis*. Leaves were sampled from wild-type and transgenic plants. WT, Wildtype; OE1-10, Eight *35S::PsneIF5A2* lines. (c) qRT-PCR analysis of *35S::PsneIF5A2* in transgenic *Arabidopsis*. Leaves were sampled from wild-type and transgenic plants. OE1-10, Ten *35S::PsneIF5A2* lines.

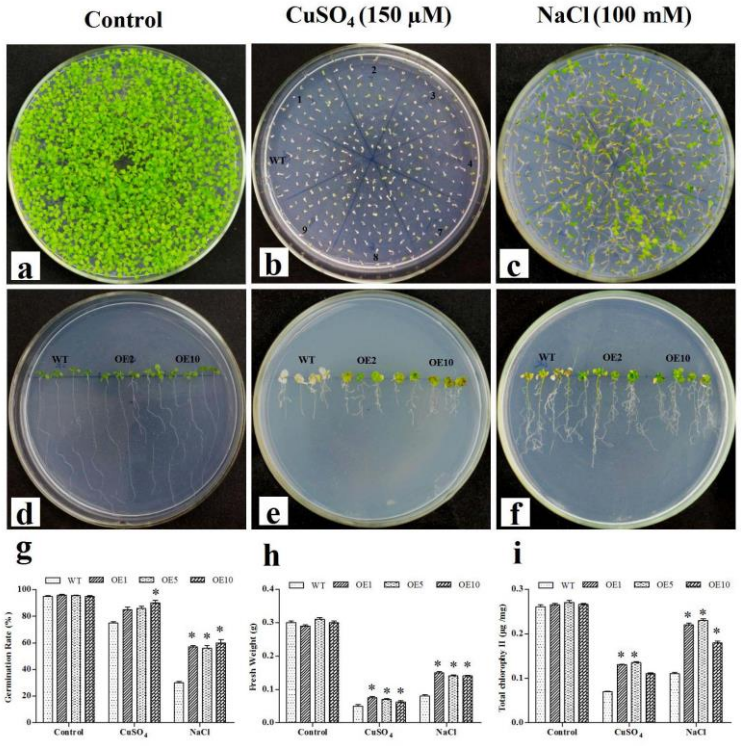

Fig 4. Heavy metal and salt stress tolerance assays of wild-type plants and transgenic *Arabidopsis*. (a-c) Germination assays using MS medium supplemented with water (a), 150 μM $CuSO_4$ (b) or 100 mM NaCl (c) for at least 14 d in stress tolerance assays of wild-type plants and transgenic *Arabidopsis*. (d-f) 6-d-old wild-type and *PsneIF5A2* transgenic lines 2 and 10 seedlings under normal conditions (MS medium) were transferred to a medium supplemented with water (d), 150 μM $CuSO_4$ (e) or 100 mM NaCl (f) for at least 10 d. (g) Germination percentage of three transgenic lines and wild-type seeds was measured after 7 d. (h) Fresh weight of 10-d-old treated seedlings. (i) Chlorophyll content was tested after treatment with 150 μM $CuSO_4$ or 100 mM NaCl for 10 d. The experiments were repeated at least three times for each treatment. The level of significance was set at $P \leq 0.05$, error bars denote SD.

Arabidopsis. We constitutively over expressed *PsneIF5A2* in transgenic *Arabidopsis* and observed that plants over expressing *PsneIF5A2* exhibited strong tolerance to $CuSO_4$ and NaCl stresses in comparison with the wild-type (Fig. 4 and 5). These results agree with the results of *RceIF5A* and *TaeIF5A* (Xu et al., 2011; Wang et al., 2012). In woody plant, Wang et al. (2012) confirmed that A WRKY (TaWRKY) and RAV (TaRAV) proteins can specifically bind to a W-box motif in the promoter of *TaeIF5A1* and activate the expression of *TaeIF5A1*. A large number of studies have shown that WRKYs confer tolerance to multiple abiotic stresses (Wang et al., 2013; Okay et al., 2014). Transgenic plants (35S::*PsneIF5A2*) produce more biomass than the wild-type (data not shown) because they get less affected under salt and heavy metal stresses and could; therefore; recover faster after the stress is removed. Because the chlorophyll pigment absorbs light energy that is vital for photosynthesis, the chlorophyll content is a good indicator of the photosynthetic functioning of plants under adverse environmental conditions. We found that the chlorophyll content in wild-type and transgenic *PsneIF5A2* plants were similar under normal conditions. However, the chlorophyll content was significantly higher in transgenic plants compared with the wild-type following 10 days of stress (Fig. 4i), which is consistent with the reports on *ThGSTZ1* transgenic *Arabidopsis* under NaCl and mannitol stress (Yang et al., 2014) and *TaeIF5A1* transgenic poplar under salt and heavy metal stress (Wang et al., 2012). Abiotic stress may lead to oxidative stress by increasing the production of ROS and/or altering antioxidant defenses in plants (Miller et al., 2010). Plants possess a large ROS-producing and scavenging network, such as SOD, POD, and CAT and can also increase protective substances such as proline (Yang et al., 2007; Tang et al., 2005). Moreover, over-expression of some *eIF5A* genes has been shown to improve oxidative stress tolerance levels in transgenic plants (Chou et al., 2004 Xu et al., 2011; Wang et al., 2012). In the current study, the over-expression of *PsneIF5A2* in *Arabidopsis* showed decreased levels of ROS accumulation. Consistently, higher activity levels were observed for SOD, POD, and lower MDA content and EC rates in plants (*35S:PsneIF5A2*), compared to wild-type *Arabidopsis*. This finding suggests that *PsneIF5A2* may act as a stress regulator through increasing the activity of antioxidant enzymes to strengthen the ROS scavenging ability or maintain ROS homeostasis. *P. simonii* x *P. nigra* as a excellent fast-growing afforestation tree species, which is widely distributed in the cold, drought and saline-alkali soils of the north China. Our results suggest the *PsneIF5A2* gene may play an important role in salt and heavy metal tolerance in plants, which contributes to our understanding of these plant tolerance mechanisms. Furthermore, this is the first report to characterize the expression profiles of the poplar *eIF5A* gene. Therefore, our results contribute to the ongoing study of *PsneIF5A* gene function and homologs, although much remains unknown. Furthermore, heavy metal stress and high salinity are responsible for dramatic reductions in crop yield worldwide, and the *PsneIF5A* genes are excellent candidates for genetic engineering to improve salt and heavy metal tolerance in agricultural plants.

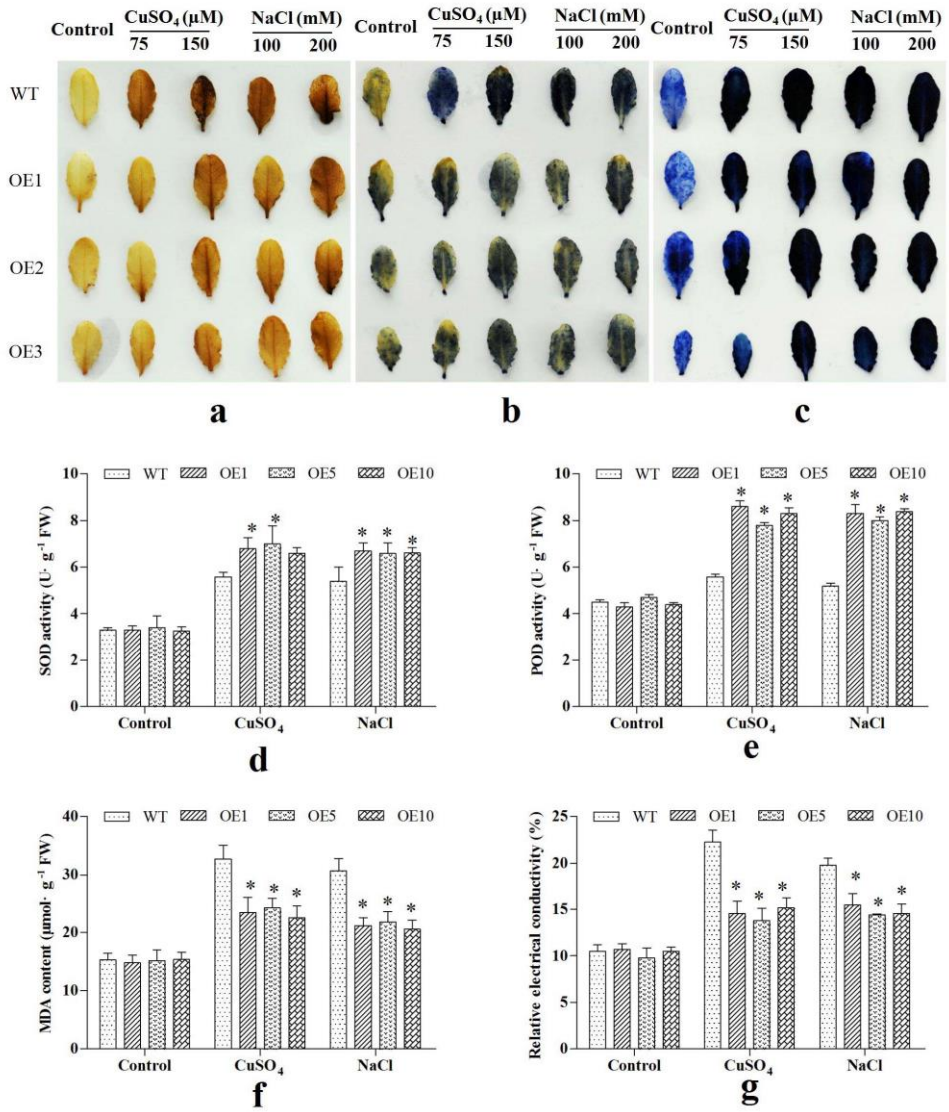

Fig 5. ROS levels, cell death, and physiological index analysis in 35S::PsneIF5A2 and wild-type Arabidopsis plants under heavy metal and salt stress conditions. DAB staining was used to detect H_2O_2 damage (a). NBT staining was used to detect O_2^- damage (b). Evans blue staining was used to detect cell death (c). SOD activity (d), POD activity (e), MDA content (f), and the relative EC (g) were quantified in transgenic and wild-type plants under 150 mM $CuSO_4$ or 100 mM NaCl for 4 d. All experiments were repeated three times. The level of significance was set at $P \leq 0.05$, error bars denote SD.

Materials and Methods

Plant material and growth conditions

The perennial *P. simonii* × *P. nigra* cross was obtained from the campus of Northeast Forestry University in Heilongjiang Province, China. For cloning the *eIF5A* gene, young leaves were sampled, immediately frozen in liquid nitrogen, and stored at -80°C before isolating total RNA. The *A. thaliana* (Col-0) plants were grown in pots containing a mixture of turf peat, vermiculite, and sand (3:1:1, *v*/*v*) in a greenhouse, which was under controlled conditions with 60-75% relative humidity and an average temperature of 22±2°C. Cool white fluorescent lights provided a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}.

Isolation of PsneIF5A

To clone the *eIF5A* gene in poplar, total RNA was isolated with RNA extraction Kit (TaKaRa, Dalian, China) from *P. simonii* × *P. nigra* young leaves. The purity and quality of RNA was checked by NanoDrop 2000c (Thermo-Scientific, USA). A 1 μg aliquot containing total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa). The full length cDNA for the ORF of *PsneIF5A2* was cloned from cDNA using RT-PCR with EX Taq^{HS} enzyme (TaKaRa). The primers used to amplify *PsneIF5A2* are listed in Table 1. The PCR cycle profile consisted of an initial denaturation at 94°C for 4 min, 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR product was cloned into pMD18-T vector (TaKaRa) and the positive clones were picked out, then the pMD18-T-*PsneIF5A2* recombinants were identified by sequencing. The deduced *PsneIF5A2* protein was characterized using Expasy tools (http://www.expasy.org/tools). Sequence alignments were performed using the ClustalX program, and phylogenetic analyses were performed using MEGA version 4.1 (Tamura et al., 2007).

Construction of plant expression vectors and plant transformation

To express the *PsneIF5A2* gene in plants, the *PsneIF5A2* gene was cloned from pMD18-T-*PsneIF5A2* using the primers listed in Table 1. It was inserted into the *Xba* I and *Sac* I sites of the binary vector pROKII under the control of a CaMV35S promoter and a NOS terminator along with a kanamycin (Km)-resistance gene as a selectable marker. The entire construction diagram and the structures of the plasmid are shown in Fig. 3a. The recombinant was identified using sequencing analysis. Then, the resulting binary vector, *i.e.*, pROKII-*PsneIF5A2*, was transferred into the *Agrobacterium tumefaciens* strain EHA105 using the freeze-thaw transformation method (Chen et al., 1994). To study gene function in a model plant, wild-type *Arabidopsis* was transformed with pROKII-*PsneIF5A2* recombinant. *Arabidopsis* flowers were dipped into *A. tumefaciens* EHA105 suspended in a solution containing 10 mM $MgCl_2$, 5% sucrose and 0.02% Silwet L-77. Invert plants and dip aerial parts of plants in the *Agrobacterium* cell suspension for 10 s with gentle agitation. Excessive bacteria liquid was absorbed with absorbent paper. Then the *Arabidopsis* plants were covered with a plastic bag and incubated in a growth chamber for 1 d. Subsequently, the plants were permitted to grow normally in the growth chamber. Finally, *Arabidopsis* seeds were harvested and selected on selection medium containing 50 mg L^{-1} Km.

qRT-PCR analysis

To detect the expression level of *PsneIF5A2* in transgenic *Arabidopsis*, rosette leaves of 30-day-old *Arabidopsis* were harvested to estimate the transcript level of *PsneIF5A2* gene in transgenic and wild-type *Arabidopsis* using qRT-PCR, with *Atactin* gene as an internal reference. Total RNA of samples was isolated with RNA extraction Kit. All RNAs were checked by NanoDrop 2000c and were reverse-transcribed as described above. 2 µl of cDNA template (equivalent to 100 ng total RNA) was used in qRT-PCR with SYBR Premix EX Taq II (TaKaRa) and the MJ Opticon 2 System (Bio-Rad, USA) according to the manufacturer's instructions. The primers used in the present study for qRT-PCR are listed in Table 1. 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 (Livak and Schmittgen, 2001).

Northern blot analysis

To further investigate the expression of the *PsneIF5A2* in *Arabidopsis*, total RNA (10 µg) of rosette leaves was separated on a 1% agarose-denaturing formaldehyde gel, transferred to a Hybond-N^+ nylon membrane and fixed with UV cross-linking (254 nm, 8 min) for northern blot analysis. The membrane was hybridized with full length *eIFA5A2* cDNA labeled with DIG-dUTP. Hybridization and detection were conducted in accordance with the manufacturer's instructions (DIG Northern starter Kit, Roche, Switzerland).

Germination and seedling growth for the stress tolerance assay

To examine the effects of $CuSO_4$ and NaCl stresses on germination, germination assays were performed on MS medium containing $CuSO_4$ (150 µM) or NaCl (100 mM). *Arabidopsis* seeds were stratified at 4°C for 3 d and then incubated at 22°C under light conditions. The number of seeds with fully-emerged radicles were counted in three independent experiments to score the seed germination, which was expressed as a percentage. For the root growth assay, seeds germinated after stratification and grew for 6 d on stress-free MS medium. Then, the seedlings were transferred to MS medium supplemented with 150 µM $CuSO_4$ or 100 mM NaCl and grown vertically.

Measurement of physiological parameters involved in stress tolerance

To determine the physiological parameters of transgenic and wild-type plants, the seeds were sterilized and sown on MS medium for 7 d and then transplanted into soil in pots and grown in a growth chamber with a 10 h light/14 h dark cycle. Three weeks later, the 28-d-old seedlings were treated with 5 L of 150 mM $CuSO_4$ or 100 mM NaCl solution in a container for 4 d and the malondialdehyde (MDA) content, superoxide dismutase (SOD) and peroxidase (POD) activities were measured following Wang et al. (2010). The electrical conductivity was measured according to Kwak et al. (2005). Each sample contained at least 9 seedlings, and all of the experiments were repeated at least three times.

In situ histochemical examination of cell death and reactive oxygen species (ROS)

A histochemical staining procedure was used to detect superoxide and hydrogen peroxide *in situ* as described by Fryer et al. (2002) and Yang et al. (2014). Leaves were sampled from transgenic and wild-type plants after 2 h of $CuSO_4$ (75µM and 150 µM) or NaCl (100 mM and 200 mM) treatments and immediately used for histochemical staining analysis. The *in situ* accumulation of O_2^- and H_2O_2, cell death, and ROS were examined based on histochemical staining using nitroblue tetrazolium (NBT), 3,30-diaminobenzidine (DAB) and Evans blue. To detect O_2^-, the leaves were immersed in 1 mg ml^{-1} fresh NBT solution (prepared in 10 mM phosphate buffer, pH 7.0) at ambient temperature until dark spots appeared. To detect H_2O_2, the leaves were immersed in 1 mg ml^{-1} fresh DAB solution (prepared in 0.2 mM phosphate buffer, pH 7.0) and incubated in the light until brown spots were observed. To determine whether $CuSO_4$ and NaCl treatment caused cell death, the leaves were incubated in 1 mg ml^{-1} fresh Evans blue solution (dissolved in water) at room temperature. The leaves were vacuumed for 10 min after dipping in the dyes. Then, the leaves were transferred to 75% ethanol plus 25% acetic acid to remove the pigment.

Statistical analysis

The data were analyzed using the SPSS software package (SPSS, Chicago, IL, USA). In all analyses, the differences between the transgenic lines and the wild-type were evaluated using SNK test. The level of significance was set at $P \leq 0.05$, and sample variability was calculated using the standard deviation (s.d.).

Conclusion

The eIF5A protein has been implicated in many vital functions in eukaryotic cells. We propose that *PsneIF5A2* increases $CuSO_4$ and NaCl stress tolerance in plants *via* several physiological pathways, including improvement of

protein synthesis, increase of activities of SOD and POD, and protection of cell membranes. Therefore, these findings suggest that *PsneIF5A2* plays a vital role in modulating responses to abiotic stress and be a useful candidate gene for improving adversity tolerance in plants.

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