RING Finger E3 Ubiquitin Ligase Gene *TaAIRP2-1B* Regulates Spike Length in Wheat

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Highlight

TaAIRP2-1B regulates spike length by facilitating TaHIPP3 degradation, haplotype *Hap*-1B-1 of *TaAIRP2-1B* is a favorable natural variation to increase spike length in wheat.

Abstract

E3 ubiquitin ligase genes play important roles in the regulation of plant development. They have been well-studied in plants, but have not been sufficiently investigated in wheat. Here, we identified a highly expressed RING finger E3 ubiquitin ligase gene *TaAIRP2-1B* (*ABA-insensitive RING protein 2*) in wheat spike. Sequence polymorphism and association analysis showed that *TaAIRP2-1B* is significantly associated with spike length under various conditions. The genotype with haplotype *Hap*-1B-1 of *TaAIRP2-1B* has longer spike than that of *Hap*-1B-2, and was positively selected in the process of wheat breeding in China. Moreover, the *TaAIRP2-1B* overexpressing rice lines have longer panicles compared to the wild-type plants. The expression levels of *TaAIRP2-1B* in *Hap*-1B-1 accessions were higher than in *Hap*-1B-2 accessions. Further study revealed that *TaAIRP2-1B's* expression was negatively *regulated* by TaERF3 (ethylene-responsive factor 3) via binding to *Hap*-1B-2 promoter, but not to *Hap*-1B-1. Additionally, several candidate genes interacting with *TaAIRP2-1B* were obtained by screening the cDNA library of wheat in yeast cells. Among which TaAIRP2-1B interacted with TaHIPP3 (heavy metal-associated isoprenylated protein 3) and promoted TaHIPP3 degradation. The present research demonstrated that *TaAIRP2-1B* regulates spike length, the haplotype *Hap*-1B-1 of *TaAIRP2-1B* is a favorable natural variation for spike length enhancement in wheat, and provided genetic resources and functional markers for wheat molecular breeding.

Introduction

Food security is an important guarantee for human health and world peace (Shewry and Hey, 2015, 2016). Wheat is a staple food crop in the world, and its production is crucial to food security. Spike size and grain number per spike are important agronomic traits that directly contribute to grain yield of wheat. Spike length is one of the main traits related to grain number per spike. Previous studies showed that spike length is positively associated with aboveground biomass, biomass per plant, harvest index and yield, and negatively correlated with spikelet density (Zhu *et al.*, 2018). Some studies have found that increasing spike length without affecting spikelet density can improve yield (Che *et al.*, 2018; Yang *et al.*, 2019). Although breeders have been focusing on the three components of yield, namely, number of spike per unit area, number of grains per spike, and 1000-grain weight, other agronomic traits, such as spike length, may also be a potential trait for future grain yield breakthroughs. Therefore*, the study of* spike length can *provide a new idea for improving the potential productivity* of wheat.

In recent years, the functions of a large number of E3 ubiquitin ligase genes have been identified, and many of them are involved in response to adverse stress, especially drought stress (Kim *et al.*, 2012; Lyzenga and Stone, 2012). Meanwhile, E3 ubiquitin ligase has also been shown to be involved in the regulation of plant growth and development, such as flowering, cell cycle, spike development (Cao *et al.*, 2008; Gu *et al.*, 2009; Zhang *et al.*, 2017; Varshney and Majee, 2022). OsDSG1, a U-box E3 ubiquitin ligase, regulates panicle, internode and root length in rice by regulating cell division and elongation (Wang *et al.*, 2017). OsFRRP1, a RING type E3 ubiquitin ligase in rice, regulates many agronomic traits, such as plant height, panicle length and grain length (Du *et al.*, 2016). *TaSDIR1-4A*, a RING finger ubiquitin E3 ligase gene contributes to regulation of grain size in wheat (Wang *et al.*,

2020). *AIRP2*, a RING type E3 ubiquitin ligase gene in *Arabidopsis thaliana*, is implicated in the response to drought stress through ABA-dependent pathway (Cho *et al.*, 2011). However, whether *TaAIRP2* is involved in the regulation of important agronomic traits in wheat remains to be elucidated.

Candidate gene association study analyzes the association between candidate genes and target traits based on the sequence polymorphism of candidate genes (Su *et al.*, 2018). This method can accurately detect mononucleotide sites affecting phenotypic variation, predict their effects and find favorable haplotype combinations, so it is a rapid and effective method for gene discovery and functional analysis (Taguchi-Shiobara *et al.*, 2019).

In our study, *TaAIRP2*-1B was highly expressed in the spike of wheat at jointing stage. Therefore, we explored whether *TaAIRP2*-1B was functional in wheat spike. The present research revealed the positive effect of *TaAIRP2-1B* in spike length regulation through association analysis. Phenotypic analysis showed that overexpression of *TaAIRP2-1B* increased panicle length of rice. Our studies exhibited that TaERF3 could inhibit the expression of *TaAIRP2-1B* by binding to the haplotype *Hap*-1B-2 promoter. TaAIRP2-1B interacts with TaHIPP3 to facilitate its degradation. This work revealed the molecular mechanism of *TaAIRP2-1B* in regulating spike length, and provided a functional marker and elite genetic resources for wheat molecular breeding.

Materials and Methods

Plant materials and cultivation

A Chinese wheat cultivar Hanxuan 10 was used for gene cloning and gene expression pattern assays. Wheat seeds were hydrocultured at 20°C for 10 days, and seedling leaves were harvested for DNA and RNA extraction. The wheat cultivar Hanxuan 10 seeds were planted

in polyvinyl chloride (PVC) pipes, and samples were collected at jointing stage for gene

expression analysis in various tissues. The samples included leaf, root base and root at

seedling stage; spike, leaf, stem, root base and root (0-30 cm, 30-60 cm, 60-90 cm, 90-120

cm, 120-150 cm, 150-180 cm) at jointing stage. For expression analysis of *TaAIRP2* gene in

genotypes with different haplotypes, six accessions of each haplotype *Hap*-1B-1 and *Hap*-

1B-2 were randomly selected from 282 common wheat accessions (Population 1), and then

cultured in water for 10 days to harvest leaves for extracting RNA.

A diverse panel consisting of 32 wheat accessions (Zhuang *et al*., 2021) was used to

clone the *TaAIRP2* gene sequences and analyze the sequence polymorphism. A collection

contained 282 wheat accessions (Population 1) from the Yellow and Huai River Valleys

Facultative Wheat Zone and Northern Winter Wheat Zone in China was employed for

association analysis between agronomic traits and genotypes (Supplementary Table S1). Two

populations consisting of 157 landraces (Population 2) (Supplementary Table S2) and 348

modern cultivars (Population 3) (Supplementary Table S3) were used to analyze the temporal

and geographical distributions of the *TaAIRP2-1B* haplotypes in China. The wheat accessions

of Population 1 were grown in Shunyi (116°56´E, 40°23´N), Beijing in 2010-2012 and 2015-

2017, and Changping (116°13´E, 40°13´N), Beijing in 2013 and 2016-2017 cropping

seasons. Field moisture management was divided into two types, drought stress (DS, rainfed)

and well-watered (WW), the agronomic traits were investigated in 30 environments (year ×

site × moisture × heat stress). Rainfall of the seven wheat growing seasons (2010, 2011,

2012, 2013, 2015, 2016 and 2017) was 131, 180, 158, 163, 173, 143 and 116 mm,

respectively. The WW plots were irrigated with 750 m^3 ha^{-1} (75 mm) at each of pre-

overwintering, booting, flowering, and grain-filling stages. The heat stress (HS) treatment

was applied by using thermal shelters, consisting of plastic film over steel frames that were

placed over the trial plots at one week post-anthesis. Each accession was evenly sowed in a

plot of four rows of 2 m in length, and 30 cm apart, with 40 seeds per row. Plants were randomly selected in the middle of each plot at harvest time to investigate their agronomic traits.

RNA extraction and qRT-PCR

Wheat RNA was extracted by TRIzol method and transcribed using reverse transcription kit (TIANGEN, Beijing, China) according to its operating protocol. Quantitative real-time PCR reaction was performed using the method provided by SYBR Premix Ex TaqTM (TaKaRa, China) and Roche LightCycler 96 Real-Time PCR System (Roche, Switzerland) to detect gene expression level. The *TaActin* was used as internal control for gene expression analysis in wheat (Zhang *et al*., 2023). The *OsActin* was used as internal control for gene expression analysis in rice (Teng *et al*., 2019). Data were collected from three biological replicates. Primers used for **qRT**-**PCR** were listed in Supplementary Table S4.

Nucleotide polymorphism analysis of *TaAIRP2* sequence

According to the genome sequences of *TaAIRP2-1A*, *TaAIRP2-1B* and *TaAIRP2-1D*, Primer Premier 5.0 software was used to design genome-specific primers AIRP2-1A-F/R, AIRP2-1B-F/R, AIRP2-1D-F/R, AIRP2-1A-CF/R, AIRP2-1B-CF/R, and AIRP2-1D-CF/R (Supplementary Table S4) to amplify the genomic sequences of *TaAIRP2-1A*, *TaAIRP2-1B* and *TaAIRP2-1D*, respectively. PCR amplification was performed using a high-fidelity DNA polymerase TransStart Fast *Pfu*, with high polymorphic population genomic DNA as templates. The PCR products were separated on 1.2% agarose gel, and the purified target fragments were ligated with *pEASY*-Blunt zero vector, and positive monoclones were selected for DNA sequencing. The genome sequences of *TaAIRP2-1A*, *TaAIRP2-1B*, and *TaAIRP2-1D* were sequenced by DNA Analyzer 3730 (ABI, USA) and assembled by Seqman software

in Lasergene 7.1 software package (DNASTAR, USA), and analyzed by MegAlign software (DNASTAR, USA) for sequence polymorphism.

Development of a dCAPS marker

According to the results of sequence alignment analysis, a dCAPS molecular marker named *TaAIRP2-1B*-dCAPS (C/A) was designed at the polymorphic site –498 bp (C/A) upstream of *TaAIRP2-1B* gene coding region. A pair of amplified primers AIRP2-Sal I-F and AIRP2-dCAP-R was designed near the polymorphic site (Supplementary Table S4). A mismatched site was introduced near the polymorphic site to form a cleavage site that can be recognized by the restriction endonuclease *Sal* I. Genomic DNA was used as the template, genome-specific primers of *TaAIRP2-1B*, AIRP2-1B-F and AIRP2-1B-R were used to conduct the first round of PCR, and the PCR product was diluted with ddH$_2$O by 100 times as the template for the second round of PCR. The PCR product of the second round was digested by restriction endonuclease *Sal* I. The products were separated on 4.0% agarose gel by electrophoresis. Genotypes of different wheat accessions were analyzed according to the fragment size after enzyme digestion.

Association analysis

The mixed linear models (MLM) program of TASSEL (version 5.2.51) software was used for association analysis with principal components and kinship matrix (K matrix) as the covariates. SPSS (version 19.0) software was used for variance analysis to detect the significance of difference.

Generation and phenotype analysis of transgenic overexpressing rice

The *TaAIRP2-1B* fragment was inserted into pCUbi1390 vector, and the constructed plasmid was transformed into EHA105 *Agrobacterium* cells for genetic transformation in Japonica rice variety Kitaake. The homozygous transgenic lines were detected by both hygromycin screening and PCR amplification using the AIRP2-1B-JC-F/R primers (Supplementary Table S4). The *TaAIRP2-1B* overexpression lines and WT rice were germinated and cultured in an artificial climate incubator for 2 weeks, then transplanted into plastic containers in late May, and cultured at Haidian (39°48´ N; 116°28´ E), Beijing in 2019 and 2020 cropping seasons in an authorized experimental base for transgenic plant materials. Each line was set up in 3 replicates with > 10 plants per replicate. The primers used for vector construction and positive transgenic rice detection are listed in Supplementary Table S4.

Subcellular localization

Primers TaAIRP2-1B-GFP-F/R were used to amplify *TaAIRP2-1B* full-length cDNA (Supplementary Table S4). The coding sequence of *TaAIRP2-1B* was fused in the pCAMBIA1300 vector with GFP label by the control of CaMV35S promoter, then the constructs were transformed into *Agrobacterium* GV3101. Tobacco cultured for about 4 weeks was used for *Agrobacterium* infection, and GFP signal was detected after culturing for 3 days under LD (long-day, 16 h light/ 8 h dark) conditions (25°C). The Zeiss LSM 700 confocal microscopy (Carl Zeiss, Germany) was used for GFP fluorescence imaging.

Yeast two-hybrid assays

The coding sequence of *TaAIRP2-1B* fused with pGBKT7 (TaAIRP2-1B-BD) was used as the bait for the yeast two-hybrid screening assay according to the operating procedures of Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, USA). For yeast two-hybrid

assay, *TaHIPP3* CDS were fused with pGADT7 (TaHIPP3-AD), then co-transformed with

TaAIRP2-1B-BD into yeast strain Y2HGold. Empty pGBKT7 and pGADT7 vectors were

used as control. The results were accessed on SD/–Trp/–Leu and SD/–Trp/–Leu/–His/–Ade

mediums at 30°C after a 3-5 d cultivation. Primers used for yeast two-hybrid assays are listed

in Supplementary Table S4.

Yeast one-hybrid assays

To perform yeast one-hybrid assays, the full-length *TaERF3* coding sequence was cloned into

the pB42AD vector (TaERF3-pB42AD). The 158 bp (–397 to –240 bp) fragments of

TaAIRP2-1B promoter region containing a single-nucleotide polymorphism site (C/A) at –

289 bp upstream of ATG were amplified from wheat cultivars Jimai 32 (*Hap*-1B-1) and Jimai

2 (*Hap*-1B-2), then cloned into pLacZ2u vector (Hap-1B-1/Hap-1B-2-pLacZ2u), separately.

Appropriate pairs of constructs were transformed into yeast strain EGY48. The yeast clones

were grown on SD/–Trp/–Leu medium at 30°C for 3 days, and then spotted onto SD medium

lacking Trp and Ura plus X-gal to detect interactions.

Protein degradation assays

For the protein degradation assay, first, the BP recombination reactions between the CDS of

TaAIRP2-1B/*TaHIPP3* and pDONR 223 vector were performed to generate entry clones

(TaAIRP2-1B-223/TaHIPP3-223), respectively. And then, the LR recombination reaction

between TaAIRP2-1B-223/TaHIPP3-223 and destination vector pEarleyGate

203/pEarleyGate 103 was conducted to generate expression clones (Myc-TaAIRP2-1B/GFP-

TaHIPP3), respectively. The plasmids Myc-TaAIRP2-1B and GFP-TaHIPP3 were

transformed into *Agrobacterium* GV3101 cells, and cultured until the concentration of

Agrobacterium OD$_{600}$ = 0.6, which were co-infiltrated into *N. benthamiana*. The proteasome

inhibitor MG132 (Sigma, USA) at a final concentration of 70 μM was applied 12 h before

sampling to prevent protein degradation. The proteins were extracted using Plant Protein

Extraction Kit (Cwbio, China), and analyzed by SDS-PAGE and Western Blotting.

Dual-luciferase assays

The coding sequence of *TaERF3* was amplified and cloned into pGreenII-62-SK vector

(TaERF3-62-sk). The 158 bp fragments of haplotypes *Hap*-1B-1 and *Hap*-1B-2 of *TaAIRP2-*

1B were amplified from wheat cultivars Jimai 32 and Jimai 2, respectively, using Hap-LUC-F

and Hap-LUC-R primers, and inserted into pGreen II 0800-LUC vector under the control of

the CaMV35S promoter to generate the recombinant plasmids *Hap*-1B-1-LUC and *Hap*-1B-

2-LUC. The plasmid *Hap*-1B-1-LUC/*Hap*-1B-2-LUC and TaERF3-62-sk were co-infiltrated

into the about 28-day-old *N. benthamiana* leaves, respectively. The luciferase activity of

firefly luciferase (LUC) and Renilla luciferase (REN) were determined using a Dual Glo$^{®}$

Luciferase Assay System (Promega, USA) following the manufacturer's instruction. The

promoter activity was calculated according to the ratio of LUC/REN.

Luciferase complementation imaging assays

The coding sequences of *TaAIRP2-1B* and *TaHIPP3* were constructed into nLUC (TaAIRP2-

1B-nLUC) and cLUC (TaHIPP3-cLUC*)*, separately, and the recombinant plasmids were co-

transformed into GV3101, which was then infiltrated into *N. benthamiana* leaves. Infiltration

buffer contains 10 mM MgCl$_2$, 10 mM MES, and 200 mM acetosyringone, pH 5.6. After 72 h

of treatment, the detached leaves were treated with beetle luciferin, and the fluorescence

signals were detected by CCD imaging apparatus (Berthold, Germany).

Results

TaAIRP2s belong to the RING type E3 ubiquitin ligase

Our previous study found that *TaAIRP2-1B* was involved in plant response to abiotic stress
(Zhang *et al*., 2020), and interestingly, the tissue expression analysis showed that the
TaAIRP2-1A, *TaAIRP2-1B* and *TaAIRP2-1D* (*TaAIRP2s*) were expressed in all tested tissues
at seedling and jointing stages, and the highest expression level was observed in the spikes at
jointing stage (Fig. 1A). These findings showed that *TaAIRP2s* might have a function in spike
development of wheat. Therefore, we investigated the effect of *TaAIRP2s* on wheat spike.
Referenced to the amino acid sequence of *AtAIRP2*, the genomic sequences of *TaAIRP2*,
TaAIRP2-1A (*TraesCS1A01G169300*, Chr1A: 301895909-301899325), *TaAIRP2-1B*
(*TraesCS1B01G187600*, Chr1B: 335971825-335976367) and *TaAIRP2-1D*
(*TraesCS1D01G167300*, Chr1D: 238329446-238332693) were cloned from hexaploid wheat
and aligned in URGI database using Chinese Spring reference sequence. The coding
sequences of *TaAIRP2-1A*, *TaAIRP2-1B* and *TaAIRP2-1D* are 741 bp, 729 bp and 741 bp,
respectively. The amino acid sequence similarities among TaAIRP2-1A, TaAIRP2-1B and
TaAIRP2-1D are more than 98%, and all three *TaAIRP2* genes contain a canonical C3HC4-
type RING finger domain, which is classified as the RING finger E3 ubiquitin ligase
subfamily (Fig. 1B).

Sequence polymorphism and association analysis

To identify the sequence polymorphisms, the genomic sequences of *TaAIRP2s* in 32 high
polymorphic wheat accessions were sequenced. The coding regions of *TaAIRP2-1A*,
TaAIRP2-1B and *TaAIRP2-1D* were highly conserved, and only four SNPs (single nucleotide
polymorphism) were detected in the upstream sequence of *TaAIRP2-1B* coding region (Fig.
2A). Based on the four SNPs, two haplotypes of *TaAIRP2-1B* were identified in the wheat

collections, namely *Hap*-1B-1 and *Hap*-1B-2 (Fig. 2B). To distinguish the two haplotypes, a

dCAPS molecular marker *TaAIRP2-1B*-dCAPS was developed based on the SNP at –498 bp

upstream of *TaAIRP2-1B* coding region (Fig. 2C).

The wheat Population 1 was genotyped using the developed marker *TaAIRP2-1B*-

dCAPS to detect the correlation between haplotypes of *TaAIRP2-1B* and yield-related traits

in 30 environments over seven years. The association analysis showed that the marker is

significantly associated with spike length in 22 environments (Supplementary Table S5). The

statistical analysis result showed that spike length of genotype *Hap*-1B-1 is significantly

longer than that of *Hap*-1B-2 in Population 1 (Fig. 2D). Therefore, *Hap*-1B-1 is a superior

haplotype to increase spike length.Since the polymorphisms are located in the promoter

region of *TaAIRP2-1B*, the expression levels in different haplotypes of *TaAIRP2-1B* was

analyzed. Six accessions with *Hap*-1B-1 and six accessions with *Hap*-1B-2 were randomly

selected from Population 1 to detect the expression levels of *TaAIRP2-1B*. The results

showed that the expression level of *TaAIRP2-1B* in wheat with *Hap*-1B-1 was significantly

higher than those with *Hap*-1B-2, suggesting that *TaAIRP2-1B* gene was correlated with long

spike (Fig. 2E).

The geographical distribution of *TaAIRP2-1B* haplotypes in the ten wheat zones of

China

According to the characteristics of wheat types, cultivation period, and natural factors such as

temperature and moisture, the wheat production areas in China are divided into 10 zones. The

TaAIRP2-1B haplotypes in Population 2 with 157 Chinese landraces and Population 3 with

348 modern cultivars were analyzed to evaluate the geographic distribution of *TaAIRP2-1B*

haplotypes in the 10 wheat zones of China. As shown in Fig. 3, compared with population 2,

the frequency of *Hap*-1B-1 was increased in most wheat zones (except Wheat Zones II and

VI) of Population 3. The proportion of *Hap*-1B-1 was 56.67% in Population 2 (landraces), and 66.67% in Population 3 (modern cultivars). *Hap*-1B-2 accounted for 43.33% in Population 2, and 33.33% Population 3 (Supplementary Fig. S1). This suggested that *Hap*-1B-1 was selected in the process of Chinese wheat breeding.

TaERF3 directly binds to the promoter of *TaAIRP2-1B* and regulates its expression

Further analysis of the promoter regions of *TaAIRP2-1B* revealed a *cis*-element difference at -289 of the single nucleotide polymorphism between the two haplotypes (http://planttfdb.cbi.pku.edu.cn/). An ERF-binding site was identified in *Hap*-1B-2 (GCTCCTCCTTCCCC**A**AGTCCG), but not in *Hap*-1B-1 (GCTCCTCCTTCCCC**C**AGTCCG) due to an A-to-C difference. Previous reports showed that OsERF3 is a transcriptional repressor containing ERF-motif and plays important roles in regulating yield-related genes (Kagale *et al.*, 2010; Wang *et al.*, 2011). The *TaERF3* (*TraesCS3A02G32800*) in wheat has high transcript levels in flower organs by expression pattern analysis of *TaERF3* from Wheat database (https://wheat.pw.usda.gov/WheatExp/) (Wang *et al.*, 2020). Therefore, we selected the TaERF3, an ortholog of OsERF3 to examine its effect on the expression of *TaAIRP2-1B*.

We conducted yeast one-hybrid assays to verify whether the promoter region of *TaAIRP2-1B* was a target of TaERF3. The full-length *TaERF3* was cloned into pB42AD vector (TaERF3-pB42AD), and two haplotypes of *TaAIRP2-1B* promoter region were separately cloned into pLacZ2u vector (Hap-1B-1/Hap-1B-2-pLacZ2u). The TaERF3-pB42AD and Hap-1B-1/Hap-1B-2-pLacZ2u plasmid were co-transformed into yeast EGY48 cells and cultured on the SD/–*Trp*/–*Ura/*x–gal yeast medium. The results showed that TaERF3 binds to promoter of *Hap*-1B-2, which contains an ERF-binding site, but not to promoter of *Hap*-1B-1 (Fig. 4A). To further verify the regulatory effect of TaERF3 on *TaAIRP2-1B*, LUC assay was

performed. The *TaAIRP2-1B* promoter was cloned into pGreen II 0800-LUC vector as a

reporter, and *TaERF3* was inserted into pGreenII-62-SK vector as effectors. The level of

LUC activity illustrated that the expression of *TaAIRP2-1B* promoter containing *Hap*-1B-2

was inhibited by TaERF3 (Fig. 4B). The results demonstrated that TaREF3 binds to *Hap*-1B-

2 promoter of *TaAIRP2-1B* and represses its transcription, but has no effect on *Hap*-1B-1.

Subcellular localization of TaAIRP2-1B

To further study the function of *TaAIRP2-1B*, we investigated its subcellular localization. The

full-length sequence encoded by *TaAIRP2-1B* was fused upstream of GFP protein in

pCAMBIA1300 expression vector under control of a CaMV35S promoter (TaAIRP2-1B-

GFP), and transformed into tobacco leaves. The fluorescence signal of TaAIRP2-1B-GFP

recombinant protein was found only in cytosolic fractions (Supplementary Fig. S2).

***TaAIRP2-1B* positively regulates spike length**

To test the effect of *TaAIRP2-1B* on plant growth and development, we cloned the full-length

CDS of *TaAIRP2-1B* and fused this fragment with pCUbi1390 vector, and obtained three rice

lines with high overexpression levels of *TaAIRP2-1B* (OE2, OE5 and OE18) by genetic

transformation (Supplementary Fig. S3). The agronomic traits of transgenic rice were

measured continuously for 2 years. As shown in Fig. 5, the *TaAIRP2-1B* overexpressing lines

exhibited longer spike length than WT. The results suggested that *TaAIRP2-1B* acts as a

positive regulator of spike length.

TaAIRP2-1B promoted TaHIPP3 degradation

To explore the mechanism of *TaAIRP2-1B*, we performed a yeast two-hybrid screen for

acquiring candidate proteins that might interact with TaAIRP2-1B. Seven candidate proteins

were identified (Table 1). Among these genes, the *AtHIPP3* (heavy metal-associated

isoprenylated protein 3) was reported as a functional role in stress response and reproductive growth (Zschiesche *et al.*, 2015). Therefore, we investigated whether TaAIRP2-1B could physically interact with TaHIPP3, homolog of AtHIPP3 in wheat. The recombinant plasmid TaHIPP3-pGADT7 (TaHIPP3-AD) and TaAIRP2-1B-BD were co-transformed into Y2H yeast cells, and the result showed that TaAIRP2-1B and TaHIPP3 interact (Fig. 6A).

We further performed LCI (Luciferase complementation imaging) assays to verify this interaction in *N. benthamiana* leaves. The interaction signal was detected only in the co-expressed sample TaAIRP2-1B-nLUC/TaHIPP3-1B-cLUC, not in the negative control (Fig. 6B). The results confirmed the interaction between TaAIRP2-1B and TaHIPP3 in vivo.

At the same time, we conducted an agroinfiltration-mediated transient expression in *N. benthamian* to check whether TaAIRP2-1B affected the protein level of TaHIPP3. TaHIPP3-GFP and TaAIRP2-1B-Myc were co-expressed in *N. benthamiana* by agroinfiltration for 72 h, then the protein levels of the TaHIPP3, TaAIRP2-1B and actin (loading control) were analyzed by Western Blotting. The results showed that the protein level of TaHIPP3-GFP was markedly decreased when co-expressed with TaAIRP2-1B-Myc compared with TaHIPP3-GFP alone, and this effect was repressed by MG132 (Fig. 6C). These results demonstrated that TaAIRP2-1B facilitated TaHIPP3 degradation.

Discussion

E3 ubiquitin ligases play important roles in plant growth, development and response to abiotic stress by ubiquitinating target proteins (Ban and Estelle, 2021; Beathard *et al.*, 2021). The number of ubiquitin ligase E3 with defined functions is less than 1% of the total number in this large family, and the functions of most members of the family remain to be characterized (Kraft *et al.*, 2005; Stone *et al.*, 2005; Zhang *et al.*, 2015a). In recent years, several E3 RING ubiquitin ligases were identified to regulate agronomic trait in plant. For

example, the RING E3 ligase Chang Li Geng 1 (CLG1) has a positive effect on rice grain length by targeting GS3 degradation (Yang *et al.*, 2021). AtAIRP2, a C3HC4 type E3 ubiquitin ligase, is a key component of ABA signaling pathway and has a positive effect on ABA-induced stress genes (Cho *et al.*, 2011). However, the effect of *AIRP2* on agronomic traits has not been reported in crop plants. Our previous study found that *TaAIRP2-1B* was highly expressed in wheat spike at jointing stage, thus we inferred that *TaAIRP2-1B* might be involved in determining the spike development in wheat. Protein alignment analysis showed that TaAIRP2 belongs to the RING domain E3 ubiquitin ligase, containing a RING finger domain. We analyzed the genomic sequence polymorphisms of *TaAIRP2-1B*. Only four SNPs were detected in the promoter region of *TaAIRP2-1B*, and *TaAIRP2-1B* was divided into two haplotypes. Association analysis showed that *TaAIRP2-1B* was significantly associated with spike length, and *Hap*-1B-1 was the favorable haplotype for increasing spike length, which was positively selected in the wheat breeding history in China.

AP2/ETHYLENE-RESPONSIVE FACTOR (ERF) *transcription factors* play important roles in growth and development *in numerous plant species*. *AtERF1* has been reported to delay *Arabidopsis* flowering by suppressing the expression of *FLOWERING LOCUS T* (Chen *et al.*, 2021). *ERF1* negatively regulates grain size in rice (Schmidt *et al.*, 2014). *OsERF115*, a transcriptional repressor in rice, plays an important role in downstream regulation of spikelet growth and endosperm development (Liu *et al.*, 2022). *Wheat FRIZZY PANICLE (WFZP) which* belongs to the AP2/ERF family and possesses a transcriptional activation, has been proved *to* directly bind to the promoters of *VRN1* and *TaHOX4* to promote the initiation and development of spikelet (Li *et al.*, 2021). *TaERF3* contains a single AP2 domain, and belongs to the ERF subfamily. TaERF3, as a transcription factor, has been confirmed to positively regulate the 1000-grain weights *by inhibiting the expression of TaSDIR1-1A in wheat (Wang et al., 2020).* Our results demonstrated that TaERF3 could negatively regulate

the expression of *TaAIRP2-1B* by binding to the ERF-binding motif in the promoter region of haplotype *Hap-1B-2*, but not in haplotype *Hap-1B-1*. Therefore, we hypothesized that TaERF3 may be a direct regulator of *TaAIRP2-1B* and affect wheat spike length.

Panicle length plays an important role in improving panicle architecture and grain yield, and can be used as an indicator for breeding (Lu *et al.*, 2015; Okada *et al.*, 2018; Sun *et al.*, 2016). Studies have shown that rice panicle length is associated to many agronomic traits, such as flowering period, grain number per panicle, and grain density (Li *et al.*, 2011; Thomson *et al.*, 2003). Panicle length directly determines the number and length of panicle branches, and thus affects the grain number per panicle (Li *et al.*, 2018; Zhang *et al.*, 2015b). Therefore, the discovery of favorable haplotypes regulating spike length genes will help breeders to develop new high-yield varieties.

In this study, we verified that *TaAIRP2-1B* positively regulates spike length using *TaAIRP2-1B* overexpressing rice lines. At the same time, *TaAIRP2-1B* transgenic lines showed no significant difference in spikelet number and grain number per spike (Supplementary Fig. S4), and other traits compared with WT. We also identified several candidate genes related to wheat growth, development and response to drought stress by yeast two-hybrid screen with library, and found that TaAIRP2-1B interacts with TaHIPP3 and triggers its degradation. The *AtHIPP3* gene in *Arabidopsis* was reported to be involved in abiotic stress response, seed and flower development (Zschiesche *et al.*, 2015). In addition, recent research has shown that AtHIPP1 interacts with CKX1 (Cytokinin-degrading) protein to trigger its degradation, which is synthesized by subjecting to ERAD (ER-associated degradation) protein (Guo *et al.*, 2021). Interestingly, *BnCKX5*, a copy of *CKX* in oilseed rape, was verified to increase silique length and determine pod development (Liu *et al.*, 2018). Moreover, we investigated the tissue expression of *TaHIPP3*, and found that *TaHIPP3s* were highly expressed in spike (Supplementary Fig. S5), indicating that TaHIPP3

might participate in the development of wheat spike. Therefore, we speculated that the interaction between TaAIRP2-1B and TaHIPP3 might increase spike length by triggering degradation of TaHIPP3. Taken together, the ERF subfamily transcription factor TaERF3 directly inhibits expression of *TaAIRP2-1B* gene by binding to the *Hap*-1B-2 promoter, and reduces the TaAIRP2-1B-mediated TaHIPP3 degradation, thus decreasing spike length. On the contrary, without ERF binding site, haplotype *Hap*-1B-1 of *TaAIRP2-1B* has longer spike (Supplementary Fig. S6).

In conclusion, *TaAIRP2-1B*, as a C3HC4-type RING E3 Ub ligase gene, is confirmed to be a regulator of spike length in wheat, and the haplotype *Hap*-1B-1 of *TaAIRP2-1B* is a favorable natural variation to increase spike length. These results enrich the new understanding of *TaAIRP2* genes and provide available genetic resources for molecular breeding of wheat.

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Author Contributions

JW and RJ designed the experiments; JZ, JW, LL conducted the experiments; XM, YX analyzed the data; JW, JZ, and RJ wrote the manuscript, CL revised the first draft.

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Conflict of Interest

The authors declare that they have no conflicts of interest in relation to the content of this manuscript.

Data Availability Statement

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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Table

Table 1 Candidate genes of screening library by yeast two-hybrid

Gene	Gene ID	Number of clones
26S proteasome non-ATPase regulatory subunit 14 homolog	XM_044483962.1	1
Nonspecific lipid transfer protein 4	XM_037616094.1	1
Ferredoxin-3, chloroplast	XM_044601495.1	1
Serine/threonine-protein kinase STY13-like	XM_044534489.1	3
Beta-glucosidase	XM_037628285.1	1
Histone H3	XM_044571407.1	2
Heavy metal-associated isoprenylated protein 3(HIPP3)	XM_044481088.1	3
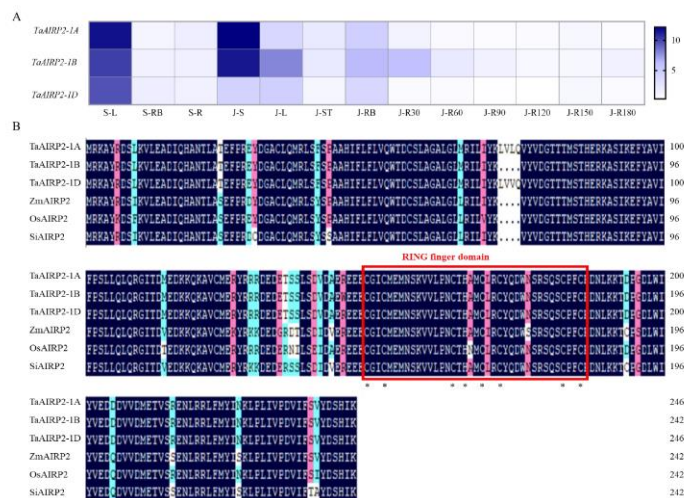
Fig. 1. *TaAIRP2s* **tissue expression pattern and sequence analysis.** (A) Expression patterns of *TaAIRP2* in wheat tissues at seedling stage and jointing stage. S, seedling stage; S-L, leaf; S-RB, root base; S-R, root. J, jointing stage; J-S, spike; J-L, leaf; J-ST, stem; J-RB, root base; J-R30, root in 0-30 cm; J-R60, root in 30-60 cm; J-R90, root in 60-90 cm; J-R120, root in 90-120 cm; J-R150, root in 120-150 cm; J-R180, root in 150-180 cm. (B) Protein sequence alignment of wheat TaAIRP2s with AIRP2 in other species. Ta, *Triticum aestivum*; Os, *Oryza sativa*; Si, *Setaria italic*; Zm, *Zea mays*. The NCBI login number: *OsAIRP2* (XM_015758962.2); *SiAIRP2* (XM_004982654.2); *ZmAIRP2* (XM_023302277.1).
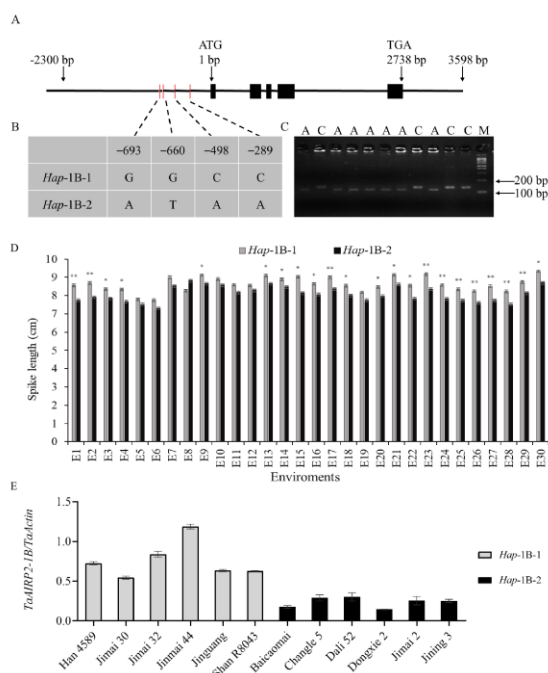
Fig. 2. Haplotypes of *TaAIRP2-1B* associated with spike length. (A) *TaAIRP2-1B* sequence polymorphisms; (B) SNPs and haplotypes of *TaAIRP2-1B*; (C) PCR products of the dCAPS marker *TaAIRP2-1B*-dCAPS (C/A) digested by *Sal* I. M, marker. (D) Phenotypic comparisons of *Hap*-1B-1 and *Hap*-1B-2 in 30 environments. E1–E30 indicate the environments. E1, 10-SY-DS; E2, 10-SY-DS-HS; E3, 10-SY-WW; E4, 10-SY-WW-HS; E5, 11-SY-DS; E6, 11-SY-DS-HS; E7, 11-SY-WW; E8, 11-SY-WW-HS; E9, 12-SY-DS; E10, 12-SY-DS-HS; E11, 12-SY-WW; E12, 12-SY-WW-HS; E13, 13-CP-DS; E14, 13-CP-WW; E15, 15-SY-DS; E16, 15-SY-DS-HS; E17, 15-SY-WW; E18, 15-SY-WW-HS; E19, 16-SY-DS; E20, 16-SY-DS-HS; E21, 16-SY-WW; E22, 16-SY-WW-HS; E23, 16-CP-DS; E24, 16-CP-WW; E25, 17-SY-DS; E26, 17-SY-DS-HS; E27, 17-SY-WW; E28, 17-SY-WW-HS; E29, 17-CP-DS; E30, 17-CP-WW. SY, Shunyi; CP, Changping; DS, drought stress; HS, heat stress; WW, well-watered; n.s., not significant; *, ** indicate significance levels at 0.05 and 0.01, respectively. Bars mean SE. (E) Expression levels of *TaAIRP2-1B* in two haplotypes accessions at seedling stage. The first six genotypes, Han 4589, Jimai 30, Jimai 32, Jinmai 44, Jinguang, and Shan R8043 possess *Hap*-1B-1. The second six genotypes, Baicaomai, Changle 5, Dali 52, Dongxie 2, Jimai 2 and Jining 3 possess the *Hap*-1B-2. Bars mean SD.
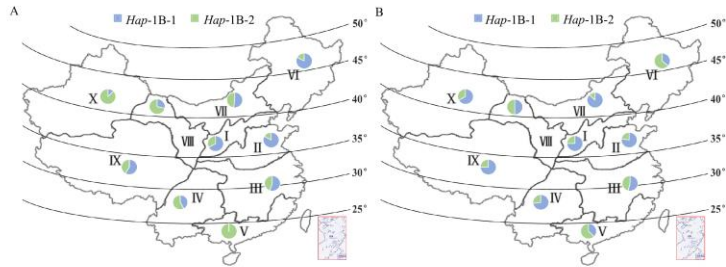
Fig. 3. The geographical distribution of *TaAIRP2-1B* haplotypes in the ten wheat zones of China. (A) Geographic distribution of *Hap*-1B-1 and *Hap*-1B-2 in 157 Chinese landraces. (B) Geographic distribution of *Hap*-1B-1 and *Hap*-1B-2 in 348 modern cultivars. I, Northern Winter Wheat Zone; II, Yellow and Huai River Valleys Winter Wheat Zone; III, Middle and Lower Yangtze Valleys Autumn-sown Spring Wheat Zone; IV, Southwestern Autumn-sown Spring Wheat Zone; V, Southern Autumn-sown Spring Wheat Zone; VI, Northeastern Spring Wheat Zone; VII, Northern Spring Wheat Zone; VIII, Northwestern Spring Wheat Zone; IX, Qinghai-Tibetan Plateau Spring-Winter Wheat Zone; X, Xinjiang Winter-Spring Wheat Zone.
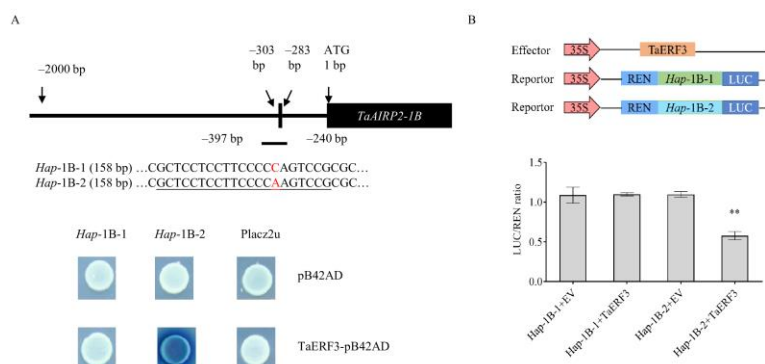
Fig. 4. TaERF3 binds to the *Hap*-1B-2 promoter region of *TaAIRP2-1B*. (A) The binding of TaERF3 and the *Hap*-1B-2 promoter in yeast. Underlined letters indicated the ERF-binding site element. Letters in red represent the single-nucleotide polymorphism sites. The fragments from *TaAIRP2-1B* promoter regions with *Hap*-1B-1 and *Hap*-1B-2 were separately cloned into the pLacZ2u vector. The full-length *TaERF3* CDS was inserted into the pB42AD vector. (B) The interaction between TaERF3 and the *TaAIRP2-1B* promoter was verified by dual-luciferase assays. The *TaERF3* was inserted into the effector construct pGreenII-62-SK and the *Hap*-1B-1/*Hap*-1B-2 promoter fragments were fused with the reporter vector pGreen II 0800-LUC. Empty pGreenII-62-SK was used as negative control. Values are means ± SD (n = 3).
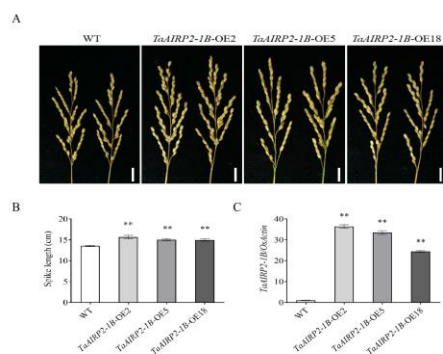
Fig. 5. *TaAIRP2-1B*-overexpressing rice lines exhibit longer spikes.
(A) Spike length of *TaAIRP2-1B* transgenic rice and WT (Kitaake) at
mature stage. (B) Comparisons of spike length. (C) The expression
levels of *TaAIRP2-1B* in overexpression transgenic rice lines. **,
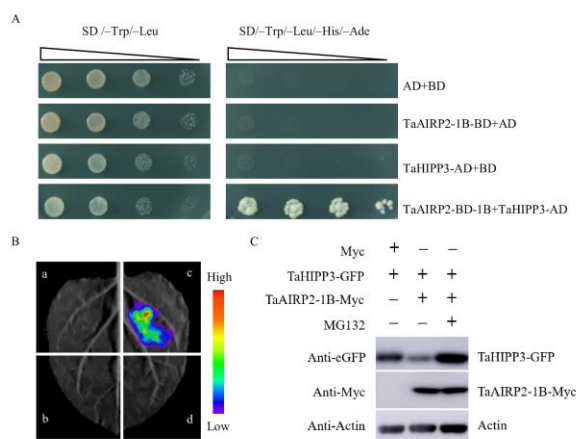indicates significance at $P < 0.01$. Values are mean $\pm$ SD.

Fig. 6. TaAIRP2-1B promoted TaHIPP3 degradation. (A) TaAIRP2-1B interacts with TaHIPP3 in yeast two-hybrid assay. The transformants were assessed on SD/–Trp/–Leu and SD/–Trp/–Leu/–His/–Ade mediums. (B) TaAIRP2-1B interacts with TaHIPP3 in LCI assay. The constructs *TaAIRP2-1B*-nLUC and *TaHIPP3*-cLUC were transferred into GV3101 cells and then infiltrated into tobacco leaves, and the interaction signals were detected at 48 h after infiltration. a: nLUC+cLUC; b: TaAIRP2-1B-nLUC+cLUC; c: TaAIRP2-1B-nLUC+TaHIPP3-cLUC; d: TaHIPP3-1B-cLUC+nLUC. (C) Protein level of GFP-TaHIPP3 in *N. benthamiana* with TaAIRP2-1B-Myc co-expression. Actin was used as the loading control. The addition of MG132 inhibited the degradation of protein in vivo.