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Screening and Bioinformatics Analysis of Proteins Interacting with StMAPKK1 in Potato by Yeast Two-Hybrid System

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Abstract Mitogen-activated protein kinase (MAPK) cascade reaction is one of important and complex signal networks involved in plant growth and development, hormone and stress response. As one of its main members, mitogen-activated protein kinase kinase (MAPKK) is located in the middle of the cascade reaction and plays a key role in signal collection and divergence. There were shown that potato (*Solanum tuberosum* L.) *StMAPKK1* (PGSC0003DMT400000744) gene responded to drought stress. Therefore, *StMAPKK1* gene was firstly selected to screen its interacting protein. In this study, the bait vector pGBKT7-StMAPKK1 was constructed by homologous recombination and used to screen potato cDNA library by yeast (*Saccharomyces cerevisiae*) two-hybrid system. Five StMAPKK1-interacting proteins, hydrolase (hydrolyzing O-glycosyl compound), RING-H2 subgroup RHE protein, cyanate hydratase, ARF GTPase activator, and a C2 domain-containing protein, were obtained through this screening and were identified by bioinformatics analysis, and the interaction was verified by small-scale hybridization verification. The results provided theoretical basis for further study on the signal pathway and biological function of potato StMAPKK1.

Keywords Potato; StMAPKK1; Yeast two-hybrid; Interacting protein

Mitogen-activated protein kinase (MAPK) cascade reaction is a kind of highly conserved biosignal transduction module in eukaryotes (MAPK Group, 2002). In plant, the MAPK signal pathway is almost involved in various growth and development processes and stress reaction of various plant biotic and abiotic stress, so that a large complex and cross-communication biological signal transduction network can be formed in the cell, the basic conditions for rapid distribution of information are provided for the growth and development in plant and stress of the external conditions in the process of it. A plurality of miscellaneous signal paths is connected, the upstream received stimulation signals are classified and transmitted to the downstream, and other functional genes which can cause physiological function changes in the plant can be started as needed.

There are three main numbers of MAPK cascade reaction, mitogen activated protein kinase kinase kinase (MAPKKK), mitogen activated protein kinase kinase (MAPKK) and mitogen activated protein kinase (MAPK). Phosphorylation at specific motifs acts on the upstream and downstream of certain members to form cascade signaling pathways (Iftikhar et al., 2017). The number of MAPKKs members is the least, and it can be divided into four subfamilies A, B, C, D. MAPKKs is a double kinase, it can be activated by upstream MAPKKKs phosphorylation and activate downsteam MAPKs through phosphorylation. MAPKK responded to drought stress in plants, AtMAPKKK18-AtMKK3-AtMPK1/2 involved in drought resistance regulation with ABA induction dependence (Li et al., 2017). Overexpression of *ZmMKK1* or *ZmMKK4* in *Arabidopsis* enhanced drought resistance (Kong et al., 2011; Cai et al., 2014). GhMKK3 in cotton enhanced drought resistance through regulating stomatal size and root growth (Wang et al., 2016). Also, studies have found that OsMKK1 were involved in salt stress signaling in rice (Wang et al., 2014) and OsMKK6 played a role in low temperature and salt stress (Xie et al., 2012).

MAPKKs family genes in potato have been identified (Liu, 2017) and five *MAPKK* genes were obtained. They were named through tomato homology analysis *StMAPKK1* (PGSC0003DMT400000744), *StMAPKK2* (PGSC0003DMT400023739), *StMAPKK3* (PGSC0003DMT400014637), *StMAPKK4* (PGSC0003DMT400083995) and *StMAPKK5* (PGSC0003DMT400039329). The quantitative results of potato *StMAPKK* genes under different treatments (including 4°C, 45°C, 20% PEG, 200 mmol/L NaCl, 10 mmol/L H_2O_2, 100 μmol/L MeJA, 100 μmol/L SA, 100 μmol/L ABA) showed that the expression of *StMAPKK1* genes increased significantly under drought stress. Therefore, the StMAPKK1 gene was selected as the experimental object, and the protein interacting with StMAPKK1 was screened by yeast two-hybrid technique, so as to explore the signal pathway of StMAPKK1 gene in potato under drought stress.

1 Results

1.1 Construction of yeast two-hybrid bait vector pGBKT7-StMAPKK1

The full length transcription sequence of *StMAPKK1* gene (PGSC0003DMT400000744) is 1666 bp. The inserted fragment designed according to the design principle of homologous recombination primers would have homologous sequence 21 bp at the end of insertion fragment 5 'and 3' respectively, so the product size is about 1708 bp, which is consistent with the results of electrophoresis (Figure 1A). pGBKT7 plasmid size is 7303 bp. The linear plasmid was obtained from pGBKT7 plasmid digestion by *Pst* I and *Nde* I. The size of the linear plasmid is about 7270 bp, and the electrophoresis detection was in accordance with the expected size (Figure 1B).

The recombinant product was transformed into *E.coli* DH5α competent cell and coated on LB solid plate (50 mg/L Kan). Six single colonies were identified by PCR, and there were obvious bands at about 1708 bp (Figure 1C). *StMAPKK1* gene had enzyme cutting sites of *Nde* I at 572 bp and 582 bp, so the recombinant plasmid pGBKT7-StMAPKK1 digestion by *Pst* I and *Nde* I should have three fragments about 7270 bp, 1126 bp and 572bp, respectively. The results of electrophoresis (Figure 1D) were in good agreement with the expected results. Mainwhile, the results of sequencing showed that *StMAPKK1* gene had been successfully inserted into pGBKT7 vector (Figure 2).
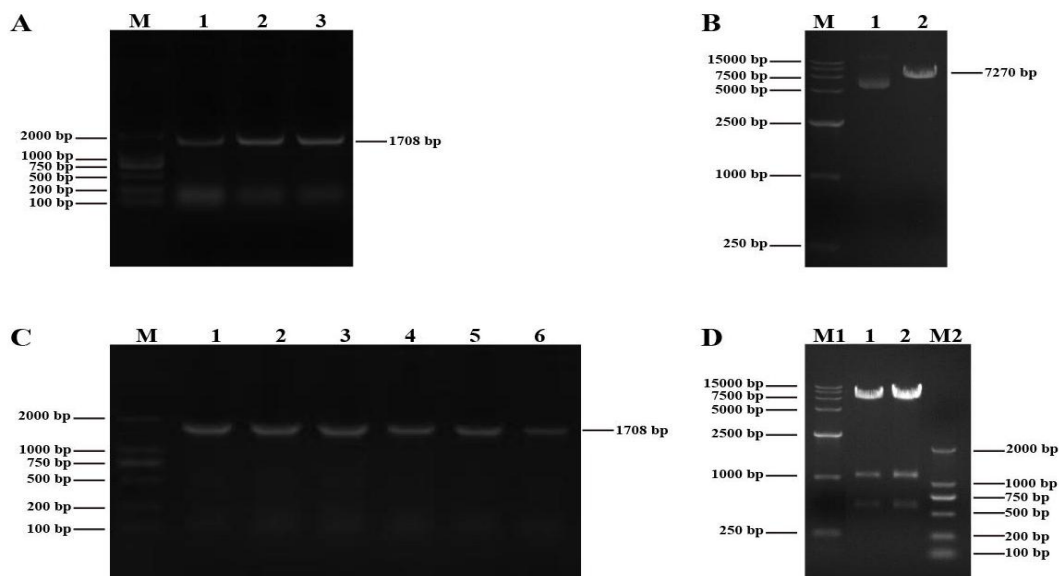

Figure 1 Construction of yeast two-hybrid bait vector pGBKT7-StMAPKK1

Note: A: Amplification of StMAPKK1 gene M: Trans2K DNA molecular marker, 1-3: PCR product of *StMAPKK1* gene; B: The double enzyme digestion linearization of pGBKT7 plasmid   M: DL15000 DNA molecular marker, 1: pGBKT7 vector plasmid, 2: *Pst* I and *Nde* I restriction endonuclease digestion to obtain the linear vector of pGBKT7; C: Identification of recombinant plasmid pBGKT7-StMAPKK1 by PCR M: Trans2K DNA molecular marker, 1-6: PCR product of single colony solution; D: Identification of the recombinant plasmid pGBKT7-StMAPKK1 digestion by *Nde* I and *Pst* I M1: DL15000 DNA molecular markers, 1 and 2: Products of pGBKT7-StMAPKK1 digestion by *Nde* I and *Pst* I, M2: Trans2K DNA molecular markers

Figure 2 Sequencing result of pGBKT7-StMAPKK1

1.2 Detection of toxicity and self-activating activity of yeast two-hybrid bait vector

Y187 yeast containing pGBKT7 empty vector or pGBKT7-StMAPKK1 bait vector was coated on single deficient medium SD/-Trp/X-α-gal. The growth number of the two strains was about the same (Figure 3), which indicated that the bait vector has no toxic to yeast cells. At the same time, the two strains did not grow on SD/-Trp/-His/X-α-gal or SD/-Trp/-Ade/X-α-gal, indicating that the strain had no self-activating activity and could not activate downstream reporter genes alone.
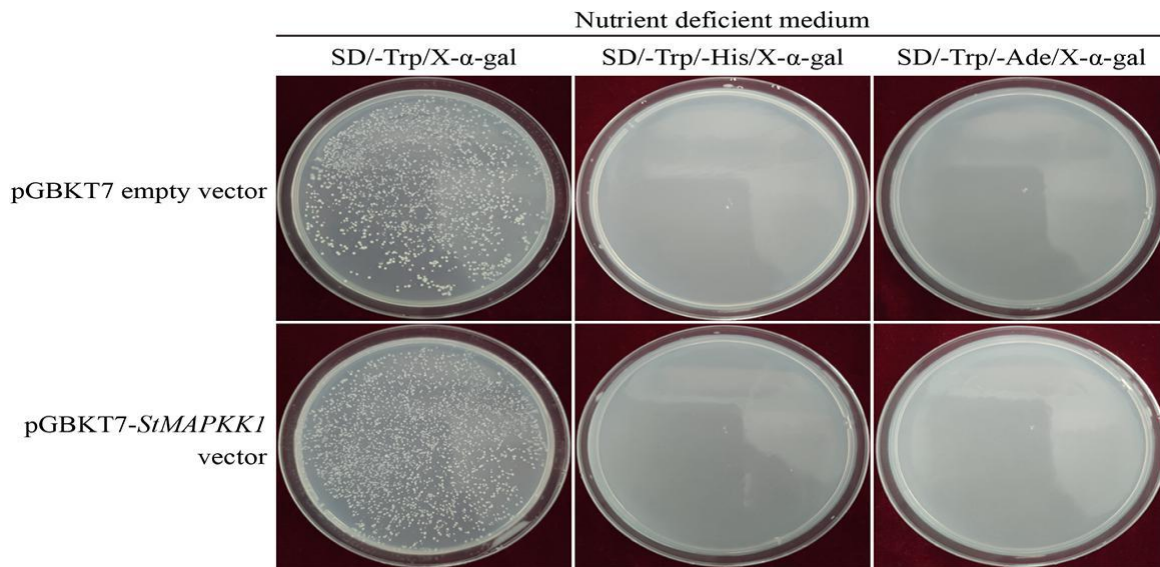
Figure 3 Detection of toxicity and self-activating activity of yeast two-hybrid bait vector pGBKT7-StMAPKK1

1.3 Small-scale hybridization verification of positive clone

The hybrid solution of bait protein and library was coated on 50 quadruple dropout media QDO. A total of 406 single colonies with large diameter were selected for identification and screening by X-α-Gal staining. 210 colonies which grew within 48 hours and displayed as blue were regarded as positive clones. The positive rate was 51.72%. Five different StMAPKK1 interacting proteins named C1 to C5, respectively, were identified by electrophoresis and sequencing. The proportion were 87%, 2.9%, 4.8%, 2.9% and 2.9%, respectively.

Five StMAPKK1 interacting protein gene plasmids were randomly selected and transferred back into AH109 yeast. The AH109 with plasmid was crossbred with Y187 containing bait vector and coated on QDO/X-α-Gal medium to culture. After two days of culture, the colonies with different blue depths grew at all five points (Figure 4). The results showed that all the five positive cloned genes could be verified by small-scale cross rotation, and the interaction between them was true and credible.
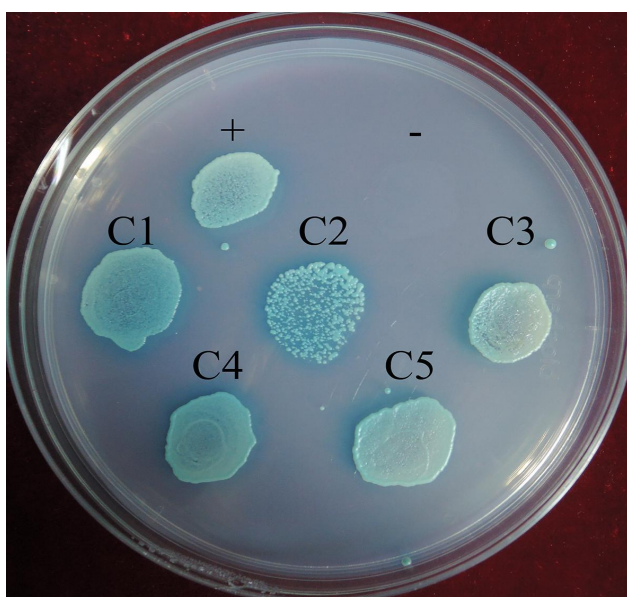
Figure 4 Small-scale hybridization verification of positive clones
Note: +: Positive control AH109 (pGADT7-RecT)×Y187 (pGBKT7-53); -: Negative control AH109 (pGADT7-RecT) ×Y187 (pGBKT7-Lam); C1~C5: Numbers of positive clones

1.4 Identification of positive clones

By sequencing and analysis of interacting protein genes, five StMAPKK1 interacting proteins C1-C5 were obtained, and then the basic physical and chemical properties of these genes were obtained (Table 1). The names of these five genes from NCBI were hydrolase, (hydrolyzing O-glycosyl compounds), RING-H2 subgroup RHE protein, cyanate hydratase, ARF GTPase activator and C2 domain-containing protein.

Table 1 Details information of proteins interacting with StMAPKK1

Clone	Gene ID	PGSC ID	Annotation of gene name	Full length of gene (bp)	Protein length (aa)	Chromosomal location
C1	102586320	PGSC0003DMG400030891	Hydrolase, hydrolyzing O-glycosyl compounds	4178	864	chr07:4096440..4101254
C2	102591415	PGSC0003DMG400011326	RING-H2 subgroupRHE protein	921	306	chr01:57283292..57284878
C3	102578903	PGSC0003DMG400017160	Cyanate hydratase	3550	162	chr09:56757059..56761063
C4	102593804	PGSC0003DMG400007759	ARF GTPase activator	2253	164	chr00:33717961..33720534
C5	102585714	PGSC0003DMG400023218	C2 domain-containing protein	3074	167	chr00:22575898..22579255

2 Discussion

After sequencing the positive clones, A total of five StMAPKK1 interacting proteins were obtained: Hydrolase (hydrolyzing O-glycosyl compounds), RING-H2 subgroup RHE protein, cyanate hydratase, ARF GTPase activator and C2 domain-containing protein.

Among them, the interacting protein C1 is hydrolase (hydrolyzing O-glycosyl compounds), and it can be also noted as galactitol-sucrose galactosyltransferase. It belongs to the GH36C subfamily of 11 subfamilies (GH36A to GH36K) in the glycoside hydrolase family 36. The members of the family can also be called raffinose synthase (RS) or seed imbibition protein I (Sip I). Raffinose synthase (EC 2.4.1.82) is the key enzyme that channels sucrose into the raffinose oligosaccharide pathway, it plays an important role in gaining drought resistance and prolonging the life of plant seeds (Peterbauer et al., 2002). In germinating maize seeds, some studies have shown that raffinose synthases are exclusively responsible for raffinose family oligosaccharides (RFO) breakdown (Andreas et al., 2008). CsRS expression was induced by low temperature and exogenous plant hormone abscisic acid (ABA) in cucumber leaves and fruits, RS activity and the content of raffinose increased gradually (Sui et al., 2012). Also, in beet (*Beta vulgaris* L.), two kinds of raffinose synthase identified in the study were proved to be involved in cold stress and salt stress responses (Kito et al., 2018). The interacting protein C2 was RING-H2 subgroup RHE protein, it is a cyclic H2 protein with E3 ubiquitin ligase activity and belongs to *ATL* (Arabidopsis toxicos para levadura) gene family (MartínezGarcía et al., 1996). It was proved that *PtaRHE1* was involved in the development of secondary phloem fibers in poplar hybrid varieties (Baldacci-Cresp et al., 2015). There are also some studies shown that the *ATL* gene family may be involved in early defense responses to pathogen attack (Salinas-Mondragón et al., 1999). The interacting protein C3 cyanate hydratase also known as cyanoate lyase (EC:4.2.1.104), is responsible for the hydrolysis of cyanate ester and exists in bacteria and plants, so that the organism with this kind of enzyme can overcome the toxicity of environmental cyanate ester (Sung and Fuchs, 1988). The interacting protein C4 ARF (ADP-ribosylation factor) GTPase activating factor is often involved in vesicle transport, especially in coatomer-coated vesicle transport between Golgi cisternae (Rothman and Wieland, 1996). In domain analysis, this activating factor contains C2 domain, which is involved in regulating membrane transport (Thomas and Rizo, 1996). At the same time, the interacting protein C5, a C2 domain-containing protein, the C2 domain could exhibit a distinct characteristic of binding to a variety of different ligands and substrates, including Ca^{2+}, phospholipids, phosphoinositides, and intracellular proteins (Cho and Stahelin, 2006). A protein,

OsPBP1, containing C2 domain in rice has been shown to regulate pollen fertility through Ca2+ and phospholipid signaling pathways (Yang et al., 2008).

In this experiment, the StMAPKK1 interacting proteins screened by yeast two-hybrid technique were different from the expected target results, other types of kinases in the MAPKs cascade reaction, such as MAPKKKs and MAPKs, were not obtained. There were some studies showed that there are other cascade pathways in addition to the typical MAPK pathway MAPKKK-MAPKK-MAPK, which may be cross-transmitted with other signal pathways.

3 Materials and Methods

3.1 Material for experiment
The yeast two-hybrid cDNA library of the potato tetraploid cultivar 'Zihuabai' (Liang, 2017), yeast (*Saccharomy-cescerevisiae*) strain Y187 and AH109, *Esche-richia coli* strain and bait vector pGBKT7 were all preserved the laboratory of the college of life science and technology, Gansu agricultural university.

3.2 Construction of yeast two-hybrid bait vector pGBKT7-StMAPKK1
Pst I and *Nde* I restriction endonuclease were used to linearize the carrier pGBKT7 and perform gel recovery. PCR response amplification of potato StMAPKK1 gene target fragment reaction system were 2×Power Taq PCR Master MIX 10 μL, Potato 'Atlantic' leaf cDNA template 1 μL, 10 μmol/L *StMAPKK1*-F (5'-<u>TCAGAGGAGGACCTGCATATG</u>GTTGCCTGGAAGCAATTTCACA-3'), *StMAPKK1*-R (5'-<u>CTAGTTATGCGGCCGCTGCAG</u>TTCATTGAAAGGTCTCTAAATTGATCTC-3') each 1 μL (The underlined part is homologous sequences of vector), ddH$_2$O 7 μL. The amplification conditions to get target fragment of *StMAPKK1* gene by PCR were as follows: 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 62℃ for 30 s, 72℃ for 67 sec, and a final extension at 72℃ for 10 min. Then the PCR products were also purified by agarose gel recovery, and the fragment size was 1708 bp. The recovered linear vector and PCR product of StMAPKK1 gene were calculated according to the ClonExpress® II One Step Cloning Kit specification from Vazyme company to establish the homologous recombination reaction system, and the reaction program was 37℃ 30 min and stored at 4℃. The recombinant product was transformed into E. coli DH5α by heat shock method and the bacteria liquid was coated with LB medium (50 mg/L Kan) for overnight culture at 37℃. A single colony was selected for PCR detection and sequencing.

3.3 Detection of toxicity and self-activating activity of yeast two-hybrid bait vector
The pGBKT7-StMAPKK1 bait vector plasmid and the pGBKT7 empty vector plasmid were transformed into the yeast Y187 by the polyethylene glycol/ lithium acetate method, respectively. The transformation products were respectively coated with SD/-Trp plates and incubated at 30℃ for 1-2 days. Choose the pGBKT7-StMAPKK1 bait vector and the pGBKT7 empty vector single colony with the size of 2-3 mm and growing at 30℃ for 2 days. Single colony uniformly was mixed into 500 μL SD/-Trp liquid culture medium, and coated 80 μL on nutrient deficient mediums SD/-Trp/X-α-Gal, SD/-His/-Trp/X-α-Gal and SD/-Ade/-Trp/X-α-Gal. The coated plates were incubated at 30℃ for 5 to 7 days. The remaining bacterial liquid was stored at 4℃. According to the growth condition of the colony, we can figure whether the bait vector has toxic or self-activating activity.

3.4 Hybridization between bait protein and yeast library
The single colony of bait vector was picked into 50 mL SDO/-Trp/Kan (20 μg/mL) medium, cultured overnight (16-24 h) at 30℃ for 230 rpm/min, and then centrifuge to get sediment and resuspend it with 5 mL SDO/-Trp liquid medium. Y187 bacteria with the bait vector solution 5 mL and library AH109 bacteria solution 1 mL were used in 2 L triangular flask. Then add 50 mL 2×YPDA/Kan (50 μg/mL), 30℃ incubated in shaking table at low speed (30-50 rpm/min) for 20 to 24 h. The 5000 rpm centrifugation of the hybrid solution for 10 min and the precipitation was resuspended with 10 mL 0.5×YPDA/Kan (50 μg/mL). The resuspending was coated on 50 quadruple dropout media QDO (SD/-Ade/-His/-Leu/-Trp), each plate was 100 μL, cultured at 30℃ for 7 days, the high rigorous interaction screening was carried out. The single colony point with larger diameter (> 2 mm) was

selected to QDO medium coated with 100 μL X-α-Gal for preliminary identification. On the selection medium QDO/X-α-Gal, the substrate X-α-Gal can be hydrolyzed and a distinct blue color is presented as a positive clone. The positive clones were identified by PCR, the primers (5'-3') were pGADT7-F TAATACGACTCACTATAGGGCGAGCG and pGADT7-R GTGAACTTGCGGGGTTTTTCAGTAT, which were designed according to sequences at both ends of restriction enzyme sites of the pGADT7 vector. The obtained PCR product is subjected to electrophoresis detection and sent to Suzhou Jinwei Biotechnology Co., Ltd. for sequencing.

3.5 Small-scale hybridization verification of positive clones
According to Xu et al. (2003), positive single clone yeast plasmids were extracted. The positive plasmid containing correct ORF was transformed into yeast AH109 receptive cells and then hybridized with yeast Y187 containing bait vector. AH109 (pGADT7-RecT)×Y187 (pGBKT7-53) and AH109 (pGADT7-RecT)×Y187 (pGBKT7-Lam) were used as positive and negative controls, respectively. 25 μL of the above hybrid solution was dripped on the fresh QDO/X-α-Gal plate. The round plaque was cultured in dark at 30℃. The growth and discoloration were observed to verify the authenticity of the interaction.

3.6 Bioinformatics analysis and identification of positive cloned genes
The sequencing results of interacting protein were identified by Blast comparison analysis in potato database of NCBI (https://www.ncbi.nlm.nih.gov/), PGSC (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml) and Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.htm), the protein name, chromosome position, full-length gene and protein sequences of interacting proteins of StMAPKK1 can be obtained.

Authors' contributions
Liao Yuqiu is the experimental designer of this research and the executor of the experimental research, carries on the data collation and the writing of the first draft of the paper; Wang Fangfang and Zhu Xi participate in part of the experiment; Zhang Ning and Si Huaijun are the project leaders to guide the experimental design, data statistics, paper writing and revision. All authors read and approved the final manuscript.

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