INTERACTION NETWORK OF *TaRHA2b* OF WHEAT *(TRITICUM AESTIVUM* L.*)* BASED ON HIGH-THROUGHPUT YEAST TWO-HYBRID SCREENING

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Abstract. To explore the signal transduction pathway that *TaRHA2b* gene is involved in regulating, yeast two-hybrid system was used to screen and identify proteins interacting with TaRHA2b. The homogenized cDNA library was constructed from the embryo of mixed barley. The decoy protein vector pGBKT7-*TaRHA2b* was constructed and transformed into yeast AH109 cells for self-activation detection. Candidate positive clones were screened by one to one yeast co-transfection, SD/-Leu/-Trp and SD/-Leu/-Trp/-Ade/-His medium screening and β-galactosidase chromogenic reaction. The in vitro co-transfection and GST-pull down experiments were used for further verification. The homogenized cDNA library of the barley embryos with titer of 1.2 10^6 cfu/ml and storage capacity of 1.1 10^6 was successfully constructed. The inserted fragment size was between 1000-3000 bp. The plasmids of the cDNA library were extracted and sequenced on a large scale, and 5000 plasmids were obtained. The decoy vector without self-activation function was successfully constructed. The x-gal filter paper of eighty-four hybrid clones showed strong blue color and forty-nine pieces of effective sequence information were obtained. Eight of them were selected for co-transfection verification. YTH2450 was selected for GST-pull down verification. The results showed that they were positive hybrid clones. TaRHA2b interacted with YTH2450 and other proteins. The information of above genes could be used for genetic improvement of crops, further improving the adaptability of crops to the environment.
Keywords: *preharvest sprouting, abscisic acid, cDNA library, self-activation, pull down assay*

Introduction

Preharvest sprouting (PHS) refers to the phenomenon of direct germination of grains on the ear in wet environment at the mature stage of wheat (*Triticum aestivum* L.). The negative effects of spike germination on wheat production are mainly reflected in decreasing yield, deteriorating quality and lower seed value. At present, the resistance of most wheat varieties to spike germination is not strong. The shortage of wheat resistance gene resources severely restricted the breeding and application of wheat varieties resistant to heading germination.

Cys-rich RING domain was identified for the first time in proteins encoded by *Really Interesting New Gene*. In *Arabidopsis thaliana* proteome, more than 5% of the predicted proteins (more than 1400 genes) are involved in ubiquitination/26S proteasome pathway (Smalle and Vierstra, 2004). Among these proteins, only a few encode E1 enzymes (two isoforms), 37 predicted proteasome components of E2, 26S, and other factors (such as deubiquitinase), while more than 1400 genes encode E3 ubiquitin ligases to participate in ubiquitination-dependent proteasome degradation pathways (Smalle and Vierstra,

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2004). A variety of E3 ubiquitin ligases involve them in specific proteasome degradation pathways. Different binding of E2 and E3 can also increase the specificity of E3 in recognizing target proteins. Therefore, the diversity of E3 and the diversity of E3 binding with E2 can not only regulate different types of ubiquitination modification, but also specifically regulate target proteins.

The *AtRHA2b* gene of *Arabidopsis thaliana* encodes E3 ubiquitinated ligase. It plays an important role in ABA (abscisic acid) signal transduction and stress. Overexpression of *AtRHA2b* gene results in ABA-related phenotypes, such as ABA sensitivity at the stage of seed germination and seedlings (Li et al., 2011).

The *TaRHA2b* (Genbank: AEQ67396.1) gene, a Ring finger protein gene, was isolated from wheat by our team (Li et al., 2019). In previous studies, much work has been done on the *TaRHA2b* and *RsRHA2b* genes. The results showed that *TaRHA2b* gene may play an important role in seed dormancy during germination, which could be used to improve the PHS resistance of wheat. The genetic distance between radish and *Arabidopsis thaliana* is very small, so the function of radish *RsRHA2b* gene should be similar to that of *Arabidopsis thaliana AtRHA2b* gene. Compared to Zhengmai 9023, wheat lines transfected with *RsRHA2b* gene showed higher dormancy, PHS resistance and sensitivity to exogenous ABA. Meanwhile, under the treatment of 0.5 μM ABA and 5 μM ABA, the expression of ABA-related genes in transgenic lines was significantly higher than that in control Zhengmai 9023. The differences in nucleotide polymorphism sites of *RsRHA2b* gene resulted in differences in the three cleavage sites of *BstD102I, Cfr10I, and HpaII/MspI*. These results facilitate the exploration of the correlation between the *TaRHA2b* gene and wheat's increased PHS resistance ability and the development of molecular markers for PHS resistance.

The protein interaction network of RHA2b can be predicted by string software. This protein interaction network includes KIN1 (AT5G15960.1), ATDI8 (AT5G66400.1), AT5G12110 (AT5G12110.1), KUOX1 (AT5G07480.1), KUF1 (AT1G31350.1), AIRP1 (AT4G23450.2), SDIR1 (AT3G550.1), KEG (AT5G13530.1), AT5G58410.1 (AT5G58410.1) and ATC019 (AT1G52890.1). RHA2b is involved in the process of protein ubiquitination, cell protein metabolism and response to acid compounds. The regulatory network of a series of proteins interacting with RHA2b involves the processes of protein ubiquitination, cell protein metabolism, low temperature tolerance, and response to water loss, ABA, acidic compounds, oxygen-containing compounds and abiotic stress (Kurkela and Franck, 1990; Haynes et al., 2002; Sajan et al., 2007; Usadel et al., 2008; Jiang et al., 2009; Nelson et al., 2010; Robinson et al., 2010; Cho et al., 2011; Zheng et al., 2012; Stone, 2014).

The complexity of transcription factors determines the diversity of their functions. However, the specific signaling pathways and mechanisms involved in RHA2b are not very clear. What proteins can interact with it? Using TaRHA2b as bait and yeast two-hybrid technique to study the proteins interacting with TaRHA2b can lay a foundation for solving the above problems. Yeast two-hybrid technology has been widely used as an effective means to study protein interaction, providing a mature technology platform for the study of protein interaction and unknown proteins.

Fields and Song's yeast two-hybrid technique is a sensitive method for analyzing protein-protein interactions (Fields and Song, 1989). Although the system is closer to the natural conformation and function of the protein, but can not avoid the "false positive" problem. The self-activation detection of decoy protein is necessary for yeast two-hybrid experiment. If the decoy itself has self-activation function, then the

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downstream reporter gene can be directly expressed, which makes no sense to use the decoy protein vector to screen the library.

In order to further understand the function of *TaRHA2b* and its mechanism of PHS resistance, some genes related to TaRHA2b were screened by yeast two-hybrid method. The present study can not only screen new stress-resistant genes, but also provide a theoretical basis for elucidating the mechanism of TaRHA2b-mediated PHS resistance.

Materials and methods

Construction and identification of homogeneous cDNA library

Wheat is hexaploid and its genome structure is too complex. Barley and wheat are both gramineous plants. The genetic distance between them is very small. Therefore, the interaction of TaRHA2b protein in barley can be studied first, and then the function of TaRHA2b in common wheat can be studied.

Preparation and purity detection of total RNA from barley (Hordeum vulgare L.) embryo

Barley seeds were treated with 5 μM ABA and distilled water, respectively. The seeds amount of each treatment was 50, and the sampling time was 0 h, 6 h, 12 h and 24 h. After the obtained materials were degerminated, mixed samples were prepared and total RNA was extracted using the RNAiso Plus Kit (Takara, Japan). Total RNA was determined by spectrophotometer at OD260/OD280 values. 1-2 μg RNA was treated with thermal denaturation (65 °C, 10 min). Then Agarose gel electrophoresis was used to detect the purity of RNA.

Synthesis of 1st strand and 2nd strand cDNA

The total RNA was taken as the template and the experimental steps were followed in the Creator Smart cDNA Library Construction Kit (Clontech, the United States). The electrophoresis on agarose gel was determined with 5 μL.

Homogenization

The obtained double-strand cDNA PCR was purified using the QIAquick PCR Purification Kit (Cat.No.28104, QIAGEN, Duesseldorf, Germany). Homogenization is performed according to trimmer-director kit (Cat#NK002, Evrogen, Moscow, Russia).

Purification, enzyme digestion, binding and library transformation of dscDNA

The dscDNA was purified using the QIAquick PCR Purification Kit. The purification of the ds cDNA was treated with *Sfi I* enzyme, 65 °C warm bath 2 h. The products were purified by CHROMA SPINTMTE-400 Column. Purified product was connected with pGADT7-Rec carrier, 16 °C, for the night. Connect the product to *E. coli* DH10B transformation, which was placed in the resistance of ammoniac benzyl LB tablet (15 cm in diameter) on 37 °C training for the night.

Identification of library

Observe and record the number of colony, then calculate its storage capacity. Thirty clones were randomly selected for PCR. The product was detected by agarose

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gel electrophoresis, the recombination rate was calculated and the size of the inserted fragment was predicted.

Extraction, sequencing and bioinformatics analysis of cDNA library plasmids

2*96 monoclonal extraction templates were selected and sequenced with ABI3730. Positive sequencing was used. The sequence was analysed by BLAST. The NCBI and Swiss-Prot databases were used for bioinformatics.

Cloning of the wheat TaRHA2b gene

The seeds of wheat variety "Zhengmai 9023" were used for total RNA extraction. Total RNA was extracted with the Plus plant total RNA extraction kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). Fast Quant RT Kit (With gDNase) (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) was used to synthesize the first strand of DNA. The reverse transcription of wheat embryo DNA was used as template. Primers (*TaRHA2b*-F1 (Bait): 5'-GAATTCATGGGGTTCCCTGG-3', *TaRHA2b*-R1 (Bait): 5'-CTGCAGTCACCAAACGCGCGGGGGGGTGAG-3') were used for PCR amplification: 94 °C, 4min; 94 °C, 40s; 61 °C, 40s; 72 °C, 50s; 72 °C, 10min; 34 cycles. The length of the target fragment was 477 bp, and the target fragment was recovered by the gel recovery kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The recovered product was linked to pMD-19T, transformed into *E. coli* DH5a and cultured overnight on ampicillin resistant LB plate 37 °C. Monoclones were selected and cultured in shaking bacteria. Plasmid DNA was extracted and identified by PCR.

Construction and self-activation detection of decoy protein vector

Screening of interacting proteins is done in accordance with Matchmaker^{TM} Gold Yeast Two-Hybrid System User Manual (PT4084-1).

Construction of bait protein vector

Both pMD19-TaRHA2b and pGBKT7 vectors were digested by *EcoR I* and *PST I* at the same time, and the corresponding target fragments and linear pGBKT7 vectors were recovered. The recombinant vector pGBKT7-TaRHA2b was finally obtained and transformed into E. coli. Monoclones were selected and plasmids were extracted after shaking bacteria culture. The obtained plasmids were identified by enzyme digestion, and the rest were stored in a refrigerator at -80 °C with 50% glycerol.

Self-activation detection of bait carrier

Yeast strain AH109 was inverted cultured on YPDA plate at 30 °C. Yeast with diameters of 2-3 mm were selected and streaked on SD/-Trp, SD/-Leu, SD/-His and SD/-Ade media, respectively. The growth of yeast was recorded by inverted culture at 30 °C for 3-5 days. The bait vectors pGBKT$_7$-TaRHA2b and pGADT$_7$ were co-transfected into yeast AH109 competent state, and were separately delineated on SD/-Trp-Leu, SD/-Trp-Leu-His-Ade medium. The positive control group was co-transformed with pGBKT$_7$-53 and pGADT$_7$-T, while the negative control group was co-transformed with pGBKT$_7$-lam and pGADT$_7$-T. The growth of yeast was recorded after 3-4 days of inverted culture at 30 °C on all co-rotating plates.

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Systematic high-throughput yeast two-hybrid assay

One-to-one transfection of bait plasmids and library plasmids into yeast competence

The bait vector pGBKT$_7$-TaRHA2b was transfected into yeast AH109 cells together with the library plasmid. The lines were drawn on SD/-Trp-Leu and SD/-Trp-Leu-His-Ade medium, respectively. At the same time, the yeasts transformed with pGBKT$_7$-53 and pGADT$_7$-T, pGBKT$_7$-lam and pGADT$_7$-T were used as positive control and negative control, respectively. The growth of yeast was observed and recorded after 3-4 days incubation at 30 °C.

Identification of yeast positive clones

The large-scale library plasmid and bait protein vector were co-transformed into yeast AH109 cells. The activity of beta-galactosidase co-transforming yeast colonies on SD/-Trp-Leu-His-Ade culture plate was detected by X-gal filter paper colorimetry. The colour reaction of yeast colonies was observed within 8 h.

Sequencing and analyzing the AD-ORF of positive candidates.

The plasmids corresponding to the yeast positive clones were sequenced. The obtained sequences were analyzed by bioinformatics. The NCBI and Swiss-Prot databases were used for bioinformatics.

The co-transfection verification

The barley DNA stored in laboratory was used as template and amplified with primers (*Table 1*). The amplification procedures were as follows: 94 °C, 5 min; 94 °C, 30 s; 57 °C, 30 s; 72 °C, 1 min 20 s; 72 °C, 10 min; 30 cycles. The size of the target fragment was 933 bp. After gel recovery, it was connected with pGEM-T and transformed into DH5a to extract plasmid. The above plasmids were digested by EcoRI/BamHI and pGADT$_7$-T by EcoRI/BamHI. The target fragment was linked with the digested pGADT$_7$-T vector by T4 ligase and transformed into DH5a. The plasmid was extracted and identified by PCR, and the corresponding candidate protein prey was finally obtained. The bait vector and prey vector co-transformed yeast were applied to SD/-Trp-Leu and SD/-Trp-Leu-His-Ade solid medium plates. Colony growth was observed after incubation for 2-4 day.

The GST-pull down verification

Cloning of YTH2450 gene and TaRHA2b gene

PGADT7-*YTH2450* was used as the template, and 59/60 primers were used for amplification. The amplification procedure was as follows: 94 °C, 5 min; 94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min 20 s; 72 °C, 10 min; 30 cycles. The size of the target fragment was 933 bp. It was linked with pGEM-T for *E. coli* transformation and plasmid extraction. Take pGBKT7-TaRHA2b as the template, The primers TaRHA2b-F1/R1 were used for amplification. Amplification procedure is as follows: 94 °C, 5 min; 94 °C, 30 s; 60 °C, 30 s; 72 °C, 45 s; 72 °C, 10 min; 30 cycles. The size of the target fragment was 477 bp. It was linked with pGEM-T for *E. coli* transformation and plasmid extraction. The primers used in the experiment are shown in *Table 2*. The design of all

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primers is based on the homologous sequences obtained by bioinformatics analysis of the corresponding fragments.

Table 1. *Primers for the amplification of positive clones*

Number	Primer sequence (5'-3')	Amplified target fragment size
831	ACCAACAGGCAACAGAACAACCTACCG	*YTH2449*
832	CGTCTCAACTTCAAATCACAGCCCATAT	663bp
833	CTAGCTCGGGACCAGTGGGAGT	*YTH2450*
834	ACACTATTACAATTTGTCGGCAAGGAT	921bp
835	CCCGATTCCATCAGGAAAGCA	*YTH2456*
836	CGACGCGAGACACTGCAAAAC	594bp
837	TTGCTCGATCTGTTCAATTACGA	*YTH2457*
838	AGGAATGAATGGCTCGGCTA	1422bp
839	CAGTTGAGTGCTCGCTGCTC	*YTH2475*
842	CGTAAATATGTATGCCTGCTGCTAT	2028bp
841	GAGGGGCACAAGGAAGAAAT	*YTH2476*
844	AAAATGGCAAAGCTCGGTTA	699bp
843	GACGATCAAGCGATGGAAAGAAGT	*YTH2496*
846	GCAAGGCGGCCAGTAGGAAT	864bp
845	GCACGCACGAACACGCACAG	*YTH2708*
848	CGCAACATTTTAGAGTATCAGGACCATTA	1092bp

Table 2. *The primers for prokaryotic expression of positive clones*

Number	Primer sequence (5'-3')	Target produce
59	GGATCCATGGCGGATGCGAAG	*YTH2450*
60	GTCGACTTAAAGGCCGACAC	
TaRHA2b-F1	GCACGCACGAACACGCACAG	*TaRHA2b*
TaRHA2b-R1	CGCAACATTTTAGAGTATCAGGACCATTA	

Construction of prokaryotic expression vector

BamHI/SalI double enzyme digestion was performed on the plasmid pGEM-T-YTH2450. At the same time, *BamHI/SalI* double enzyme digestion was performed on the pMAL vector, and the target products were recovered after electrophoresis. The target fragment and the target vector were linked with T4 ligase, and the linked product was transformed into *E. coli*. The plasmid pMAL-YTH2450 was extracted. *BamH I/Xho I* double enzyme digestion was performed on the plasmid pGEMT-T-TaRHA2b. At the same time, the pLEICS vector was subjected to *BamH I/Xho I* double enzyme digestion, and the target products were recovered after electrophoresis detection. The target fragment and the target vector were linked with T4 ligase, and the linked product was transformed into *E. coli*. The plasmid pLEICS-TaRHA2b was extracted.

Induced expression and purification of fusion protein

Transfer 500 μL positive bacteria fluid to 10 ml LB liquid medium containing 50 μg/ml ampicillin, 37 °C for the night. Transfer 2 ml bacteria liquid to 50 ml LB

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liquid medium containing 50 μg/ml ampicillin, 37 °C for the night. Take 1 ml bacteria liquid as a control, to add IPTG residual cultures to the final concentration of 0.3 mM, 37 °C to continue to develop. 1 ml of bacterial fluid was collected at different induction stages (1 h, 2 h, 3 h, 5 h). Centrifuge for 2 min, discard the supernatant, and suspend the cells with 100 L buffer solution. The samples were boiled in boiling water for 5 min, centrifuged for 1 min, and the cells were suspended with buffer solution. PAGE electrophoresis was performed on the obtained samples. Freeze thawing at room temperature, place on ice immediately, add 10-20 ml of bacterial lysate (PBS + l%Triton-100 + PMSF) for every 500 ml of medium, and mix well. Ultrasonic crushing on ice, open 2 s, stop 9 s, total 40-60 min. Until the lysate is sufficiently cool. 11000 rpm, 15 min, 4 °C on the centrifugal separation, -80 °C saved for later use.

GST (glutathione S-transferase) pull down

Three purified columns containing Glutathione sepharose 4B were balanced by adding a binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl). The bacterial lysate was added by ultrasonic crushing and centrifugal filtration. Two of them were added with pLEICS-TaRHA2b to induce GST-TaRHA2b lysates in large quantities, while the other one was added with pLEICS to induce GST lysates in large quantities. Placed in 4 °C, horizontal oscillation apparatus, slow upside down with rights and cracking liquid contact with column material, promote the combination. After 2-4 h, discard the lysate and rinse 5-8 times with pre-cooled combined buffer. A large amount of pMAL-YTH2450 induced bacterial lysate (ultrasonic crushing, centrifugal filtration) was added to the GST-TaRHA2b column and the GST-bound column respectively. Also 4 °C, fluctuation slow oscillation 4 h, abandon cracking fluid, clean with combined buffer after 5-8 times, with the elution buffer (50 mM Tris HCl, pH 7.4, 10 mM NaCl, 10 mM reducing GSH) elution 3 purification column, collect samples for SDS-PAGE analysis.

Statistical analysis

The GraphPad Prism 8 was used for statistical analysis and drawing. For comparing results of different treatments, Variance analysis is followed by a post-hoc test in order to determine pairwise differences. Differences were considered significant for $P < 0.05$.

Results

Construction and identification of homogeneous cDNA library of barley embryos

When the prepared RNA was detected by spectrophotometer, the OD260/OD280 value was 2.03, which indicated that the purity of the RNA was very high. When RNA agarose gel electrophoresis, the brightness of the two bands of 28S and 18S is close to 2:1 (*Fig. 1A*). Normal proportion indicates that the integrity of the RNA is good. In summary, the prepared RNA can be further tested. The size of double-stranded DNA fragments was uniformly distributed in the range of 300-5000 bp by LD-PCR (*Fig. 1B*), with slightly high abundance gene bands.

The storage capacity of the plate is about 1200. The titer of the library was $1.2\ 10^6$ cfu/ml and the storage capacity was $1.1\ 10^6$. The results of colony PCR identification of randomly selected clones in the library showed that the size of inserted fragments in the

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library ranged from 1000 to 3000 bp (*Fig. 1C*). The results showed that the library was of high quality and could be used in the follow-up yeast two-hybrid screening library.

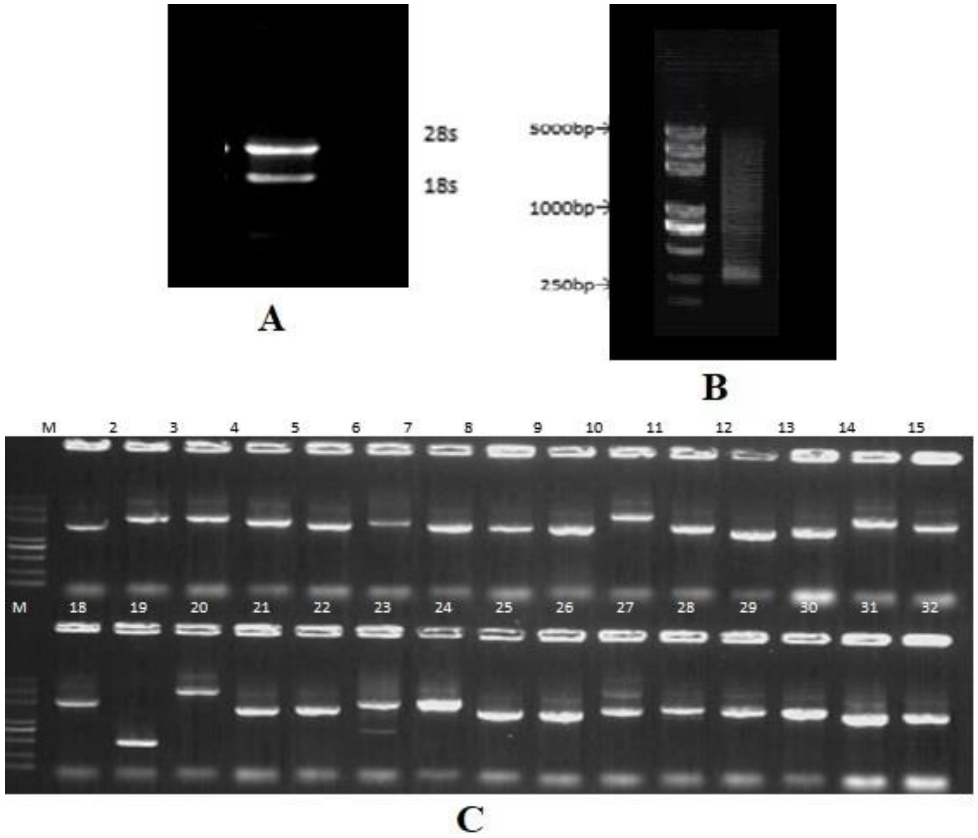

Figure 1. *Construction and identification of homogeneous cDNA library of barley embryos.* ***A*** *Detection of total DNA by 1.0% agorose;* ***B*** *The result of second cDNA ecletrophoresis. M: DL2, 000 plus marker, 1: the second Cdna;* ***C*** *The PCR identification of plasmid in the library. M: DL 2,000 plus marker, 2-32: the PCR products of the clonies*

Large-scale extraction, sequencing and bioinformatics analysis of library plasmids

The bacterial solution of barley cDNA library was amplified by PCR, and 5000 plasmids were finally extracted. Most of the amplified fragments are over 1000bp in size (*Fig. 2*). Considering the integrity of the DNA fragments, 120 plasmids with longer amplified fragments were selected and sequenced. Sequence data of 80 plasmids were obtained. A total of 71 genes were obtained after the duplication was removed (*Table 3*). The information about the sequences includes query coverage, percentage of identity, accession number, species and domain description. Information about 51 of these genes is known. Domain functions of some genes are related to Aldo/keto reductase family, EPSIN1, Zinc finger B-box type profile, Serine/Threonine, protein kinases active-site signature, AGD5, TPR repeat profile, NAF domain profile, AP2/ERF domain profile, Myb-type HTH DNA-binding domain profile, BARE-2, SAC9, Leucine-rich repeat profile, HEAT repeat profile, UBX domain profile, GRAS family profile, autophagy-related protein 7c (ATG7c), delta-1-pyrroline-5-carboxylate synthase, MO25-like protein and so on (*Table 3*). Information about 20 of these genes is unknown.

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Stress-resistant genes mining is an important work in molecular breeding. The acquisition of these data lays a foundation for the discovery of genes.

Table 3. *Blast analysis of plasmid in library (from NCBI and Swiss-Prot databases). The symbol "-"indicates no matching information after blast analysis*

Number	Query coverage	Percentage of identity	Accession number	Species	Domain description
1	99%	98.73%	AK368673.1	*Hordeum vulgare*	*Aldo/keto reductase family*
4	90%	81%	MG560142.1	*Triticum aestivum*	*Fructose-1-6-bisphosphatase active site*
5	100%	99.87%	AK376197.1	*Hordeum vulgare*	Unknown
6	99%	100%	AK353866.1	*Hordeum vulgare*	*Trp-Asp (WD) repeats circular profile*
21	94%	97.19%	AK371436.1	*Hordeum vulgare*	*R3H domain profile*
23	99%	94.96%	AK248592.1	*Hordeum vulgare*	EPSIN1
24	94%	97.01%	AK355971.1	*Hordeum vulgare*	*Zinc finger B-box type profile*
26	82%	80.52%	AF474072.1	*Hordeum vulgare*	*Serine/Threonine protein kinases active-site signature*
27	100%	96.34%	AK372413.1	*Hordeum vulgare*	AGD5
29	95%	97.66%	AK371180.1	*Hordeum vulgare*	*TPR repeat profile*
30	95%	97.82%	AK359851.1	*Hordeum vulgare*	*NAF domain profile*
32	84%	80.08%	AB749310.1	*Triticum aestivum*	*AP2/ERF domain profile*
36	96%	97.09%	AK250324.1	*Hordeum vulgare*	Unknown
37	67%	95.24%	AK354512.1	*Hordeum vulgare*	Unknown
38	99%	95.39%	AM180263.1	*Hordeum vulgare*	*Myb-type HTH DNA-binding domain profile*
39	95%	96.70%	AK371597.1	*Hordeum vulgare*	*Armadillo/plakoglobin ARM repeat profile*
40	88%	95.04%	XM_020324227.1	*Aegilops tauschii*	Ras-group-related LRR protein 5-like
41	45%	94.92%	AK367111.1	*Hordeum vulgare*	Unknown
42	85%	84.37%	AJ279072.1	*Hordeum vulgare*	BARE-2
44	93%	97.63%	AK251198.1	*Hordeum vulgare*	Unknown
45	42%	99.36%	AK370277.1	*Hordeum vulgare*	Unknown

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48	90%	98.92%	AK373080.1	*Hordeum vulgare*	*Type-1 KH domain profile*
49	68%	99.74%	AK357985.1	*Hordeum vulgare*	Unknown
50	64%	94.92%	AK252282.1	*Hordeum vulgare*	*Nucleolar GTP-binding protein 2*
51	99%	96.65%	AK365218.1	*Hordeum vulgare*	*Zinc-binding region signature*
53	99%	96.60%	AK365203.1	*Hordeum vulgare*	*Eukaryotic RNA Recognition Motif (RRM) profile*
55	98%	93.54%	AK447559.1	*Triticum aestivum*	*Putative DNA ligase 4-like (LOC100844955)*
57	76%	95.22%	AK365868.1	*Hordeum vulgare*	Unknown
58	99%	97.34%	AK364108.1	*Hordeum vulgare*	Unknown
59	27%	99.59%	AK371752.1	*Hordeum vulgare*	Beta-glucosidase BoGH3B-like
61	97%	92.31%	AB678347.1	*Hordeum vulgare*	Unknown
62	97%	97.00%	AK374311.1	*Hordeum vulgare*	Acyl-CoA-binding domain-containing protein
63	91%	91.80%	AP009567.1	*Hordeum vulgare*	*SPX domain profile*
64	98%	96.79%	AK358311.1	*Hordeum vulgare*	*AAA-protein family signature*
67	85%	96.98%	AK249586.1	*Hordeum vulgare*	Unknown
68	84%	92.81%	XM_020337903.1	*Aegilops tauschii*	*Solute carrier (Solcar) repeat profile*
69	56%	94.55%	AK360644.1	*Hordeum vulgare*	*Trp-Asp (WD) repeats circular profile*
70	85%	80.25%	AK370729.1	*Hordeum vulgare*	Unknown
73	99%	99.60%	AK357076.1	*Hordeum vulgare*	Mechanosensitive ion channel protein 10
74	99%	99.42%	AK252829.1	*Hordeum vulgare*	26S proteasome regulatory subunit 7A
75	99%	96.22%	XM_020340817.1	*Aegilops tauschii*	ABC transporter C family member 2
76	99%	99.02%	AK356202.1	*Hordeum vulgare*	Cycloeucalenol cycloisomerase
77	99%	98.77%	AK371292.1	*Hordeum vulgare*	Phosphatidylinositol 4-kinase gamma 6-like
78	91%	99.78%	AK356216.1	*Hordeum vulgare*	*DCUN1 domain profile*
79	99%	99.32%	AK372829.1	*Hordeum vulgare*	Unknown
80	98%	94.91%	AY268139.1	*Hordeum vulgare*	*Protein kinases ATP-binding region signature*

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81	39%	95.96%	AK334556.1	*Triticum aestivum*	SAC9
83	99%	98.52%	AK250364.1	*Hordeum vulgare*	Unknown
84	98%	92.57%	MG560140.1	*Triticum aestivum*	Unknown
85	99%	97.33%	AP017301.1	*Hordeum vulgare*	*NADH dehydrogenase subunit 5 gene*
86	99%	96.78%	AK333961.1	*Triticum aestivum*	Unknown
88	99%	98.52%	AK365825.1	*Hordeum vulgare*	Unknown
89	91%	98.99%	AK364487.1	*Hordeum vulgare*	Unknown
90	97%	98.96%	AP017301.1	*Hordeum vulgare*	Mitochondrial DNA
91	79%	99.53%	AK355938.1	*Hordeum vulgare*	*RNA-binding protein Luc7-like 2*
92	99%	98.98%	AK362481.1	*Hordeum vulgare*	Signal recognition particle subunit SRP72
93	99%	98.74%	AK248376.1	*Hordeum vulgare*	Mitochondrial phosphate carrier protein 3
94	99%	83.66%	AP009567.1	*Hordeum vulgare*	*Leucine-rich repeat profile*
95	99%	99.24%	AK357387.1	*Hordeum vulgare*	*HEAT repeat profile*
96	99%	98.91%	AK364716.1	*Hordeum vulgare*	*UBX domain profile*
98	99%	97.81%	EF115541.1	*Hordeum vulgare*	*Ribosomal protein S12 signature*
100	99%	82.79%	AC256253.1	*Hordeum vulgare*	Unknown
102	99%	98.65%	AK359701.1	*Hordeum vulgare*	*GRAS family profile*
103	99%	99.41%	AK357819.1	*Hordeum vulgare*	*W2 domain profile*
104	99%	95.61%	KF294806.1	*Triticum aestivum*	Autophagy-related protein 7c (ATG7c)
108	72%	100%	AY552749.1	*Hordeum vulgare*	18S ribosomal RNA gene
111	94%	97.35%	AK251855.1	*Hordeum vulgare*	Delta-1-pyrroline-5-carboxylate synthase
113	99%	97.57%	AK248531.1	*Hordeum vulgare*	MO25-like protein
115	99%	95.72%	FM164415.1	*Hordeum vulgare*	HOX1
118	99%	94.87%	XM_020329084.1	*Aegilops tauschii*	*DBINO domain profile*
120	98%	99.12%	AK252011.1	*Hordeum vulgare*	Unknown

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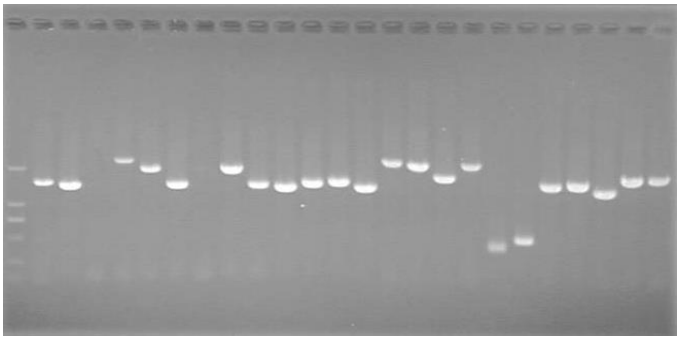

Figure 2. *The PCR results of liquid bacterial germ in the cDNA library*

Construction and self-activation detection of recombinant vector of decoy protein

RT-PCR was used to obtain a fragment size close to 500 bp, which was consistent with the expected fragment size (*Fig. 3A*). After sequencing, the size was confirmed to be 477 bp, which was found to be consistent with *TaRHA2b* gene sequence of wheat by sequence blast. The enzyme digestion results of decoy vector pGBKT7-*TaRHA2b* were shown in *Figure 3B*. After plasmid sequencing, sequence blast was compared and found to be consistent with the *TaRHA2b* gene sequence of wheat, indicating that *TaRHA2b* fragment was correctly integrated into the polyclonal site of pGBKT7 vector.

Yeast strains AH109 on SD/-Leu/-Trp/-Ade/-His medium did not grow after 3-5 d (*Fig. 3C*). This indicated that the yeast strain AH109 had no phenotypic mutation in the secondary culture. The experimental group, positive control group and negative control group grew on the SD/-Leu/-Trp medium (*Fig. 3D*). The positive control grew on the SD/-Leu/-Trp/-His/-Ade medium, but neither the experimental group nor the negative control grew on the SD/-Leu/-Trp/-His/-Ade medium (*Fig. 3E*). The results show that the decoy carrier pGBKT7-*TaRHA2b* has no self-activation function.

cDNA library was screened by decoy protein pGBKT7-RsRHA2b

A total of 1000 plasmids of a pair of transgenic yeast AH109 cells were tested (*Fig. 4A–C*), and the preliminary positive plasmids were 84 and 49 were effectively sequenced, all of which were analyzed by bioinformatics (*Table 4*). The information about the sequences includes query coverage, percentage of identity, accession number, species and domain description. The RHA2b interacting proteins include RGA, MYB, SF2, USP1, Lks2, Rp120, SBE starch branching enzyme, RPR44, TUDOR1, TEF1, auxin response factor 12, Hox-1, CBF9, TIP1, AP2/EREBP, MYB and ARF GTPase activator (*Table 4*).

The focus of the present research was plasmids in the following codes: 311, 611, 1065, 2433, 2437, 2438, 2443, 2449, 2450, 2456, 2457, 2475, 2476, 2496, 2708, 3019. In the process of in vitro and in vitro verification of yeast two-hybrid candidate proteins, 8 of them were finally selected, with codes 2449, 2450, 2456, 2457, 2475, 2476, 2496, 2708.

A pair of positive plasmids were co-transformation for in vitro validation

pGADT$_7$-*YTH2450* and pGBKT$_7$-*TaRHA2b* were co-transformed into yeast AH109 cells and then cultured on SD/–Trp–Leu–His–Ade medium for 3d (*Fig. 5*). The results showed that YTH2450 and RHA2b had a strong interaction.

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The primers were designed according to the homologous sequences obtained by bioinformatics analysis of *YTH2450*. The obtained plasmid pMAL-*YTH2450* was sequenced. After bioinformatics analysis has determined that the fragment is correct, subsequent operations can be carried out. The final pMAL-*YTH2450* recombinant plasmid and its enzyme digestion identification results showed that it was correct (*Fig. 6A*). PCR was performed on the bacterial solution containing *pLEICS-TaRHA2b* plasmid, and the results were shown in *Figure 6B*. The size of the target fragment was consistent with the expectation. Sequencing was performed, bioinformatics analysis was performed to determine the correct fragment, and subsequent operations were carried out.

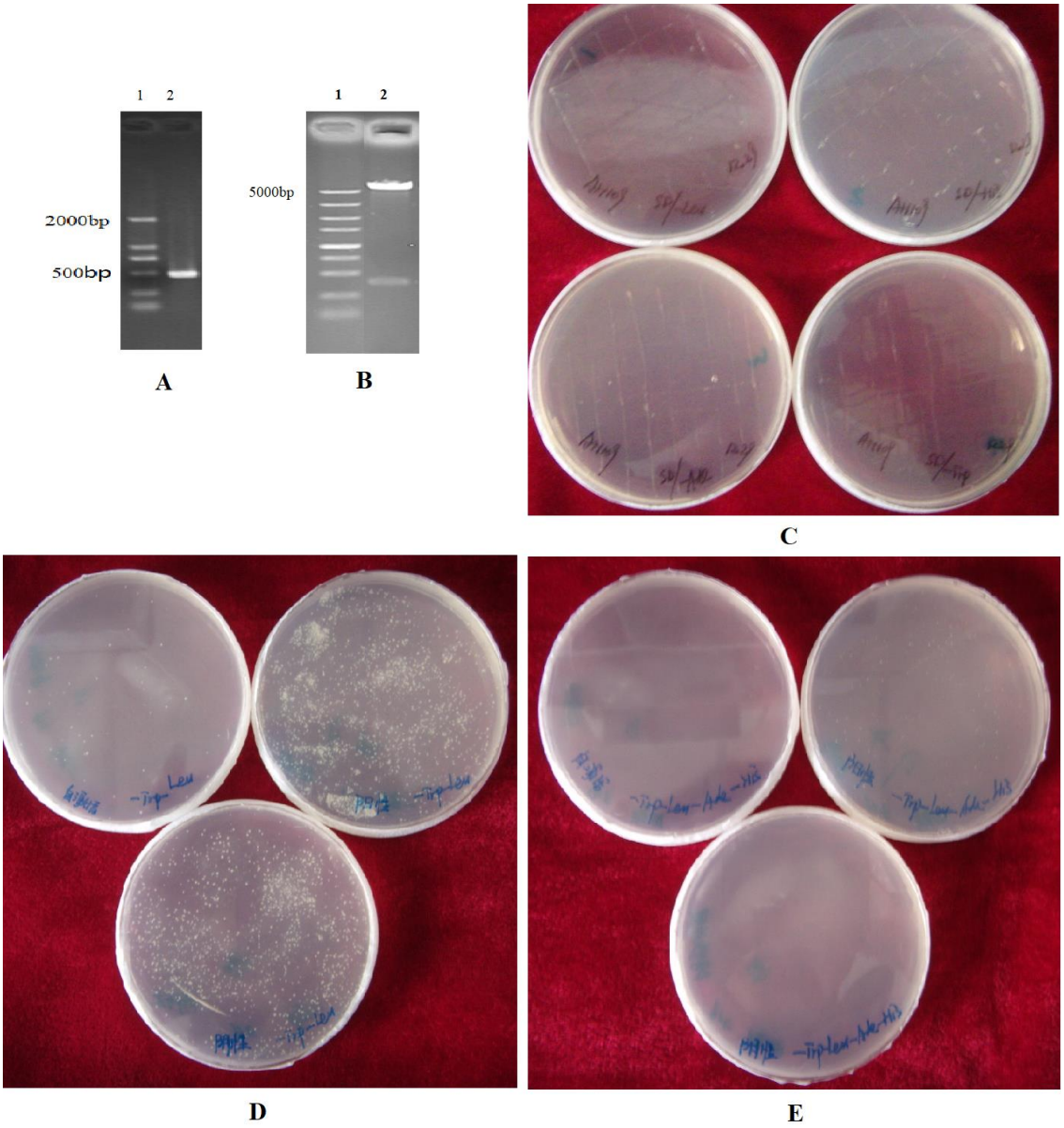

Figure 3. *Construction and self-activation detection of recombinant vector of decoy protein.* ***A*** *Clone of TaRHA2b gene, 1, Marker 2000; 2, the target fragment.* ***B*** *Enzyme digestion identification of pGBKT7-TaRHA2b, 1, Marker 5000; 2, the target fragment.* ***C*** *Growth of AH109 on each single defect medium.* ***D*** *The growth of the experimental group and the control group on SD/-Leu/-Trp medium.* ***E*** *The growth of the experimental group and the control group on SD/-Leu/-Trp/-His/-Ade medium*

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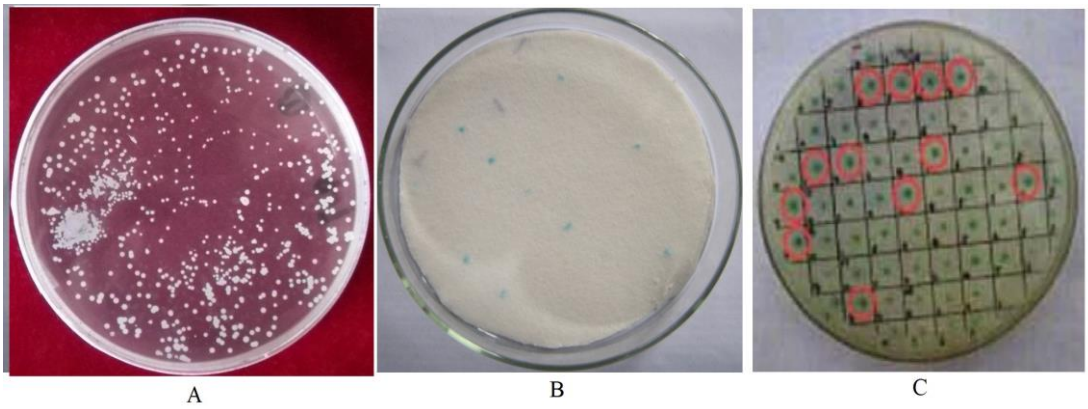

Figure 4. *cDNA library was screened by decoy protein pGBKT7-RsRHA2b.* ***A*** *The growth of co-transformation prey/bait AH109 on SD/-Leu/-Trp/-His/-Ade medium.* ***B*** *β-galactosidase color reaction of positive clones.* ***C*** *β-galactosidase color reaction of positive clones (circled with red lines)*

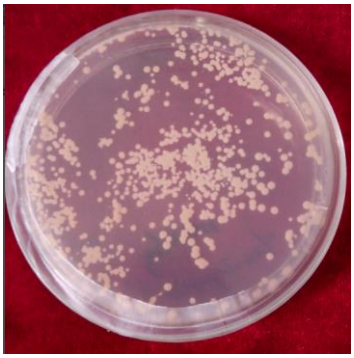

Figure 5. *The growth of co-transformation prey/bait AH109 in SD/-Leu/-Trp/-His/-Ade medium. pGADT$_7$-YTH2450 and pGBKT$_7$-TaRHA2b were co-transformed into yeast*

Table 4. *Blast analysis of positive plasmids (from NCBI and Swiss-Prot databases). The symbol "-"indicates no matching information after blast analysis*

Plasmid number	Barley			Wheat			Domain description
	Query coverage	Percentage of identity	Accession number	Query coverage	Percentage of identity	Accession number	
266	68%	78.80%	AK370200.1	91%	94.34%	AK455561.1	Mucin like protein
267	83%	99.89%	AK375235.1	-	-	-	NADH ubiquinone oxidoreductase subunit, NDUFA12
269	88%	99.22%	AK248821.1	-	-	-	RGA
271	52%	98.24%	AK372313.1	-	-	-	MYB
277	76%	94.49%	AY641412.1	69%	87.22%	KX447407.1	SF2, USP1
282	90%	99.19%	AK362421.1	24%	87.23%	MG560142.1	NADH dehydrogenase subunit 5
285	47%	78.10%	AC256251.1	-	-	-	Unknown
286	90%	92.41%	AB678347.1	18%	89.57%	CD903273.1	Lks2
291	89%	99.68%	AK370771.1	89%	96.88%	AK447925.1	Mechanosensitive ion channel protein
292	89%	99.03%	AK251731.1	89%	86.30%	AJ315040.1	18S rRNA gene (EST)
293	89%	99.34%	AK252316.1	89%	96.7%	BT009474.1	Rp120
302	67%	99.29%	AK355938.1	-	-	-	Putative RNA-binding protein Luc7-like 2-like
306	91%	99.25%	AK357819.1	91%	97.37%	JX978407.1	T16 eIF5-mimic protein (5MP) mRNA

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311	90%	99.95%	FN179383.1	90%	98.53%	BT008928.1	SBE starch branching enzyme
594	89%	99.55%	AK359743.1	88%	90.78%	HE996524.1	PPR repeat family
611	90%	99.59%	AK363669.1	89%	95.74%	AK331993.1	Pyridine nucleotide-disulphide oxidoreductase
633	89%	88.96%	AF453665.1	-	-	-	Sukkula retrotransposon
1063	23%	97.49%	KC472759.1	76%	92.74%	AK333658.1	RPR44
1065	89%	99.37%	AK252316.1	89%	94.04%	AK453829.1	TUDOR1
1068	21%	80.66%	EU812563.1	21%	79.37%	AB201446.1	Rpg
1069	58%	98.71%	AK250775.1	40%	96.52%	AK336110.1	Dehydrogenase/reductase SDR family member 7-like
1072	90%	97.82%	AK357798.1	89%	93.48%	JN412070.1	60S ribosomal protein L5 mRNA
1078	88%	98.78%	AK250680.1	88%	95.96%	AK454722.1	TEF1
2433	64%	99.30%	AK372028.1	-	-	-	DDT domain protein
2436	88%	92.16%	AK249066.1	84%	83.64%	AK452430.1	Chitobiase/beta-hexosaminidase C-terminal domain
2437	78%	98.68%	AK358361.1	89%	95.15%	KY795022.1	Auxin response factor 12
2438	70%	92.90%	AK364219.1	84%	96.08%	AK332649.1	E3 UFM1-protein ligase 1 homolog
2443	89%	94.29%	DQ249273.1	88%	82.43%	EU660898.1	E3 UFM1-protein ligase 1 homolog
2449	89%	92.06%	FJ477091.1	89%	77.69%	FN564428.1	Hox-1
2450	89%	99.68%	AK357510.1	89%	96.50%	AK452889.1	2-oxoglutarate/malate translocator
2456	58%	98.57%	AK251632.1	39%	90.48%	GU452718.1	Rho-related protein from plants (Rop)-like
2457	91%	98.85%	KC912689.1	91%	97.39%	KC912694.1	PS II 43,44 kDa protein (psbC) mRNA
2461	79%	99.73%	AK366008.1	-	-	-	Cytochrome P450
2462	89%	94.56%	DQ445253.1	89%	78.15%	MG560140.1	CBF9
2463	89%	93.41%	FJ477091.1	89%	77.32%	FN564428.1	Hox1
2473	-	-	-	89%	82.37%	FN564429.1	Unknown
2475	86%	99.67%	AK362415.1	86%	96.42%	AK455067.1	NADB_Rossmann Superfamily
2476	90%	95.56%	AF254799.1	90%	82.55%	FN564431.1	TIP1, TIP2, Rar1
2486	91%	100%	NC_042692.1	91%	99.79%	MH051715.1	Ribosomal_L2_C
2487	90%	99.48%	AK371367.1	90%	96.90%	AK330835.1	Ribosomal_L10_P0
2495	89%	99.79%	AM055812.1	88%	92.48%	CJ834723.1	GPH_sucrose
2496	89%	99.68%	HQ647352.1	80%	84.63%	FJ560496.1	AP2/EREBP protein
2708	82%	99.6%	AK371673.1	-	-	-	MYB
3005	90%	98.30%	AK363781.1	90%	91.68%	AK331286.1	Lipase
3014	91%	99.59%	AK363781.1	91%	96.85%	AK331992.1	40S ribosomal protein S4 mRNA
3018	83%	96.89%	NC_043837.1	68%	96.89%	MK253668.1	Chloroplast
3019	90%	100%	AK364064.1	91%	95.15%	AK332298.1	SINT5
3039	91%	99.12%	AK370200.1	57%	93.57%	GU563378.1	ARF GTPase activator

The size of the expressed protein was about 75.0 KDa, which was consistent with the expected size of 75.0 KDa, indicating the successful expression of prokaryotic protein (*Fig. 6C*). The size of the expressed protein was about 63.0 KDa, which was consistent with the expected size of 63.0 KDa, indicating the successful expression of prokaryotic protein (*Fig. 6D*).

GST-*TaRHA2b* was fixed to Glutathione-Sepharose4B column and GST (inducing expression of empty vector pLEICS-14) was fixed as negative control. After repeated washing, SDS-PAGE analysis was performed. YTH2450 can bind specifically to GST-TaRHA2b (swim lane 1), but not to negative control GST (swim lane 2) (*Fig. 6E*). It indicates that there is an interaction between TaRHA2b and YTH2450 in vitro.

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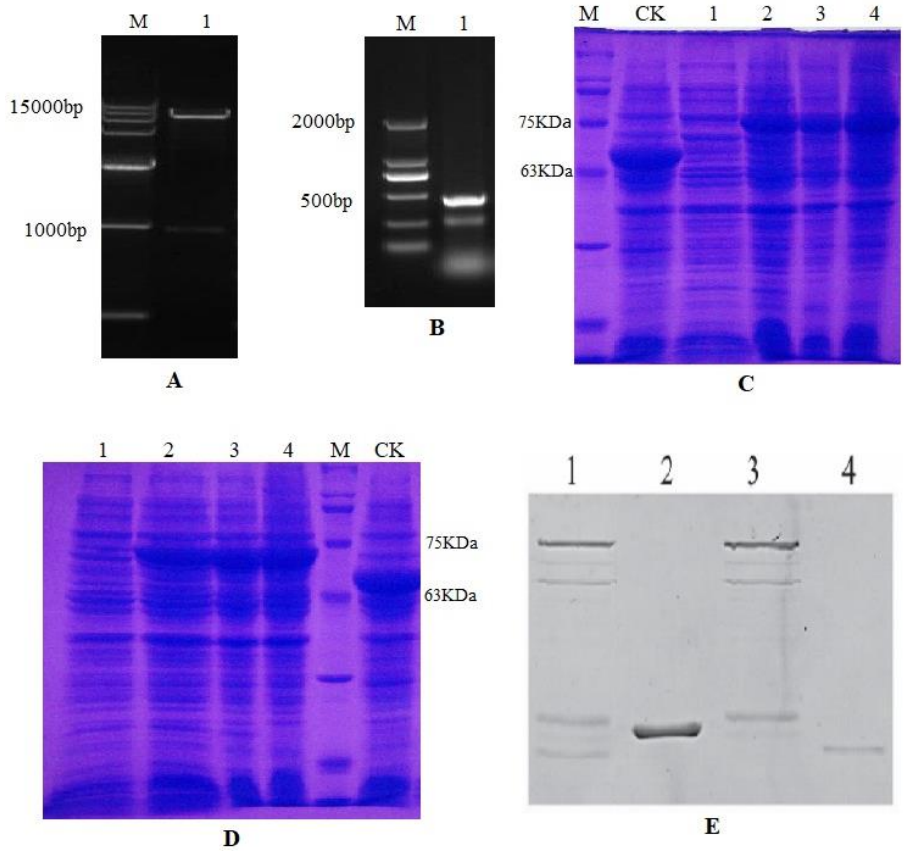

Figure 6. *GST-pull down validation.* ***A*** *Recombinant plasmid and enzyme digestion identification of pMAL-YTH2450. M: DL15000; 1: target product.* ***B*** *PCR results of BL21 containing pLEICS-TaRHA2b recombinant plasmid. M: DL2000, 1, the target fragment.* ***C*** *Prokaryotic expression of YTH2450. M: 11 kDa-245 kDa, CK, pMAL empty vector, 1-4, IPTG induction for 1 h,2 h,3 h and 5 h, respectively.* ***D*** *Prokaryotic expression of TaRHA2b. M: 11 kDa-245 kDa, CK, pMAL empty vector, 1-4, IPTG induction for 1 h,2 h,3 h and 5 h, respectively.* ***E*** *Pull-down assay of TaRHA2b and YTH2450. 1. pull down product of GST-TaRHA2b and YTH2450; 2. pull down product of GST and YTH2450; 3. GST-TaRHA2b for the pull down experiment; 4. the purified YTH2450*

Discussion

The development of cDNA library technology provides an important tool for functional genome research (Shao et al., 2009). At present, cDNA libraries are widely used in the research of model organisms and important crops, including tobacco, peanut and soybean (Ke et al., 2011; Cai et al., 2007). cDNA libraries provide information that reflects the level of gene expression at the RNA level. There are not many studies on the cDNA library of barley, and the construction of the cDNA library based on the embryo of barley treated by ABA treatment has not been studied. This project aims to explore the specific regulatory network of *TaRHA2b* gene in ABA signal transduction pathway and study the interaction between proteins. The yeast two-hybrid technique is the first choice. The construction of high-quality homogeneous cDNA library is the basis of subsequent experiments. Homogenization can ensure the existence of low-abundance mRNA as far as possible, which is necessary for the successful construction of cDNA library. In SMART technology, LD PCR method is adopted to obtain dscDNA, which

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can obtain longer fragments as far as possible, fully reflecting the integrity of mRNA (Schuler, 1997; Wellenreuther et al., 2004). In this experiment, the homogeneous cDNA library was constructed with a capacity of 1.1 10^6. Random clone sequencing results showed that the fragment size was within the range of 1000-3000 bp. The storage capacity requirement of cDNA library is above 1.7 10^5 (Thanh et al., 2011). Only when this standard is reached, the low abundance mRNA can be well processed and the constructed library can be used for the yeast two-hybrid screening library experiment.

In the present study, the yeast AH109 was deficient in the synthesis ability of Leu, Trp, His and Ade. The synthesis of Leu and Trp was remedied when the plasmid was transformed into yeast. As for His, there is a possibility of background expression in yeast. However, it was found that AH109 could not grow in the SD/-His medium without 3-AT in the defect validation experiment on yeast. Therefore, 3-AT was not added in this experiment. In this experiment, the decoy vector pGBKT$_7$-TaRHA2b for yeast two-hybrid system was successfully constructed. The decoy vector can be expressed normally in yeast without toxicity. It is proved that it has no self-activation in the system.

Yeast two-hybrid screening consists of two methods. The first method was to screen AH109 by co-transposing decoy protein granules with ds cDNAs. Although this method is simple, the library can only be used once and it is difficult to screen the interacting proteins. The second was screened by conjugation of two inverters, including Y187 yeast inverter containing decoy protein and AH109 yeast inverter containing pGADT$_7$-Rec and ds-cDNAs. The operation of this method is complex. In this experiment, a pair of total transformed yeast AH109 was used. If positive interaction was confirmed, specific plasmids could be directly corresponding. The follow-up operation is more efficient.

Using the protein expressed by the limited PHS resistance gene *RHA2b* as bait, we screened some corresponding fragments of TaRHA2b-related proteins by yeast two-hybrid technology, which not only has important significance in searching for new PHS resistance genes, but also provides new information for studying the mechanism of TaRHA2b-mediated PHS resistance.

The interaction between TaRHA2b and interacting proteins was fully demonstrated by positive clones identification, sequencing, verification of the pair of repeated co-transformation and GST-pull down.

These conclusions lay a foundation for studying the interaction and mechanism of TaRHA2b/YTH2450, TaRHA2b/YTH2456 and TaRHA2b/YTH2476 in the stress tolerance pathway of wheat. The complex formed by yeast hybrid candidate proteins and TaRHA2b may act on different signal transduction pathways. Bioinformatics analysis showed that the function of YTH2450 may be malate/ketoglutarate transporter, the function of YTH2456 may be RACD protein, and the function of YTH2476 may be TIP1 protein.

TaRHA2b is a protein of the type of transcription factor positively regulated by ABA signal transduction, which is essentially an ubiquitinated ligase and mainly participates in the process of protein degradation in cells. RHA2b targeted MYB30 degradation to regulate ABA signal transduction (Zheng et al., 2018).

Ketoglutaric acid/malate transporter was involved in the tricarboxylic acid cycle. The present study showed a strong interaction between ketoglutarate/malate transporter and TaRHA2b. The process of ubiquitination involves the formation of multiple complexes and changes in energy metabolism. α-ketoglutarate carrier protein (OGCP), located on

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mitochondria, encodes 314 amino acids and has the following important functions: scavenging endogenous oxygen free radicals in mitochondria and participating in cellular energy metabolism (De Palma et al., 2010; Regalado et al., 2013). These results indicated that the complex formed by ketoglutarate transporters and TaRHA2b could be involved in ABA signal transduction.

RAC/ROP involved in plant (ROP) family is related to small molecule G protein, which is a molecular switch of signal transduction in many cells, regulating the development process of cells and the response to the environment. RAC/ROP has three conformations, including an active GTP binding state, a transient free state, and a GDP bound state. In the active GTP binding state, RAC/ROP interacts with effector proteins to initiate downstream signaling (Haitina et al., 2006). These results indicate that the complex formed by RAC/ROP and TaRHA2b can activate the downstream signal and then play a role in a specific pathway.

TIP1 protein is a water channel protein, which can theoretically be expressed in all tissues, but the expression amount is not the same (Wudick et al., 2009). Therefore, in response to ABA, an abiotic stress, or in the climate where spikelet germination occurs, it is very likely that the complex formed by TIP1 and RHA2b participates in these regulatory pathways. These results lay a foundation for further study on the interaction and mechanism of TaRHA2b and protein-protein interaction in wheat stress resistance pathway.

Conclusion

The information of cDNA library lays a foundation for the discovery of stress resistance genes. TaRHA2b interact with YTH2450 and other proteins. The TaRHA2b interacting proteins could be used for genetic improvement of crops, further improving the adaptability of crops to the environment. The detailed regulatory mechanism mediated by TaRHA2b and interacting proteins needs to be further studied.

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Conflict of interests. The authors state no conflict of interests.

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