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Full Length Article

Improvement of Salt Tolerance on *Escherichia coli* by Expression of Agglutinin from *Amygdalus mira*

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Abstract

Amygdalus mira (Koehne) Yü et Lu, also called Tibetan peach, is a wild species belonging to the genus *Amygdalus*, from family Roseceae. It is distributed in Tibet and possesses tolerance to extreme environment, such as salt, cold and drought and also possesses resistance to disease. In present research, we cloned the *Am*AGG gene in the root of *A. mira* and analyzed its expression pattern in drought roots of *A. mira* (Koehne) Yü et Lu by qRT-PCR. The result showed the expression of *Am*AGG increased under drought and decreased after re-watered, indicating that the *Am*AGG was induced by drought. Accordinlgy, the recombined *Am*AGG protein (pET-*Am*AGG) was induced and purified from *E. coli* strain (Rossetta). The optimal expression temperature was 28°C, the optimal induction time was 6 h and the optimum concentration of IPTG was 0.1 m*M*, through which the protein was induced and expressed with soluble form in Rossetta strain. The salt tolerance of *E. coli* expressing the agglutinin was estimated by measuring at OD_{600} (optical density at 600 nm) value of Rossetta by supplementing different concentrations of NaCl into the culture media. The results indicated that Rossetta strain harboring the *Am*AGG protein was more tolerant to salt stress than that of harbored pET-15b empty vector, revealed the *Am*AGG might improve the salt tolerance of Rossetta cells. The data suggested that *Am*AGG plays a crucial role in tolerance against drought and salinity stresses.

Keywords: *Amygdalus mira* (Koehne) Yü et Lu; Agglutinin; Expression; Purification; Salt stress; Tolerance

Introduction

Biotic and abiotic stress such as drought and salinity greatly impact agricultural development (Farhad *et al*. 2011). Salinity limit water absorb and increase accumulation of sodium in plants. Therefore, it is a top priority to solving the problem of salinity (Hossain *et al*. 2012).

To successfully survive in their environment, plants have carefully evolved and able to confront complex and adverse environmental conditions including salt, drought, cold, heat, insect and pathogen infections. Now, salt stress is one of the main adverse environmental factors which limit plant growth, development, distribution and production worldwide. Currently, much researches are focused on the molecular mechanisms that control plant response to salt stress. Therefore, many related genes and/or signaling pathways have been discovered and identified (Liu *et al*. 2019; Wu *et al*. 2019).

To date, the role of agglutinin in protecting cells against damage related to biotic and abiotic stresses has been well proved in plants (Jin *et al*. 2012; Suzuki *et al*. 2014; He *et al*. 2017; Eggermont *et al*. 2018; Mostofa *et al*. 2018; Mostafa *et al*. 2018). Agglutinin is a non-immune protein or glycoprotein that is found in plants, animals, microorganisms, and viruses and it specifically combines with different carbohydrates (Nakata *et al*. 2017). Among them, plant agglutinin is easily purified and their many features have been extensively studied (Umadevi *et al*. 2017). These specific features may help us to follow up with a more in-depth understanding. However, there are large researches on the role of agglutinin in response to environmental stresses in different plants, studies on agglutinin of *Amygdalus mira* (Koehne) Yü et Lu have been rarely reported (Kil'dibekova *et al*. 2004; Bezrukova *et al*. 2008).

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A. mira is an important wild peach species that possesses strong adaptability to cold, drought, barrenness and high ultraviolet, which has additional long life (over 1000 years), abundant fruit and other excellent features, means that it is a valuable resource with high ecological and economic merits and is reputed as an ideal wild peach germplasm for improving cultivated peach plants (Liu *et al*. 2009; Peng *et al*. 2015; Cao *et al*. 2017). As a wild species, it can be used to regain vegetation and control soil run off while showing a tolerance to various environmental stresses (Rzepka-Plevnes *et al*. 2007; Xing *et al*. 2015).

Recombinant DNA technologies have made it possible to obtain a broad range of diverse proteins in various types living organisms, even mutant proteins that do not exist in nature (Gessler and Patocchi 2007; Fujita *et al*. 2016; Misbah *et al*. 2017). Accordingly, recombinant proteins expressions and purification have become fundamental techniques in many aspects of life science research (Noi and Chung 2017). However, the results of these techniques often remain frustratingly elusive (Jensen 1996). The efficient expression of soluble functional proteins isolated from highly complex crude protein lysates in the host, is necessary for successful purification of recombinant proteins. Currently, *E. coli* was one of the most frequently used host organisms for recombinant protein expression. However, over-expression of eukaryotes proteins in *E. coli* often yields inadequate soluble protein (Chae *et al*. 2017).

Materials and Methods

Plant culture and drought treatment

The experiments were carried out at Northeast Forestry University. All seeds were obtained from the Tibetan plateau. After being rinsed and immersed in water for 24 h, the seeds were embedded with wet sand and stored at 4°C for 3 months. After the seeds germinated, the seedlings were planted into the polyvinyl chloride (PVC) pots of (12 cm diameter and 12 cm height) filled with wet sand. Young and fresh roots of water deficit treated for 4, 8, 12, 16 and 20 days were gathered and frozen in liquid nitrogen, then stored at -80°C for total RNA extraction. Three independent repetitions were performed for each treatment.

Gene cloning and bioinformatics analysis

The total RNA of the *A. mira* roots was extracted using a plant RNA extraction kit (TSINGKE, China) according to the instructions. cDNA was composited using reverse transcriptase (promega, U.S.A.). Specific primers for *Am*AGG cloning were designed by using Premier 5.0 and BioEdit. The *Am*AGG-F (sequence: 5′ -ATGGCAGACTTACCAAAGTTTTTATGGCAGACTTACCAAAGTTTTT - 3′) and *Am*AGG-R (sequence: 5′ -TCATCCTGGAAGTTTTTCGAC -3′) were synthesized as the forward and reverse primer, respectively. cDNA of *A. mira* roots for 16 days of drought were used as the templates for *Am*AGG cloning. The obtained PCR fragment was purified and ligated with the pMD18-T vector (Takara, Japan), and then transformed into the *E.* coli (JM109 strain), and the colonies containing the *Am*AGG were sequenced by TSINGKE biological company.

The *Am*AGG protein sequence was analyzed using the ExPASy website (https://www.expasy.org/). Protein hydrophobicity, signal prediction were performed. The phylogenetic tree of *Am*AGG was constructed using MEGA version 5 (Tamura, Peterson, Stecher).

Expression of the *Am*AGG in root of *A. mira*

The qRT-PCR was performed using SYBR Premix Ex Taq II Real-time PCR Master Mix (TAKARA, U.S.A.) with a LightCycler480 (Roche, U.S.A.) in a total volume of 20 μL. Master Mix are composed of *TaKaRa Ex Taq*® HS, dNTP Mixture, Mg^{2+}, Tli RNaseH, SYBR® Green I. The relative expression of the target genes was analysed by the ΔΔCT method. Specific primer pairs were determined using Primer Premier 5.0 software. The forward primer was *Am*AGG1-F: 5′- GAAGACAGCCCGTGGATT-3′ and reverse primer was *Am*AGG1-R: 5′-GGCATTCCTCGTITGAAGACC-3′.

Expression and purification of the *Am*AGG protein in *E. coli* cells

For protein prokaryotic expression vector construction, the *Am*AGG was amplified with forward primer *Am*AGGp-F: 5′ -CTCGAGATGGCAGACTTACCAA-3 ′ and reverse primer *Am*AGGp-R: 5′ -GGATCCTCATCCAAGTTTT-3′ (the underlined are Xho1 and BamH1 sites, respectively) and the purified fragment was constructed into the prokaryotic expression vector pET-15b to generate the recombinant expression plasmid pET-*Am*AGG. Then the recombinant plasmid was transformed into the *E. coli* strain Rossetta, which was cultured at 37°C with shaking at 180 rpm. The *E. coli* cells grew until the OD_{600} value reached about 0.5, using the IPTG in the bacterium solution to induce the protein expression. To obtain the appropriate induction conditions, a time gradient and IPTG concentration gradient were set. To obtain the optimal IPTG concentration, 0.1, 0.5 and 1.0 m*M* were added for protein induction, the time gradient was set for 0, 15, 30, 60, 120, 240, 360 min, respectively.

The Rossetta cells induced by IPTG at 28°C were collected at 13000 rpm for 2 min and then adding a protein loading buffer on an ice bath for 1 h. The loading buffer contained 125 m*M* Tris-HCl (pH 6.8), 4% SDS, 20% Glycerol and 200 m*M* DTT and a trace of bromophenol blue, then the cell suspension was centrifuged and the supernatant was soluble protein. The precipitation was dissolved in a

protein lysis buffer and was classified as insoluble protein. The lysis buffer contained 7 mol/L urea, 2 mol/L thiourea, 2% Chaps, 50 m*M* Tris (pH 8.0), trace bromophenol blue and DTT. The recombinant fusion protein was dissociated by 14.5% SDS-PAGE and visualized Coomassie brilliant blue (CBB) staining.

To obtain the purified *Am*AGG recombinant protein, the Rossetta bacteria solution were cultured at 180 rpm for 2.5 h at 37°C until the OD_{600}nm of the bacterial solution reached 0.4–0.6. Later 0.1 m*M* IPTG was added to bacterial solution and incubated at 28°C, 180 rpm for 6 h. The bacteria culture was harvested and resuspended in the binding buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole, pH 8.0) and the crude protein mixture was centrifuged at 10000 rpm for 10 min. Then, the supernatant was the sample for the recombinant protein to be purified using Novagen's Nitia following the manufacture's guidelines. In brief, 6 mL of 50% Nitia was added to the column and equilibrated with five resin-bed volume of binding buffer, the crude protein was loaded onto the resin-bed. After the resin-bed was washed with washing buffer containing 30, 50 and 80 m*M* imidazole successively, the purified *Am*AGG protein was eluted by five resin-bed volume of elution buffer containing 150 m*M* imidazole. The samples of all purification steps were collected for SDS-PAGE analysis.

The purity of the purified *Am*AGG recombinant protein was assessed with Quantity One v4.62 and then the concentration was measured by the Bradford method with bovine serum albumin as a standard.

Salt stress tolerance assay of *E. coli* expressing the agglutinin protein

Tolerance to salt stress of OD_{600} was tested in an LB (Luria-Bertani) medium supplemented with different concentration of NaCl. The OD_{600} reached a value of 0.5 and was induced with 0.1 m*M* IPTG for 6 h as described above. Then, the samples were diluted 100-fold by LB medium supplemented with NaCl to the final concentration of 0, 200, 400, 600, 800 m*M*, respectively. Empty vector transformed Rossetta cells was used as control and the bacteria growth rate was determined by measuring the OD_{600} value at 0, 2, 4, 6, 8 and 10 h, respectively.

Results

Gene cloning and bioinformatical analysis of *Am*AGG

The *Am*AGG gene was amplified using cDNA libraries from drought roots of *A. mira* by ordinary PCR. The obtained *Am*AGG fragment was then ligated into the PET-15b prokaryotic expression vector and was sequenced.

A multiple sequence alignment of *Am*AGG protein and its bioinformatical characters are shown in Fig. 1A-D. The alignment of the *Am*AGG amino acid sequence and

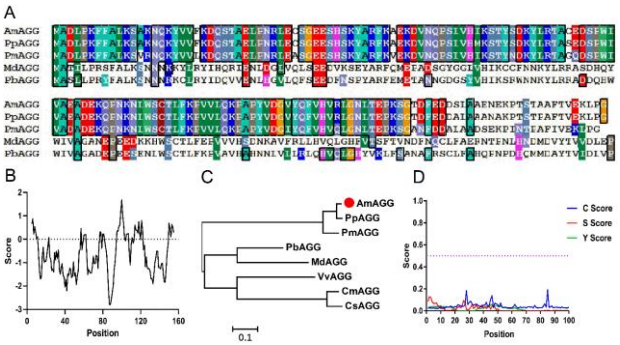

Fig. 1: Multiple sequence alignment and bioinformatical analysis of *Am*AGG. (**A**) Alignment of *Am*AGG protein and other agglutinins from *Rosaceae*. The $\chi 2$ test statistic was 0.00 (P = 1.00000 with 1 degree[s] of freedom). P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages. The analysis involved 3 amino acid sequences. All positions containing gaps and missing data were eliminated. There are a total of 158 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. (**B**) *Am*AGG protein hydrophobicity and hydrophilicity prediction and (**D**) signal peptide prediction of *Am*AGG protein (**C**) Phylogenetic tree analysis of the deduced *Am*AGG protein. *Prunus pcrsica* (*Amygdalus persica* Linn. var. persica f. duplex Rehd), *P. mume* (*Armeniaca mume* Sieb), *Malus domestica* (*M. pumila* Mill), *Pyrus* × *bretschneideri* (*P. bretschneideri*), *Cucumis sativus*, *Cucumis melo*, *Vitis vinifera*

other agglutinins from *Rosaceae* show that the *A. mira* was closest to the *P. pcrsica*, with only one amino acid difference between them, *i.e.*, the 74^{cys} in *A. mira* and 74^{ser} in *P. pcrsica* (Fig. 1A). Phylogenetic tree analysis of the amino acid sequence of *Am*AGG indicates that, compared with *Malus domestica*, *Pyrus* × *bretschneideri*, *Cucumis sativus*, *Cucumis melo* and *Vitis vinifera*, *Am*AGG of *A. mira* was closest to that of *P. pcrsica* and *P. mume* (Fig. 1C).

Protein hydrophobic amino acid composition is the main driving force of protein folding and hydrophilic prediction can reflect the folding of the protein. Hydrophobic/hydrophobic analysis of the *Am*AGG protein was performed using the Kyte and Doolittle algorithm of ProtScale (> 0.5). The hydrophobic region was found in the region of < 0.5, and the hydrophilic region was in the range of < -0.5 and between + 0.5 and - 0.5 Gender area. Hydrophobicity and Hydrophilicity prediction of the *Am*AGG protein was shows that the *Am*AGG protein had 8 hydrophobic regions and 8 hydrophilic regions (Fig. 1B). Signal peptides prediction results indicated that there was no signal peptide was found in N-terminal of the *Am*AGG protein (Fig. 1D).

Expression of the *Am*AGG in root of *A. mira*

A qRT-PCR analysis of the *Am*AGG was performed to determine changes at the *Am*AGG transcription levels were associated with drought (Fig. 2). To verify how drought influenced the expression of the *Am*AGG in the of *A. mira*

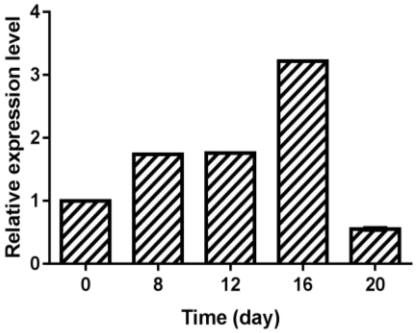

Fig. 2: Quantitative real-time PCR analysis of *Am*AGG in root tissues in response to drought at different time intervals. 0,8,12,16 means day 0,8,12,16 under drought treatment, 20 means day 4 under re-watering

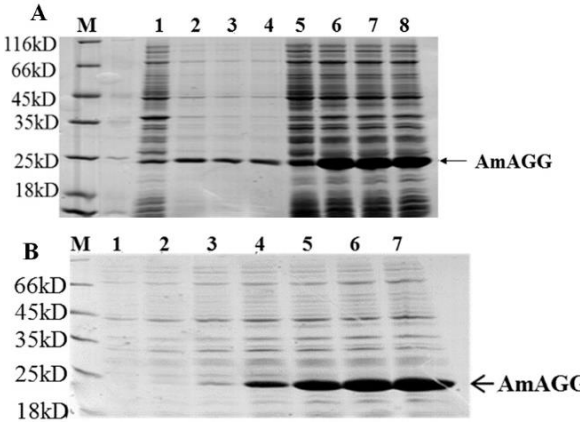

Fig. 3: Expression of *Am*AGG fusion protein. (**A**) Solubility analysis of expressed *Am*AGG. Coomassie-stained gel was used in SDS-PAGE analysis. Lane M, protein ladder; lane1, 2, 3, 4 are insoluble protein induced with 0, 0.1, 0.5 and 1.0 mM ITPG respectively; lane 5, 6, 7, 8 are soluble protein induced with 0, 0.1, 0.5 and 1.0 mM ITPG respectively. (**B**) Time-course of *Am*AGG expression. Lane M, protein ladder; lane 1, 2, 3, 4, 5, 6 and 7 are induced by 0.1 mM IPTG for 0, 15, 30, 60, 120, 240, 360 min respectively

root, we observed the change of expression of *Am*AGG. In Fig. 2, The expression of *Am*AGG was up-regulated in response to drought and exhibited the highest expression at day 16, recovering at day 20. These results suggested that the *Am*AGG was induced by drought.

Expression and purification of the *Am*AGG recombinant protein in *E. coli*

In our study, the prokaryotic expression vector pET-*Am*AGG was successfully constructed by genetic engineering method, and the expression condition of pET-*Am*AGG recombinant protein was optimized by designing a series of induction time gradient and inducer concentration gradients. The optimal expression temperature of pET-*Am*AGG was 28°C, the optimal induction time was 6 h, and the optimum concentration of IPTG was 0.1 m*M*, through which the protein was induced and expressed in a soluble form in the Rossetta strain.

To optimize the expression conditions of recombinant the *Am*AGG protein, solubility, IPTG concentration gradient and time course were performed. The results of SDS-PAGE are shown in Fig. 3. In Fig 3A, at 0.1, 0.5 and 1.0 m*M* IPTG induction, there was a markedly enhanced protein band (below 25 kD), whereas in the control (lane 1 and lane 5) there was no band, indicating that this band may be target protein. Furthermore, most of the recombinant *Am*AGG protein was soluble and a small part was the insoluble inclusion body. Soluble expression can greatly simplify the following protein purification and activity analysis. To obtain the optimal IPTG concentration, 0.1, 0.5 and 1.0 m*M* were added for protein induction, yet there was no difference in soluble *Am*AGG protein expression with the increase of IPTG concentration (lane 6, 7 and 8). To reduce the damage of IPTG to the cells of Rossetta strain, 0.1 m*M* was confirmed as the optimum concentration for IPTG induction. In Fig. 3B, a time gradient was set to optimize the induction time of the recombinant protein and provide a guarantee for the subsequent induction and purification. The expression of the target protein increased with the increase of induction time (0–360 min), indicating that 360 min was the optimal induction time. The optimal over-expression was obtained with the pET-*Am*AGG after 6 h of induction with IPTG at 28°C.

A Large-scale purification of the induced *Am*AGG recombinant protein with a concentration gradient of imidazole in washing buffer was performed with column chromatography and samples corresponding to each stage subjected to SDS-PAGE are shown in Fig. 4.I In Fig. 4, the *Am*AGG recombinant protein was purified after washing with 30 m*M* imidazole in a washing buffer, and the purified protein was obtained via elution with 50 and/or 80 m*M* imidazole in a washing buffer. At this point, the elution was not complete and the remaining purified target protein could be further eluted with 150 m*M* imidazole in an elution buffer. These results indicated that 30 m*M* imidazole in a washing buffer and 150 m*M* imidazole in an elution buffer might be the optimal conditions for *Am*AGG recombinant protein purification. The purity of the purified *Am*AGG recombinant protein was assessed with Quantity One v4.62 (lane 6–12) and the purity was about 95%. The purified *Am*AGG recombinant protein was then collected and quantified and the results showed that the concentration of the purified protein was 0.28 mg/mL.

Stress tolerance assay of *E. coli* expressing the agglutinin protein

To identify the *Am*AGG expression to salt tolerance in *E. coli*, the effect of *Am*AGG expression on salt tolerance of *E.*

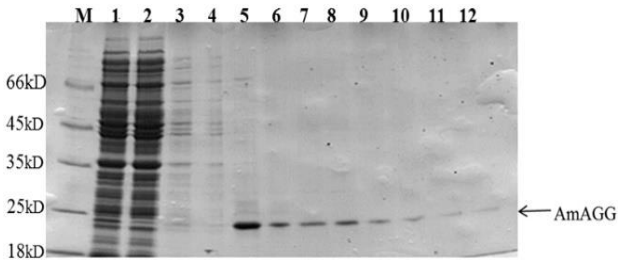

Fig. 4: Purification of *Am*AGG recombinant protein expressed in *E. coli.* Lane M, protein ladder; lane 1, crude protein sample extract from E. coli.; lane 2, sample flow through the column (FT); lane 3-5, lane 6-7 and lane 8-9, washing with 30, 50 and 80 mM imidazole in the washing buffer respectively; lane 10-12, eluting with 150 m*M* imidazole in the elution buffer

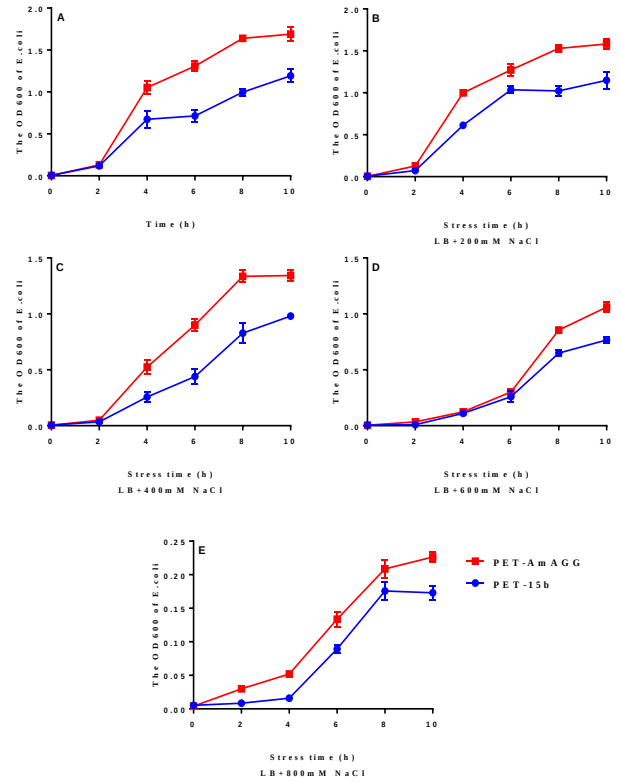

Fig. 5: The effects of *Am*AGG expression on the growth of *E. coli* under salt stress conditions. Compared to the control of *E. coli* in normal condition (**A**), *E. coli* cell growth after 200 mM NaCl treatment (**B**), 400 mM NaCl treatment (**C**), 600 mM NaCl treatment (**D**) and 800 mM NaCl treatment (**E**) was determined. pET-15b denoted *E. coli* with empty vector and represented control in (A–E). pET-*Am*AGG denoted *E. coli* with pET-*Am*AGG. The mean value of each transgenic line represented a statistically significant difference with respect to the control, as determined by the LSD-t test ($P \leq 0.05$). Vertical bars indicate the mean ± SE of three biological independent experiments

coli was determined (Fig. 5). In the control experiment, as shown in Fig. 5A, there was no obvious difference in cell survival between the *Am*AGG-expressing strain and the empty vector control strain in 0–2 h, yet in 2–10 h, the control strain grew better than the *Am*AGG-expressing strain. After treatment with, the *Am*AGG-expressing strain showed higher under 200–800 m*M* NaCl ($P < 0.05$) survival compared to the control strain, indicating that the expression of *Am*AGG might be conducive for the survival and growth of *E. coli* NaCl stress (Fig. 5 B–E). Specifically, under 800 m*M* NaCl, both the empty vector and *Am*AGG-expressing strain showed sharply lower OD_{600} value than other conditions throughout the growing period, which means they both suffered from serious salt stress. Compared with the empty vector control strain, however, the *Am*AGG-expressing strain showed a significantly higher OD_{600} value during 2–10 h treatment, especially in 0–4 h, which almost no growth for the control strain, while the *Am*AGG-expressing strain could grow slowly (Fig. 5E). These results indicated that the empty vector was inhibited more severely than the *Am*AGG-expressing strain under salt stress. In other words, the expression of *Am*AGG might increase the salt tolerance of *E. coli* to salt stress.

Discussion

Plants are often influenced by abiotic stresses, such as salinity, drought and low temperature. These stresses can cause adversely affected in plant growth and crop productivity. Therefore, to research the mechanisms of plant adjust to these stresses, many genes have been examined (Deng *et al*. 2013).

Even though the research on the role of agglutinin in response to environmental stresses in diverse plants, few studies on *A. mira* have been reported. As a tolerant drought and salt stress plant, there is no genome and transcriptional information of *A. mira* in the database (Bai *et al*. 2009; Guan *et al*. 2014). Therefore, currently we cannot explain the mechanism of peptide expression in *E. coli*, the agglutinin gene has been reported to award tolerance to drought and salinity (Alqahtany *et al*. 2019). In previous studies, some drought response proteins have been identified by proteomic analysis (Cao *et al*. 2017). To obtain the gene sequences of these drought response proteins, a series of primers were designed according to the results of previous proteomic sequencing and the transcriptional information of *Prunus persica* in NCBI database, which is close to the *A. mira* in phylogenies and might share high protein sequences identity with *A. mira.* Thus, we cloned the *Am*AGG gene from *A. mira* and deduced amino acid of the full-length sequence of agglutinin gene from *A. mira*, and its expression pattern under drought was also examined. It showed only one amino acid different between *A. mira* and *P. persica*, the 74^{cys} in *A. mira* and 74^{ser} in *P. persica*, which maybe the reason for different resistance in *A. mira* and *P. persica.*

As salt and drought share the similar response pathways (Song *et al*. 2019), we presumed the agglutinin from *A. mira* might also respond to salt stress and play

some roles in salt tolerance of *A. mira*. Recombined *Am*AGG protein was induced and purified from *E. coli*. In the exploration of the conditions of expression, although 37°C is a typically used bacteria breeding temperature, a lower temperature of 28°C was selected as the choice according to experience. Generally, under relatively high temperature (37°C), bacteria are prone to form inclusion bodies showing low or no biological activity. The purified *Am*AGG recombinant protein might provide an essential foundation for exploring the characters and activities of the *Am*AGG protein.

Functional identification of genes in *E. coli* and yeast has been widely used for studying salt-sensitive (Latef *et al*. 2019). The salt tolerance of *E. coli* expressing the agglutinin was estimated via measurement of the OD_{600} value of the *E. coli* strain which was supplied with different concentrations of NaCl into the cultural media (Udawat *et al*. 2014; Pathak and Ikeno 2017; Biradar *et al*. 2018; Daldoul *et al*. 2018). Compared with empty vector controls, *Am*AGG-expressing *E. coli* showed increased survival rates following salt stress. Under the same concentration of salt treatment, the growth state of *Am*AGG-expressing *E. coli* was better than that of empty vector, which may be due to increased tolerance to salt stress after recombinant protein expression. It was shown that exogenous supplement of WGA, a wheat germ agglutinin, can reduce the oxidative stress level of wheat seedlings and have a protecting effect on the cell growth in wheat seedling roots under salinity (Kil'dibekova *et al*. 2004; Bezrukova *et al*. 2008). Therefore, in our study，the *Am*AGG might have a similar effect on the salt tolerance of plants, and was presumed to be involved in the salt response and play a pivotal role in plant stress tolerance. However, the molecular mechanism has not been completely explained and further experiments are necessary to explore how *Am*AGG is induced in these abiotic stress responses.

Conclusion

Expression of the *Am*AGG from drought leaves of *A. mira* may improve the salt tolerance of *E. coli*. This study has provided a foundation for further study of the activity and characteristics of agglutinin.

Acknowledgements

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