OsSPL10, a SBP-Box Gene, Plays a Dual Role in Salt Tolerance and Trichome Formation in Rice (Oryza sativa L.)

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ABSTRACT  Salinity is one of the major abiotic stress factors limiting rice production. Glabrousness is a trait of agronomic importance in rice (Oryza sativa L.). We previously found a single-gene recessive mutant sst, which displayed increased salt tolerance and glabrous leaf and glume without trichomes, and identified an SBP-box gene OsSPL10 as the candidate of the SST gene. In this study, OsSPL10-knockout and OsSPL10-overexpression mutants were created to check the function of the gene. The knockout mutants exhibited enhanced salt tolerance and glabrous leaves and glumes as expected, while the overexpression mutants showed opposite phenotypes, in which both salt sensitivity and trichome density on leaf and glume were increased. These results clearly confirmed that OsSPL10 is SST, and suggested that OsSPL10 controls the initiation rather than the elongation of trichomes. In addition, expression analysis indicated that OsSPL10 was preferentially expressed in young panicle and stem, and protein OsSPL10 was localized in nucleus. Taken together, OsSPL10 negatively controls salt tolerance but positively controls trichome formation in rice.

Rice is sensitive to salt stress, which suppresses rice growth and development, causing severe yield loss. Salinity tolerance is a quantitative trait controlled by multiple genes in rice (Ren et al. 2005). Salt tolerance mechanism is complex in rice, involving many pathways. Certain apoplastic proteins are involved in the initial phase of salt stress response (Zhang et al. 2009). Some receptor-like kinases (RLKs) mediate salt sensitivity or improve salt tolerance by regulating ethylene homeostasis or H2O2 homeostasis (Li et al. 2014; Zhou et al. 2018). G-protein and small G-protein also play roles in salt-induced cellular senescence and other salt sensitivity (Urano et al. 2014; Zang et al. 2010). Calcineurin B-like protein (CBLs), CBL-interacting protein kinases (CIPKs), and calcium-dependent protein kinases (CPKs) function in salt signal transduction (Martinez-Atienza et al. 2007; Campo et al. 2014). Some cation transporters of plasma membrane, such as OsSOS1, OsHKT1;5 (SKCl), OsKAT1, OsHAK1, and OsMGT1, which are sodium, potassium and magnesium transporter (or channel), respectively, are related to salt tolerance (Martinez-Atienza et al. 2007; Ren et al. 2005; Obata et al. 2007; Chen et al. 2015; Chen et al. 2017). Many different transcription factors are involved in salinity tolerance or sensitivity, including NAC, OsbZIP23, DST, OsWRKY45-2, DREB1B, OsMYB2, SERF1 and so on (Hu et al. 2006; Xiang et al. 2008; Huang et al. 2009; Tao et al. 2011; Datta et al. 2012; Yang et al. 2012; Schmidt et al. 2013). In addition, epigenetics is also involved in salt tolerance in rice (Yuan et al. 2015; Srivastava et al. 2016; Wang et al. 2017).

Glabrous rice varieties have glabrous leaves and glumes without trichomes. In most terrestrial plants, trichomes are specialized structures, which originate from the above-ground epidermal tissues and develop into hair-like projections extending from the epidermal surfaces through growth, differentiation or cell division (Johnson 1975). As trichomes can lead to the generation of dust during harvesting and grain manipulating processes in rice production, glabrous leaves and glumes are a desirable characteristic. Only two glabrous genes have been cloned in rice so far, namely, OsWOX3B (DEP, NUDA/GL-1, GLR1) and OsPLT2 (HL6). The former is a WUSCHEL-like homeobox gene (Angeles-Shim et al. 2012; Zhang et al. 2012;
Li et al. 2012), while the latter encodes an AP2/ERF transcription factor, which physically interacts with OsWOX3B (Sun et al. 2017). SQUAMOSA Promoter-Binding Protein (SBP) and SBP-Like (SPL) proteins are putative transcription factors, which have a plant-specific SBP domain consisting of 76 amino acids in length (Cardon et al. 1997). SBP genes (SBP1 and SBP2) were first isolated from Antirrhinum majus and found to control early flower development by regulating the MADS-box gene SQUAMOSA (Klein et al. 1996). Then, SPL3 involved in floral transition was isolated from Arabidopsis thaliana (Cardon et al. 1997) and LG1 with SBP domain was found to be required for induction of ligules and auricles during maize leaf organogenesis (Moreno et al. 1997). SPL gene family is not large, with only 17 members in Arabidopsis and 19 in rice (Xie et al. 2006). SPL genes have been shown to play numerous important roles in plant growth and development, including trichome development and fertility (Unete et al. 2003; Xing et al. 2010), lateral root development (Yu et al. 2015), fruit ripening (Manning et al. 2006), plastochron length, flowering pathway and organ size (Wang et al. 2008, 2009; He et al. 2018), yield (Chuck et al. 2014; Si et al. 2016; Zhang et al. 2017), copper homeostasis (Yamasaki et al. 2009; Yan et al. 2017), and so on. Some SPL genes are related to abiotic stress tolerance. SPL1 and SPL12 confer thermostolerance at reproductive stage in Arabidopsis (Chao et al. 2017). Down-regulation of MsSPL8 leads to enhanced salt and drought tolerance in alfalfa (Gou et al. 2018).

In rice, it has been found that OsSPL genes control a large range of processes underlying plant growth and development (Wang and Zhang 2017). For example, OsSPL8 (OsLGI1) controls ligule development and inflorescence architecture (Lee et al. 2007; Ishii et al. 2013; Zhu et al. 2013). OsSPL13 (GLW7) controls grain size (Si et al. 2016). OsSPL14 (IPA1, WFP) affects tiller number and panicle branching (Jiao et al. 2010; Miura et al. 2010) and promotes immunity (Wang et al. 2018). OsSPL16 controls grain size, shape and quality (Wang et al. 2012) and plays a role in panicle cell death during ER stress (Wang et al. 2018). OsSPL18 controls grain weight and grain number in rice (Yuan et al. 2019).

We previously obtained a rice mutant sst showing salt tolerance and glabrous leaves and glumes from a restorer line R401. We found that sst was controlled by a recessive gene, which was likely to result from a deletion of one nucleotide in OsSPL10 (LOC_Os06g44860, Os06g0659100), an SBP-box gene (Wang et al. 2013; Lan et al. 2015; Song et al. 2016). In this study, we confirmed the function of OsSPL10 as the candidate of SST through gene knockout and overexpression, investigated the expression pattern of OsSPL10, and analyzed the subcellular localization of OsSPL10, aiming to lay a foundation for deep studies of the molecular mechanism of OsSPL10 function in salt tolerance and trichome development.

MATERIALS AND METHODS

Plant materials
The following plant materials were used or created in this study: indica rice cultivars R401 and Huanghuazhan (HHZ); japonica rice cultivars Nippombare and Zhonghua 11 (ZH11); the salt-tolerant and glabrous-leaf mutant sst obtained from R401 by radiation mutagenesis (Wang et al. 2013; Lan et al. 2015; Song et al. 2016); OsSPL10-knockout mutant plants from HHZ and ZH11; and OsSPL10-overexpression plants from ZH11. All rice plants were grown in plastic trays with paddy soil under a long day condition (approximately 14 h light/day) in the growth chamber (with cool-white light 300 μmol m⁻² s⁻¹).

Knockout of OsSPL10
The CRISPR/Cas9 editing system were used to knock out OsSPL10 in ZH11 and HHZ. Two target sites (5'-GTTCGGGGGGATGCAGGCG-3' and 5'-CACCCACCACATGCTAGCA-3') upstream of the SST mutation site in the first exon of OsSPL10 were selected and isolated according to the rules of low off-target score and high sgRNA score (http://cbi.hzau.edu.cn/crispr/), and then inserted into the VK005-01 binary vector containing the rice U6 promoter (Viewsolid Biotechnology Company of Beijing). The construct was introduced into ZH11 and HHZ using the stable transformation method (Hiei and Komari 2008). To examine mutations occurred in positive transgenic (T₀) plants, a 400-bp genomic DNA fragment harboring the two target sites was amplified from them by PCR using primers 5'-AGCTCCACCTTCGTTGGAAGCCA-3' and 5'-GGGACGCTGTAGCACGCTT-3' and then sequenced. Homozygous mutants obtained in
T1 generation were phenotyped for salt tolerance and trichomes on leaves and glumes.

**Overexpression of OsSPL10**

Total RNA was extracted from the young panicles (<5 cm) of Nipponbare and converted into cDNA by reverse transcription. RNA extraction was performed using TRIzol reagent (Invitrogen, USA). PrimeScriptTM RT reagent Kit (Takara, Japan) was used to synthesize the first strand of cDNA with OligoT primer. The 1.2-kb coding sequence of OsSPL10 was amplified from the cDNA by PCR using primers 5’-ATGATGAGCGGTAGGATGAA-3’ and 5’-CTACATGAAGTCGACCTCGA-3’, and then inserted into the pCXUN vector containing the maize ubiquitin promoter. The construct was introduced into ZH11 using the stable transformation method (Hiei and Komari 2008). The positive transgenic plants overexpressing OsSPL10 were phenotyped for salt tolerance and trichomes on leaves and glumes.

**Measurement of salt tolerance**

Rice seeds were sown on paddy soil in plastic trays (36x28x4.5 cm3) after pregermination and allowed to grow at 26°C under a photoperiod of 14 h light/10 h dark in a growth chamber. Salt stress treatment began from late two-leaf stage. During the treatment, 200 mL of either NaCl solution (150 mM) or fresh water was added into each tray every day. The treatment procedure for the OsSPL10-knockout seedlings was: 7 d NaCl/3 dwater/7 d NaCl/3 dwater, while that for the OsSPL10-overexpression seedlings was: 7 d NaCl/3 d water/4 d NaCl. The survival rate of seedlings was investigated at the end of the treatment.

**Measurement of leaf trichome density**

The penultimate leaves of individual plants were collected at tillering stage or heading stage. The adaxial surface of the middle part of each leaf was observed with scanning electron microscopy (SEM). The number of trichomes within a field of vision was counted, and three fields of vision were investigated on each leaf.

**Quantitative RT-PCR of OsSPL10**

Total RNA was extracted from seedlings as well as flag leaf blades, flag leaf sheaths, mature (second) leaf blades, mature leaf sheaths, stems, pre-emergence inflorescences and young panicles at the booting stage. RNA extraction and cDNA synthesis were conducted...
using the same methods as described above. The qRT-PCR was performed using SYBR Premix Ex Taq™ (Tli RNaseH Plus) (Takara, Japan) on a Prism 7500 96 Real-time PCR System (ABI, USA). The primers for OsSPL10 were 5′-ACAACGACAACAGCCACAACAA-3′ and 5′-ACACGAACACATGGTAGGATCGA-3′. The actin mRNA level was used as internal reference, for which the primers were 5′-AGTGCGACGTGGATATTAGG-3′ and 5′-TGGCTTAGCATTCTTGTTG-3′. Three independent biological replicates were analyzed by qRT-PCR in triplicate. The changes in gene expression were calculated using the 2−ΔΔCt method.

Subcellular localization of OsSPL10

GFP cDNA was fused to the C-terminus of OsSPL10 cDNA (without terminator) in the pMDC202 vector through BP and LR recombination (Lambda integrase/excisionase; Elpis-Biotech), resulting in the 35S::OsSPL10-GFP plasmid. The fusion construct as well as the control (empty pMDC202 vector; 35S::GFP) were infiltrated into tobacco (Nicotiana benthamiana) leaves using a needleless syringe.

For agroinfiltration, agrobacteria were grown overnight in Luria–Bertani containing the appropriate antibiotics. The agrobacteria were collected by centrifugation and then re-suspended in 10 mM MgCl2 containing 100 mM acetosyringone. After incubated for a minimum of 2 h at room temperature, the culture was diluted to an OD600 of 0.2. Tobacco plants were agroinfiltrated with appropriate agrobacterial cultures, and the agroinfiltrated plants were maintained under normal growth conditions for 12 to 72 h. The DAPI (4′, 6-diamidino-2-phenylindole) was used to confirm nucleus. The tobacco cell layers were examined with a confocal laser scanning microscope. (TCS SP8, Leica, Germany). Data availability All data generated or analyzed during this study are included in this published article.

RESULTS

Knockout of OsSPL10 enhances salt tolerance but inhibits trichome development

In the experiment of CRISP/Cas9 editing of OsSPL10, 28 and 20 positive transgenic (T0) plants were obtained from ZH11 and HHZ.
respectively. Among the T₀ plants, 25 (89.3%) from ZH11 and 15 (75%) from HHZ had mutations at either or both of the target sites, with 7 from ZH11 and 3 from HHZ being homozygous with the mutant allele. Protein sequence analysis predicted that all of the mutations resulted in a premature stop codon. Therefore, the mutants obtained were all OsSPL10-knockout mutants (denoted as ZH11-KO and HHZ-KO, respectively). We chose two mutants, one from ZH11 and HHZ each, named ZH11-KO-2 and HHZ-KO-4, respectively (Figure 1), to investigate the effects of OsSPL10 mutation on salt tolerance and trichome development.

Both ZH11-KO-2 and HHZ-KO-4 showed significantly higher tolerance to salt stress than their corresponding wild types in the experiment. While all of the ZH11 and HHZ seedlings died (survival rate = 0%) at the end of salt treatment, the ZH11-KO-2 and HHZ-KO-4 seedlings still all kept alive (survival rate = 100%), similar to the case of sst vs. R401 (Figure 2A, B and 3A, B). Meanwhile, both ZH11-KO-2 and HHZ-KO-4 displayed glabrous leaves and glumes without or with very few trichomes as expected (Figure 2C-E and 3C-E). SEM observation showed that the trichome density on leaf surface (number of trichomes per vision) at heading stage was ~43 in ZH11 (Figure 2F and H) and ~88 in HHZ (Figure 3F and H), respectively, whereas the density was only ~2 in ZH11-KO-2 (Figure 2G and H) and nearly 0 in HHZ-KO-4 (Figure 3G and H), respectively. These results indicated that loss of OsSPL10 function can result in higher salt tolerance as well as glabrous leaves and glumes, confirming that OsSPL10 is SST. In addition, the ZH11-KO-2, HHZ-KO-4 and sst seedlings all appeared to be a little taller than those of their corresponding wild types (Figure 2A and 3A), suggesting that loss of OsSPL10 function has an effect of promoting plant growth.

**Overexpression of OsSPL10 reduces salt tolerance but promotes trichome development**

A total of 22 positive transgenic (T₀) plants overexpressing OsSPL10 were acquired, among which plant ZH11-OE-12 showed the highest level of OsSPL10 expression, followed by plant ZH11-OE-19. We examined the phenotypes of the stably-inherited homozygous progeny lines of ZH11-OE-12 and ZH11-OE-19. Contrary to the OsSPL10-knocked-out seedlings, the OsSPL10-overexpressed seedlings were a little shorter (Figure 4A) but more sensitive to salt stress than the wild-type seedlings (Figure 4B). At the end of salt treatment there were still ~44% ZH11 seedlings alive, while the ZH11-OE-12 seedlings all died and only ~9% ZH11-OE-19 seedlings survived (Figure 4C). Since OsSPL10 expression was stronger in ZH11-OE-12 than in ZH11-OE-19, the result suggested that higher OsSPL10 expression level would lead to higher sensitivity to salt. In addition, SEM observation at tillering stage indicated that the OsSPL10-overexpressed plants had higher density of macrohairs on leaf (Figure 4E) than wild type (Figure 4D), and the
density also appeared to be positively proportional to the level of OsSPL10 expression (Figure 4F). These results indicated that OsSPL10 overexpression had exactly the opposite effect to that of OsSPL10 knockout, and the effect increased with the increase of OsSPL10 expression. This validated the function of OsSPL10 known from its loss-of-function mutants, further confirming that OsSPL10 is SST.

OsSPL10 is preferentially expressed in young panicle and stem
To examine the potential tissue specificity of OsSPL10, we used qRT-PCR to analyze the expression pattern of OsSPL10 at the booting stage. We found that OsSPL10 was preferentially expressed in early young panicles (< 5 cm) and stem, while its expression levels in late young panicles (pre-emergence inflorescence, 5-10 cm), leaf blades and leaf sheaths were generally low or very weak (Figure 5). These results suggested that OsSPL10 is probably involved in the early development of inflorescence, or in the phase transition from vegetative growth to reproductive development.

OsSPL10 is localized in nucleus
Some SPL genes playing important roles in growth and development have been found to function as transcription factors (Wang et al. 2009; Jiao et al. 2010). Therefore, we predicted that OsSPL10 protein might be also a transcription factor, which should be sorted to nucleus. Transient expression of 35S::OsSPL10-GFP in the epidermal cells of Nicotiana benthamiana (tobacco) leaves clearly showed that the GFP signal of SST-GFP fusion protein was observed only in nucleus (Figure 6A-D). By contrast, the GFP signal due to transformation of 35S::GFP was observed everywhere in the cell without specificity (Figure 6E-H). These results supported our prediction that SST is localized in the nucleus, suggesting that SST possibly functions as a transcription factor.

DISCUSSION
In this study, we confirmed through gene knockout and gene overexpression that OsSPL10 is SST, which plays a negative role in salt tolerance but a positive role in trichome formation and has a small negative effect on seedling growth as well in rice. In addition, the result of subcellular localization supported the prediction that OsSPL10 probably functions as a transcription factor like other OsSPL proteins.

There are 19 OsSPL genes in rice, including one pseudogene. Among them, 11 genes (not including OsSPL10) are the targets of miR156 (Xie et al. 2006). OsSPL10 is the first OsSPL gene confirmed to control salt tolerance in rice. However, there could be other OsSPL genes related to salt tolerance. It has been found that the abundance of miR156 increases in rice plants when subjected to salt stress, and the transgenic rice seedlings overexpressing miR156 show higher salt tolerance (Cui et al. 2014). This implies that there might be some OsSPL genes targeted by miR156 negatively controlling salt tolerance in rice. If this is true, there will be two different pathways of OsSPL-mediated salt tolerance regulation in rice. One is miR156-dependent, the other is miR156-independent (e.g., mediated by OsSPL10). But no matter in what pathways, the OsSPL genes involved all function as a negative regulator.

To date, two genes controlling trichome development have been reported in rice, namely, OsWOX3B (DEP, NUDA/GL-1, GLR1) and OsPLT2 (HL6) (Angeles-Shim et al. 2012; Zhang et al. 2012; Li et al. 2012; Sun et al. 2017). OsWOX3B belongs to the WOX3 family of plant-specific homeobox transcription factors (Angeles-Shim et al. 2012),
while OsPLT2 is an AP2/ERF transcription factor. OsPLT2 regulates trichome elongation, which is dependent on functional OsWOX3B that acts as a key regulator in trichome initiation (Sun et al. 2017). In this study, we found that knockout of OsSPL10 exhibited glabrous leaves and glumes, while overexpression of OsSPL10 increased the density of trichomes on leaves. In Arabidopsis, SPLs have also been found to be involved in the development and distribution of trichomes (Unte et al. 2003; Yu et al. 2010). The effect of OsSPL10 on trichome development found in this study is more similar to that of OsWOX3B than that of OsPLT2. Therefore, we speculate that OsSPL10 is likely to control trichome initiation. As an SBP-box gene with its protein being localized in nucleus (Figure 6), OsSPL10 probably function as transcription factor in trichome initiation. Further research is needed to clarify how OsSPL10 regulates trichome development and what relationship exists among OsWOX3B, OsPLT2, and OsSPL10 in rice.

CONCLUSION
OsSPL10 plays a dual role in salt tolerance and trichome formation in rice. It negatively controls salt tolerance but positively controls trichome initiation. The results of this study will help further research and better understanding of the mechanisms of salt tolerance and trichome formation in rice.

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