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Poaceae Orthologs of Rice OsSGL, DUF1645 Domain-Containing Genes, Positively Regulate Drought Tolerance, Grain Length and Weight in Rice

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Abstract

Background

Grain yield is a polygenic trait influenced by environmental and genetic interactions at all growth stages of the cereal plant. However, the molecular mechanisms responsible for coordinating the trade-off or cross-talk between these traits remain elusive.

Results

We characterized the hitherto unknown function of four *STRESS_tolerance and GRAIN_LENGTH* (*OsSGL*) *Poaceae* ortholog genes, all encoding DUF1645 domain-containing proteins, in simultaneous regulation of grain length, grain weight, and drought stress-tolerance in rice. In normal growth conditions, the four ortholog genes were mainly expressed in the developing roots and panicles of the corresponding species. Over-expressing or heterologous high-level expressing *Poaceae OsSGL* ortholog genes conferred remarkably increased grain length, weight, and seed setting percentage, as well as significantly improved drought-stress tolerance in transgenic rice. Microscopical analysis also showed that the transgene expression promoted cell division and development. RNA-seq and qRT-PCR analyses revealed 73.8% (18,711) overlapped DEGs in all transgenic plants. Moreover, GO and KEGG analyses of different comparisons revealed that the key DEGs participating in drought stress-response belonged to hormone (especially auxin and cytokinin) pathways, and signaling processes were apparently affected in the young panicles.

Conclusion

Together, these results suggest the four *OsSGL* orthologs perform a conserved function in regulating stress-tolerance and cell growth by acting via a hormone biosynthesis and signaling pathway. It may also induce a strategy for tailor-made crop yield improvement.

Background

Sufficient food supply is critically important to humans with more than 60% of total worldwide agricultural production being provided by domesticated graminaceous cereal crops such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), maize (*Zea mays*), barley (*Hordeum vulgare* L.), sorghum (*Sorghum biocolor*), and small millets (*Setaria italica* L.) [1]. Crop yield is a complex trait that is controlled simultaneously by multiple genes (i.e., non-allelic genes and quantitative trait loci [QTLs]) and heavily influenced by the surrounding environment [2, 3]. Numerous genes have been identified that participate in regulating plant stress-tolerance at various developmental processes and stages in different pathways through diverse mechanisms [4, 5]. Therefore, it is necessary to increase our understanding of the underlying genetic and molecular mechanism of grain yield and stress response in these crops, and equally important to enrich the cultivated gene pool for enhancing crop productivity and stress-tolerance

in tailored breeding of crop varieties, in order to ensure food and nutritional security under stressful changes in the environment [6, 7, 8].

The Pfam database provides a collection of protein families formed on the basis of domain sequence similarity, each presented by multiple sequence alignments and hidden Markov models (HMMs). Over 3,000 domains of unknown function (DUFs) protein families, representing 26.5% of all families, remain poorly characterized in the Pfam database. Providing functional annotation for such domains is required for complete characterization of the protein family space, and will aid in the annotation of whole genome sequences [9, 10, 11]. Some highly conserved plant-specific DUF proteins play important roles in different biological processes of plant growth and development, defense response to diseases and insect pests, and adaption response to abiotic stresses. DUF26 (PF01657), a duplicated domain in the rice root meander curling protein (OsRMC), which also has been found in ginkbilobin-2 from *Ginkgo biloba*, are annotated as salt stress response/antifungal family [12, 13]. DUF221 proteins are a family of osmotic-sensing calcium-permeable cation channels. DUF221 domain-containing genes (*DDP gene*s) play important roles in developmental biology, hormone signaling transduction, and responses to abiotic stress [14, 15, 16]. *REL2* (*Rolling and erect leaf 2*) encodes a protein containing DUF630 and DUF632 domains. Its functions in the control of rice leaf morphology might involve multiple biological processes such as bulliform cell development, auxin synthesis and transport [17]. DUF640 containing genes *G1*, *TH1* (*Triangular Hull 1*) / *BSG1* (*Beak-Shaped Grain1*) and *AFD1* (*Abnormal Flower And Dwarf 1*), mainly expressed in young inflorescences of rice as well as in the spikelet lemmas and paleas, affect plant height, floral development and grain yield by regulating the expression of cell division and expansion related genes [18, 19, 20, 21]. *OsDSR2*, a new stress-repressive gene in the DUF966 gene family, negatively regulated rice responses to salt and simulated drought stresses as well as ABA signaling. Overexpression of *OsDSR2A* resulted in enhanced sensitivity to ABA-dependent salt and simulated drought stresses by down-regulating the expression of multiple stress-responsive genes [22]. DUF1618 genes are only present in several monocot plants, and the transcription levels of some DUF1618 genes varied in different cultivars, suggesting important roles for development and environmental fitness in rice [23]. The DUF1644 genes *OsSIDP366* and *OsSIDP409* may function as regulators of the processing bodies/ stress granules and positively regulate salt and drought resistance in rice [24]. Overexpression of a novel ABA-responsive *TaSRHP* (*Triticum aestivum* salt-related hypothetical protein) gene, encoding a conserved DUF581 domain, enhanced resistance to salt stress in *Arabidopsis thaliana* [25]. These studies indicate that DUF proteins play important roles in plant growth and stress adaptions, although little is known about their molecular mechanisms.

Rice has been heralded as a model cereal not only because of its vast acreage and importance as a staple food crop but also due to its small genome size, amenability to high efficiency transformation techniques, high synteny and co-linearity to other cereal crops, and availability of high resolution linkage maps [26, 27]. Homologs of 98% of the known maize, wheat, and barley proteins are found in rice, despite its long period of independent evolution [1]. Comparative QTL mapping studies have shown that some QTLs for many traits, including grain yield, are located on collinear chromosomes in different species [28, 29, 30]. Grain size, one of the major components determining grain yield and quality, is an important

selective target during domestication and breeding. To date, several genes or QTLs for the trait have been identified and extensively studied using homology-based cloning approaches. *GS3*, the first characterized QTL for grain size regulated, encodes a putative phosphatidylethanolamine-binding protein which negatively controls grain size [31, 32, 33]. Its maize ortholog, *ZmGS3*, has domains in common with the rice GS3 protein, and is involved in maize kernel development but with different functional polymorphisms, possibly different mechanisms [34]. *GW2*, encoding a RING-type E3 ubiquitin ligase, negatively regulates rice grain weight [35]. Two of its maize co-orthologs *ZmGW2-CHR4/5*, control some of the phenotypic variation for kernel size and weight in maize [36]. Research on three wheat homologs, *TaGW2-A/B/D*, has been extensive and includes gene cloning, expression analysis, evolution, functional marker development, and elucidation of the genetic effects of each homolog [37, 38, 39, 40, 41]. *TGW6*, another negative regulator of grain size and weight, hydrolyzes indole-3-acetic acid (IAA)-glucose into IAA and glucose [42]. Its homolog in wheat, *TaTGW6-a/b/c*, is considered as a candidate gene related to grain development [43, 44]. In addition, *GS5* encodes a putative serine carboxypeptidase which functions as a positive regulator of grain size. Higher expression of *GS5* increases grain width and grain yield by accelerating cell division and cell expansion in the spikelet hull [45, 46]. Maize ortholog, *ZmGS5* affects kernel development, suggesting a conserved function among different plant species [47]. *TaGS5* homoeologues in wheat have been isolated and mapped on Chr 3A, 3B and 3D. *TaGS5-3A* is a positive regulator of grain size and its favoured allele *TaGS5-3A-T* exhibits a larger potential application in wheat high-yield breeding [48, 49]. These pieces of evidence indicate that different plant species share similar mechanisms to control seed and organ growth. Therefore, studies on rice grain size could help us to understand the mechanisms of seed size control in other crops.

In our previous study, a DUF1645 (Pfam PF07816) domain-encoding protein, *OsSGL* (*Oryza sativaSTRESS_tolerance and GRAIN_LENGTH*), was indenified as a pleiotropic powerful gene in positively regulation of rice grain length, grain weight and drought resistance [50, 51]. The *OsSGL* homolog in sorghum, *SbSGL* also shows conserved functions in seed size control by regulating cell division [52]. DUF1645 proteins are widespread throughout monocots and eudicots, and bio-informatics analysis results suggested that gene *OsSGL* is quite conserved in rice genomes, and that its functions may be more or less conserved in plants closely related to *Oryza* species. Thus, we hypothesized that *Poaceae* orthologs of *OsSGL* may play a conserved role in the increase of grain length and water-deficit stress tolerance in cereal plants. To test this, four entire DUF1645 domain-containing *OsSGL* orthologs from sorghum (*SbSGL*), maize (*ZmSGL*), millet (*SiSGL*), and a second ortholog from rice (*OsSGL2*) were cloned, and functionally characterized by heterologous/over-expression in a *Xian* rice cultivar. Broadly similar phenotypic changes were observed in rice plants transgenic for the *OsSGL* orthologs, including increased grain size, grain weight, enhanced water-deficit stress tolerance during the seedling and vegetative stages, enlarged root systems, higher osmolyte contents, and highly overlapped altered transcript levels of cell cycle genes and stress-responsive genes.

Results

DUF1645 domain is highly conserved in *Poaceae* OsSGL orthologs

Four *OsSGL* orthologs each containing an entire DUF1645 domain were cloned from sorghum (*SbSGL*), maize (Zm*SGL*), millet (*SiSGL*), and rice (*OsSGL2*) (Supplementary Table S1, 2). *ZmSGL* (NCBI Accession KT626002) is located in maize chromosome 4. Its ORF is 810 bp, encoding a putative 269-aa protein with a molecular mass of 27.6 kDa, and predicted to be a proline-rich receptor-like protein kinase PERK7. The cDNA of *SiSGL* (NCBI Accession XM_004951915.4) is 1,300 bp long and contains an open reading frame (ORF) of 876 bp flanked by a 5' UTR (232 bp) and a 3' UTR (192 bp). This gene encodes a putative 291-aa protein with a molecular mass of 30.3 kDa. The cDNA of *OsSGL2* (NCBI Accession XM_015770529.2) is 1,424 bp long and contains an ORF of 747 bp flanked by a 5' UTR (133 bp) and a 3' UTR (544 bp). This gene encodes a putative 248-aa protein with a molecular mass of 26.7 kDa. Low DNA similarities (only up to 31% with sorghum) were revealed when aligning the *OsSGL* nucleotide sequence and its ORF to those of the non-*Oryza* species, whereas the OsSGL protein is relatively closely related (up to 53.3% with maize) to its homologs for the highly conserved DUF1645 domain [50]. However, the relationship between the two rice orthologs (OsSGL and OsSGL2) and other rice DUF1645 proteins is more divergent, suggesting that the latter play different roles in rice (Supplementary Figure S1).

A nucleotide sequence diversity and haplotype study of the *OsSGL* locus (LOC_Os02g04130) in cultivated varieties and wild rice (WT) accessions with RiceVarMap v2.0, detected only four SNPs in the ORF region corresponding to synonymous mutations (http://ricevarmap.ncpgr.cn/v2/). Therefore, we speculated that as gene *OsSGL* was quite conserved and specific in rice genome, its DUF1645 domain-containing orthologs may play similar roles in cereal crops (Supplementary Table S2) .

The spatial and temporal expression patterns of the four *OsSGL* orthologs were assessed in the corresponding cereal plants growing under normal conditions, including in young roots and leaves of seven day-old seedlings, the first leaf below the flag leaf and the young stems at stem elongation stage, the flag leaf blades, mature stems at booting stage, and young panicles (maize cob) at the early heading stage. The quantitative real-time PCR (qRT-PCR) results showed that all genes were expressed in almost all tissues examined, with expression being greatest in the young roots and panicles (Supplementary Figure S2). The high levels of expression in these tissues suggest that *OsSGL* orthologs may play an important role in regulating early germination and reproductive development in their respective species.

Poaceae OsSGL orthologs have a conserved function in increasing grain size and yield in rice

To elucidate the possible conserved function of the four complete DUF1645 domain-containing *OsSGL* orthologs, their effects on plant development were analyzed in transgenic rice lines harboring the corresponding over/heterologous-expression construct (OE). A *Xian* rice cultivar, KH2, was used for transformation with the OEs. The *OsSGL* ortholog transcripts were abundant in the transgenic lines with large differences among them (Supplementary Figure S3). Phenotypic analysis showed that transgenic plants expressing *ZmSGL* (OE-*ZmSGL*) showed slightly lower stature, but the grains were on average 7.97% longer, 6.41% narrower and 8.75% heavier than those of WT, leading to an average 13.84% increase in grain yield (Fig. 1d,h, Supplementary Table S4). The smallest grain size changes were observed in OE-*SiSGL* lines, with 5.43% longer in length, 3.74% narrower in width and 7.21% heavier in weight than those

of WT (Fig. 1h, Supplementary Table S4). In total, the average grain length, width and 1000-grain weight in transgenic lines were dramatically increased. Nevertheless, the heights of the transgenic plants were decreased relative to WT. These results indicate that expression of *OsSGL* orthologs promotes the formation of slightly dwarfed plants with more slender but heavier grains (Fig. 1i-k, g-k).

Given that the over/heterologous-expression transgenic lines have dramatically enlarged grains and floral organs, we analyzed the effects of over/heterologous-expression of each gene on cell number and size in palea/lemma of the transgenic rice plants. Before fertilization, the spikelet hulls formed by transgenic lines were much more slender than those of WT (Fig. 2a). Transverse sections of the central parts of the palea/lemma of florets were microscopically inspected before fertilization and compared between WT and transgenic lines to investigate the cellular basis of the increased organ size (Fig. 2b-k). Observation of a cross-section of the spikelets revealed that the inner parenchyma cell layer of palea/lemma in OE-*ZmSGL* contained 23.7 - 32.2% more cells than in the WT hull and its cells were 12.6-34.6% larger (Fig. 2d,i,l). Furthermore, we compared the center part of the spikelet hull (lemma) at maturity in transgenic lines and the corresponding control using scanning electron microscopy (SEM). The spikelet hull cells of OE-*ZmSGL* were larger (~21.7%) in size and significantly longer (~24.9%) in longitudinal orientation than those of the control, although the cell width were decreased (~3.2%), however, the estimated total cell number was slightly lower in OE-*ZmSGL* (Fig. 2d,i,m,n). These findings implied that the formation of the longer and narrower spikelet hull of OE-*ZmSGL* resulted from increased cell length and cell size in the longitudinal direction, but decreased cell width and increased cell division in transverse direction for epidermal cells of the outer and inner glumes. Broadly similar phenotypic changes were also observed with the OE-*SiOSGL* and OE-*OsSGL2* transgenic plants. Such observations were consistent with our previously reported results [50, 52].

To further seeking to uncover the cytological basis underlying the regulation of grain size by *OsSGL*, 14 independent knock-out rice mutants lines with CRISPR-Cas9-edited *OsSGL* expression (Cas9-*OsSGL*) were successfully produced with *Xian* rice cultivar KH2. In our previous studies, there were no obvious phenotype differences between WT and *OsSGL*-RNAi transgenic plants during growth and development, and the average grain size and length of lines with down-regulated *OsSGL* expression were comparable to those of WT [50]. In contrast the knockout Cas9-*OsSGL* lines showed abnormal grain shape with dramatically decreased size and length compared to control plants (Supplementary Figure S4). Consistent with the above results, the sizes and lengths of cells in spikelet hulls of Cas9-*OsSGL* plants were significantly smaller, shorter (~30.1%) and longitudinally narrower (~15.0%) as revealed by SEM (Supplementary Figure S4). In addition, substantially decreased plant height, number of tillers, grain number per panicle and flag leaf length, as well as very low fertility were observed in Cas9-*OsSGL* plants (view detail in a separate manuscript). Together the data suggest that *OsSGL* orthologs have a conserved function in positively regulating grain shape by changing cell division patterns and cell size in both longitudinal and transverse directions leading to the enhanced longitudinal growth of the rice grain.

Up-regulation of *OsSGL* orthologs also slightly increased the grain-filling rate. There were no differences observed in either endosperm fresh weight (FW) or dry weight (DW) between transgenic and WT plants 6

days after fertilization (daf). However, starting at 6 daf, both the FW and DW of transgenic lines increased significantly faster, and from 12 daf, the FW of transgenic plants were slightly heavier than that of WT. The endosperm FW and DW of transgenic plants were obviously heavier than those of WT starting at 24 daf and reached their maximum at 36 daf, consistent with their longer ovaries, slender grains, and increased rice endosperm weight (Supplementary Figure S5). The RiceXPro expression data on *OsSGL* (https://ricexpro.dna.affrc.go.jp/field-development.php?featurenum=16985) shows that it is specially high in reproductive organs (inflorescence, anther, pistill, lemma, and palea) during development stage, and in embryo and endosperm at ripening stage. Thus, *OsSGL* orthologs might also play a positive role in dry matter accumulation during grain milk filling and in improving rice endosperm growth, thereby regulating grain weight.

Poaceae OsSGL orthologs have a conserved function in increasing water-deficit stress tolerance in rice

Consistent with the results obtained with the *OsSGL* heterologous/over-expressing transgenic rice and *Arabidopsis* plants [51], similar osmotic-tolerance phenotypes were observed in other *OsSGL* orthologs over/heterologous-transgenic rice lines. In comparison to WT, the average shoot length of homozygous T3 transgenic rice lines were indistinguishable under normal growth conditions. However, the transgenic lines exhibited longer shoots under osmotic stress at the post-germination stage. Also, the average length of roots in transgenic lines were longer than that of WT under both normal and osmotic conditions (Supplementary Figure S6). The contents of three drought stress-relevant parameters, namely proline, soluble sugar and MDA were investigated between transgenic rice lines and WT at seeding stage. Under normal growing conditions, no differences of contents were observed between WT and transgenic rice. However, under osmotic stress the proline content and soluble sugar content were significantly elevated in all genotypes, but the increases were significantly larger in the transgenic plants (144.9-187.3 mg/g FW and 10.96-13.2 mg/g FW, respectively) than in WT (86.4 mg/g FW, 7.44 mg/g FW respectively). Moreover, the MDA contents in transgenic plants (13.4-16.7 nmol/g FW) were significantly lower than that in the WT (24.6 nmol/g FW) (Supplementary Figure S6).

To further assess drought tolerance at the vegetative stage, we withheld irrigation for a short period before returning to a normal watering regimen. After 12 days of simulated drought treatment, the leaves of WT rice were severely rolling and wilting, while the transgenic lines were less affected. After 5 days following resuming irrigation, the transgenic plants grew more vigorously than WT rice (Fig. 3a). Following 3 weeks of recovery, no more than 34.4% of the WT plants still displayed green tissues, while the survival ratios were much higher in transgenic lines, up to 32.5% with OE-*OsSGL2* and 67.1% with OE-*ZmSGL* (Fig. 3b). All of the OE transgenic plants for *OsSGL* and its *Poaceae* orthologs conferred significantly improved drought tolerance in rice.

qRT-PCR analyses were performed using 1-week-old sorghum and maize seedlings exposed to osmotic stress. Just as those of well-known drought-related genes (*SOD*, *POD*), the expression levels of *SbSGL* and *ZmSGL* were highly up-regulated under drought treatment (Supplementary Fig. S7). Considering our previous research results, we speculate that *OsSGL* orthologs may promote root growth irrespective of

exposure to different levels of drought stress, and respond to drought stress in their corresponding species [51].

Four *OsSGL* orthologs function in regulating expression of genes responsible for cell division, root development, hormone response and signaling

To further explore the possible mechanisms and genes involved in the yield and stress-tolerance performance of the transgenic lines, the genome-wide transcriptional profiles of four transgenic rice lines (OE-*SbSGL*, OE-*ZmSGL*, OE-*SiSGL*, OE-*SGL2*) and WT were sequenced using the SOLiD next-generation sequencing libraries, including three biological replicates for young inflorescence buds at early developmental stages at which the spikelet numbers were determined and the floret shape were changing fast. We also identified a stack of nonadditively expressed genes (20,916-23,232) that differ significantly from the WT, including more than 3,000 differentially expressed genes (DEGs) ($P<0.05$) out of 25,372 genes expressed in the panicle between any two cultivars (Supplementary Table S5). Gene ontology (GO) analysis showed that genes affected by the over/heterologous-expression of the four genes were significantly enriched in cellular macromolecule biosynthetic processes, gene expression, cell part and organelle which were related to cell division and elongation (Fig. 4d). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for the nonadditively expressed genes in each stage revealed that they are involved in a variety of biological pathways. Most of the enriched pathways are for ribosome pathway, RNA transport, RNA polymerase and DNA replication (Fig. 4). Interestingly, as shown in volcano plots, the up-regulated (red) and down-regulated (blue) genes were distributed in almost the same area (Fig. 4a). Furthermore, the Venn diagram analysis results also showed that only 8.3% of DEGs were specific to a construct, with most of DEGs overlapping, including 73.8% of DEGs overlapping in all different comparisons (Fig. 4b,c). Thus the expression of the four transgene *OsSGL* orthologs in rice led to a similar effect on transcriptome composition, suggesting that the function of DUF1645 in difference cereal crops may be very similar and conserved.

To confirm the RNA sequencing (RNA-seq) data and obtain further insight into the function of *OsSGL* orthologs, based on the functional annotation, 63 key DEGs that participate in hormone response, and biosynthesis and signaling pathways were selected for expression analyses by qRT-PCR in four OE transgenic lines and WT (Supplementary Table S6, Supplementary Figure S8). Compared with the WT, the transcript levels of two auxin transport genes, *OsPIN1* and *OsPIN2*, were obviously decreased while three auxin synthesis genes, *YUCCA4*, *YUCCA6* and *YUCCA9*, were greatly increased in the young panicles of OE transgenic lines. The expression levels of five cytokinin (CK) signaling pathway-related genes, *OsCKX4*, *RR1*, *RR5*, *RR7* and *RR10*, were considerably changed in the transgenic plants. The other genes analyzed showed no significant differences in expression levels between the transgenic and WT samples. In summary, validating the RNA-seq data for these genes by qRT-PCR supported the reliability of our transcriptome data. The altered transcript levels of the genes analyzed in the over-/heterologous-expression lines together with our previous findings suggested that the four *OsSGL* orthologs may play a conserved dual role in regulating stress response and meristematic activity through a plant hormone-mediated pathway.

Discussion

DUF1645 domain-containing *OsSGL* orthologs enhance drought-tolerance and grain length in rice

A suite of abiotic and biotic stresses result in 30%-60% yield losses in crop plants globally each year [53]. Ensuring future food supplies necessitates the development of crop varieties for breaking the current yield barrier either through a better response to stress factors or improved yield parameters. Therefore, understanding the molecular mechanisms regulating grain size and stress resistance would enable the development of new strategies for yield improvement in cereal crops. In this study, over/heterologous-expression of four DUF1645-containing *OsSGL* orthologs positively increased cell numbers and size by promoting mitotic division and cell expansion in the inflorescence meristem, ultimately resulting in increase grain length and yield. Moreover, it also significantly enhanced drought tolerance presumably due to promotion of more extensive root systems, increased accumulation of osmolytes, and altered transcript levels of stress-responsive genes (Supplementary Figure S9a, b). This study provides a important basis for the functional analysis of DUF1645-containing *OsSGL* ortholog genes in regulating rice stress-tolerance and grain length and a potential means to improve crop yield.

The intactness of the DUF1645 domain is essential for its conserved function

Leaf rolling, influencing the yield through the manipulation of photosynthetic capacity and transpiration, is an important and highly complex agronomic trait, especially in crop plants. A coil at the base of the flag leaf was exclusively present in all OE-transgenic plants except in OE-*OsSGL2* (Supplementary Figure S9c). Alignment of the polypeptide sequences of the five *OsSGL* orthologs using CLUSTALW showed that only the N-terminal of DUF1645 domain in OsSGL2 is obviously different from the corresponding region of the other four proteins (Supplementary Figure S9d). This dissects the functions of the DUF1645 domain. The structural and functional features of the OsSGL protein, and its regulation of both long grain and curling flag leaf shape are currently under investigation.

Our recently obtained data from domain deletion analysis indicates the intactness of the DUF1645 domain is essential for the effect of *PoaceaeOsSGL* orthologs, and that the N-terminal region of DUF1645 may control the flag leaf curling in rice (manuscript in preparation). Our recent studies have also shown that *OsSGL* overexpression and/or knock-out mutants lines with CRISPR-Cas9-edited (Cas9-*OsSGL*) can induce adaxially screw flag leaf-at-bottom due to abnormal cell wall formation, impaired epidermis and water deficit in flag leaves and have revealed the role of *OsSGL* in modulating leaf abaxial cell development and in sustaining abaxial characteristics during flag leaf development (manuscript in preparation).

OsSGL orthologs may regulate grain size and drought tolerance via plant hormone response/biosynthesis/signaling/crosstalking

Phytohormones such as auxins and cytokinins regulate numerous biological processes, including cell proliferation and expansion, reproductive development, grain size, seed yield, abiotic stress responses,

root development and growth, and root architecture, via a complex signaling network [54, 55, 56, 57]. For example, *BIG GRAIN 1 (BG1)* encodes a novel membrane-localized protein, which is involved in auxin transport, could increase cell proliferation and elongation in spikelet hulls [58]. *TGW6* negatively regulated the endosperm growth or grain filling via modulating the endogenous auxin level in rice [42]. Moreover, an appropriate root architecture is a vital component for plants, providing a secure supply of nutrients and water in response to drought [55, 56]. Auxin-cytokinin crosstalk signaling plays key roles in root development and can coordinately regulate a series of genes [56, 57]. Our data also showed that some auxin and CKs responsive/ biosynthesis/signaling/crosstalking genes are either activated or repressed in OE transgenic rice lines. Based on these data and our previous research results [50, 51, 52], we speculate that the DUF1645-containing *OsSGL* orthologs may share the same functions in the *Gramineae* family, acting as a positive modulator upstream of auxin and/or CKs signaling or in an indirect manner affect the plant hormone pathway, and positively regulate both yield component traits (grain length, grain yield) and drought stress-tolerance. It will be fascinating to investigate the possibility that DUF1645-containing proteins may interact with similar protein or gene targets in their regulation of cell proliferation and cell size in the inflorescence meristem for grain elongation, and of flag leaf blade/root system development for abiotic stress-tolerance.

Conclusion

This study provides fundamental information concerning the grain length, yield and abiotic-stress response in the *Gramineae* family , and enhances our the understanding of the molecular mechanisms of DUF1645 in *Poaceae* plants. Our findings present the first evidence, to our knowledge, that *OsSGL Poaceae* orthologs probably have a highly conserved function in regulating both drought stress-tolerance and yield component traits in rice through plant hormone responses, biosynthesis, signaling, and crosstalking pathways. We speculate that this highly conserved DUF1645 family in cereal crops have not been subject to natural or artificial selection during domestication and breeding. These findings bridge the gap in the understanding of the plant hormone cross-talk between environmental stress and genetic interactions influencing grain yield.

Methods

Plant materials and growth conditions

The seeds of KH2 (*O. sativa* L. *ssp. xian*), Nipponbare (*O. sativa* L. *ssp. geng*), sorghum Xingxiangliang 2 (*S. bicolor*), maize Zhongnuo 1 (*Z. mays*), millet Yugu 1 (*S. italica*), *OsSGL* mutant lines and transgenic plants were provided by the Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences in Changsha, Hunan Province, China. Seeds were surface sterilized with 70% ethanol for 1 min and then with 2% (w/v) sodium hypochlorite for 15 min, washed 3 times with sterile distilled water and soaked in water for 3 days at 25^{o}C (with daily water changes). Field-grown plants were raised under natural field conditions during the standard season in the experimental research field/paddy field located at the Institute of Subtropical Agriculture, CAS, Changsha,

Hunan, China. The planting density was 20 cm between plants in each row, and the rows were 20 cm apart, with one plant per hill. Field management, including irrigation, fertilizer application and pest control, followed the normal agricultural practice. For rice root measurement, sterilized seeds were germinated on 1/2 MS medium for 7 days at 28^{o}C under a 12h light/12h darkness photoperiod.

Cloning of gene *ZmSGL*, *SbSGL*, *SiSGL*, *OsSGL2* and sequence analysis

To identify genes homologous to *OsSGL* in *Poaceae*, we first performed a BLAST search with the *OsSGL* sequence against the National Center for Biotechnology Information (NCBI) database (http://www.ncib.nlm.nih.gov/BLAST/). Then the conserved DUF1645 domain of OsSGL-homologous proteins from *Poaceae* were searched and analysed using the NCBI Batch Web CD-Search Tool (https://www.ncbi.nlm.nih. gov/Structure/bwrpsb/bwrpsb.cgi). The cDNA fragments containing the whole ORFs, including entire DUF1645 domains, of *OsSGL*, *SbSGL*, *ZmSGL*, *SiSGL* and *OsSGL2* were respectively amplified from total RNA preparations of KH2, sorghum Xingxiangliang 2, maize Zhongnuo 1, millet Yugu 1, and Nipponbare, respectively, with specific forward and reverse primers, using an RT-PCR kit (Promega, Madison, WI, USA) according to the manufacturer's instructions (Supplementary Table S1). The details of all 24 OsSGL *Poaceae* homologous proteins are listed in Supplementary Table S2. The nucleotide sequence diversity of *OsSGL* (LOC_Os02g04130) was analyzed with RiceVarMap v2.0 (http://ricevarmap.ncpgr.cn/v2/) (Supplementary Table S3). The nucleotide and amino acid sequences of *OsSGL* were aligned with their homologs of other cereal species with CLUSTALW2 (http://www.ebi.ac.uk/tools/clustalw2). The phylogenetic tree was constructed by multiple sequences alignment via MEGA6 using the Neighbor-Joining method with 1,000 replicates bootstrap analysis.

Plasmid construction and transformation

After sequence verification, the various amplified DNA fragments were inserted into the multi-cloning sites of the binary expression vector *pCAMBIA1300-pJITI63*. The resulting overexpression constructs *pCaMV35S::X::NOS* (*X=OsSGL, SbSGL, ZmSGL, SiSGL or OsSGL2*), which carried *hpt* II (hygromycin resistance gene) as a selection marker, was introduced into *Agrobacterium tumefaciens* strain EH105 cells by electroporation. All the final constructs were sequenced to ensure the correctness of the introduced segments and then were used to transform immature embryogenic calli induced from rice KH2. Hygromycin resistance was used to screen positive transgenic plants. In total, we obtained 67 independent transgenic plants for *OsSGL*, 29 for *SbSGL*, 24 for *ZmSGL*, 14 for *SiSGL* and 21 for *OsSGL2*. Using primers specific for the hygromycin phosphotransferase gene, we performed PCR to confirm the presence of the T-DNA in the transformants. The expression levels of *OsSGL*, *SbSGL*, *ZmSGL*, *SiSGL* and *OsSGL2* in corresponding T_0 positive transgenic plants were assessed using the middle part of the 7th young leaf from different independent transgenic lines by qRT-PCR with *Actin2* being used as the internal control. The transgene transcripts were abundant in all transgenic lines but with large differences among among lines. Homozygous T3 plants were used in subsequent experiments.

RNA extraction and qRT-PCR analysis

Total RNAs were extracted with TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Afterwards, 1 μg of DNAase-treated RNA was reverse transcribed using a PrimeScript^{TM} 1st Strand cDNA synthesis kit (Takara) according to the manufacturer's protocol. The qRT-PCR analysis was conducted with Fast Start Universal SYBR Green Master (Roche), and reactions were performed in an ABI 7900HT (Applied Biosystems) at 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 s and 58^{o}C for 30 s. The rice housekeeping gene *OsActin* (Accession NO. AB047313) was used as the internal control. Threshold cycles for each of the target genes and control were adjusted manually. Real-time PCR efficiencies (E) were calculated from the slopes of standard curves for each gene. Samples from the unstressed group were selected as calibrators. After normalization with the *OsActin* transcript, the relative transcript levels in the various strains were averaged from three independent replicates of each sample and relative amounts of mRNA were calculated per the comparative threshold cycle method.

Phenotype measurements

Harvested rice grains were air-dried and stored at room temperature for at least two months before testing. Fully filled seeds (with hull) were used for measuring grain length, width and weight. In detail, sixty full seeds were randomly selected from each cultivar/line and divided into three groups equally. All seeds from each group were lined up length-wise along a Vernier caliper to measure seed length and then arranged breadthwise to measure seed width. Seed length and width were determined by averaging three measurements. Seed thickness was measured individually by using a Vernier caliper. More than 600 seeds of each plant were used to determine the one hundred-seed weight, which was then converted to 1,000-seed weight. For the measurement of panicle traits, three medium-sized main panicles were obtained from each transgenic and corresponding control plants. We measured the panicle length, and counted the number of primary rachis branches, the number of secondary rachis branches, and the number of grains per panicle. For the measurement of the flag leaf traits, sixty healthy plants were randomly selected from each cultivar/line and divided into three groups equally. The flag leaf length (FLL, cm) and flag leaf width (FLW, cm) of the first two panicles were measured, and the flag leaf area (FLA, cm^2) was calculated using the formula: FLA=FLL*FLW*0.75. Duncan or Dunnett tests were performed to compare the means of all traits for different allelic groups or cultivars/lines using SPSS 19.0 (SPSS Inc, IBM Company).

Histological analysis and microscopy observation

Young spikelet hulls were fixed in FAA (50% ethanol, 5% glacial acetic acid, and 5% formaldehyde) for 48 h, and sent to Servicebio Company (http://www.servicebio.cn/) for paraffin sectioning according to their protocol. The sections were observed under a microscope (Leica DMR) and scanned by Pannoramic MIDI (3D HISTECH). Area measurements of vascular elements were performed using both the Pannoramic Scanner and Caseviewer (C.V 2.3) software. For glume cell observation, samples were fixed in 2.5% glutaraldehyde (30.5% 2 M Na_2HPO_4, 19.5% 2 M NaH_2PO_4, and 2.5% glutaraldehyde) for 48 h, then truncated at the longitudinally middle position of the spikelet hulls, followed by vacuum sputtering of

gold nano-particles on their surfaces, and observed by SEM (HITACHI, S-3000N) at an accelerating voltage of 10 kv.

Osmotic and drought treatments

To determine drought tolerance in transgenic rice, T3 homozygous seeds were used. For osmotic stress at the post-germination stage, sterilized seeds were sown on 1/2 MS medium for 5 days, then healthy germinated seeds were transferred to 1/2 MS medium containing 0 or 400 mM mannitol for 6 days in a growth room. Three replicates were performed. Lengths of shoot, primary, adventitious and lateral roots were measured at the end of the treatments. For the determination of adventitious root, the five longest adventitious roots on each seedling were counted. Similar to measurement of adventitious root length, lateral root length was determined with the 15 longest lateral roots on each primary root.

Drought assays were performed in a controlled growth chamber PGC15.5 (Percival, Perry, IA, USA). For drought assays at seedling stage, germinated seeds were transferred to an incubator with a photoperiod of 12 h light (30^{o}C)/12 h dark (25^{o}C) for 5 days, then 20 similar germinated seeds for each transgenic line were planted in three rows (one plot) along with the WT control after a randomized complete block design with three replicates. All plants were grown in PVC (polyvinyl chloride) pots (diameter 30 cm and height 45 cm) under natural conditions prior to stress treatment. At the four-leaf-seedling stage watering was withheld for all plants for several days (~14 days) until 90% of the leaves coiled, and then resumed. After 1-week recovery, survival rates were determined.

Determining the contents of proline, soluble sugars and MDA

After 6 days of osmotic treatment, shoots of WT and of transgenic plants were used for biochemical analysis. Proline and soluble sugar contents in harvested tissue samples were measured according to the sulphosalicylic acid method and the anthrone method, respectively [59, 60]. The levels of MDA were determined with thiobarbituric acid [61].

RNA sequencing

Total RNA was isolated with TRIzol reagent from 2-4 cm-long young panicles of relevant over- or heterologous-expressing transgenic and WT KH2 rice plants at early booting stage as described earlier. Material from 10 plants of each genotype was pooled for RNA extraction. RNA quantification, qualification, library preparation for strand-specific transcriptome sequencing, clustering and sequencing, data analysis and quality control were conducted by Novogene (http://www.novogene.com/) according to their protocol. Differential expression analysis of the genes in the mutant, transgenic and WT samples were performed using the DESeq R package (version 1.18.0). DESeq provides statistical routines for determining differential expression in a digital gene expression data set using a model based on the negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini and Hochberg method for controlling the false discovery rate. Genes with an adjusted *P*-value <0.05 found by DESeq

were assigned as differentially expressed. After quantifying gene expression level, gene ontology and genome enrichment analyses of differentially expressed genes were performed.

Abbreviations

DAF: Days after fertilization

DUF: Domains of unknown function

DW: Drought weight

FW: Fresh weight

GO: Gene ontology

KEGG: Kyoto encyclopedia of genes and genomes

MDA: Malondialdehyde

ORF: open reading frame

qRT-PCR: Quantitative real-time PCR

QTL: Quantitative trait loci

RNA-seq: RNA sequencing

SEM: Scanning electron microscopy

UTR: Untranslated region

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Datasets used in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's Contributions

M.L.W. designed the experiments and revised the manuscript. M.L.W. and K.L. collected data, performed data analysis, conducted experiments and wrote the manuscript. Y.C.C. and X.M.Y. conducted the qRT-PCR experiments. M.J.L. and B.Z. conducted the gene cloning, vector construction and genetic transformation. The authors have read and approved the manuscript and declare no conflict of interest.

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Not applicable

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Supplementary Information

Additional file 1: Figure S1.

Sequence homology and conserved DUF1645 domains in *OsSGL Poaceae* orthologs.

Additional file 2: Figure S2.

The expression pattern of the five *OsSGL* orthologs in the corresponding cereal plants.

Additional file 3: Figure S3.

The expression levels of *OsSGL* orthologs in overexpression transgenic rice.

Additional file 4: Figure S4.

OsSGL regulates grain length. **a** Gross morphology of rice grains in WT and transgenic lines.

Additional file 5: Figure S5.

OsSGL orthologs regulate endosperm size and grain milk filling in KH2 and transgenic lines at indicated days after fertilization (DAF).

Additional file 6: Figure S6.

Osmotic treatment in transgenic and wild type rice at seedling stage.

Additional file 7: Figure S7.

The expression levels of *SbSGL*, *ZmSGL* and well-known stress-related genes in sorghum and maize at seedling stage under normal condition (CK) and drought-treatment.

Additional file 8: Figure S8.

Validation of the RNA sequencing (RNA-seq) data by qRT-PCR.

Additional file 9: Figure S9.

The intactness of the DUF1645 domain is essential for the effect of *Poaceae* OsSGL orthologs.

Additional file 10: Table S1.

PCR primers used for cloning of OsSGL Poaceae orthologs.

Additional file 11: Table S2.

OsSGL Poaceae orthologs and their corresponding proteins in DUF1645 super-family.

Additional file 12: Table S3.

Sequence and haplotype analysis on the *OsSGL* locus in cultivated varieties and wild rice accessions.

Additional file 13: Table S4.

Phenotypical measurements (mean±s.e.m) of wild type rice KH2 and corresponding over-/heterologous-expression transgenic rice plants.

Additional file 14: Table S5.

Up- and down-regulated DEGs in any two cultivars.

Additional file 15: Table S6.

Primer pairs used for qRT-PCR analysis.

Figures

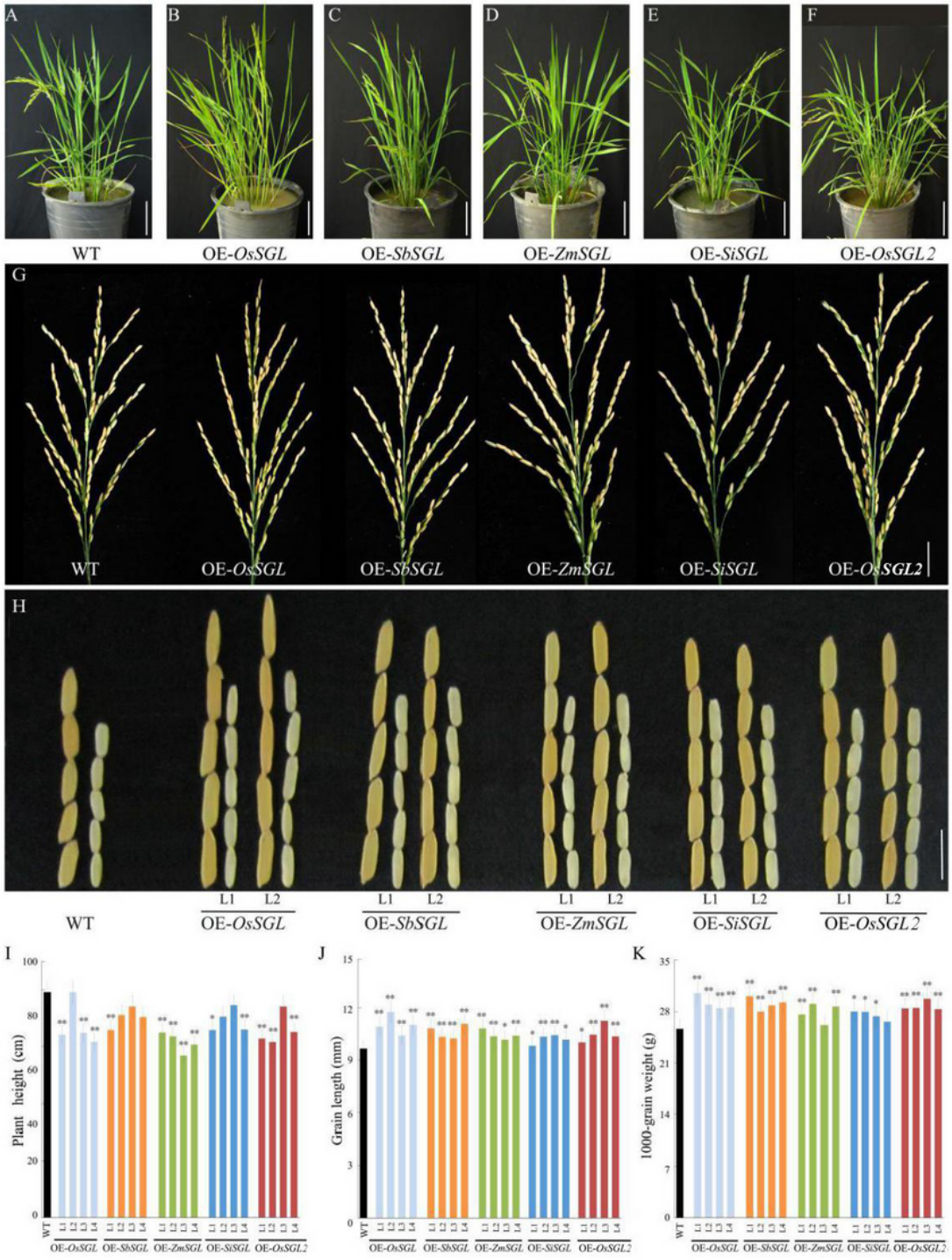


Figure 1

Comparisons of agronomic traits of mature plants, panicles and grains between wild type (WT) and transgenic rice lines. a-h Gross morphology of WT and relevant transgenic mature plants (a-f), panicles (g) and grains (h). Scale bars, 20 cm, 2 cm and 1 cm, respectively. i-k Comparisons of plant height (i), grain length (j), 1,000-grain weight (k) between WT and transgenic rice lines. WT, Xian rice cultivar KH2; OE-OsSGL, OsSGL over-expression transgenic lines; OE-SbSGL, SbSGL heterologous-expression transgenic lines; OE-ZmSGL, ZmSGL heterologous-expression transgenic lines; OE-SiSGL, SiSGL heterologous-expression transgenic lines; OE-OsSGL2, OsSGL2 over-expression transgenic lines. L1-L4, over/heterologous-expression transgenic rice lines 1 to 4 of T3 generation. Student's t-test was used to generate the P values; * $P<0.05$, ** $P<0.01$. All phenotypic data were measured from paddy-grown plants under normal cultivation conditions.

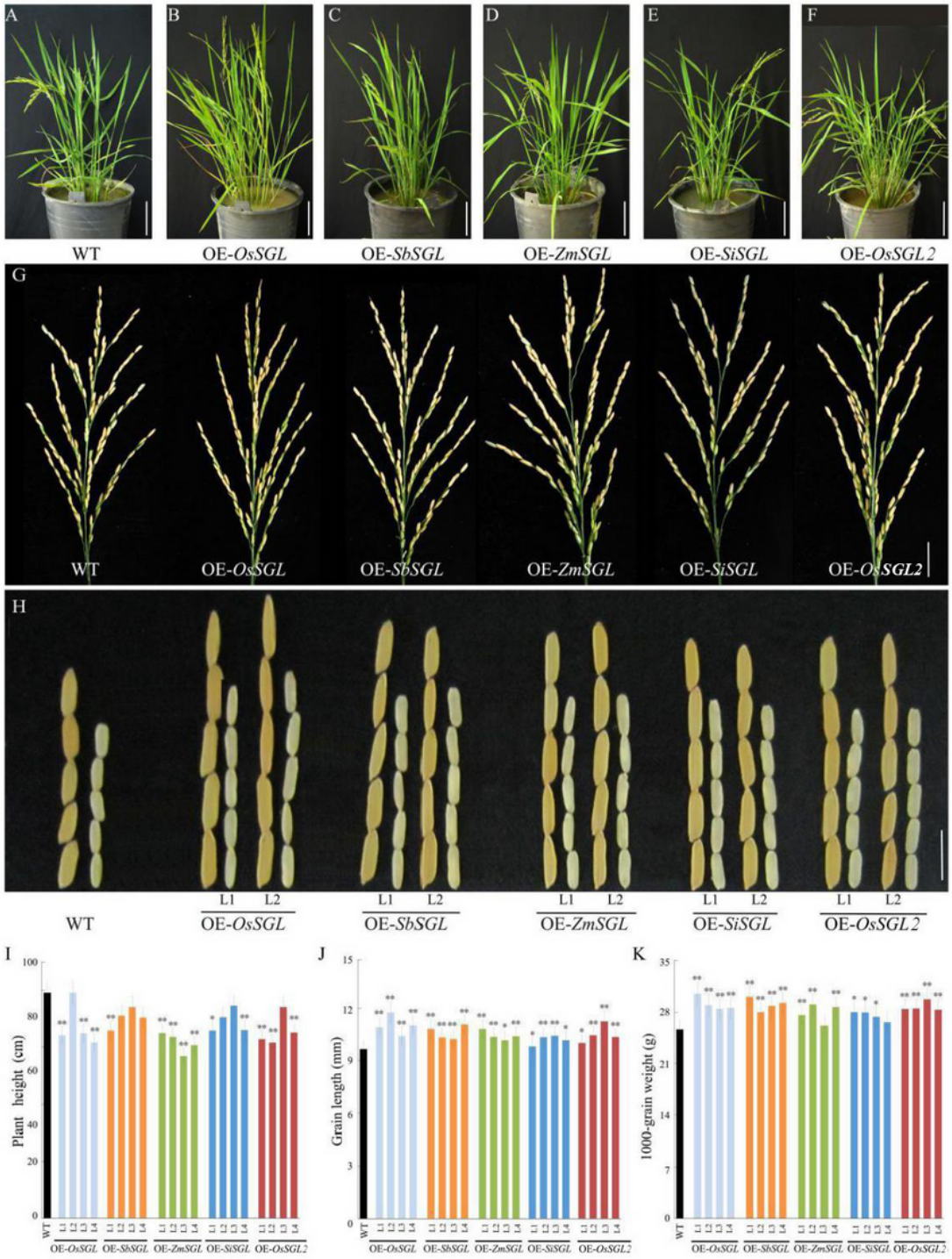


Figure 1

Comparisons of agronomic traits of mature plants, panicles and grains between wild type (WT) and transgenic rice lines. a-h Gross morphology of WT and relevant transgenic mature plants (a-f), panicles (g) and grains (h). Scale bars, 20 cm, 2 cm and 1 cm, respectively. i-k Comparisons of plant height (i), grain length (j), 1,000-grain weight (k) between WT and transgenic rice lines. WT, Xian rice cultivar KH2; OE-OsSGL, OsSGL over-expression transgenic lines; OE-SbSGL, SbSGL heterologous-expression

transgenic lines; OE-ZmSGL, ZmSGL heterologous-expression transgenic lines; OE-SiSGL, SiSGL heterologous-expression transgenic lines; OE-OsSGL2, OsSGL2 over-expression transgenic lines. L1-L4, over/heterologous-expression transgenic rice lines 1 to 4 of T3 generation. Student's t-test was used to generate the P values; * P<0.05, ** P<0.01. All phenotypic data were measured from paddy-grown plants under normal cultivation conditions.

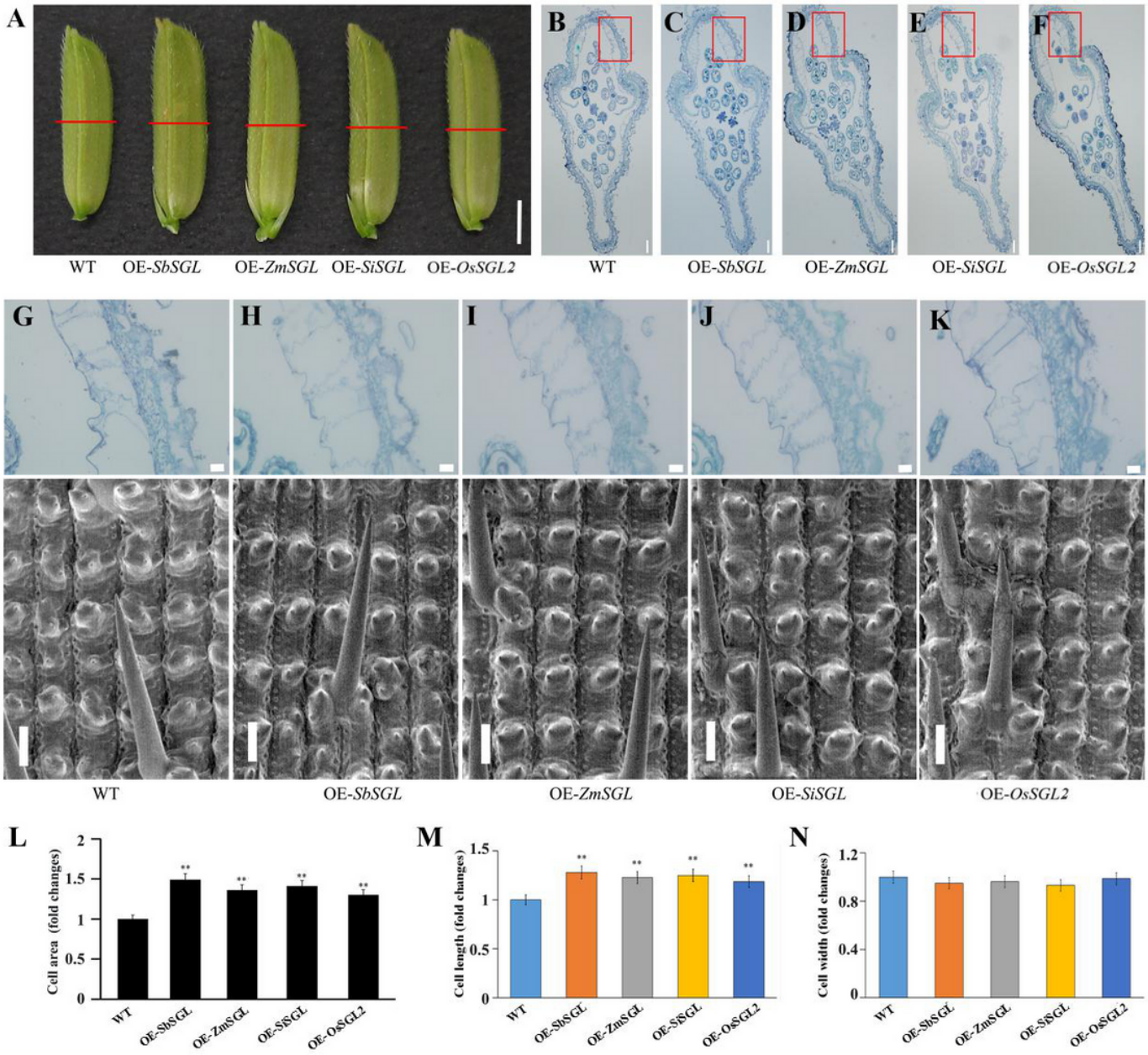
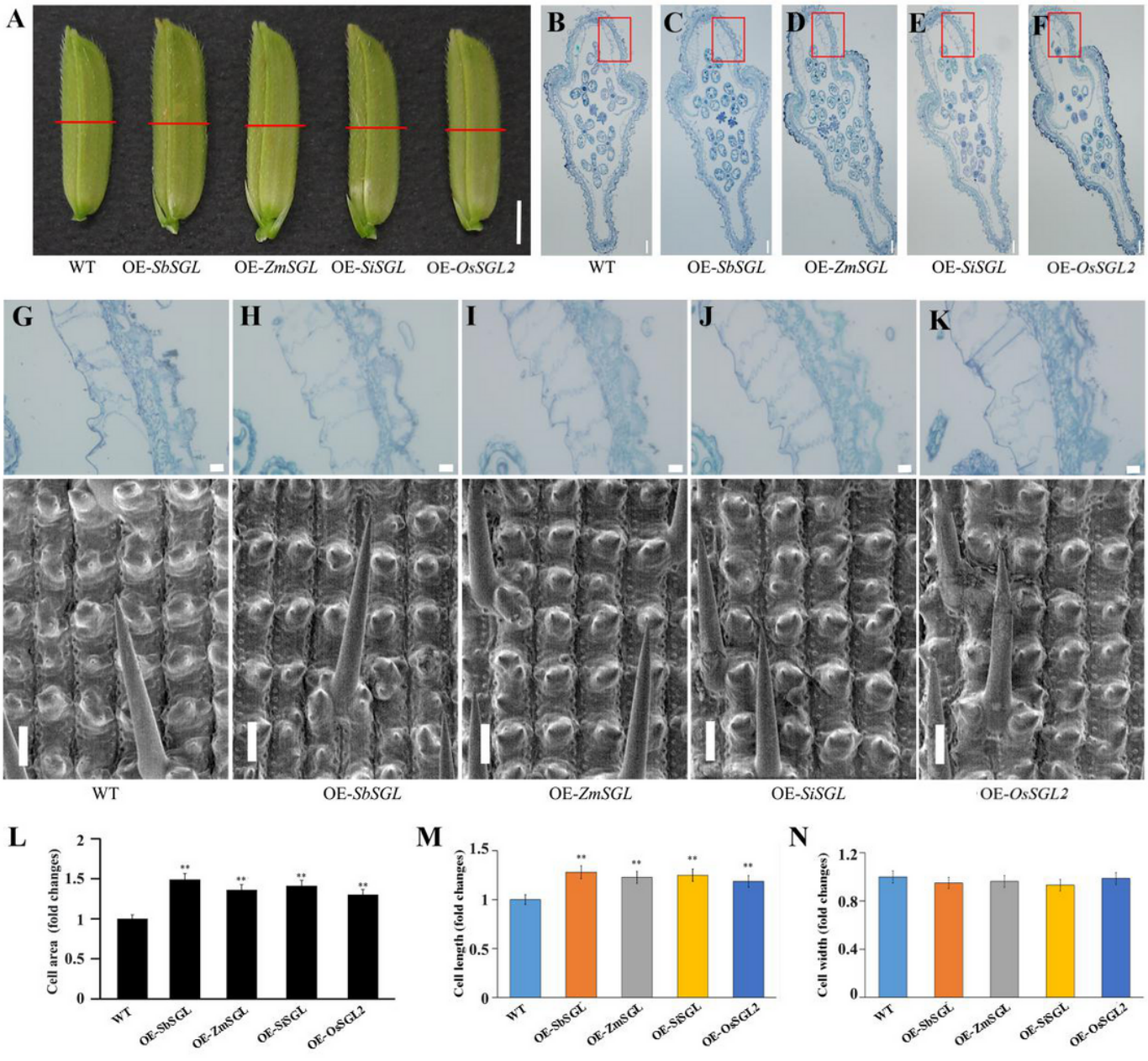


Figure 2

Histological analysis of spikelet hulls of WT and the four over/heterologous expression transgenic rice. a Young spikelet hulls of WT and transgenic rice lines 6 days before heading. The red line indicates indicates the sites of the cross sections. Scale bar, 2.5 mm. b-f Cross-section cut horizontally at the

middle of of spikelet hulls as show in a. Scale bar, 200 μm. g-k Microscopic inspection inside and outside of spikelet hulls. Magnification of indicated cross-section area boxed in b-f, the upper panels. SEM analysis of the outer surfaces of spikelet hulls, the bottom panels. Scale bars, 20 μm and 150 μm, respectively. i Comparison analysis of cell area in the inner parenchyma layer shown in upper panels of g-k. m-n Comparison analyses of cell length (m) and width (n) of outer epidermal cells shown in bottom panels of g-k. Student's t-test was used to generate the P values; *P<0.05, **P<0.01. WT, Xian rice cultivar KH2; OE-OsSGL, OsSGL over-expression transgenic lines; OE-SbSGL, SbSGL heterologous-expression transgenic lines; OE-ZmSGL, ZmSGL heterologous-expression transgenic lines; OE-SiSGL, SiSGL heterologous-expression transgenic lines; OE-OsSGL2, OsSGL2 over-expression transgenic lines.



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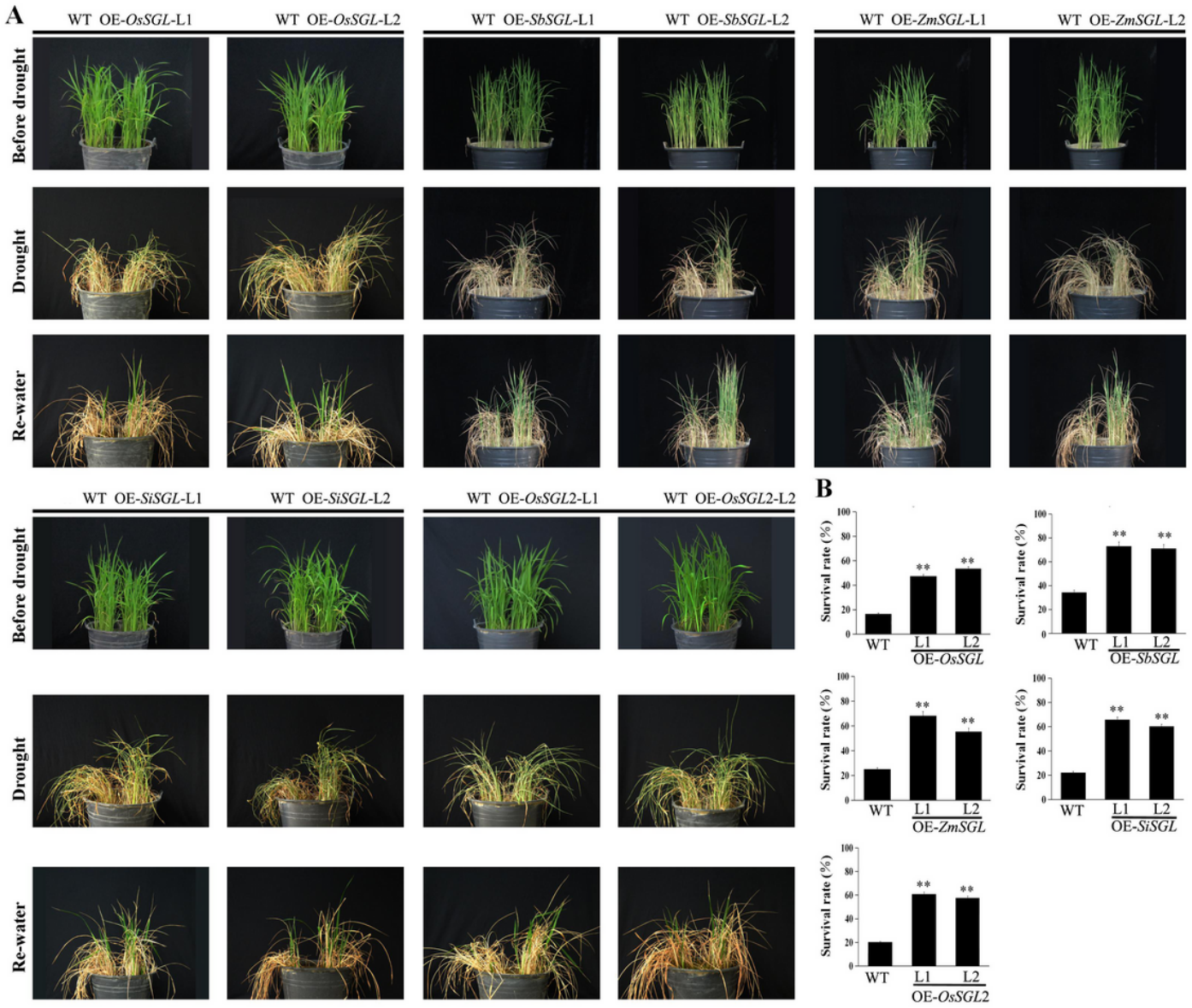
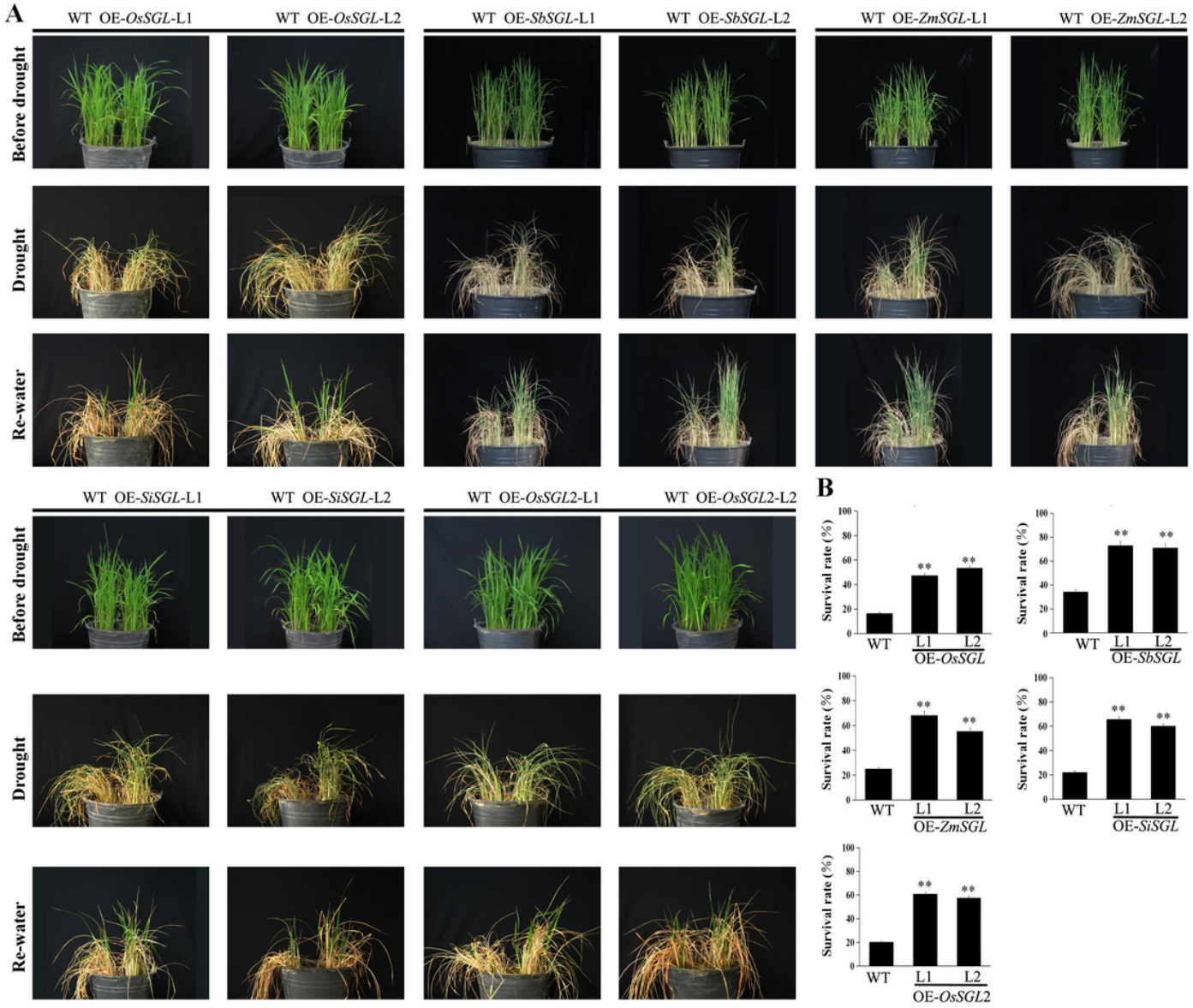


Figure 3

OsSGL orthologs improve drought tolerance in rice. a Phenotype comparison of WT and transgenic rice under drought treatment at four-leaf seedling stage. b The survival rate (ratio of surviving plants to total number of plants after re-watering) of WT and transgenic rice. Data are given as means SD. Student's t-test was used to generate the P values; * P<0.05, ** P<0.01. WT, wild type, Xian rice cultivar KH2; OE-OsSGL-L1/2, OsSGL over-expression transgenic lines; OE-SbSGL-L1/2, SbSGL heterologous-expression transgenic lines; OE-ZmSGL-L1/2, ZmSGL heterologous-expression transgenic lines; OE-SiSGL-L1/2, SiSGL heterologous-expression transgenic lines; OE-OsSGL2-L1/2, OsSGL2 over-expression transgenic lines.



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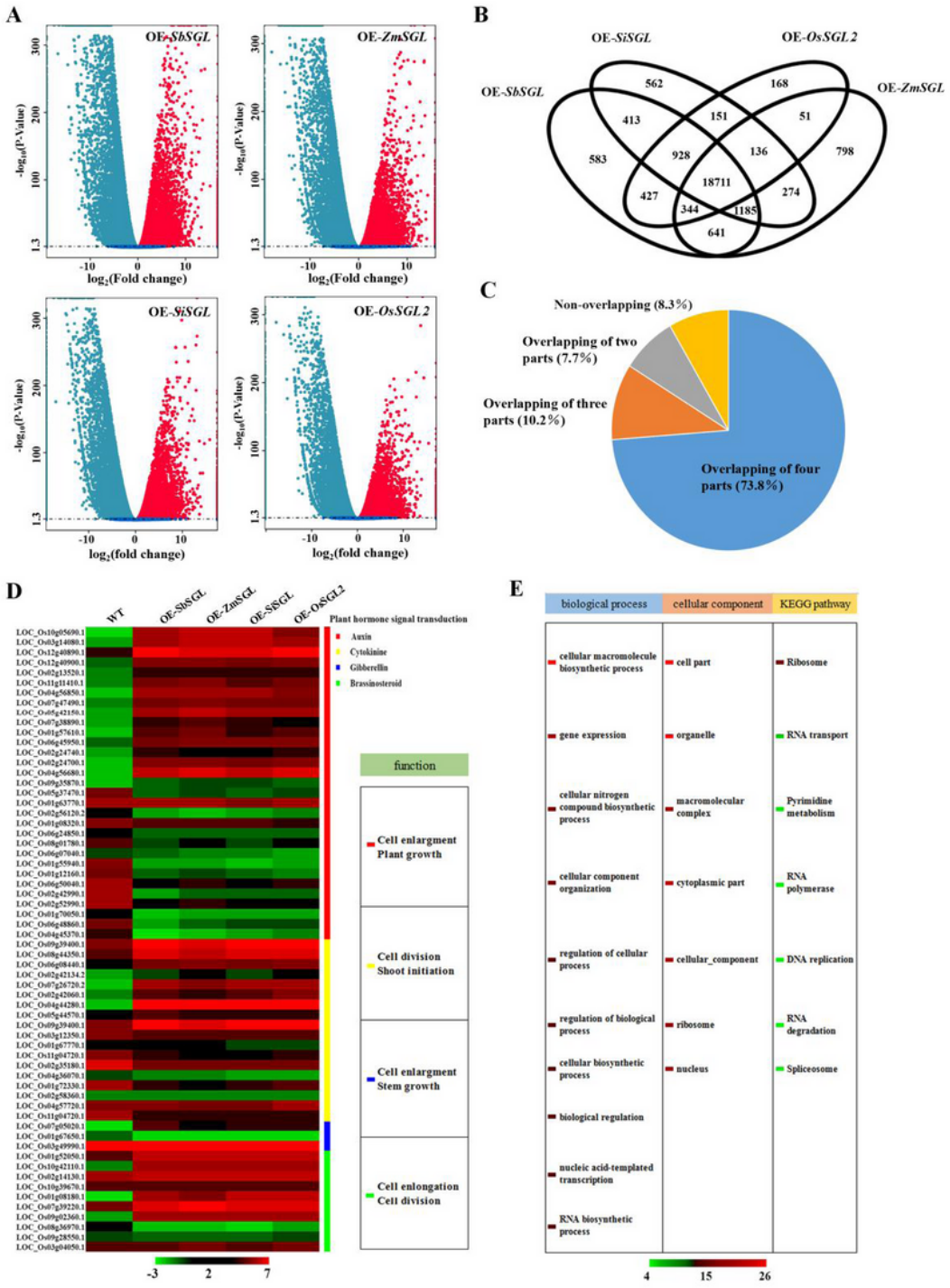
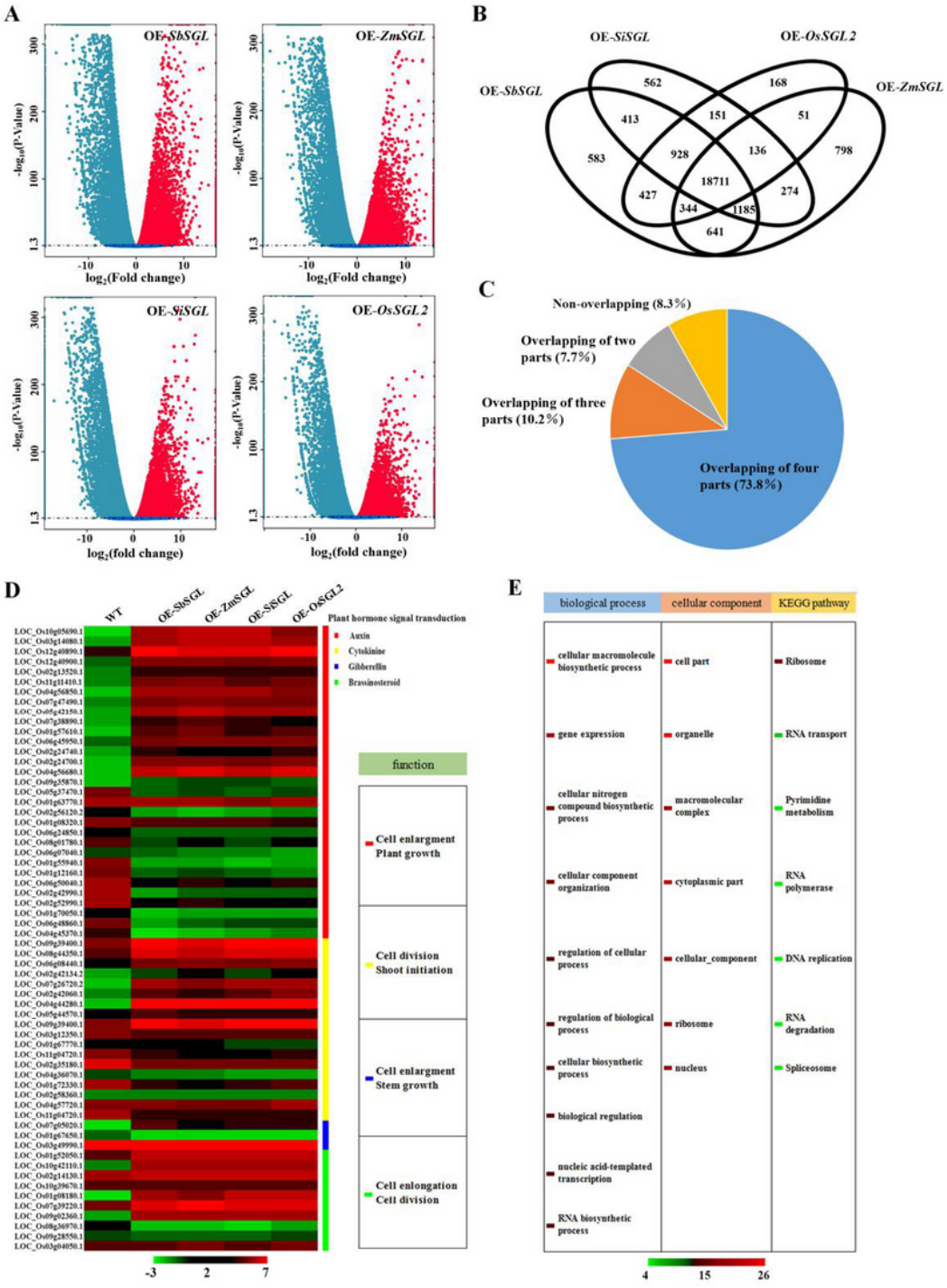


Figure 4

Genome-wide transcriptional profiles analyses for young inflorescence buds (1-2 cm in length) before heading in WT and four OE transgenic plants. a Volcano Plot analysis of DEGs in different comparisons. b,c Analysis DEGs with Venn diagram (b) and percentage chart (c). d Heatmap of partial DEGs related to plant hormone signal transduction pathway. WT, wild type, Xian rice cultivar KH2; OE-SbSGL, Sb, SbSGL heterologous-expression transgenic lines; OE-ZmSGL, Zm, ZmSGL heterologous-expression transgenic lines; OE-SiSGL, Si, SiSGL heterologous-expression transgenic lines; OE-OsSGL2, OsSGL2 over-expression transgenic lines.



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