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The Serine Proteinase Inhibitor OsSerpin Is a Potent Tillering Regulator in Rice

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Tillering in rice (*Oryza sativa* L.) is an important agronomic trait that enhances grain production. A tiller is a specialized grain-bearing branch that is formed on a non-elongated basal internode that grows independently of the mother stem. Transgenic rice over-expressing the transcription factor *OsTB1*, a homologue of maize *TB1* (*Teosinte Branched 1*), exhibits markedly reduced lateral branching without the propagation of axillary buds being affected. However, the tillering mechanism remains unknown. Therefore, to further understand that mechanism, we applied proteomics methodology to isolate the proteins involved. Using two-dimensional gel electrophoresis and mass spectrometry, our analysis of the basal nodes from two rice cultivars that differ in their numbers of tillers showed that a rice serine proteinase inhibitor, OsSerpin, accumulates in great amounts in high-tillering 'Hwachung' rice. Northern blot analysis revealed that much more *OsSerpin* transcript is found in 'Hwachung' than in relatively low-tillering 'Hanmaeum', likely because of high levels of transcription. Therefore, our data suggest that OsSerpin content determines the extent of lateral branching.

Keywords: basal node, OsSerpin, proteomics, rice, tillering

Shoot branching plays a critical role in determining primary shoot architecture, thereby influencing the productivity of a plant and the final morphology of its aboveground organs. The first step in this process is the formation of axillary meristems in the axil leaves. This is followed by the outgrowth of the axillary buds. The number and extent of growth by the lateral buds are controlled by developmental and genetic signals, as well as environmental factors (Shimizu-Sato and Mori, 2001; Beveridge et al., 2003; Leyser, 2003).

Auxin inhibits this outgrowth of axillary meristems whereas cytokinin participates, as a potential second messenger, in axillary bud development (Morris, 1977; Napoli et al., 1999). In bushy mutants of *Arabidopsis*, pea, and petunia, a novel graft-transmissible signal may act to inhibit bud growth (Beveridge et al., 1994; Napoli, 1996; Stirnberg et al., 2002). Interestingly, molecular characterization of *Arabidopsis* bushy mutants (*max1* to *max4*) has demonstrated that branching is also regulated by a carotenoid-derived hormone (Booker et al., 2005). Two *Arabidopsis* MAX2/ORE9 orthologues -- *D3* and *Htd1* -- have now been found in rice (Ishikawa et al., 2005; Zou et al., 2005), as well as a series of reduced culm number (*rcn*) rice mutants -- *rcn1* to *rcn9* (Takamure and Kinoshita, 1985; Takamure, 1994; Jiang et al., 2006). Disruption in the *TEOSINTE BRANCHED 1* (*TB1*) gene causes enhanced lateral branching in maize, suggesting that TB1 functions as a negative regulator in the growth of axillary buds (Doebley et al., 1995). A rice homologue, *OsTB1*, has been identified based on its structural similarity to maize *TB1* (Lukens and Doebley, 2001); overexpression of *OsTB1* in rice negatively regulates tillering (Takeda et al., 2003). Although many tillering-related mutants have now been isolated, a regulatory mechanism has remained obscure. Therefore, we used proteomics methods to isolate proteins that are involved in tillering in two rice cultivars. Transcript levels were monitored in the basal nodes.

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MATERIALS AND METHODS

Materials

Experimental materials were obtained from the following sources: pharmalyte (pH 3.5 to 10.0), Amersham Biosciences; IPG DryStrips (pH 4 to 10NL, 24 cm), Genomine, Inc.; modified porcine trypsin (sequencing grade), Promega; supplies for growth media, BD Biosciences; restriction enzymes and modifying enzymes, Takara Bio, Inc.; reverse transcriptase, Invitrogen; GST-gene fusion system and radiochemicals, GE Healthcare Life Sciences; other reagents, Sigma-Aldrich; membranes, Perkin-Elmer; rabbit anti-GST antibody, Santa Cruz Biotechnology; Donkey anti-rabbit IgG HRP-linked whole antibodies, GE Healthcare Life Sciences; chemiluminescent substrate, PIERCE; and X-ray film, AGFA.

Seeds of two rice cultivars -- high-tillering 'Hwachung' and relatively low-tillering 'Hanmaeum' -- were sown in the field. Tiller numbers were recorded for each type at 30 and 45 days after seeding (DAS). For our proteomics analysis, 0.5-cm sections from the basal nodes were harvested at 45 DAS.

E. coli Strains and Growth Media

Escherichia coli (*E. coli*) strain DH10B was used for the

propagation of plasmids. Cells were grown in LB media (1% tryptone, 0.5% yeast extract, and 0.5% NaCl; pH 7.4) at 37°C. Ampicillin (50 μg ml^{-1}) was added to select and amplify DH10B cells harboring the recombinant plasmid during culture. Each GST-fusion protein was expressed in *E. coli* BL21(DE3) pLysS cells.

Preparation of Total Proteins from Rice Basal Nodes

Basal nodes from 'Hwachung' and 'Hanmaeum' rice were homogenized in lysis buffer (7 M urea, 2 M Thiourea, 4% CHAPS, 1% DTT, 2% pharmalyte, and 1 mM benzamidine). Proteins were extracted by vortexing at room temperature for 1 h. After centrifugation at 15,000×g for 1 h at 15°C, soluble fractions were used for two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). The protein concentrations were determined by using the Bradford (1976) assay.

2-D PAGE and Image Analysis

IPG (Immobilized pH gradient) dry strips were equilibrated for 12 h with IPG equilibration buffer (7 M urea, 2 M Thiourea, 2% CHAPS, 1% DTT, and 1% pharmalyte), then loaded with 200 μg of protein. Isoelectric focusing was performed at 20°C using a Multiphor II electrophoresis unit according to the manufacturer's protocol (Bio-Rad). Prior to the second dimension, strips were incubated for 10 min in equilibration buffer [50 mM Tris-Cl (pH 6.8), 6 M Urea, 2% SDS, and 30% glycerol], first with 1% DTT and then with 2.5% iodoacetamide. The equilibrated strips were inserted onto SDS-polyacrylamide gels (20 × 24 cm, 10% to 16%), and the 2-D gels were run at 20°C for 1,700 Vh before being stained with silver. Quantitative analysis of the digitized images was carried out with PDQuest software (Bio-Rad, version 7.0). The quantity of each spot was determined after normalization to the total valid spot intensity. Protein spots corresponding to 'Hwachung' and 'Hanmaeum' were selected and accumulated for comparison.

Protein Identification by Mass Spectrometry

Seven protein spots were digested in-gel using modified porcine trypsin. Protein identification was performed using an Ettan MALDI-TOF (Amersham Biosciences). Taking a delayed extraction approach, we evaporated the peptides with an N_2 laser at 337 nm. They were accelerated with a 20 kV injection pulse for time-of-flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for protein identification via peptide mass fingerprinting. Spectra were calibrated using the trypsin auto-digestion

Table 1. Numbers of tillers from two rice cultivars.

	'Hwachung'	'Hanmaeum'
Time		
30 DAS	15	10
45 DAS	19	14

DAS: Days after seeding

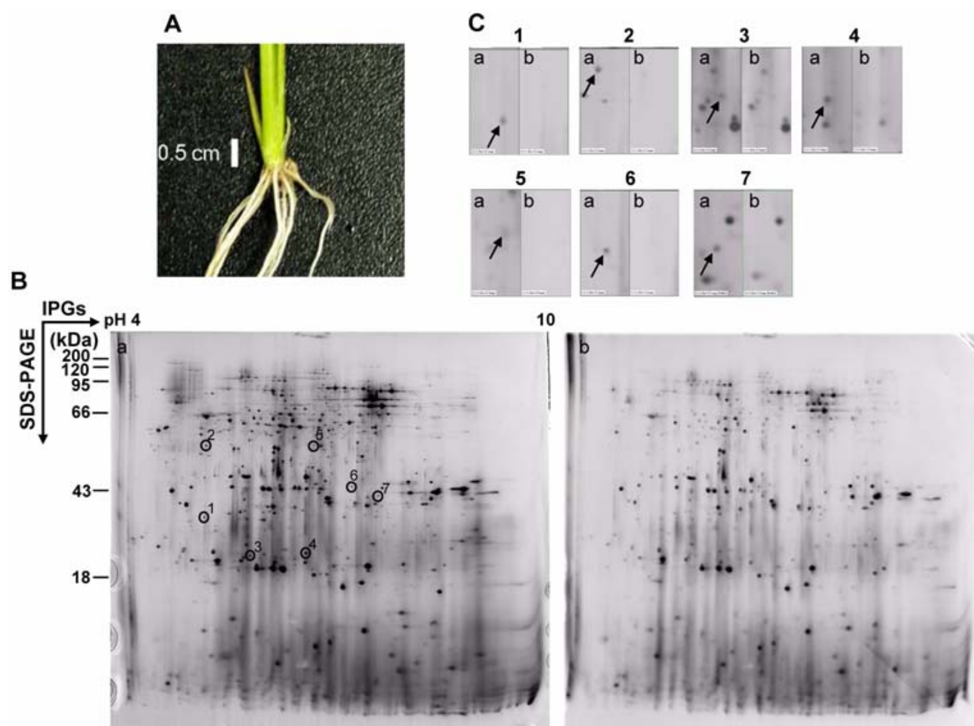

Figure 1. Proteomics analysis of above ground rice basal nodes. **A**, 0.5-cm samples were harvested. **B,** Total soluble proteins were extracted from high-tillering 'Hwachung' (a) and relatively low-tillering 'Hanmaeum' (b), and were analyzed by two-dimensional gel electrophoresis (2-DE). Each number indicates protein spot that specifically accumulated in 'Hwachung'. 1, thiamin biosynthesis protein; 2, vacuolar ATPase B subunit; 3, expansin; 4, 6-phosphogluconolactonase; 5, UDP-glucose pyrophosphorylase; 6, serine proteinase inhibitor (Serpin); 7, oxidoreductase. **C,** Gel regions, in which spots analyzed by 2-DE are magnified. Arrows indicate specific protein spots.

ion peak *m/z* (842.510, 2211.1046) as an internal standard.

Northern Blot Analysis

Total RNAs were isolated from the basal nodes of 'Hwachung' and 'Hanmaeum' according to a phenol-SDS/LiCl precipitation procedure (Wang et al., 2007). Five micrograms of RNA was separated on 1.3% agarose-formaldehyde gels and transferred to nylon membranes, which were hybridized with a ^{32}P-labeled OsSerpin cDNA probe (nucleotide sequences 425 through 995). After washing, the membranes were exposed to X-ray film.

RESULTS

Proteomics Analysis of Basal Nodes from Two Rice Cultivars with Differing Tiller Numbers

Proteomics is a useful method for identifying organ- or development-specifically accumulated proteins (Cho et al., 2006). These techniques were used here to isolate and analyze tillering-related proteins. At 30 DAS, field-grown high-tillering 'Hwachung' and relatively low-tillering 'Hanmaeum' rice had 15 and 11 tillers, respectively; at 45 DAS, those respective numbers were 18 and 14 (Table 1). Soluble fractions were extracted from 0.5-cm sections of the basal nodes (Fig. 1A), and were run with a pH gradient (4 to 10) in first-dimension IEF, followed by SDS-PAGE in 10% to 16% gels (Fig. 1B). The protein spots in parallel gels were cross-matched, demonstrating reproducibility. After electrophoresis, the gels were analyzed with PDQuest software, and the 2-D gels were systematically compared.

Seven protein spots were selected that differed significantly in their two expression patterns, and that were characteristic of the Hwachung cultivar (Fig. 1C). These were identified by performing Ettan MALDI-TOF, which was followed by a homology search. The spots consisted of thiamin biosynthesis protein, vacuolar ATPase subunit, expansin, 6-phosphogluconolactonase, UDP-glucose pyrophosphorylase, oxidoreductase, and Serpin (serine proteinase inhibitor) (Table 2).

Identification of an *OsSerpin* Gene

Based on these proteomics results, we focused further attention on Serpin, which was especially accumulated in high-tillering 'Hwachung'. To characterize the *OsSerpin* (rice Serpin) gene, we isolated *OsSerpin* cDNA by RT-PCR using gene-specific primers designed on the basis of the NCBI

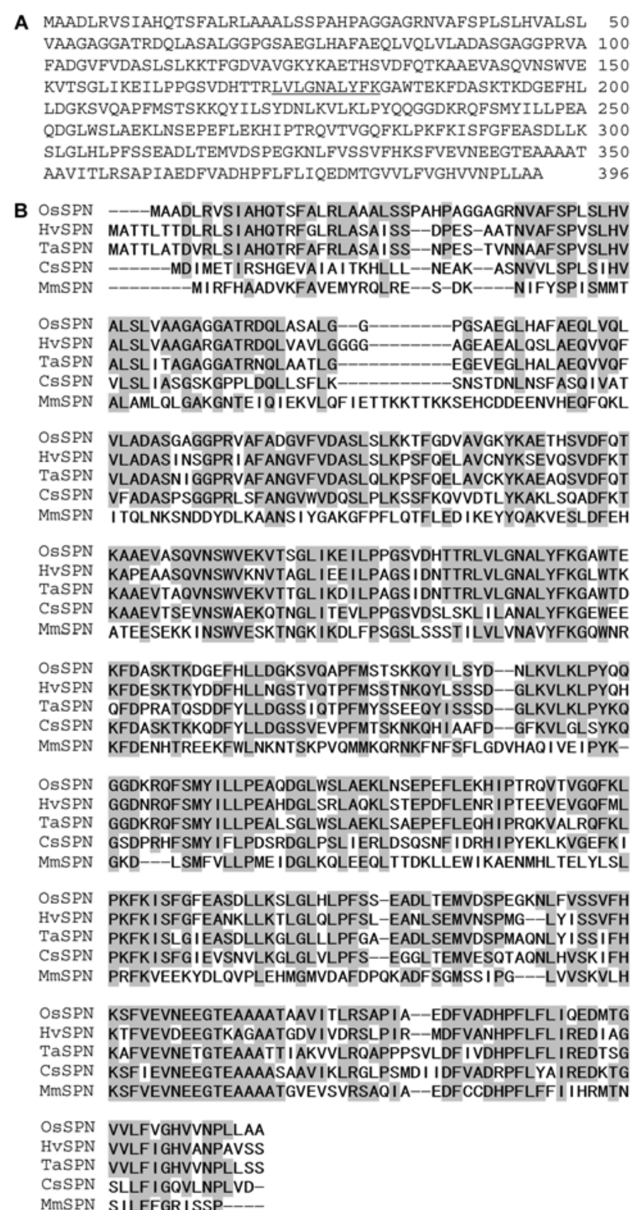

Figure 2. Comparisons of amino acid sequences of rice Serpin (OsSerpin) with other Serpins. **A,** Amino acid sequences of putative rice Serpin identified by proteomics analysis. Amino acids mapped by MALDI-TOF are underlined. **B,** Alignment of amino acid sequences via Clustal W. Rice OsSerpin (*Oryza sativa*, gi37700305), wheat TaSerpin (*Triticum aestivum*, gi1885346), barley HvSerpin (*Hordeum vulgare*, gi1197577), cucumber CsSerpin (*Cucumis sativus*, gi58416137), mouse MmSerpin (*Mus musculus*, gi68534939).

Table 2. Proteins that were specifically accumulated in high-tillering 'Hwachung'.

Spot ID	Mr	pI	Sequence	Homologous protein	Protein name
1212	37.2	5.4	LLARPNVK	Thiamin biosynthesis protein	Os07g0529600
1511	54.2	5.1	YQEIVNIR	Vacuolar ATPase B subunit	Os06g0568200
2114	29.6	5.5	YQEIVNIR	Beta-Expansin EXPB3	Os10g0555900
3106	29.1	5.5	WVTYIK	6-phosphogluconolactonase	Os09g0529100
4524	51.8	5.4	VANFLAR	UDP-glucose pyrophosphorylase	Os09g0553200
5309	42.1	5.8	LVLGNALYFK	Serine proteinase inhibitor	Os03g0610800
6323	38.5	6.0	GFFSAGAK	Oxidoreductases	Os04g0337500

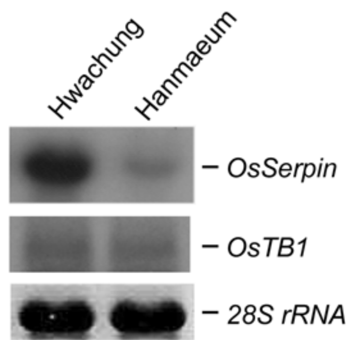

Figure 3. Expression of *OsSerpin* in basal node. Total RNAs were isolated from same samples as for 2-DE. Full-length cDNA of *OsSerpin* or *OsTB1* was labeled with ^{32}P and used as probe. 28S rRNA served as loading control.

database. The cDNA encodes a polypeptide comprising 396 amino acids (Fig. 2A). Comparisons of these amino acid sequences with those of other Serpin proteins showed that they are highly conserved (Fig. 2B), suggesting that they are involved in both plant and animal development.

Accumulation of *OsSerpin* Transcript in High-tillering 'Hwachung'

These proteomics data showed that the OsSerpin protein was found at significant levels in high-tillering 'Hwachung' rice. Therefore, we examined the transcript of *OsSerpin* to determine whether levels were regulated at the transcription or the translation stage. Northern blot analysis revealed that *OsSerpin* is highly expressed in 'Hwachung' compared with 'Hanmaeum' (Fig. 3), suggesting a positive correlation between protein content and level of expression at the transcript level. Because OsTB1 is a transcription factor that represses rice tillering, its transcript also was examined by northern blotting; levels, however, were the same for both cultivars (Fig. 3).

DISCUSSION

Due to the agronomic importance of lateral branching, many branching mutants have been investigated and characterized in several crops, including rice and *Arabidopsis* (Beveridge et al., 1994; Napoli, 1996; Stirnberg et al., 2002; Booker et al., 2005; Ishikawa et al., 2005; Zou et al., 2005; Takamure and Kinoshita, 1985; Takamure, 1994; Jiang et al., 2006; Doebley et al., 1995; Li et al., 2003). In rice, the number of lateral branches is one of the determining factors for the number of seeds produced. Therefore, knowledge of how such branching is controlled is of great practical and scientific interest. Our northern blot analysis showed that transcript levels of *OsSerpin*, a gene for the serine proteinase inhibitor, were much higher in the high-tillering Hwachung cultivar than in the relatively low-tillering Hanmaeum cultivar (Fig. 3), suggesting that the latter possesses a specific repressor for *OsSerpin* expression.

Lateral branching involves two developmental steps -- the formation and the outgrowth of axillary buds. Takeda et al. (2003) have proposed that OsTB1 plays an important role in the latter step because it is still propagated even in *OsTB1*-overproducing transgenic plants, and because *OsTB1* is expressed in the entire axillary bud. In addition, OsTB1 is considered a transcription repressor (Takeda et al., 2003). Therefore, we postulate that OsTB1 inhibits the growth of axillary buds, after their formation, by repressing *OsSerpin* expression. Nevertheless, the levels of *OsTB1* transcript were nearly the same in 'Hwachung' and 'Hanmaeum' (Fig. 3), implying that another factor may be involved in the control of *OsSerpin* expression.

At present, eight classes of proteinase inhibitors are known in plants (Christeller and Laing, 2005), with six being unique to that kingdom. There is evidence that proteinase inhibitors form tight binding complexes with their target proteinases and that they follow a standard mechanism for inhibition. However, it remains to be uncovered how serine proteinases (serpins) form complexes with, and inhibit, target proteinases. Isolation of the target proteinase of OsSerpin and analysis of *OsSerpin*-mutant rice should help to elucidate the OsSerpin-mediated tillering mechanism and to explain its mode of action.

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