

Pleiotropic function of SQUAMOSA PROMOTER-BINDING PROTEIN-BOX gene TaSPL14 on plant architecture of wheat

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Abstract

Background

SQUAMOSA PROMOTER-BINDING PROTEIN-BOX gene *OsSPL 14* from rice is evaluated as the major gene for ideal plant architecture consisting of few unproductive tillers, more grains per spike and high lodging resistance stems. However, the function of its orthologous gene *TaSPL 14* in wheat is still unknown.

Results

Here, we reported the similarity and variation between *TaSPL 14* and *OsSPL 14*. Similar to *OsSPL 14*, *TaSPL 14* knock-out mutants exhibited decreased plant height, spike length, spikelet number, thousand-grain weight. Different from *OsSPL 14*, *TaSPL 14* had no effect on tiller number. Transcriptome analysis genes related to ethylene response were significantly decreased in young spikes of *TaSPL 14* knockout mutants, compared with wild type. *TaSPL 14* directly binds to the promoters of the ethylene response gene *TaEIL 1* (*EIN3-LIKE 1*), *TaRAP2.11* (*ETHYLENE-RESPNSIVE TRANSCRIPTION FACTOR 2.11*) and *TaERF1* (*ETHYLENE-RESPNSIVE TRANSCRIPTION FACTOR 1*) and activities their expression, suggesting that *TaSPL 14* might regulate wheat spike development through ethylene response pathway.

Conclusions

TaSPL 14 had similar function with *OsSPL 14* in regulating plant height, spike length, spikelet number and thousand-grain weight of wheat, and had different function in tiller development. *TaSPL 14* might regulate spike development through *TaEIL 1*, *TaRAP2.11* and *TaERF1*, not *TaDEP1*. The elucidation of *TaSPL 14* will contribute to exploring the molecular mechanisms underlying plant architecture of wheat.

Background

Rice *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL*) gene *OsSPL 14* (*Ideal Plant Architecture 1*, *IPA1*) encodes a plant-specific transcription factor and is a new "Green Revolution" gene, which plays a critical role in regulating rice ideal plant architecture [1–3]. Therefore, the optimal increased expression of *OsSPL 14* in rice could confer ideal plant architecture including reduced tiller number, increased panicle primary branching, elevated lodging resistance of stems, at the same time increased thousand-grain weight [4–6]. Three natural alleles of *OsSPL 14* were identified from different high yield rice lines. The *ipa1-1D* allele contains a nucleotide substitution (C to A) affecting miR156 targeting and leads to the increased expression in shoot apex and the ideal plant architecture [7, 8]. *WEALTHY FARMER'S PANICLE* (*WFP*) epigenetic allele shows optimal increased expression of *OsSPL 14* in young spikes due to reduced DNA methylation in the promoter region, which has strong positive effect on primary branch and grain number [9]. The *ipa1-2D* epigenetic allele exhibits elevated expression of *OsSPL 14* due to tandem repeat sequences in promoter region producing an open chromatin structure, which controls tiller number, stem

diameter and panicle primary branch number in a dosage-dependent manner [4]. In addition, base deletions in the exon of *OsSPL14* by gene editing causes a frameshift mutation and the inactivation of *OsSPL14* leads to dwarf phenotype with an extremely enhanced number of tillers and decreased plant height, panicle length and spikelet number [10]. Taken together, these findings show that *OsSPL14* plays an important role in regulating ideal plant architecture in rice.

Previously, reported that *OsSPL14* regulates ideal plant architecture through controlling downstream genes expression by directly binds the GTAC core motif in promoter [11]. One of target genes of *OsSPL14*, *DENSE AND ERECT PANICLE1 (DEP1)* controls rice panicle size [12, 13]. The expression level of *DEP1* were significantly increased in shoot apices and slightly elevated in young panicles with higher expression of *OsSPL14* [14]. The panicle length of RIL-*IPA1/dep1* lines was significantly shorter than that in the RIL-*IPA1/DEP1* lines, suggesting that *OsSPL14* positively regulated *DEP1* in adjusting panicle length in rice [12, 14]. *OsSPL14* regulates tiller number by directly activating *TEOSINTE BRANCHED1 (TB1)*, which is a negative regulator of tillering in rice [14, 15]. The expression level of *OsTB1* were significantly raised in axillary buds in NIL-*ipa1* line with higher expression of *OsSPL14*, and the RIL-*ipa1/tb1* line could suppress the tiller phenotype of NIL-*ipa1* plants, indicating that the direct activation of *OsTB1* by *OsSPL14* contributing to few tiller number of NIL-*ipa1* plants [14, 16].

In wheat, only a couple of genes have been reported to affect plant architecture related traits: genes including *tin1 (tiller inhibition gene 1)*, *tin2 (tiller inhibition gene 2)*, *tin3 (tiller inhibition gene 3)* and *ftin (fertile tiller inhibition)* gene, are regulators for tiller inhibition [17–21]; *Q* gene is associated with spike compactness [22]. However, the effects of *OsSPL14* ortholog, *TaSPL14* on wheat plant architecture and yield is still largely unexplored. In this study, we aimed to analyze the function of *TaSPL14* in developing ideal plant architecture of wheat. We constructed *TaSPL14* knock-out mutants (*taspl14*) by using CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9) system, and phenotypic analysis of mutants exhibited reduced plant height, spike length, spikelet number, thousand-grain weight but similar tiller number compared with wild type Fielder. Comparison of the transcriptome of young spikes between the *taspl14* mutants and the wild type indicated that the genes involved in ethylene response were downregulated in mutants, and *TaSPL14* could directly activate the expression of the *TaEIL1*, *TaERF1* and *TaRAP2.11* involved in ethylene signaling pathway. It is different from *OsSPL14*, *TaSPL14* regulated spike length of wheat through regulating expression levels of genes associated with ethylene response, but not the *TaDEP1*. Therefore, our data provides new clues about exploring the functions and mechanisms of *TaSPL14* in regulating plant architecture in wheat.

Results

Identification of *TaSPL14* in wheat

We annotated 56 sequences containing SBP domain from wheat genome sequence (TGACv1 version) [23]. Since common wheat (BBAADD) is a typical hexaploid species that generated from three diploid ancestral species *Triticumurartu* (AA), *Aegilopsspeltoides* (BB), and *Aegilopstauschii* (DD) [24–27]. The

homoeologous genes from three subgenomes among these 56 sequences were grouped to 19 unique *TaSPL* genes, among which 10 had been previously isolated, named as previous report [28] (Table S1). A phylogenetic tree was performed based on the SBP-domain of 19 *OsSPLs* from rice and 19 *TaSPLs* from wheat [29–31]. According to the phylogenetic tree, the remaining 9 genes were named by orthologous genes of rice (Fig. 1A). We found three homoeologous genes including *TraesCS5A02G265900*, *TraesCS5B02G265600* and *TraesCS5D02G273900*, designated *TaSPL14-A*, *TaSPL14-B* and *TaSPL14-D*, which showed high amino acid similarity with *OsSPL14*. Previous studies suggested that *OsSPL14* was the target gene of *OsmiR156* [7]. To verify whether *miR156* could regulate *TaSPL14 in vivo*, we performed modified RNA ligase-mediated 5'-rapid amplification of cDNA ends (RLM-5'-RACE), and the results indicated that the *TaSPL14* occurred cleavage at the 10th nucleotide from the 5' end of *miR156* (Fig. 1B).

Three homoeologous genes of *TaSPL14* were observed to be located on chromosome 5A, 5B and 5D, respectively, through amplifying from genomic DNA of Chinese Spring (CS) nullisomic-tetrasomic of chromosome group 5. The results showed the PCR amplification bands disappeared when the corresponding chromosome was deleted from CS lines (Fig. 1C). The expression levels of *TaSPL14-A*, *TaSPL14-B*, and *TaSPL14-D* among different tissues including seeding roots (SR), stems in jointing stage (SJ), seeding leaves (SL), grains at 4, 15, and 25 days after pollination (GDAP), young spikes with 5 mm (YS5), 10 mm (YS10), 20 mm (YS20) and 30 mm (YS30) in length were measured through reverse transcription quantitative real-time PCR (RT-qPCR) (Fig. 1D). The results indicated that the expression levels of *TaSPL14-B* or *TaSPL14-D* were higher than *TaSPL14-A* in measured tissues, *TaSPL14-A* and *TaSPL14-D* were mostly abundant in stems and young spikes, *TaSPL14-B* were mostly abundant in stems, leaves, seeds and young spikes, suggesting that three homoeologous genes of *TaSPL14* might differently contribute to wheat development.

Functional comparison of *TaSPL14* and *OsSPL14*

In order to investigate the biological function of *TaSPL14* in wheat, we generated *TaSPL14* knock-out mutant using the CRISPR/Cas9 system. In order to simultaneously knock out three homoeologous genes *TaSPL14-A/B/D*, we designed a specific sgRNA to target conserved region within first exon of the *TaSPL14-A*, *TaSPL14-B* and *TaSPL14-D* (Fig. 2A). The CRISPR/Cas9 vector pBUE411::sgRNA was transformed into wild type Fielder by *Agrobacterium*-mediated transformation [32]. Three independent transgenic T₀ plants were obtained. DNA sequencing for *TaSPL14* revealed that three homoeologous genes mutated simultaneously with frameshift mutations in mutant line #5 and #12. The mutant line #13 showed a 3-base deletion of *TaSPL14-5A* without shifting mutation of protein coding region, while *TaSPL14-5B* and *TaSPL14-5D* were mutated simultaneously resulting code-shifting mutations (Fig. 2B). Homozygous T₂ generation plants #5 – 3, #12 – 4 and #13 – 4 were generated and planted in field.

We found all of three *taspl14* mutant lines showed significantly decreased plant height and spike length compared to wide-type Fielder (Fig. 2C). Further observations revealed that the average length of rachis internode and spikelet number of *taspl14* mutants were significantly decreased compared with Fielder

(Fig. 2D). These results indicated that *TaSPL14* affected wheat spike development through affecting both rachis internode elongation and spikelet formation. Thousand-grain weight (TGW) of mutant line #5 - 3, #12 - 4 and #13 - 4 was markedly decreased 17.3%, 22.88% and 23.7%g respectively compared to wild-type, indicating that the *TaSPL14* influenced the grain weight of wheat (Fig. 2E). *osspl14 (ipa1)* mutants with a frame shift in protein and inactivation of OsSPL14, which caused rice mutants with notable decreased plant height, panicle length and number of flowers [10]. Moreover, thousand-grain weight increased in NIL OsSPL14^{ipa1} with higher expression level of *OsSPL14* [7]. Thus, our results indicated that *TaSPL14* acts as a conserved regulator with *OsSPL14* to pleiotropically regulate plant height, spike length, spikelet number and thousand grain weight.

However, different from *OsSPL14*, which is a regulator of rice tillering [5], *TaSPL14* is not associated with tiller development in wheat. We found that *taspl14* mutant line #5 - 3, #12 - 4, #13 - 4 showed similar tiller number as wild type Fielder in heading stage (Fig. 2F). These data indicated that *TaSPL14* showed functional variation from *OsSPL14* in tiller development.

Downstream genes affected by TaSPL14

To understand how *TaSPL14* regulated spike development, RNA sequencing (RNA-seq) experiment was performed with young spikes (20–30 mm) of wild-type Fielder and mutant line *taspl14-#13 - 4* with two biological replications. Totally 1103 and 228 genes were down- and up-regulated in *taspl14-#13 - 4* compared with Fielder, respectively (Table S2). Further analysis of Gene Ontology of differentially expressed genes showed that upregulated genes were mainly enriched in the pathways related to red or far red light response and jasmonic acid response, while the downregulated genes were mainly enriched in the pathways related to meristem maintenance, meristems growth, cell proliferation and ethylene response (Fig. 3A, Table S3).

Among the down-regulated genes, we observed that 7 genes including *TaERF1* (*ETHYLENE-RESPNSIVE TRANSCRIPTION FACTOR 1*), *TaRAP2.11* (*ETHYLENE-RESPNSIVE TRANSCRIPTION FACTOR 2*), *TaTCP* (*TEOSINTE BRANCHED1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR*), *TaRAP2-1A* (*ETHYLENE-RESPNSIVE TRANSCRIPTION FACTOR 1*), *TaEIN4* (*ETHYLENE INSENSITIVE 4*), *TaRAP2-1D* (*ETHYLENE-RESPNSIVE TRANSCRIPTION FACTOR 1*), *TaEIL1* (*EIN3-LIKE 1*), which associated with ethylene response exhibited a statistically significant decreased expression in *taspl14-#13 - 4* compared to Fielder (Fig. 3B). The down regulation of these genes in *taspl14-#13 - 4* was further validated by performing RT-qPCR analysis (Fig. 3C-3I). Therefore, these results indicated that *TaSPL14* regulated the expression of genes related to ethylene response.

It was reported that *OsSPL14* was able to bind GTAC or TGGGCC/T core motif in promoter region of its target genes [14], which reminded us to detect whether *TaSPL14* regulate genes related to ethylene response through binding to their promoters. The core GTAC motif was identified from promoter sequence of *TaEIL1*, *TaRAP2.11* and *TaERF1*, we employed the electrophoretic mobility shift assays (EMSAs) to determine whether the *TaSPL14* binds on these genes. Specific labeled and unlabeled probes

were synthesized from promoter region of *TaEIL1*, *TaRAP2.11* and *TaERF1* respectively. As the results showed, the specificity bindings were observed in the combinations between TaSPL14- GST fusion proteins and labeled probes, and excessive molar of unlabeled probes prevented the band shift, but mutated unlabeled probes did not, indicating the TaSPL14 bound the GTAC core motif from the promoter of *TaEIL1*, *TaRAP2.11* and *TaERF1* respectively (Fig. 4A, 4B, 4C). To confirm the regulatory effect of the TaSPL14 on *TaEIL1*, *TaRAP2.11* and *TaERF1* *in vivo*, the transcriptional activity assays by *Agrobacterium tumefaciens*-mediated transient infiltration were carried out in *Nicotiana benthamiana* leaves. We generated three plasmids containing the 2 kb promoter of *TaEIL1* (*pTaEIL1::LUC*), *TaRAP2.11* (*pTaRAP2.11::LUC*), *TaERF1* (*pTaERF1::LUC*) driving the expression of the luciferase (LUC) reporter gene respectively (Fig. 4D, 4E and 4F). At the same time, we constructed a plasmid overexpressing *TaSPL14* (*35S::TaSPL14*) as effector. These results confidently illustrated that co-expression of *35S::TaSPL14* with *pTaEIL1::LUC*, *pTaRAP2.11::LUC* and *pTaERF1::LUC* led to significantly elevates the LUC reporter activity compared with that in the empty vector controls. The above results suggested that TaSPL14 could directly and dramatically elevate expression of *TaEIL1*, *TaRAP2.11* and *TaERF1* (Fig. 4D, 4E and 4F).

OsDEP1, directly and positively regulated by OsSPL14, is an important regulatory gene that affects panicle architecture [12, 14, 33] In order to detect whether SPL14-*DEP1* regulatory module is conserved between rice and wheat, we detected the expression of wheat *TaDEP1* between *taspl14-#13 - 4* and wild type Fielder. *TaDEP1-A* (*TraesCS5A01G215100*), *TaDEP1-B* (*TraesCS5B01G208700*) and *TaDEP1-D* (*TraesCS5D01G216900*) has been identified in wheat [34, 35]. Based on the results of RNA-seq, these three homoeologous genes were not differentially expressed in young spikes between mutant line *taspl14-#13 - 4* and wild type Fielder, implying that TaSPL14 might regulate spike development through other genes such as related to ethylene response not *TaDEP1* (Figure S1).

Discussion

OsSPL14 is an essential component for regulating rice ideal plant architecture, including tiller development, panicle architecture and stems lodging resistant, substantially contributing to grain yield [14, 36]. However, the function and molecular mechanism of its orthologs, *TaSPL14* is largely unknown, our study provided information about the role and mechanisms of *TaSPL14* in regulating plant architecture of wheat.

Similar to OsSPL14, TaSPL14 acts as one of the major regulators for spike morphology. The *taspl14* mutants showed reduction of spike length and spikelet number, but not altered the panicle branching (Fig. 2C, 2D). OsSPL14 regulates panicle architecture not only changing the panicle length and spikelet number, but also has strong effect on primary branching of rice [7, 9, 37]. Therefore, TaSPL14 and OsSPL14 showed the similar function in regulating length of spike and number of spikelet per panicle. It is worthy to note that OsSPL14 functions as a positive regulator of *DEP1*, which is an important component in regulating panicle architecture [14]. However, in our study the expression level of *TaDEP1* was not altered in *taspl14* mutant, compared with wild type (Figure S1), implying that TaSPL14 might regulate spike development not through *DEP1*, but rather other pathways. Here we revealed that the

expression levels of 7 genes associated with ethylene response exhibited a significantly decrease in *taspl14-#13 - 4* compared to Fielder and TaSPL14 could directly and dramatically elevate the driving activities of promoters of *TaEIL1*, *TaERF1* and *TaRAP2.11*. This indicated that TaSPL14 might involve in spike development through regulating ethylene response pathway (Fig. 5).

Different from *OsSPL14*, *TaSPL14* has no effect on tillering in wheat. The *osspl14* mutants showed two contrasting phenotypes of tiller number in rice, *osspl14* mutant plants with amino acid deletions in protein coding sequence and still maintained the activity of the protein, which showed fewer tillers than wild type [10]. Nevertheless, *osspl14* mutants with a frame shift which may completely lost the action of OsSPL14, the mutants showed dwarf phenotype with an elevated number of tillers compare with wild type [10]. In this study, we found the tiller number of all *taspl14* mutant lines was similar with wild type Fielder in the heading stage in the field (Fig. 2F), indicating the functional variation between *OsSPL14* and *TaSPL14* (Fig. 5).

OsSPL14 contains three yield-positive alleles *IPA1* (*ipa1-1D* allele), *WEALTHY FARMER'S PANICLE* (*WFP*) epigenetic allele and *ipa1-2D* epigenetic allele, which all applied in rice breeding [4, 7, 9]. Introduction of the *OsSPL14*^{ipa1} allele into Xiushui11 a japonica rice variety, increased the grain yield by ~ 10% in the test plot [7]. Introgression of *OsSPL14*^{WFP} allele into different rice varieties could significantly increase primary branches and grains per plant [9, 37–39]. For *ipa1-2D* epigenetic allele, JYZK-3 and JYZK-4 are two hybrid varieties bear *ipa1-2D*, markedly improved yield in comparison with the control variety at different locations [4]. Thus, it is meaningful to identify the natural variation related to optimal expression levels of *TaSPL14* in young panicle, which could be used to design and generate high-yielding new varieties by using molecular assisted selection.

Conclusion

In this study, we indicated that *TaSPL14* acts as a conserved regulator with *OsSPL14* to pleiotropically regulate plant height, spike length, spikelet number and thousand-grain weight of wheat. And *TaSPL14* showed functional variation from *OsSPL14* in tiller development. TaSPL14 might regulate spike development through ethylene response genes, including *TaEIL1*, *TaRAP2.11* and *TaERF1*, not *TaDEP1*. Our findings will be useful for programs that improve grain yield by manipulating wheat architecture.

Abbreviations

SPL: SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE; IPA1: Ideal Plant Architecture 1; WFP: WEALTHY FARMER'S PANICLE; RIL: Recombinant inbred lines; TB1: TEOSINTE BRANCHED1; NIL: Near isogenic lines; tin: tiller inhibition gene; ftin: fertile tiller inhibition; CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9; SBP: SQUAMOSA BINDING PROTEIN; RLM-5'-RACE: RNA ligase-mediated 5'-rapid amplification of cDNA ends; PCR: Polymerase Chain Reaction; RNA-seq: RNA sequencing; RT-qPCR: Reverse transcription quantitative real-time PCR; EMSAs: electrophoretic mobility shift assays; GST: glutathione S-transferase; LUC: luciferase.

Declarations

Availability of Data and Materials

The datasets generated and analysed during the current study are available in the NCBI (PRJNA629470) repository, <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA629470>.

Consent for Publication

Not applicable

Ethics Approval and Consent to Participate

Not applicable

Competing interests

The Authors declare that there is no conflict of interest.

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Authors' contributions

Z.N, Q.S, Y.Y, and D.J designed the project and all experiments; J.C, K.L, W.S, and N.Z carried out the experiments and analysis experimental data; M.X, H.P, and Z.H responsible for the wheat transformation; J.C, Y.Y, and D.J contributed to article writing and reversion. All authors approved the contents of this manuscript.

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Methods

Plant materials and growth conditions

Wild type Fielder and mutant *taspl14* lines were grown in the experimental field of China Agricultural University in Beijing (39°57′N, 116°17′E) for seed reproduction, visible phenotypic and molecular analyses. Young spikes around 20–30 mm in length of wild type and mutants were collected, and immediately frozen in liquid nitrogen, then stored at -80 °C for following experiments. For molecular analyses, wheat materials were grown in artificial chamber. The conditions of artificial chamber is 45% humidity, 26/20°C day/night temperatures, 3000lux illumination (Master GreenPower CG T 400W E40, PHILIPS).

Identification of TaSPL genes in wheat

To identify the novel *SPL* gene members in wheat, we downloaded SBP domain (PF03110) of SQUAMOSA-PROMOTER BINDING PROTEIN from Pfam (<http://pfam.xfam.org/>). All of sequences with SBP domain were obtained in wheat genome database (<http://plants.ensembl.org/biomart/>) based on Hidden Markov Model (HMM) profile with a cut of E-value $< 1 \times 10^{-5}$ [40–42]. Each predicted gene was confirmed by CDD website (<http://www.ncbi.nih.gov/cdd>) for conserved SBP domain analysis. Analysis of homoeologous genes was performed by using Ensembl Plant website (<http://plants.ensembl.org/index.html>).

Phylogenetic analysis of SPL genes

For phylogenetic analysis, 19 *OsSPLs* were obtained from GRAP [30]. Phylogenetic analysis was developed by using amino acid sequence of conserved SBP domain. We used CDD website to confirm the SBP domain sequence of SBP-box genes in rice and wheat, the ClustalW algorithm of MEGA5.02 to perform the multiple sequences alignment. A phylogenetic tree was constructed by using Neighbor-joining method, and bootstrapping with 1000 replicates. The accession number of *OsSPLs* and *TaSPLs* were shown in the Table S1.

Reverse transcription quantitative real-time PCR (RT-qPCR) analysis

According to the manufacturer, total RNA was isolated by using Trizol (Invitrogen, USA), and the first strand cDNA was synthesized by using the M-MLV Reverse Transcriptase kit (TaKaRa). We used the wheat *ACTIN* (*TraesCS5B01G124100*) gene as a reference for normalization. RT-qPCR were executed on the CFX96 real-time system (Bio-Rad) in 10 µl reactions containing 2 µl of each gene-specific primer, 1 µl of cDNA template, 2 µl of ddH₂O, and 5 µl of SYBR Premix EXtaq II (TaKaRa, Dalian, China). The RT-qPCR conditions were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 15 s, and 72 °C for 15 s, after that a melting curve was performed for each sample to analysis the specificity of the reactions.

The relative expression levels were calculated using the $2^{-\Delta CT}$ method normalized to CT value of wheat *ACTIN* gene, the RT-qPCR amplification of each sample was repeated three times. Gene-specific primers were shown in Table S4.

Vector construction and wheat transformation

We used CRISPR/Cas9 system to edit the genome sequence of *TaSPL14* [43], and E-CRISP Design website (<http://www.e-crisp.org/E-CRISP/designcrispr.html>) to design small guide RNA (sgRNA). Later, two reverse complementary sgRNA sequences with BsaI site were synthesized. Intermediate vector (pCBC-MT1T2) connected to sgRNA sequences by using PCR, then, inserted into the terminal vector pBUE411 as previously described [43]. Plasmids were transformed into wild type Fielder by *Agrobacterium*-mediated transformation, EHA105 as *Agrobacterium* strain. The primers for vector construction were shown in Table S4.

RNA sequencing

We collected 5 young spikes with 20 mm-30 mm in length of wild type Fielder and *taspl14* mutant line #13 – 4 for RNA sequencing. The extraction method of total RNA has been described before, and each RNA sample divided into two groups for two biological replicates. At least 5 µg total RNA of each replication was constructed cDNA libraries by using Illumina Poly-A Purification TruSeq library reagents, and performed on NovaSeq platform. The detailed analyze of RNA-seq data as previously described [44–46]. The differentially expressed genes between mutant line *taspl14*-#13 – 4 and the wild type Fielder were characterized by the Bioconductor package “edgeR” [47]. We used agriGO v.2.0 (cutoff of $P < 0.05$) to carried out GO analysis [48].

EMSA

The DNA-binding domain of TaSPL14 was expressed in BL21 *Escherichia coli* (Transgene) by cloning full-length of ORF sequence of *TaSPL14* into the pGEX6P-1 with GST sequence. The detailed processes of induction and purification of proteins were as previously described [49, 50]. The labeled probes were 5' end with modified biotin, and the unlabeled probes without biotin modification in 5' end, which including competed probes that have same sequence as labeled probes, and mutant probes with changed core motif. All of probes were synthesized by company (Invitrogen), and to be double-stranded by cooling from 100 °C to room temperature in annealing buffer. We used the LightShift® Chemiluminescent EMSA Kit (Thermo) for EMSA, and the detailed processes of EMSA assays were as previously described [49]. Shifted complexes of labeled fragments and proteins, and free probes were visualized in nylon membrane by chemiluminescence camera (Tanon). Primers of EMSA were listed in Table S4.

Transcriptional Activity Assays in *N. benthamiana*

For reporter constructions, the 2 kb promoter sequences of *TaEIL1*, *TaERF1* and *TaRAP2.11* were cloned, and generated in the plant binary vector pGreen that has the luciferase reporter gene, respectively, and separately introduced into *Agrobacterium tumefaciens* strains GV3101 with pSoup. The full length of coding sequence of *TaSPL14-B* subgenome was cloned and generated into overexpression vector Supper1300 to generate effector construct, and introduced into *Agrobacterium tumefaciens* strains GV3101 [51]. The *Agrobacterium tumefaciens* strains GV3101 with reporters or effector were co-infiltrated *N. benthamiana* leaves. After 48 h infiltration, collected leaves and detected LUC activities by Dual-Luciferase Reporter Assay System (Promega). Each experiment has three biological replications. The primers of transcriptional activity assays were shown in Table S4.

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Figures

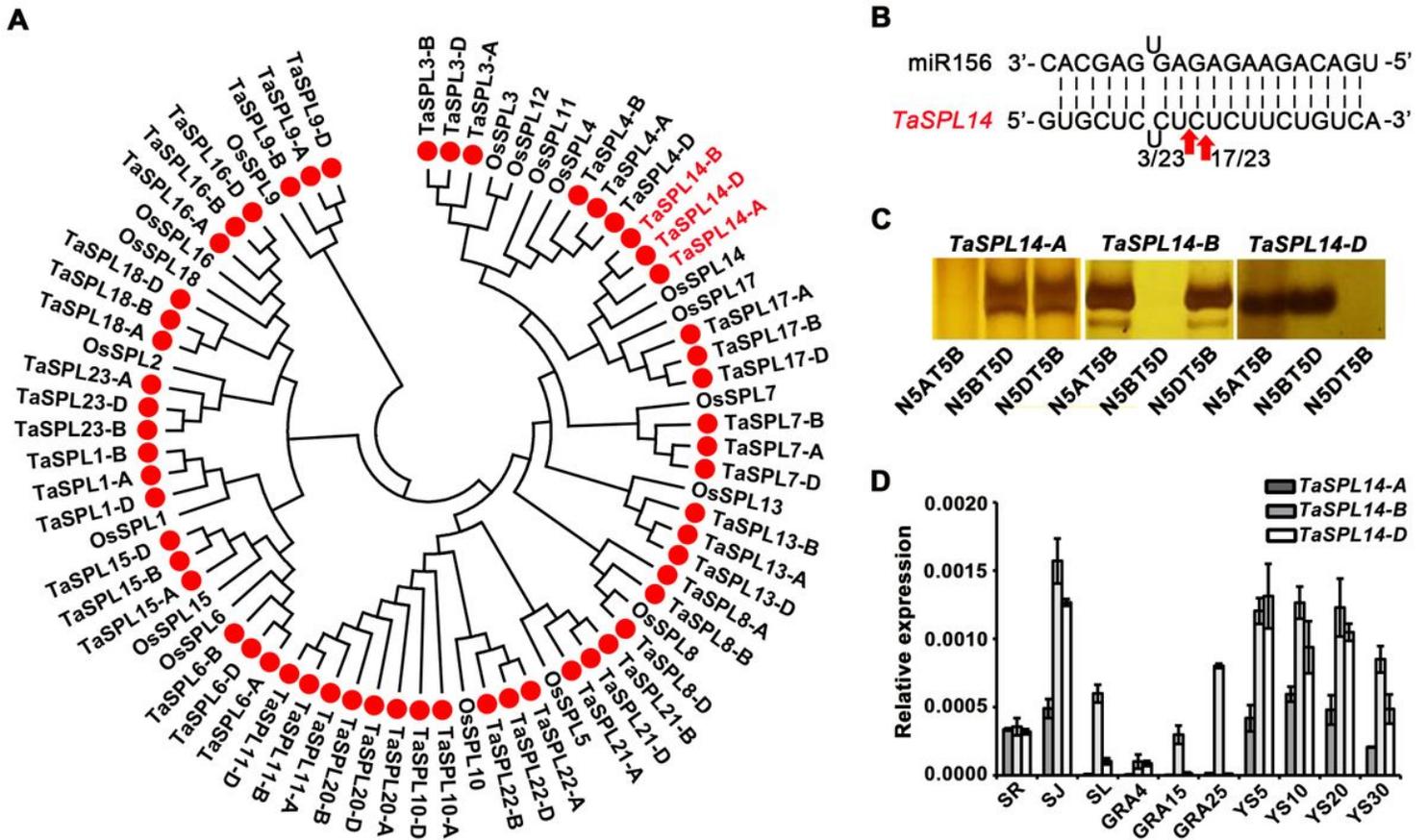


Figure 1

The identification and expression pattern of TaSPL14 in wheat. (A) Phylogenetic analysis of SBP-box genes in rice and wheat. Phylogenetic tree was constructed based on amino acid of SBP domain of OsSPL and TaSPL proteins by using MEGA5.02 with the neighbour-joining method, and the bootstrap test was performed with 1000 replicates. (B) The result of RLM-5'RACE of miR156 and TaSPL14. The red arrows represent the cleavage sites of miR156, and 3/23 and 17/23 represent the ratios of the cleaved clones from this site out of total clones identified by sequencing. (C) Chromosomes locations of three homoeologous genes of TaSPL14. The binds indicate PCR amplification fragments of homoeologous genes from the Chinese Spring (CS) null-tetrasomic lines. N5AT5B represents nullisomic 5A-tetrasomic5B, the others are similar. All PCR electrophoreses were performed simultaneously, and cropped from same gel. (D) Tissue expression patterns of TaSPL14-A, TaSPL14-B, and TaSPL14-D. SR, seeding roots; SJ, stems in jointing stage; SL, seeding leaves; GDAP4, GDAP15 and GDAP25 represent grains at 4, 15, and 25 days after pollination; YS5, YS10, YS20 and YS30 represent young spikes with 5mm, 10mm, 20 mm and 30mm in length. The ordinate represents the relative expression of TaSPL14 homoeologues genes. The reference gene is wheat ACTIN.

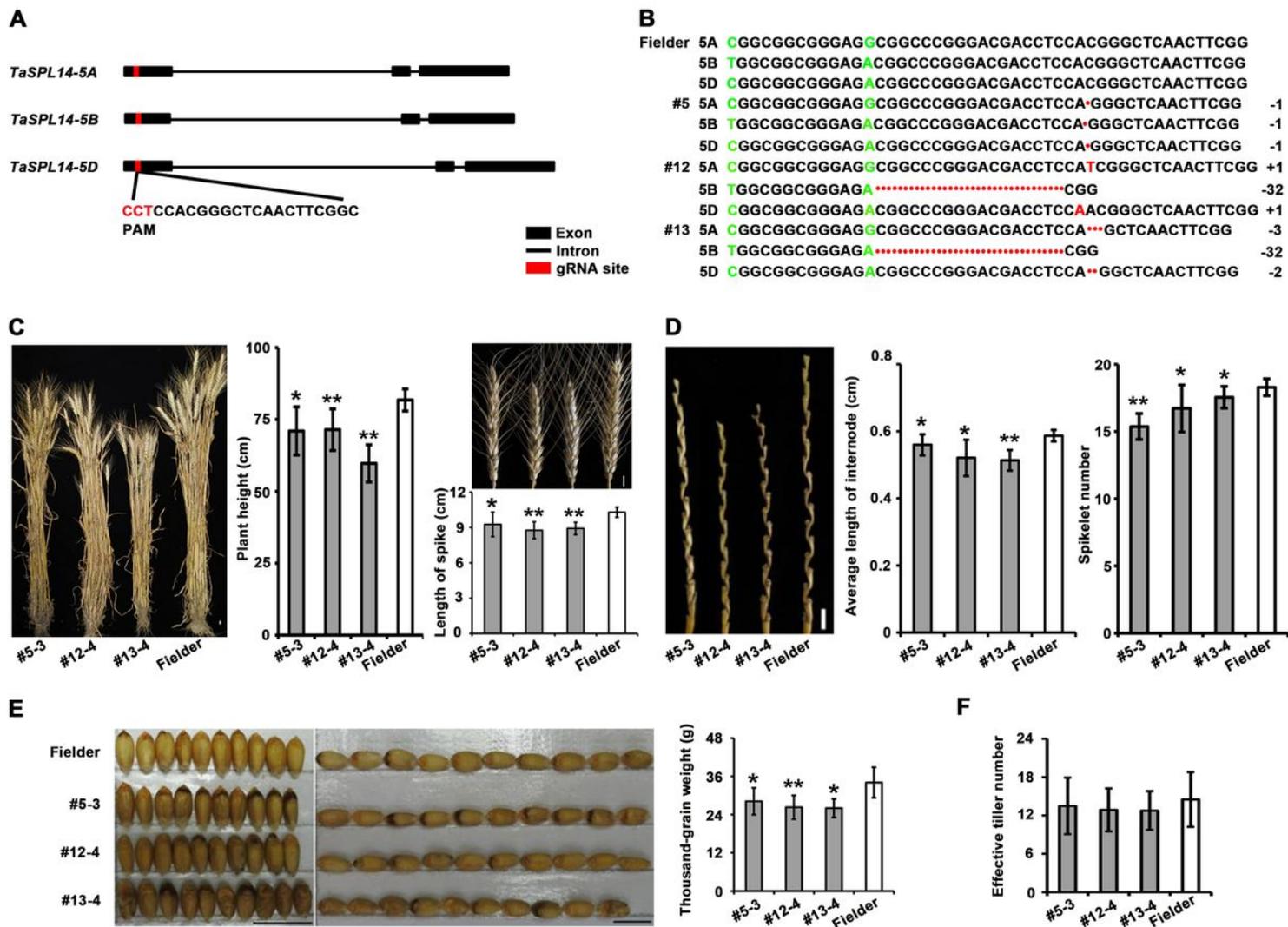


Figure 2

Phenotypic analysis of TaSPL14 knock-out mutant lines. (A) Sketch map of sgRNA location in three TaSPL14 homoeologues. Black square boxes and lines represent exons and introns respectively, red square boxes represent gRNA site. The protospacer-adjacent motif (PAM) sequence is highlighted in red, and the sgRNA sequence is in black. (B) The genotypes of three mutant lines of TaSPL14. #5, #12 and #13 represents three mutant line of TaSPL14 respectively. Fielder is wild type. "+" and "-" indicates the insertion or deletion caused by CRISPR/Cas9-induced mutations, respectively. Numbers indicates the length of the insertion or deletion. (C) Plant height and spike length of TaSPL14 mutant lines (#5-3, #12-4 and #13-4) and wild type (Fielder). $n \geq 8$. Bar = 1 cm. (D) Average length of internode and spikelet number of TaSPL14 mutant lines (#5-3, #12-4 and #13-4) and wild type (Fielder). $n \geq 8$. Bar = 1 cm. (E) Thousand-grain weight of TaSPL14 mutant lines (#5-3, #12-4 and #13-4) and wild type (Fielder). $n \geq 8$. Bar = 1 cm. (F) Effective tiller number of TaSPL14 mutant lines (#5-3, #12-4 and #13-4) and wild type (Fielder). $n \geq 8$. Statistical significant differences determined by Student's t-test (* $P < 0.05$, ** $P < 0.01$).

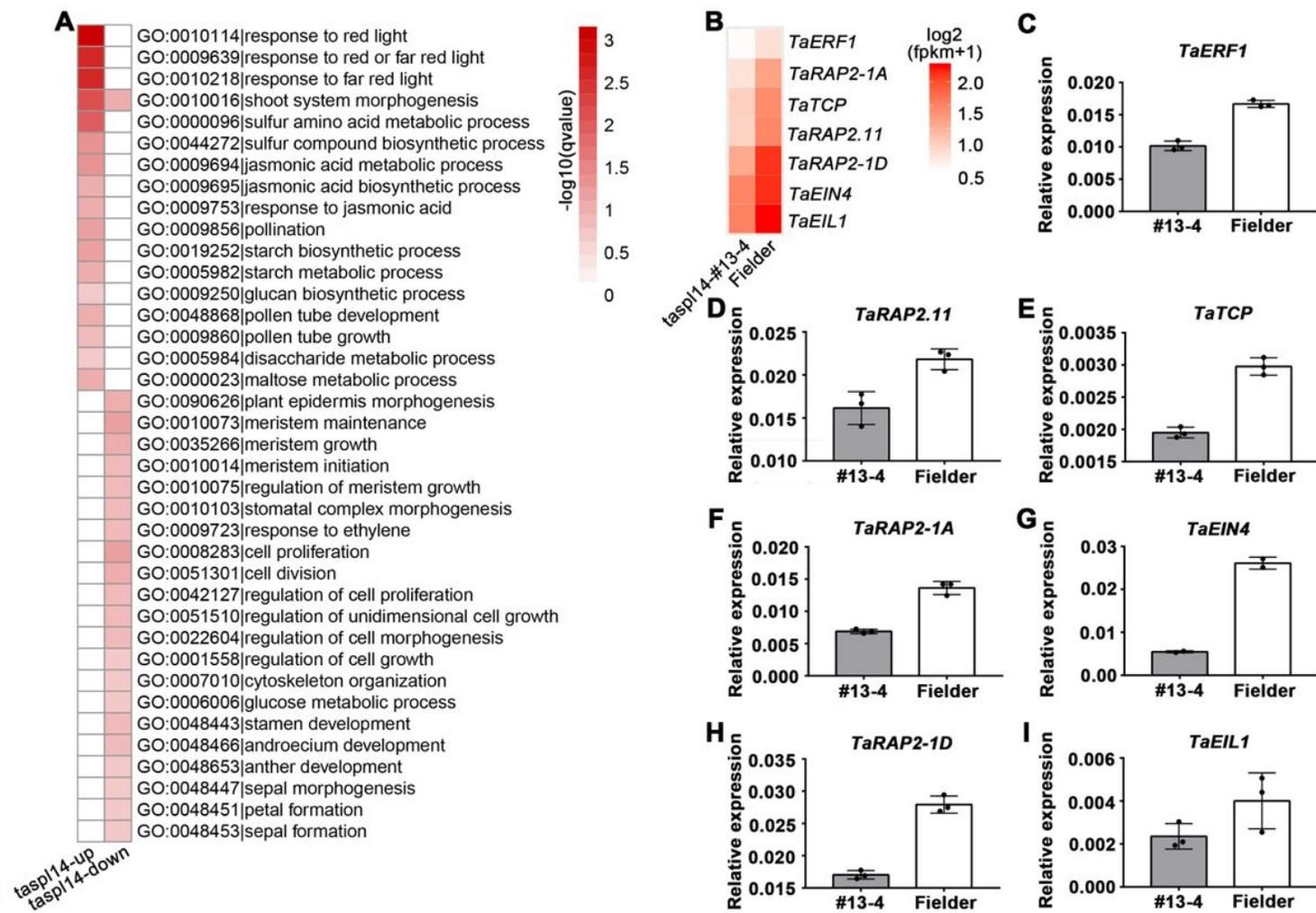


Figure 3

GO analysis and expression levels of differentially expressed ethylene response genes in *taspl14-#13-4* mutant line and wild type Fielder. (A) GO analyze of ethylene response genes differentially expressed in mutant line *taspl14-#13-4* and wild type Fielder. The color in each cell indicates \log_{10} (P values) of the GO enrichment. (B) Heat-map of the differentially expressed genes involved in ethylene response in the mutant line *taspl14-#13-4* and wild type Fielder. (C)-(I) Expression levels of differentially expressed genes involved in ethylene response in the mutant line #13-4 and wild type Fielder. The ordinate represents the relative expression of ethylene response genes. The reference gene is wheat ACTIN. Each bar in the graph represents the mean value of three repetitions, which were shown as circles.

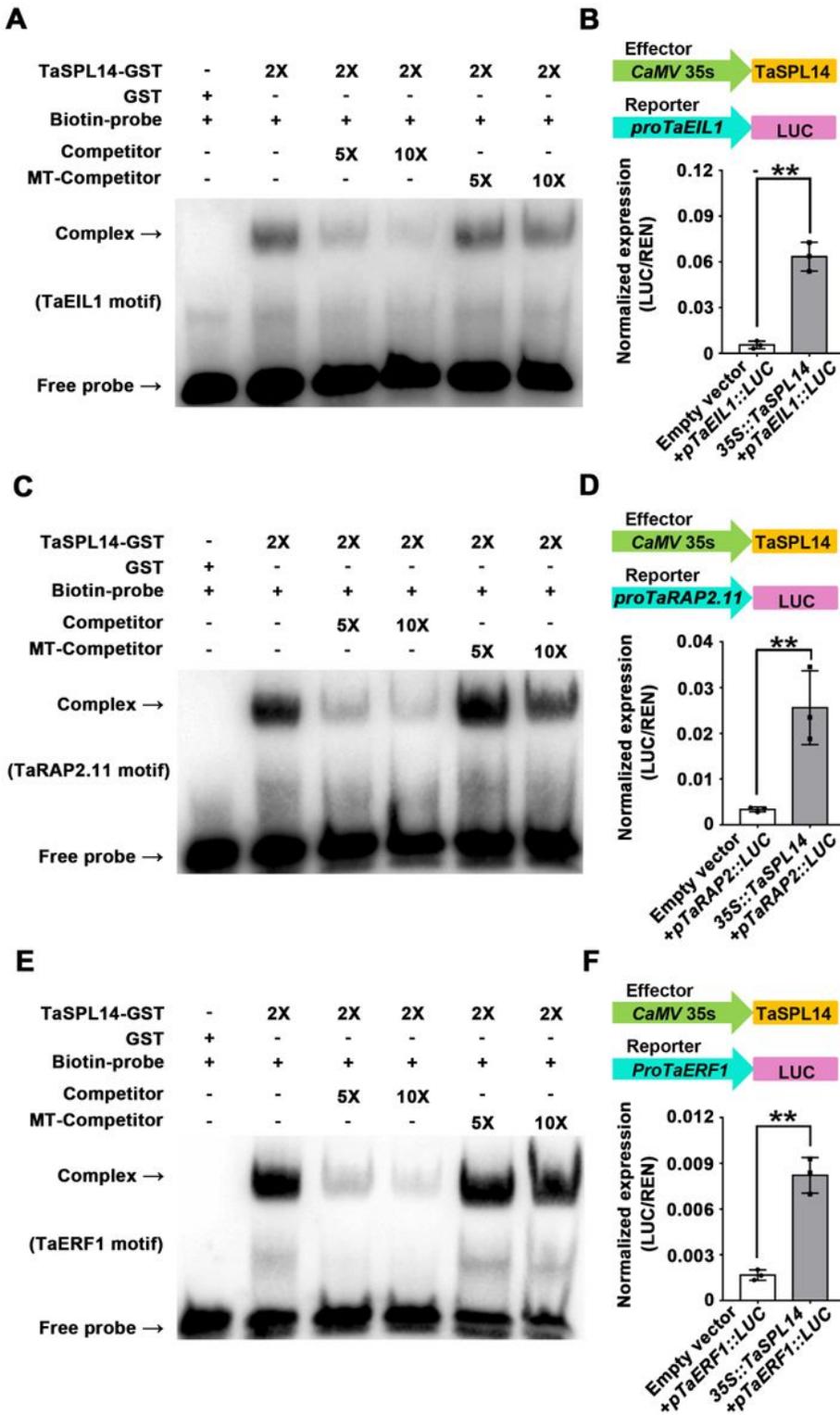


Figure 4

TaSPL14 directly binds and activates the expression of TaEIL1, TaRAP2.11 and TaERF1. (A), (C), (E) EMSAs to identify the TaSPL14 binds to the GTAC core motif from promoter region of TaEIL1, TaRAP2.11 and TaERF1. TaSPL14-GST means the recombinant protein of TaSPL14 fused to GST. Three labeled probes with modification of biotin in 5'end, competitor probes (without biotin modification in 5'end), and mutant competitor probes (without biotin modification in 5'end) come from the TaEIL1, TaERF1 and

TaRAP2.11 promoters respectively. "+" and "-" indicate the "add" and "not add" the TaSPL14-GST or corresponding probes. "2x" indicate the amount of TaSPL14-GST in reaction systems. "5x" and "10x" indicate 5- and 10-fold excessive molar of competitor or mutated competitor (MT) probes relative to labeled probes respectively. (B), (D), (F) TaSPL14 directly activated the expression of TaEIL1, TaRAP2.11 and TaERF1. Transcriptional activity assays with effector (CaMV35s::TaSPL14) and different reporter (proTaEIL1::LUC, proTaRAP2.11::LUC and proTaERF1::LUC) were performed in *Nicotiana benthamiana* leaves. The horizontal ordinate represents the different combinations of effector and reporter, and controls (empty effector and reporter). The ordinate represents the ratios of LUC out of corresponding REN. Each bar in the graph indicates the mean value of three repetitions, which were shown as circles. Statistical significant differences determined by Student's t-test (*P < 0.05, **P < 0.01).

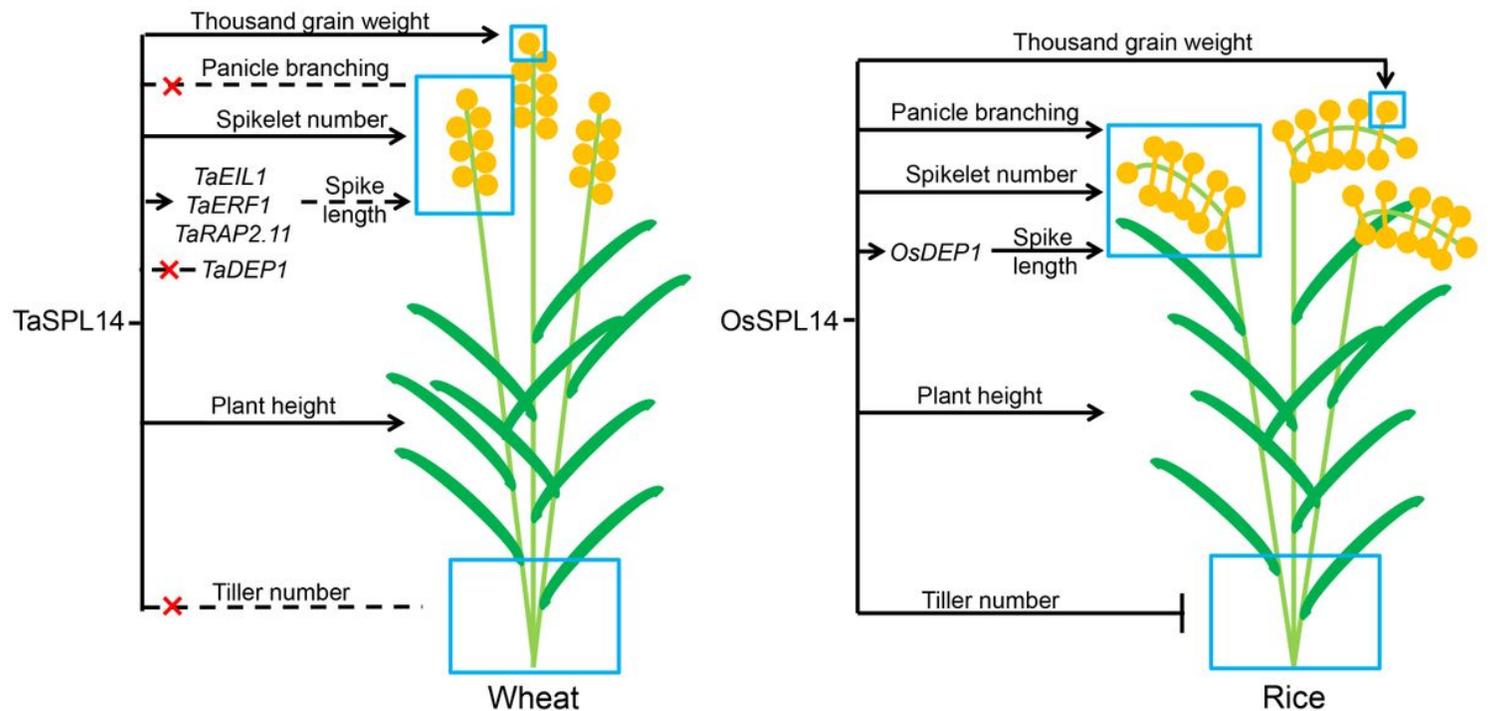


Figure 5

The similarity and variation between TaSPL14 and OsSPL14. Similar to OsSPL14, TaSPL14 regulates plant height, spike length, spikelet number and thousand-grain weight. Different from OsSPL14, TaSPL14 had no effect on panicle branching and tiller number. OsSPL14 regulated spike length by positively activating OsDEP1. But TaSPL14 might regulate wheat spike development through ethylene response related genes TaEIL1, TaERF1 and TaRAP2.11, not the TaDEP1.

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