Comprehensive study of Serine/Arginine-Rich (SR) gene family in rice: Characterization, evolution and expression analysis (#84270)

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Comprehensive study of Serine/Arginine-Rich (SR) gene family in rice: Characterization, evolution and expression analysis

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As important regulators of alternative splicing (AS) events, serine/arginine (SR)-rich proteins play indispensable roles in the growth and development of organisms. Till now, the study of SR genes is still lacking in plants. In the current study, we performed genome-wide analysis of the SR gene family in rice. A total of 24 *OsSR* genes were phylogenetically classified into seven groups, corresponding to 7 subfamilies: SCL, SC, SR45, RS2Z, RSZ, RS and SR. The *OsSR* genes' structures, distribution of conserved domains and protein tertiary structure of OsSR were conserved within each subfamily. The synteny analysis revealed that there were six pairs of segmental duplicated genes (12 *OsSR* genes), suggesting that segmental duplication events were critical for the expansion of the *OsSR* gene family. Besides, interspecific synteny revealed the distribution of orthologous SR gene pairs between rice and *Arabidopsis*, sorghum, wheat, and maize, inferring these genes may originate from the same ancestor. Among all the *OsSR* genes, 14 genes exhibited NAGNAG acceptors and only four *OsSR* genes had AS events on the NAGNAG acceptors. Furthermore, *OsSR* genes showed distinct tissue-specific expression patterns, indicating that different *OsSR* genes may function in different developmental stages in rice. The RT-PCR experiments confirmed that *OsSR* genes underwent AS. The AS patterns on the same *OsSR* gene were variable among the root, stem, leaf, and grains at different filling stages, and some isoforms could only be detected in one or few of tested tissues. Meanwhile, our results showed that the expression of some *OsSR* genes changed dramatically under ABA, GA, salt, drought, cold or heat treatment , which were related to the wide distribution of corresponding *cis*-elements in their promoter regions, suggesting their specific roles in stress and hormone response. This research facilitates our understanding of SR gene family in rice and provides clues for further exploring of the function of *OsSR* genes.

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Abstract

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expression of some *OsSR* genes changed dramatically under ABA, GA, salt, drought, cold or heat treatment, which were related to the wide distribution of corresponding *cis*-elements in their promoter regions, suggesting their specific roles in stress and hormone response. This research facilitates our understanding of SR gene family in rice and provides clues for further exploring of the function of *OsSR* genes.

Keywords

Rice (*Oryza sativa* L.); *OsSR* genes; Alternative splicing; Expression profile; Stress

1. Introduction

Splicing of pre-mRNA is an important post-transcriptional regulatory mechanism in eukaryotes, a pre-mRNA can produce different transcripts by splicing at different splicing sites. It was reported that more than 40% genes in plants undergo AS (Chen et al., 2019a). The serine/arginine (SR) proteins are well-known splicing factors that play important roles in both the assembly of spliceosomes and the regulation of alternative splicing (AS) (Long & Caceres, 2009). After transcription, the splicing of pre-mRNA is crucial to the production of mature mRNA, which takes place in the spliceosome. The core of the spliceosome was composed of five small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6 snRNPs) and numerous non-snRNP ~~proteins~~. SR proteins were important non-snRNPs~~proteins~~ that regulate splicing of pre-mRNA (Long & Caceres, 2009; Wang & Brendel, 2004).

The serine/arginine (SR) proteins were characterized by the presence of RNA recognition motif (RRM) and the consecutive serine and arginine dipeptides repeats and RNA recognition motif (RRM) (Barta et al., 2010). The consecutive serine and arginine dipeptides repeat functioned as a protein-interaction domain and the RRM provided RNA-binding specificity for SR proteins. Besides, some SR proteins had specific domains which were less well understood, like Zn-knuckles and RGG box (Jin, 2022). The results of plant SR proteins sequence comparison and

phylogenetic analysis showed that plant SR proteins were classified into 7 different subfamilies, SR, SR45, RSZ, SC, SCL, RS2Z and RS, and the last three of these subfamilies were plant-specific which had unique domains, while the other four subfamilies had orthologs in animals (Reddy & Shad Ali, 2011; Richardson et al., 2011). SR proteins in SCL subfamily had an RRM with a charged extension at the N-terminus. The members of SCL subfamily included dicotyledons, monocotyledons, mosses and green algae. The RS2Z subfamily was found in dicotyledons and monocotyledons, and two Zn-knuckles domains and an extra SP-rich region were present on the proteins in this subfamily. The members of RS subfamily in plants contained two RRMs and the RS domains which were rich in RS dipeptides. The RS subfamily was mainly composed of photosynthetic eukaryotes (Xie et al., 2022).

The SR gene itself undergoes extensive AS. It was shown that 18 SR genes in *Arabidopsis thaliana* could produce more than 90 transcripts, and the precursor mRNA from rice SR gene also underwent extensive AS (Reddy & Shad Ali, 2011). Alternative splicing (AS) events that occurred at the NAGNAG acceptor were termed the AS-NAGNAG events, which would cause NAG insertion-deletions in transcripts (Iida et al., 2008). In the NAGNAG motif, the first AG is termed the E-acceptor and the second AG was termed I-acceptor (Hiller et al., 2004). AS-NAGNAG events were widespread in mammals and plants, which contributes to the diversity of transcriptome and proteome in different species. It has been reported that AS-NAGNAG acceptors were overrepresented in genes which coded RRM-containing proteins. Genes coding for RNA binding proteins were preferentially equipped with NAGNAG acceptors in human (Akerman & Mandel-Gutfreund, 2006; Iida et al., 2008). In *Arabidopsis thaliana*, NAGNAG acceptors were frequently found genome, particularly in the *AtSR* genes (Schindler et al., 2008; Shi et al., 2014).

Because of the indispensable role played by SR proteins in both constitutive splicing and alternative splicing of precursor mRNA, it is likely that they will be instrumental in modulating the expression of genes which are essential for plants in different developmental stages. Until now, some studies have revealed that members of the SR gene family played important roles in various biological processes in different species, such as the hormone signal transduction and response to

stress. In *Arabidopsis*, 19 *AtSR* genes have been identified (Barta et al., 2010). The function of *SR45* has been extensively studied in *Arabidopsis thaliana*. Studies have shown that *SR45* negatively regulated glucose-induced growth by inhibiting abscisic acid (ABA) accumulation and signaling, thereby inhibiting seedling establishment under adverse conditions (Ali et al., 2007; Carvalho et al., 2010). *AtSR45a* was detected to undergo AS and produced two alternative splicing variants, *AtSR45a-1a* and *AtSR45a-1b*. *AtSR45a* could regulated the response to salt stress in *Arabidopsis thaliana* by increasing the expression these two variants and interacting with the cap-binding complex (Li et al., 2021). *AtRS40*, *AtRS41* and *AtSCL30* participated in response to ABA and salt stress in *Arabidopsis* (Chen et al., 2013; Cruz et al., 2014). And *AtSR* genes including *AtRS40*, *AtSR34a*, *AtRSZ22* and *AtSR45a* etc. were reported to participate in response to heat stress, they underwent specific AS and produced specific mRNA variants under high temperature stress (Filichkin et al., 2010; Ling et al., 2021; Ling et al., 2018).

In rice, 24 SR genes have been identified (Barta et al., 2010). The *OsRSp29*, *OsRSZp23* and *OsSCL26* played roles in stimulating pre-mRNA splicing and promoting splicing efficiencies of downstream genes (Isshiki et al., 2006). The OsSR45 functioned in regulating the response to various stresses, including temperature stress and reactive oxygen species stress at the post transcriptional level by interacting with OsFKBP20-1b which belonged to immunophilin family in rice (Park et al., 2020). *OsSR40*, *OsSCL57* and *OsSCL25* played crucial roles in regulating mineral element absorption and homeostasis in rice by participating in alternative splicing of ~~related genes'~~ pre-mRNA (Dong et al., 2018).

Although SR genes have been identified in rice, there is still lacking ~~of~~ further study about their biological function. A comprehensive analysis of SR genes in rice was performed in the present study. The genetic relationship among *OsSR* genes was analyzed ~~firstly, and~~ then we analyzed structures of OsSR proteins, collinear relationship, as well as the promoter ~~region~~ sequence, NAGNAG acceptors, expression and alternative splicing patterns in both vegetative ~~organs~~ and reproductive organs of *OsSR* genes and their responses to hormones and abiotic stresses. This study ~~addressed~~ a better understanding and established the foundation for further functions

elucidation of *OsSR* genes.

2. Materials and methods

2.1 Identification and acquisition of information of *OsSR* genes

According to the accession provided in Jin's article (Jin, 2022), we extracted the sequences of *OsSR* genes and the corresponding protein sequence of each gene from Rice Genome Annotation Project Database (https://rapdb.dna.affrc.go.jp/) (Kawahara et al., 2013; Sakai et al., 2013).

2.2 Bioinformatics analysis of SR gene family genes

2.2.1 Phylogenetic analysis, conserved motifs, gene structure, tertiary structure prediction

Based on the results of the multiple amino acid sequence alignment done by ClustalW, MEGA 7.0 was used in this study to perform the phylogenetic analysis ~~of~~ with maximum-likelihood (ML) method and 1,000 bootstrap replicates (Kumar et al., 2016). Exon and intron positions in *OsSR* genes were mapped and gene structures were deduced using the Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015). Conserved motifs of OsSR proteins were analyzed using SMART (Simple Modular Architecture Research Tool) online tool (http://meme-suite.org/tools/meme) (Letunic & Bork, 2018), and then visualized using the TBtools (Chen et al., 2020a). The tertiary structures of OsSR proteins were predicted by SWISS-MODEL (https://swissmodel.expasy.org/) (Waterhouse et al., 2018).

2.2.2 Physicochemical properties and subcellular localizations

The physicochemical properties and subcellular localizations of OsSR proteins were predicted using ExPASy Protparam online tool (https://web.expasy.org/protparam/) and BUSCA (http://busca.biocomp.unibo.it) (Savojardo et al., 2018), respectively. The NetPhos3.1 service was used to predict the OsSR proteins' phosphorylation sites

(https://services.healthtech.dtu.dk/service.php?NetPhos-3.1) (Blom et al., 1999).

2.2.3 Syntenic relationships

From the Ensembl plants database the genomic information for rice, *Arabidopsis*, sorghum, maize, soybean, and wheat was retrieved (http://plants.ensembl.org/index.html). Furthermore, segment duplication events of *OsSR* genes and synteny relationship ~~of~~ between rice and other specie were analyzed by the MCScanX program, and the results were visualized using TBtools (Chen et al., 2020a; Wang et al., 2012).

2.2.4 Cis-acting elements

The key promoter regions (2000 bp sequences upstream of the translation start codons) of the *OsSR* genes were retrieved from RAP-DB (https://rapdb.dna.affrc.go.jp/) and submitted to the PlantCARE online software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to predict and analyze the regulatory *cis*-acting elements in promoter regions (Lescot et al., 2002).

2.3 Plant materials and growth conditions

Rice (*Oryza sativa* L. spp. Japonica, var Nipponbare) plants were used in this study. The seeds were sterilized with 20% NaClO solution, soaked with sterile water and incubated at 37℃ for germination. ~~Then~~ a portion of the~~m~~ were transferred to the field with normal water and fertilizer management to continue growing until maturity. Meanwhile, the other ~~part of seeds were~~ transplanted to the 96-well PCR plates with the bottom removed, and ~~then grew~~ in the Hoagland solution ($CaNO_3 \cdot 4H_2O$ 945 mg/L, KNO_3 506 mg/L, NH_4NO 380 mg/L, KH_2PO_4 136 mg/L, $MgSO_4 \cdot 7H_2O$ 493 mg/L, iron salt solution 2.5 mL/L (2.78 g $FeSO_4 \cdot 7H_2O$, 500 mL distilled water, 3.73 g EDTA-2Na pH 5.5), microelement 5mL/L (KI 0.83mg/L, H_3BO_3 6.2mg/L, $MnSO_4$ 22.3 mg/L, $ZnSO_4$ 8.6 mg/L, Na_2MoO_4 0.25mg/L, $CuSO_4$ 0.025mg/L, $CoCl_2$ 0.025mg/L), pH 6.0) in the greenhouse with a photoperiod of 14/10 h at 28 ℃/26 ℃ (day/night) and relative humidity of 65%. The Hoagland solution was renewed every 2 days.

For tissue-specific expression analysis, the different tissues of rice planted in the field were sampled from vegetative organs and spikelets at different filling stages. For the salt, drought and phytohormone treatment, the seedlings at 4-leaf stage were subjected to Hoagland solution with 200 mM NaCl, 20% PEG6000, 100 μM gibberellin (GA) (100 μM) or abscisic acid (ABA), respectively. For cold or heat stress, the seedlings at 4-leaf stage were moved to incubator with the temperature ~~keeping~~ at 4 ℃ and 37 ℃, respectively. And the 2rd and 3rd leaves from the seedlings were dissected as samples at different time points under various treatments.

2.4 RNA isolation, RT-PCR and *q*RT-PCR

Total RNA from different samples was extracted by Total RNA Extractor (Trizol) (Sangon Biotech) and reverse transcribed into cDNA using Hifair® Ⅲ Reverse Transcriptase (YEASEN) according to the instruction book. The cDNA was used for following PCR amplification.

Semi-quantitative RT-PCR was performed using PrimerSTAR MaxDNA Polymerase (TaKaRa, Japan). ~~Choosing~~ the appropriate annealing temperature for PCR amplification according to the property of primer pairs for different genes. The number of amplification reaction cycles in this study was 30. The PCR products were determined using electrophoresis on the 1% agarose gels. Specific primers of different *OsSR* genes and control gene *OsActin* used for RT-PCR ~~were~~ listed in Table S6.

For *q*RT-PCR experiment, SYBR Green qPCR Master Mix (TOROIVD) was used, and the experiment was conducted on LightCycler 480 II (Roche). The data was analyzed as previously described using *OsActin* as the internal standard (Livak & Schmittgen, 2001). The *q*RT-PCR experiment was carried out using 3 biological replicates, and 3 technical replicates were performed for each biological replicate. The *q*RT-PCR data was calculated using $2^{-\Delta\Delta CT}$ method and Student's t-test. The primer sequences for *q*RT-PCR were listed in Table S5.

3. Results

3.1 Identification of *OsSR* genes and their characteristics

Until now, SR proteins in SR gene family have been identified in rice (Jin, 2022). Here, we performed the prediction and analysis of the physicochemical properties of the OsSR proteins (Table 1). The results revealed that OsSR proteins ranged in length from 185 amino acids (OsRSZ21a and OsRSZ21) to 502 amino acids (OsSCL57), while the molecular weight varied from 21.02 kDa (OsRSZ21) to 56.83 kDa (OsSCL57). Notably, all of the rice SR proteins were alkaline proteins with the isoelectric point ranging from 8.67 (OsSR40) to 12.37 (OsSR45) (Table 1).

According to the BUSCA prediction analysis, except for OsSR45, which was predicted to localize in the chloroplast, the remaining 23 OsSR proteins were localized in nucleus. ~~By the experiments,~~ OsSR45 was observed to co-express and physically interact with OsFKBP20-1b in the nucleus and cytoplasm in vivo (Park et al., 2020). There was experiment proved that OsSCL30, which was the SR protein in rice, was visible only in the nucleus (Zhang et al., 2022). The results indicated that different OsSR proteins together with their interaction proteins could function in different intracellular partitions.

According to previous research, the mobility of SR proteins was regulated by phosphorylation in *Arabidopsis* (Tillemans et al., 2006). In OsSR proteins, we found a number of phosphorylation sites (Table 1), which varied considerably among the OsSR proteins, ranging from 25 (OsSC25) to 86 (OsSR45a). The results inferred that OsSR proteins could be regulated by phosphorylation as well. The difference in physicochemical properties is suggestive of functional differences among the OsSR proteins.

3.2 Phylogenetic, motif composition, structure of OsSR proteins and gene structure analysis of *OsSR* genes

To analyze evolutionary relationships among the SR families in rice, a maximum-likelihood

phylogenetic tree ~~was constructed~~ using amino acid sequences of 24 OsSR proteins (Fig. 1). A total of 24 *OsSR* genes could be classified into 7 distinct subgroups based on the evolutionary relationships, and the results were consistent with the reported 7 subfamilies: SCL, SC, SR45, RS2Z, RSZ, RS, and SR, indicating their conservation within the subfamilies during their evolution. These 7 subgroups contained 6, 3, 2, 4, 3, 2, and 4 members, respectively. Among them, SR, RSZ, and SC subfamily are common between plants and animal, the remaining subfamilies are plant-specific (Reddy & Shad Ali, 2011), indicating the SR proteins diverged along with the speciation.

Conserved domains usually have important functions and are closely related to the completion of physiological functions of proteins. Analysis of the conserved domain of OsSR proteins showed that the OsSR proteins within the same subfamily were highly conserved in the type and distribution of conserved domains. (Fig. 1b). The members of SCL and SC subfamily contained only one RRM domain near the N-terminal, while a consecutive serine and arginine dipeptides repeats (SR domain) located on the C-terminal of the proteins (Jin, 2022). Besides, the OsSR proteins in the SCL subfamily also had a domain which could be diverse at the N-terminal. As for two members of SR45 subfamily, both of them contained one RRM domain and SR domains which existed in both the N- and C-terminus of the proteins. Unlike OsSR proteins in other subfamily, two and one ZnF_C2HC motif were contained in members of RS2Z and RSZ subfamily besides RRM and SR domains, respectively. The members in RS subfamily contained two RRMs and the RS domain. Similarly, the four proteins of the SR subfamily also contained two RRM domains, but unlike the RS subfamily, the C-terminal of these proteins was SR domain.

Furthermore, ~~we performed~~ the prediction of the tertiary structure of these proteins using SWISS-MODEL online server ~~to further understand the properties of OsSR proteins. It showed~~ that OsSR proteins were mainly composed of α-helices, β-folds and random coils (Fig. S1 and Table S1). It was speculated that proteins with different tertiary structures may determine the diversity functions of *OsSR* genes. We noticed that OsSR protein structures showed differences among different subfamilies, especially among RS subfamily, SR subfamily and other subfamilies

(Fig. S1). While in most cases, the OsSR proteins in the same subfamily had similar tertiary structures (Fig. S1). For example, OsSCL30a, OsSCL30, OsSCL57, OsSCL28 and OsSCL25 had identical structures, indicating that they have similar function.

Gene structural variety might function as a form of evolution for numerous genes (Fedorov et al., 2002). And the conservation of gene structure is related to the number of introns in eukaryotes (Rogozin et al., 2003). ~~In order to further explore~~ the structural difference and conserved relationship among these *OsSR* genes, the genetic structure of 24 *OsSR* genes was analyzed using GSDS online tool. Notably, the *OsSR* genes differed in nucleotide sequence, but they contained the similar number of exons and introns in the same subfamily except for the genes in SCL subfamily. The number of introns ranged from 3 to 13 (Fig. 1c). The genes, containing the most introns and exons and fewest introns and exons, were *OsRSZ23* and *OsSR33a*, belonging to RSZ and the SR subfamily, respectively. The *OsSR* genes in SC subfamily usually contained 6 or 7 introns, while the members of SR45 subfamily contained 10 or 11 introns. All the members of RS2Z and RS subfamily contained 5 and 4 introns, respectively. The number of introns of *OsSR* genes in the RSZ subfamily is 4 or 3. The intron number in SR subfamily was up to 12 or 13.

The analysis of domain and protein structural characteristics laid the foundation for further understanding the function of OsSR. Meanwhile, *OsSR* genes with closer evolutionary relationship were similar in protein domain distribution, protein structure and gene structure, thus we speculated that *OsSR* genes belonging to the same subfamily would have similar function.

3.3 Segment duplication analysis of *OsSR* genes

Segmental duplication is considered as one of the main factors driving ~~expanding~~ of gene families during evolution in plants (Cannon et al., 2004). As shown in Fig. 2, 12 genes (6 pairs), including *OsRS2Z37* and *OsRS2Z39*, *OsSR40* and *OsSR33a*, *OsSR45* and *OsSR45a*, *OsRSZ21* and *OsRSZ21a*, *OsSR32* and *OsSR33*, *OsSCL57* and *OsSCL30*, were implicated in segmental duplication events. Totally, 50% members of *OsSR* genes showed collinear relationships, indicating that segmental duplication was primarily responsible for the expansions of the SR gene

family in rice. The ratio of Ka/Ks that exists between gene duplication pairs provides insight into whether or not there is selection pressure existing on protein-coding genes (Hurst, 2002). Additionally, we found that the *Ka/Ks* ratios of all *OsSR* gene duplication pairs were less than 1.0 (Table S2), suggesting that these six duplication pairs underwent purifying selection.

3.4 Synteny and orthologous gene pairs of SR genes

Synteny refers to the distribution or arrangement of homologous genes within one specie or among different species (McCouch, 2001). The syntenic relationship of the SR genes between rice and five plant species (*Sorghum bicolor*, *Arabidopsis thaliana*, *Zea mays*, *Triticum aestivum* and *Glycine max*) was examined in this study. There were no orthologous genes between rice and soybean (Fig. S2). And only four pairs of orthologous genes were identified between rice and *Arabidopsis* (*OsSR33a* and *AtSR34*, *OsSR33* and *AtSR34*, *OsRS2Z36* and *AtRS2Z33*, *OsRS2Z36* and *AtRS2Z32*) (Fig. 3). This might due to the distant evolutionary genetic relationship between dicotyledon and monocotyledon. In addition, a total of 20, 40, 64 orthologous SR gene pairs were identified between rice and sorghum, maize, wheat, respectively (Fig. 3 and Table S3). These orthologous SR genes in different species may have similar functions that involved in constitutive and alternative pre-mRNA splicing, and post-splicing activities.

3.5 NAGNAG acceptors in *OsSR* genes

NAGNAG splicing produces two distinct isoforms that are distinguished by three nucleotides (NAG, N = A, C, G, T). NAGNAG acceptors were termed based on the existence of a NAGNAG acceptor motif, and alternative splicing at NAGNAG acceptors was widespread in the genome of animals and plants (Akerman & Mandel-Gutfreund, 2006).

A scan of *OsSR* gene products from the information on the RAP-DB ~~was carried out~~ for signatures associated with NAGNAG acceptors. ~~It showed~~ that 14 out of 24 *OsSR* genes exhibited NAGNAG acceptors. Among these 14 genes, *OsSCL26*, *OsRS2Z36*, *OsRSZ21a* and *OsRSZ21* contained 2, 2, 3, 2 NAGNAG acceptors, respectively, and the other 10 genes contained only one

(Table 3). Furthermore, we focused on whether alternative splicing occurred on these NAGNAG acceptors. We found that only four *OsSR* genes, including *OsSCL26*, *OsSC25*, *OsSR45a* and *OsSR45*, had AS-NAGNAG event on the NAGNAG acceptor, which led to the deletion of a single amino acid at the protein level (Table S7).

3.6 The prediction of *cis*-acting elements on *OsSR* genes' promoters

Inducing or inhibiting the binding of transcription factors to the corresponding *c*is-acting sites in the gene promoter region to regulate the expression of downstream gene is an important mechanism to response to environmental changes (Riechmann et al., 2000). The identification of *cis*-elements might provide clues for determining gene expression patterns under different kinds of stresses. We performed the analysis on the promoter regions of *OsSR* genes. Then, 6 types *cis*-regulatory elements were identified, including promoter/enhancer elements and elements related to light responsiveness, stress, hormone response, development/tissue specificity or circadian control (Fig. 4 and Table S4). The proportion of *cis*-acting elements in each of these categories was 9.1%, 45.5%, 9.1%, 18.2%, 16.4%, and 1.8%, respectively.

The *cis*-acting elements in 'promoter/enhancer element' category, which ensure the correct location and start of transcription, were ubiquitously identified in all *OsSR* genes' promoters, including CAAT-box and TATA-box (Fig. 4). The ARE and MBS in 'stress' category, which are involved in anaerobic induction and drought responsiveness, respectively, were harbored in most of *OsSR* genes' promoters. In 'hormone response' category, the *cis*-acting elements respond to ABA, auxin, GA, MeJA and salicylic acid were identified. The ABA, salicylic acid and MeJA responsiveness elements, including ABA responsive element (ABRE), TCA-motif, TGACG-motif and CGTCA-motif, widely presented in *OsSR* genes' promoters. Notably, ABRE was the most widely distributed hormone-responsive element, which presented in almost all the promoters of the 24 *OsSR* genes. Moreover, among these hormone-responsive elements, GA responsive elements were the most abundant. We found that 3 out of 9 identified hormone-responsive elements were GA response elements (Fig. 4 and Table S4), including P-box, TATC-box and

GARE-motif. As for the 'development/tissue specificity' category, GCN4_motif, RY-element and AACA_motif were identified which were seed and endosperm development-related (Fig. 4 and Table S4). In addition, only *OsSCL30a* and *OsRS29* contained circadian control related elements in their promoters. Based on these findings, it indicates that *OsSR* genes may have roles in respond differently to different hormones and environmental stresses.

3.7 Expression patterns and AS of *OsSR* genes

The identification of *c*is-acting elements revealed that *OsSR* genes may play roles in growth and development, as well as response to abiotic and hormone in plants. We examined the expression profiles and AS patterns of *OsSR* genes in several tissues or under abiotic conditions (drought, salt, cold, and heat) and hormone treatments (GA and ABA) in this section.

3.7.1 Tissue expression profiles of the OsSR genes

To further characterize the potential biological function, *q*RT-PCR was used to conduct issue-specific expression analyses of *OsSR* genes. Totally, we detected all 24 *OsSR* genes in 8 tissues and organs including root, stem, leaf and spikelets before fertilization, at flowering and 5, 10 and 20 days after fertilization (Table S8). Our results showed that the expression of the *OsSR* genes was tissue-specific and development phase-dependent (Fig. 5 and Fig. S3). The genes in SCL subfamily mainly expressed in stems, leaves and young panicles, and showed a lower expression in grains after 5 days of pollination. Notably, the expression of *OsSC32* and *OsSC34* in SC subfamily showed leaf preferential expression, whereas the expression of *OSC25* was very low in the tested tissues. The *OsSR45a* and *OsSR45* in SR45 subfamily were specifically higher expressed in panicles at DBF, DF and 5 DAF. As for the genes in RS2Z subfamily, the expression of *OsRS2Z39* was almost failed to be detected in both vegetative and reproductive organs, indicating that this gene might be luxury gene. The rest 3 genes mainly expressed in stem and leaves, while *OsRS2Z36* was also highly detected in panicles at DF. The ubiquitous expression of three genes in the RSZ subfamily was observed in 8 tissues with relatively high levels, especially in stems, leaves

and grains after 10 days of fertilization. Furthermore, 2 RS subfamily genes highly expressed in panicles at different developmental stages. And high expression in leaves and panicles at DBF were observed for 4 genes in SR subfamily.

3.7.2 Alternative splicing of OsSR genes in different tissues

It has been reported that the pre-mRNA of the SR gene which encoded the splicing regulator in different species would undergo extensive AS themselves (Chen et al., 2019b; Isshiki et al., 2006). Although alternative splicing of *OsSR* genes has been reported, the alternative splicing pattern of SR gene and the expression pattern of corresponding transcripts in different tissues at different development stages are still poorly understood. We summarized the alternative splicing of all 24 *OsSR* genes according to the information on the RAP-DB (Table S7), and the schematic diagrams of alternatively spliced transcripts of the *OsSR* genes mentioned in the following experiment were drawn according to the sequence information provided by the database.

To analyze the expression patterns of different transcripts produced by the alternative splicing of *OsSR* genes, we performed the semi-quantitative RT-PCR using the primers which were specific to the target genes (Table 2 and Table S6). For 11 selected *OsSR* genes, RT-PCR analysis was conducted in roots, stems, leaves, panicles at different development stages. The results showed except *OsSCL25* and *OsRS2Z36*, the remaining 9 genes exhibited AS (Fig. 6).

The *OsSCL30a* belonging to SCL subfamily produced four transcripts, but the expression of the isoform 1 was dominant compared with other transcripts. The expression of isoform 1 could be observed in various tissues, while isoform 2, isoform 3 and isoform 4 accumulated only in the vegetative tissues including root, stem and leaf. (Fig. 6). *OsSC34* from SC subfamily produced three transcripts, among which the expression of the isoform 1 was much more abundant than the other transcripts, which were mainly accumulated in the leaf and stem (Fig. 6). As for three genes in RS2Z subfamily, *OsRS2Z36* produced only one transcript (Fig. 6). *OsRS2Z38* produced two transcripts and the isoform 2 was predominantly accumulated in all tissues. There were four different transcripts that produced by *OsRS2Z37*, the isoforms 1 and 2 were observed in all the

tissues while the isoform 3 and isoform 4 were detected in different tissues except the root. Moreover, compared to the other two isoforms, the isoform 1 and isoform 2 of *OsRS2Z37* were more abundant in all tissues. The AS pattern of *OsRSZ21* belonging to RSZ subfamily in different tissues was analyzed (Fig. 6). Two transcripts of *OsRSZ21* were observed, and the isoform 1 expressed more abundant. The AS expression pattern of two SR genes belonging to RS subfamily in rice was detected in various tissues (Fig. 6). The isoform 1 and isoform 2 generated by *OsRS29* were more abundant in all tested tissues. *OsRS33* generated four transcripts which could be detected in different tissues except the root, and the isoform 1 expressed more abundant than the others. In the SR subfamily, *OsSR32* mainly produced isoform 1 and 2 in various tissues (Fig. 6). It was observed that the two variants generated by *OsSR33a* had equivalent expression levels in the stem, leaf and spikelet at 5 days after flowering, while isoform 1 was much more abundant in other tissues.

Taken together, the alternative splicing patterns of *OsSR* genes were tissue-specific, which means the expression levels of different transcripts produced by the same *OsSR* genes varied greatly in different tissues, but most *OsSR* genes mainly express one transcript in each tissue.

3.7.3 Expression of OsSR genes in response to abiotic stresses

The analysis of *cis*-acting elements suggesting that *OsSR* genes may have roles in response to abiotic stress in rice (Fig. 4). Here, the response of 24 *OsSR* genes to different environmental stress were examined (Table S9-S13).

OsSR genes exhibited different expression patterns in response to the salt stress. *OsSCL28*, *OsSC32*, *OsSR45a*, *OsRS2Z37* and *OsRSZ21* displayed similar response patterns to salt stress (Fig. 7a). After being exposed to salt stress for 1 h to 2 days, the expression of these five genes was significantly down-regulated compared to the control the mock treatment. The expression levels of *OsRS2Z38*, *OsRS2Z21a*, *OsRSZ23*, *OsRS29*, *OsRS33* and *OsSR32* were significantly induced by salt treatment, having a stably elevated expression level after 1 h treatment. The response of *OsSCL26* to salt stress appeared after 9 hours of treatment, the expression of *OsSCL26* was down-

regulated dramatically. The significant and steady induction or inhibition of expression levels were not observed in other *OsSR* genes, which had the similar expression patterns to the mock treatment (Fig. S4).

Under drought stress (Fig. 7b), there was the evident increase in the expression level of *OsSCL28*, *OsSCL26*, *OsSR45a*, and *OsRSZ21a* after treatment for 1h to 2 days. Different *OsSR* genes were responsive to drought with various degrees. The expression levels of *OsSC25*, *OsSR45*, *OsRSZ21* and *OsRS33* were considerably up-regulated from 2 h, 9 h, 9 h and 4 h after treatment, respectively, while *OsSCL30a* was up-regulated within 4 h of treatment and the suppressed expression of *OsSCL30a* was observed after drought treatment for 9 h. Other *OsSR* genes exhibited no obvious patterns in response to drought stress (Fig. S5).

The expression of *OsSC25*, *OsSR45a*, *OsRSZ21a* and *OsSR33* were induced by the cold treatment (Fig. 8a). The induced expression of *OsSC25* peaked at 6 h after treatment. Moreover, the induction of *OsSR45a* was strong, the expression level of *OsSR45a* increased by more than 10 times compared to the control within 1 hour to 9 hours after treatment. Under cold stress (Fig. 8a), the expression levels of *OsSCL30*, *OsSCL28*, *OsSCL26* and *OsRSZ23* were remarkably decreased. The *OsRSZ23* showed an exaggerated response to the cold treatment, its expression was almost completely suppressed under low temperature. Furthermore, the expression levels of other *OsSR* genes fluctuated, but the changes were slight between treatment and control (Fig. S6).

OsSR genes were responsive to high temperature with various patterns and degrees (Fig. 8b and Fig. S7). Heat treatment induced the significant down-regulation of *OsSCL30a*, *OsSCL30*, *OsSCL26*, *OsRS2Z37*, *OsRSZ23* and *OsRS33* (Fig. 8b). Notably, the expression of *OsSCL26* gene was almost completely suppressed under heat stress. The results showed that heat treatment significantly upregulated the expression of *OsSCL25* and *OsSC32* for 1 h to 2 days (Fig. 8b). After exposure to heat stress within 6 h, the expression of *OsSR45a* was remarkably induced, and it was observed to be down-regulated after 9 h of treatment. The expression of *OsRS29* was up-regulated within 1 day of heat treatment and began to decrease after 1 day. The response of *OsRSZ21* and *OsSR33* to heat stress appeared after 9 hours of treatment, showing a significant down-regulation

(Fig. 8b).

In summary, the results above indicated that response patterns to abiotic stresses of *OsSR* genes were time-dependent and varied among different genes.

3.7.4 Expression of OsSR genes in response to hormones

According to the above analysis, the promoter regions of *OsSR* genes contained abundant *cis*-elements that related to hormones (Fig. 4), especially ABA-responsive and GA-responsive elements. Thus, we focused on two hormones, ABA and GA, which are essential for plant growth and development. The expression patterns of *OsSR* genes under different phytohormone treatments were investigated by *q*RT-PCR (Fig. 9, Fig. S8, Fig. S9, Table S9 and Table S14-S15). *OsSCL25*, *OsRSZ21* and *OsSR33a* were significantly induced by GA. *OsSCL28*, *OsSR45a*, *OsSR32* and *OsSR33* were induced by GA after treatment for 1 h to 12 h (Fig. 9a), the induced peak values appeared at about 9 h. After being treated with GA for approximately 5 h, there was a considerable decrease in the expression level of *OsRS2Z38*, then the level gradually increased from 24 h time point and recovered to the similar level compared to the control at 48 h time point (Fig. 9a).

Only a few *OsSR* genes showed obvious response patterns under exogenous ABA treatment. As compared with the expression under mock treatment, the ABA treatment resulted in a significant increase in that of *OsSCL25* and *OsSR45a*, while the change in expression of *OsRS2Z36* was found to be inverse, which was shown to be suppressed by ABA treatment (Fig. 9b).

4. Discussion

SR proteins which working as the splicing regulator play indispensable role in splicing ~~of~~ especially in AS on pre-mRNA. The SR gene family has been identified in many plant species, such as *Arabidopsis*, rice, wheat, maize, cotton and longan (Chen et al., 2019b; Chen et al., 2020b; Jin, 2022; Wei et al., 2022). In this study, we focused on the 24 SR genes in rice, including their classification, gene and protein structure, chromosomal location, evolution, *cis*-elements, expression profiles, and response to abiotic stress and hormone.

4.1 Domains and physicochemical properties of OsSR proteins

The SR proteins were evolutionarily conserved at the structural level. Previous studies have shown that these conserved domains are essential for the protein function properly in plants. In *Arabidopsis*, N- or C-RS domains were necessary for the accurate nuclear localization for atSR30 and atSR45a (Mori et al., 2012). It has been reported that RSZp22, which was the member of the RSZ subfamily in *Arabidopsis*, displayed speckle-like distribution and localization in nuclear, and the realization of this accurate localization was inseparable from the presence of RRM and zinc-knuckle in the protein sequence of RSZp22 (Rausin et al., 2010). We found that the OsSR proteins in the same subfamily showed similar motif arrangements and considerable variation among different subfamilies (Fig. 1b). All OsSR proteins were found to have the RRM domain, and the Zn_C_2HC domain was contained in the OsSR proteins belonging to RSZ and RS2Z subfamilies (Fig. 1). In addition, the distribution of domains in OsSR proteins were significantly different among different subfamily, which may be related to the functional differentiation of OsSR proteins. However, there are few studies on the effect of conservative domains on the properly function of OsSR proteins. Further research could be conducted by targeted editing of regions encoding RRM, SR or Zn_C2HC domain *OsSR* genes with gene editing technology and analysis the corresponding transgenic materials.

The SR proteins are essential nuclear localized proteins that function as splicing factors in splicing of precursor mRNA (Misteli et al., 1997). In the current study, subcellular localization of most of OsSR proteins was in nucleus (Table 1), indicating the OsSR proteins could function as splicing factors as those in other species. Intriguingly, SR proteins were also involved in post-splicing activities, which were achieved through continuous shuttle between the nucleus and the cytoplasm (Huang & Steitz, 2001; Michlewski et al., 2008; Swartz et al., 2007). The state of phosphorylation and dephosphorylation was the key factor affecting the dynamic subcellular localization of SR proteins (Jin, 2022; Mori et al., 2012). For example, studies have proved that RSZp22 in *Arabidopsis thaliana* was a nucleocyto-plasmic shuttling protein, and the nucleocytoplasmic shuttling properties of RSZp22 have been analyzed in detail (Rausin et al.,

2010; Tillemans et al., 2006). Nevertheless, there is a lack of research on the determinants of SR protein dynamic regulation especially in plants. The results of this study showed that OsSR proteins contained different amounts of phosphorylation sites (Table 1). Further research is needed on dynamic distribution of OsSR proteins and how this process affects post-splicing activities including mRNA export.

4.2 The orthologous SR gene pairs between rice and other species provide insights into the evolution and function of the *OsSR* genes

Collinearity analysis in this study revealed the distribution of orthologous genes of SR genes between rice and other species, which helped us further understand the origin of the *OsSR* genes. Compared to a larger number of orthologous SR gene pairs identified between rice and other monocotyledonous plants, and only 4 orthologs of *OsSR* genes were found in soybean and *Arabidopsis*, respectively (Fig. S1, Fig. 3 and Table S3), suggesting that the development of orthologous SR pairs was more probable to occur after the divergence of dicots and monocots. Evidently, multiple *TaSR* genes were identified as orthologs of one single *OsSR* gene. For instance, *TaSR4D*, *TaSR7A*, *TaSR6B*, *TaSR7D* and *TaSR14D* were the orthologs of *OsSR33* (Table S3), indicating the expansion of *OsSR* genes may occur before that of wheat.

The orthologous genes in different species may originated from a common ancestor, the sequencings of orthologous genes are conserved, indicating the conservation of function of these genes (Tang et al., 2008). Understanding the function of these orthologous genes is helpful to reveal and explore the function of *OsSR* genes. Among the orthologous SR genes identified in other species in this study, there have been some reports on their gene functions. *AtSR34* in *Arabidopsis*, the orthologous gene of rice *OsSR33a* and *OsSR33* has been reported to be related to heat stress response (Ling et al., 2018). ~~So far, there are few researches about the function of the SR gene in rice and other species such as *Arabidopsis*, wheat and maize, and which is worthy of further study.~~

4.3 AS-NAGNAG events were not frequent on *OsSR* genes

~~As splicing factors, SR proteins could participate in the assembly and function of spliceosome (Graveley, 2000). Due to the fact that SR genes would undergo alternative splicing, one SR gene can generate transcripts that encode different proteins, which give the spliceosome greater spatial flexibility, and influence the outcome of splicing. Protein diversity induced by the AS-NAGNAG contributes to this flexibility to some extent. NAGNAG acceptor motifs were frequent in human genes and SR genes in *Arabidopsis* (Hiller et al., 2004; Schindler et al., 2008). Here, we screened for NAGNAG acceptor tandems in *OsSR* genes. A total of 19 NAGNAG acceptors were identified in 14 *OsSR* genes, belonging to 7 subfamilies (Table 3).~~ ~~However~~, AS-NAGNAG events were only observed at the location of the 3 acceptors. Notably, the different tissues and the change of environmental conditions could affect the alternative splicing rate occurring at the NAGNAG acceptor in *Arabidopsis*, which suggested that the differential splicing of NAGNAG acceptor in *Arabidopsis* may mediated by the organ and condition specific differences of composed of the spliceosome (Schindler et al., 2008). However, the factors affecting NAGNAG alternative splicing in *OsSR* genes are remaining to be evaluated.

4.4 The AS pattern of *OsSR* genes varied with different tissues

The SR genes themselves undergo extensive alternative splicing (Reddy & Shad Ali, 2011). This study investigated the alternative splicing pattern as well as the expression of different transcripts produced by the *OsSR* genes in both vegetative and reproductive tissues (Fig. 6). Previous study performed the investigation of all splicing variants of SR genes in *Arabidopsis*. Notably, the majority of these alternative splicing occurred within the coding region of SR genes, and the AS type on SR genes in *Arabidopsis* was mainly intron retention (Palusa et al., 2007). Interestingly, we found in most cases, AS events of *OsSR* genes occurred in the 3' or 5' untranslated regions, which would not cause the corresponding genes to generate new protein coding sequences (Fig. 6 and Table S7). We speculated that such splicing may have an impact on the expression and stability of precursor mRNA (Jin, 2022). Nevertheless, Some *OsSR* genes such as *OsSCL30a*,

OsRS2Z37, *OsRS2Z38*, etc., could undergo alternative splicing in the coding region and generated transcripts with different CDS, which means they could encode different proteins (Fig. 6 and Table S7). Moreover, these different transcripts produced by the same *OsSR* gene had tissue expression specificity, and some transcripts could only be detected in specific tissues. For example, isoform 3 and isoform 4 produced by *OsRS29* was only detected in stem, leaf and spikelets at 10 days after flowering (Fig. 6), indicating that the proteins encoded by these transcripts only expressed in specific tissues. Studies have shown that different transcripts of one gene produced by alternative splicing may perform distinct functions. The SR gene *SR45* in *Arabidopsis* could produce two transcripts, and *SR45.1* played a role in flower development, while *SR45.2* was involved in regulating root growth and development (Zhang & Mount, 2009). Whether there are functional differences between different transcripts produced by the same *OsSR* gene in rice needs further exploration and research.

4.5 *OsSR* genes may function on plant growth, response to hormones and abiotic stresses

In the current study, we have found most of *OsSR* genes extensively expressed with high level in the stem, leaf or spikelet. The expression patterns of different *OsSR* genes were tissue and development stage dependent, indicating their specific functions. Based on the detection results of gene tissue-specific expression (Fig. 5), we speculated that SCL, SC, and RS2Z subfamily genes may be involved in regulating the development of vegetative organs in rice, while the *OsSR* genes in SR45, RSZ, and RS subfamily were more likely to participate in regulating the formation and filling of grains in rice.

The growth of plants could be profoundly influenced by a variety of environmental conditions. The transcription levels of related genes could be induced, repressed or regulated by various stress (Palusa et al., 2007). However, the expression profiles of *OsSR* genes under various stresses have not been detected till now. The promoter analysis in this study suggested that *OsSR* genes played important roles in various stress responses in rice. In the promoter region of *OsSR* genes, different types of *c*is-acting elements were discovered, including 10 hormone-responsive and 5 stress-

responsive elements (Fig. 4 and Table S4), and our results showed the expression of some of *OsSR* genes were changed after abiotic or hormone treatment (Fig. 7, Fig. 8 and Fig. 9), indicating that they modulated the response to stresses in rice. These results lay a foundation for further understanding the function of *OsSR* genes. For example, our experiment showed that under salt treatment, the expression of *OsRS33* was significantly upregulated (Fig. 7), which was consistent with the previous study that *OsRS33* gene knockout lines were more sensitive to salt stress compared with the wild type (Butt et al., 2022). We found *OsSCL30* was obviously and continuously suppressed by the cold treatment, and in fact, *OsSCL30* was related to cold tolerance in rice, overexpression of *OsSCL30* reduced the tolerance of rice seedlings to low temperature (Zhang et al., 2022).

Besides, we observed that some *OsSR* genes respond to a variety of stresses simultaneously. *OsSCL30*, *OsSCL26* and *OsRSZ23* responded to both cold and heat stress, suggesting that the expression of these genes was affected by ambient temperature (Fig. 8). Moreover, the expression of *OsSC25* and *OsRSZ21a* genes were affected by both drought and cold stress, while the expression of *OsSC32* and *OsRSZ23* were affected by both salt and temperature stress (Fig. 7 and Fig. 8). However, no *OsSR* gene was found to respond to GA and ABA simultaneously (Fig. 9). In *Arabidopsis*, *SR45a* responded to ABA and abiotic stresses (Cruz et al., 2014; Ling et al., 2021). Consistently, we found that *OsSR45a*, a member in SR45 subfamily, could also respond to multiple stresses simultaneously. The results showed that ABA, GA, salt, drought and temperature stress significantly affected the expression level of *OsSR45a* (Fig. 7, Fig. 8 and Fig. 9), indicating that expression pattern of *OsSR45a* were stress-dependent. Altogether, these results strongly suggest that *OsSR* genes are critical in response to environmental signals in rice, and the function and mechanism of *OsSR* genes could be further studied based on the results in this study.

5. Conclusions

In this study, the comprehensive analysis on *OsSR* genes gave some insights on their characteristic and function. It showed that 24 *OsSR* genes were distributed in 7 different

subfamilies based on the phylogenetic analysis. Gene structures of *OsSR* genes, distribution of domains and protein structure of OsSR were conserved within each subfamily. There were 6 segmental duplicated *OsSR* gene pairs (50%) in rice genome, indicating segmental duplication played an overwhelming role in the expansion of SR gene family in rice. Most of *OsSR* genes would undergo AS and the AS patterns varied with different tissues. The majority of *OsSR* genes ~~would~~ express in different tissues, while their expression level varied substantially among different organs, suggesting their extensive functions in vegetative growth or spikelet development in rice. Furthermore, the different expression patterns of *OsSR* genes displayed between abiotic stress or hormone treatment and mock treatment indicating that *OsSR* genes may participate in rice hormone/abiotic stress signaling pathway. The current results will be helpful for better understanding and further study of *OsSR* genes.

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Figure 1

Schematic representation of the phylogenetic relationship, gene structures and conserved motifs in *OsSR* genes.

(**a**). Maximum-likelihood (ML) phylogenetic tree of OsSR proteins. (**b**). Distribution of conserved motifs in OsSR proteins. (**c**). Exon/introns and untranslated regions (UTRs) of *OsSR* genes. Green boxes denote UTR (untranslated region); yellow boxes denote exon; black lines denote introns. The length of gene can be estimated using the scale at the bottom.

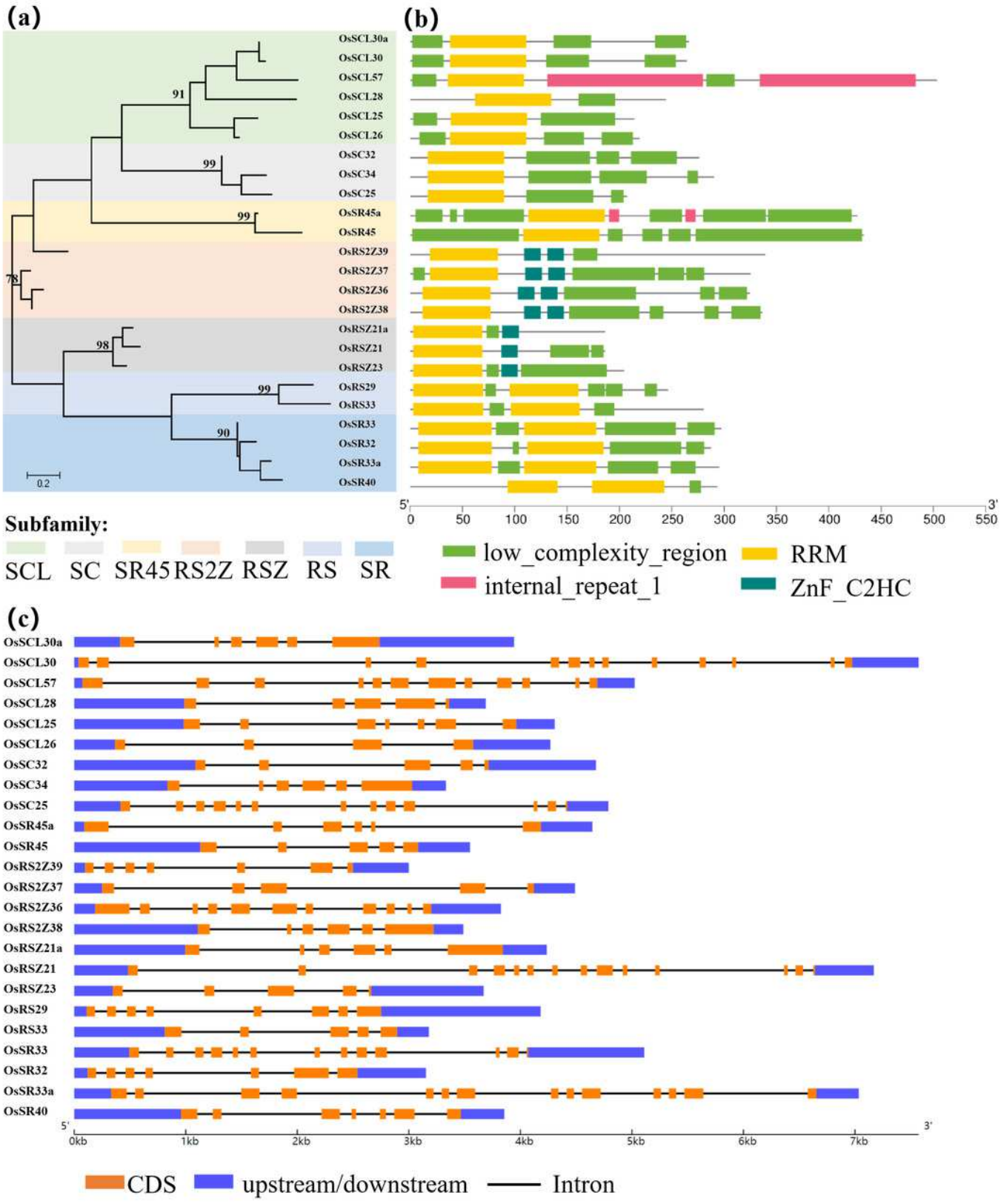
(a)
OsSCL30a
OsSCL30
OsSCL57
OsSCL28
OsSCL25
OsSCL26
OsSC32
OsSC34
OsSC25
OsSR45a
OsSR45
OsRS2Z39
OsRS2Z37
OsRS2Z36
OsRS2Z38
OsRSZ21a
OsRSZ21
OsRSZ23
OsRS29
OsRS33
OsSR33
OsSR32
OsSR33a
OsSR40
91
99
99
78
98
99
90
0.2
(b)
5'
3'
0
50
100
150
200
250
300
350
400
450
500
550
Subfamily:
SCL
SC
SR45
RS2Z
RSZ
RS
SR
low_complexity_region
RRM
internal_repeat_1
ZnF_C2HC
(c)
OsSCL30a
OsSCL30
OsSCL57
OsSCL28
OsSCL25
OsSCL26
OsSC32
OsSC34
OsSC25
OsSR45a
OsSR45
OsRS2Z39
OsRS2Z37
OsRS2Z36
OsRS2Z38
OsRSZ21a
OsRSZ21
OsRSZ23
OsRS29
OsRS33
OsSR33
OsSR32
OsSR33a
OsSR40
5'
3'
0kb
1kb
2kb
3kb
4kb
5kb
6kb
7kb
CDS
upstream/downstream
Intron

Figure 2

Schematic representations for the chromosomal locations and segment duplications of *OsSR* genes.

A total of 24 *OsSR* genes were mapped onto the chromosomes on the basis of their physical location. 1-12 were the chromosome numbers (Chr1- Chr24). The gray lines indicated duplicated blocks. The duplicated *OsSR* gene pairs were highlighted in green lines.

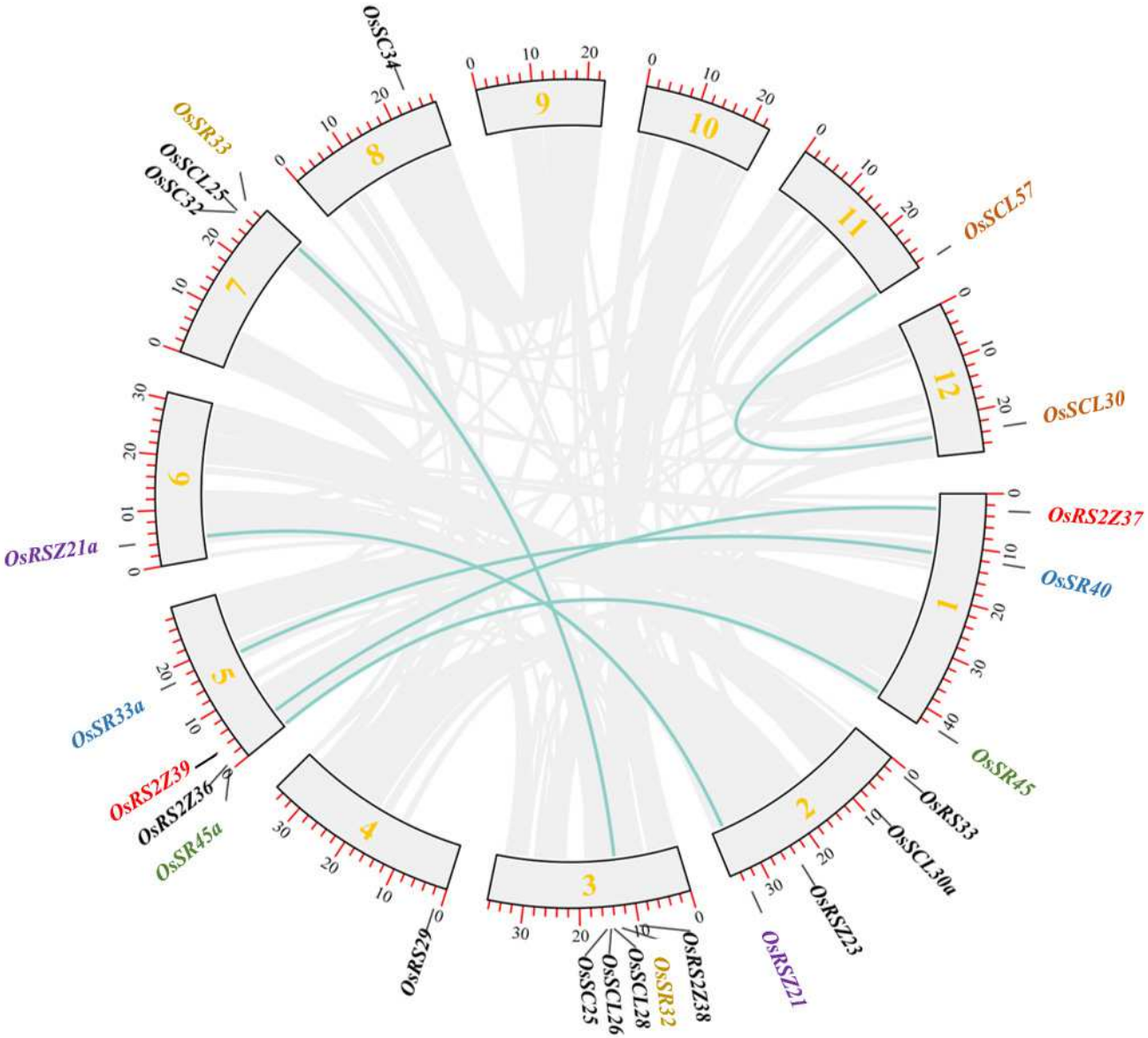

Figure 3

Synteny analyses of SR genes between rice and four plant species (*Arabidopsis thaliana*, *Sorghum bicolor*, *Zea mays*, and *Triticum aestivum*).

The gray lines indicated collinear blocks and syntenic SR gene pairs would be highlighted in blue lines.

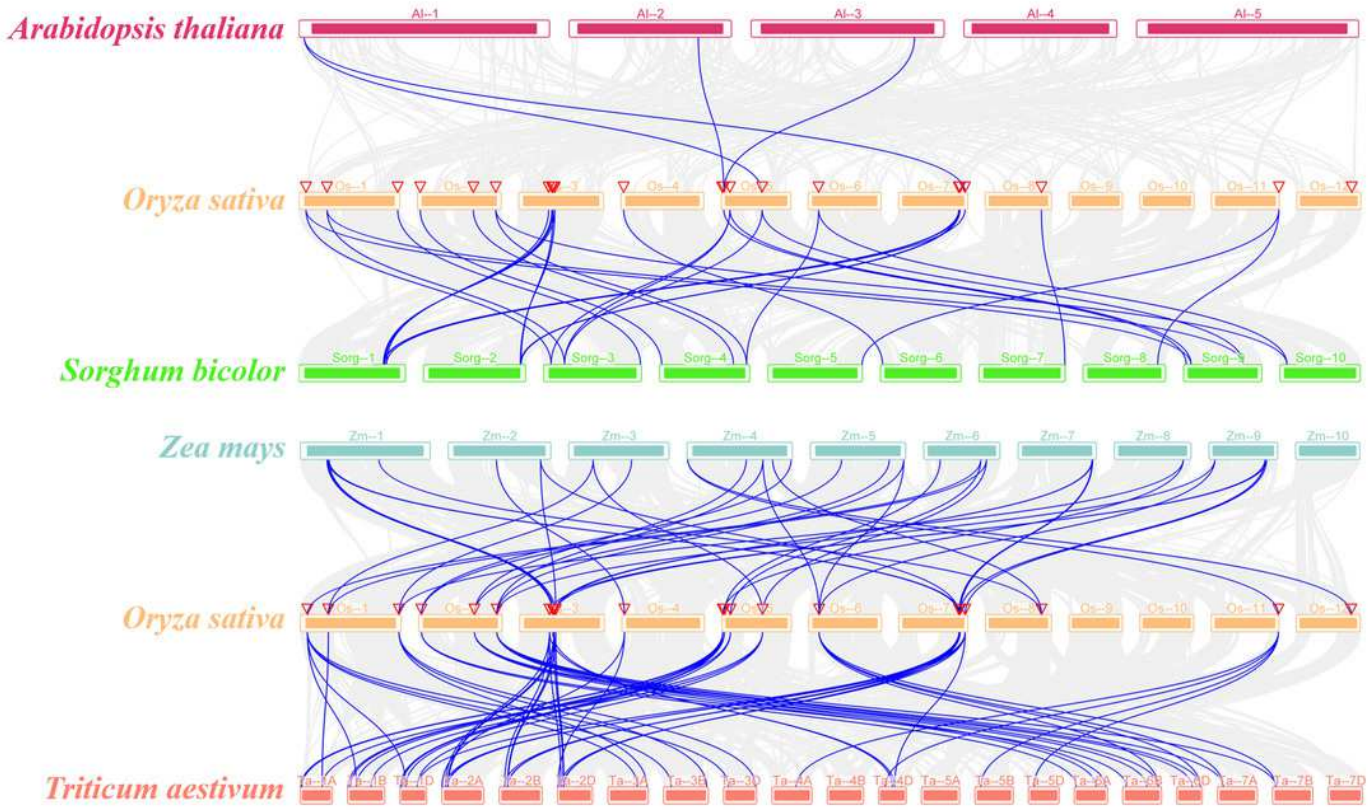

Figure 4

Cis-acting elements in promoter regions of *OsSR* genes.

Cis-acting elements were predicted based on 2 kb sequences upstream of coding sequences. The quantity of *cis*-acting elements was normalized by log 10 (number + 1) and then used for heatmap construction.

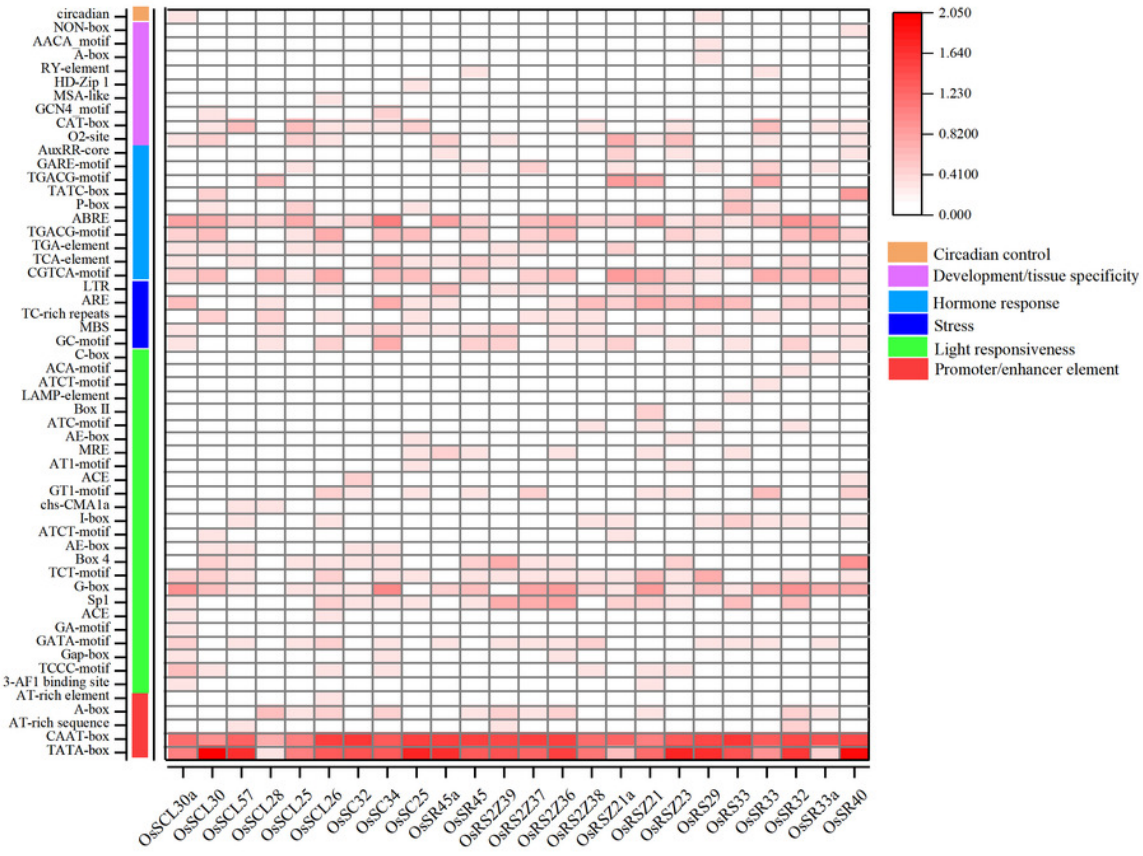

Figure 5

Expression profiling of the *OsSR* genes in 8 tissues based on *q*RT-PCR

DBF, Day before fertilization; DF, Day of flowering; DAF, Day after fertilization. *OsActin* was used as control, and each set of data contained three replicates. The comparative ΔCT values of *OsSR* genes were transformed by log2 to build the heatmap.

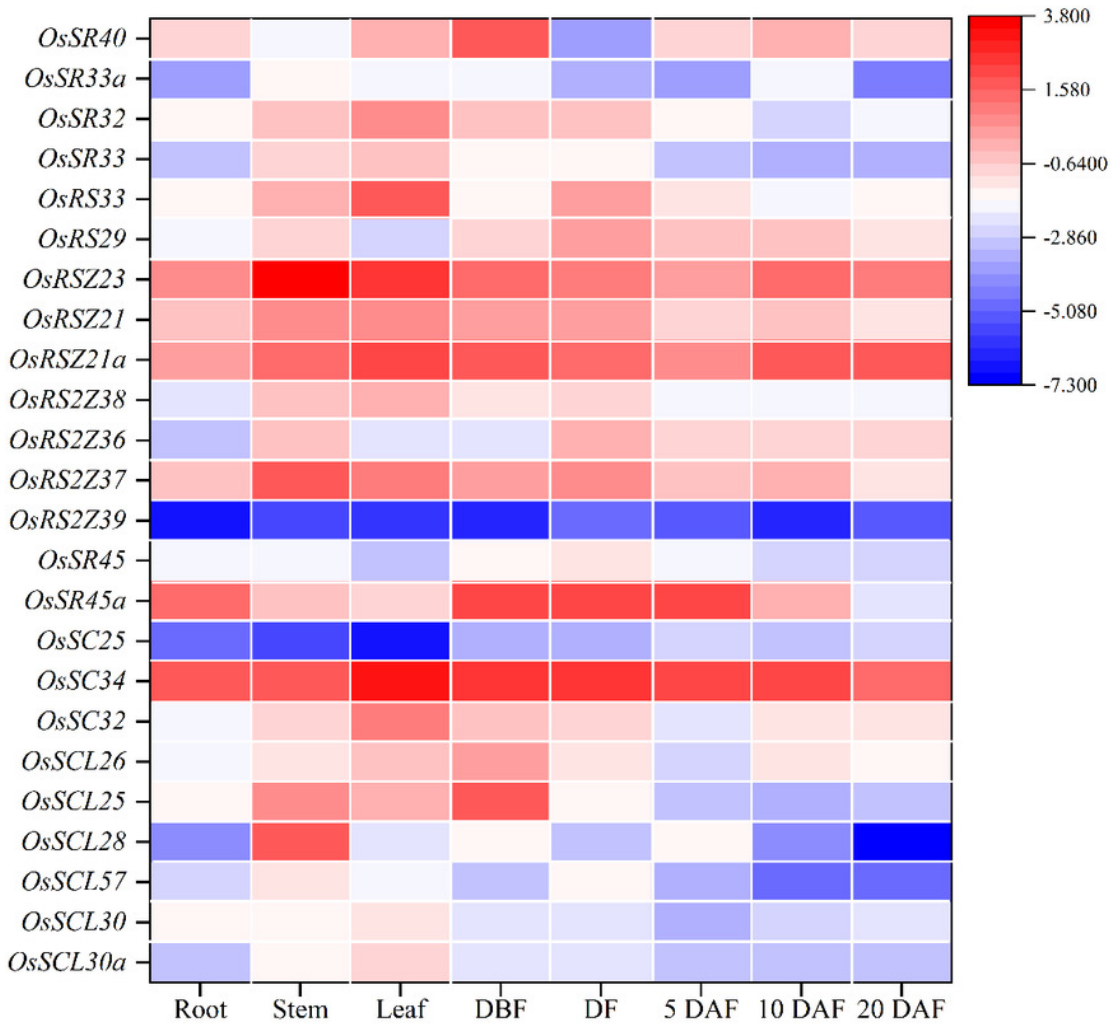

Figure 6

Expression and AS patterns of *OsSR* genes.

DBF, Day before fertilization; DF, Day of flowering; DAF, Day after fertilization. The numbers after the black arrows indicate the size of the amplification products. The diagrams on the right are schematic diagrams of alternatively spliced transcripts, red arrows indicate primers, the numbers on the right indicate the expected size of products.

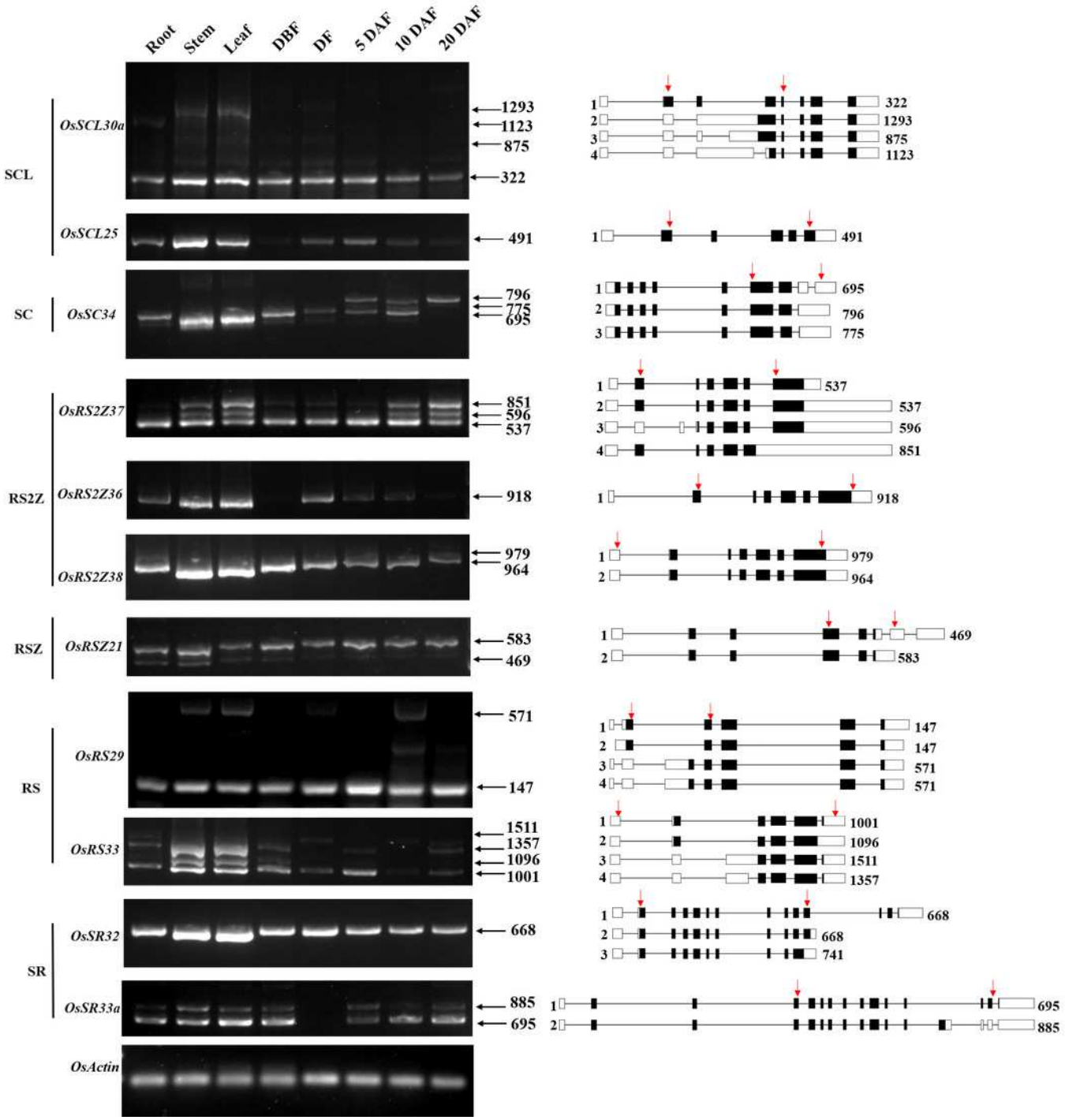
Root
Stem
Leaf
DBF
DF
5 DAF
10 DAF
20 DAF
SCL
OsSCL30a
1293
1123
875
322
OsSCL25
491
SC
OsSC34
796
775
695
RS2Z
OsRS2Z37
851
596
537
OsRS2Z36
918
OsRS2Z38
979
964
RSZ
OsRSZ21
583
469
RS
OsRS29
571
147
OsRS33
1511
1357
1096
1001
SR
OsSR32
668
OsSR33a
885
695
OsActin
322
1293
875
1123
491
695
796
775
537
537
596
851
918
979
964
469
583
147
147
571
571
1001
1096
1511
1357
668
668
741
695
885

Figure 7

Expression of *OsSR* genes in response to salt (a) and drought (b) stress.

OsActin was used as control. Error bars represent mean ± SE of three biological replicates. *P < 0.05 and **P < 0.01 indicate significant differences compared with CK determined by Student's t-test.

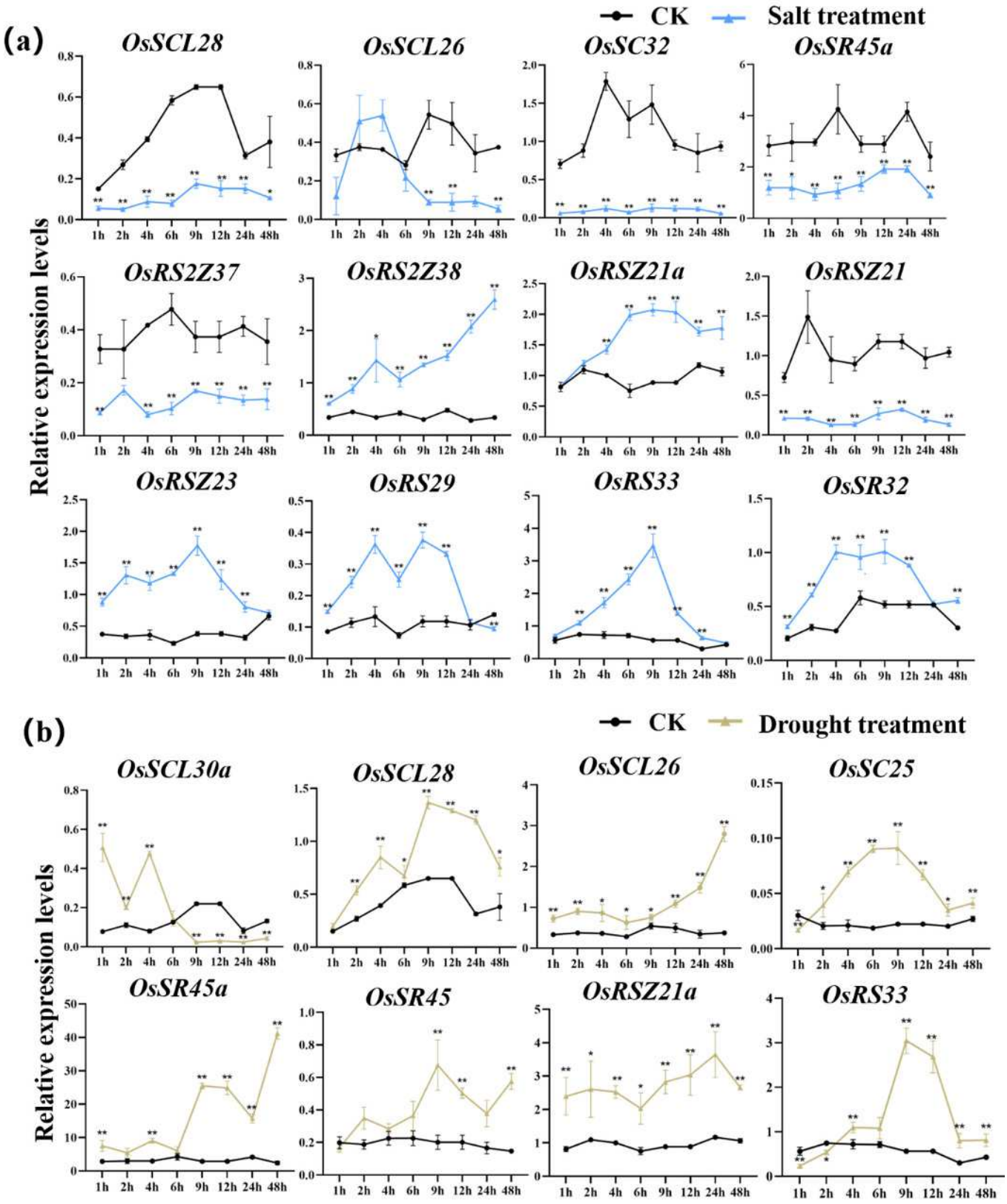
(a)
CK
Salt treatment
OsSCL28
OsSCL26
OsSC32
OsSR45a
OsRS2Z37
OsRS2Z38
OsRSZ21a
OsRSZ21
OsRSZ23
OsRS29
OsRS33
OsSR32
Relative expression levels
1h 2h 4h 6h 9h 12h 24h 48h
(b)
CK
Drought treatment
OsSCL30a
OsSCL28
OsSCL26
OsSC25
OsSR45a
OsSR45
OsRSZ21a
OsRS33
Relative expression levels
1h 2h 4h 6h 9h 12h 24h 48h

Figure 8

Expression of *OsSR* genes in response to cold (a) and hot (b) stress.

OsActin was used as control. Error bars represent mean ± SE of three biological replicates., *P < 0.05 and **P < 0.01 indicate significant differences compared with CK determined by Student's t-test.

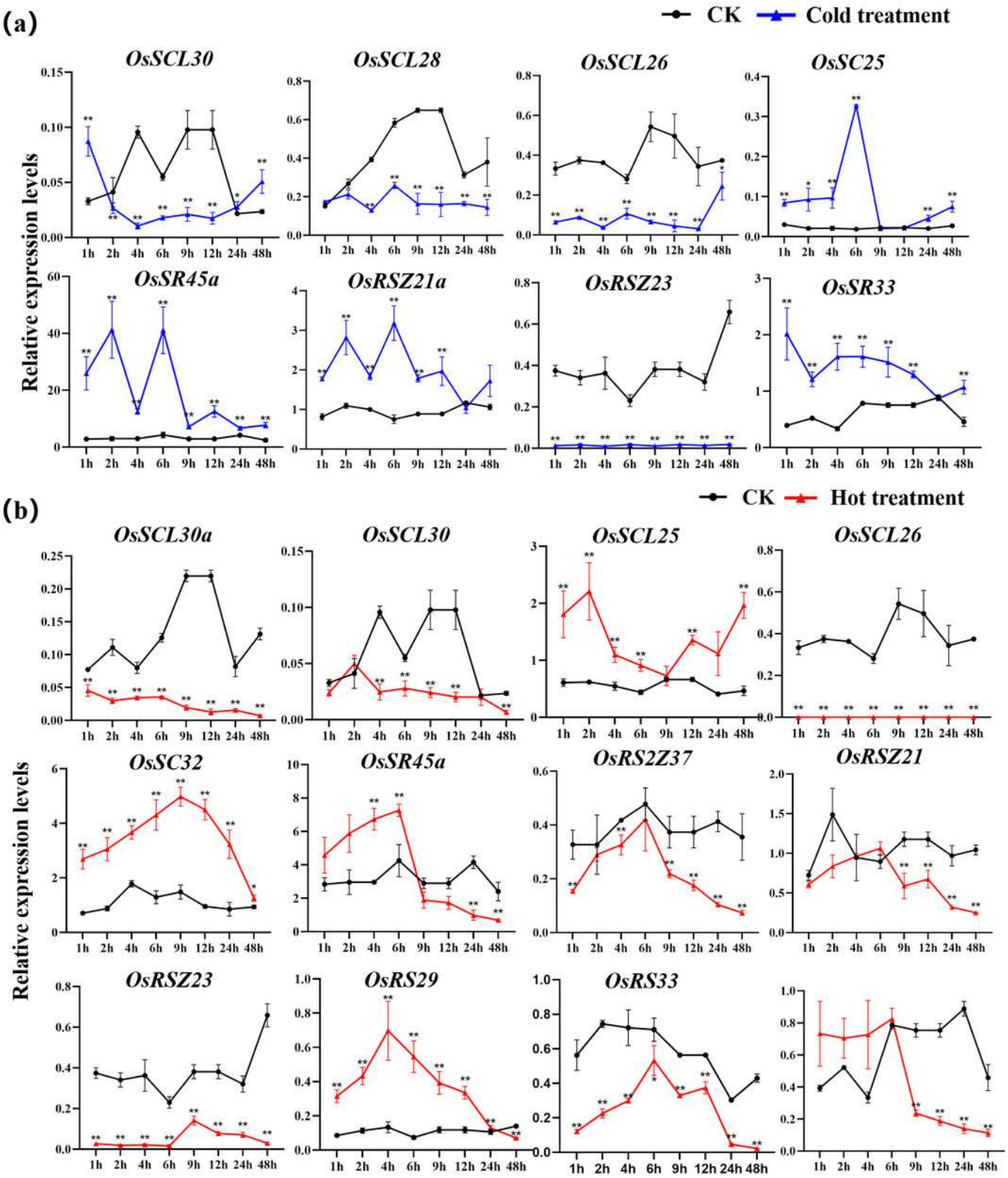
(a)
CK
Cold treatment
OsSCL30
OsSCL28
OsSCL26
OsSC25
OsSR45a
OsRSZ21a
OsRSZ23
OsSR33
Relative expression levels
(b)
CK
Hot treatment
OsSCL30a
OsSCL30
OsSCL25
OsSCL26
OsSC32
OsSR45a
OsRS2Z37
OsRSZ21
OsRSZ23
OsRS29
OsRS33
Relative expression levels
1h 2h 4h 6h 9h 12h 24h 48h

Figure 9

Expression of *OsSR* genes in response to hormones.

OsActin was used as control. Error bars represent mean ± SE of three biological replicates. *P < 0.05 and **P < 0.01 indicate significant differences compared with CK determined by Student's t-test.

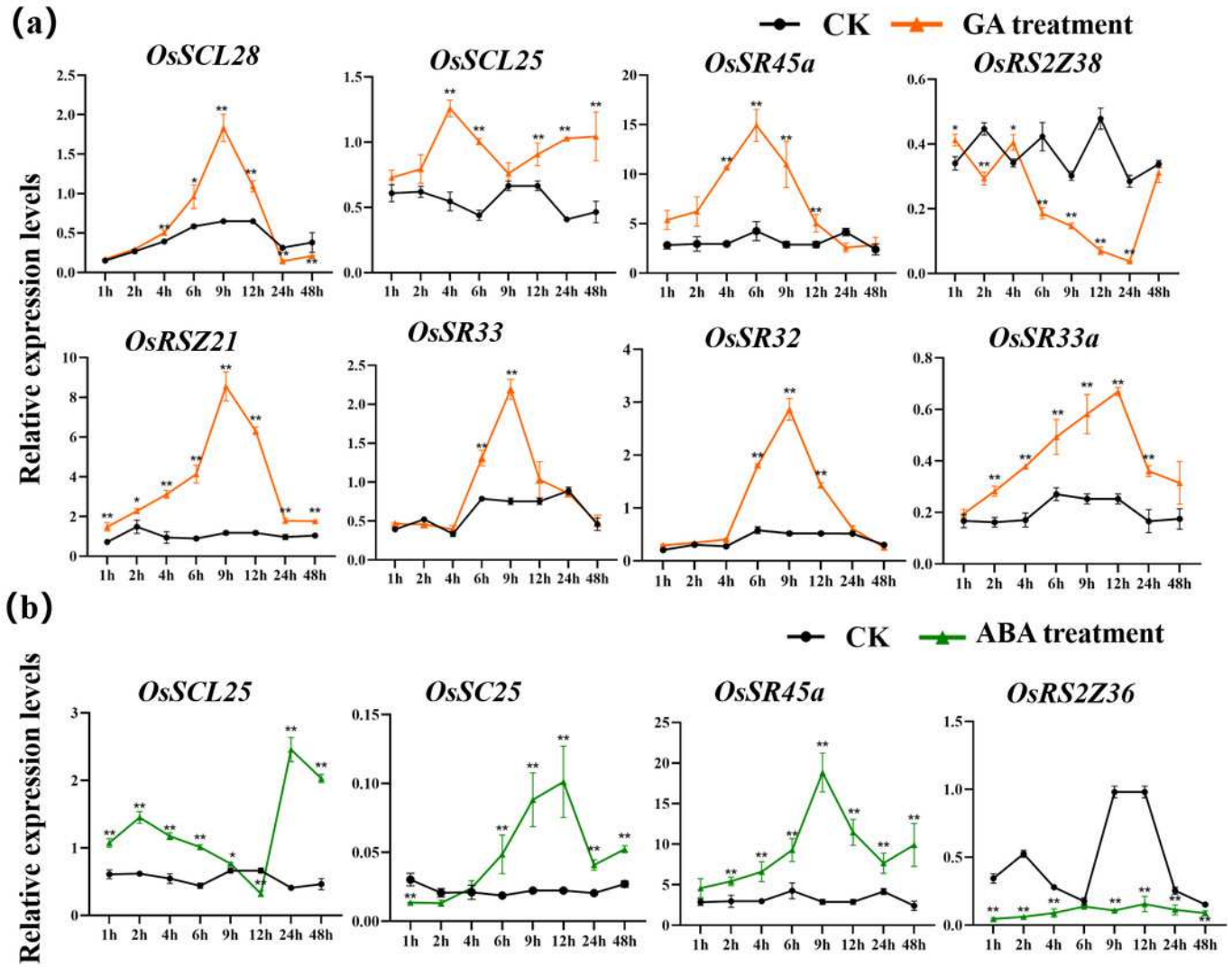

Table 1(on next page)

Basic information of *OsSR* gene family members.

Subfamily	Revised Nomenclature	Alternative Splice Form	Length (bp)	Intron	Exon	Protein length (aa)	Molecular Weight (kDa)	Isoelectric point	Instability index	Predicted Subcellular location	phosphorylation sites
SCL	*OsSCL30a*	LOC_Os02g15310.1	4309	6	7	265	30.47	11.09	111.87	nucleus	45
	OsSCL30	LOC_Os12g38430.1	3856	6	7	263	30.19	10.9	110.93	nucleus	47
	OsSCL57	LOC_Os11g47830.1	7034	13	14	502	56.83	10.01	100.02	nucleus	82
	OsSCL28	LOC_Os03g24890.1	4646	5	6	243	27.78	10.83	88.03	nucleus	35
	OsSCL25	LOC_Os07g43950.1	3180	4	5	213	24.82	10.68	103.01	nucleus	50
	OsSCL26	LOC_Os03g25770.1	3549	4	5	218	25.68	11.17	114.71	nucleus	40
SC	*OsSC32*	LOC_Os07g43050.1	4182	7	8	275	32.24	11.35	118.57	nucleus	59
	OsSC34	LOC_Os08g37960.1	3154	6	7	289	33.54	11.8	112.71	nucleus	56
	OsSC25	LOC_Os03g27030.1	3000	6	7	206	24.90	10.33	64.44	nucleus	25
SR45	*OsSR45a*	LOC_Os05g01540.1	3826	10	11	426	47.75	12.19	133.86	nucleus	86
	OsSR45	LOC_Os01g72890.1	5025	11	12	432	48.11	12.37	160.81	chloroplast	77
RS2Z	*OsRS2Z39*	LOC_Os05g07000.1	4237	5	6	338	39.02	9.83	64.03	nucleus	45
	OsRS2Z37	LOC_Os01g06290.1	3944	5	6	324	36.89	11.27	96.97	nucleus	57

	OsRS2Z36	LOC_Os05g02880.1	3489	5	6	323	36.22	10.83	101.82	nucleus	61
	OsRS2Z38	LOC_Os03g17710.1	3333	5	6	335	37.52	11	100.05	nucleus	60
RSZ	*OsRSZ21a*	LOC_Os06g08840.1	3671	4	5	185	21.18	11.29	103.17	nucleus	34
	OsRSZ21	LOC_Os02g54770.1	4678	4	5	185	21.02	11.24	93.82	nucleus	29
	OsRSZ23	LOC_Os02g39720.2	4269	3	4	203	23.20	11.33	112.07	nucleus	35
RS	*OsRS29*	LOC_Os04g02870.1	4490	4	5	245	28.78	9.94	68.28	nucleus	31
	OsRS33	LOC_Os02g03040.1	3691	4	5	279	32.54	9.88	60.31	nucleus	30
SR	*OsSR33*	LOC_Os07g47630.1	5111	12	13	296	33.14	10.64	104.32	nucleus	59
	OsSR32	LOC_Os03g22380.1	4789	12	13	286	31.94	10.54	98.23	nucleus	55
	OsSR33a	LOC_Os05g30140.1	7169	13	14	294	33.42	10.92	102.83	nucleus	64
	OsSR40	LOC_Os01g21420.1	7570	12	13	292	33.50	8.67	48.14	nucleus	29

1

Table 2(on next page)

Alternative splicing pattern of 11 selected *OsSR* genes.

SR gene	Size of all predicted transcripts (bp)	Size of amplification product on genome (bp)
OsSCL30a	1) 1269 (322), 2) 2241 (1293), 3) 1822 (875), 4) 2071 (1123)	1738
OsSCL25	1) 1091 (491)	1931
OsSC34	1) 1403 (695), 2) 1416 (796), 3) 1392 (775)	970
OsRS2Z37	1) 1332 (537), 2) 2351 (537), 3) 2417 (596), 4) 2672 (851)	1887
OsRS2Z36	1) 1312 (918)	2059
OsRS2Z38	1) 1456 (979), 2) 1442 (964)	2855
OsRSZ21	1) 1398 (469), 2) 991 (583)	957
OsRS29	1) 1234 (650), 2) 1183(650), 3) 1560(1074), 4) 1579(1083)	3782
OsRS33	1) 1349 (1001), 2) 1422 (1096), 3) 1859 (1511), 4) 1705(1357)	3343
OsSR32	1) 1042 (668), 2) 1003 (668), 3) 1076 (741)	2572
OsSR33a	1) 1500 (695), 2) 1690 (885)	2974

(1) The number in the parenthesis indicates the product size corresponding to the amplified primer used in this experiment.

Table 3(on next page)

NAGNAG acceptors in *OsSR* genes.

	mRNA		
SR gene	**E**	**I**	**Motif**
OsSCL30a	1		CAGGAG
OsSCL30	1		CAGGAG
OsSCL26	2		CAGTAG, TAGCAG
OsSC25	1		TAGCAG
OsSR45a	1		CAGCAG
OsSR45	1		CAGCAG
OsRS2Z39	1		CAGCAG
OsRS2Z36	2		CAGGAG
OsRS2Z38	1		CAGGAG
OsRSZ21a	1	2	CAGAAG, GAGCAG, AAGCAG
OsRSZ21	2		CAGAAG
OsRSZ23	1		TAGGAG
OsRS33	1		CAGGAG
OsSR33	1		CAGAAG

(1) Observed NAGNAG motifs and E and I acceptors confirmed by mRNA (from RefSeq) are shown.