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ORIGINAL ARTICLE

Development of Functional Modules Based on Co-expression Patterns for Cell-wall Biosynthesis Related Genes in Rice

Anil Kumar Nalini Chandran[1,†], Ho Young Jeong[2,†], Ki-Hong Jung[1,*] and Chanhui Lee[2,*]

[1]*Graduate School of Biotechnology and Crop Biotech Institute, Kyung Hee University, Yongin 446-701, Korea*
[2]*Department of Plant and Environmental New Resources, Kyung Hee University, Yongin 446-701, Korea*

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Abstract The functional elucidation of plant cell wall biosynthesis (CWB) related genes is important for understanding various stress tolerance responses as well as enhancing biomass in plants. Despite their significant role in physiology and growth of the plant, the function of a limited number of CWB related genes have been identified. Major obstacles such as functional redundancy and limited functional information pose challenges in the characterization of CWB genes. Here, a genome-wide analysis of CWB genes using meta-expression data revealed their roles in stress tolerance and developmental processes. The identification of co-expressed CWB genes suggests functional modules for plant cell wall biosynthesis associated with specific tissue types, biotic stress, abiotic stress, and hormone responses. More interestingly, we identified that glycosyl hydrolases are specialized for root and pollen development, glycosyltransferases for ubiquitous function and leaf development, and carbohydrate esterases for pollen development. A T-DNA insertional mutant of *OsCESA9* showing internode preferred expression revealed severe dwarfism and a co-expression network analysis of *OsCESA9* in *oscesa9* mutant suggest downstream pathways for secondary cell wall biosynthesis and DNA repair processes. Data from our studies will facilitate functional genomic studies of CWB genes in rice and contribute to the enhancement of biomass and yield in crop plants.

Key words: Cell wall biosynthesis, Co-expression analysis, Meta-expression analysis, Rice, T-DNA insertional mutants

Introduction

Plant cell walls are highly heterogeneous composites mainly made of polysaccharides and phenolic compounds as well as cell wall-bound proteins (Carpita and Gibeaut 1993). Cell wall composition and relative abundance varies among tissues and even among cell types within a species. Thus, formation of the final architecture of cell walls is temporally and spatially regulated during plant growth and development. Recent studies have demonstrated that transcription profiling data for cell wall genes are different according to applied biotic or abiotic stresses, suggesting that the alteration of wall composition and abundance play an important role in plant defenses (Cheong et al. 2002). Cell wall formation requires the coordinated expression of a number of genes encoding cell wall-related transcription factors, cell wall biosynthetic enzymes, and remodeling/assembling hydrolases (Lombard et al. 2014). Although it is estimated that about 10% of the plant genome is dedicated to cell wall biology, only a few genes have been functionally characterized in plants.

Rice is an agriculturally important grain crop and serves as a model organism of the grass family with a completed genome sequence and the availability of a T-DNA mutant collection (Chandran and Jung 2014). Recently, extensive studies have focused on understanding the molecular/cellular mechanisms underlying cell wall biosynthesis with the hope to increase lignocellulosic biomass of grasses, which can be a useful source for the development of novel nanomaterials and for bioethanol production. Considering that cell wall polymers constitute more than 80% of plant biomass (Pauly and Keegstra 2010), detailed knowledge about cell wall biogenesis will provide facile strategies to increase or modify wall polysaccharides by plant breeding and bioengineering.

Functional analysis of CWB genes pose challenges in terms of proper screening of the phenotypic effects. ‘Chemical

†These authors contributed equally to this work.

*Corresponding author; Ki-Hong Jung, Chanhui Lee
Tel : +82-031-201-3474, +82-031-201-3480
E-mail : khjung2010@khu.ac.kr, chan521@khu.ac.kr

genetics' provides a promising method that results in observable phenotypic effects for loss of function CWB gene mutants. In Arabidopsis, liquid culturing of mutant plants in the presence of xyloglucanase led to the identification of 23 genetic loci responsible for alterations in cell wall composition (Gille et al. 2009). Xyloglucanase acts on major cross-linking glycan in the plant extra cellular matrix and has been used to identify distinguishable phenotypic changes of CWB mutants. The advantage of the oligosaccharide fingerprinting technique is the identification of structural variation in cell wall polymerase (Lerouxel et al. 2002). In comparison with advancements in the chemical genetics of Arabidopsis, high throughput techniques to mediate the functional identification of CWB genes in rice are still in their infancy.

Cell wall biosynthetic genes include glycosyltransferases (GTs), glycosyl hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and carbohydrate-binding modules (CBMs). GTs are responsible for backbone polymerization and the addition of side chains and functional groups using activated UDP or ADP donor sugars. Once synthesized by the action of GTs, each polymer is subjected to different modifications (partial degradation or removal of functional groups) by a suite of GHs, PLs, and CEs in order to obtain functional cellular structures. Tremendous effort including mutagenesis and transcriptome analysis has been devoted to studying cell wall biogenesis in the model eudicot *Arabidopsis thaliana* (Arabidopsis) and the woody model *Populus trichocarpa* (poplar), leading to the identification of multiple genes and mutants relating to cell wall biosynthesis. It has been concluded that cell wall biogenesis is highly conserved between Arabidopsis and poplar. Compared with Arabidopsis, where the primary cell walls are type I consisting of cellulose microfibrils, xyloglucan, and pectin, rice has a typical type II cell wall with low amounts of xyloglucan and pectin, but high quantities of mixed linkage glucans (MLGs) and glucuronoarabinoxylan (GAX). Moreover, the type and relative abundance of secondary wall components differ between dicot and monocot species. Apart from cell wall composition and distribution, distinct anatomical features such as an absence of woody tissues in monocots are also found. Because of these distinctions and anatomical differences between dicot and monocot species, there are limitations in using the knowledge from dicots to understand cell wall biosynthesis in monocot species.

Transcriptome analysis has been widely used as an initial step to obtain a list of candidate genes participating in cell wall synthesis and modification in diverse species. Particularly, this approach has been very effective in exploring secondary wall-related genes in Arabidopsis and poplar (Brown et al. 2005; Dharmawardhana et al. 2010). Transcriptional regulation and gene expression pattern that are part of a specific biosynthesis pathway tend to cluster together (Jung et al. 2008a;Jung et al. 2008b). Recently, co-expression analysis has been conducted using different rice tissues at various developmental stages and resulted in the identification of secondary wall-specific GTs and GHs in rice (Hirano et al. 2013). Genes involved in xylan and lignin biosynthesis were identified as co-expressed with secondary cell wall related *cellulose synthase* genes (Ruprecht and Persson 2012; Mutwil et al. 2009). Moreover, there is evidence about the conservation of secondary cell wall synthesis transcriptional regulatory network in plants (Wang et al. 2014b; Zhong et al. 2010). Also, deep sequencing of developing wheat endosperms, in which arabinoxylan is the major cell wall structure has contributed to the identification of xylan arabinosyltransferases in wheat and rice (Anders et al. 2012). Besides tissue specific expression, gene transcripts encoding cell wall enzymes have also been shown as differentially expressed due to biotic and abiotic stresses. Considering that cell walls represent the first barrier against external stimuli, plants have evolved to fine tune cell wall type and abundance of each plant component to minimize cellular defects caused by such stresses. However, our understanding on the adaptive alteration of cell walls against external stresses is very limited.

Although a large number of rice GTs, GHs, PLs, CEs, and CBMs were identified and recorded in the Carbohydrate-Active Enzyme (CAZy) database (http://www.cazy.org/) (Lombard et al. 2014), their biological and biochemical roles in cell wall genesis and defense mechanisms against environmental challenges are poorly understood, especially in crop plants. Here, we have analyzed and suggest the putative roles of cell wall biosynthesis (CWB) related genes in rice identified from the CAZy enzyme database using meta-expression analysis in terms of anatomy, abiotic stress, biotic stress, and hormone response. Identification of CWB related genes with featured expression patterns suggest functional modules for diverse anatomical development, abiotic stresses, biotic stresses, and hormone responses. Detailed data analysis and discussion are presented.

Results and Discussion

Identification of Cell Wall Biosynthesis (CWB) Related Genes

We retrieved 1,048 non-redundant CWB related rice genes categorized in six major enzyme classes from the CAZy database (http://www.cazy.org/). Among the CWB genes, 555 belong to the glycosyltransferase (GT) class, 399 genes from the glycoside hydrolase (GH) class, 51 genes from the carbohydrate esterase (CE) class, 28 genes from the carbohydrate-binding module (CBM) class, 13 genes from the polysaccharide lyase (PL) class, and the remaining two

Table 1. Summary of known genes out of rice genes belonging to CAZY enzyme families

Locus_ID	Gene	Gene_symbol	Character_major	Character_ Minor	Featured expression cluster*	Isolation	Reference
LOC_Os01g54620.1	brittle culm 7(t)	Bc7(t)	Morphological trait	Culm leaf		Mutant	Yan et al. 2007
LOC_Os01g70200.1	-	OsIRX10	Morphological trait	Culm leaf		Mutant	Chen et al. 2013
LOC_Os03g48740.1	IAA-glucose synthase gene	OsIAGLU	Morphological trait	Culm leaf		Overexpression	Choi et al. 2012
LOC_Os09g25490.1	brittle culm 6	bc6	Morphological trait	Culm leaf	N	Mutant	Kotake et al. 2010
LOC_Os10g32980.1	cellulose synthase catalytic subunit A7	OsCesA7	Morphological trait	Culm leaf		Mutant	Tanaka et al. 2003
LOC_Os12g36890.1	narrow leaf and dwarf1	nd1	Morphological trait	Culm leaf		Mutant	Li et al. 2009
LOC_Os08g06380.1	Cellulose synthase-like F 6	cslf6	Morphological trait	Culm leaf	S	Mutant	Vega-Sánchez et al. 2012
LOC_Os03g21210.1	endo-1,4-b-D-glucanase1	glu1	Morphological trait	Dwarf	U,C	Mutant	Zhou et al. 2006
LOC_Os01g54620.1	cellulose synthase catalytic subunit A4	OsCesA4	Morphological trait	Dwarf		Mutant	Tanaka et al. 2003
LOC_Os03g26044.1	cellulose synthase A5	CESA5	Morphological trait	Dwarf	D, ABA	Others	Budot et al. 2014
LOC_Os03g48740.1	IAA-glucose synthase gene	OsIAGLU	Morphological trait	Dwarf		Overexpression	Choi et al. 2012
LOC_Os04g46980.1	cZ-O-glucosyltransferase 1	cZOGT1	Morphological trait	Dwarf	C, JA	Overexpression	Kudo et al. 2012
LOC_Os06g42020.1	cellulose synthase-like A9	CSLA9	Morphological trait	Dwarf		Others	Budot et al.,2014
LOC_Os07g14850.1	cellulose synthase A6	CESA6	Morphological trait	Dwarf		Others	Budot et al. 2014
LOC_Os08g44510.1	SPINDLY	OsSPY	Morphological trait	Dwarf	U	Knockdown	Shimada et al. 2006
LOC_Os09g25490.1	brittle culm 6	bc6	Morphological trait	Dwarf	N	Mutant	Kotake et al. 2010
LOC_Os10g32980.1	cellulose synthase catalytic subunit A7	OsCesA7	Morphological trait	Dwarf		Mutant	Tanaka et al. 2003
LOC_Os12g36890.1	narrow leaf and dwarf1	nd1	Morphological trait	Dwarf		Mutant	Li et al. 2009
LOC_Os04g41970.1	endo-1,4-b-D-glucanase	OsGLU3	Morphological trait	Root	R	Mutant	Zhang et al. 2012
LOC_Os01g69210.1	mannosyl-oligosaccharide glucosidase	OsMOGS	Morphological trait	Root		Mutant	Wang et al. 2014
LOC_Os02g34560.1	-	OsCyt-inv1	Morphological trait	Root	U, X	Mutant	Jia et al. 2008
LOC_Os03g18820.1	xyloglucan (XyG) 6-xylosyltransferase 1	OsXXT1	Morphological trait	Root	C	Mutant	Wang et al. 2014
LOC_Os04g46980.1	cZ-O-glucosyltransferase 1	cZOGT1	Morphological trait	Root	C, JA	Overexpression	Kudo et al. 2012
LOC_Os10g42750.1	Oryza sativa cellulose synthase-like D1	OsCSLD1	Morphological trait	Root	R, AP	Mutant	Kim et al. 2007
LOC_Os03g55090.1	Plastidial phosphorylase1	pho1	Morphological trait	Seed		Mutant	Satoh et al. 2008
LOC_Os04g33740.1	GRAIN INCOMPLETE FILLING 1	GIF1	Morphological trait	Seed		Natural variation	Wang et al. 2008
LOC_Os02g52710.1	Alpha-amylase I-1	AmyI-1	Morphological trait	Shoot seedling		Knockdown Overexpression	Asatsuma et al. 2005
LOC_Os07g32060.1	UDP-glycosyl transferase	-	Others	Others		Overexpression	Chen et al. 2014
LOC_Os09g28400.1	Alpha-amylase 3A	Amy3A	Physiological trait	Eating quality		Knockdown	Hakata et al. 2012
LOC_Os02g52710.1	Alpha-amylase 1A	Amy1A	Physiological trait	Eating quality		Knockdown	Hakata et al. 2012
LOC_Os09g28420.1	Alpha-amylase 3B	Amy3B	Physiological trait	Eating quality	S	Knockdown	Hakata et al. 2012
LOC_Os08g40930.1	isoamylase1	OsISA1	Physiological trait	Eating quality	AF, X	Overexpression	Utsumi et al. 2011
LOC_Os03g55090.1	Plastidial phosphorylase1	pho1	Physiological trait	Eating quality		Mutant	Satoh et al. 2008
LOC_Os05g32710.1	isoamylase2	OsISA2	Physiological trait	Eating quality		Overexpression	Utsumi et al. 2011
LOC_Os09g29404.1	isoamylase3	isa3	Physiological trait	Eating quality		Mutant	Yun et al. 2011
LOC_Os08g09230.1	Soluble starch synthase IIIa	SSIIIa	Physiological trait	Eating quality	AF	Knockdown	Zhang et al. 2011

Table 1. Continued

Locus_ID	Gene	Gene_symbol	Character_major	Character_ Minor	Featured expression cluster*	Isolation	Reference
LOC_Os06g04200.1	waxy	wx	Physiological trait	Eating quality	AF	Natural variation	Wang et al. 1995
LOC_Os06g12450.1	Soluble starch synthase IIa	SSIIa	Physiological trait	Eating quality	AF	Knockdown	Zhang et al. 2011
LOC_Os06g06560.1	starch synthase I	OsSSI	Physiological trait	Eating quality	D, ABA, X	Mutant	Fujita et al. 2006
LOC_Os02g34560.1	-	Oscyt-inv1	Physiological trait	Flowering	U, X	Mutant	Jia et al. 2008
LOC_Os02g52710.1	Alpha-amylase I-1	AmyI-1	Physiological trait	Germination dormancy		Knockdown Overexpression	Asatsuma et al. 2005
LOC_Os01g69030.1	sucrose phosphate synthase 1	OsSPS1	Physiological trait	Panicle flower		Mutant	Hirose et al. 2014
LOC_Os02g32660.1	Branch enzyme isozyme	BE2b	Physiological trait	Source activity	D	Others	Abe et al. 2014
LOC_Os03g48170.1	FLUORY ENDOSPERM6	FLO6	Physiological trait	Source activity		Mutant	Peng et al. 2014
LOC_Os01g57854.1	pectin methylesterase1	PME1	Physiological trait	Source activity	X	Knockdown Overexpression	Kang et al. ,2011
LOC_Os06g06560.1	Starch synthase 1	SS1	Physiological trait	Source activity	D, ABA, X	Others	Abe et al. 2014
LOC_Os06g51084.1	Branch enzyme isozyme	BE1	Physiological trait	Source activity	D	Others	Abe et al. 2014
LOC_Os01g71930.1	Beta-1,3-glucanase 1	Osg1	Physiological trait	Sterility		Knockdown	Wan et al. 2011
LOC_Os01g15780.1	GLYCOSYLTRANSFERASE1	OsGT1	Physiological trait	Sterility		Mutant	Moon et al. 2012
LOC_Os02g34560.1	-	Oscyt-inv1	Physiological trait	Sterility	U, X	Mutant	Jia et al. 2008
LOC_Os12g36890.1	NARROW AND ROLLED LEAF 1	nrl1	Physiological trait	Sterility		Mutant	Wu et al. 2010
LOC_Os05g31140.1	Beta-glucanase1	Gns1	Resistance or Tolerance	Blast resistance	ABA	Overexpression	Nishizawa et al. 2003
LOC_Os09g34250.1	salicylic acid glucosyltransferase1	OsSGT1	Resistance or Tolerance	Blast resistance	JA	Knockdown	Umemura et al. 2009
LOC_Os11g47580.1	Rice xylanase inhibitor	RIXI	Resistance or Tolerance	Blast resistance		Others	Hou et al. 2014
LOC_Os05g44210.1	trehalose-6-phosphate synthase1	OsTPS1	Resistance or Tolerance	Cold tolerance		Overexpression	Li et al. 2011
LOC_Os05g44210.1	trehalose-6-phosphate synthase1	OsTPS1	Resistance or Tolerance	Drought tolerance		Overexpression	Li et al. 2011
LOC_Os01g54620.1	cellulose synthase catalytic subunit A4	OsCesA4	Resistance or Tolerance	Lodging resistance		Mutant	Tanaka et al. 2003
LOC_Os09g25490.1	brittle culm 6	bc6	Resistance or Tolerance	Lodging resistance	N	Mutant	Kotake et al. 2010
LOC_Os09g32080.1	brittle culm 15/Oryza sativa Chitinase-like 1	BC15/OsCTL1	Resistance or Tolerance	Lodging resistance	U	Mutant	Wu et al. 2012
LOC_Os10g32980.1	cellulose synthase catalytic subunit A7	OsCesA7	Resistance or Tolerance	Lodging resistance		Mutant	Tanaka et al.,2003
LOC_Os08g06380.1	Cellulose synthase-like F 6	cslf6	Resistance or Tolerance	Lodging resistance	S	Mutant	Vega-Sánchez et al. 2012
LOC_Os05g15770.1	rice xylanase-inhibiting protein (XIP)-type gene	OsHI-XIP	Resistance or Tolerance	Other stress resistance	D, BPH	Overexpression	Xin et al. 2014
LOC_Os05g44210.1	trehalose-6-phosphate synthase1	OsTPS1	Resistance or Tolerance	Salinity tolerance		Overexpression	Li et al. 2011

*Indicates the meta-expression based featured cluster of known genes. Abbreviations: N indicates Node, internode collar and lamina joint preferred expression patterns; U, Ubiquitous expression; R, Root preferred; AF, Anther and flower preferred; D, Drought induced; S, Salt induced; C, Cold induced; ABA, ABA induced; JA, JA induced; AP, All pathogen induced; BPH, BPH induced; X, XOO induced.

genes were classified with auxiliary activity (AA). The entire gene list is presented in Table S1.

Analysis of the Functional Roles of CWB Genes Using Known Genes

To identify the functional roles for the 1,048 CWB genes analyzed in this investigation, we searched the OGRO database (http://qtaro.abr.affrc.go.jp/ogro) that summarizes recent rice mutant studies (Yamamoto et al. 2012). Forty-four CWB genes were identified from this database; among them, 11 genes were involved in more than one trait category with multiple phenotypic effects, 27 genes were related to morphological traits, 23 were related to physiological traits, 12 were related to stress resistance or tolerance, and one gene with unclassified function (Table 1).

Regarding morphological traits, 19 CWB genes were related to plant height or culm strength/development, six genes in root growth, and two genes in seed development. Major physiological effects of the CWB genes were observed in the panicle, in which 11 genes were involved in regulating eating quality, two genes involved in flowering, and four genes related to sterility and source activity. The functional identification of stress related genes clearly indicate that the CWB genes play roles in stress tolerance responses. Among the resistance conferring candidates, three were associated with biotic stress and the *OsTPS1* (trehalose-6-phosphate synthase1) gene confers abiotic stress resistance, five genes attributed to lodging resistance, and one gene was related with other kinds of stress resistance.

Based on the analysis of known CWB genes, we speculate that the CWB genes in rice might play diverse biological and physiological functions. To obtain functional clues for the remaining CWB genes, a meta-expression analysis based on a large collection of transcriptome data was undertaken. The rice oligonucleotide array database, RiceXPro and Genevestigator provide diverse meta-expression pattern data for rice genes (Grennan 2006; Sato et al. 2011; Cao et al. 2012). Loss of function studies of seven GT genes resulted in an alteration of cell wall constituency and fragile culm with or without morphological phenotypes. For example, mutations in the *cellulose synthase-like F6* (*Cslf6*, *LOC_Os08g06380*) gene, which mediates the biosynthesis of cell wall polymerase MGL, resulted in reduced stem diameter and a reduction in plant height (Vega-Sanchez et al. 2012). Ten out of 11 gene mutants exhibited dwarf phenotypes that also belong to the GT class, demonstrating the importance of GT class genes in plant skeletal structure. Therefore, modulating the activity of CWB genes might be a feasible mechanism for improving biomass and lodging resistance in plants. Among the genes related to root traits, defects in root hair elongation were observed in *OsGLU3* (*LOC_Os04g41970*), *OsMOGS* (*LOC_Os01g69210*) and *OsXXT1* (*LOC_Os03g18820*) mutants (Zhang et al. 2012; Wang et al. 2014a; Wang et al. 2014c). Among them, the *osglu3* and *osmogs* mutants showed a decrease in root cellulose content. The role of CWB genes associated with seed development was identified by the variation of total endosperm starch content in mutants. In addition, the stress dependent function of CWB genes was evident from functional studies. For example, a mutation in the *Pho1* gene (*LOC_Os03g55090*) produced normal seeds at 30°C, while the plants at 20°C caused changes in seed morphology with low starch accumulation (Satoh et al. 2008). These results indicate that CWB genes are important in diverse developmental processes such as seed development, culm strength, and biomass increase. Meta-expression analyses might provide useful clues for further functional studies.

Meta-expression Analysis of CWB Genes

Meta-expression analysis based on a large collection of microarray data is a useful way to provide gene expression data in diverse aspects according to the feature of samples in the meta-expression database (Jung et al. 2011). Currently, more than 5,000 rice microarray data are available from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO, http://www.ncbi.nlm.nih.gov/geo/) (Barrett et al. 2011; Jung et al. 2011; Chandran and Jung 2014). Based on rice microarray data from GEO, we developed meta-expression databases for anatomical expression, abiotic stress response, and hormone response analyses (Materials and Methods) (Nguyen et al. 2013). These databases cover more than 35,000 rice loci (MSU-locus identifier) including isoforms that were annotated by the rice genome annotation project team (Ouyang et al. 2007). Out of the 1,048 rice CWB genes information from the CAZy database, 929 genes have probes in the Affymetrix array platform. Expression analysis of these 929 CWB genes using meta-expression databases revealed diverse groups (functional modules) of CWB genes showing similar tissue/organ-specific or abiotic stress-specific expression patterns. For featured hormone responsive expression patterns, we used Agilent 44K array data and 979 of 1,048 rice CWB genes were analyzed.

Identification of Rice CWB Genes Showing Featured Anatomical Expression Patterns

k-means clustering (KMC) analysis and manual editing of Affymetrix anatomical-meta expression data generated five groups with featured anatomical expression comprising 84 genes; 20 genes showed ubiquitous expression regardless of the tissue/organ type, 33 were preferentially expressed in the anther/pollen/endosperm, 11 genes showed leaf, shoot and

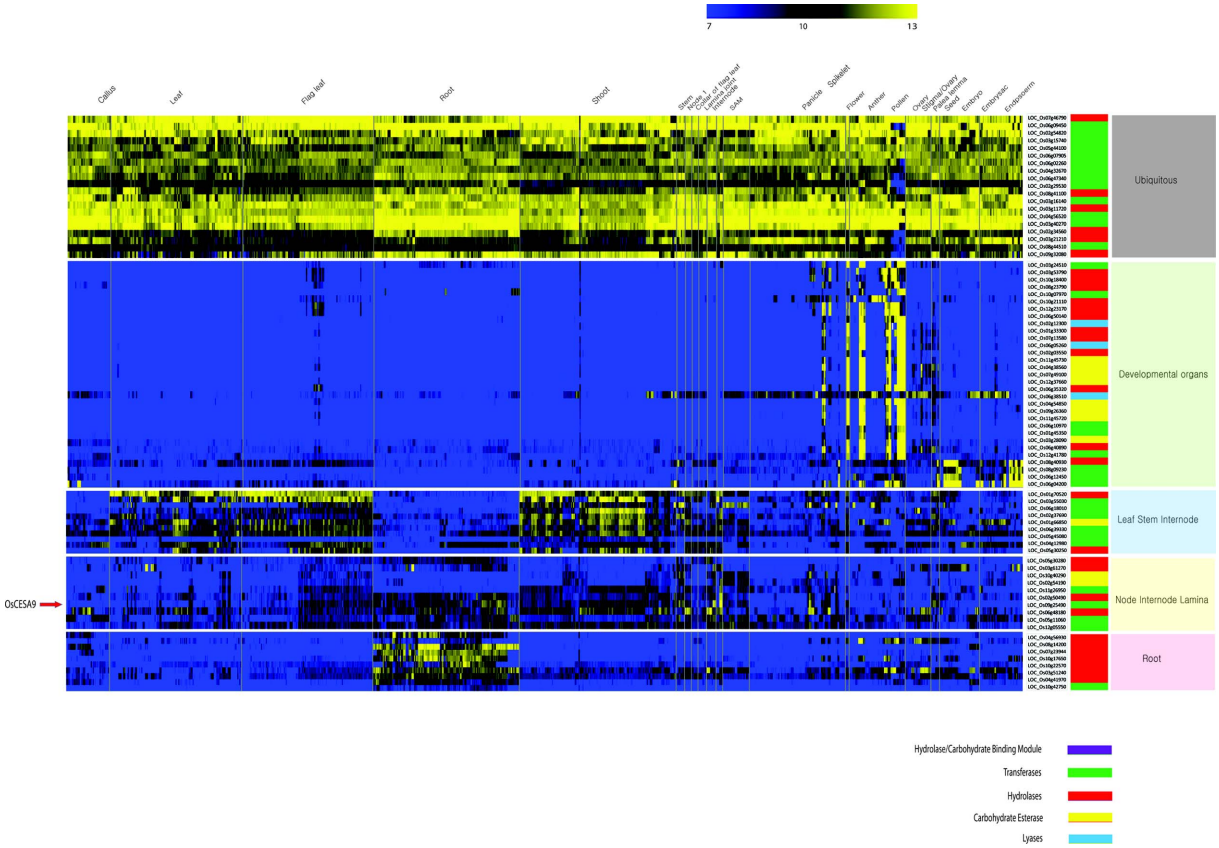

Fig. 1. Meta-expression analysis of CWB genes showing featured anatomical expression patterns. The red arrow indicates the position of *OsCESA9*. Color scale for the heatmap is shown on the top. Five clusters were generated and the corresponding genes are indicated. The enzyme class of each gene is represented using a different color code below the heatmap.

internode preferred expression, 10 genes were preferentially expressed in the node/ internode/collar/lamina joint, and 10 genes were expressed in the roots (Fig. 1, Table S2). The remaining genes did not show clear expression patterns.

In the ubiquitously expressed functional module, six genes were found to belong to the GH class and 14 genes encoded GT enzymes. Callose wall synthesis is important for pollen fertility and 10 genes encoding *callose synthase* (CS) were reported in rice previously (Yamaguchi et al. 2006), whereas the Arabidopsis genome encodes 12 *callose synthase* genes (Jacobs et al. 2003; Xie et al. 2012). Among the previously reported rice CS genes, we found that *LOC_Os06g02260* is ubiquitously expressed and might have an impact on pollen tube development. The monocot divergent transcript *LOC_Os08g41100* encodes a chitinase family protein (CHIT12) and anatomical expression of the putative chitinase family genes indicated that *LOC_Os08g41100* is a ubiquitously expressed gene.

Of the known CWB genes included in the featured anatomical expression associated with stem, *SPINDLY* (*SPY, LOC_Os08g44510*) encoding GT enzyme negatively regulates elongation of rice lower internodes (Shimada et al. 2006) and is a potential target for increasing plant biomass through the genome editing mediating loss of function method. Loss of function of ubiquitously expressed *Brittle culm 15* (*BC15, LOC_Os09g32080*) revealed its significance in cell wall synthesis evidenced by reduced cellulose content and low mechanical strength in *BC15* knock down plants (Wu et al. 2012). From a previous report on genome-wide duplicated genes in rice (Jiang et al. 2013), it was found that the *CHIT12* encoding chitinase family protein precursor (*LOC_Os08g41100*), a monocot divergent candidate, is a possible segmental duplicate (SD) of *BC15*. Based on integrated anatomical expression patterns and phylogenic relationship of the chitinase family, we conclude that both genes show ubiquitous expression patterns and have functional redundancy (Figure S1). *OsCyt-inv1* (*LOC_Os02g34560*) encoding the neutral invertase domain protein is primarily involved in root cell elongation and flowering. Accumulation of sucrose in the mutants and the recovery of growth defects in the mutants after a supply of glucose indicate the role of *OsCyt-inv1* in glucans (MLGs) and glucuronoarabinoxylan rich type II cell wall biosynthesis (Jia et al. 2008).

Thirty-one genes were categorized in the anther/pollen/

endosperm preferred expression functional module. Of them, 12, nine, eight, and three genes were of the GH, GT, CE, and lyase enzyme family, respectively. Seventeen genes belonged to the monocot-divergent group, indicating that unique mechanisms for cell wall biosynthesis are important during the development of reproductive organs in monocots.

Pollen tube growth in rice is exclusive at its tip, which is composed of a single layer of pectin. Pectin metabolism and associated modification is crucial for proper pollen tube elongation (Tian et al. 2006). In line with this assumption, eight genes were identified that encode pectinesterase and three genes encode precursors of pectate lyase. Based on enzyme class and the anatomical features of expression pattern, it is likely that these genes might take part in rice pollen tube elongation by mediating pectin biosynthesis. Eleven genes were preferentially expressed in stem parts like the leaf, shoot, and internode, and were categorized as six GTs, four GHs, and one CE. Among them, five genes contain UDP-glucoronosyl and UDP-glucosyl transferase domains, which are associated with sugar metabolism (Huang et al. 2008). Notably, nine of them were monocot divergent genes. Expression of a group of 10 genes was comparatively high at the lamina joint, node, or internode and therefore might be significant candidates for plant height or biomass traits. Four genes belonged to the GH class and the remaining two genes were CE class genes. Among the internode-preferred genes, *brittle culm 6* (*bc6*, *LOC_Os09g25490*) encoded a cell wall specific CesA protein that is important for secondary cell wall cellulose deposition. In the *culm of Bc6* mutant, cellulose was decreased by 38%. In the group showing root preferred expression, eight out of 10 genes belonged to the GH class and the remaining two genes belonged to the GT class. Among these genes, the activity of *OsCSLD1* (*LOC_Os10g42750*) is required for root hair elongation (Kim et al. 2007).

Identification of Rice CWB Genes Showing Featured Abiotic Expression Patterns

The plant cell wall immediately responds to environmental challenges and structural modification of the cell wall is essential in the defense response against stress. To estimate candidate genes important for the abiotic stress response mediated by the cell wall system, we analyzed the expression pattern of CWB genes across multiple sets of abiotic stress data (Nguyen et al. 2013). Clustering analysis revealed 78 drought-induced genes that were grouped into four enzyme classes, namely, GH (49 genes), GT (24 genes), CBM (four genes), and one CE gene (Fig. S2; Table S3). A major role for the GH group is in remodeling of the cell wall and the functional role of these genes in abiotic stress has been elucidated previously (Minic and Jouanin 2006; Tyler et al. 2010). In our analysis, the transcripts of 14 putative GH family proteins (annotated by RGAP) were induced under water stress, providing a hint that their function is related to alterations in cell wall elasticity under water stress. In total, 62 genes were significantly induced under salt stress (Table S3), where 33 were GHs class, 28 were GTs, and one belonged to the CE class. Of them, 24 genes were also induced under drought stress and might be due to overlapping signal transduction under drought and salt conditions.

Abiotic stress database analysis revealed 80 cold induced CWB genes that were distributed into GH (32 genes), GT (42 genes), CE (four genes) and CBM (two genes) groups (Table S3). Of these, 10 and 14 genes were also induced by drought and salt stress, respectively. Using anatomical expression patterns, we further classified cold responsive genes to three groups, including the expression of 14 genes that were significantly high in the flower and related organs, suggesting the role in response against the stress acclimation in these organs. Nine genes showed root preferred expression and 16 genes displayed ubiquitous expression patterns.

We found that the function of three genes that featured abiotic stress responses were previously reported. Drought induced *starch synthase I* (*OsSSI*, *LOC_Os06g06560*) in coordinated action with other starch synthase genes, influences the gelatin temperature of the endosperm by altering amylopectin chain length (Oikawa et al. 2010). By comparing anatomical meta-expression patterns with abiotic stress expression, it was found that the root preferred GH family 17 gene (*LOC_Os03g51240*) significantly accumulated under drought stress and suggests role of coping with drought stress in the root. Heterologous expression of rice cell wall invertase in Arabidopsis exhibited an increase in root mass as well as entire plant biomass (von Schweinichen and Büttner 2005). In our analysis, transcript levels of two putative cell wall invertases, *LOC_Os03g20020* and *LOC_Os04g33490*, were observed as stimulated under drought stress, suggesting the potential role of these genes under drought stress. Low levels of active cytokinins in plants under cold stress might be a result of its conjugation by zeatin O-glycosyltransferase (Li et al. 2000). We identified a cold stress induced *cZOGT* gene (*LOC_Os04g47720*) that might be related to cytokinin regulation under cold stress.

Identification of Rice CWB Genes Showing Featured Hormonal Responses

Hormone signaling plays a significant role in various stress responses and developmental progresses (Smekalova et al. 2014). Expression profiling of CWB genes under six hormone treatments identified three groups of CWB genes with featured expression patterns, including ABA induced genes, ABA and JA induced genes, and JA induced genes

(Fig. S3, Table S4).

Regarding the ABA response, we identified 46 CWB genes (group 1) that were significantly induced by ABA treatment. Of them, 20 genes belonged to the GH class, 25 to GT, and one to CBM. The ABA response of these genes was more obvious at 3 hrs or 6 hrs treatments relative to the initial treatment for 30 min (Fig. S4). Induction of gene expression was higher in the root tissues than in the shoot. In comparison with abiotic stress responses, 25 ABA induced genes were also responsive to drought stress and 11 genes were also induced under salt stress, explaining 80% (36/45) of all the ABA inducible CWB genes. A higher proportion of drought or salt inducible ABA responsive genes indicates that endogenous ABA level increases in plants that are subjected to drought or salt stress. Previous studies identified the antagonistic role of ABA and JA signaling in the stress response and plant defense system (Anderson et al. 2004). In parallel with these studies, expression of ABA responsive CWB genes in JA treatment was low, especially in the roots. Interplay between two cis regulatory elements (CREs) is required for ABA responsiveness (Gomez-Porras et al. 2007). Based on previous genome-wide analysis of ABA responsive elements in the rice genome (Gomez-Porras et al. 2007), we identified that six ABA responsive genes in our study have ABRE pairs at the promoter region. ABRE-ABRE pairs can functionally act as ABA-responsive complexes (ABRCs).

Fifty-six CWB genes (group 2) showed significant induction in response to JA treatment and of them, 20 genes belong to the GH, 35 to GT, and one gene was from the CBM class. JA plays a crucial role in the biotic stress-mediated defense response. Studies using cell wall mutants have revealed the link between cell wall synthesis and the JA signaling pathway (Cruz et al. 2013). Due to the JA-dependent expression patterns, genes from group 2 were assigned to the pathogen-mediated defense response. As expected, biotic stress expression analysis of genes in group 2 revealed that 21 genes showed an induced expression pattern in response to *M. grisea*, *M. oryzae*, *X. oryzae* and brown planthopper (Fig. S5). Interestingly, 11 CWB genes were significantly induced under ABA and JA treatment in roots, with seven genes belonging to the GT class, three to GH, and one to CBM. Genes from group 3 were regulated by a synergistic relationship between ABA and JA, and are also important in drought or cold stress responses. In addition to the antagonistic relationship between ABA and JA in the defense response (Anderson et al. 2004), a synergistic role of ABA in the JA-dependent biotic stress defense response was identified (Adie et al. 2007).

Two genes (*LOC_Os05g31140* and *LOC_Os09g34250*) identified in the hormone responsive gene module were previously characterized and those genes are known to be associated with disease resistance. As we mentioned earlier, this observation confirms the multi-dimensional roles of cell wall related genes and their involvement in biotic stress. Plants overexpressing beta-glucanase1 (*Gns1*, *LOC_Os05g31140*) developed resistance type lesions on the leaves when infected with a virulent strain of *M. grisea*. The development of brown specks and activation of the defense related genes *PR-1* and *PBZ1* in transgenic plants revealed the biotic stress resistance role of these genes (Nishizawa et al. 2003). *LOC_Os09g34250,* which encodes salicylic acid glucosyl-transferase 1 (*OsSGT1*), converts free salicylic acid (SA) into SA O-b-glucoside (SAG). SAG is a chemical stress resistant agent and SAG production mediated by *OsSGT1* is the key component of the SA-mediated defense response (Umemura et al. 2009). Induction of *OsSGT1* levels under JA treatment in our analysis suggests a role for JA in the *OsSGT1* mediated stress defense and furthermore, crosstalk between JA and SA.

Identification of Rice CWB Genes Showing Biotic Stress Expression Patterns

Plants are known to trigger multi-level events based on cell wall rearrangement in order to defend against pathogen attack. Elucidation of candidate genes conferring cell wall integrity (CWI) is essential for developing disease resistant plants. To reveal pathogen responsive CWB genes, we carried out a meta-expression analysis of CWB genes using biotic stress database. This analysis clustered CWB genes into three categories based on response to pathogen type. The expression of 50 genes was highly stimulated under BPH infection, 111 genes were induced under *Xoo* infection, and the induction of 43 genes was observed under all types of pathogen treatments (Fig. S6, Table S5). Among the BPH induced genes, role of xylanase inhibitor protein (OsHI-XIP, *LOC_Os05g15770*) that functions against larval growth of rice striped stem borer (SSB) was previously studied (Xin et al. 2013). In concert with our analysis, OsHI-XIP was also associated with BPH pathogenicity where overexpression of the protein decreased oviposition preferences of BPH. Hence, there might be additional BPH induced genes in signaling events for tolerance against herbivore attack. Interplay between hormone signaling and biotic stress is well established in plant species (O'Brien and Benkova 2013). In addition, there is evidence for the synergistic or antagonistic action of SA and JA during plant-pathogen interaction (Santino et al. 2013). We identified that 24 biotic stress induced genes were also induced under ABA treatment, 49 genes with JA treatment, 26 genes were induced under both ABA and JA treatment, and seven genes were expressed under ABA, IAA, and JA treatment. Therefore, we propose that genes in this category might contribute to pathogen

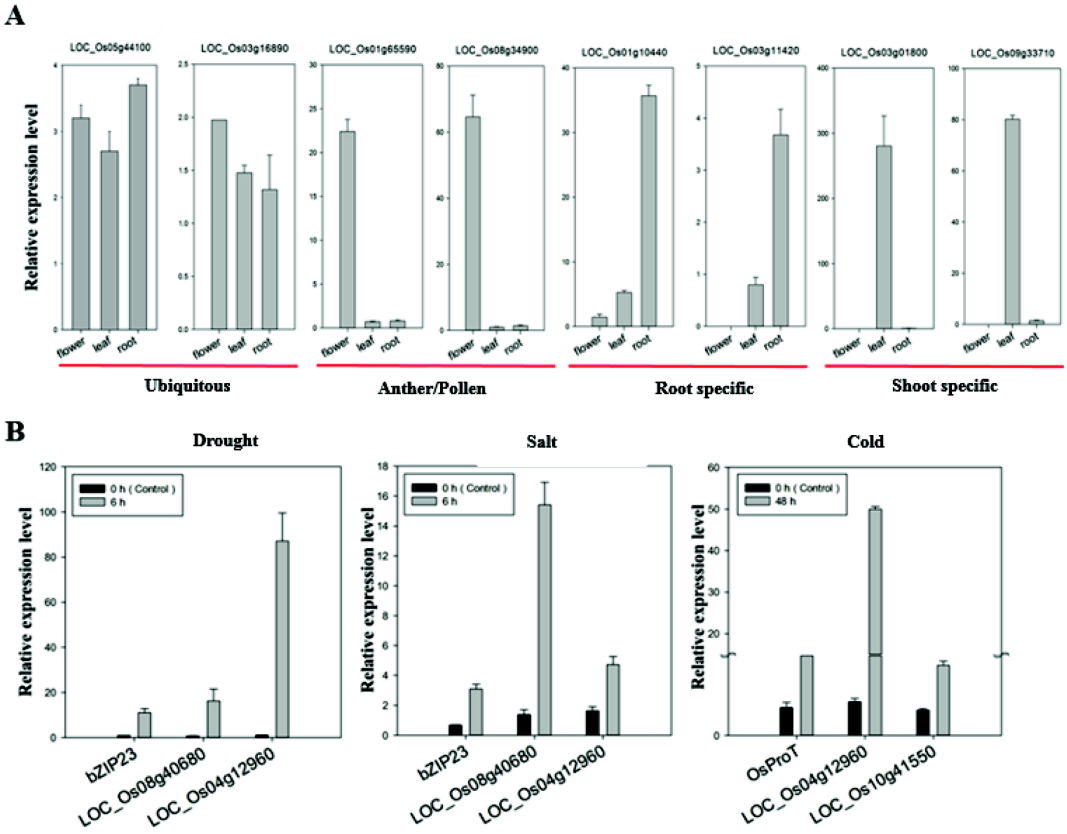

Fig. 2. Validation of expression patterns for rice cell wall related genes with preferential expression levels in tissues or in response to abiotic stress by RT-PCR. Two genes were randomly chosen for each featured group. (A) Quantitative PCR analysis of the gene expression in different tissues. The lowest expression level of each gene among the tissues analyzed was set to 1. (B) Quantitative PCR analysis of the expression of genes in response to different abiotic stresses. *OsbZIP* was used as marker cDNA control for drought and salt stress. The *OsProT* gene was used as marker cDNA control for cold stress. Error bars denote the standard error (SE) of three biological replicates.

adaptation associated with hormone signaling.

Validation of fEatured Gene Expression Patterns

To confirm the expression pattern of CWB genes with featured expression groups, we carried out RT-PCR analyses using various anatomical tissues and organs and diverse abiotic stress treatments. We tested the expression of two genes in each of the ubiquitous, anther/pollen/endosperm preferred, root-preferred, and shoot-preferred anatomical featured groups. Subsequently, we confirmed the featured anatomical expression patterns of the following genes, including ubiquitous expression of *LOC_Os05g44100* encoding trehalose synthase and *LOC_Os03g16890* encoding N-acetylglucosaminyltransferase. *LOC_Os01g65590* encoding galactosyltransferase and *LOC_Os08g34900* encoding pectinesterase showed preferred expression in flower including anther/pollen. In addition, *LOC_Os01g10440* coding xylosyltransferase and *LOC_Os03g11420* encoding Os3bglu6 beta-glucosidase showed root preferred expression, and *LOC_Os03g01800* encoding a GH family 16 protein and *LOC_Os09g33710* encoding Os9bglu33 beta-glucosidase showed preferred expression in the shoot (Fig. 2A, Table 2).

To evaluate and confirm CWB gene expression patterns with featured abiotic stress responses, we also tested the expression patterns of two genes in each of three abiotic stresses including drought, salt, and cold. Before testing expression patterns of the candidate genes for abiotic stress response, we first tried to confirm the expression pattern of marker genes for each stress to evaluate the applied abiotic stress. For drought and salt stress, we analyzed the expression pattern of *basic leucine zipper23* gene (*bZIP23*, *LOC_Os02g52780*) as the molecular marker (Xiang et al. 2008) and for cold stress, we used rice proline transporter gene *OsProT* (AB022783) (Igarashi et al. 2000) (Fig. 2B). Subsequently, we identified that expression of the *bZIP23* gene was significantly upregulated at 6 hrs after drought or salt stress treatment and *OsProT* was significantly upregulated at 48 hrs after cold stress treatment. *LOC_Os08g40680* encoding a GH enzyme showed a similar expression pattern with *bZIP23*, indicating that *LOC_Os08g40680* plays a significant role in the abiotic stress response. The expression

Table 2. Summary of selected genes for validating the featured expression patterns using RT-PCR analysis

Locus_id	Featured Cluster	RT-PCR Result	RGAP Annotation
LOC_Os05g44100	Ubiquitous expression	Flower Leaf and Root expression	Trehalose synthase, putative, expressed
LOC_Os03g16890	Ubiquitous expression	Flower Leaf and Root expression	N-acetylglucosaminyltransferase, putative, expressed
LOC_Os01g65590	Anther Flower	Flower preferred expression	Galactosyltransferase, putative, expressed
LOC_Os08g34900	Anther Flower	Flower preferred expression	Pectinesterase, putative, expressed
LOC_Os01g10440	Root	Root preferred expression	Xylosyltransferase, putative, expressed
LOC_Os03g11420	Root	Root preferred expression	Os3bglu6 - beta-glucosidase/beta-fucosidase/beta-galactosidase, expressed
LOC_Os03g01800	LEAF SHOOT	Leaf preferred expression	Glycosyl hydrolases family 16, putative, expressed
LOC_Os09g33710	LEAF SHOOT	Leaf preferred expression	Os9bglu33 - beta-glucosidase homologue, similar to G. max hydroxyisourate hydrolase, expressed
LOC_Os08g40680	Drought and Salt induced	Drought and Salt induced	Glycosyl hydrolase, putative, expressed
LOC_Os04g12960	Drought, Salt and Cold induced	Drought, Salt and Cold induced	UDP-glucoronosyl/UDP-glucosyl transferase, putative, expressed
LOC_Os10g41550	Cold and Salt induced	Cold induced	Beta-amylase, putative, expressed

of *LOC_Os04g12960* encoding a GT enzyme was significantly stimulated under the three abiotic stress conditions and indicates the role for multi-abiotic stress responses. Expression of the glycosyl hydrolase 14 family gene *LOC_Os10g41550* encoding beta-amylase showed significant upregulation under cold stress conditions, informing the potential involvement of this enzyme in the cold stress response (Table 2). Also, reports are available on the induction of beta-amylase in correlation with maltose accumulation in response to acute temperature stress (Kaplan and Guy 2004). Consistent with that report, *LOC_Os10g41550* also demonstrated a drought-induced pattern in our meta-expression analysis, supporting the reliability of the abiotic stress response of CWB genes in rice.

Construction of a Co-expression Network Mediated by *OsCESA9* for Cell Wall Biosynthesis

For analyzing cell wall biosynthesis in rice, we used a T-DNA insertional mutant in the *OsCESA9* (*LOC_Os09g25490*) gene because of its demonstrated role in cell wall biosynthesis and plant growth (Kotake et al. 2011). The *oscesa9* mutant has a T-DNA insertion in exon 11 and showed severe defects in growth and cell wall biosynthesis compared to wild-type alleles of this gene (Fig. 3). In this mutant, we confirmed its role in secondary cell wall biosynthesis by observing the collapsed meta-xylem (indicated by red arrows in Fig. 3D) due to reduced wall thickness.

To get insight into the molecular mechanism of *OsCESA9,* we first adopted a method of co-expression network using anatomical meta-expression data from Genevestigator (Grennan 2006) and constructed a network of *OsCESA9* with 25 co-expressed genes (Fig. 4, Table S6). Of them, we tested the expression patterns of four genes with annotated functions in the *oscesa9* mutant and compared the results with those of the wild type (Fig. 4). As a result, we found that the

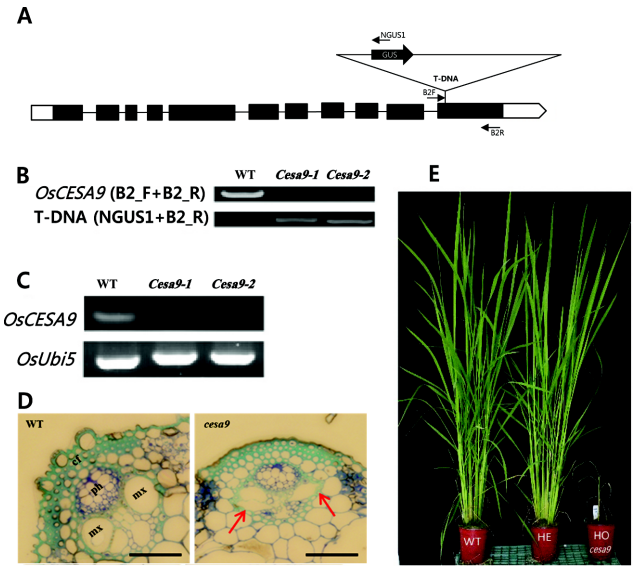

Fig. 3. Identification and phenotypic characterization of the *OsCESA9* T-DNA mutant. (A) A diagram showing the site of T-DNA insertion in the *OsCESA9* (*LOC_Os09g25490*) gene. (B) PCR identification of a homozygous *oscesa9* mutant. Amplified *OsCESA9* fragment with primers spanning the T-DNA insertion site is shown. T-DNA indicates the PCR product with a T-DNA left border primer and an *OsCESA9* flanking primer. WT, wild type. (C) RT-PCR results showing no amplification of the *OsCESA9* gene product in homozygous mutants (*oscesa9-1* and *osces9-2*). *OsUbiquitin5* was used as quantitative cDNA control. (D) Cross sections of wild type and mutant leaves. Note the collapsed metaxylem (indicated by red arrows) in the homozygous mutant due to reduced wall thickness. The middle parts of two-month-old leaves were sectioned and stained with toluidine blue for anatomy. cf, cortical fiber; ph, phloem; mx, metaxylem. Bars = 100 μm. (E) Morphology of two-month-old wild type and *oscesa9* mutant. Note the dwarf phenotype in the *oscesa9* mutant. HE, heterozygous line of *Oscesa9* mutant; HO, homozygous line of *Oscesa9* mutant

expression of four genes was significantly reduced in the *oscesa9* mutant, suggesting downstream events affected by

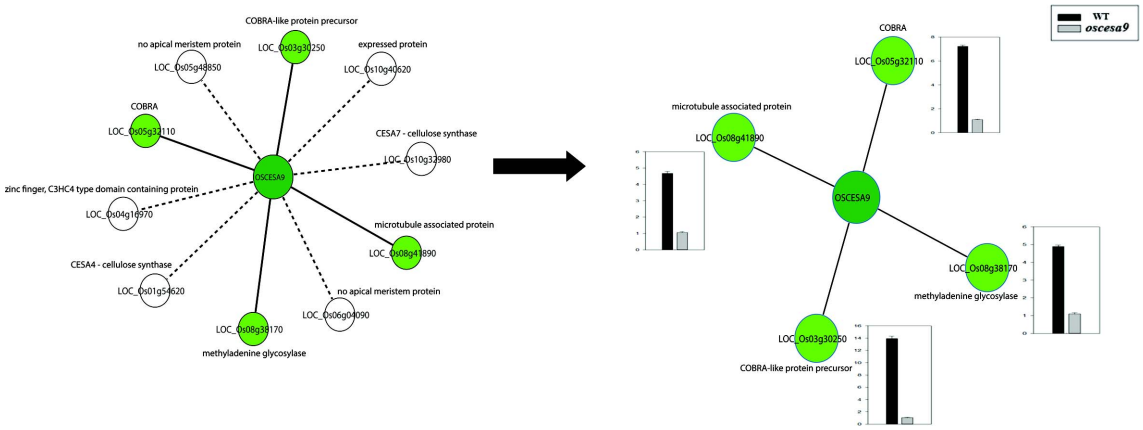

Fig. 4. Co-expression network of *OsCESA9* and construction of a refined network using the *oscesa9* mutant. Genevestigator was used to find the most co-expressed genes in *OsCESA9* and the primary functional module was generated using Cytoscape. The network was further refined using differential expression data in the *oscesa9* mutant compared to the wild type plant of selected co-expressed genes. Expression patterns of four genes co-expressed with the *OsCESA9* gene were significantly downregulated. cDNAs were prepared from two-month-old leaves of the wild type (black bar) and the *oscesa9* T-DNA mutant and the expression patterns of four genes using quantitative RT-PCR were examined. Error bars indicate the standard deviation of three biological replicates.

mutation in the *OsCESA9* gene. Of them, *LOC_Os03g30250* and *LOC_Os05g32110* encoding COBRA protein has gene ontology (GO) terms relating to secondary cell wall biogenesis and lignin metabolic process. Thus, we expect that *LOC_Os03g30250* and *LOC_Os05g32110* function in secondary cell wall biogenesis and lignin metabolic processes associated with *OsCESA9*. Consistently, in Arabidopsis, *COBRA* (*COB*) encodes a putative GPI-anchored protein and *COB* RNA level is remarkably high in the root elongation zone. Mutation of the gene revealed its role as a regulator of oriented cell expansion in the root (Schindelman et al. 2001). Mutation in the *Bk2* gene encoding maize Cobra-like protein exhibited a reduction in stalk tissue mechanical strength, which was accompanied by a reduction in cellulose content and uneven secondary cell wall material (Ching et al. 2006). Both studies indicate the significance of COBRA in the proper elongation and morphology of the root and shoot. *LOC_Os08g38170* encodes methyladenine glycosylase is related to DNA repair based on GO biological processes. Downregulation of the methyladenine glycosylase gene in the *oscesa9* mutant indicated that the DNA repair process is located at a pathway downstream of *OsCESA9*. Previous analysis on the Arabidopsis 3-methyladenine glycosylase gene *(aMAG*) indicated that expression of the gene is high in rapidly dividing tissues and in addition to the DNA replication and repair process, *aMAG* also facilitates cell growth (Shi et al. 1997). Another *oscesa9* mutant dependent downregulated gene *LOC_Os08g41890* encoding microtubule associated protein has a GO term relating to the cytoskeleton in cellular component. Downregulation of the microtubule associated protein gene showed that *OsCESA9* might be also important for the cell division process. Consistently, genetic analysis of the *cellulose synthase-like D* gene (*CslD*) from maize indicated the role of *CslD* in cell division where a mutation of this gene was observed with reduced leaf width, the number of cell fibers across the blade, and other narrow-organ phenotypes (Hunter et al. 2012) Co-expression network analysis of *OsCESA9* and the construction of a refined network using the *oscesa9* mutant and RT-PCR analysis quickly suggested the potential downstream molecular mechanism. Further studies will be required to clarify the model suggested by our study.

Evolutionary Role of OsCESA9 using Phylogenomic Analysis

To understand the evolutionary relationship and the functional area of cellulose synthase genes, we conducted a phylogenomics analysis using putative cellulose synthase protein sequences. RGAP annotated 11 putative genes and among them, two genes (*LOC_Os6g39970* and *LOC_Os12g29300*) were not present in the CAZy database. After omitting these entries, a phylogenetic tree was constructed using the maximum likelihood method with 500 bootstraps using MEGA 6 software (Tamura et al. 2013) (Fig. S7). Further, we integrated the anatomical expression data into the tree context. In the analysis, OsCESA9 was closely linked to OsCESA3, OsCESA5 and OsCESA6, which form a clade. OsCESA1, OsCESA2 and OsCESA8 were connected to this group and formed an additional subclade. OsCESA7 and OsCESA8 are divergent from both of these groups. Expression pattern data covering developmental or anatomical samples throughout the plant lifecycle can be a much clearer source to determine functional roles. Expression of *OsCESA3, OsCESA5, OsCESA6, OsCESA8,* and *OsCESA1* genes are high at many of the analyzed tissues/organs indicating functional redundancy among these genes for ubiquitous function. Expression of *OsCESA9* is high in the internode, node, and stem with trace

expression in other tissues. Notably, *OsCESA7* and *OsCESA4* showed very similar expression patterns with a significant level of expression in the root, shoot parts, and flower. Sequence analysis showed that both genes (*OsCESA7* and *OsCESA4*) are paralogous genes and it is likely that these genes retained their function after the duplication event.

Materials and Methods

Collection of Cell Wall Biosynthesis Related Genes in Rice

The Carbohydrate-Active Enzymes database (CAZy; http://www.cazy.org) is a well-organized, regularly updated resource that provides systematic nomenclature information of carbohydrate-active enzymes (Lombard et al. 2014). We downloaded the rice cell wall biosynthesis related genes from CAZy based on five enzyme modules. After omitting entries with a lack of locus ID information and excluding multiple splice variants of genes, unique CWB genes were chosen for further analysis.

Meta-expression Analysis

The Affymetrix anatomical meta-expression database, Affymetrix biotic stress response database, Affymetrix abiotic stress response database, and the Agilent 44K hormone response database were used for anatomical, biotic, abiotic, and hormone analysis of the CWB genes, respectively. Various rice anatomical and stress treated microarray samples were downloaded from NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/). Downloaded data were normalized using the Affy package available under the BioC software in the R program (http://www.r-project.org/). The Affy package provides a probe level analysis of Affymetrix GeneChip data (Gautier et al. 2004). Normalized data were $\log_2$ transformed using Microsoft Excel. Probes were then mapped to the corresponding RGAP locus id. For genes with multiple splice variants, the expression profile of representative transcripts annotated in the RGAP website were taken.

For identifying the tissue preferential expression, probe intensity values were k-mean clustered using euclidian distance method. Genes in the generated groups showed higher intensity value in a specific tissue type compared to other samples. These sets were manually edited and used for tissue preferential featured group. For identifying the significant upregulated genes under different stress conditions including biotic, abiotic and hormone treatment, we first generated the log2 fold change (FC) values of each samples and genes with log2 FC greater than 1.5 fold in average across samples was chosen. Co-expression clustering based on k-means euclidian distance was employed. Genes in co-expressed cluster was further manually edited based on their consistent expression across multiple experiments.

Anatomy

The Affymetrix anatomy database covers the expression profile from major tissues types including the callus, root, collar, leaf, flag leaf, root, stem, node and internode, shoot apical meristem (SAM), panicle, flower, developmental organs, palea, lemma, spikelet, embryo, and endosperm.

Abiotic Stress

Plants subjected to drought, salt, and low temperature stresses were included in the Affymetrix abiotic stress database. Among them, expression profiles from drought treated roots, leaves, panicles, and whole seedlings of different cultivars were categorized under drought stress. Plants treated with different molar concentrations of NaCl comprised the salt stress. Samples from plants subjected to varying levels of low temperature (12°C - 4°C) at regular intervals (ranging from 2 h to 48 h) were used for the cold treatment gene expression profile.

Hormone Response

Abscisic acid, gibberellic acid, indole-3-acetic acid (IAA), jasmonic acid (JA), trans-zeatin (tZ), and brassinolide (BL) are the major hormones or growth promoters used for the Agilent Hormone response database. Each hormone was applied for an interval of 15 min, 30 min, 1 h, 3 h, and 6 h for roots. In the case of shoots, profiling was done at intervals of 1 h, 6 h, and 12 h of treatment.

Biotic Stress

Affymetrix expression profiling of rice plants treated with pathogens *Magnaporthe grisea*, *Magnaporthe oryzae*, *Xanthomonas oryzae* (*XOO*) and Brown planthopper (BPH) were integrated to create the biotic stress database.

Plant Growth

We searched mutant lines with T-DNA insertions within cell wall biosynthesis related genes from our T-DNA insertion mutant population (Jeon et al. 2000; Sorrells et al. 2003). In total, six lines were randomly selected and seedlings were grown in half-strength Murashige and Skoog (MS) medium. Seeds of transgenic rice were collected from the Crop Biotech Institute of Kyung Hee University, South Korea. Rice seeds were sterilized using 50% (v/v) sodium hypochlorite solution for 30 min at room temperature and washed five times with distilled water. Sterilized seeds were inoculated in 1/2 MS media and kept in an incubator for growth at 24 °C for 7 days with a photoperiod of 16 h light and 70% humidity. After germination, plants were transferred into pots filled with nutrient soil in greenhouse.

Genotyping Analysis

DNA was prepared from seven-day-old plants via the hexadecyl-trimethylammonium (CTAB) method. Genotypes were determined by PCR using gene-specific primers and T-DNA primers (Table S7). T-DNA insertional lines with homozygous, heterozygous, and wild type progenies were further analyzed to determine the anatomical and biochemical phenotypes.

Tissue Fixation, Embedding, and Visualization

Two-month-old stems were cut and fixed in 2% glutaraldehyde (Sigma Aldrich, St. Louis, MO, USA) in 1X PBS (10 mM phosphate buffer, pH 7.2, 138 mM NaCl, and 3 mM KCl) at 4°C overnight. After washing in a PBS solution three times, tissues were dehydrated through a gradient series (10% to 100%) of ethanol and finally embedded in LR white resin (Ted Pella Inc., Redding, CA, USA). Tissues embedded in 100% LR white resin were polymerized in a UV chamber (PELCO UVC2 Cryo Chamber, Ted Pella Inc.) at 4°C. Thin sections (0.5 ìm thickness) were prepared using a microtome, and stained with 0.1% toluidine blue. Sections were observed under a compound microscope (Leica DMRB, Bensheim, Germany) using bright-field illumination.

RNA Isolation and RT-PCR Analysis

Total RNAs were extracted with Tri Reagent (MRC Inc., Cincinnati, OH, USA). For synthesis of cDNA, 1 mg of total RNA was reacted with M-MLV reverse transcriptase (Promega, Madison, WI, USA), 2.5 mM dNTP, and 10 ng of oligo(dT). To evaluate the expression

patterns of the eight CWB genes in Fig. 2, we prepared samples from seven-day-old seedling shoots, seven-day-old seedling roots, and mature flowers for anatomical expression analysis, and from ten-day-old seedlings under drought, salt, and cold stress conditions. For drought treatment, we placed seedlings on a paper towel for 6 h; for salt treatment, we incubated the seedlings in a 250 mM salt solution for 6 h; and for cold treatment, we incubated the seedlings in a growth incubator at 4×C for 48 h. To evaluate each stress treatment, we used well-known marker genes for drought and salt stress, including *basic leucine zipper23* gene (*bZIP23*, *LOC_Os02g52780*) and for cold, the proline transporter gene *OsProT* (AB022783). In addition, we used the *rice ubiquitin 5* (*OsUbi5*) gene as an internal control. The primers used for this analysis are summarized in Table S7.

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Author's Contributions

AKNC and HYJ carried out experiments and data analysis in figures and tables. KHJ and CL designed experiments. AKNC, HYJ, KHJ and CL wrote manuscript.

Supporting Information

Fig. S1. Phylogenomics analysis of chitinase family genes in rice.
Fig. S2. Meta-expression analysis of CWB genes showing featured abiotic expression patterns.
Fig. S3. Meta-expression analysis of CWB genes showing featured hormone expression patterns.
Fig. S4. ABA responsiveness of the genes in the 'ABA induced group' at different time points.
Fig. S5. Biotic stress response of 21 jasmonic acid (JA) induced genes is shown.
Fig. S6. Meta-expression analysis of CWB genes showing featured biotic expression patterns.
Fig. S7. Phylogenomics analysis of the rice cellulose synthase (OsCESA) family genes in rice.
Table S1. List of cell wall biosynthesis (CWB) related genes in rice and their enzyme classification.
Table S2. List of genes showing tissue preferential expression and their classification.
Table S3. List of abiotic stress induced genes and their classification.
Table S4. List of hormone responsive genes and their classification.
Table S5. List of biotic stress induced genes and their classification.
Table S6. Top 25 co-expressed genes of *OSCESA9.*
Table S7. Genes and primers used for RT-PCR analysis.

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