SmallOrgan 1 plays an essential role in cell proliferation, cell expansion and cadmium uptake in rice

Peng Qin 1, Jiangbo Hu 1, Weilan Chen 1, Guohua Zhang 1, Jian Li 1, Shijun Fan 1, Bin Tu 1, Xuewei Chen 1, Yuping Wang 1, Shigui Li 1, Bingtian Ma Correspond. 1

1 Rice Research Institute, Sichuan Agricultural University, China

Corresponding Author: Bingtian Ma
Email address: btma02@sicau.edu.cn

Background: Organ size is determined by the number and size of cells, which are controlled by cell proliferation and cell expansion. Organ size in rice plays a crucial role in determination of rice yield. Cadmium (Cd), one of the major environmental pollutants, is harmful to human health. Cd-polluted rice grains have been a major source of human Cd-intake, thus reducing Cd-uptake into rice grains is important. Strigolactone (SL), a hormone, has been well-studied in terms of regulating plant architecture and in its signal perception, but its biological roles in cell proliferation, cell expansion and Cd-uptake have not been uncovered.

Methods: We comprehensively investigated agronomic traits and cell size between WT and so1. We treated WT and so1 with Cd, and analyzed Cd content in rice grain using inductively coupled plasma mass spectrometry (ICP-MS). We used bulk segregation analysis for SO1 mapping, and analyzed sequences surrounding SO1 in rufipogo, indica, japonica and intermedia varieties of rice.

Results: We identified a spontaneous rice mutant, small organs 1 (so1), which exhibited small organs, such as small leaves, panicles and grains. Cytological analysis indicated that the small organs of so1 were due to both impaired cell proliferation and expansion. We found that so1 grains highly accumulated Cd after Cd treatment. In the so1 mutant, an 8bp insertion resulting in a premature stop codon was identified in LOC_Os04g46470 (High-Tillering Dwarf1) which is involved in Strigolactone (SL) synthesis. Because both htd1 and so1 mutants exhibited dwarf and excessive tillers, the 8bp insertion was responsible for the so1 phenotype. SO1/HTD1 was ubiquitously expressed, most highly in stem and sheath. Very few SNP polymorphisms surrounding SO1/HTD1 were identified in japonica group, whereas SNPs were abundant in the rufipogon, indica and intermedia groups.

Discussion: Our work showed the novel biological roles of SO1/HTD1 in controlling rice organ size and Cd-uptake, and indicated that the SL signaling pathway is involved in cell proliferation, cell expansion, and Cd-uptake, a role completely different from the previously reported role of HTD1. Therefore, our study is helpful for...
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\textsuperscript{A}Rice Research Institute of Sichuan Agricultural University, Chengdu Wenjiang, Sichuan, 611130, China.
\textsuperscript{B}State Key Laboratory of Hybrid Rice, Sichuan Agricultural University, Chengdu Wenjiang, Sichuan, 611130, China.

\textsuperscript{1}These authors contributed equally to this work.

*Corresponding author: Bingtian Ma and Shigui Li

Email: btma02@sicau.edu.cn; lishigui@sicau.edu.cn
Abstract

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Discussion: Our work showed the novel biological roles of SO1/HTD1 in controlling rice organ size and Cd-uptake, and indicated that the SL signaling pathway is involved in cell proliferation, cell expansion, and Cd-uptake, a role completely different from the previously reported role of HTD1. Therefore, our study is helpful for understanding the molecular functions of SO1/HTD1 and SL during rice development.

Introduction

Organ size is determined by the number and size of cells, which are controlled by cell proliferation and cell expansion (Jiang et al. 2012). Organ size in rice, such as panicle and grain size, plays a crucial role in determination of rice yield. So far, various signaling pathway have been reported to be involved in plant cell proliferation and expansion, including plant hormones, such as gibberellin, which can promote rice internode elongation in deep water (Sauter et al. 1995); DA1, involved in ABA signaling, which controls organ size by regulating the cell cycle (Li et al. 2008); ARGOS functions in auxin signaling pathway upstream of ANT (Hu et al. 2003); and Xiao, which is involved in the control of organ size by participating in brassinosteroid signaling (Jiang et al. 2012). An enzyme responsible for cytokinin biosynthesis controls panicle size (Kurakawa et al. 2007). SL (Strigolactone), another hormone, has been well-studied in regulating plant architecture and its signaling perception (Al-Babili & Bouwmeester 2015). However, its biological roles in cell proliferation or cell expansion have not been uncovered.

Cd (Cadmium), one of the major environmental pollutants, is harmful to human health. Cd-polluted rice grains has been a major source of human Cd intake (Uraguchi & Fujiwara 2012). Controlling Cd uptake in rice grains has been an important issue in agriculture. So far, several approaches to reduce Cd accumulation in rice grains have been reported, but those approaches are costly, ineffective or unstable (Clemens et al. 2013; Ueno et al. 2009). Genetic improvement for reduced Cd in rice grain was thought be effective, but not cost-effective costly in Cd-polluted areas. Several genes, such as OsLCT1 (Uraguchi et al. 2014), OsHMA3n, OsHMA3a (Kumagai et al. 2014; Miyadate et al. 2011; Sasaki et al. 2014), OsNramp1 (Takahashi et al. 2011) and OsIRT1
(Lee & An 2009), have been reported to be involved in the Cd uptake pathway in rice. However, the molecular mechanism of Cd uptake in rice was largely unknown.

Here, we identified a spontaneous mutant, so1, which exhibited small organs. Cytological analysis showed that SO1 is involved in cell proliferation and expansion. Grains of the so1 mutant accumulated Cd. Mapping-based cloning showed that an 8bp insertion resulting in a premature stop codon was identified in LOC_Os04g46470 (HTD1) whose mutant exhibits similar phenotype with so1, and is involved in Strigolactone (SL) synthesis. SO1 was ubiquitously expressed, and highly expressed in stem and sheath. Very few SNP polymorphisms surrounding SO1 were identified in the japonica group, whereas SNPs were abundant in the rufipogon, indica and intermedia groups. Together, our work highlights biological roles of SO1/HTD1 in a way different from those previously reported, and is helpful for understanding the molecular functions of SL signaling pathways during rice development.

Methods and material

Plant materials
The so1 mutant was a spontaneous mutant, and was noticed in an indica rice breeding program. For evaluation of all agronomic traits, the wild-type and so1 were grown in a paddy at Chengdu, Sichuan in 2015, and mean values from three measurements were used for analysis. The tiller number of WT and so1 was counted once every 7 days from the beginning of tillering (39 day after sowing) to the end of tillering.

Semi-section and SEM
To observe epidermal cell size in the culm, we performed semi-section of the first internode as described by (Qin et al. 2013); the measurement of cell length and width was performed using Image J software. To document cell size of the vascular bundles and glume cells of the spikelet, we used SEM following the protocol described by (Qin et al. 2013). Briefly, the culm at the mature stage and glume at the filling stage were fixed in 0.1M sodium phosphate buffer (2.5% glutaraldehyde, pH=6.8) at 4°C for overnight, then washed in 0.1M phosphate buffer (pH 6.8)
twice, then fixed in 0.1M sodium phosphate buffer (2% osmium tetroxide) at 4°C overnight. We used an acetone series from 30-100% to dehydrate, and used liquid CO₂ to dry samples out, and then coated them with gold powder, then observed with Scanning Electron Microscope JEM-1200 EX (Hitachi).

Cadmium treatment and measurement
For cadmium treatment, WT and sol plants were first grown in soil without added Cd (background level of Cd is 0.05mg/Kg) for 30 days, then transferred to soil with 1mg/Kg Cd. Mature grains were collected for cadmium measurement, according to (Miyadate et al. 2011).

Gene Mapping
Bulk segregation analysis was used for gene mapping. Briefly, we analyzed the polymorphisms of 608 SSR markers on 12 chromosomes between sol and HuaB. 61 SSR markers with polymorphisms were further used to analyze two DNA pools which contained 10 F2 individual with or without excessive tiller phenotype. The markers RM5749, RM6365 and RM6748 on Chr.4 showed polymorphism between the two pools. More markers with polymorphism adjacent to these three markers were analyzed for 463 F2 individuals with excessive tillers.

Expression analysis
Total RNAs of different tissues in Fig. S4 were isolated using Trizol (Invitrogen). For the qPCR assay, we used 600ng total RNA for reverse transcription, with a Primescript RT reagent kit with gDNA eraser (TakaRa), and 200ng/µL cDNA, gene-specific primer pairs and SsoFast EvaGreen supermix (Bio-Rad), with the Bio-Rad CFX96 real-time system. All primers are listed in supplementary table 1.

SNP polymorphism analysis
The O.rufipogon haplotype sequence in Hap3 (Huang et al. 2012) and the Indica, Japonica and Intermedia re-sequencing data of 3K rice accessions (project 2014) were used for analyzing
polymorphisms in the adjacent SO1 region using fastPHASE36 (Scheet & Stephens 2006).

Results

The so1 mutant exhibited small organs

The so1 mutant was a spontaneous mutant in the indica background. The so1 mutant was dwarf and had excessive tillers (A). The dwarf phenotype of so1 was due to equally shortened internodes (Fig. S1A and B). The so1 mutant increased its tiller number until 112 days after sowing, whereas the tiller number of WT did not increase after 53 days after sowing (Fig. S2A). The number of so1 tillers at the mature stage was up to 91, while only 8 tillers were seen for WT. In addition to the dwarf and excessive tiller phenotype, the organ size of so1 was much smaller than in WT, such as leaf, panicle grain size and each internode (Fig. 1B-F). Analysis of agronomic traits showed that except for tiller number per plant, all traits, such as plant height, 1000-grain weight, panicle length, grain length and grain width, were significantly reduced in the so1 mutant (Fig. 1G-N).

SO1 was involved in cell proliferation and expansion

To investigate the cytological reason for the small organ in so1, we performed semi-sections and SEM (scanning electron microscopy) of the first internode and outer parenchyma layer of the spikelet hulls, respectively. The number and size of the vascular bundles of the so1 stem was obviously decreased (Fig. 2A-D). The number and length of the culm epidermal cells was also significantly decreased in so1 (Fig. 2E, F, I and J). The cell length and width of the spikelet outer epidermal cells were reduced around 18.4% and 13% in so1 (Fig. 2E, F and K). The number of longitudinal and horizontal cells was increased 14.2% and 4.4% in the spikelet outer epidermis (Fig. 2G, H and L), respectively. We investigated the expression of genes involved in cell proliferation and expansion using stem apex meristem. Consistent with the decreased cell length and number of so1 culm, most cell proliferation-related genes were significantly down-regulated, and three of six cell expansion-related genes were down-regulated in so1 (Fig. 2 M and N).
The grain of \textit{so1} mutant accumulated Cd

During the course of mutant screening for aberrant Cd accumulation in rice grain, we surprisingly found that Cd concentration in \textit{so1} was about 2.8 fold more than in WT when 1mg/Kg Cd was added, whereas no difference was found between WT and \textit{so1} without Cd addition (Fig.3A).

Genetic analysis and mapping of \textit{SO1}

All F1 plants generated from a cross between \textit{so1} and WT exhibited normal tiller number and plant height as WT, and in the F2 population, 331 and 1324 plants exhibited excessive tillers and normal tillers, respectively ($\chi^2(3:1)\text{}=2.95 < \chi^2(0.05)=3.84$), indicating a single recessive mutation was responsible for the \textit{so1} phenotype.

A bulk segregation approach was performed to map the target mutation of \textit{so1} using a population generated from the cross between \textit{so1} and HuaB. The target mutation was mapping to a 749kb region between AG234 and RM470 on Chr.4 by preliminary mapping, and then narrowed to a 76.8kb region by fine mapping. An 8bp insertion was identified in the second exon of \textit{Loc_Os06g46470}, leading a premature stop codon in \textit{so1} (Fig. 4A). Mutants of \textit{Loc_Os06g46470}, including \textit{hdt1} and \textit{osccd7}, exhibited similar phenotype with \textit{so1} (Kulkarni et al. 2014; Zou et al. 2006), we therefore concluded that the \textit{so1} phenotype is due to the 8bp insertion in \textit{Loc_Os04g46470}.

Expression analysis of \textit{SO1} and genes involved in strigolactone pathway

We then investigated the expression pattern of \textit{so1} among a number of tissues using RT-qPCR. \textit{SO1/HTD1} was ubiquitously expressed in most tissues, and highly expressed in stem and leaf sheath (Fig. 5A). The expression of \textit{D14/D88/HDT2} and \textit{D53} involved in SL signal perception pathway were respectively up-regulated and down-regulated, and no change for \textit{D3}. Both \textit{D10} and \textit{D27} involved in SL synthesis was up-regulated, whereas \textit{SO1/HDT1} was down-regulated (Fig. 5B), respectively.
SNP diversity surrounding SO1/HTD1
Given that the expression difference of D88, involved in SL signaling transduction, was proposed to contribute to the difference of panicle branch number between 9311 and PA64s (Peng et al. 2014), we analyzed SNP polymorphisms of SO1/HTD1 among 3K rice accessions, interestingly, SNP polymorphisms of SO1/HTD1 were similar among rufipogon, indica and intermedia group, whereas very few SNP polymorphisms were identified in the japonica group (Fig. 6A), indicating that SO1/HTD1 might have been domesticated during japonica breeding selection.

Discussion
HTD1 encodes a carotenoid cleavage dioxygenase involved in SL synthesis, which negatively regulates the growth of axillary buds (Kulkarni et al. 2014; Zou et al. 2006). However, whether and how HDT1 functions in other tissues was unknown. In this study, we identified a mutant, so1, which is allelic to hdt1. Phenotypic characterization indicated that SO1/HTD1 functions in most organs, such as stem, leaf, panicle and seed, and positively regulates organ size. SO1/HTD1 is involved in SL synthesis pathway, therefore, we determined that SL signaling pathway is involved in cell proliferation and cell expansion. Additionally, we showed that SO1/HTD1 negatively regulates Cd accumulation in rice grain, the first indication that SL signaling participates in the pathway of Cd accumulation in rice grain. Our study expands understanding of SL signaling pathway in rice development processes. Also, our results suggest a possibility of increasing organ size and reducing Cd accumulation in rice grain by manipulating SO1/HTD1.

The mutation of 8bp insertion in SO1/HTD1, which caused a premature stop codon in the so1 mutant, is different from the reported mutation in HTD1. Therefore, the allelic mutation in so1 provided a novel haplotype for studying the protein function of SO1/HTD1.

As D88/HDT2/D14 functions in the SL signaling pathway, its natural variation was proposed to be responsible for panicle branch number differences between 9311 and PA64s (Peng et al. 2014). Natural SNP polymorphisms surrounding SO1/HTD1 were few in japonica, whereas diversified in rufipogon, indica and intermedia, suggesting that the SNP haplotype of SO1/HTD1 in japonica
was selected during *Japonica* rice breeding, and is possibly important for some important agronomic traits.

**Conclusion**

The *so1* mutant exhibited small organs, such as small leaf, panicle and grain size. *SO1* controls organ size by regulating cell proliferation and expansion. *SO1* also negatively regulates Cd accumulation in rice grain. An 8bp insertion in *Loc_Os06g46470* is responsible for the *so1* phenotype, and *so1* is allelic to *htd1*. The *japonica* haplotype of *SO1/HTD1* was selected during rice breeding.

**Contribution**

Peng Qin and Jiangbo Hu performed most experiments, wrote the manuscript and contributed equally to this work. Weilan Chen performed the Cd measurements. Guohua Zhang and Jing Li performed the sequence diversity analysis. Shijun Fan, Bin Tu, Xuewei Chen and Yuping Wang generated the population for mapping, and Shigui Li and Bingtian Ma designed and supervised the research.

**Acknowledgements**

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**References**


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indica rice (Oryza sativa). Physiol Plant 151:339-347. 10.1111/ppl.12189


Supplementary Table
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Figure 1 (on next page)

Figure 1-6
Fig. 1 Comparison of plant (A), leaf (B), panicle (C), grain length (D), grain width (E) and each internode (F) between WT and so1. The sequence of internodes from top to bottom is the same with that of each plant internode. The agronomic traits between WT and so1, (G) Plant height; (H) Tiller number; (I) Grain number per plant; (G) 1000-grain weight; (K) Grain width; (L) Grain length; (M) Panicle length; (N) Seed setting rate.
Fig. 2 Section comparison of the first internode between WT (A) and so1 (B). Cell size comparison of vascular bundle by SEM between WT (C) and so1 (D). Comparison of cell size and number of the first internode epidermal cell by longitudinal section between WT (E) and so1 (F). Comparison of cell size and number of spikelet hull epidermal cell by SEM between WT (G) and so1 (H). Quantitative analysis of longitudinal cell length (I) and cell number (J) of the first internode. Quantitative analysis of cell length and width (K), longitudinal and horizontal cell number (L) between WT and so1 spikelet hull. Expression analysis of genes related cell expansion (M) and cell proliferation (N) between WT and so1.

CL: cell length, CW: cell width, LCN: longitudinal cell number, HCN: horizontal cell number.
Fig. 3 The comparison of Cd concentration in grain between WT and so1 (A).
Fig. 4 Gene mapping schematic of *SO1* (A).
Fig. 5 (A) Expression pattern of SO1 among various tissues. (B) Expression analysis of genes related SL biosynthesis and signal perception between WT and so1.
Fig. 6 Sequence diversity surrounding SO1-HTD1 among Indica, Intermediate, Japonica and O. Rufipogon (A).