Expression profiling and integrative analysis of the CESAL superfamily in rice

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Abstract

Background: The cellulose synthase and cellulose synthase-like gene superfamily (CESA/CSL) is proposed to encode enzymes for cellulose and non-cellulosic matrix polysaccharide synthesis in plants. Although the rice (Oryza sativa L.) genome has been sequenced for a few years, the global expression profiling patterns and functions of the OsCESA/CSL superfamily remain largely unknown.

Results: A total of 45 identified members of OsCESA/CSL were classified into two clusters based on phylogeny and motif constitution. Duplication events contributed largely to the expansion of this superfamily, with Cluster I and II mainly attributed to tandem and segmental duplication, respectively. With microarray data of 33 tissue samples covering the entire life cycle of rice, fairly high OsCESA gene expression and rather variable OsCSL expression were observed. While some members from each CSL family (A1, C9, D2, E1, F6 and H1) were expressed in all tissues examined, many of OsCSL genes were expressed in specific tissues (stamen and radicles). The expression pattern of OsCESA/CSL and OsBC1L which extensively co-expressed with OsCESA/CSL can be divided into three major groups with ten subgroups, each showing a distinct co-expression in tissues representing typically distinct cell wall constitutions. In particular, OsCESA1, -3 & -8 and OsCESA4, -7 & -9 were strongly co-expressed in tissues typical of primary and secondary cell walls, suggesting that they form as a cellulose synthase complex; these results are similar to the findings in Arabidopsis. OsCESA5/OsCESA6 is likely partially redundant with OsCESA3 for OsCESA complex organization in the specific tissues (plumule and radicle). Moreover, the phylogenetic comparison in rice, Arabidopsis and other species can provide clues for the prediction of orthologous gene expression patterns.

Conclusions: The study characterized the CESAL of rice using an integrated approach comprised of phylogeny, transcriptional profiling and co-expression analyses. These investigations revealed very useful clues on the major roles of CESAL, their potentially functional complement and their associations for appropriate cell wall synthesis in higher plants.

Background

Plant cell walls make up the most abundant renewable biomass on the earth. Of the main wall polysaccharides, cellulose is synthesized at the plasma membrane whereas non-cellulosic polysaccharides (pectins and hemicelluloses) are made in the Golgi body. In higher plants, CESAL was first isolated from developing cotton fibers, and it was further characterized in Arabidopsis as catalytic subunits of cellulose synthase complexes (CSCs) that locate within the plasma membrane [1,2]. The CSCs are believed to be a rosette structure holding as many as 36 individual CESAL proteins. In Arabidopsis, at least three CESAL isoforms are required for the synthesis of primary (AtCESA1, -3 & -6) and secondary (AtCESA4, -7 & -8) cell walls. Mutant and co-immunoprecipitation analysis demonstrates that AtCESA2 & -5 are partially redundant with AtCESA6 [3-5]. Consequently, the CESAL family has been identified in other plants, such as maize [6], barley [7], poplar [8,9], pine [10], moss [11] and rice [12]. Those higher plants appear to have many more CESAL family members, but...
very little is known about their functions in comparison to those from Arabidopsis.

A large number of cellulose synthase-like (CSL) genes showing sequence similarity to CESA have been identified. In Arabidopsis, a total of 30 CSL genes are classified into the six following families: CSLA, B, C, D, E and G [13]. Based on the common motif DXD, D, Q/RXXRW, all CSL proteins are predicted to encode processive glycosyl transferases (GTs) [14-17]. There are increasing lines of evidence supporting CSL as catalytic enzymes for non-cellulosic polysaccharide synthesis. In Arabidopsis and guar, genes of the CSLA family are demonstrated to encode (1,4)-β-D-mannan synthases [16-19]; in rice, genes of the CSLF family have been implicated in the biosynthesis of (1,3;1,4)-β-D-glucans [20]. More recently, it has also been established that barley CSLH genes, like CSLF, are able to direct mixed-linkage β-glucan biosynthesis [21]. In addition, the CSLC family contains a glucan synthase involved in the synthesis of the backbone of xyloglucan [22,23], and several CSLD mutants have been characterized for their potential roles in wall polysaccharide (xylan and homogalacturonan) synthesis [24-27]. However, even though there are a number of CSLD mutants in Arabidopsis and rice displaying interesting phenotypes, very little is known about the biochemical function(s) of CSLD proteins. The detailed functions of these CSL genes, especially those of families CSLB, E and G, remain to be clarified.

Rice, one of the major food crops across the world, is a model species for the functional genomic characterization of monocotyledonous plants. With the completion of the rice genome sequence, the CESA/CSL superfamily has been identified in rice http://waltonlab.prl.msu.edu/CSL_updates.htm. This rice superfamily has shown a striking difference in the CSL families between rice and Arabidopsis, reflecting the distinct cell wall compositions of dicots and monocots [28]. In contrast, several orthologs of the AtCESA genes exhibited a similar function in rice [29]. But, the OsCESA/CSL functions still remain largely unknown.

In this work, we utilized an innovative approach for the characterization of genes of the CESA/CSL superfamily in higher plants. We first performed a phylogenetic and structural analysis to determine their potential functions. Then, we focused on an integrative analysis of co-expression profiling and regulations using 33 tissue samples from the entire life cycle of two rice varieties. We further carried out a comparative analysis of CESA/CSL in rice and Arabidopsis.

**Methods**

**Database searches for OsCESA/CSL genes in rice**
The Hidden Markov Model (HMM) profile of the cellulose synthase domain (PF03552) was downloaded from PFam http://pfam.sanger.ac.uk/. We employed a name search and the protein family ID PF03552 for the identification of OsCESA/CSL genes from the rice genome. Information about the chromosomal localization, coding sequence (CDS), amino acid (AA) and full length cDNA accessions was obtained from TIGR http://www.tigr.org and KOME http://cdna01.dna.affrc.go.jp/cDNA. The corresponding protein sequences were confirmed by the Pfam database http://www.sanger.ac.uk/Software/Pfam/search.shtml.

**Sequence and structure analysis**

We performed our exon-intron structure analysis using GSDS http://gsds.cbi.pku.edu.cn/[30]. The protein transmembrane helices were predicted by the TMHMM Server V2.0 http://www.cbs.dtu.dk/services/TMHMM/ [31,32]. Protein subcellular locations were analyzed using WoLF PSORT http://psort.nibb.ac.jp/ [33], an extension of the PSORT II program http://www.psort.org.

**Phylogenetic analyses and motif identification**
The multiple alignment analysis was performed using the Clustal X program (version 1.83) [34] and MAFFT [35]. The unrooted phylogenetic trees were constructed with the MEGA3.1 program and the neighbor joining method [36] with 1,000 bootstrap replicates. Protein sequences were analyzed using the MEME program http://meme.sdsc.edu/meme/cgi-bin/meme.cgi for the confirmation of the motifs. The MEME program (version 4.0) was employed with the following parameters: number of repetitions, any; maximum number of motifs, 25; optimum motif width set to >6 and <200. The motifs were annotated using the InterProScan http://www.ebi.ac.uk/Tools/InterProScan/ search program.

**Chromosomal localization and gene duplication**
The OsCESA/CSL genes were mapped on chromosomes by identifying their chromosomal positions given in the TIGR rice database. The duplicated genes were elucidated from the segmental genome duplication of rice http://www.tigr.org/tdb/e2k1/osal/segmental_dup/100. The DAGchainer program [37] was used to determine the segmental duplications with following parameters: V = 5 B = 5 E = 1e-10-filter seg and distance = 100 kb. Genes separated by five or fewer genes were considered to be tandem duplicates. The distance between these genes on the chromosomes was calculated, and the percentage of protein sequence similarity was determined by the MegAlign software 4.0.

**Genome-wide expression analysis of OsCESA/CSL and OsBC1L in rice and AtCESA/CSL and AtCOBL in Arabidopsis**
The expression profile data of OsCESA/CSL in 33 tissue examples (Additional file 1) of Zhenshan 97 (ZS97) and
Minghui 63 (MH63) were obtained from the CREP database http://crep.ncpgr.cn and from a rice transcriptome project using the Affymetrix Rice GeneChip microarray (Additional file 2). Massively parallel signature sequencing (MPSS) data http://mpss.udel.edu/rice/ was used to determine the expression profiles of the genes with conflicting probe set signals. The expression values were log-transformed, and cluster analyses were performed using a software cluster with Euclidean distances and the hierarchical cluster method of “complete linkage clustering.” The clustering tree was constructed and viewed in Java Treeview. The same method was used in the “artificial mutant” analysis. However, in the hierarchical cluster of the “artificial mutant” analysis, the expression data for regarding gene(s) or tissues were deleted. All Arabidopsis microarray data were downloaded from the Gene Expression Omnibus database http://www.ncbi.nlm.nih.gov/geo/ using the GSE series accession numbers GSE5629, GSE5630, GSE5631, GSE5632, GSE5633 and GSE5634 (Additional file 3 and 4). Subsequent analysis of the gene expression data was performed in the statistical computing language R http://www.r-project.org using packages available from the Bioconductor project http://www.bioconductor.org. The raw data were processed with the Affymetrix Microarray Analysis Suite (MAS Version 5).

RT-PCR analysis of representative genes of the OsCESA/CSLD family

The primers designed for the RT-PCR analysis are listed in Additional file 5. Samples were collected from Zhenshan 97 (ZS97), one of the varieties used in microarray. The samples were ground in liquid nitrogen using a mortar and pestle. Total RNA (4 μg) was isolated using a RNA extraction kit (TransZol reagent, TransGen) and treated with RNase-free DNase I (Invitrogen) for 15 min to eliminate possible contaminating DNA. Then, first strand cDNA was reverse transcribed from total RNA with an oligo(dT)18 primer in a 50 μl reaction (diluted to 200 μl before use) using an M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s instructions. For the PCR amplification of the reverse transcription product, the PCR reaction was performed in a volume of 25 μl containing 2 μl of template. The reactions were conducted with rTaq polymerase (Takara Biotechnology, Japan) on a Bio-rad MyCycler thermal cycler using the following program: 3 min at 95°C for pre-denaturation, followed by 29 cycles of 20 s at 95°C, 20 s at 60°C and 30 s at 72°C, and a final 5 min extension at 72°C.

Plant cell wall fractionation and polysaccharide colorimetric assays

The plant tissues were firstly heated at 110–120°C for about 10 min to inactivate the enzymes, before they were fully ground in a mortar and pestle with liquid nitrogen and dried to constant weight at 65°C for about 2 days. The extraction and fractionation of the cell wall polysaccharides were performed with 0.5 M phosphate buffer, chloroform-methanol (1:1, V/V), DMSO-water (9:1, V/V), 0.5% ammonium oxalate, 4 M KOH, acetic acid-nitric acid-water (8:1:2, V/V/V) and 72% (w/w) H2SO4, and the extraction was measured using colorimetric assays according the method reported in a previous study [39].

Results

OsCESA/CSL superfamily in rice

Searching the TIGR database revealed 45 sequences that significantly matched to CESA/CSL superfamily, out of which eleven are predicted as OsCESA and 34 as OsCSL http://waltonlab.prl.msu.edu/CSL_updates.htm (Table 1). The sequences of OsCESA10 were short and appeared to be truncated. Of the 11 OsCESA sequences, CESA 1-9 contained a cellulose synthase domain (CS) and zinc finger structure, whereas CESA 10 & -11 only harbored a CS domain. When referring to the CSL classification in Arabidopsis, the 34 OsCSL proteins with a CS domain could be divided into six groups (Table 1). In addition, 31 genes had KOME cDNA support, and probes for 41 genes could be found in the CREP database (Table 1). The “DXD, D, QXXRW” motif is typically in the OsCESA/CSL family, but OsCSLA10 and OsCSL2 showed alternative motifs (“DXD, D, RXXRW” and “DXD, D, LXXRW”); OsCESA10, 11 and CSLH3 contained only “DXD” and lacked “D, LXXRW” (Additional file 6). Besides the “DXD, D, LXXRW” motif, some novel conserved amino acid residues (G, E, G, P and G) with unknown biochemical functions were also detected in this region.

Structural and phylogenetic analyses of OsCESA/CSL

An unrooted phylogenetic tree was generated from the alignments of 45 OsCESA/CSL protein sequences with two distinct clusters (Figure 1). Cluster I was resolved into five branches, namely Cluster IA (OsCESA), Cluster IB (OsCSLD), Cluster IC (OsCSLF), Cluster ID (OsCSLE) and Cluster IE (OsCSLH), whereas Cluster II had two branches, Cluster IIA (OsCSLA) and Cluster IIB (OsCSLC). In Cluster I, OsCESA had the most introns, and the OsCSLD had the fewest number of introns. In Cluster II, OsCSLA had more introns than OsCSLC. The analysis of motif composition was in agreement with the above OsCESA/CSL family classification (Additional files 7 and 8). Of the total 25 motifs predicted, Cluster I contained 18 motifs and Cluster II had 10 conserved motifs, of which three were in common.
| No. | Genes   | Accession Number | KOME cDNA        | Probsets\(^a\) | Protein characteristics |
|-----|---------|------------------|------------------|----------------|-------------------------|
| 1   | OsCESA1 | LOC_Os05g08370   | AK100188         | Os.10183.1.S2_at | 8 Zinc finger, CS (PF03552) |
| 2   | OsCESA2 | LOC_Os03g59340   | AK069196         | Os.14979.1.S1_at | 6 Zinc finger, CS (PF03552) |
| 3   | OsCESA3 | LOC_Os07g24190   | AK073561         | Os.10178.2.S1_at | 8 Zinc finger, CS (PF03552) |
| 4   | OsCESA4 | LOC_Os01g54620   | AK100755         | Os.18724.2.S1_x_at | 8 Zinc finger, CS (PF03552) |
| 5   | OsCESA5 | LOC_Os03g62090   | AK100877         | Os.4857.1.S1_at | 8 Zinc finger, CS (PF03552) |
| 6   | OsCESA6 | LOC_Os07g14850   | AK100914         | Os.10926.1.S1_at | 8 Zinc finger, CS (PF03552) |
| 7   | OsCESA7 | LOC_Os10g32980   | AK072259         | Os.3206.1.S1_at | 6 Zinc finger, CS (PF03552) |
| 8   | OsCESA8 | LOC_Os07g10770   | AK100914         | Os.10176.1.S1_at | 8 Zinc finger, CS (PF03552) |
| 9   | OsCESA9 | LOC_Os09g25490   | AK100877         | Os.10206.1.S1_at | 6 Zinc finger, CS (PF03552) |
| 10  | OsCESA10| LOC_Os12g29300   | NF / 0           | CS, cellulose synthase; GT, glycosyl transferase |
| 11  | OsCESA11| LOC_Os06g39970   | NF               | OsAffx.15853.1.S1_at | 6 CS (PF03552) |
| 12  | OsCSLA1 | LOC_Os02g09930   | AK102694         | Os.24972.1.S1_at | 5 GT family 2 (PF00535) |
| 13  | OsCSLA2 | LOC_Os10g26630   | NF               | Os.15231.1.S1_at | 5 GT family 2 (PF00535) |
| 14  | OsCSLA3 | LOC_Os06g12460   | NF               | OsAffx.15891.1.S1_at | 5 GT family 2 (PF00535) |
| 15  | OsCSLA4 | LOC_Os03g07350   | NF               | OsAffx.12764.2.S1_x_at | 5 GT family 2 (PF00535) |
| 16  | OsCSLA5 | LOC_Os03g26044   | AK111424         | Os.56873.1.S1_at | 6 GT family 2 (PF00535) |
| 17  | OsCSLA6 | LOC_Os02g51060   | AK058756         | Os.6170.1.S1_at | 5 GT family 2 (PF00535) |
| 18  | OsCSLA7 | LOC_Os07g43710   | AK112106         | Os.8080.1.S1_at | 6 GT family 2 (PF00535) |
| 19  | OsCSLA9 | LOC_Os06g42020   | AK242831         | Os.48268.1.S1_at | 5 GT family 2 (PF00535) |
| 20  | OsCSLA11| LOC_Os08g33740   | NF               | OsAffx.6015.1.S1_at | 5 GT family 2 (PF00535) |
| 21  | OsCSLC1 | LOC_Os01g56130   | AK100759         | Os.29016.1.S1_at | 5 GT family 2 (PF00535) |
| 22  | OsCSLC2 | LOC_Os09g25900   | NF               | Os.18770.1.S1_at | 4 GT family 2 (PF00535) |
| 23  | OsCSLC3 | LOC_Os08g15420   | AK108045         | Os.55417.1.S1_at | 4 GT family 2 (PF00535) |
| 24  | OsCSLC7 | LOC_Os05g43530   | AK243206         | Os.15705.1.S1_x_at | 2 GT family 2 (PF00535) |
| 25  | OsCSLC9 | LOC_Os03g56060   | AK121805         | Os.10855.1.S1_at | 3 GT family 2 (PF00535) |
| 26  | OsCSLC10| LOC_Os07g03260   | NF               | OsAffx.28245.1.S1_at | 2 GT family 2 (PF00535) |
| 27  | OsCSLD1 | LOC_Os10g42750   | AK110534         | Os.46811.1.S1_at | 8 CS (PF03552) |
| 28  | OsCSLD2 | LOC_Os06g02180   | AK105393         | Os.25614.1.S1_at | 6 CS (PF03552) |
| 29  | OsCSLD3 | LOC_Os08g25710   | NF               | OsAffx.17155.1.S1_x_at | 6 CS (PF03552) |
| 30  | OsCSLD4 | LOC_Os12g36890   | AK242601         | Os.57510.1.S1_x_at, Os.57510.1.A1_at | 6 CS (PF03552) |
| 31  | OsCSLD5 | LOC_Os06g22980   | AK072260         | Os.53359.1.S1_at | 8 CS (PF03552) |
| 32  | OsCSLD6 | LOC_Os09g30130   | NF               | Os.26822.1.S1_at | 2 CS (PF03552) |
| 33  | OsCSLD7 | LOC_Os07g36700   | NF               | Os.26822.1.S1_at | 2 CS (PF03552) |
| 34  | OsCSLF1 | LOC_Os07g36700   | NF               | Os.26822.1.S1_at | 2 CS (PF03552) |
| 35  | OsCSLF2 | LOC_Os07g36690   | AK100523         | Os.15704.1.S1_at | 8 CS (PF03552) |
| 36  | OsCSLF3 | LOC_Os07g36750   | NF               | OsAffx.5550.1.S1_at | 8 CS (PF03552) |
| 37  | OsCSLF4 | LOC_Os07g36740   | NF               | Os.52482.1.S1_at | 8 CS (PF03552) |
| 38  | OsCSLF6 | LOC_Os08g06380   | AK065259         | Os.9709.1.A1_at, Os.9709.2.S1_at | 9 CS (PF03552) |
| 39  | OsCSLF7 | LOC_Os10g20260   | AK110467         | Os.46814.1.S1_at | 7 CS (PF03552) |
| 40  | OsCSLF8 | LOC_Os07g36630   | AK067424         | Os.52482.1.S1_at | 8 CS (PF03552) |
| 41  | OsCSLF9 | LOC_Os07g36610   | AK242890         | OsAffx.15856.1.S1_x_at | 8 CS (PF03552) |
| 42  | OsCSLH1 | LOC_Os10g20090   | AK069071         | Os.11623.1.S1_x_at | 6 CS (PF03552) |
| 43  | OsCSLH2 | LOC_Os04g35010   | NF               | Os.45970.1.S1_at | 8 CS (PF03552) |
| 44  | OsCSLH3 | LOC_Os04g35030   | NF               | Os.26822.1.S1_at | 2 CS (PF03552) |

\(^a\) Probeset ID of OsCESA/CSL genes
\(^b\) The number of transmembrane helices predicted by the TMHMM server V2.0
\(^c\) CS, cellulose synthase; GT, glycosyl transferase

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Tandem and segmental genome duplications of OsCESA/CSL

The OsCESA/CSL members are distributed on 12 chromosomes of rice (Figure 2). As reported by Burton et al. (2006) [20], members of the OsCLSF (9, 8, 2, 1, 4, &3) are physically linked within a region of approximately 118 kb of rice chromosome 7. We discovered two additional tandem duplication sets (OsCSLH2/CSLH3 and OsCSLE1/CSLE6) and seven segmental duplication sets (OsCESA2/CESA8, OsCSLA1/CSLA9, OsCSLA2/CSLA4, OsCSLA5/CSLA7, OsCSLA6/CSLA3, OsCSLC9/CSLC10 and OsCSLE2/CSLE6) that were assigned to the TIGR segmental duplication blocks at a maximal length distance permitted between collinear gene pairs of 100 kb. In most sets, both members (genes) in a segmental duplication set were from same family. The extreme
example is from CSLA family; eight of nine members in this family are in duplicated regions. Moreover, most of the duplicated genes have a relatively close phylogenetic relationship; in particular, in the four sets OsCESA2/CESA8, OsCSLA2/CESA4, OsCSLA5/CESA7, and OsCSLC9/CSLC10, two member genes are phylogenetically closest to each other (Figure 1A). Interestingly, the two pairs of segmental sets (OsCESA2/CESA8 and OsCSLC9/CSLC10) join closely in two chromosomes (Figure 2). Of the 45 OsCESA/CSL genes, 23 are involved in duplication events. Therefore, segmental and large-scale tandem duplication events contributed largely to the expansion of this superfamily. Cluster I families were mainly attributed to tandem duplication, whereas Cluster II likely resulted from segmental genome duplication.

OsCESA/CSL expressions

A microarray analysis was conducted for the expression of OsCESA/CSL genes in two rice varieties (Additional file 2), and the expression patterns of OsCESA and OsCSLD families were further verified by RT-PCR analysis (Figure 3, Additional file 9). We also demonstrated the expression of OsCESA/CSL genes in both individual and collective levels (Figure 4). Generally, OsCESA genes, with the exception of the OsCESA11, exhibited an extensively high expression in most of the tissues examined; in particular, OsCESA1 and OsCESA3 demonstrated extremely high expression in many tissues over different developmental stages of the life cycle (Figures 3 and 4). In addition, the accumulative OsCESA expression levels were highest in the stem and root, but were relatively low in the flag leaf and stamen (Figure 4). Of the OsCSL families, six OsCSL members (CSLA1, CSLC9, CSLD2, CSLF6 and CSLH1) were expressed in all of the tissues examined. In contrast, other OsCSL genes showed tissue-specific expression. For instance, CSLD3 & -5, CSLH2 and CSLC9 showed high stamen-specific expression, whereas CSLA5, CSLD1 and CSLD4 were specific in the endosperm, radicle and plumule, respectively. The accumulative expression of all the CSL genes in a family is also depicted in Figure 4. The overall expression of the family of CSLD genes is highest in the stamen and lowest in the shoot of seedlings with two tillers. The total expression of the CSLA genes was highest in plumes (mostly contributed by CSLA1 and 6) and was followed by high expression in radicles (roots) and calli, with the lowest expression detected in flag leaves. The total expression of CSLC was higher in the stamen and plumule/radicles, but was lower in leaves. Collectively the expression of the genes of the whole family often accumulated to high levels in one or more of the tissues for which the CSL members showed preferences. This may indicate functional homoplasy among the members in a family although most of them exhibit different expression patterns.

Expression divergence of OsCESA/CSL genes in duplication

We further observed the expression profiling of the duplicated OsCESA and OsCSL genes. The expression of the two duplication sets OsCSLE1/OsCSLE6 and OsCSLE2/OsCSLE6 were not included in the analysis because we lacked the corresponding probe set of OsCSLE6. The expression profile of the eight remaining sets of OsCESA/CSL genes (two tandem duplication sets and six segmental duplication sets) with the corresponding probes was analyzed. We found a divergent expression pattern within a
OsCESA/CSL co-expression profiling

Because many genes of COBRA-like proteins, including the brittle culm1 like family (OsBC1L), have been investigated for cell wall biosynthesis in Arabidopsis and rice [41-44], the OsBC1L genes were referred as markers of OsCESA/CSL co-expression patterns in this study. Based on the hierarchical cluster analysis, the OsCESA/CSL family can be classified into three major groups with ten distinct groups that exhibit a complementary expression pattern spanning 33 tissues from entire life cycle of two rice varieties (Figure 6). Each group consists of multiple OsCESA/CSL members, which show predominant co-expression in tissues with distinct cell wall constitutions (Table 2).

Generally, Group IA showed high co-expression in the young vegetative tissues (M7/Z7-M11/Z11) typical of the primary cell wall, and Group IB exhibited additional co-expression in other vegetative tissues (e.g., seedlings, young shoots and stems). Five OsCESAs (5, -6 and -7, -8) were strongly co-expressed in those two groups, suggesting that OsCESA1, -3 & -8 may form a cellulose synthase complex for primary cell wall biosynthesis. However, while OsCESA1 and OsCESA8 are tightly co-expressed, there are some differences in expression between OsCESA3 and OsCESA1 &-8 (Figure 6). We observed that OsCESA3 had exceptionally low expression in the plumule and radicle (M8/Z8-M11/Z11), where the expression of OsCESA5/OsCESA6 is relatively high (Figure 6). This observation might indicate the partial complementation of OsCESA3 by OsCESA5 &-6 in the expression pattern. In comparison to Group I, Group II showed co-expression in three tissues rich in secondary cell walls (old panicle, hull and spikelet) (Figure 6). However, three OsCESAs (CESA4, -7 & -9) in the group also showed a co-expression pattern that overlapped with Group IB in young and old stem tissues, which represent the transition stage from primary to secondary cell wall synthesis. Thus, OsCESA4, -7 & -9 may be organized as a cellulose synthase complex involved in secondary cell wall synthesis.

In contrast, Group III appeared to show co-expression in diverse tissues harboring specific cell wall structures. For instance, five OsCSL genes of Group III B demonstrated high co-expression in the stamen (M31/Z31), a tissue that contains extremely high levels of pectins (Table 2), and Group III C showed co-expressions in four early stages of panicle development. Co-expression was detected between the OsCESA and OsCSL families in all ten groups; we also observed strong co-expression between the OsCESA/CSL and OsBC1L families in seven groups, each containing at least one OsBC1L family gene. For instance, OsBC1 and OsBC1L5 both have correlation coefficients (r values) above 0.94 with respect to their relevant OsCESA/CSL genes. Interestingly, this extensive co-expression was only found between BC1L and OsCESA/CSL. There are no such extensive relationships found between OsCESA/CSL

Figure 3 OsCESA and OsCSLD gene expression patterns by RT-PCR analysis

duplicated set (Figure 5). The pairwise expression correlation coefficients (r values) of the duplicated OsCESA/CSL genes were below the level of significance at P = 0.05 (data not shown). Of the nine gene sets, only CSLA2 and CSLA4 in a segmental duplication set (CSLA2/CSLA4) exhibited a relatively similar expression pattern. The fate of four pairs (CSLH2/CSLH3, CESLA2/CESLA8, and CSLC9/CSLC10) could be described as nonfunctionalization, where one member of the set lost expression in all tissues, while the other showed strong expression. In the other duplication sets, the expression patterns of both member genes were partial complementary and/or overlapped. Comparison of expression pattern shifts of the duplicated genes of the OsCESA/CSL superfamily could reflect the divergence hypotheses that a duplicate gene pair might be involved in: nonfunctionalization, subfunctionalization and neofunctionalization [40].
with other gene families, such as cellulase (including Korrigan), lignins and expansins (data not shown).

**Comparative co-expression analyses with Arabidopsis**

Using the *Arabidopsis* public database, we presented a co-expression profiling of 63 tissue samples, and compared it with rice (Figure 7, Table 3). Based on hierarchical clustering, the expression pattern of the *AtCESA*/*AtCSL* genes could also be divided into three major groups (Figure 7). In contrast, the expression patterns of the *CESA*/*CSL* genes in both species are summarized in Table 3. Clearly, the expression patterns of the genes of the *AtCESA*/*AtCSL* superfamily fell into groups similar to those of the *OsCESA*/*CSL* genes. As an example of genes showing a similar expression pattern, *AtCESA1*, -3 & -6 showed high co-expression in the tissues of the primary cell wall, whereas *AtCESA4*, -7 & -8 were co-expressed in the secondary cell wall tissues. As an example of genes showing a different expression pattern, there was no *AtCESA* gene, like *OsCESA3*, showing an exceptionally low expression level. In addition, distinct CSL co-expressions were compared between rice and

Figure 4  Accumulative expressions of *OsCESA*/CSL genes in representative tissues of rice  The y-axis indicates the relative expression level of the genes (signal values from the microarray data) and it is arbitrary. The x-axis indicates the tissues across development stages with 1-3: Calli; 4: Seed imbibition; 5: Young panicle stages 3-5; 6: Young panicle; 7: Plumule; 8: Stem; 9: Young leaf and root; 10: Shoot; 11: Radicle and root; 12: Stamen; 13: Flag leaf; 14: Endosperm 1, 2, 3; 15: Sheath; 16: Old Leaf; 17: Hull; 18: Old panicle; 19: Spikelet.
Arabidopsis (Table 3). For example, a group of IC genes (AtCSLG1, -2, & -3 and AtCSLB2) was specifically expressed in flower organs (carpels or sepals) in Arabidopsis, while the OsCSLF genes (OsCSLF2 & -7) were preferentially expressed in the hull of rice. Thus, the gene expression pattern may reflect both the similarities and differences in the cell wall composition of rice and Arabidopsis.

**Discussion**

The previous characterization of the rice OsCESA/CSL family was focused on phylogenetic and gene structure analyses [12,28]. Hazen et al. (2002) identified 37 OsCSL genes [28]; however, some of the CSL genes are pseudogenes, and these have now been updated http://waltonlab.pll.msu.edu/CSL_updates.htm. For example, CSLC4, -5, -6 & -8 were verified as pseudogenes and were not included in this study.
The OsCSLA8 (LOC_Os09g39920.1) gene was recently annotated as a retrotransposon in TIGR version 6.1, while OsCSLA10 (DAA01745.1) identified in the NCBI database was actually the same as OsCSLA4 and now has been excluded. These updated OsCESA/CSL genes were indentified and characterized in this study. We performed expression, co-expression and comparative co-expression analyses of this superfamily. The results, coupled with the bioinformatic analysis of phylogeny, gene structure, motif constitution, genome organization and gene duplication,

Table 2  Cell wall composition (%) of seven representative tissues in rice

| Tissues      | Cellulose | Hemicelluloses | Pectins |
|--------------|-----------|----------------|---------|
|              | Hexose    | Pentose        | Total   | Hexose  | Pentose | UroA | Total |
| Calli        | 23.8 (4.2)* | 35.1           | 64.9    | 65.4 (11.5) | 23.0 | 23.9 | 53.0 | 10.8 (1.9) |
| Seedling leaves | 48.8 (15.7) | 31.1           | 68.9    | 44.8 (14.4) | 33.1 | 26.5 | 40.4 | 6.4 (2.1) |
| Seedling roots | 54.0 (20.5) | 35.1           | 64.9    | 42.5 (16.1) | 45.3 | 30.9 | 23.8 | 3.5 (1.3) |
| Young stem   | 33.8 (11.1) | 64.0           | 36.0    | 63.5 (20.9) | 34.5 | 27.5 | 38.0 | 2.7 (0.9) |
| Old stem     | 38.3 (20.6) | 67.3           | 32.7    | 60.1 (32.3) | 30.3 | 21.1 | 48.5 | 1.7 (0.9) |
| Hull         | 56.4 (26.6) | 22.7           | 77.3    | 41.1 (19.4) | 36.1 | 30.1 | 33.8 | 2.5 (1.2) |
| Stamen       | 29.7 (2.3)  | 24.9           | 75.1    | 29.0 (2.3)  | 34.3 | 30.0 | 35.7 | 41.3 (3.3) |

* % of wall polysaccharide based on the tissue dry weight; the absolute values are bracketed.
could provide an innovative approach and important clues toward understanding the roles of the CESA/CSL superfamily in cell wall biosynthesis in higher plants.

**CESA/CSL evolution and classification**

In principle, gene families are extended by three major mechanisms: segmental duplication, tandem duplication and retroposition [45,46]. Here we confirmed that both tandem and segmental duplication events were largely responsible for the expansion of the OsCESA/CSL family. Interestingly, we characterized two clusters of OsCESA/CSL and concluded that they not only differ in phylogeny and motif constitution, but that they also expanded in the following distinct ways: Cluster I (OsCESA/CSLD, E, F and H) arose mainly from the tandem duplication, and Cluster II (CSLA/CSLC) resulted from the segmental duplication. These results support a previous report claiming that CSLA/CSLC has a different evolutionary origin compared to other CSL families [12]. In terms of the duplicated gene expression, we observed that two genes in a duplication set show a strongly contrasting expression pattern. The fate of duplicated genes in OsCESA/CSL could be described as nonfunctionalization, subfunctionalization and neofunctionalization. None of the genes in a segmental duplication set have similar expression patterns. The latter findings are consistent with a previous report whereby growth-related genes were sensitive to high dosage of gene expressions, and stress responsive genes were tolerant to high dosage [47].

The comparison of the CESA expression patterns among seven plant species (rice, barley, maize, poplar, cotton, eucalyptus and Arabidopsis) is depicted in the unrooted neighbor-joining tree (Additional file 10). Most clusters contain genes from both monocot and dicot plants, and most orthologs show a higher similarity than paralogs in the CESA family, indicating that some gene expansion may have arisen earlier than when the divergence(s) of the species occurred. The latter result is supported by reports whereby the orthologous genes in a cluster show a similar expression pattern in primary and secondary cell walls [48,49]. Furthermore,
we compared the expression patterns of some CSL homologs in Arabidopsis, rice, barley and other species, and a striking similarity was observed in the close orthologous genes across species (Additional file 11). We also observed similarities of CSL orthologs in other aspects such as gene duplication and intron-exon structure (data not shown). Thus, such observations could be helpful in the prediction of gene expression patterns of orthologs in cereal species and other higher plants.

Analysis of OsCESA functions
Patterns of co-expression can reveal networks of functionally related genes and provide a deeper understanding of the processes required to produce multiple gene products [50]. The genome-wide expression analysis of the CESA family could provide insights into the potential functions of its members in cell wall biosynthesis. Almost all OsCESA genes are expressed in the tissues we examined, confirming their major roles in the biosynthesis of cellulose, the main component of plant cell walls. The co-expression profiling of the CESA genes can somehow indicate their protein interaction/association as an essential synthase complex for cellulose biosynthesis. Despite the use of the mutant analysis and co-immunoprecipitation in Arabidopsis [3,5,51], the application of these approaches in the identification of the CESA complex in other higher plants, such as rice, maize and barley has not been reported.

In this work, therefore, we utilized an alternative approach via the integrative analysis of gene co-expression profiling and developmental regulations. First, we confirmed the formation of two distinct cellulose synthase complexes, AtCESA1, -3, & -6 and AtCESA4, -7, & -8 in Arabidopsis from our AtCESA co-expression profiling data (Figure 7). Similarly, we can assume that OsCESA1, -3 & -8 and OsCESA4, -7 & -9 may be two synthase complexes involved in primary and secondary cell wall synthesis in rice, respectively (Figure 6, Table 2), which provides clues on the physical interactions of proteins in the synthase complexes. The co-expression profiling in Arabidopsis in this study, however, could not further verify the previous finding of AtCESA6 as partial redundant gene with AtCESA2 & -5 [4,5], probably because of the lack of essential expression data of Arabidopsis tissues from the public microarray data (Figure 7). Similarly, we can assume OsCESA1, -3 & -8 and OsCESA4, -7 & -9 may be two synthase complexes involved in primary and secondary cell wall synthesis in rice, respectively (Figure 6, Table 2), which provides clues on the physical interactions of proteins in the synthase complexes. The co-expression profiling in Arabidopsis in this study, however, could not further verify the previous finding of AtCESA6 as partial redundant gene with AtCESA2 & -5 [4,5], probably because of the lack of essential expression data of Arabidopsis tissues from the public microarray data (Figure 7). Similarly, we can assume OsCESA3 to be a partially redundant candidate gene with OsCESA5/ OsCESA6 given its low transcript level in specific tissues (plumule and radicle), where the expression of OsCESAS/OsCESA6 is relatively high (Figure 6). In other words, OsCESA5 or -6 may be partially redundant with OsCESA3 in those specific tissues. Eventually, we
demonstrated the partial redundancy of OsCESA5 or -6 with OsCESA3 by a novel approach, the “artificial-mutant” analysis of gene co-expression profiling (Figures 8 and 9, Additional file 12 and 13). While OsCESA3 was artificially deleted, the hierarchical cluster analysis showed that OsCESA1 &-8 clustered together with OsCESA5 and OsCESA6. This result might indicate that OsCESA1 & -8 form a synthase complex with OsCESA5 or OsCESA6 (Figure 8). However, deleting either OsCESA1 or OsCESA8 did not disrupt the above organization (Figure 8). Even after the double deletion of OsCESA3/OsCESA1 or OsCESA3/OsCESA8, OsCESA5 and OsCESA6 could somehow still organize a complex with either OsCESA1 or OsCESA8 (Figure 8). Clearly, the data are in support of our assumption. When the gene expression data in the plumule and radicle tissues were not included in the hierarchical cluster analysis, OsCESA1 &-8 could not form a group with OsCESA5 or OsCESA6 when OsCESA3 was artificially deleted (Figure 9). Thus, we believe that partial redundancy occurs in the specific development stages/tissues (such as plumule and radicle) of rice.

Characterization of the OsCSL family

Several OsCSL genes were demonstrated to exhibit relatively tissue-specific expression, indicating their specific/unique roles for wall polysaccharides synthesis or their potentially functional complements for appropriate cell wall synthesis. For instance, in the pectin-rich and cellulose-less stamen tissue (Table 2), all OsCESAs have a relatively low transcript level, but three OsCSLs (OsCSLC9, OsCSLD5 and OsCSLH2) exhibit specifically high expression. In addition, all six OsCSL families appear to have at least one highly expressed gene (CSLA1, CSLC9, CSLD2, CSL1E1, CSLF6 and CSLH1) in all the tissues we examined, therefore suggesting that the entire OsCSL family is essential for cell wall biosynthesis.

The analysis of co-expression profiling and developmental regulations, together with a comparison with Arabidopsis, can be used for the characterization of OsCSLs. As described above, we concluded that ten co-expressed groups are expressed in cells/tissues with different cell wall constitution. Based on this information, we could find clues about the predominant roles of OsCSL genes in cell wall biosynthesis. For example, OsCSLF2 and OsCSLF7 in Group IIA may have quite a different role from other OsCSLF genes in Groups IB, IIBD and IIE (Figure 6). OsCSLF2 and OsCSLF7 show a uniquely high co-expression pattern with OsCESA4, -7 & -9 in the hull/spikelet tissue typical of secondary cell walls (Figure 6); however, they both have a much lower transcript level than OsCSLF6 and OsCSLF8 (Figure 4). Because there are pentose-rich hemicelluloses in the hull tissue (Table 2), we assume that OsCSLF2 and OsCSLF7 may also encode other synthase enzymes besides the β-(1,3-1,4)-glucan synthase that was previously characterized. In addition, comparison of co-expression profiling in the stamen tissue between rice (Group IIIB) and Arabidopsis (Group IIC) suggests that
OsCSLH2 and AtCSLA9 may play a similar or replaceable role in cell wall synthesis (Table 3). We can also infer the functional meanings from the developmental regulations of the gene expression. For example, the higher expression of OsCSLD2 and OsCSLE1 was found in older leaves versus young leaves. This result was consistent with the report that AtCSLD2 and AtCSLE1 apparently exhibit strong increases in expression in old leaves versus young leaves in Arabidopsis [25]. The authors proposed that the changes in expression of these two genes may reflect a role in homogalacturonan synthesis, which accumulated to a high level in old leaves. The availability of more detailed information about cell wall composition (e.g., monosaccharide) will help in establishing links between CESA/CSL proteins and the carbohydrates they might synthesize.

Conclusions

Previous analysis of the functions of CESA/CSL members on plant cell wall biosynthesis has been focused on biochemical and genetic approaches in the model plant Arabidopsis. Here, we performed a validated approach that is applicable in higher plants and successful at finding out useful clues on OsCESA/CSL protein interaction or association. Our approach not only relies on a comprehensive phylogenetic analysis, but it also integrates the characterization of co-expression profiling and regulations, which can reveal very useful clues on the dynamic organization of OsCESA proteins as distinct cellulose synthase complexes in primary and secondary cell wall biosynthesis. We also conclude that the co-expression profiling of OsCESA/OsCSL and OsBC1L can be associated with ten distinct groups in specific cell wall polysaccharide synthesis. In a word, our results provide insights into functional analyses of CESA/CSL family and of other GT families or cell wall-related genes in rice and other higher plant species.

Additional material

Additional file 1: Tissues and developmental stages throughout the life cycle of two rice varieties.

Additional file 2: Signal intensities of the probe sets for the OsCESA/CSL and OsBC1L families.

Additional file 3: Tissues sampled from different developmental stages throughout the life cycle of Arabidopsis.

Additional file 4: Signal intensities of the probe sets for the AtCESA/CSL and AtCOBL families.

Additional file 5: Primers of the OsCESA/OsCSLD genes used for RT-PCR analysis.

Additional file 6: Conserved amino acids in the ”D, D, D, QXXRW” motif (depicted in red) of OsCESA/CSL in rice.

Additional file 7: Motif composition of the OsCESA and CSL protein families.

Additional file 8: Details of the 25 putative motifs.

Additional file 9: Expression patterns of the individual genes from OsCESA (up) and OsCSLD (below) families in representative tissues of rice. The y-axis indicates the relative expression level of the genes (signal values from the microarray data) and it is arbitrary. The x-axis indicates the tissues across development stages with 1-3: Calli; 4: Seed imbibition; 5: Young panicle stages 3-5; 6: Young panicle; 7: Plumule; 8: Stem; 9: Young leaf and root; 10: Shoot; 11: Radicle and root; 12: Stamen; 13: Flag
leaf; 14: Endosperm I, 1, 2, 3; 15: Sheath; 16: Old Leaf; 17: Hull; 18: Old panicle; 19: Spikelet.

Additional file 1: Unrooted phylogenetic tree subjected to the alignment of the deduced amino acid sequences of the OsCESA family genes with full-length CESA protein sequences from other species. At = Arabidopsis thaliana; Eg = Eucalyptus grandis; Gs = Gossypium hirsutum; Hv = Hordeum vulgare; Os = Oryza sativa; Pt = Populus tremuloides; and Zm = Zea mays. ‘PCW’ and ‘SCW’ indicate primary cell wall and secondary cell wall, respectively. Information about CESA refers to: At [1], [2], [4-9], [12-14], [16-17], [23-24], [26-29], Zm [6], [7], [9], [10].

Additional file 11: Comparative analysis of the expression patterns of the CSL homologs (CSLD, CSLF, CSLC and CSLA) in Arabidopsis, rice, barley and other species. Os: rice, At: Arabidopsis, Hv: barley, Pt(p): popcorn, Na: tobacco. The plus signs indicate the preferential expression, while the minus sign indicates lower expression; The asterisks indicate the genes expressed throughout the tissues examined; The numbers in parentheses indicate the duplicated genes of OsCESA/CSL. The expression data refer to: AtCESA/CSLA [25,53], HvCSLF [54], HvCSLC [22], PtCESA [18], PtCSLD and NaCSLD1 [55].

Additional file 12: Gene co-expression profiling of OsCESA by "Artificial-mutant" analysis in all the tissues examined.

Additional file 13: Gene co-expression profiling of OsCESA by "Artificial-mutant" analysis; data from the plumule and radicle tissues were excluded.

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Authors’ contributions
LW performed all data analyses and drafted the manuscript. KG conducted all data collection and analyses. YT and HH completed chemical tests. YL, BW and XH participated in the growing of the rice and in data interpretation. LP supervised the project and finalized the paper. All authors have read and approved the final manuscript.

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