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Abiotic Stress Resistance, a Novel Moonlighting Function of Ribosomal Protein RPL44 in the Halophilic Fungus *Aspergillus glaucus*

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Ribosomal proteins are highly conserved components of basal cellular organelles, primarily involved in the translation of mRNA leading to protein synthesis. However, certain ribosomal proteins moonlight in the development and differentiation of organisms. In this study, the ribosomal protein L44 (RPL44), associated with salt resistance, was screened from the halophilic fungus *Aspergillus glaucus* (AgRPL44), and its activity was investigated in *Saccharomyces cerevisiae* and *Nicotiana tabacum*. Sequence alignment revealed that AgRPL44 is one of the proteins of the large ribosomal subunit 60S. Expression of *AgRPL44* was upregulated via treatment with salt, sorbitol, or heavy metals to demonstrate its response to osmotic stress. A homologous sequence from the model fungus *Magnaporthe oryzae*, MoRPL44, was cloned and compared with *AgRPL44* in a yeast expression system. The results indicated that yeast cells with overexpressed *AgRPL44* were more resistant to salt, drought, and heavy metals than were yeast cells expressing *MoRPL44* at a similar level of stress. When *AgRPL44* was introduced into *M. oryzae*, the transformants displayed obviously enhanced tolerance to salt and drought, indicating the potential value of *AgRPL44* for genetic applications. To verify the value of its application in plants, tobacco was transformed with *AgRPL44*, and the results were similar. Taken together, we conclude that *AgRPL44* supports abiotic stress resistance and may have value for genetic application.

Halotolerant and halophilic microorganisms are widely distributed in saline environments such as solar salterns, seawater, and concentrated brines (1–4). These microorganisms can adapt to extreme concentrations of sodium chloride (NaCl) and often to high concentrations of other ions, high UV irradiation, and in some cases, extremes of pH (3, 5). These characteristics make halotolerant and halophilic microorganisms valuable resources for genes that confer stress tolerance and the investigation of stress resistance mechanisms. Halophilic and halotolerant microbes are also of interest for their potential industrial applications, including as a source of transgenes for improving osmotolerance of industrially important yeasts. The gene source from these halophilic and halotolerant organisms might also provide aids to enhance the halotolerance of some plant species and help overcome the global problem of salinization of agricultural areas (5–7).

Previously, we isolated the halophilic fungus *Aspergillus glaucus* from air-dried wild vegetation at the surface periphery of a solar salt field. The salinity range of growth for this isolate, defined *in vitro*, was from 0 to 32% (saturation) NaCl, with a broad optimum from 5 to 15% NaCl (8). To elucidate the saline stress response mechanisms, a cDNA yeast library was constructed from mycelia exposed to NaCl for 24 h. By screening the cDNA library on potato dextrose agar supplemented with different concentrations of NaCl, we isolated some genes that have strong saline resistance. Among the identified sequences, a cDNA fragment representing an *A. glaucus* ribosomal protein (RP) L44 gene (designated *AgRPL44*) was found.

Ribosomal proteins make up the protein portion of the ribosome and with rRNA are essential for protein synthesis (9, 10). However, recent studies have shown that some ribosomal proteins may also participate in cellular events apart from protein biosynthesis, and this has inspired research interest in the genes that encode them. Such extraribosomal functions include DNA reparation, RNA chaperone activity, cell development, regulation of differentiation, pathogen resistance mechanisms, and tumor repression (10–14). RPS3e is involved in DNA repair and selective gene regulation via the NF-κB signaling pathway (15). Plastid-specific ribosomal protein 2 (PSRP2), with RNA chaperone activity, participates as a negative regulator in seed germination and seedling growth under abiotic stress conditions and thus has a role in the abiotic stress response (16). Overexpressed StoL13a in potato (*Solanum tuberosum*) was shown to enhance disease resistance against *Verticillium dahliae* infection in transgenic potato plants (17). An antimicrobial peptide purified from skin secretions and epithelial cells of rainbow trout (likely the 40S ribosomal protein S30) has a protective function against intracellular or extracellular pathogens (18). Research on ribosomal proteins RPL24, RPL31, and RPS23 of the giant panda (*Ailuropoda melanoleuca*) shows that they have anticancer activity (19–21). Overexpression of human ribosomal protein RPL36A may be associated with hepatocarcinogenesis and functionally relates to tumor cell proliferation; RPL36A may thus be a potential target gene for anticancer therapy in hepatocellular carcinoma (22). In summary, it is clear that the functions of ribosomal proteins extend beyond the ribosome, and such moonlighting may alert the cell to stresses or defects in ribosome protein synthesis itself. Indeed, there is increasing evidence that among ribosomal proteins extraribosomal moonlighting is widespread.

Ribosomal protein genes are regulated in response to environmental stresses (16, 23–25). However, the mechanism of their

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resistance to abiotic stresses has not been described. In the present study, to gain further insight into the function of the *AgRPL44* gene, its expression patterns were measured in response to NaCl, sorbitol, and $CuSO_4$ stresses. We used a yeast expression system to generate a transgenic *Saccharomyces cerevisiae* strain that overexpresses *AgRPL44* or *MoRPL44* (a homologous sequence from *Magnaporthe oryzae*), and then abiotic stress resistance of transgenic yeast was examined. To verify the value of its application in genetic engineering, we transformed *AgRPL44* into the model fungus *M. oryzae* and model plant tobacco. Overexpressing *AgRPL44* enhanced tolerance to salt and drought in transgenic *M. oryzae* and transgenic tobacco. The present study provides a candidate gene for genetic engineering of enhanced stress tolerance in crops and explores the possibility of a new screening marker for construction of an *M. oryzae* mutant library.

MATERIALS AND METHODS

Strains and culture conditions. Spores of *Aspergillus glaucus* were cultured (10^6 to 10^{10} spores/ml) in potato dextrose medium containing 10% NaCl at 30°C for about 6 days. The *Escherichia coli* strains employed in this study were DH5α and BL21. Standard procedures were used for manipulating bacterial cells and recombinant DNA (26, 27). The *Saccharomyces cerevisiae* strains used in the present study were His-, Leu-, Trp-, and Ura-AH109. Yeast cells were grown in yeast potato dextrose (YPD) containing 2% peptone, 1% yeast extract, and 2% glucose. Yeast strain maintenance was achieved by plating onto yeast nitrogen base-glucose medium supplemented with 2% (wt/vol) agar.

Isolation and sequence analysis of *AgRPL44*. A yeast expression library containing full-length cDNAs of *A. glaucus* was constructed in our lab. Briefly, total RNAs of *A. glaucus* treated by different concentrations of NaCl (5%, 10%, and 20% [wt/vol]) were isolated and purified together. Then, full-length cDNAs were synthesized and cloned into pDONR222 and pYES2-DEST52 through the Gateway cloning system (Invitrogen, Shanghai, China). Lastly, the library was introduced into *S. cerevisiae*. The titer of the library was 1.28×10^6 CFU/ml, and the number of total colonies was 1.28×10^7 CFU. The rate of positive recombinants clones was 93%, and the size of the average insert cDNA was 1 kb. Salt-tolerant transformants were isolated by screening the yeast expression library. The inserted cDNA fragments were identified via PCR amplification using primers S1/S2. After purification, the PCR products were combined with the plasmid pMD-18T (TaKaRa, Dalian, China) and sequenced. Molecular weight (MW) and isoelectronic point (pI) predictions for the deduced AgRPL44 protein were performed using the ProtParam tool (http://www.expasy.org/tools/protparam.html). Multiple sequence alignment was performed with ClustalW and MEGA5.05.

***AgRPL44* gene expression profiles in response to abiotic stresses.** Total RNA from *A. glaucus* was extracted before and after treatment with 20% NaCl, 2 M sorbitol, or 10 mM $CuSO_4$ using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. First-strand cDNAs were synthesized from 2 μg of total RNA (pretreated with DNase I) with avian myeloblastosis virus reverse transcriptase (TaKaRa). Semiquantitative reverse transcription (RT)-PCR was used to determine expression of the *AgRPL44* gene. Primers R1/R2 (see Table S1 in the supplemental material) used in RT-PCR had high specificity, as determined by agarose gel electrophoresis, and were confirmed by sequencing. The RT-PCRs were performed using TaKaRa DNA polymerase (TaKaRa, Dalian, China). The cycle numbers of the PCRs were adjusted for each gene to obtain visible bands on agarose gels. The *ACTB* (β-*actin*) gene was used as the internal control.

Experiments for adverse tolerance analysis in yeast. Ribosomal protein is highly conserved. We found that the *M. oryzae* ribosomal protein gene *MoRPL44* is 85.85% homologous with *AgRPL44* (see Fig. S1 in the supplemental material). To further investigate the salt tolerance of 60S ribosomal protein L44, we also cloned the *MoRPL44* gene. The *AgRPL44* open reading frame (ORF) was amplified from the *A. glaucus* cDNA clone via PCR using the primers Y1/Y2. The *MoRPL44* ORF was amplified through RT-PCR using primers Y3/Y4. The PCR-amplified product was digested with BamHI-HindIII and directionally cloned into the yeast expression vector pURL129, which contains the URA3 selection marker, and was identified by PCR and sequencing. Sequence analysis confirmed that *AgRPL44* or *MoRPL44* was inserted into the pURL129 plasmid. The recombinant plasmid pURL129-*AgRPL44* or pURL129-*MoRPL44* was transformed into competent *S. cerevisiae* AH109 cells. Selection and growth of transformants were performed in synthetic medium (yeast nitrogen base, 2% glucose) lacking uracil.

For growth assays, yeast cells harboring the empty vector pURL129 (as a control) or recombinant plasmid were pregrown for 2 days in YPD and then resuspended in YPD to an optical density at 600 nm (OD_{600}) of 0.5 and diluted in a 10-fold dilution series, of which 4 μl was spotted onto agar plates supplemented with 10%, 15%, or 20% NaCl, 1 M or 2 M sorbitol, or 10 mM $CuSO_4$. Growth conditions were maintained for 2 to 5 days at 30°C.

Analysis of salt and drought tolerance in *Magnaporthe oryzae*. *M. oryzae* and *A. glaucus* both are filamentous fungi. *M. oryzae* is often used as a model fungus, and the method for its genetic transformation has been well refined. We transformed the *AgRPL44* gene into *M. oryzae* to verify its tolerance to salt and drought. The expression vector introduced into *M. oryzae* was constructed for expression of the *AgRPL44* gene by cloning it (using primers M1/M2) at the XbaI site into the pGFPGUS*Plus* vector (28). The *M. oryzae* strain 70-15 was cultured on complete medium (CM), 5× yeast extract-glucose (YEG; 0.5% yeast extract and 2% glucose), and oatmeal agar as previously described (29). To enhance conidiation, culture plates were grown under constant fluorescent light at room temperature. Protoplasts were prepared and transformed by polyethylene glycol treatment as described previously (30). Transformants were selected on TB3 medium (0.3% yeast extract, 0.3% Casamino Acids, and 20% glucose) with 250 mg ml^{-1} hygromycin B (Calbiochem). Transformants overexpressing *AgRPL44* were further confirmed by Western blot assay.

Mycelium blocks 10 mm in diameter from wild-type *M. oryzae* or transformed *M. oryzae* with an empty vector or *AgRPL44* were obtained using a round punch, after which they were inoculated on 0%, 5%, 10%, or 15% NaCl and 5% sorbitol solid CM culture medium and cultured 7 to 10 days under normal conditions. Vegetative growth and mycelium growth diameters of wild-type and transformed *M. oryzae* were determined to investigate salt tolerance and drought resistance.

Western blot assay. The ORF of *AgRPL44* was constructed into vector pET32a (Novagen, Billerica, MA, USA) to generate a fusion protein with a hexahistidine tag. The PCR product (using primers A1/A2) was digested with BamHI and HindIII. The identity of the recombinant pET32a-*AgRPL44* construct was confirmed by DNA sequencing and then transformed into *E. coli* BL21 cells. The BL21 cells were induced by 1 mM isopropyl-beta-D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. Cells were then harvested by centrifugation and disrupted by physical fragmentation. Inclusion bodies were dissolved with 6 M urea on ice. The supernatant was filtered through a 0.45-μm membrane and purified by affinity chromatography using a nickel-nitrilotriacetic acid column (Ni-NTA agarose; Roche). The protein was renatured through step dialysis at 4°C for 12 h. The primary polyclonal antibody was produced via the immunization of a New Zealand rabbit with AgRPL44 protein (31).

The total proteins of *M. oryzae* overexpressing *AgRPL44* were extracted and resolved via SDS-PAGE. After separation, proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked overnight at 4°C in Tris-buffered saline with Tween 20 (TBST) buffer with 5% nonfat milk. The membrane was incubated with primary rabbit anti-AgRPL44 antibody (dilution, 1:10,000 in TBST) at room temperature for 2 h. The membrane was washed with TBST buffer three times and then incubated with 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at 37°C (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

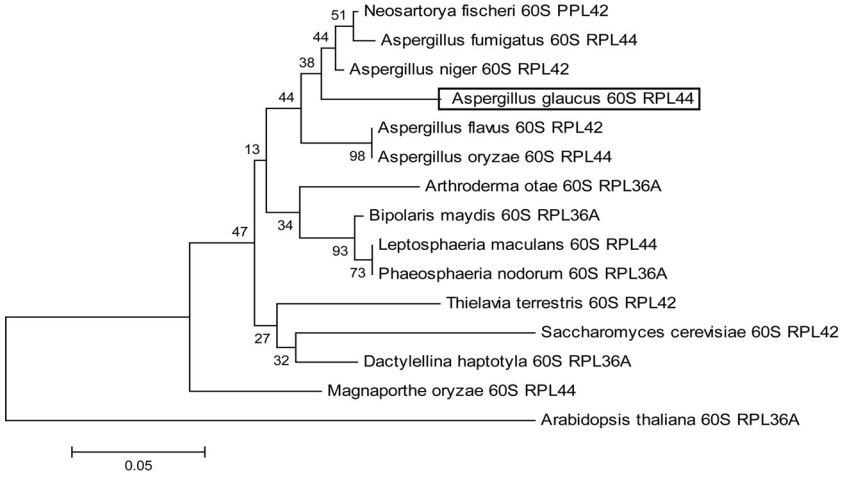

FIG 1 Phylogenetic tree of *AgRPL44* from various species. The multiple alignments generated by ClustalW and the phylogenetic tree were constructed with MEGA5.05 using a bootstrap test of phylogeny with the minimum evolution test and default parameters. GenBank accession numbers of RPL44 proteins used for drawing the phylogenetic tree are shown in Table S1 in the supplemental material.

Plant transformation and salt resistance assay, postgermination. The role of the ribosomal protein RPL44 gene in salt stress tolerance was investigated by generating transgenic tobacco plants overexpressing *AgRPL44*. The transformation vector was constructed for expression of the ribosomal protein *RPL44* gene by cloning it at the BamHI and SacI sites into plasmid pBI121 using primers P1/P2 and a plant expression vector carrying a cauliflower mosaic virus 35S (CaMV35S) promoter and nopaline synthase (NOS) terminator (see Fig. 5A). The construct was introduced into competent cells of *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method. Tobacco (*Nicotiana tabacum* L.) plants were transformed using the *Agrobacterium*-mediated leaf disc method. The regenerated seedlings were selected on Murashige and Skoog medium containing a final concentration of 40 mg/liter of kanamycin. The transgenic lines were also confirmed by RT-PCR using primers E1/E2. Three independent transgenic homozygous T2 line (TG) seedlings and the pBI121 vector control (VC) lines were used for subsequent experiments.

For early growth assessments, well-grown tobacco plants (VC) and three transgenic line seedlings of 3 weeks were placed in Hoagland's nutrient solution containing NaCl at different concentrations (0 mM, 100 mM, or 200 mM). Tobacco plants were maintained vertically under normal conditions. The root length and fresh weight were monitored after the 15th day.

Nucleotide sequence accession number. The sequence of the *AgRPL44* gene determined in this study has been deposited in GenBank under accession number KJ679499.

RESULTS

Isolation and sequence analysis of the ribosomal protein *AgRPL44* gene. *S. cerevisiae* is an excellent single-cellular model for quickly identifying functional genes. Based on screening of the yeast expression library constructed in our lab, a yeast colony that was able to grow on YPD solid medium supplemented with 10%, 15%, or 20% NaCl was selected. The cDNA fragments inserted from the salt-tolerant transformants were identified by PCR amplification and then sequenced. Protein conservative domains were analyzed via NCBI BLAST alignment. We found that a protein encoded by *AgRPL44* belongs to the 60S ribosomal protein family. The ribosomal protein RPL44 cDNA is 462 bp in length, including a complete ORF of 321 bp encoding a putative protein of 106 amino acids (predicted relative molecular mass, 12.09 kDa; theoretical pI, 10.43).

Analyzing the evolutionary relationships among the various species of RPL44 might provide clues to the evolution of their function. To further characterize the AgRPL44 protein, 15 RPL44 proteins in different species were aligned. In the phylogenetic tree (Fig. 1), AgRPL44 and other 60S ribosomal proteins of five *Aspergillus* species were on the same branch, indicating that AgRPL44 is closely related to the *Aspergillus* 60S ribosomal protein RPL44.

Ribosomal protein-encoding *AgRPL44* is more resistant to abiotic stresses than *MoRPL44* in yeast. Wild-type yeast cells (AH109) and yeast transformants harboring the empty vector pURL129, pURL129-*AgRPL44*, or pURL129-*MoRPL44* were spotted in a 1:10 dilution series on defined medium supplemented with NaCl or sorbitol as indicated and incubated for 3 to 5 days at 30°C. Each experiment was repeated at least three times, and a representative example is shown.

The data show that *AgRPL44*- or *MoRPL44*-transformed yeast could survive on 10% and 15% NaCl while the wild-type yeast could not. However, only *AgRPL44*-transformed yeast could survive on 20% NaCl YPD medium. At the same salt concentration, *AgRPL44*-transformed yeast had stronger vitality than *MoRPL44*-transformed yeast. In addition, *AgRPL44*-transformed yeast was more resistant to drought and heavy metals than *MoRPL44*-transformed yeast at a similar level of stress (Fig. 2).

Expression of *AgRPL44* in response to different abiotic stresses. The expression of *AgRPL44* in response to NaCl, sorbitol, or $CuSO_4$ in *A. glaucus* was investigated using semiquantitative RT-PCR. *AgRPL44* was induced at 1 h and reached a maximum at 8 h after treatment with NaCl (Fig. 3). During sorbitol or $CuSO_4$ treatment, the expression of *AgRPL44* peaked at 4 h. These results suggest that expression of the *AgRPL44* gene is induced by NaCl, sorbitol, and $CuSO_4$ treatments.

***AgRPL44* gene can promote adverse-stress resistance levels in *M. oryzae*.** We constructed an expression recombinant plasmid, pGFPGUS*Plus*::*AgRPL44*, for *M. oryzae* and introduced it or an empty vector into *M. oryzae*. The *AgRPL44* transformant showed high expression of the AgRPL44 protein, as confirmed by Western blotting (Fig. 4A).

We tested transgenic strains' responses to adverse stresses after introducing the *AgRPL44* gene. The result showed that transfor-

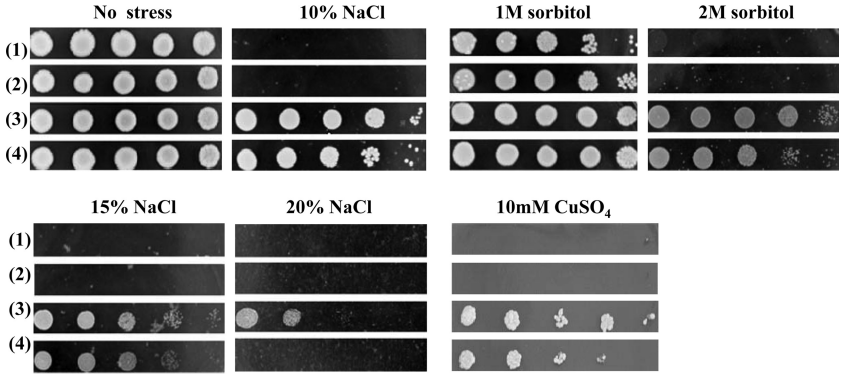

FIG 2 Expression of *AgRPL44* or *MoRPL44* in yeast enhances growth at high osmolarity. At high osmolarity, yeast cells (AH109) expressing *AgRPL44* or *MoRPL44* show increased growth compared with control cells transformed with an empty vector (pURL129). Transformants were spotted in 1:10 dilution series on YPD medium supplemented with NaCl, sorbitol, or $CuSO_4$ as indicated and incubated for 3 to 5 days at 30°C. Rows: 1, wild-type yeast AH109; 2, transformants with empty vector (pURL129); 3, transformants with *AgRPL44*; 4, transformants with *MoRPL44*. Each experiment was replicated at least three times. A representative example is shown.

mation with *AgRPL44* was associated with greater salt and drought tolerance in transgenic *M. oryzae*. Under the same culture conditions (temperature, humidity, and time), on both the 5% and 10% NaCl solid CM, the wild-type *M. oryzae* and empty vector transformant remained alive, but the *AgRPL44* transformant had greater salt tolerance and viability than either of the other two groups. On the 5% NaCl solid CM, the mycelium growth diameters of the wild type and empty vector transformant were 24.6% and 32% that of the *AgRPL44* transformant, respectively. On the 10% NaCl solid CM, the mycelium growth diameters of the wild type and empty vector transformant were only 15% and 9% that of the *AgRPL44* transformant, respectively (Fig. 4C). On the 15% NaCl solid CM, the *AgRPL44* transformant could still grow, but the wild type and empty vector transformant could hardly stay alive (Fig. 4B).

We also inoculated the wild-type and transformed *M. oryzae* in 5% sorbitol solid CM under 28°C culture 7 to 10 days. Compared with that of the wild type, the mycelial growth area of the *AgRPL44*-transformed *M. oryzae* was large (Fig. 4B) and the mycelium growth diameters of the wild type and empty vector transformant were 25.3% and 55.9% that of the *AgRPL44* transformant, respectively (Fig. 4C). These results show that *AgRPL44* has a very important role in salt and drought tolerance. *AgRPL44* in *M. oryzae* might provide a new, simple, and low-cost screening marker for *M. oryzae* mutant library screening.

Overexpression of *AgRPL44* improves tolerance of transgenic tobacco plants to salt stress. Tobacco leaf disks were transformed with the *AgRPL44* gene. Transgenic lines were confirmed by RT-PCR (Fig. 5B). Three independent T2 lines (TG 2, TG 5, and TG 9) of the *AgRPL44* transgenic plants were chosen for further analysis.

To evaluate the stress tolerance of the control (VC) and transgenic (TG) tobacco lines, seedlings were exposed to different concentrations of NaCl for salt tolerance analysis. The growths of the VC and TG lines under normal conditions were similar (data not shown). However, when treated with NaCl, the TG plants adapted much better to the stress than did the control lines (Fig. 5C). Under normal and 100 mM or 200 mM NaCl conditions, the root lengths and fresh weights of the VC and TG plants were comparable (Fig. 5D and E). As the NaCl concentration increased, the root lengths of the control lines were significantly arrested, whereas the roots of the TG plants continued to grow. At the NaCl concentrations of 100 and 200 mM, the root lengths of the control lines were 50.4% and 48.6% those of the TG lines, respectively, showing that the transgenic seedlings were significantly more salt resistant. It was easily observed that the health of the transgenic plants was superior to that of the control lines. The growth of the transgenic plants was robust, and their leaves were bright green, but the growth of the seedlings of the control lines was significantly inhibited and their leaves were chlorotic.

Transgenic plants also exhibited greater fresh weight than the VC. The fresh weight of the wild type was only 55.4% that of the transgenic lines, a significant difference between the two. At the NaCl concentration of 200 mM, the fresh weight of the wild type was 37.8% that of the transgenic lines (Fig. 5E). These results indicate that overexpression of *AgRPL44* enhanced the salt stress tolerance of transgenic tobacco after germination.

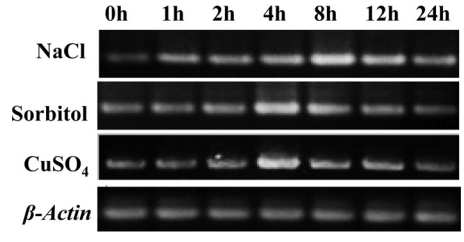

FIG 3 Semiquantitative RT-PCR analysis of *AgRPL44* expression under different abiotic stresses. RT-PCR experiments were conducted with total RNA isolated from *A. glaucus* before and after stress treatments consisting of 20% NaCl, 2 M sorbitol, or 10 mM $CuSO_4$ for the indicated times.

DISCUSSION

In the present study, a ribosomal protein gene (*AgRPL44*) was isolated by screening an *A. glaucus* cDNA library. Sequence alignment revealed that AgRPL44 belongs to the 60S ribosomal protein RPL44. *AgRPL44* expression was strongly induced in *A. glaucus* by exposure to salinity (NaCl), drought (sorbitol), or heavy metal ($CuSO_4$), suggesting that *AgRPL44* has an important role in responses to these abiotic stressors. The yeast transformed with *RPL44* exhibited significantly elevated tolerance to salt, drought,

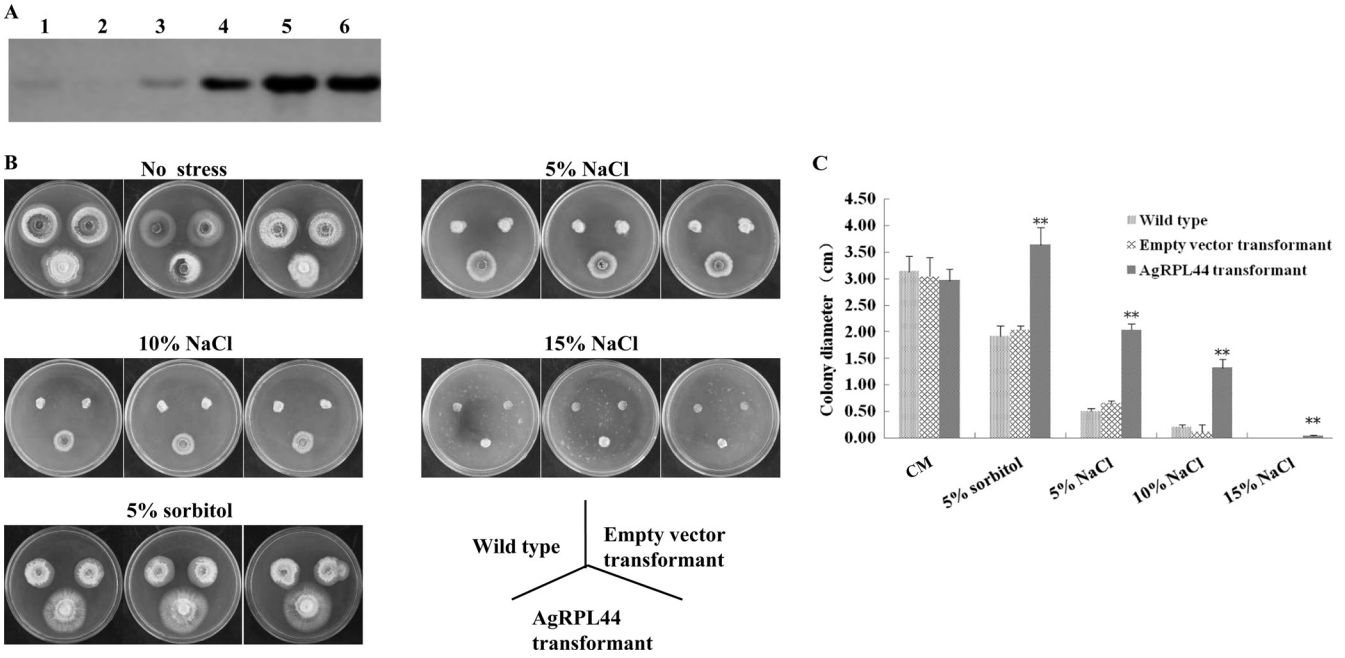

FIG 4 Analysis of stress resistance in *Magnaporthe oryzae*. (A) Western blot confirmation of *AgRPL44* protein expression. Lane 1, wild-type *M. oryzae*; lane 2, *M. oryzae* transformed with empty vector; lane 3, halophilic *A. glaucus*; lanes 4 to 6, three *M. oryzae* strains transformed with *AgRPL44*. (B) The wild type and transformants were incubated on complete medium alone (CM) or CM supplemented with NaCl or sorbitol as indicated for 7 to 10 days at 30°C. (C) Mycelium growth diameter of wild-type and transformed *M. oryzae*. Each experiment was replicated at least three times. A representative example is shown. The data represent means ± standard deviations (SD) of results from three experiments performed. Significant differences between the *AgRPL44* transformants and control groups are indicated with asterisks (**, $P < 0.01$).

and heavy metal stresses. Interestingly, the tolerance to these conditions in the transformed *AgRPL44* yeast strain was stronger than that of the *MoRPL44* strain. This suggests that *AgRPL44* expression confers tolerance to a variety of abiotic stresses. When *AgRPL44* was introduced into *M. oryzae*, transformants displayed obviously enhanced tolerance to salt and drought, revealing a genetic application for *AgRPL44*. In addition, overexpression of *AgRPL44* in tobacco resulted in enhanced salt stress tolerance.

Ribosomal protein (RP) gene expression can be dramatically altered in response to growth stimuli and environmental stresses. For example, plant ribosomal proteins S14, S15a, S16, L10, L13a, and L30 can be induced by diverse phytohormones, including auxin (32), cytokinin (32, 33), and abscisic acid (33), and by stress-inducing stimuli, including heat (32), cold (32, 34), and UVB stress (35). Recent analysis of the expression of RP genes and the differential accumulation of RPs in *Arabidopsis* roots upon phosphate or iron deficiency revealed that these 579 RP genes were associated mainly with abiotic stress responses. Some of the highly expressed RP genes were also involved in responses to cold, UVB, and salt stresses (36). It might thus be assumed that some ribosomal proteins have protective functions against multiple abiotic and nutrient stresses.

Although the ribosomal protein RPL44 is highly conserved in many fungal strains, under the same abiotic stresses *AgRPL44* was associated with greater stress tolerance than *MoRPL44*, especially for salt; only the *AgRPL44* transformant could survive on 20% NaCl YPD. Comparing the protein sequences of AgRPL44 and MoRPL44, it was found that they shared identical motifs: their amino acid residues 1 to 9, 24 to 33, 36 to 74, and 84 to 105 are nearly identical. Therefore, we could deduce that these regions were probably essential for the stress resistance function of RPL44. Although the two RP genes are highly conserved, there are some specific amino acid residues in the AgRPL44 sequence, for instance, lysines 12 and 80, histidine 17, glutamines 21 and 75, leucine 34, or serines 35 and 83, which may play a very key role in its remarkable abiotic-stress resistance. We will further proceed with site-specific mutagenesis to verify our deduction.

A. glaucus is halophilic and therefore grows more slowly under low-salt conditions than it does under high salt (8). However, higher salt no doubt affects the growth of most eukaryotic organisms. The expression patterns (Fig. 3) of *AgRPL44* may in part explain the resistance to high salt in *A. glaucus*. Thus, we presume that when *A. glaucus* is under osmotic stresses, to eliminate saline damage, it enhances the expression levels of *AgRPL44*. Overexpression of *AgRPL44* in *M. oryzae* markedly increased the transformants' ability to withstand salinity via exposure to NaCl. Moreover, mycelial growth, conidiation, and pathogenicity were evaluated, and there was no difference between the wild type and the transformed strains under normal conditions (data not shown). Thus, we could explore a new simple, practical, and low-cost selection marker for genetic transformation. Some ribosomal proteins have been utilized as selectable markers. For example, the small ribosomal protein S12P is used as an efficient positive-selection marker in allelic exchange mutation systems for *Corynebacterium glutamicum* (37, 38). A mutant protein of RPL44 from *Aurantiochytrium* sp. strain KRS101 affords cycloheximide resistance to strains, serving as a novel selection marker (39). The present *M. oryzae* transformants were selected with a high concentration of hygromycin B or phleomycin D1 (Zeocin) (40), both of which are toxic and expensive. In our research, we might use

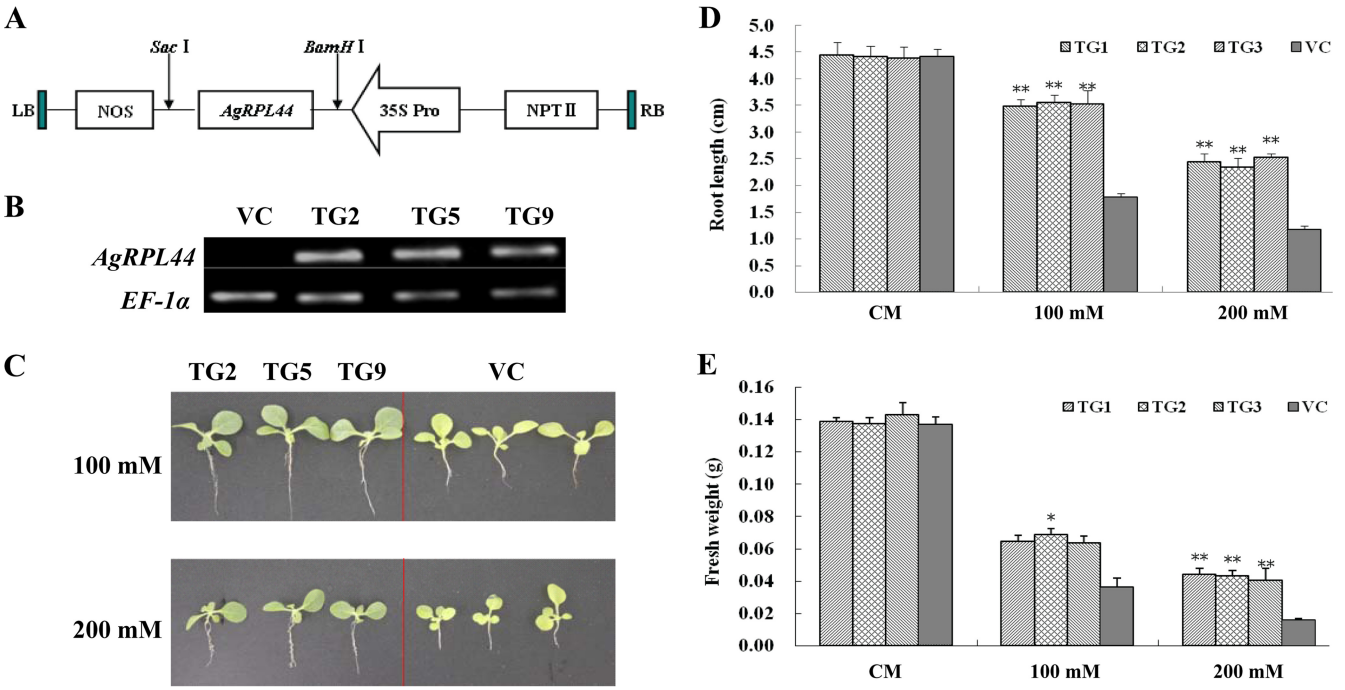

FIG 5 Assay of early development of *AgRPL44*-transformed tobacco plants. (A) Schematic of the vector used for plant transformation. (B) RT-PCR analysis of transgenic lines with the pBI121::*AgRPL44* construct. (C) Phenotype of wild-type and TG tobacco after 2 weeks of 100 mM or 200 mM NaCl treatment. (D) Root lengths of tobacco plants overexpressing the *AgRPL44* gene. (E) Fresh weights of tobacco plants overexpressing the *AgRPL44* gene. The data represent means ± SD of results from three experiments performed. Significant differences between the TG and control lines are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

RPL44 to create a dominant selectable marker expressed with the aid of native transcriptional and translational machinery, and it is safe in biological transformation. Our results may be significant for *M. oryzae* mutation library screening. Despite this, further work is required. The optimization of transformation conditions and integration into various regions of ribosomal DNA (rDNA) may further increase conversion efficiency.

In summary, our work presented here has demonstrated ribosomal protein AgRPL44 as being involved in the response to abiotic stresses and revealed a previously unrecognized role of abiotic stress resistance in transgenic yeasts and transgenic plants. We might explore a new, simple, and safe screening marker for *M. oryzae* mutant library screening. However, more work will be needed to measure physiological indices involved in abiotic stresses of mature transgenic tobacco, analyze the expression of stress-related genes to verify whether these genes are directly regulated by *AgRPL44*, and explore its protecting mechanisms against abiotic stresses in transgenic plants.

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