

Transcriptional dynamics during cell wall removal and regeneration reveals key genes involved in cell wall development in rice

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Abstract Efficient and cost-effective conversion of plant biomass to usable forms of energy requires a thorough understanding of cell wall biosynthesis, modification and degradation. To elucidate these processes, we assessed the expression dynamics during enzymatic removal and regeneration of rice cell walls in suspension cells over time. In total, 928 genes exhibited significant up-regulation during cell wall removal, whereas, 79 genes were up-regulated during cell wall regeneration. Both gene sets are enriched for kinases, transcription factors and genes predicted to be involved in cell wall-related functions. Integration of the gene expression datasets with a catalog of known and/or predicted biochemical pathways from rice, revealed metabolic and hormonal pathways involved in cell wall degradation and regeneration. Rice lines carrying Tos17 mutations in genes up-regulated during cell wall removal exhibit dwarf phenotypes. Many of the genes

up-regulated during cell wall development are also up-regulated in response to infection and environmental perturbations indicating a coordinated response to diverse types of stress.

Keywords Cell wall · Defense response · Expression · Microarray · Protoplast · Stress

Abbreviations

MeV	MultiExperiment Viewer
CAZy	Carbohydrate-Active enZymes
GEO	Gene expression omnibus
ERF	Ethylene response factors
JA	Jasmonic acid
GT	Glycosyltransferase
GH	Glycoside hydrolase

Introduction

Plant cell walls display a unique combination of strength and plasticity with an astonishing capability to sense and respond to external stimuli (Manfield et al. 2004; Pilling and Hofte 2003; Somerville et al. 2004). In addition to their role in plant development and survival, plant cell walls are essential for important commercial industries such as textile, paper and pulp manufacturing, malting, and brewing. Plant cell walls will also be key to the success of a future environment friendly and renewable transportation fuel industry (Carroll and Somerville 2009; Farrokhi et al. 2006). To optimize the structure, amount and composition of cell walls for efficient biofuel production, it is important to understand the molecular mechanisms underlying cell wall biosynthesis, modification and degradation. With an estimated 10% of plant genomes encoding proteins

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involved in cell wall-related functions, a concerted effort to elucidate their functions is needed (Yong et al. 2005). Various high-throughput transcriptomics and proteomics-based technologies have been employed to shortlist candidate genes for detailed investigation (Bonetta et al. 2002; Imoto et al. 2005; Manfield et al. 2004; Yang et al. 2008; Cao et al. 2008). However, so far only a handful of these genes have been functionally characterized mainly using T-DNA populations or expression in heterologous systems (Bouton et al. 2002; Burton et al. 2006; Fagard et al. 2000; Liepman et al. 2005; Turner et al. 2001). Moreover, due to redundancy in the cell wall-related genes, subtle alterations in cell wall components often do not lead to visible phenotypes under normal growth conditions or remain undetectable using current techniques (Fagard et al. 2000).

Because of their ability to regenerate cell walls and their homogenous nature, protoplasts have emerged as a versatile investigative tool for understanding the physiological and genetic aspects of plants in response to experimental treatments and cell wall perturbations (Burgess 1983; Cazale et al. 1998; Manfield et al. 2004; Pojnar and Cocking 1967; Shea et al. 1989; Skopelitis et al. 2006; Wang et al. 2007; Bart et al. 2010). Cell wall regeneration around protoplasts varies in different species ranging from as soon as 10 min following transfer of cells to regeneration medium to ~5 days (Amstel and Kengen 1996; Burgess and Fleming 1974; Kwon et al. 2005; Yang et al. 2008). Using *Arabidopsis* protoplasts undergoing cell wall regeneration, Kwon et al. (2005) identified candidate proteins involved in cell wall expansion, synthesis and post-translational modifications within 3 h of incubation. Conversely, Yang et al. (2008) reported complete regeneration of cell wall using cotton protoplasts within 48 h of incubation with significant accumulation of cell wall-related genes. These results indicate that the protoplast system is useful for cell wall-related studies.

Recently, Tan et al. (2011) used proteomics approaches in rice suspension cells to examine the cellular response to enzymatic removal of the cell wall and subsequent regeneration. Substantial chromatin decondensation/reorganization and histone modification were observed during cell wall removal and regeneration. They also observed initiation of wall synthesis at multiple sites indicating a novel mechanism for wall regeneration in protoplasts as compared to wall synthesis during cytokinesis (Tan et al. 2011). We took advantage of this well-developed system to assess transcript-level variations during cell wall removal and regeneration in rice suspension cells using a microarray-based time-course analysis. In this system, cell walls are completely removed in less than 9 h following enzyme treatment. Wall regeneration is observed within 48 h of incubation in regeneration medium (Tan et al. 2011). For this reason, two different time points (2 and 6 h) were

selected for identifying the genes induced during wall removal. In addition, expression profiles were analyzed following 4, 12 and 48 h of incubation in regeneration medium. Expression profiling of cells undergoing wall removal allowed us to identify candidate genes involved in sensing, signaling and feedback regulation in response to external perturbations. The data obtained in this study conforms to the earlier findings reported using proteomics analysis (Tan et al. 2011). The comparison of up-regulated genes with gene sets induced in response to various biotic and abiotic stress treatments elucidates the multifunctional nature of the genes involved in cell wall removal and regeneration. Based on these results, we propose a molecular genetic model for maintenance of cell wall integrity.

Materials and methods

Plant material and culture conditions

Homogenous cultures of suspension cells from NB2P line of *Japonica* rice cv. Nipponbare (Lee et al. 2004) were used for isolation of protoplasts. The suspension culture was maintained in the dark and weekly sub-cultured at a dilution of 1:5 (cells: fresh medium). Cells were grown at 24°C with constant shaking on a gyratory shaker at 150 rpm in B5 organic medium (pH 5.7) supplemented with 20 g/L sucrose, 0.5 g/L MES, 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 2 g/L casein enzymatic hydrolysate and 0.005% pectinase as reported (Tan et al. 2011). Cell walls of suspension cells were subjected to enzymatic hydrolysis by adding filter-sterilized enzyme solution containing 2.5% Cellulase RS (Onozuka RS) and 1% Macerozyme R10 (Research Products International) as described (Tan et al. 2007). The cells were harvested after 0 (S), 2 (D1) and 6 h (D2) of incubation. After enzymatic digestion, protoplasts were adjusted to a final concentration of 5×10^5 /mL, and cultured in dark using protoplast medium (PTM) containing 0.4 M mannitol, 20 mM CaCl₂, 0.125 mM MgCl₂, and 0.5 mM MES and 2 g/L N-Z-Amine A in B5 organic medium plus 2.0 mg/L 2,4-D at pH 5.6. The protoplasts were harvested after 0 (P), 4 (R1), 12 (R2) and 48 h (R3) of incubation. For each time point of cell wall removal and protoplasts undergoing cell wall regeneration, three biological replicates were collected. Suspension cells treated with PTM medium for 9 h were used as a control. The experiments were carried out in triplicates.

RNA Isolation and microarray experiments

The RNA was isolated by TRIzol method and DNase (Invitrogen) treated as per manufacturer's instructions.

Poly-A RNA was extracted using Qiagen oligotex kit. The quantity and quality of RNA was determined by measuring absorbance at 260 and 280 nm using a nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). The samples with A260/A280 ratio of 2.0–2.2 were used for microarray experiments. RT-PCR was carried out as described earlier (Jung et al. 2008). Amino-allyl UTP was incorporated using aRNA amplification followed by coupling with fluorescent dyes (Cy3 or Cy5). Probe labeling, hybridizations and scanning were carried out at Arraycore microarray facility (UC, Davis) using NSF45K arrays as described previously (Jung et al. 2008). Axon GenePix Pro 4.0 image analysis software was used to quantify the spot intensities and generate data files.

Data analysis

Data was normalized using the Lowess normalization method in the LMGene Package of R and log transformed (Berger et al. 2004; Lu et al. 2008). To avoid variations caused by media composition, the data was further normalized with respect to suspension cells incubated in protoplasts medium (PTM). The resulting log ratios were uploaded in the MeV data analysis tool (TIGR) as a tab separated file (<http://www.tm4.org/mev/>). For differential expression analysis, Student's *t* test was carried out assuming equal variance without any correction using an overall alpha (critical *P* value) of 0.001. From the resulting list of significant genes, those exhibiting \geq two-fold change were used for further analysis. Differential expression analysis was performed during cell wall removal using suspension cells (S) as control. For differential expression analysis during wall regeneration, protoplasts (P) were used as reference. Hierarchical clustering with selected set of genes was performed in MeV using default parameters.

Functional categorization and pathway analysis

Rice annotations available from the MSU rice genome annotation database v 6.1 (<http://rice.plantbiology.msu.edu/>) and GO annotation in rice array database (http://www.ricearray.org/analysis/go_enrichment.shtml) were downloaded and used for functional classification of differentially expressed genes. The sequence information for genes associated with synthesis and modification of cell wall polysaccharides and glycan chains associated with cell wall proteins was downloaded from CAZy database (www.cazy.org) and in-house generated GT and GH databases for rice (<http://phyloomics.ucdavis.edu/citing.shtml>).

The omics viewer tool (<http://pathway.gramene.org/expression.html>) in the RiceCyc database, a catalog

of known and/or predicted biochemical pathways from rice developed and curated by Gramene (Jaiswal et al. 2006), was used to identify the pathways altered during cell wall removal (Jung et al. 2011). The Tos17 (<http://tos.nias.affrc.go.jp/~miyao/pub/tos17/index.html.en>) mutant database was searched for phenotypes associated with mutations in cell wall-related genes.

Comparison with previously published studies

Expression profiles of differentially expressed genes were compared with those reported as differentially expressed during cell wall regeneration or stress response in published reports. The data was compared with the genes induced in response to sucrose starvation in rice suspension cells (Wang et al. 2007) and cellulase treatment to rice leaves (Jha et al. 2010). Microarray analysis has also been reported to identify genes induced during cell elongation (Kwon et al. 2005) and cell wall regeneration using suspension cells in *Arabidopsis* (Irshad et al. 2008). We identified rice orthologs of up-regulated *Arabidopsis* genes, in these two studies, using the Blast tool available from the MSU rice annotation database and then compared with the list of genes up-regulated during cell wall removal and regeneration in our study. For comparing with those induced or suppressed in response to various stress treatments in rice, the Affymetrix-based expression data were downloaded from GEO database under series numbers GSE8216, GSE16793, GSE14275, GSE10373, GSE6901, GSE18361 and GSE19024, normalized using MAS 5.0 algorithm and differential expression analysis was performed using MeV package as explained before. Genes, exhibiting \geq two folds up-regulation at *P* value \leq 0.01, were compared with those up-regulated in our dataset using Microsoft excel.

Real time quantitative PCR

Six genes exhibiting varied expression profiles were selected for validation of the microarray data. The PCR was carried out for two biological and three technical replicates in a Bio-Rad cycler using default PCR conditions as recommended by the manufacturer (<http://www.bio-rad.com>). The gene-specific primers were designed using Beacon designer software and synthesized commercially (Sigma-Biosys). The list of primers is given in Online Resource 1. The specificity of each primer set was determined by melt curve analysis after amplification. The abundance of each gene was determined relative to *elongation factor 1* using $\Delta\Delta$ Ct method. Data was further normalized to assist profile matching with the signal values obtained using microarrays.

Results

Transcriptional dynamics during cell wall removal and regeneration in rice

A time-course experiment was designed to monitor transcriptional dynamics during enzymatic removal of cell wall and its regeneration in rice suspension cells using NSF45K arrays (Jung et al. 2008). Differential expression analysis at P value ≤ 0.001 revealed 1,162 genes differentially expressed during cell wall removal with respect to suspension cells. Conversely, only 136 genes were differentially expressed during wall regeneration with respect to protoplasts. Out of 1,162 differentially expressed genes during cell wall removal, 928 were up-regulated, whereas, only 234 genes were down-regulated (Fig. 1a; Online Resource 2). During cell wall regeneration, 79 and 57 genes showed up- and down-regulation, respectively (Fig. 1a; Online Resource 3). The high number of up-regulated genes during cell wall removal suggests significant transcriptional activation in response to cell wall removal, which was also evident from the chromatin decondensation/reorganization during cell wall removal in rice suspension cells, reported earlier (Tan et al. 2011). Because suspension cells would already be undergoing active cell division and wall synthesis, protoplasts were used as reference to identify differentially expressed genes during wall regeneration. The lower number of differentially expressed genes during wall regeneration is probably due to high expression of most of the stress-associated genes in protoplasts as well (Yang et al. 2008).

The comparison of up-regulated genes in response to cell wall removal and regeneration showed merely five genes

common between both datasets including a RING Zn finger (LOC_Os10g31850), an RD kinase (LOC_Os11g34624) and three stress-associated genes (LOC_Os11g26790, LOC_Os10g22050, LOC_Os02g27480; Fig. 1a). RD kinases are characterized by a conserved arginine (R) in subdomain VI, which is required for their catalytic activity and are mainly implicated in growth and development (Dardick and Ronald 2006). One gene encoding an RD-type calmodulin-dependent kinase (LOC_Os12g07230), down-regulated during cell wall removal, was significantly up-regulated during cell wall regeneration (Fig. 1a). Blast analysis revealed its 91% identity with the phospholipid-regulated *Zea mays* protein, ZmCPK11 induced by wounding stress (Szczegieliński et al. 2005). A RICENET (<http://www.functionalnet.org/ricenet/search.html>; Lee et al., *under revision* for PNAS) search predicted the association of this gene with those involved in activation of MAPK pathway (*MAPK1*, 2, 5 and 6), progression of cell cycle and jasmonic acid mediated signaling suggesting its potential involvement in cell wall regeneration. Nine genes encoding two transcription factors (NAM, LOC_Os05g34830; ERF, LOC_Os05g34730), an RD kinase (LOC_Os06g34960), an F box family gene (LOC_Os09g22460), ribosome inactivating protein (LOC_Os12g07520), a WD domain containing protein (LOC_Os01g28680), a TE-related gene (LOC_Os05g45680) and two amino acid transporters (LOC_Os12g08090, LOC_Os12g08130), up-regulated during cell wall removal were down-regulated during wall regeneration (Fig. 1a). These genes might be involved in degradation and transport of cell wall components. Fig. 1b presents the kinetics of up-regulated genes during cell wall removal and regeneration. During cell wall removal, ca. 60% (573) of the genes were up-regulated only after 6 h of incubation with the

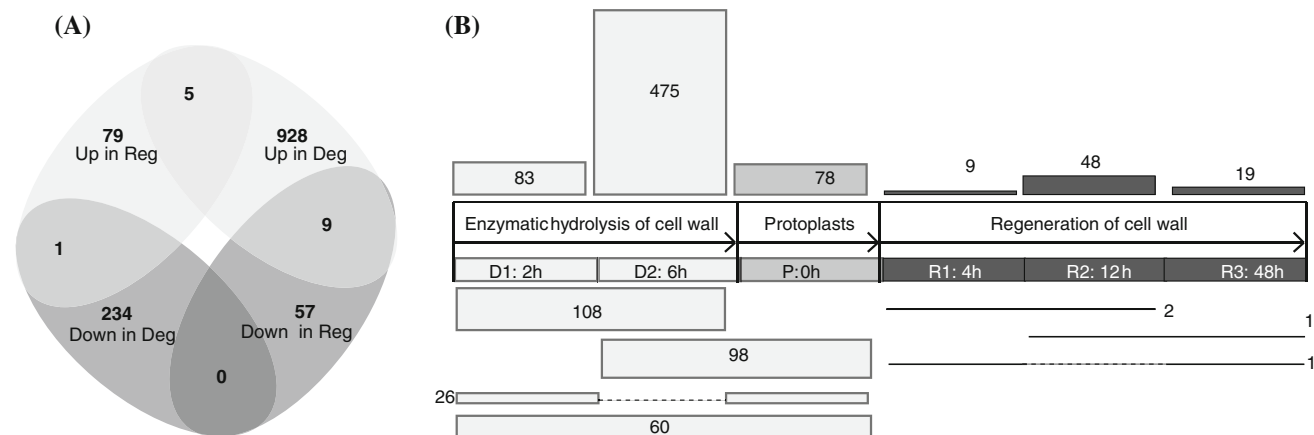


Fig. 1 **a** Diagram showing overlap between differentially expressed genes and, **b** Expression dynamics of up-regulated genes during cell wall removal and regeneration. Bars represent the number of up-regulated genes exhibiting varied expression patterns at different time points during cell wall removal (D1, D2) and regeneration

(R1, R2 and R3). The bars above the timeline represent the number of genes exhibiting specific up-regulation in any one stage and bars below the timeline represent number of genes exhibiting specific up-regulation in two or more stages. The size of each bar is scaled to number of genes and gene numbers are given in each box

enzyme (D2). Similarly, during wall regeneration, the majority of genes (62%) were up-regulated after at least 12 h of incubation suggesting that 6 and 12 h are crucial time points for initiation of transcriptional activity during wall removal and synthesis, respectively.

Very few genes were identified that were commonly up-regulated at different time points during cell wall regeneration (Fig. 1b). The ephemeral expression of most of the genes up-regulated during wall regeneration without any overlap at different time points suggests a role for these genes in maintaining short-term cell wall integrity instead of long-term developmental response.

Functional categorization of genes differentially expressed during wall removal and regeneration

Based on the putative functions assigned (<http://rice.plantbiology.msu.edu/>) and GO annotations available in the rice array database (http://www.ricearray.org/analysis/go_enrichment.shtml), the differentially expressed genes were divided into 13 functional categories (Fig. 2). Because many genes are predicted to be regulated in response to both biotic and abiotic stress based on GO annotations (Fujita et al. 2006), these genes are grouped into one category. Transcription factors, stress and, defense response-related genes, and signal transduction components are highly represented among genes up-regulated during cell wall removal. Notable of them are kinases (74), phosphatases (13), glycoside hydrolases (13), genes involved in ubiquitin-mediated degradation (16) and calcium signaling (12). Genes involved in protein synthesis, folding and transport as well as carbohydrate and energy metabolism are down-regulated during wall removal (Fig. 2a).

Among the genes encoding transcription factors up-regulated during cell wall removal, those containing zinc finger motifs were most highly represented. Next highly represented include ERFs, MYBs (v-myb avian myeloblastosis viral oncogene homolog) and WRKY family genes (inset, Fig. 2a). Members of these families have also been previously implicated in cell wall-related functions by regulating hormonal and other metabolic pathways (Marsch-Martinez et al. 2006; Wang et al. 2010; Zhong and Ye 2007; Fukao et al. 2006; Xu et al. 2006). Strikingly, a large number of genes implicated in cell wall-related functions, summarized in Table 1, were also up-regulated during cell wall removal. The hierarchical cluster map of log₂ fold changes exhibited by putative cell wall-associated genes during cell wall removal and regeneration is presented in Online Resource 4. Based on their functions, these could be placed into following categories:

Nucleotide-sugar interconversion pathway

Genes involved in nucleotide-sugar interconversion pathway form the basic building blocks of cell wall carbohydrates (Penning et al. 2009; Seifert et al. 2004). We found that three genes, belonging to each of three groups of C-4 epimerases including GAE-like (UDP-glucuronate-4-epimerase; LOC_Os08g41440), UXE-like (GDP-mannose 4,6-dehydratase; LOC_Os07g04690) and UGE-like (UDP-glucose-4-epimerase; LOC_Os09g35800) were up-regulated during cell wall removal (Table 1).

Hemicellulose biosynthesis

Genes belonging to GT2 (LOC_Os03g56060), 10 (LOC_Os08g36840), 31 (LOC_Os03g16334, LOC_Os09g26310, LOC_Os09g27950), 37 (LOC_Os02g52610), 43 (LOC_Os10g13810) and 61 (LOC_Os01g02910, LOC_Os01g72610, LOC_Os12g13640) subfamilies were also up-regulated during cell wall removal. Members of these families have been implicated in synthesis of xylans, xyloglucans, glucuronoarabinoxylans and other grass-specific cell wall components (Cao et al. 2008; Cocuron et al. 2007; Mitchell et al. 2007; Strassera et al. 2007).

Pectin biosynthesis

Four genes belonging to the GT8 (LOC_Os02g41520, LOC_Os04g43700, LOC_Os10g40640, LOC_Os12g38930) and a GT47 family gene (LOC_Os04g32670) were also up-regulated during cell wall removal. Members of the GT8 family have been implicated in pectin (Sterling et al. 2006; Bouton et al. 2002) biosynthesis. Harholt et al. (2006) identified a GT47 family gene encoding arabinosyltransferase involved in synthesis of pectin arabinan in *Arabidopsis*.

Lignin biosynthesis

A significant number of genes involved in lignin biosynthesis including cinnamoyl-CoA reductase (CCR; LOC_Os02g08420), 4-coumarate-CoA ligase (4CL; LOC_Os01g51920), hydroxycinnamoyl transferase (HCT; LOC_Os02g39850) and dihydroflavonol-4-reductase (DFR; LOC_Os02g56690, LOC_Os07g41070) were up-regulated during wall removal. CCR2 catalyzes the first committed step in lignin biosynthesis. Rice *snl6* and *Arabidopsis irx4* mutants, defective in CCR encoding genes, display altered lignin composition (Jones et al. 2001; Bart et al. 2010). HCT catalyzes generation of p-coumaroyl-CoA from p-coumaric acid and transfer of caffeoyl moiety of caffeoyl-quinic acid and caffeoyl-shikimate to CoA (Hoffmann et al. 2003; Penning et al. 2009).

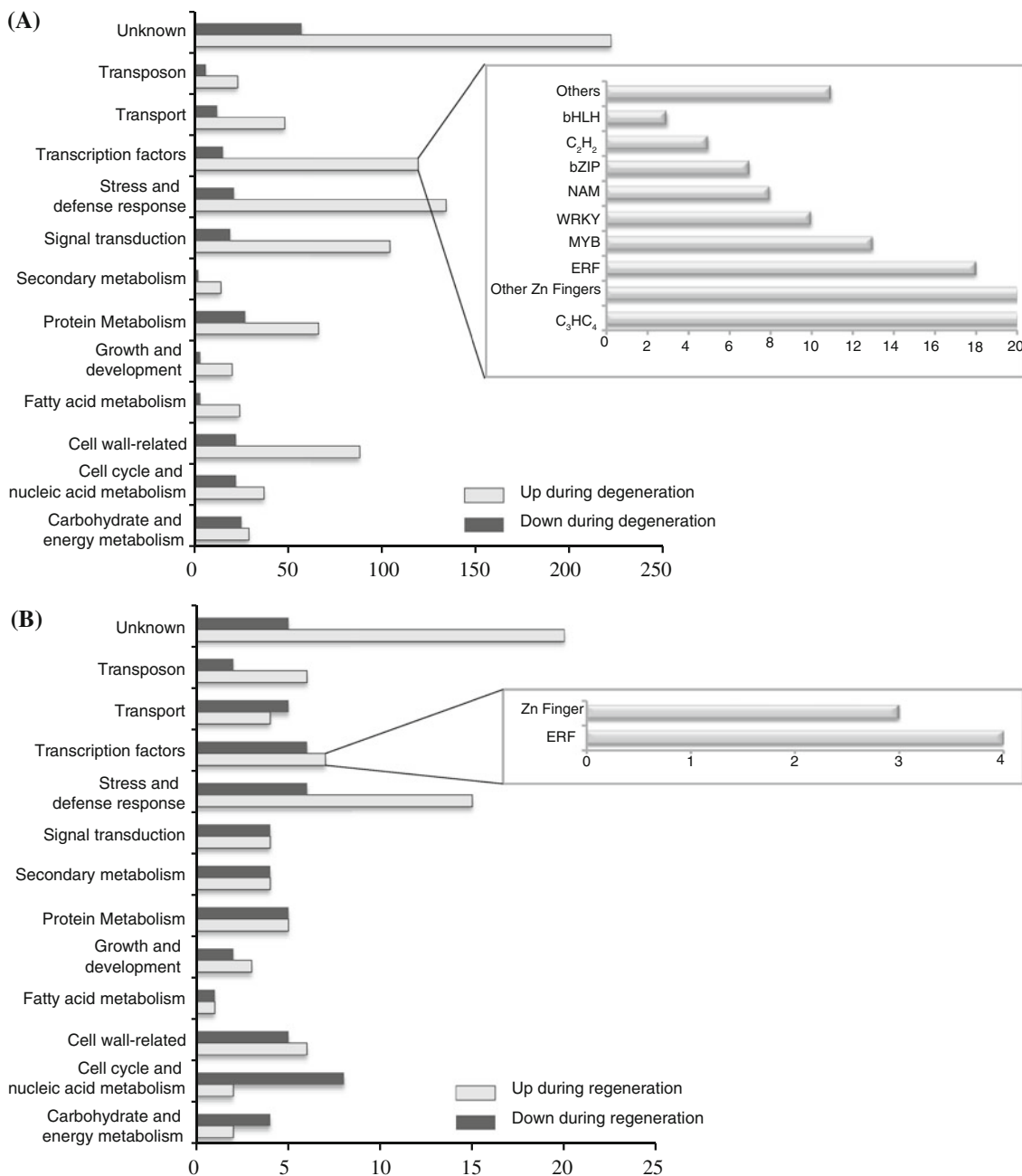


Fig. 2 Functional categorization of differentially expressed genes during cell wall **a** removal and **b** regeneration. The sub-categorization of transcription factors has been presented as an inset

Structural proteins

Various genes encoding structural proteins including expansin, proline, hydroxyproline and glycine-rich proteins were up-regulated. Expansins, based on in vitro assays have been suggested to disrupt hydrogen bonds between wall polymers in addition to their role in cell wall extension (Cho and Cosgrove 2000; McQueen-Mason and Cosgrove 1994). A cobra-like protein 7 (LOC_Os03g18910) is also up-regulated during

cell wall removal. Blast analysis showed its 93% similarity with *roothairless3*, implicated in root hair elongation (Hochholdinger et al. 2008) and 76% similarity with *brittle stalk 2*-like protein of maize (Wen and Schnable 1994). Cobra-like proteins seem to be involved in maintaining proper orientation of cellulose microfibrils and thus would be important candidates for further investigation (Roudier et al. 2005). In addition, genes encoding annexins, ankyrins, actin assembly and depolymerizing factors were also up-regulated.

Table 1 List of cell wall-associated genes up-regulated during cell wall removal

S. No.	Locus ID	Gene name	Tos17 Line	Position of insertion	Tos17 phenotype
<i>Nucleotide-sugar interconversion pathway</i>					
1	LOC_Os07g04690	GDP-mannose 4,6-dehydratase			
2	LOC_Os09g35800	UDP-glucose 4-epimerase			
3	LOC_Os08g41440	UDP-glucuronate 4-epimerase			
<i>Hemicellulose biosynthesis</i>					
4	LOC_Os03g56060	CSLC9—cellulose synthase-like family C			
5	LOC_Os03g16334	Glycosyltransferase family 31			
6	LOC_Os09g26310	Glycosyltransferase family 31			
7	LOC_Os09g27950	Glycosyltransferase family 31	T15902T	Exon	Dwarf
8	LOC_Os02g52610	Galactoside 2-L-fucosyltransferase			
9	LOC_Os10g13810	Glycosyltransferase family 43			
10	LOC_Os01g02910	β -1,2-xylosyltransferase			
11	LOC_Os01g72610	β -1,2-xylosyltransferase			
12	LOC_Os12g13640	β -1,2-xylosyltransferase			
13	LOC_Os08g36840	Glycoprotein 3-alpha-L-fucosyltransferase A			
<i>Pectin biosynthesis</i>					
14	LOC_Os02g41520	Glycosyltransferase family 8			
15	LOC_Os04g43700	Glycosyltransferase family 8			
16	LOC_Os10g40640	Glycosyltransferase family 8	T07048T	Exon	Withering, dwarf, yellow, sterile
17	LOC_Os12g38930	Glycosyltransferase family 8			
18	LOC_Os04g32670	Glycosyltransferase family 47			
<i>Lignin biosynthesis</i>					
19	LOC_Os02g08420	Cinnamoyl-CoA reductase			
20	LOC_Os02g56690	Dihydroflavonol-4-reductase			
21	LOC_Os07g41070	Dihydroflavonol-4-reductase			
22	LOC_Os01g51920	4-coumarate-CoA ligase	NE5037_0_703_1A	3' UTR	Sterile
23	LOC_Os02g56460	Cinnamoyl CoA reductase			
24	LOC_Os02g39850	Hydroxycinnamoyl transferase			
<i>Structural proteins</i>					
25	LOC_Os10g39640	Expansin			
26	LOC_Os05g02780	Glycine-rich protein	T11772T	Promoter	None
27	LOC_Os03g57210	Glycine-rich protein			
28	LOC_Os04g32370	Hydroxyproline-rich glycoprotein			
29	LOC_Os02g31080	Hydroxyproline-rich glycoprotein			
30	LOC_Os05g33400	Glycoprotein			
31	LOC_Os02g44300	Proline-rich protein			
32	LOC_Os05g30950	Proline-rich protein			
<i>Cell Wall modification and degradation</i>					
33	LOC_Os01g03710	Mannose-6-phosphate isomerase			
34	LOC_Os01g53420	Anthocyanidin 5,3-O-glucosyltransferase			
35	LOC_Os09g34230	UDP-glucuronosyl/UDP-glucosyl transferase			
36	LOC_Os02g38140	β -1,4-mannosyl-glycoprotein β -1,4-N-acetylglucosaminyltransferase			

Table 1 continued

S. No.	Locus ID	Gene name	Tos17 Line	Position of insertion	Tos17 phenotype
37	LOC_Os10g33420	Glucosylceramidase			
38	LOC_Os08g39350	Phosphodiesterase	NC0218_0_504_1A	Exon	Dwarf
39	LOC_Os12g38760	Phosphodiesterase	T01385T	Exon	Dwarf
40	LOC_Os10g38060	Phospholipase D			
41	LOC_Os01g63200	Laccase			
42	LOC_Os09g32080	Chitinase			
43	LOC_Os05g15850	Chitinase			
44	LOC_Os06g25010	Chitinase			
45	LOC_Os11g47510	Chitinase			
46	LOC_Os11g47520	Chitinase			
47	LOC_Os11g47530	Chitinase			
48	LOC_Os11g47590	Chitinase			
49	LOC_Os02g33110	Glycosyl hydrolase family 32			
50	LOC_Os01g20980	Pectinesterase			
51	LOC_Os01g66840	Pectin acetylsterases	NE7046_0_703_1A	Exon	Dwarf
52	LOC_Os01g53990	Pectin methylesterase			
53	LOC_Os05g46510	Polygalacturonase			
54	LOC_Os06g48180	Xyloglucan endo-transglycosylase			
55	LOC_Os10g37660	α,α -trehalase	T26142T	Exon	Sterile
56	LOC_Os03g22790	β -amylase			
57	LOC_Os01g22900	β -fructofuranosidase			
58	LOC_Os01g54560	α,α -trehalose-phosphate synthase	T39935T	Exon	None
59	LOC_Os02g54820	α,α -trehalose-phosphate synthase			
60	LOC_Os08g34580	α,α -trehalose-phosphate synthase			

Cell wall modification and degradation

Various genes encoding enzymes involved in modification and degradation of cell wall components, including seven chitinases, were also up-regulated during cell wall removal (Table 1). Though chitin is absent in plants, plant chitinases have been implicated in defense response as well as cell wall signaling pathways (Zhong et al. 2002).

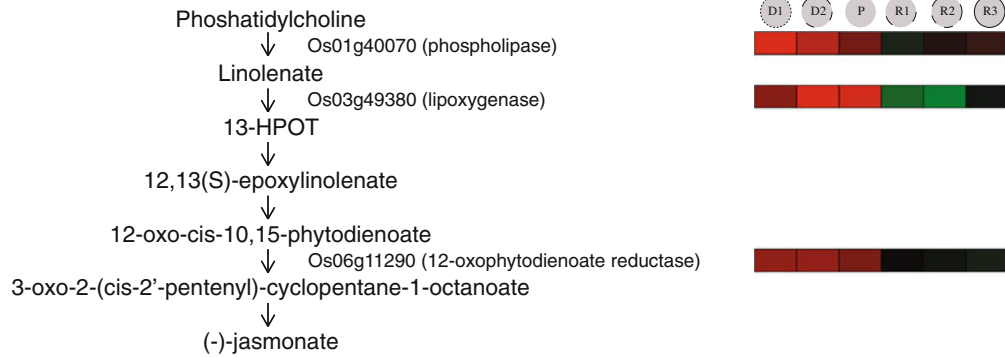
During wall regeneration, genes associated with cell wall-related functions, stress response and transcription are up-regulated (Fig. 2b). Conversely, transcription factors and genes regulating cell cycle and nucleic acid metabolism are down-regulated during wall regeneration (Fig. 2b). Among the up-regulated transcription factors were three Zn fingers and four ERFs (inset, Fig. 2b). Four genes encoding glycosyl hydrolases, three kinases, three cytochrome P 450 proteins and three dehydrins were also up-regulated. In addition, seven genes associated with cell wall biosynthesis including an alpha amylase (LOC_Os09g28400), beta amylase (LOC_Os10g32810), UDP-glycosyltransferase family 1 protein (LOC_Os11g38650), phenyl ammonia-lyase (LOC_Os02g41650), chitinase (LOC_Os08g40690), GH36 family gene (LOC_Os06g07600) and

phosphodiesterase (LOC_Os09g32840) were exclusively up-regulated during wall regeneration. Several transposons-related genes were also up-regulated during cell wall regeneration (Fig. 2b).

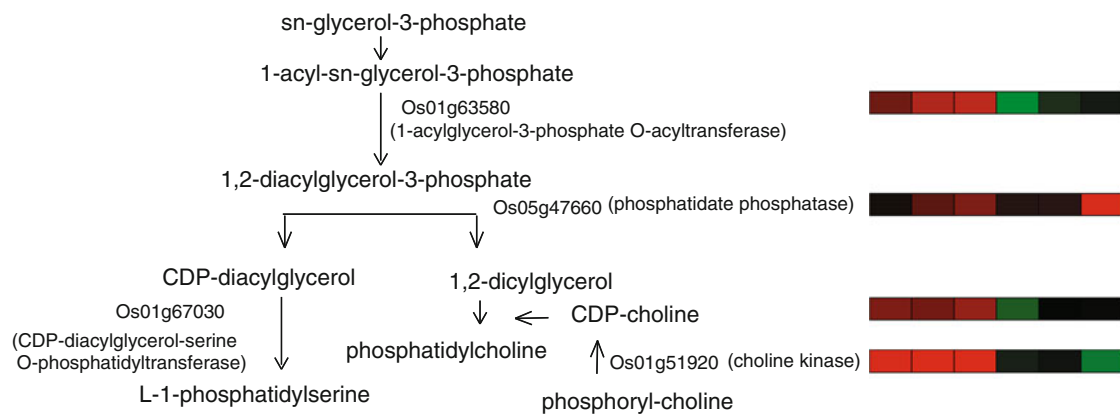
Metabolic pathways affected during cell wall removal

To identify the metabolic pathways affected during cell wall removal, the set of up-regulated genes was analyzed using the RiceCyc Pathway Tools Omics Viewer from the Gramene database (Jaiswal et al. 2006). Genes involved in JA, trehalose and phospholipid biosynthesis as well as triacylglycerol and homogalacturonan degradation pathways were significantly up-regulated during cell wall removal (Fig. 3). Because jasmonic acid is known to be involved in response to wounding and pathogen infection (Hu et al. 2009), the induction of the JA biosynthetic machinery could be triggered by signaling molecules released during cell wall removal. JA may in turn induce defense-related genes, which could also be important for maintaining cell wall integrity. Induction of genes involved in trehalose biosynthesis would be important for maintaining the integrity of cell, as trehalose forms a gel phase

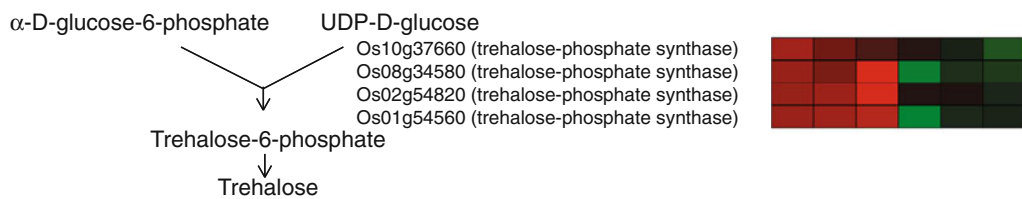
Jasmonic acid biosynthesis



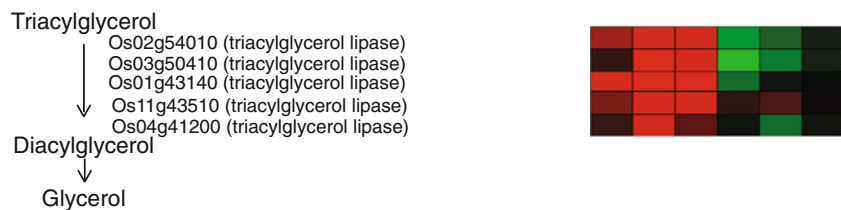
Phospholipid biosynthesis



Trehalose biosynthesis



Triacylglycerol degradation



Homogalacturonan degradation

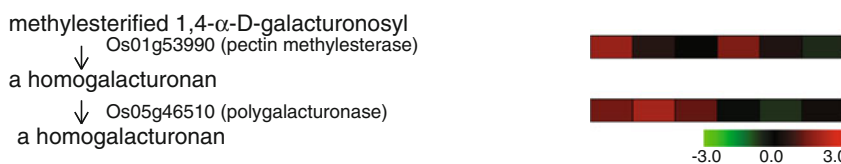


Fig. 3 Metabolic pathways up-regulated during cell wall removal. The locus IDs of up-regulated genes predicted/known to be involved in these pathways have been given with the corresponding enzymes encoded. The heat map on the right shows the log₂ fold ratios

exhibited by each gene with respect to suspension cells and protoplasts during stages of wall removal and regeneration, respectively. The green color depicts down-regulation and red indicates up-regulation in respective stages

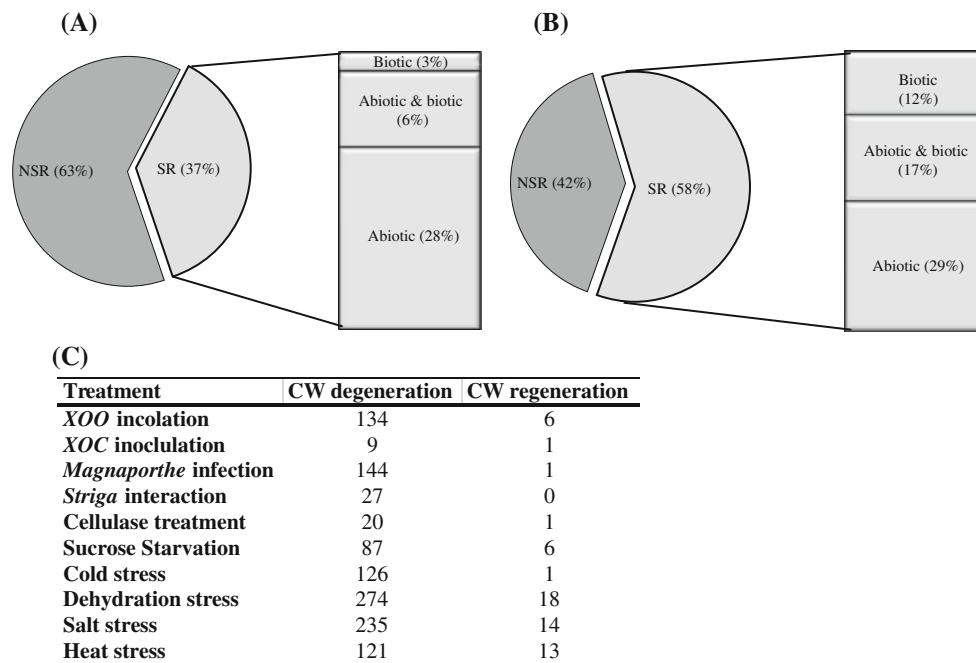


Fig. 4 Bar of pie chart showing distribution of stress responsive (SR) genes during cell wall **a** removal and **b** regeneration. **c** Table showing number of genes up-regulated during wall removal and regeneration

and in response to various treatments. SR, Stress-responsive, NSR-Non-stress responsive

while the cell dehydrates, thereby preventing rupture of cell organelles.

During homogalacturonan degradation, pectin methyl-esterases assist in formation of interchain salt bridges of calcium by removal of methyl groups. Polygalacturonases hydrolyze the α -1,4-D galacturonan backbone (Brummell and Harpster 2001; Somerville et al. 2004). Reduction of de-methyl-esterified homogalacturonan by inhibition of pectin methyl-esterases improves saccharification efficiency in *Arabidopsis* (Lionetti et al. 2009). Genes involved in degradation of triacylglycerols are also up-regulated during cell wall removal (Fig. 3). Degradation of triacylglycerols results in release of diacylglycerols, which in turn may act as secondary messengers to induce downstream signaling pathways.

Coordinated expression of genes up-regulated during cell wall removal with stress-response related genes

To identify genes that function in other cellular processes as well, publicly available microarray data of rice tissues undergoing biotic and, abiotic stress treatments were downloaded and compared with genes up-regulated during wall removal. The datasets used for comparison include sucrose starvation in suspension cells (Wang et al. 2007); cellulase treatment to leaves (Jha et al. 2010); *Xanthomonas oryzae* pv. *oryzae* (*XOO*) and *Xanthomonas oryzae* pv.

oryzicola (*XOC*) inoculations; *Magnaporthe* infection; parasitic interaction with a root parasitic plant *Striga hermonthica*; heat shock to 14-day-old seedlings and, cold, salt and dehydration stress to 7-day-old seedlings in rice (Arora et al. 2007). We found that 58% of the genes up-regulated during cell wall removal were also induced by various stress treatments (SR, Fig. 4a). In total, 29% of the genes were responsive to abiotic stress only, mainly including dehydration and salt stress (Fig. 4c). About 17% genes were up-regulated in response to both abiotic and biotic stress treatments, whereas, rest 12% genes were responsive to biotic stress only (Online Resource 5).

Among the genes up-regulated during cell wall regeneration, about 37% genes were stress-responsive (SR, Fig. 4b). A majority of these (28%) were up-regulated only in response to abiotic stress (dehydration, salt and heat stress), with 6% genes responding to both biotic and abiotic stress. Only 2 genes were exclusively induced in response to *Xoo* inoculation (Fig. 4c; Online Resource 6). Twenty and 17% of the stress-responsive genes were up-regulated during cell wall removal and regeneration, respectively (Online Resource 5 and 6).

Validation of microarray data using QPCR and cross-study comparisons

High-throughput technologies such as microarrays provide researchers with the ability to analyze thousands of genes

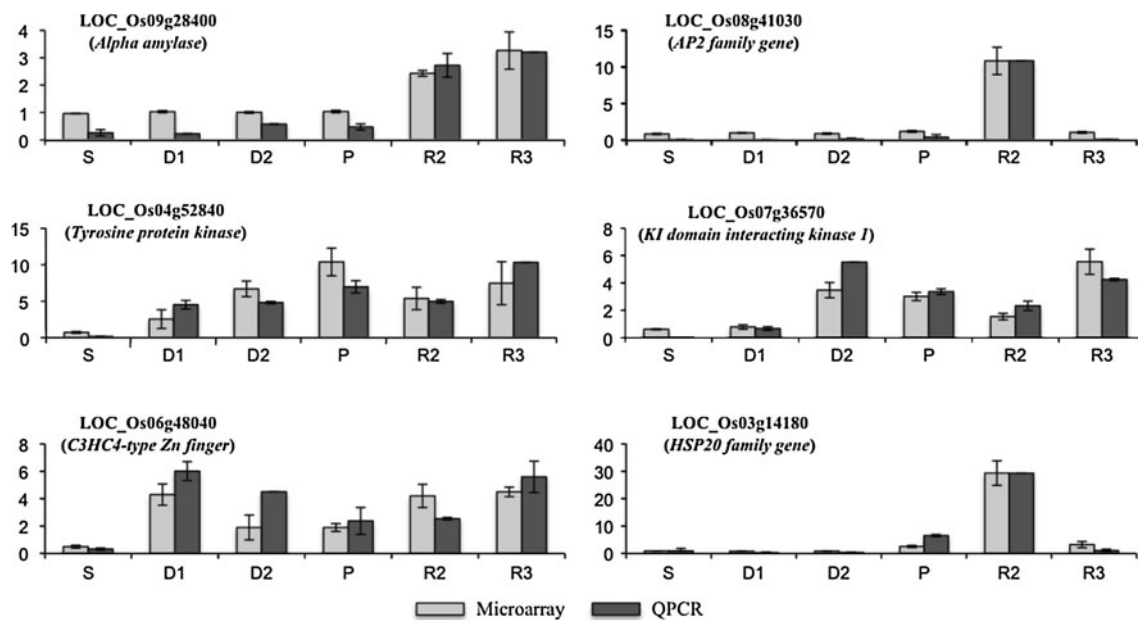


Fig. 5 Validation of microarray data using QPCR. The *black bars* represent expression data obtained using QPCR and *grey bars* represent microarray expression values. The *error bars* show the

standard error between three and two biological replicates used for microarray and QPCR experiments, respectively

simultaneously. Despite this advantage, one drawback is that such large datasets can include false positive or negative results. To assess the quality of our datasets, we chose six genes that exhibit varied expression patterns for validation using quantitative real time PCR. QPCR and microarray analysis correlated well for all six genes, indicating that the microarray results are robust and reproducible (Fig. 5).

Proteomic analysis of elongating hypocotyls of *Arabidopsis* identified genes associated with cell wall-related functions (Irshad et al. 2008). Comparison of this *Arabidopsis* dataset with the up-regulated gene set from our study revealed four genes that were common between both the datasets (Irshad et al. 2008). These include an aspartyl protease homologue (LOC_Os05g33400), an expansin like A (LOC_Os10g39640), receptor like kinase homologue (LOC_Os05g41370) and serpin homologue (serpine protease inhibitor; LOC_Os01g56010). In a separate study, proteins secreted during *Arabidopsis* protoplasts during cell wall regeneration were analyzed (Kwon et al. 2005). Putative rice orthologs corresponding to eight of these *Arabidopsis* genes were also up-regulated during cell wall removal and/or regeneration in our study. These include a glycoprotein (LOC_Os05g33400), an expansin (LOC_Os10g39640), four chitinases (LOC_Os11g47510, LOC_Os11g47520, LOC_Os11g47530, LOC_Os11g47590), a fruktokinase (LOC_Os01g66940) and a phosphodiesterase (LOC_Os12g38760). The identification of genes common to both rice and *Arabidopsis* datasets further validate the

usefulness of our data for investigating cell wall biosynthesis.

Identification and phenotypic analysis of rice lines carrying mutations in genes up-regulated during cell wall removal

To assess the function of up-regulated genes, we queried the Tos17 database (<http://tos.nias.affrc.go.jp/>) to search for phenotypes of the up-regulated genes listed in Table 1. We identified mutant lines associated with nine genes. Rice lines carrying mutations in five of these genes exhibit dwarf phenotypes. Rice lines carrying mutations in two genes were sterile, whereas, mutations in the other two genes did not exhibit any phenotypic abnormality. The positions of the insertions and the corresponding phenotypes are listed in Table 1. The observed dwarf phenotypes suggest that these genes might be involved in cell wall-related functions.

Discussion

Cell wall perturbations trigger activation of stress response and cell wall-related genes

To elucidate genetic molecular pathways induced in response to cell wall perturbations, we subjected rice suspension cells to enzymatic degradation. Microarray-based

expression profiling revealed significant up-regulation of cell wall-related genes (Table 1). These include genes that are predicted/known to regulate cell wall biosynthesis and stress response. Genes involved in lignin and pectin biosynthesis are also significantly up-regulated during cell wall removal. Our results support previous studies that lignin deposition is a response to cell wall perturbations (Cano-Delgado et al. 2003; His et al. 2001; Vaughn and Turley 2001; Zhong et al. 2002).

The significant overlap observed in the genes up-regulated during cell wall removal/regeneration and those induced in response to stress could be partially attributed to osmolarity imbalances (Brett and Waldron 1996; Iraki et al. 1989a, b, c; Ouyang et al. 2007). Because buffer conditions were kept constant in the suspension cells used for these experiments and the data was normalized to minimize differences due to osmotic stress, osmotic stress could not be solely responsible for differential accumulation of stress-related genes. Therefore, we hypothesize that these coordinately regulated genes are involved both in maintenance of cell wall integrity and the rice stress response.

Induction of genes controlling jasmonic acid biosynthesis and genes that respond to stress may be a result of decreased cellulose content in the protoplasts (Cano-Delgado et al. 2003; Ellis et al. 2002a, b). Support for this hypothesis comes from studies of the *Arabidopsis cev1* mutant, which carries a recessive mutation in the cellulose synthase gene, *CeSA3* of *Arabidopsis* and displays reduced root cellulose content. The *cev1* mutant also exhibits enhanced resistance to pathogens and increased production of jasmonic acid and ethylene, thereby suggesting a link between cell wall signaling, jasmonate and ethylene responses (Ellis et al. 2002a, b). Similarly, the *Arabidopsis* cellulose synthase mutants, *cesa8/lirx1* (*irregular xylem 1*), *cesa7/lirx3* and *cesa4/lirx5* also exhibit enhanced resistance to bacterial and fungal pathogens (Hernandez-Blanco et al. 2007). Together with our results, these studies indicate coordinated activation of cell wall-related and stress-responsive genes in response to cell wall removal. Characterization of genes regulating both cell wall development and response to stress will be useful for future development of biofuel crops that have enhanced cell wall properties and can withstand stress.

Genes involved in cell wall regeneration

Among the genes up-regulated during wall regeneration, we identified four glycosyl hydrolases (alpha amylase, beta amylase, chitinase and GH36 family gene), four ERF transcription factor family genes, three kinases, three zinc fingers, phenyl ammonia-lyase and phosphodiesterase encoding genes. The classic function of glycosyl

hydrolases into hydrolyze plant wall polysaccharides. A role for these proteins in cell wall biosynthesis and remodelling has also recently been reported (Bosch et al. 2011; Lopez-Casado et al. 2008). Upregulation of genes encoding zinc finger proteins has been observed in cotton protoplasts undergoing wall regeneration as well (Yang et al. 2008). Ethylene response factors have also been implicated in wall division and expansion (Marsch-Martinez et al. 2006; Fukao et al. 2006). Detailed functional characterization of these genes is needed to decipher their precise roles in cell wall development.

In *Arabidopsis* suspension cells, epigenetic restructuring of genomes in immortalized cell cultures leads to activation of transposable elements (Tanurdzic et al. 2008). These results suggest that the induction of transposon-related genes during wall regeneration, observed here, could be due to chromatin rearrangement in rice suspension cells (Tan et al. 2011).

Proposed model for transcriptional dynamics during cell wall removal

Based on the studies in yeast, it has been hypothesized that plants sense structural perturbations in the cell and in response, activate a cascade of signaling components to maintain wall integrity (Garcia et al. 2004; Somerville et al. 2004; Humphrey et al. 2007). Although there has been much speculation about the nature of sensors and downstream candidates involved in this process, still little is known. In this study, we have identified candidate genes/pathways modulated in response to cell wall removal and propose a model for the transcriptional dynamics operating in the cell that might be involved in maintaining cell wall integrity (Fig. 6).

Receptor kinases are ideal candidates for monitoring cell wall integrity due to their cell membrane localization and ability to activate downstream signaling through the kinase domain (Humphrey et al. 2007; Steinwand and Kieber 2010; Seifert and Blaukopf 2010). Our identification of receptor kinases that are up-regulated in response to cell wall removal and regeneration suggests that these kinases play a key role in sensing cell wall perturbations. The activated kinases would in turn phosphorylate downstream signal transduction components and transcription factors, which would mediate cell wall biosynthesis/repair and stress signaling pathways (Baluska et al. 2003; Chivasa et al. 2002; Decreux and Messiaen 2005; Guo et al. 2009a, b; Hematy and Hofte 2008; Hematy et al. 2007; Kwon et al. 2005; Lally et al. 2001; Xu et al. 2008).

In addition to post-translational modification, kinases have also been shown to play a role in chromatin decondensation by phosphorylating histones, transcription factors and chromatin remodeling enzymes or directly interacting

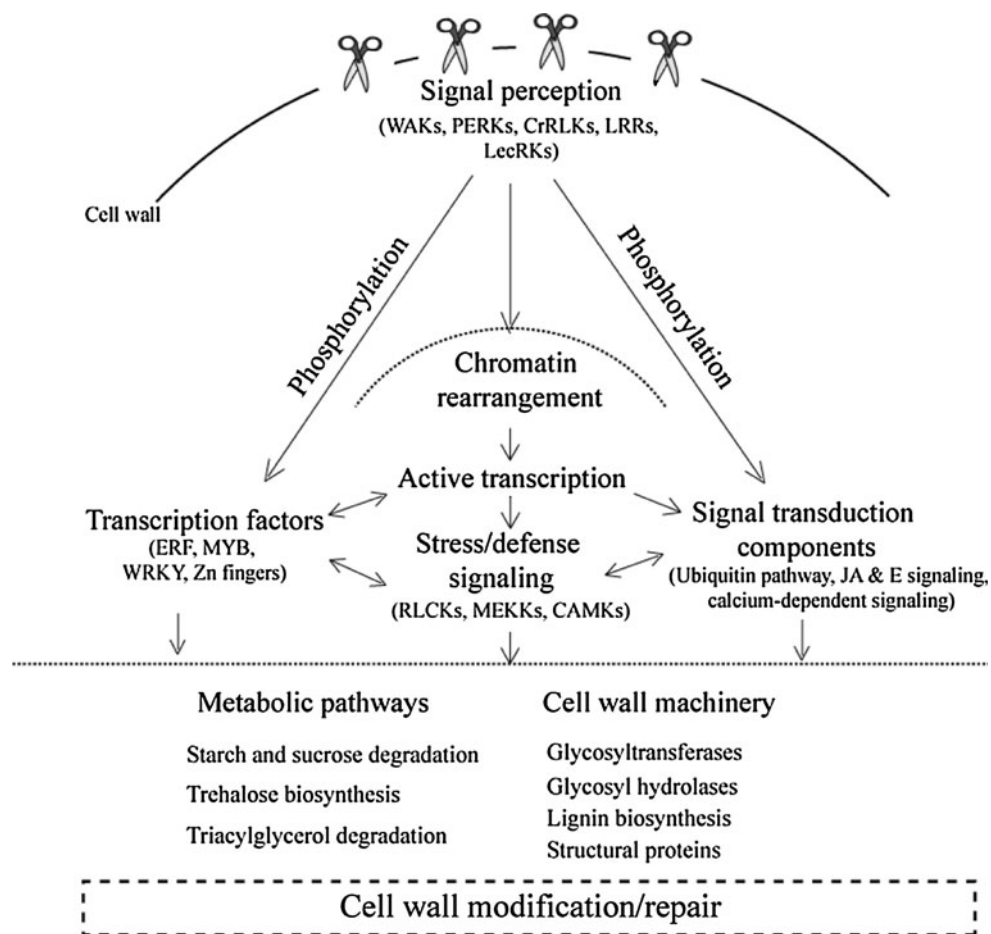


Fig. 6 Model showing the function of key genes involved in maintenance of cell wall integrity. Cell wall perturbations are perceived by cell wall-associated receptor like kinases (RLKs). The up-regulated RLKs identified in this study include: Wall-associated kinases (WAKs), Pro-rich extensin-like receptor kinases (PERKs), Catharanthus roseus-like RLKs (CrRLKs), Leucine-rich repeat RLKs and Leguminous L-type lectin RLKs (LecRKs). We also identified a few receptor-like cytoplasmic kinases (RLCKs), MAPK activating kinase kinases (MEKKs) and Ca²⁺/calmodulin-dependent kinases (CAMKs), which are predicted to function in intracellular signaling pathways. Activation of these kinases leads to induction of transcription factors (e.g. ERF, MYB, WRKY and Zn fingers) and other signal

transduction components (e.g. Ubiquitin machinery). Activation of genes controlling jasmonic acid/ethylene signaling, ubiquitination and calcium-dependent signaling leads to induction of genes controlling response to stress. Activation of metabolic pathways controlling degradation of starch, sucrose and triacylglycerols as well as trehalose biosynthesis, and regulation of cell wall-related genes (Glycosyltransferases, glycosyl hydrolases, structural proteins) prepare the cell to respond to stress and to catalyze cell wall regeneration/repair. Up-regulation of genes involved in lignin biosynthesis makes cell walls more resistant to mechanical injury. Scissors represent enzymes degrading the cell wall

with chromatin throughout the entire transcribed region of target gene (Alexandrow and Hamlin 2005; Chow and Davis 2006). Our data indicates that activation transcription factors (e.g. WRKY, MYB, ERF, Zn fingers) leads to activation of downstream pathways involved in maintaining cell wall integrity (see Fig. 6 for details).

The results presented here facilitate prioritization of candidate genes for detailed investigation into the molecular mechanisms underlying cell wall dynamics.

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