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Note

Overexpression of *SRS5* improves grain size of brassinosteroid-related dwarf mutants in rice (*Oryza sativa* L.)

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Grain size is a trait that is important for rice (*Oryza sativa* L.) yield potential. Many genes regulating grain size have been identified, deepening our understanding of molecular mechanisms of grain size determination in rice. Previously, we cloned *SMALL AND ROUND SEED 5* (*SRS5*) gene (encoding alpha-tubulin) from a small and round seed mutant and revealed that this gene regulates grain length independently of the brassinosteroid (BR) signaling pathway, although BR-related mutants set small grain. In this study, we showed that overexpression of *SRS5* can promote grain length and demonstrated that the overexpression of *SRS5* in BR-related mutants rescued the shortened grain length, which is an unfavorable phenotype in the yield potential of BR-related mutants, while preserving the useful semi-dwarf and erect leaf phenotypes.

Key Words: *Oryza sativa* L., grain size, *SRS5*, brassinosteroid, molecular breeding.

Introduction

Grain size is an important trait that determines rice (*Oryza sativa* L.) yield potential. Many genes regulating grain size in rice were identified by quantitative trait locus analysis, namely, *GS3*, *GW2*, *qSW5/GW5*, *GS5*, *GW8*, *GL3.1*, *TGW6*, *GW6a*, and *GL7/GW7* (Fan *et al.* 2006, Ishimaru *et al.* 2013, Li *et al.* 2011, Qi *et al.* 2012, Shomura *et al.* 2008, Song *et al.* 2007, 2015, Wang *et al.* 2012, 2015a, Wang *et al.* 2015b, Weng *et al.* 2008, Zhang *et al.* 2012). Other genes regulating grain size were cloned from short grain rice mutants, *d1*, *brd1*, *d2*, *d11*, *d61*, *srs1*, *srs3*, *Srs5*, *Sg1*, and *tud1* (Abe *et al.* 2010, Ashikari *et al.* 1999, Fujisawa *et al.* 1999, Hong *et al.* 2002, 2003, Hu *et al.* 2013, Kitagawa *et al.* 2010, Nakagawa *et al.* 2012, Segami *et al.* 2012, Tanabe *et al.* 2005, Yamamuro *et al.* 2000), and recently, *GLW7* was identified by a genome-wide association study (Si *et al.* 2016). These genes are classified into two groups, those controlling cell elongation (*GL7/GW7*, *D2*, *D11*, *D61*, *BRD1*, *SRS1*, *SRS3*, *SRS5*, and *GLW7*) and those controlling cell division (*GS3*, *GW2*, *qSW5/GW5*, *GS5*, *GW8*, *GL3.1*, *TGW6*, *D1*, *SG1*, and *TUD1*). Among these genes, GW8 protein was reported to directly interact with the promoter of *GW7*, negatively regulating *GW7* expression (Wang *et al.* 2015a). The GLW7 protein was similarly reported to directly interact with the promoter of *SRS5* and positively regulate the *SRS5* expression (Si *et al.* 2016). However, Si *et al.* (2016) did not present genetic evidence that correlates the higher expression of *SRS5* with increased grain length that they observed. Previously, we reported that short grain and dwarf phenotypes of the *Srs5* (encoding alpha-tubulin) mutant and brassinosteroid (BR)-related mutants were controlled by independent signal transduction pathways (Segami *et al.* 2012). Semi-dwarfism in BR-related mutants renders them resistant to lodging and their erect leaves improve their photosynthetic ability. Thus, in barley (*Hordeum vulgare*), a change in one amino acid due to a single nucleotide substitution in *HvBRI1*, the homologous gene of *D61*, creates a mutant with low sensitivity to BR and semi-dwarf stature and erect leaves, which is widely used as a high yielding variety (Chono *et al.* 2003). In rice, a mild mutation in the BR biosynthetic gene *OsDWARF4* that does not affect grain size was reported to increase grain yield under conditions of high density and high fertilizer concentrations but not under normal conditions (Sakamoto *et al.* 2006). Because of the pleiotropic effect of genes on reduced grain size, BR-related mutants have been used only rarely in rice breeding programs.

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In the present study, we produced a plant overexpressing *SRS5* to test whether higher expression of *SRS5* increases grain length and grain yield potential. Further, since *SRS5* promotes grain length via a pathway independent from BR-related genes, we hypothesized that overexpression of *SRS5* in the BR-related mutant background will improve grain length, while preserving useful dwarfism and erect leaf phenotypes.

Materials and Methods

Plant materials and growth conditions

A *japonica* rice cultivar, Taichung 65, was used as wild-type (WT) plant. The BR biosynthesis mutant *d2-2* and BR insensitive mutant *d61-2* were obtained by MNU treatment of Taichung 65. All transgenic plants were grown in a closed greenhouse under natural sunlight. Room temperature was maintained at 30°C from 09:00 to 18:00 and 25°C from 18:00 to 09:00.

Production of transgenic plants

Total RNA was extracted from ripening spikelets of WT plants and used to synthesize cDNA. The coding sequence of *SRS5* was amplified by PCR using this cDNA as a template and the primers cacc + SRS51stATG-F: 5′-CACC ATGAGGGAGTGCATCTCGAT-3′ and SRS5-3UTR-R: 5′-CGCCAACTAAAGGTCACAAT-3′. The amplicon was subcloned into an entry vector pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). The DNA fragment containing the full-length *SRS5* cDNA in pENTER/D-TOPO was inserted into a binary vector p2KG by the gateway method described in the manual of the pENTR/D-TOPO cloning kit (Invitrogen, USA). The *SRS5* cDNA, controlled by the ubiquitin promoter in p2KG, was introduced into the *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.* 1993) by electroporation and transformed into the WT, *d2-2*, and *d61-2* as reported previously (Ashikari *et al.* 2005). The WT, *d2-2*, and *d61-2* containing empty vectors were used as controls. Over 30 plants were obtained from each regenerated plant. Of those, over 10 plants carrying the transgene were selected by PCR using hygromycin resistance gene (*HPT*)-specific primers, HPT-L: 5′-CGTATATGCTCCGCATTGGT-3′ and HPT-R: 5′-ATTTGTGTACGCCCGACAGT-3′.

RT-PCR analysis

Total RNA was extracted from ripening spikelets using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and cDNAs were synthesized from the total RNA using a SuperScript III system (Invitrogen, USA). To quantify the SRS5 mRNA, real-time reverse transcription PCR (RT-PCR) was conducted using SYBR Premix Ex Taq II (TAKARA Bio, Inc., Tokyo, Japan). The primer set RT-alpha-tub-F: 5′-ATGA GGGAGTGCATCTCGAT-3′ and RT-alpha-tub-R: 5′-CAA GATCGACGAAGACAGCA-3′ was used to quantify the

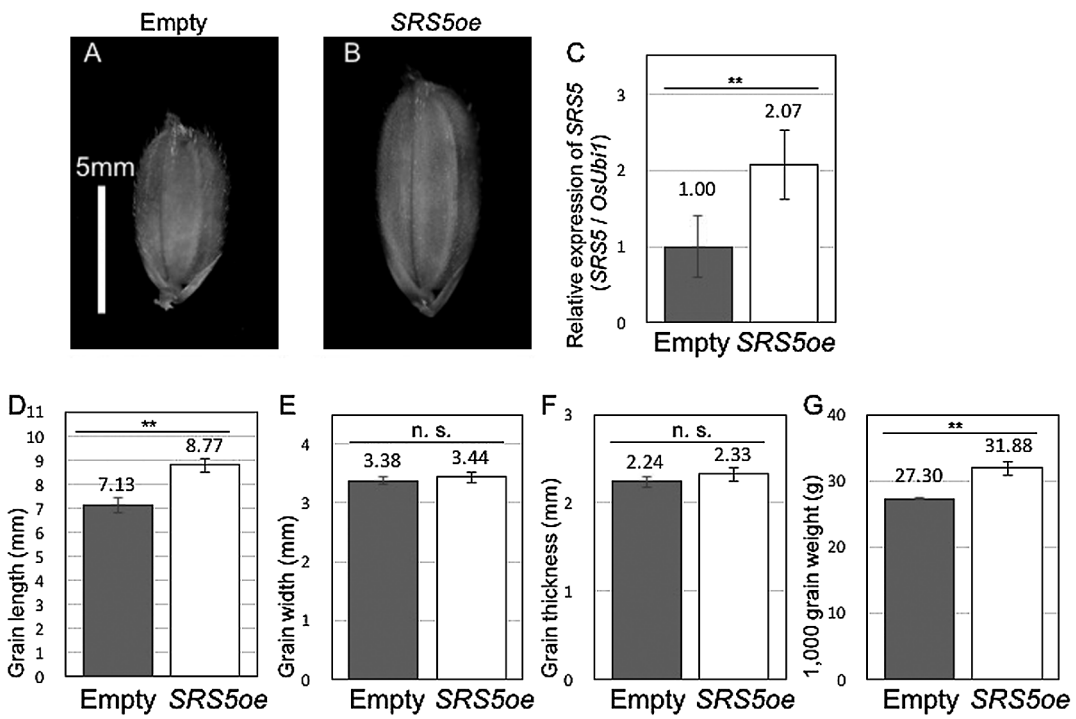

Fig. 1. Grain phenotypes of the wildtype (WT) overexpressing *SRS5*. Grain morphology of the WT transformed with empty vector (Empty) (A) and the WT overexpressing *SRS5* (*SRS5oe*) (B). Relative expression of *SRS5* was calculated using *OsUbi1* as internal control (C). Relative amount of *SRS5* was normalized by Empty. Comparison of the grain length (D), grain width (E), grain thickness (F), and 1000-grain weight (G) of Empty and *SRS5oe*. The analysis was based on 10 Empty and 13 *SRS5oe*. Error bars indicate standard deviation. The results of the Student's *t*-test are indicated above the graph bars. **: $p < 0.01$, n.s.: not significant ($p > 0.05$).

expression of *Srs5* (GenBank accession no. NC_029266), and another primer set, RT-OsUbiquitin1-F: 5′-CTTGGT CGTGTCCCGTTTC-3′ and RT-OsUbiquitin1-R: 5′-TTCT TCCATGCTGCTCTACCAC-3′, was used to quantify the expression of *OsUbiquitin1* (GenBank accession no. NC_ 029261). A Thermal Cycler Dice Real Time System (TAKARA Bio, Inc., Japan) was used for quantification in real-time RT-PCR.

Results

Overexpression of SRS5 leads to longer grain in rice

To confirm whether higher expression of *SRS5* increases grain length and grain yield potential, we produced plants overexpressing *SRS5* controlled by the *OsUbi1* promoter and selected at least 10 transgene-inserted plants by PCR using *HPT*-specific primers (data not shown). All of the selected plants overexpressing *SRS5* (*SRS5oe*) produced longer grains than those of the control plants that were transformed with empty vectors (**Fig. 1A**, **1B**). Real time quantitative RT-PCR analysis detected a twofold higher expression of *SRS5* in *SRS5oe* plants (**Fig. 1C**). Overexpression of *SRS5* affected grain length, but not grain width and grain thickness (**Fig. 1D–1F**). The 1000-grain weight of *SRS5oe* was also higher than that of the WT (**Fig. 1G**). These results support the presumption stated by Si *et al.* (2016) that the higher expression of *SRS5* promotes longitudinal grain elongation.

Overexpression of SRS5 does not affect useful traits of BR-related mutants

To test whether overexpression of *SRS5* in the BR-related mutant background can improve grain size while

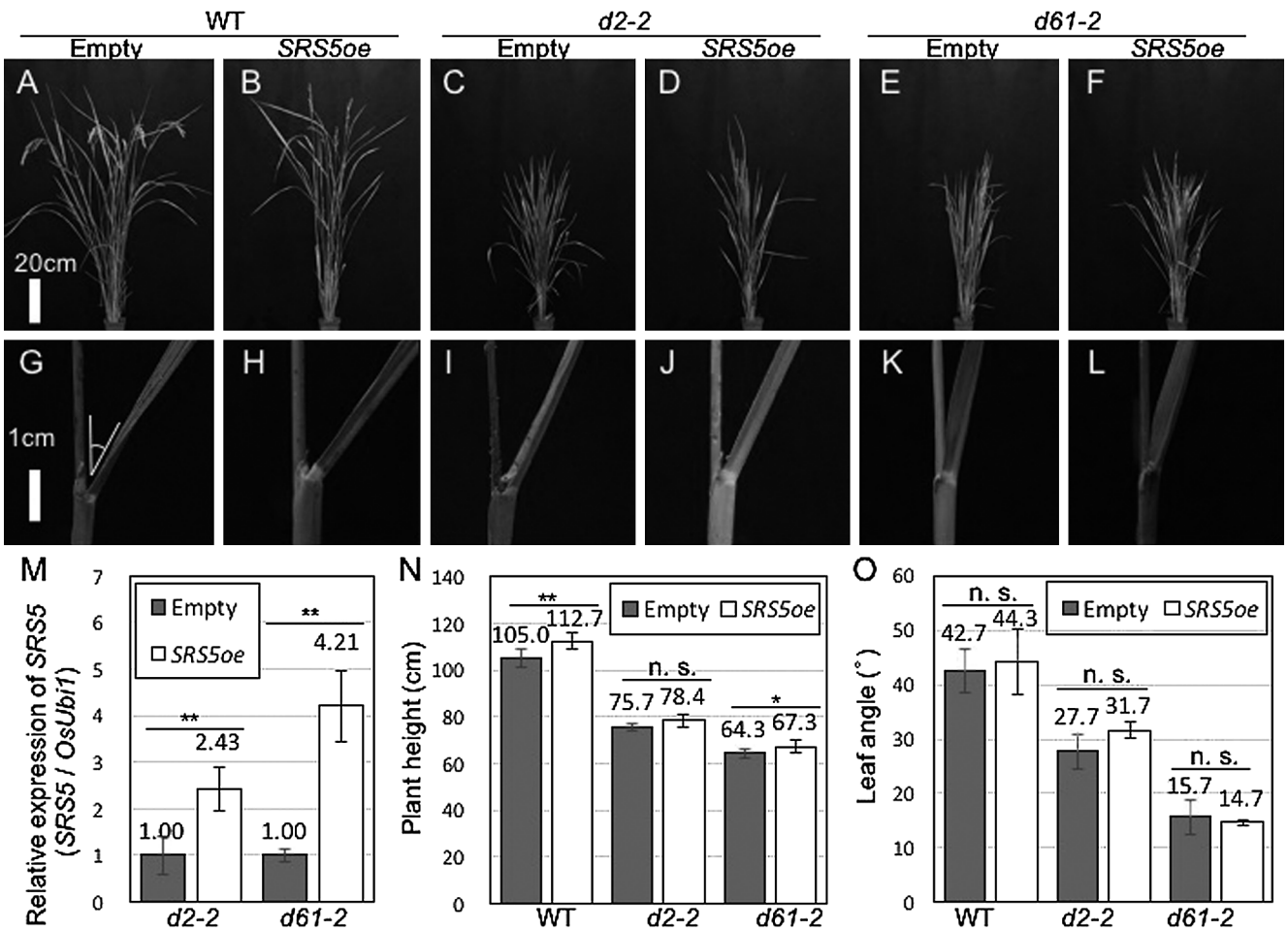

Fig. 2. Gross morphology and leaf angle of *SRS5oe* in brassinosteroid (BR)-related mutant background. Gross morphologies of the wildtype (WT) (A), *d2-2* (C), and *d61-2* (E) background transformed with empty vector used as control (Empty). Gross morphologies of *SRS5* overexpression (*SRS5*oe) in WT (B), *d2-2* (D), and *d61-2* (F) background. Leaf angle of the flag leaf at ripening stage of Empty in WT (G), *d2-2* (I), and *d61-2* (K) background. Leaf angle of the flag leaf at ripening stage of *SRS5oe* in WT (H), *d2-2* (J), and *d61-2* (L) background. Relative expression of *SRS5* was calculated using *OsUbi1* as internal control (M). Relative amount of *SRS5* was normalized by Empty. Comparison of plant height (N) and leaf angle (O). The analyses were based on 10 Empty and 13 *SRS5oe* in the WT background, 10 Empty and 13 *SRS5oe* in the *d2-2* background, and 10 Empty and 11 *SRS5oe* in the *d61-2* background. Error bars indicate standard deviation. The results of the Student's *t*-test are indicated above the graph bars. **: $p < 0.01$, *: $0.01 < p < 0.05$, n. s.: not significant ($p > 0.05$).

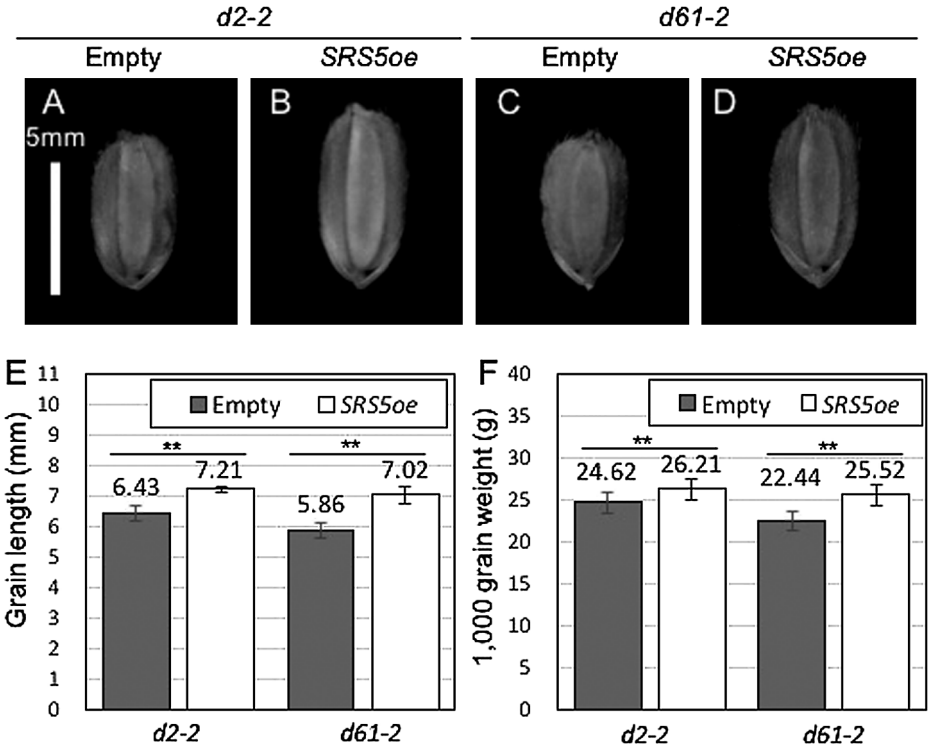

Fig. 3. Grain phenotypes of plants overexpressing *SRS5* in brassinosteroid (BR)-related background. Grain morphology of *d2-2* (A) and *d61-2* (C) background transformed with empty vector used as control (Empty). Grain morphology of *SRS5* overexpression (*SRS5oe*) in *d2-2* (B) and *d61-2* (D) background. Comparison of grain length (E) and 1000-grain weight (F). The analysis was based on 10 Empty and 13 *SRS5oe* in the *d2-2* background and 10 Empty and 11 *SRS5oe* in the *d61-2* background. Error bars indicate standard deviation. The results of the Student's *t*-test are indicated above the graph bars. **: $p < 0.01$.

preserving the useful dwarf and erect leaf traits, we overexpressed *SRS5* in *d2-2* and *d61-2* mutants. The overexpression of *SRS5* in the WT slightly increased plant height but did not affect the leaf angle (**Fig. 2A**, **2B**, **2G**, **2H**, **2N**, **2O**). Although the overexpression of *SRS5* in *d2-2* and *d61-2* resulted in 2.43- and 4.21-fold higher transcription of *SRS5*, respectively (**Fig. 2M**), the height of *d2-2* plants did not differ significantly from that in the control and it was only slightly increased in *d61-2* (**Fig. 2C–2F**, **2M**). The leaf angle in *d2-2* and *d61-2* overexpressing *SRS5* was not affected (**Fig. 2I–2L**, **2O**). These results indicate that overexpression of *SRS5* in BR-related mutants slightly increased plant height and preserved dwarfism and the erect leaf phenotype.

Overexpression of SRS5 rescues grain length in BR-related mutants

The grain length increased in *d2-2* and *d61-2* overexpressing *SRS5* (**Fig. 3A–3E**). These grains were almost the same length as those of WT plants (**Figs. 1A**, **1C**, **3B**, **3D**, **3E**). Further, with increasing grain length, the 1000 grain-weight of *SRS5oe* in the *d2-2* and *d61-2* backgrounds was also restored significantly (**Fig. 3F**). Thus, overexpression of *SRS5* in the BR-mutant background is a useful combination that produces normal grain size with semi-dwarf and erect leaf phenotypes.

Discussion

In this study, we demonstrated that overexpression of *SRS5* in the WT as well as in the BR-related mutant background increases grain length and yield potential. These results are consistent with the hypothesis that higher expression of *SRS5* has a potential to promote grain size (Si *et al.* 2016) and that *SRS5* regulates grain length via cell elongation independently from the BR signaling transduction pathway (Segami *et al.* 2012). Therefore, when novel genes regulating grain size are identified, it is important to analyze genetic interactions between novel and known genes. If the genes are completely epistatic and one masks the effect of the other, their interaction would contribute to our understanding of the molecular mechanisms of grain size regulation. If the genes are not epistatic, or they are partially epistatic, we can utilize these genes simultaneously in the gene-pyramiding approach, as we demonstrated in this study. Although a number of genes regulating grain size are identified, their genetic interactions are rarely demonstrated. Therefore, it is important to clarify genetic interactions of known genes regulating grain size for cloning of novel genes in future studies.

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