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Transcriptional Profiling of Rice Leaves Undergoing a Hypersensitive Response Like Reaction Induced by *Xanthomonas oryzae* pv. *oryzae* Cellulase

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Abstract A secreted cellulase of *Xanthomonas oryzae* pv. *oryzae* induces innate immune responses in rice including a hypersensitive response (HR) like reaction. Microarray analysis was conducted using RNA isolated 12 h following ClsA treatment of leaves. BLAST searches were performed for the 267 (152 up- and 115 downregulated) differentially expressed (≥2-fold) genes. A number of defense and stress-response functions are upregulated while a number of functions involved in metabolism and transport are down-regulated following induction of HR. A significant proportion of the differentially expressed genes (41/267) are predicted to encode transcription factors. Co-infiltration of *X. oryzae* pv. *oryzae* suppresses ClsA-induced expression of two transcription factors, *OsAP2*/ethylene response factor (*ERF*) and *OsRERJ1*, that are predicted to be involved in the jasmonic-acid-mediated defense pathway. Transient transfer of *OsAP2/ERF* via Agrobacterium results in the induction of callose deposition, programmed cell death, and resistance against subsequent *X. oryzae* pv. *oryzae* infection.

Keywords Cell wall degrading enzymes · Innate immunity · Plant defense response · Jasmonic acid · Hypersensitive response · Microarray · Programmed cell death

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Introduction

Plants have powerful innate immune responses that help them ward off most potential pathogens. These immune responses are triggered following recognition of common microbe-associated molecular pattern molecules (MAMPS) or pathogen-associated molecular pattern molecules (He et al. 2007; Bent and Mackey 2007). For bacterial pathogens, such MAMPS include elicitors such as lipopolysaccharides (Newman et al. 2002), cold shock protein (Felix and Boller 2003), flagellin (Felix et al. 1999), elongation factor Tu (Kunze et al. 2004), an extracellular signaling molecule called AvrXa21 (Lee et al. 2006), etc. The recognition of these signals occurs through receptors at the plant cell surface (Zipfel et al. 2004, 2006; Lee et al. 2006; He et al. 2007; Bent and Mackey 2007). Bacterial pathogens have the capacity to suppress innate immune responses of plants (Palva et al. 1993; Keshavarzi et al. 2004; Li et al. 2005; Jha et al. 2007). This ability of plant pathogenic bacteria to suppress host innate immunity is considered to be a precondition for their ability to cause disease (Grant et al. 2006; Bent and Mackey 2007; Jha et al. 2007; He et al. 2007).

The plant cell wall is a formidable barrier for potential pathogens. Cell wall degrading enzymes are important virulence factors of phytopathogenic bacteria (reviewed in Jha et al. 2005). Conversely, the oligosaccharides that are

released following the action of these enzymes on plant cell walls elicit potent innate immune responses (Darvill and Albersheim 1984; Ryan and Farmer 1991). These immune responses are in turn suppressible by plant pathogenic bacteria (Palva et al. 1993; Jha et al. 2007). Prior treatment of tobacco with purified preparations of *Erwinia carotovora* subsp. *carotovora* pectate lyase and polygalacturonase induced resistance against subsequent infection by the same pathogen (Palva et al. 1993). These defense responses are mediated by the jasmonic acid (JA)- and ethylene (ET)-dependent pathways but not the salicylic acid (SA) pathway (Norman-Setterblad et al. 2000; Vidal et al. 1998).

The rice pathogen, *Xanthomonas oryzae* pv. *oryzae*, uses a bacterial Type 2 secretion system (T2S) to secrete several cell-wall-degrading enzymes including two cellulases (ClsA and EglXoB), cellobiosidase (CbsA), lipase/esterase (LipA), and xylanase (Rajeshwari et al. 2005; Jha et al. 2007; Hu et al. 2007). The EglXoB protein is expressed only during in planta growth, but all five enzymes are important for *X. oryzae* pv. *oryzae* virulence. Conversely, treatment with either ClsA or CbsA or LipA induces innate immune responses such as HR like reaction and callose deposition in rice (Jha et al. 2007). These defense responses are so potent that prior treatment with these cell-wall-degrading enzymes induces resistance against subsequent infection by *X. oryzae* pv. *oryzae*. It appears that soluble elicitors that are released by the action of these enzymes on rice walls are the actual elicitors of innate immune responses. These defense responses are suppressed by *X. oryzae* pv. *oryzae* using proteins that are secreted through the type 3 secretion system (T3S). Prior treatment with a T3S^{-} *X. oryzae* pv. *oryzae* mutant induces resistance against subsequent infection by the wild-type pathogen indicating that defense suppression is crucial for the ability of *X. oryzae* pv. *oryzae* to cause disease. A T2S^{-} T3S^{-} double mutant of *X. oryzae* pv. *oryzae* is very much compromised in the ability to elicit rice innate immune responses indicating that a functional T2S and presumably the cell-wall-degrading enzymes that are secreted through this system play an important role in elicitation of innate immunity during growth within the plant (Jha et al. 2007).

Secreted ClsA is a 48-kDa protein that exhibits endoglucanase as well as exoglucanase activities. In order to understand the molecular events associated with ClsA-induced innate immune responses in rice, we have performed transcriptome analysis following treatment with the ClsA protein at concentrations that would induce HR. Our results indicated that a number of functions that are related to defense and stress are upregulated while those that are related to metabolism and transport are downregulated following ClsA treatment. A number of transcription factor genes are differentially expressed during cellulase-induced HR. Transient transfer of one of these transcription factor genes, *OsAP2*/ethylene response factor (*ERF*), via Agrobacterium, results in induction of callose deposition in rice leaves, programmed cell death (PCD) in rice roots, and enhanced resistance against subsequent *X. oryzae* pv. *oryzae* infection.

Results

Microarray analysis of ClsA-induced transcriptome of rice

In order to understand gene expression changes in rice leaves undergoing a ClsA-induced HR, we performed microarray analysis using the Affymetrix GeneChip rice genome array. Each array contains probe sets for 51,279 rice transcripts; out of which, around 48,564 are from japonica and 1,260 from indica cultivars. The adaxial surfaces of leaves of 15 days old Taichung Native-1 (TN-1) rice seedlings were infiltrated using a needleless syringe with either *X. oryzae* pv. *oryzae* ClsA (500 μg/ml) or buffer. Approximately, 10 μl of the solution is infiltrated into each leaf. The zone of infiltration is approximately 1 cm^2. This means that 5 μg of ClsA is infiltrated into an area of approximately 1 cm^2. Total RNA was isolated, 12 h after treatment, from leaf tissues encompassing the zone of infiltration. The samples from 20–30 infiltrated leaves were pooled together to obtain enough RNA for the analysis. Following a series of steps (as described in the "Methods"), the RNA was converted into biotin-labeled cRNA and hybridized to the rice gene chip. Separate gene chips were hybridized for the ClsA and buffer-treated samples, and the experiments were repeated with three independent biological replicates. All the raw data files (CEL files; three for ClsA and three for buffer treatment) obtained from GeneChip Operating Software (GCOS; Affymetrix) were subjected to AVADIS™ Software (Strand Life Science, Bangalore, India) for further analysis. The Probe Logarithmic Intensity Error (PLIER; Affymetrix Inc 2005) and Robust Multichip Average (RMA; Irizarry et al. 2003a, b) algorithms used in the analysis picked different numbers of genes at $p<0.05$. PLIER identified 1,582 genes as differentially expressed with ≥1.5-fold change and 427 genes with ≥2.0-fold change at $p<0.05$ whereas RMA picked 1,004 and 302 genes with 1.5- and 2.0-fold change, respectively, at $p<0.05$. In order to reduce the possibility of false discovery, we adopted a stringent criterion wherein we considered only those genes as differentially expressed, which were selected by both the algorithms. A total of 862 genes were common to both lists at 1.5-fold change and $p<0.05$. Q values were calculated from p values of all 862 genes, and q-value-based significance analysis suggested that the expected false positives are only four genes out of this list. The gene ontology (GO) enrichment analysis of ≥1.5-fold change rice genes differentially expressed upon ClsA treatment have revealed that genes with GO terms associated

with response to stress are overrepresented among upregulated genes while those associated with primary and secondary metabolism, photosynthesis, chloroplast, and plastids are overrepresented among downregulated genes. Genes with GO terms associated with calcium and magnesium ion binding are also overrepresented in upregulated genes while the genes involved in iron ion binding, transporter activity, lipid metabolism, and fatty acid biosynthesis are overrepresented among downregulated genes.

All the six raw CEL files and the corresponding Avadis™ processed RMA and PLIER normalized data files have been deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE8216.

Rice metabolic pathway analysis

In order to understand the changes associated with metabolic pathways during ClsA-induced HR, pathway analysis was performed using RiceCYC 1.2 Software (http://dev.gramene.org/pathway). For this analysis, we selected genes that were identified as being differentially expressed at ≥1.5-fold and $p \leq 0.05$ by both PLIER and RMA algorithms. Out of the 862 genes deemed to be differentially expressed (363 upregulated and 499 downregulated), we could retrieve the requisite locus information for only 709 genes from the TIGR rice genome database. Out of these, 197 genes could be associated with different metabolic pathways using the RiceCYC 1.2. Interestingly, two paralogous genes encoding putative 12-oxo phytodienoate reductases that are predicted to be involved in JA biosynthesis as well as four different glutathione-*S*-transferases were upregulated following ClsA treatment (Supplementary Table S1). A number of plant glutathione-*S*-transferases have been reported to be upregulated following pathogen infection (Marrs 1996), and silencing of the gene for a glutathione-*S*-transferase of *Nicotiana benthamiana* has been shown to lead to enhanced susceptibility to *Colletotrichum orbiculare* (Dean et al. 2005). Five putative lipases that are predicted to be involved in triacylglycerol degradation were downregulated. Several lipase-like functions have been shown to be associated with the SA-mediated defense pathway (Falk et al. 1999; Jirage et al. 1999; Kumar and Klessig 2003).

Functional categorization of the differentially expressed genes

A majority of the differentially expressed rice genes that were identified from above were not functionally annotated in public databases. We performed BLASTX-based homology searches for 267 genes that were identified as being differentially expressed using both PLIER and RMA at ≥2.0-fold change and $p<0.05$ at the NCBI server (Altschul et al. 1997) and the function of the protein with which they demonstrated significant homology ($E \geq 10^{-7}$) was ascribed to them. We used the 2.0-fold change cutoff for this analysis as each gene was individually analyzed and the smaller number of genes (267 with 2.0-fold cutoff as opposed to 862 obtained with 1.5-fold cutoff) was easier for us to handle. By this method, we could assign a putative function to nearly 80% (215/267) of the differentially expressed genes. Of these 215 genes, an $E > 10^{-10}$ was used to assign a function for 205 genes, and a value in the range of 10^{-7} to 10^{-10} had to be used to assign a function for about 10 genes. For the remaining genes, a function could not be assigned even if we considered less stringent *E* values, and these genes were put in the category of "others." Based upon the assigned function, the differentially expressed genes were categorized into 14 different functional categories (Fig. 1; Tables 1 and 2). The functional categories of Bevan et al. (1998) were used with some minor changes as described in "Methods." A large number of putative transcription factors were present among the upregulated (26/152; 17%) as well as downregulated (15/115; 13%) genes. In comparison, approximately 6% of the total genes in the rice genome are predicted to encode transcription factors (Gao et al. 2006). Interestingly, disease/defense and stress-related genes constituted 14% and 9% of the upregulated genes, respectively, whereas each of them constituted 6% of the ClsA-downregulated genes. The genes under the categories of metabolism and transport constituted 19% and 10% of the downregulated genes, respectively. These categories constituted 7% and 2% of the upregulated genes. Furthermore, genes involved in protein synthesis/turnover constituted 4% of the ClsA-upregulated genes and ~1% of the downregulated genes. Several metallothioneins and stress responsive proteins like heat shock proteins as well as a peroxidase and glutathione *S*-transferase were significantly upregulated (Table 1). The gene for rice ortholog of *Arabidopsis GUN4*, a positive regulator of chlorophyll synthesis (Larkin et al. 2003) as well as several genes encoding chlorophyll A/B binding proteins, glycine rich proteins, cytokinin dehydrogenases, and auxin responsive proteins were downregulated during ClsA-induced HR (Table 2). An important host susceptibility factor (Os8N3), whose expression is highly upregulated during *X. oryzae* pv. *oryzae* pathogenesis in rice (Yang et al. 2006), was ~2.6-fold downregulated during ClsA-induced HR. Also downregulated were several condensing enzymes (Os.7669.1.S1_at, Os.27713.1.A1_at, Os.9724.1.S1_at) and a ß-ketoacyl reductase (Os.12839.1.S1_at; Table 2) that are predicted to function in long chain fatty acid synthesis.

Increased expression of functions involved in the JA-mediated defense pathway during ClsA-induced HR

A rice transcription factor (*OsRERJ1*) gene that had been previously found to be induced by methyl JA treatment

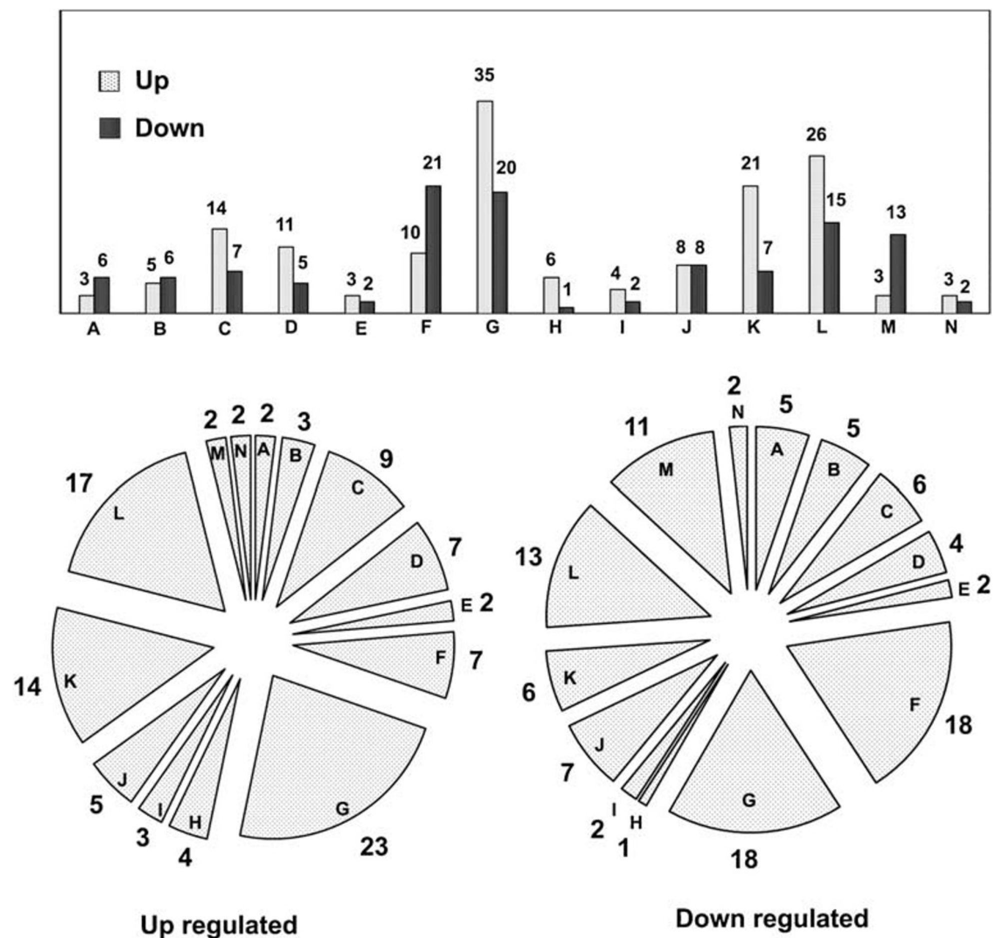

Fig. 1 Functional categorization of differentially expressed rice genes. The functions of differentially expressed genes (≥2-fold change) were classified into 14 different categories based upon BLASTX analysis. The number of up- and downregulated genes for each functional category is shown in the histogram. Percentage representations of the number of genes present per individual category with respect to total up- or downregulated genes, respectively, are shown in the pie charts. The functional categories are: *a* cell growth/division; *b* cell structure; *c* disease/defense; *d* energy; *e* intracellular traffic; *f* metabolism; *g* other; *h* protein synthesis/turnover; *i* secondary metabolism; *j* signal transduction; *k* stress; *l* transcription; *m* transporter; *n* transposons.

(Kiribuchi et al. 2004) was upregulated (~5.3-fold) following ClsA treatment. The gene for a putative ethylene binding protein/APETALA2 type of transcription factor (*OsAP2/ERF*) was upregulated (4- to 7-fold) following ClsA treatment. Two different probe sets for this gene are present on this chip: one probe set (Os.15849.1.S1_s_at) exhibited a 4.3-fold upregulation while the other (OsAffx.17366.1.S1_at) exhibited a 6.5-fold upregulation. This gene is homologous to subfamily 3 of *AP2/ERF* transcription factors in *Arabidopsis*; one member of this subfamily (*AtERF1*) is involved in integration of the JA and ET pathways (Lorenzo et al. 2003) whereas another member (*AtERF2*) functions as an activator in JA signaling (McGrath et al. 2005). The genes for five ZIM motif family proteins are upregulated following ClsA treatment (Table 3). The *Arabidopsis* homologs of four of these proteins have been shown to be upregulated by JA (Chini et al. 2007; Thines et al. 2007). These proteins function as negative regulators of the JA signaling pathway, and their upregulation has been attributed to serve in dampening the JA pathway after its initial induction. Furthermore, the ZIM family proteins repress a MYC transcription factor, which is the key activator of the JA signaling pathway (Lorenzo et al. 2004). A gene for the rice ortholog (Os.46443.1.s1_at) of this MYC factor (*OsMYC*) is upregulated 1.49-fold by ClsA treatment.

Allene oxide synthase is a key enzyme in the JA biosynthetic pathway (Turner et al. 2002). The Affymetrix rice array contains two paralogous genes that encode allene oxide synthase. One of them (Affymetrix probe set ID, Os.8266.1.A1_at) was found to be upregulated ~2.5-fold by the PLIER algorithm and ~1.95-fold by RMA but was not identified at $p<0.05$. The other gene (Affymetrix probe set ID, Os.7678.1.S1_at) has been earlier shown to be involved in JA biosynthesis in rice (Mei et al. 2006). It was identified as being upregulated by the PLIER algorithm (~1.7-fold at $p<0.05$) but was not selected as being upregulated using RMA. We assessed the expression of this gene (Os.7678.1.S1_at) by quantitative real-time PCR and found that it was upregulated ~2.0-fold at $p<0.05$ following ClsA treatment (Fig. 3; Supplementary Table S2, for the list of primers). Furthermore, as described above, pathway analysis had revealed the upregulation of two 12-oxophytodienoate reductase genes (*OsOPR2*, Os.50513.1.S1_at; *OsOPR1*, Os.8778.1.S1_a_at) that are predicted to be involved in JA biosynthesis (Table 3). ClsA treatment also induced the expression of two paralogous

Table 1 Rice Genes Upregulated (≥2 Fold) Following Cellulase Treatment

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
Cell growth/division				
Os.10388.1.S1_at	AK120374	2.74	MEE59 (maternal effect embryo arrest 59)	NP_195447 (3e-22)
OsAffx.7876.1.S1_s_at	9640.m04245	2.46	Os12g0626500 (Seed maturation protein domain containing protein)	NP_001067326 (2e-35)
Os.38249.2.S1_at	CB638262	2.39	Putative early flowering 3	BAA83571 (6e-28)
Cell structure				
OsAffx.22303.1.S1_at	9635.m03076	5.06	Expansin-like B1 precursor (OsEXLB1) (Expansin-related 1)	Q850K7 (3e-147)
Os.37723.1.S1_at	AK072531	2.27	Putative tonoplast membrane integral protein	AAS98488 (3e-95)
Os.5754.1.S1_at	AK099590	2.23	Putative plastid developmental DAG protein	BAD03018 (8e-91)
Os.36975.3.S1_x_at	NM_189551	2.17	Putative MAR binding filament-like protein 1	BAD68082 (0.0)
Os.24307.1.S1_at	AK066849	2.01	TMP14 (THYLAKOID MEMBRANE PHOSPHOPROTEIN OF 14 KDA)	NP_566086 (1e-24)
Disease/defense				
Os.53444.1.S1_at	AK072661	4.61	Putative pathogen-induced protein 2–4	BAD08038 (7e-17)
Os.11575.3.S1_x_at	AK065363	3.04	Ankyrin-repeat protein/OsNPR3	ABE11617 (0.0)
Os.10546.1.S1_s_at	AU183565	2.92	Putative UDP-glucose:salicylic acid glucosyltransferase	BAD34356 (2e-33)
OsAffx.30138.1.S1_at	9637.m02954	2.9	Putative UDP-glucose:salicylic acid glucosyltransferase	BAD34356 (0.0)
Os.8760.1.S1_a_at	AK099869	2.6	Putative nodulin-like protein	BAD34227 (0.0)
Os.36969.1.S1_at	AK120328	2.43	NB-ARC domain containing protein	EAY99786 (3e-25)
Os.11179.1.S1_at	CB647801	2.37	Ankyrin repeat family protein, putative, expressed	ABF95250 (1e-43)
Os.25266.1.S1_at	AK058891	2.34	Putative beta 1,3-glucanase	BAB63855 (2e-167)
OsAffx.23277.2.S1_at	9629.m01656	2.3	Ribosome inactivating protein, expressed	ABF98182 (1e-08)
Os.38249.1.S1_at	AK065173	2.23	Nematode responsive protein-like	BAD45081 (0.0)
Os.4327.1.S1_at	AF384030	2.21	Seven transmembrane protein MLO2	AF384030_1 (0.0)
Os.11831.1.S1_at	AK062130	2.12	Putative benzothiadiazole-induced S-adenosyl-L-methionine:salicylic acid Carboxyl methyltransferase1	BAD45797 (0.0)
Os.11575.1.S1_at	AK065952	2.09	Ankyrin-repeat protein/OsNPR3	ABE11617 (0.0)
Os.15355.1.S1_x_at	AK067000	2.07	Putative bacterial-induced peroxidase precursor	BAD28871 (2e-166)
Energy				
OsAffx.12379.1.S1_at	9630.m03520	3.26	Putative oxygen-evolving enhancer protein 3-2, chloroplast precursor (OEE3)	BAD29563 (8e-61)
OsAffx.32230.1.A1_at	X15901	2.96	ATP synthase CF1 epsilon subunit	NP_039389 (4e-65)
Os.52588.1.S1_at	AK068191	2.85	2Fe-2S iron-sulfur cluster protein-like/ferredoxin-related	BAC80058 (5e-86)
Os.19061.1.S1_s_at	AK099499	2.77	4Fe-4S ferredoxin, iron-sulfur binding; Heat shock protein DnaJ	ABP02426 (2e-21)
Os.26744.1.A1_at	CA762807	2.66	Chloroplast photosystem I assembly protein Ycf3	BAD81968 (7e-16)
Os.44475.1.S1_x_at	AK121213	2.66	Putative NADH dehydrogenase (ubiquinone oxidoreductase)	BAC15811 (0.0)
Os.29056.1.S2_at	AK110782	2.54	Apocytochrome f precursor, putative	AAP53263.2 (4e-54)
Os.27830.1.S1_at	AK068377	2.54	PsbQ domain protein family/oxygen evolving enhancer 3	BAC10375 (4e-105)
OsAffx.32225.1.A1_at	X15901	2.5	Photosystem I assembly protein Ycf3	NP_039384 (3e-95)
Os.3406.1.S1_at	AB004865	2.48	Alternative oxidase	BAA28771 (0.0)
Os.11570.1.S1_s_at	AK101484	2.22	Putative glycerol-3-phosphate dehydrogenase	AAU44049 (0.0)
Intracellular traffic				
Os.8700.1.S1_at	AK066218	2.74	Dynein light chain type 1-like	BAD28635 (2e-36)
Os.31464.1.S1_a_at	AK101489	2.6	Os04g0527400	NP_001053367 (0.0)
Os.40002.1.S1_s_at	CF315096	2.17	Got1-like family	NP_001060166 (1e-07)
Metabolism				
Os.18490.1.S1_x_at	AK120238	3.16	Thiamine biosynthesis protein ThiC, putative, expressed	ABF98201 (0.0)
Os.56682.1.S1_at	AK110292	3.1	GDSL-lipase-like	BAD61697 (0.0)
Os.23154.2.S1_at	AK111360	2.68	Putative proline-rich protein APG	BAD15755 (1e-61)

Table 1 (continued)

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
			(GDSL-lipase-like)	
Os.52414.1.S1_at	AK066972	2.41	Hydrolase, alpha/beta fold family protein-like	BAD23358 (8e-145)
OsAffx.27752.1.S1_s_at	9634.m02096	2.41	Putative aldose 1-epimerase	BAD05401 (4e-149)
Os.50951.1.S1_at	AK059666	2.21	Metallo-beta-lactamase protein-like	BAD28843 (6e-136)
OsAffx.32255.1.A1_at	X15901	2.19	Acetyl-coa carboxylase beta subunit	NP_039394 (5e-56)
Os.8957.1.S1_at	AK071523	2.19	Phosphatidic acid phosphatase-related/ PAP2-related	NP_190970 (6e-128)
Os.47381.2.S1_at	AK120916	2.12	Putative 3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplast precursor	BAD35225 (0.0)
Os.8957.1.S1_a_at	AK071523	2.06	Phosphatidic acid phosphatase-related/PAP2-related	NP_190970 (3e-128)
Other[f]				
OsAffx.5746.1.S1_s_at	9636.m00746	8.72	Hypothetical protein OsJ_025189	EAZ41706 (3e-20)
Os.4618.1.S1_at	AK062310	4.71	Hypothetical protein OsI_002101	EAY74254 (2e-50)
Os.40007.1.S1_x_at	CF318727	3.86	Os03g0120600	NP_001048790 (4e-18)
Os.6288.1.S1_at	AK106356	3.58	Unknown protein/Os08g0412700	NP_001061796 (2e-169)
Os.20548.1.S1_at	AK066753	3.58	Hypothetical protein OsI_016689	EAY95456 (9e-34)
Os.30528.1.S1_at	AK108716	3.45	Unknown protein/Os08g0412800	NP_001061797 (0.0)
Os.46544.1.A1_at	AK066066	3.38	Expressed protein/Os10g0389500	NP_001064501 (3e-113)
Os.21260.1.S1_at	AK067400	3.38	Os03g0184100	NP_001049187 (2e-120)
Os.6516.1.S1_at	CF303902	3.37	No significant similarity found	
Os.52004.1.S1_at	AK064520	3.31	Hypothetical protein LOC_Os12g17920	ABA97088 (3e-06)
Os.27218.1.A1_at	CB649864	3.23	Os05g0575000	NP_001056395 (5e-33)
Os.57343.1.S1_at	AK111335	3.06	Os02g0733900	NP_001048037 (2e-36)
Os.22703.1.S1_at	AK066033	3.03	Unknown protein /Os07g0618700	BAC79871 (1e-112)
Os.27764.1.S1_at	CB629248	3	Os05g0464200	NP_001055778 (4e-04)
Os.51529.1.S1_at	AK063010	2.99	Os12g0174200	NP_001066286 (1e-16)
Os.39942.2.S1_x_at	CF321166	2.81	Osjnbb0058j09.9	CAD39870 (9e-06)
Os.7944.1.S1_at	AK120788	2.76	No significant similarity found	
Os.5823.1.S1_at	AK109216	2.75	Os02g0718200	NP_001047938 (0.003)
Os.51299.1.S1_at	AK062470	2.75	Os05g0464300	NP_001055779 (2e-14)
Os.4773.1.S1_at	CF325704	2.67	Os06g0133500	NP_001056709 (3e-30)
Os.57191.1.S1_at	AK111114	2.63	Os06g0147300	NP_001056802 (2e-50)
Os.18712.1.S1_at	AK108985	2.63	Hypothetical protein OsI_028900	EAZ07668 (7e-127)
Os.2426.1.A1_at	CA759372	2.49	Expressed protein	ABA92028 (2e-21)
Os.12342.1.S2_at	CB668460	2.43	No significant similarity found	
Os.39725.1.S1_at	CA763989	2.35	No significant similarity found	
Os.7130.2.S1_x_at	AU032629	2.27	No significant similarity found	
OsAffx.7530.1.S1_s_at	9640.m00831	2.26	Expressed protein	ABA96042 (6e-24)
Os.38992.1.A1_x_at	D43281	2.23	No significant similarity found	
OsAffx.27741.1.S1_s_at	9634.m02034	2.23	Hypothetical protein	BAD62179 (2e-21)
Os.50952.1.S1_at	AK059668	2.19	No Significant similarity	
Os.10823.2.S1_at	AK064162	2.07	Os05g0487300/unknown protein	NP_001055881 (1e-05)
Os.55407.1.S1_at	AK108023	2.02	Os03g0799300	NP_001051576 (3e-26)
Protein synthesis/Turnover				
Os.55628.1.S1_at	AK108443	3.69	Eukaryotic translation initiation factor SUI1, putative	NP_175831 (3e-30)
OsAffx.26671.1.S1_x_at	9632.m05851	3.34	Eukaryotic translation initiation factor SUI1, putative	NP_175831 (4e-30)
Os.8724.1.S1_at	AK072982	3.02	Cyclin-like-F-box family protein	NP_567629 (5e-09)
OsAffx.24862.1.S1_at	9630.m05377	2.43	F-box domain containing protein	AAP54337.2 (1e-27)
Os.46325.1.A1_at	AK120658	2.32	Zinc finger (C3HC4-type RING finger) family protein	NP_568096 (3e-146)
Os.27569.1.S2_at	AK121706	2.26	Calcium-binding EF-hand family protein-like	BAD08916 (8e-49)
Secondary metabolism				

Table 1 (continued)

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
Os.50053.1.A1_at	AK119780	3.9	9-cis-epoxycarotenoid dioxygenase 4	AAW21320 (0.0)
Os.6376.1.S1_at	AK121461	3.41	Chalcone-flavanone isomerase	NP_001057086 (0.0)
Os.27507.1.S1_at	AK109673	2.97	Reticuline oxidase precursor, putative/berberine bridge enzyme-like protein	ABA93766 (1e-132)
OsAffx.32219.1.A1_x_at	X15901	2.61	Terpene synthase family, metal binding domain containing protein	ABF96083 (0.0)
Signal transduction				
Os.1606.1.S1_at	AK111969	5.05	Putative receptor-like protein kinase	AAL87185 (8e-137)
Os.15247.1.S1_s_at	AB125310	3.15	Serine/threonine-protein kinase SAPK9	BAD18005 (0.0)
Os.10254.1.S1_at	AK066150	3.12	Copine I-like	BAD10026 (0.0)
Os.9585.1.S1_at	AB060552	3.04	Calmodulin-2	BAB69673 (8e-66)
Os.26226.1.S1_at	AK103704	2.96	Putative serine/threonine protein kinase	BAD08131 (0.0)
Os.9585.1.S1_s_at	AB060552	2.62	Calmodulin, putative, expressed	ABA99816 (5e-66)
Os.35013.1.S1_at	AK069274	2.16	Putative Serine/threonine phosphatases	AAP06902 (0.0)
OsAffx.11760.1.S1_at	9629.m06614	2	Putative S-receptor kinase	BAD82381 (0.0)
Stress				
Os.51546.1.S1_at	AK063042	10.2	Senescence-associated protein-related	NP_197570 (1e-08)
OsAffx.32080.1.S1_at	9640.m03722	7.34	Metallothionein-like protein 1, putative, expressed	ABA99660 (8e-30)
Os.37783.1.S1_a_at	AK105219	4.52	Metallothionein-like protein 4C (OsMT-I-4c)	Q2QNC3 (2e-22)
OsAffx.7826.2.S1_at	9640.m03694	4.02	Metallothionein-like protein 1, putative, expressed	ABA99599 (1e-10)
Os.37773.1.S1_at	AU165294	3.82	Low molecular mass heat shock protein	AAC78392 (2e-37)
Os.37783.2.S1_x_at	AU070898	3.73	Metallothionein-like protein 1, putative, expressed	ABA99639 (4e-24)
Os.14105.1.S1_at	AK106404	3.63	Cytochrome P450	AAX92767 (0.0)
Os.4671.1.S1_a_at	AF435970	3.47	Jacalin-related lectin like /Protein mannose-binding lectin	P24120 (2e-78)
Os.37773.1.S1_x_at	AU165294	3.46	Alpha-crystallin-Hsps/17.4 kda class I heat shock protein, putative	AAP06883 (6e-38)
Os.6092.1.S1_at	AB120515	3.17	Trehalose-6-phosphate phosphatase	BAD12596 (0.0)
Os.7756.1.S1_at	AK060639	2.7	Putative stress-responsive protein (pectinesterase inhibitor domain containing)	AAM94917 (2e-83)
Os.15428.1.S1_at	AK065414	2.61	Putative wound inducive gene	BAD81783 (2e-174)
Os.8741.1.S1_at	AK102303	2.57	RADICAL-INDUCED CELL DEATH1	ABF94777 (0.0)
Os.33675.1.S1_at	AK099569	2.5	Rbcx protein having a possible chaperonin-like function	NP_001061837 (2e-82)
Os.8774.1.S1_at	AK071331	2.43	Peroxidase-like protein	BAD82471 (7e-127)
Os.5363.1.S1_at	AU174652	2.4	Putative cadmium-induced protein	BAC19956 (1e-29)
Os.53236.1.S1_at	AK071507	2.28	Putative zinc finger protein/Salt tolerance-like protein	BAD25819 (7e-95)
Os.49030.1.A1_s_at	CR292984	2.27	Putative glutathione S-transferase OsGSTU5	AF309377_1 (3e-09)
Transcription				
OsAffx.17366.1.S1_at	9636.m03709	6.54	Ethylene-binding protein-AP2 domain containing	BAD38371 (4e-41)
Os.6043.1.S1_at	AB040744	5.3	RERJ1(Helix-loop-helix domain containing transcriptin factor)	AB040744 (8e-111)
OsAffx.27442.1.S1_at	NM_185471	5.23	Transcription factor CBF1	BAA90812 (3e-73)
Os.4463.1.S1_s_at	AY327040	4.57	Transcription factor CBF1	BAA90812 (3e-73)
Os.15849.1.S1_s_at	AK062882	4.34	AP2 domain-containing transcription factor-like	BAD17116 (4e-15)
Os.49746.1.S1_at	AY581256	3.87	Induced protein mgi1(MYB-like DNA-binding domain)	AAS90600 (1e-76)
Os.55259.1.S1_at	AK107750	3.77	ZIM motif family protein, expressed	ABF94311 (1e-07)
Os.46849.1.S1_at	AK107854	3.46	ZIM motif family protein, expressed	AAP53563 (7e-23)
Os.54501.1.S1_at	AK105440	3.44	PHD-finger, putative	AAX94898 (0.0)
Os.5335.1.S1_at	AK101209	3.19	Putative MYB transcription factor	BAD61826 (1e-33)
Os.3400.1.S1_s_at	AB001888	3.05	Zinc finger protein	BAA33206 (0.0)

Table 1 (continued)

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
Os.12977.1.S1_at	AK072192	2.96	Zinc finger protein/COL9 (CONSTANS-LIKE 9); transcription factor	BAA33206 (4e-141)
Os.55550.1.S1_at	AK108297	2.88	Transcription factor GT-3b	AAP13348 (3e-04)
Os.9923.1.S1_s_at	AK120087	2.58	ZIM motif family protein, expressed	ABF94311 (9e-57)
Os.35681.1.S1_at	AY344493	2.54	Putative heat shock transcription factor 8	BAB68070 (2e-124)
Os.35343.1.A1_at	AK067922	2.51	No apical meristem protein (NAC domain containing)	ABA95706 (2e-116)
Os.10411.1.S1_at	AY077725	2.45	C2H2 zinc finger protein	BAD81699 (2e-72)
Os.23778.1.S1_at	AK101934	2.44	Putative heat stress transcription factor	BAD25410 (0.0)
Os.7130.1.S1_at	AK073475	2.42	Transcription factor jumonji, putative, expressed	ABB48025 (0.0)
Os.8031.1.S1_at	AK111775	2.38	Putative ethylene response factor 2/AP2 domain-containing transcription factor	BAB92777 (1e-73)
Os.49787.1.S1_at	AK111571	2.36	Putative MYB-like transcription factor	AAT69605 (6e-50)
Os.20750.1.S1_at	CB635910	2.33	bZIP transcription factor-like	BAB89012 (5e-54)
Os.19899.1.S1_x_at	AK106830	2.12	Transcription factor PCF8	BAC01119 (4e-38)
Os.11046.1.S1_at	AK119729	2.1	Putative ethylene-responsive transcriptional coactivator	BAD32863 (8e-60)
Os.10356.1.S1_at	AK073589	2.09	ZIM motif family protein, expressed	ABF94310 (2e-82)
OsAffx.3006.1.S1_at	9630.m04817	2.08	MYB-like transcription factor-like	BAD13038 (1e-15)
Transporter				
Os.47946.1.S1_s_at	AK070124	4.22	Putative glucose-6-phosphate/phosphate-translocator precursor	BAC57673 (2e-90)
Os.11800.1.S1_s_at	AK108373	3.37	MDR-like ABC transporter	CAD59587 (3e-141)
Os.11800.1.S1_at	AK108373	3.13	MDR-like ABC transporter	CAD59587 (4e-140)
Transposons				
Os.18078.1.S1_at	C97547	3.91	Rtac1	AAX95969 (0.17)
Os.39994.1.S1_at	CF319063	3.8	Rtac1	AAL86017 (1e-10)
Os.6901.1.S1_at	AK102892	2.72	Os10g0378500/retrotransposon protein, putative	NP_001064482 (2e-31)

[a] Affymetrix Probe set ID of upregulated gene

[b] The public representative ID of these genes obtained from Affymetrix NetAffx analysis center

[c] PLIER normalized absolute fold change value for upregulation

[d] The putative function of the gene based on BLASTX analysis

[e] Accession ID of the homologous protein based upon which the function has been assigned. The corresponding BLASTX E score is shown in brackets

[f] Genes encoding hypothetical proteins or those with no significant matches in the databases are kept in this category

rice chitinase (*PR-3*) genes whose *Arabidopsis* ortholog is involved in a JA-mediated defense pathway (Thomma et al. 1998). ClsA treatment led to increased expression of a rice ortholog of the *Arabidopsis RADICAL-INDUCED CELL DEATH-1* gene which has been postulated to modulate methyl JA responses (Ahlfors et al. 2004). A gene encoding a jacalin-related lectin like protein which is homologous to a JA-induced protein of wheat and barley (Lee et al. 1996; Wang and Ma 2005) was ~3.5-fold induced by ClsA treatment.

The gene for the rice ortholog of *Arabidopsis COPINE1* (a negative regulator of SA-associated systemic-acquired resistance; Jambunathan et al. 2001) was ~3-fold upregulated during ClsA-induced HR. Interestingly, genes that are putatively involved in SA modification were upregulated. Two different probe sets (Os.10546.1.S1_s_at and OsAffx.30138.1.S1_at) for a gene encoding a putative UDP-glucose/salicylic acid glucosyltransferase (*OsSGT*; predicted to be involved in glucosylation and inactivation of SA; Chen et al. 1995) were upregulated ~2.9-fold. The gene for a putative benzothiadiazole-induced *S*-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase1 (*OsBSMT1;* which is predicted to be involved in volatilizing SA; Koo et al. 2007) was ~2.0-fold upregulated. Glycosylated SA is an inactive storage form of SA (Chen et al. 1995) whereas methylated SA is volatile (Koo et al. 2007); thus, both modifications might lead to overall reduction of the cellular pool of SA. Increased expression of the JA pathway has been suggested to lead to a downregulation of the SA pathway (Takahashi et al. 2004; Kunkel and Brooks 2002; Spoel et al. 2003).

Table 2 Rice Genes Downregulated (≥2-Fold) Following Cellulase Treatment

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
Cell growth/division				
Os.27224.2.A1_at	AK103510	3.66	Putative cytokinin dehydrogenase	BAD09964 (8e-59)
OsAffx.29543.1.S1_x_at	9636.m03606	3.05	Putative cytokinin dehydrogenase	BAD09964 (0.0)
Os.43596.1.S1_at	CB620528	2.62	Cytokinin oxidase/dehydrogenase	BAE16612 (3e-39)
Os.9392.1.S1_at	AK071762	2.16	Auxin/aluminum-responsive protein putative	NP_197415 (3e-86)
Os.55455.1.S1_at	AK108118	2.06	Auxin-responsive protein-like	BAD25785 (2e-26)
Os.12201.1.S1_at	AK065039	2.05	Putative 1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1)	BAD38208 (1e-171)
Cell structure				
Os.49281.1.S1_s_at	AK062457	4.40	Putative glycine rich protein	BAD61563 (1e-11)
Os.38152.1.S1_at	AK065615	3.41	LrgB-like family protein expressed	AAP55171 (1e-73)
OsAffx.11838.1.S1_at	9629.m07254	2.53	Putative bundle sheath cell specific protein 1	BAB63632 (8e-60)
Os.55444.1.S1_at	AK108103	2.34	Glycine-rich protein	NP_193893 (4e-08)
Os.10370.1.S1_at	AK062370	2.32	Putative glycine rich protein	BAD61558 (1e-14)
Os.10370.1.S1_x_at	AK062370	2.27	Putative glycine rich protein	BAD61558 (1e-14)
Disease/defense				
Os.10401.1.S1_s_at	AK070510	2.65	Putative MtN3 (disease resistant allele XA13)	BAD13168 (1e-141)
Os.51641.1.S1_at	AK063248	2.55	Putative pathogenesis-related protein/PR1 like	BAC84818 (2e-67)
Os.37893.1.S1_at	AK100346	2.46	Phenylalanine ammonia-lyase	AAO72666 (0.0)
Os.37890.1.S1_s_at	AK061288	2.09	Protease inhibitor/seed storage/Lipid Transfer Protein family putative	AAX96637 (4e-39)
Os.9679.1.S1_at	AK060077	2.03	Disease resistance-responsive family protein putative expressed	ABA91787 (1e-90)
Os.46305.1.S1_at	AK122171	2.01	Leucine Rich Repeat family protein expressed	ABA96188 (0.0)
Os.51641.1.S1_x_at	AK063248	2.86	Putative pathogenesis-related protein/ PR1 like	BAC84818 (5e-68)
Energy				
Os.12181.1.S1_s_at	AK098872	3.26	Chlorophyll a/b-binding protein CP26 precursor - maize	AAX95978 (7e-146)
Os.37713.1.S1_at	AK070051	3.23	Putative photosystem I antenna protein	BAC83072 (5e-55)
Os.28216.2.S1_a_at	AK119545	2.59	Putative chlorophyll A-B binding protein of LHCII type III chloroplast precursor (CAB)	BAC83393 (2e-153)
OsAffx.18836.1.S1_at	9639.m01283	2.58	Chlorophyll a/b-binding protein CP26 precursor - maize	AAX95980 (9e-113)
Os.5869.3.S1_x_at	CB667246	2.02	Chlorophyll a/b-binding protein CP24 precursor	AAD27882 (8e-78)
Intracellular traffic				
Os.2759.1.S1_s_at	AK099409	2.46	Mitochondrial carnitine/acylcarnitine carrier putative expressed	AAP55124 (3e-116)
Os.11654.1.S1_at	AK065544	2.26	SOUL heme-binding protein-like	BAD32927 (8e-174)
Metabolism				
Os.11789.1.S1_at	AK099538	3.18	Putative lysine decarboxylase-like protein	AAN61486 (1e-111)
Os.26441.2.S1_at	AK119461	2.84	Putative beta-glycosidase	CAD36515 (0.0)
Os.7669.1.S1_at	CB643976	2.69	Putative fatty acid elongase	AAR96244 (5e-57)
Os.6595.1.S1_a_at	AK062262	2.65	Magnesium-chelatase subunit H family protein expressed	ABF95686 (0.0)
Os.14145.1.A1_at	AK070312	2.56	Ferric reductase	BAD18962 (0.0)
Os.27713.1.A1_at	AK120567	2.54	Beta-ketoacyl-coA synthase putative expressed	ABA94525 (0.0)
Os.12025.1.S1_a_at	AK099966	2.43	Hydrolase/alpha/beta fold family protein	NP_175660 (3e-153)
Os.15570.1.S1_at	AK068586	2.40	Putative glucosyltransferase-3 UDP-glucoronosyl and UDP-glucosyl transferase	BAC83960 (0.0)
Os.25109.1.S1_at	AK105536	2.35	Mannan endo-1 4-beta-mannosidase 7 (OsMAN7)	Q2RBB1 (0.0)
Os.17814.2.S1_x_at	AK068064	2.33	Putative malate dehydrogenase	BAC20686 (0.0)
Os.12839.1.S1_at	AK060512	2.26	Putative beta-keto acyl reductase	BAD22122 (1e-103)
Os.26486.1.S1_at	AK120227	2.15	Putative glossy1 protein/Sterol desaturase	BAD33619 (0.0)
OsAffx.27508.100.S1_s_at	AK108972	2.15	Putative methionyl aminopeptidase	BAD08973 (3e-18)
Os.9494.1.S1_s_at	CF292249	2.14	Short-chain dehydrogenase/reductase SDR family protein	NP_001053404 (6e-18)

Table 2 (continued)

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
Os.9724.1.S1_at	AK104714	2.14	Very-long-chain fatty acid condensing enzyme putative expressed	ABF94686 (0.0)
Os.22241.1.S1_s_at	AK099444	2.13	Nitrilase-associated protein putative expressed	ABA98636 (2e-13)
Os.14118.1.S1_at	AK099064	2.09	Putative lipase	AAP33477 (3e-100)
Os.36960.1.S1_at	AK062283	2.08	Glucosyltransferase NTGT2-like	BAD82532 (0.0)
Os.11403.1.S1_at	AK073262	2.07	Putative N-carbamyl-L-amino acid amidohydrolase	BAD45389 (0.0)
Os.8569.1.S1_at	AK060559	2.01	Beta-carotene hydroxylase putative expressed	ABF93742 (9e-98)
Os.20755.1.S1_at	AK068040	2.13	UDP-glucoronosyl/UDP-glucosyl transferase family protein	NP_196793 (2e-87)
Other[f]				
Os.51579.1.S1_at	AK063100	5.03	No significant similarity found	
Os.37849.1.A1_at	AK068289	3.73	Unnamed protein product	BAA90498 (2e-07)
Os.17536.1.S1_at	AK063180	2.90	Unknown protein	BAD36443 (3e-63)
Os.34400.1.S1_at	AK071545	2.61	Protein domain with at least 5 transmembrane alpha-helices	NP_001042652 (2e-102)
OsAffx.24703.1.S1_s_at	9630.m04191	2.60	No significant similarity found	
Os.11944.1.S1_at	AK070758	2.53	No significant similarity found	
Os.55256.1.S1_at	AK107743	2.51	Expressed protein	AAP54198 (8e-09)
Os.24338.1.A1_at	AK111667	2.47	Os01g0908300 .	NP_001045142 (8e-51)
Os.5066.1.S1_at	AK073193	2.45	Os01g0219300	NP_001042419 (4e-44)
Os.8395.1.S1_at	AK069514	2.31	Expressed protein	ABA98963 (3e-70)
OsAffx.23005.1.S1_x_at	AK108349	2.31	Unknown protein	BAD67880 (2e-17)
OsAffx.23005.1.S1_at	AK108349	2.30	Unknown protein	BAD67880 (2e-17)
Os.10615.1.S1_x_at	AK058921	2.22	No significant similarity found	
Os.10524.2.S1_at	AK060098	2.21	FHA domain containing protein expressed	ABA95708 (4e-83)
Os.5648.1.S1_at	AU172533	2.21	No significant similarity found	
OsAffx.14410.1.S1_s_at	9632.m05269	2.13	Os04g0635000 .	NP_001054004 (6e-11)
Os.25052.1.S1_at	CA763439	2.10	Hypothetical protein OsJ_000675	EAZ10850 (2e-23)
Os.49304.1.A1_at	AK063613	2.08	Hypothetical protein OsI_013772	EAY92539 (1e-34)
Os.27659.1.S1_x_at	AK069734	2.03	TMS membrane protein/tumour differentially expressed protein	ABE91594 (4e-133)
Os.11190.1.S1_at	AK058995	2.01	Os04g0402700/Hypothetical protein	NP_001052695 (7e-28)
Protein synthesis/turnover				
Os.11707.1.A1_at	CB656443	4.46	Putative cysteine proteinase	AAM34401 (7e-37)
Secondary metabolism				
Os.9685.1.S1_a_at	AK067949	2.51	Putative cinnamoyl CoA reductase	BAD28656 (2e-171)
Os.46551.1.S1_at	AK064736	2.31	Putative chalcone flavonoid 3′ - hydroxylase	AAN04937 (0.0)
Signal transduction				
OsAffx.16737.1.S1_at	AK067579	3.35	Phototropic-responsive protein putative/a light-activated serine-threonine protein kinase	NP_187478 (3e-31)
Os.12110.1.S1_at	AK064985	2.88	RPT2 expressed /nonphototropic hypocotyl 1 (NPH1)/a serine-threonine protein kinase	ABA91174 (0.0)
Os.55822.1.A1_at	AK108850	2.39	Putative serine/threonine protein phosphatase 2A (PP2A) regulatory subunit B’	BAD67828 (1e-95)
Os.7676.1.S1_at	AY476807	2.30	Extracellular calcium sensing receptor	AAS00828 (1e-146)
Os.7140.1.S1_at	AK061014	2.28	GUN4-like family protein expressed/regulator of chlorophyll synthesis and intracellular signaling	ABA92587 (4e-110)
Os.45531.1.S1_at	CB621884	2.19	Receptor-like kinase	AAF78017 (1e-48)
Os.7304.1.S1_at	AK069537	2.16	Putative protein kinase	BAB92217 (0.0)
Os.28255.1.S1_at	AK070519	2.07	ABC1 family protein /Mn2+-dependent serine/threonine protein kinase	NP_565025 (0.0)
Stress				
Os.53572.1.S1_at	AK073356	3.66	Universal stress protein USP1-like protein	BAC16006 (2e-84)
Os.37549.1.S1_at	AK102998	3.35	Auxin/aluminum-responsive protein putative	NP_197415 (3e-10)
Os.15692.1.S1_at	AK069017	2.68	Cytochrome P450 family protein expressed	ABF97430 (0.0)

Table 2 (continued)

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
Os.5377.1.S1_at	AK105891	2.52	Selenium-binding protein-like (pentatricopeptide repeat-containing protein)	BAD07861 (1e-152)
Os.34316.1.S1_at	AK071882	2.37	Hypothetical protein OsJ_021319/DnaJ domains	EAZ37836 (9e-21)
Os.17037.1.S2_a_at	CB671899	2.20	Putative Altered Response to Gravity/DnaJ domain	BAD73072 (7e-21)
Os.35448.1.S1_at	AK067480	2.20	Thioredoxin h-like protein	AAN63618 (4e-17)
Transcription				
Os.46600.1.S1_at	AK058809	3.77	Helix-loop-helix DNA-binding domain containing protein	AAP53429 (7e-113)
Os.56880.1.S1_at	AK110526	3.66	Basic-leucine zipper (bZIP) transcription factor domain containing protein	NP_001043273 (5e-39)
Os.31716.2.A1_at	BI305433	3.03	Scl1 protein/GRAS family transcription factor	AAF00139 (2e-24)
Os.49848.1.S1_at	AK111960	2.71	Putative MYB-related protein 1	BAD07916 (4e-71)
Os.25588.2.S1_x_at	AK101674	2.67	Putative MYB protein	BAC79723 (5e-88)
Os.3386.1.S1_x_at	AK062487	2.63	MYB protein	BAD36195 (7e-26)
Os.27085.1.A1_at	CB656188	2.34	Putative typical P-type R2R3 MYB protein/SANT domain containing protein	BAD10148 (2e-12)
Os.46081.1.S1_at	AK060177	2.18	Hypothetical protein OsJ_029817/transcription factor activity	EAZ15608 (2e-96)
Os.16025.1.S1_s_at	CA764046	2.14	Putative bZIP protein HY5	BAD32844 (1e-07)
Os.19539.1.S1_at	AK058932	2.04	Putative DNA helicase	BAD08079 (0.0)
Os.11409.1.S1_at	AK063523	2.04	Basic helix-loop-helix putative expressed	ABF93851 (6e-36)
Os.17902.1.S1_at	AK111634	2.03	MYB17 protein	CAD44611 (7e-34)
Os.26437.1.A1_s_at	CB673064	2.02	bZIP transcription factor family protein expressed	ABA99796 (6e-51)
Os.23030.1.S1_at	AK060509	2.05	Arabidopsis NAC domain containing protein 83; transcription factor	NP_196822 (4e-24)
Transporter				
Os.11804.1.S1_at	CB624264	3.27	Tryptophan/tyrosine permease family protein expressed .	ABF95016 (3e-111)
Os.52259.1.S1_at	AK065945	2.55	Major Facilitator Superfamily protein expressed	ABA91097 (0.0)
Os.5210.1.S1_at	AK072183	2.49	Putative sodium-dicarboxylate cotransporter	BAD09278 (0.0)
Os.19896.2.S1_x_at	CB623166	2.47	Putative sodium transporter	CAD37186 (1e-64)
Os.41637.1.S1_at	CA766063	2.40	MDR-like ABC transporter	CAD59585 (8e-14)
Os.27393.1.S1_s_at	AK061521	2.39	MDR-like ABC transporter	CAD59590 (0.0)
Os.57530.1.S1_x_at	AJ491855	2.27	Putative sodium transporter	CAD37198 (0.0)
Os.25449.1.S1_at	AK108531	2.21	Integral membrane transporter family protein	NP_180886 (1e-62)
Os.17299.1.S1_at	CB655994	2.18	Putative glycerol 3-phosphate permease	AAS82603 (7e-112)
Os.52960.1.S1_x_at	AK072316	2.17	Putative amino acid permease	AAP46198 (3e-146)
Os.17681.1.S1_at	AK072059	2.14	Monosaccharide transporter 1	BAB19862 (4e-158)
Os.21635.1.S1_at	AF543419	2.11	Major facilitator superfamily antiporter	AAN33182 (0.0)
Os.52621.1.S1_x_at	AK068351	2.04	Nitrate transporter putative	AAT85061 (8e-162)
Transposons				
Os.16593.1.S1_at	BI803439	2.14	Retrotransposon protein putative Ty3-gypsy subclass expressed	ABA92095 (1e-34)
Os.24273.1.A1_at	AK102986	2.07	Hypothetical protein osi_009106/transposase activity	EAY87873 (5e-30)

[a] Affymetrix Probe set ID of downregulated gene

[b] The public representative ID of these genes obtained from Affymetrix NetAffx analysis center

[c] PLIER normalized absolute fold change value for downregulation

[d] The putative function of the gene based on BLASTX analysis

[e] Accession ID of the homologous protein based upon which the function has been assigned. The corresponding BLASTX E- score is shown in brackets

[f] Genes encoding hypothetical proteins or those with no significant matches in the databases are kept in this category

Table 3 List of Cellulase Responsive Rice Genes Associated with Jasmonic-Acid-Mediated Defense Pathway

Probe set ID[a]	Public ID[b]	FCA[c]	Gene description[d]	Homolog (E score)[e]
OsAffx.17366.1.S1_at [f]	9636.m03709	6.54	Ethylene-binding protein-AP2 domain containing	BAD38371 (4e-41)
Os.6043.1.S1_at	AB040744.1	5.30	RERJ1(Helix-loop-helix domain containing transcriptin factor)	AB040744 (8e-111)
Os.15849.1.S1_s_at [f]	AK062882.1	4.34	AP2 domain-containing transcription factor-like	BAD17116 (4e-15)
Os.55259.1.S1_at	AK107750.1	3.77	ZIM motif family protein, expressed	ABF94311 (1e-07)
Os.4671.1.S1_a_at	AF435970.1	3.47	Jacalin lectin family protein, putative	P24120 (2e-78)
Os.46849.1.S1_at	AK107854	3.46	ZIM motif family protein, expressed	AAP53563 (7e-23)
Os.9923.1.S1_s_at	AK120087.1	2.58	ZIM motif family protein, expressed	ABF94311 (9e-57)
Os.8741.1.S1_at	AK102303.1	2.57	Poly polymerase catalytic domain containing protein/RADICAL-INDUCED CELL DEATH1	ABF94777 (0.0)
Os.35343.1.A1_at	AK067922.1	2.51	No apical meristem protein (NAC domain containing)	ABA95706 (2e-116)
Os.10356.1.S1_at	AK073589.1	2.09	ZIM motif family protein, expressed	ABF94310 (2e-82)
Os.50513.1.S1_at	AK121554.1	2.05	Putative 12-oxophytodienoate reductase (OPR2)	BAD61319 (0.0)
Os.12012.1.S1_at	AK061602.1	1.97	ZIM motif family protein, expressed	AAP53568 (5e-83)
Os.22000.1.S1_at	AK061042.1	1.95	Endochitinase/ putative PR3 like	BAA03749 (9e-166)
Os.8778.1.S1_a_at	AB040743.1	1.63	Cis-12-oxo-phytodienoic acid-reductase 1 (OPR1)	BAD26703 (0.0)
Os.3415.1.S1_s_at	AB016497.1	1.51	Chitinase/ putative PR3 like	BAA31997 (2e-135)
Os.46443.1.S1_at	AY536428.1	1.46	MYC protein/ATMYC2 (JASMONATE INSENSITIVE 1) ortholog	AAS66204 (0.0)

[a] Affymetrix Probe set ID

[b] The public representative ID obtained from Affymetrix NetAffx analysis center

[c] PLIER normalized absolute fold change value

[d] The putative function of the gene based on BLASTX analysis

[e] Accession ID of the homologous protein based upon which the function has been assigned. The corresponding BLASTX E score is shown in brackets

[f] These are two probe set IDs for the same gene

Validation of differentially expressed biologically significant genes by real-time PCR analysis

The expression profile of a few selected biologically significant genes was also examined by SYBR green based real-time PCR. Primers were designed to amplify 130–170 bp gene fragments of eight upregulated genes (*OsAP2/ERF, OsRERJ1*, *OsMYC*, *OsOPR2*, *OsAOS*, *OsCOPINE1*, *OsSGT*, and *OsBSMT1*) and one downregulated gene (*OsMtN3*; a host susceptibility factor; see Supplementary Table S2; for list of primers). The results indicate that *OsAP2/ERF*, *OsRERJ1*, *OsMYC, OsOPR2*, *OsAOS, OsCOPINE1, OsSGT*, and *OsBSMT1* are upregulated while the *OsMtN3* gene is down regulated following ClsA treatment (Fig. 2).

Wild-type *X. oryzae* pv. *oryzae* suppressed ClsA-induced expression of *OsAP2/ERF* and *OsRERJ1*

Rice seedlings were infiltrated with either ClsA or ClsA along with wild-type *X. oryzae* pv. *oryzae* in order to determine if the ClsA-induced expression of *OsAP2/ERF*, and *OsRERJ1* is suppressed by co-treatment with the bacterium. Expression of these genes was analyzed by SYBR green-based quantitative real-time PCR in three independent biological replicates. The relative fold change was calculated in comparison to their expression in buffer-treated leaves and the *OsGAPDH* gene as an endogenous control. Although there was variability between individual experiments, overall, the results indicated that co-treatment with wild-type *X. oryzae* pv. *oryzae* suppressed the induction of expression of *OsAP2/ERF* and *OsRERJ1* by ClsA at significance level $p<0.05$ (Table 4).

Sequence features of *OsAP2/ERF*

The *OsAP2* (APETALA2)/ERF gene that is upregulated following ClsA treatment is an 849 bp intron less gene encoding a 282 amino acid protein. Sequence analysis of the *OsAP2/ERF* using prosite (http://www.expasy.ch/prosite) has revealed that it contains a single AP2/ERF domain which spans from 138–195 amino acid residues (Supplementary Fig. S2). The presence of A and D amino acid at position 151 and 156 is a characteristic of AP2/ERF proteins which distinguishes them from related DREB proteins wherein the V and E amino acids are present at the corresponding positions (Sakuma et al. 2002). AP2/ERF domain has two sub-domains: YRG, involved in DNA binding and RAYD,

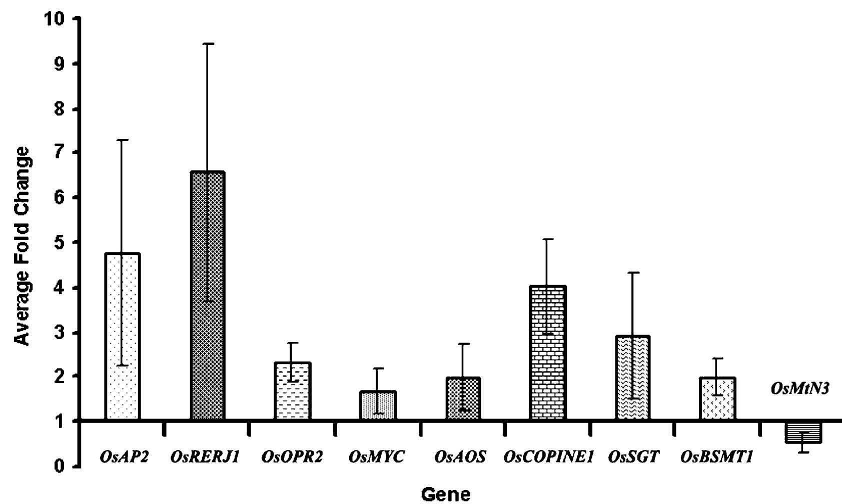

Fig. 2 Real-time PCR analysis of some differentially expressed genes. Total RNA was isolated from either ClsA- or buffer-treated rice leaves; first-strand cDNA was synthesized, and gene expression was assayed by SYBR green-based real-time PCR. The genes that were assayed are the following: *OsAP2* (AP2/ERF transcription factor), *OsRERJ1* (methyl JA induced helix loop helix rice transcription factor), *OsOPR2* (a 12-oxophytodienoate reductase), *OsMYC* (a key transcription factor in the JA-mediated defense pathway), *OsAOS* (Allene Oxide Synthase), *OsCOPINE1*, *OsSGT* (salicylic acid glucosyltransferase), *OsBSMT1* (benzothiadiazole-induced *S*-adenosyl-L methionine:salicylic acid carboxyl methyltransferase1), and *OsMtN3* (*Os8N3*; a host susceptibility factor). The relative fold changes were calculated by using 2^{-ddC}T method using the expression of buffer treated leaves as a comparison. The *OsGAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) gene was used as an endogenous control. Mean and standard deviation from at least four biological replicates for each gene are presented.

which is probably involved in DNA binding through the interaction of its hydrophobic phase with the major groove of DNA (Okamuro et al. 1997). The protein also contains nuclear localization signal sequences. The protein is predicted to contain two α helices and four β sheets. Amino acid residues in the pink box are predicted to be in direct contact with DNA (Allen et al. 1998; Nakano et al. 2006). Besides this, presence of a putative zinc finger motif (C×2C×4C×2~4C) on the N-terminal region of the protein suggests its involvement in DNA binding or protein–protein interactions. Due to presence of this motif, the protein has been previously classified to be a member of Xb subgroup of AP2/ERF proteins of rice (Nakano et al. 2006).

Agrobacterium-mediated transient transfer of *OsAP2/ERF* induces defense responses such as a PCD reaction in rice roots and callose deposition in leaves

PCD is a form of plant defense response (Pennell and Lamb 1997). We treated rice roots with Agrobacterium strain LBA4404/p*OsAP2/ERF* and stained with propidium iodide (PI) followed by confocal microscopy in order to determine if transient transfer of *OsAP2/ERF* induces PCD. Cell-wall-associated fluorescence was observed in roots treated with Agrobacterium strain LBA4404/pSB11; very few cells took up PI stain, and the distribution of the stain indicated that the nucleus remained compact (Fig. 3). In contrast, the PI

Table 4 Suppression of Cellulase-induced Expression of *OsRERJ1* and *OsAP2/ERF* Transcription Factor Genes by Wild-type *X. oryzae* pv. *oryzae*

Genes[a]	Experiment-1[b]		Experiment-2[b]		Experiment-3[b]	
	ClsA[c]	ClsA + Xoo[c]	ClsA[c]	ClsA + Xoo[c]	ClsA[c]	ClsA + Xoo[c]
OsAP2/ERF	5.56	1.33	5.11	1.43	2.64	0.66
OsRERJ1	8.26	1.48	9.45	1.7	5.86	1.36

[a] Rice genes upregulated following cellulase treatment

[b] Fifteen days old rice seedlings were infiltrated with either cellulase (ClsA) or cellulase along with wild type *X. oryzae* pv. *oryzae* (ClsA+Xoo). Buffer-treated leaves were used as control. Total RNA was isolated from each of the samples, converted into cDNA and subjected to real-time PCR analysis

[c] The relative fold changes were calculated by 2^{-ddC}T method in comparison to expression in buffer treated leaves. The *OsGAPDH* (Glyceraldehyde-3-phosphate dehydro-genase) gene was used as an endogenous control

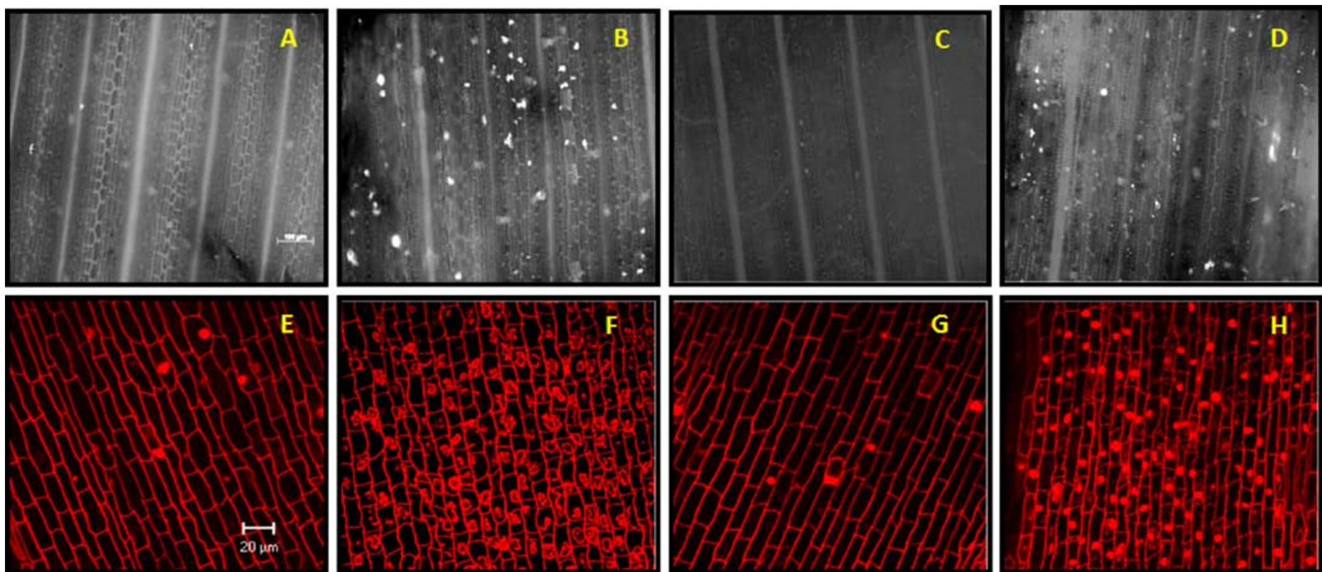

Fig. 3 Transient transfer of *OsAP2/ERF* induces callose deposition in rice leaves and cell death in rice roots. Rice leaves were syringe infiltrated with one of the following: water, ClsA, LBA4404/ pSB11, and *LBA4404/pOsAP2/ERF*. After 15 h, they were stained with aniline blue and examined under epifluorescence microscope. *White spots* in these pictures are indicative of callose deposition. **b** cellulase and **d** *LBA4404/pOsAP2/ERF* induce increased callose deposition compared with either **a** water or **c** LBA4404/pSB11. Rice roots were also treated either with water, cellulase, LBA4404/pSB11, *LBA4404/pOsAP2/ERF* and stained with propidium iodide (PI), and examined under a confocal microscope. Treatment with either water (**e**) or LBA4404/ pSB11 (**g**) did not induce cell death (intake of PI), whereas treatment with cellulase (**f**) and *LBA4404/pOsAP2/ERF* (**h**) induced cell death and extensive nuclear fragmentation. Similar results were obtained in at least three independent experiments.

stain was taken up by all the cells in rice roots treated with LBA4404/p*OsAP2/ERF* indicating that extensive cell death has been induced (Fig. 3). As expected, cell death was not observed in roots treated with water, and extensive cell death was observed in roots treated with ClsA. Syringe infiltration of rice leaves with LBA4404/p*OsAP2/ERF* strain but not LBA4404/pSB11 strain induced callose deposition in rice leaves. As expected, infiltration with ClsA induced callose deposition while only background levels of callose deposition were observed in leaves infiltrated with water (Fig. 3, Table 5).

Prior treatment with LBA4404/p*OsAP2/ERF* strain induces resistance to subsequent *X. oryzae* pv. *oryzae* infection in rice

The midveins of leaves from 40-day-old rice plants were preinjected with one of the following: water and saturated culture of LBA4404/pSB11 and LBA4404/p*OsAP2/ERF*. All leaves were subsequently infected with *X. oryzae* pv. *oryzae*. Across four independent experiments, all the leaves (100%) that had been preinjected with water exhibited lesions induced by *X. oryzae* pv. *oryzae*. About 72–77% of the

Table 5 Transient Transfer of *OsAP2/ERF* Induces Callose Deposition in Rice Leaves

Mean number of callose deposits/0.6mm^2 area ± SD[a]				
Exp[b]	Water	Cellulase	LBA4404/pSB11	LBA4404/p*OsAP2/ERF*
1	13.30±4.08	141.20±37.43	23.10±10.07	87.00±15.29
2	11.70±3.09	154.00±37.21	29.00±15.81	97.50±35.73
3	14.40±3.50	126.10±29.17	25.50±6.43	80.00±16.47

[a] Rice leaves were syringe infiltrated with one of the following: water, cellulase (100 μg ml^{-1}), LBA4404/pSB11, and LBA4404/p*OsAP2/ERF*, stained with aniline blue, and examined under epifluorescence microscope. Mean and standard deviation were calculated for number of callose deposits from a leaf area of 0.60 mm^2

[b] Data were collected from at least ten leaves in each experiment. ANOVA was performed, and the values obtained after infiltration with LBA4404/p*OsAP2/ERF* were found to be significantly different ($p<0.05$) as compared to all other treatments

Table 6 Transient Transfer of *OsAP2/ERF* Induces Resistance Against *Xanthomonas oryzae* pv. *oryzae* Infection

Treatments[a]	% of infected leaves (no. of infected leaves/total no. of leaves)[b]				Mean lesion length of leaves showing disease symptoms (cm) ± SD[c]			
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 1	Exp 2	Exp 3	Exp 4
Water	100 (19/19)	100 (28/28)	100 (21/21)	100 (25/25)	11.2±7.01	10.50±6.42	11.86±4.82	10.18±3.66
LBA4404/ pSB11	72 (18/25)	75 (21/28)	77 (14/18)	76 (19/25)	10.8±7.36	9.38±5.93	7.71±5.30	8.68±5.75
LBA4404/ p*OsAP2/ERF*	30 (7/23)	50 (10/20)	50 (9/18)	48 (12/25)	3.57±1.51	5.0±1.56	3.56±1.24	5.58±1.73

[a] Midveins of leaves of 40 days old rice plants (TN-1) were preinjected with either water alone, or water containing ~10^9 cells of LBA4404/pSB11 or LBA4404/p*OsAP2/ERF*. After 6 h, *Xanthomonas oryzae* pv. *oryzae* was inoculated onto the midvein, 2 to 3 cm below the point of initial injection, by pricking with a needle that had been used to touch a freshly grown colony of *Xanthomonas oryzae* pv. *oryzae*. Lesion lengths were measured 10 days after inoculation

[b] 18–28 leaves were used in each experiment and four independent replications were performed

[c] Lesion lengths were measured 10 days after inoculation. Mean and standard deviation were calculated for each of the treatments. ANOVA was performed and the values obtained after treatment with LBA4404/p*OsAP2/ERF* were found to be significantly different ($p<0.05$) as compared to all other treatments

leaves which were preinjected with LBA4404/pSB11 exhibited *X. oryzae* pv. *oryzae*-induced lesions. Interestingly, only 30–50% of leaves that were preinjected with LBA4404/p*OsAP2/ERF* exhibited disease lesions (Table 6). Among the leaves that exhibited disease lesions, the lesion lengths were 3.56±1.24 to 5.58±1.73 cm, 7.71±5.30 to 10.8±7.36 cm, and 10.18±3.66 to 11.86±4.82 cm long following preinjection with LBA4404/p*OsAP2/ERF*, LBA4404/pSB11, and water, respectively.

Discussion

X. oryzae pv. *oryzae* secretes a cocktail of at least five cell-wall-degrading enzymes, including ClsA, to damage the plant cell wall during infection. Besides these five enzymes, the *X. oryzae* pv. *oryzae* genome additionally encodes five cellulases, three xylanases, one pectinase as well as several other categories of cell-wall-degrading enzymes (Lee et al. 2005). It is possible that many of these enzymes are expressed only during in planta growth, and it is likely that the action of each one of these enzymes will induce rice innate immune responses. In spite of the apparent importance of cell-wall-degrading enzymes for induction of host innate immunity (Jha et al. 2007), very little information is available about plant functions that participate in the elaboration of innate responses that are induced following their action.

To address this lacuna, we have performed transcriptional profiling of rice gene expression changes that occur following cell-wall damage initiated by treatment with *X. oryzae* pv. *oryzae* ClsA. In our experiments, we have infiltrated 5 μg of ClsA protein into approximately 1 cm^2 of leaf area although we find that infiltration of even 4 μg of enzyme is sufficient to induce a HR like response. In the laboratory, approximately 4 μg of ClsA is secreted per milliliter of a saturated culture (approx. 1×10^9 cell) of *X. oryzae* pv. *oryzae*. During infection, 1 cm^2 of leaf area supports 1×10^9 cells of *X. oryzae* pv. *oryzae*, and we expect that these cells would secrete about 4 μg of ClsA. As a number of other cell-wall-degrading enzymes, besides ClsA, are secreted by *X. oryzae* pv. *oryzae*, we expect that the total amounts of cell-wall-degrading enzymes secreted by *X. oryzae* pv. *oryzae* is going to be much higher than that used in this experiment. Besides the above, there is evidence that secretion by T2S of *V. cholerae* and *P. aeruginosa* occurs in a polar manner because the T2S apparatus is localized to one pole of the cell, possibly at the point of attachment to host surface (Scott et al. 2001; Senf et al. 2008). If the T2S of *X. oryzae* pv. *oryzae* is similarly localized, the local concentrations of cell-wall-degrading enzymes are likely to be quite high during infection.

We have performed homology searches using BLASTX for each of the 267 genes that were found to be differentially expressed (≥2-fold at $p<0.05$) following ClsA treatment. A striking observation is that a large number of putative transcription factors (26 upregulated and 15 downregulated) are differentially expressed following ClsA treatment. This is suggestive of global changes in rice gene expression during the HR like reaction. The microarray analysis has revealed the downregulation of genes for several chlorophyll a/b binding proteins and suppression of the rice ortholog of *GUN4*, a critical activator of chlorophyll synthesis in *Arabidopsis* (Larkin et al. 2003). The gene ontology enrichment analysis also indicated that functions involved in photosynthesis are downregulated. This suggests an overall downregulation of synthesis of chlorophyll and chlorophyll-binding proteins during ClsA-induced cell death and a possible reduction in photosynthetic efficiency during this process. It was also interesting to note that the gene which exhibits maximum upregulation in our microarray is

homologous to a gene encoding a senescence associated protein of *Arabidopsis* (AT1G74940.1) suggesting a possible correlation between some events associated with senescence and HR. Three condensing enzymes and a ß-ketoacyl reductase that are homologous to *Arabidopsis* functions that participate in cuticle development were also downregulated following ClsA treatment. Defects in cuticle development have been shown to cause enhanced resistance to *Botrytis cinareae* and enhanced disease symptoms in response to an avirulent strain of *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana* (Chassot et al. 2007; Bessire et al. 2007; Tang et al. 2007).

We had earlier demonstrated that co-treatment with wild-type *X. oryzae* pv. *oryzae* results in suppression of ClsA-induced rice defense responses. We demonstrate here, using real-time PCR, that co-treatment with wild-type *X. oryzae* pv. *oryzae* also results in suppression of ClsA-induced expression of the rice transcription factor genes *OsAP2/ERF* and *OsRERJ1*. AP2/ERF members are downstream components of both the ethylene and jasmonatic acid (JA) pathways and are key to the integration of both signals (Lorenzo et al. 2003; Zhang et al. 2004a, b). The *OsRERJ1* gene is upregulated by JA (Kiribuchi et al. 2004). Chini et al. (2007) and Thines et al. (2007) have demonstrated that several *Arabidopsis* ZIM family transcription factors serve as negative regulators of the JA pathway through repression of a MYC transcription factor (*JASMONATE INSENSITIVE1*, *JIN1;* Lorenzo et al. 2004), which is the key activator of JA responsive genes. JA promotes ubiquitination and degradation of these ZIM transcription factors, resulting in expression of MYC and activation of the JA responsive genes. Transcription of the genes for these ZIM transcription factors is upregulated by JA, and this has been attributed to serve in damping the JA pathway (Chini et al. 2007; Thines et al. 2007). The microarray data presented here indicates that rice homologs of these ZIM transcription factors and *OsMYC* (or *OsJIN1*) are upregulated by ClsA treatment. Besides the above, we have found that several genes that encode functions predicted to be involved in JA biosynthesis are upregulated following ClsA treatment. These results, taken together, suggest that the JA-mediated pathway might be associated with ClsA-induced HR. This needs to be confirmed using rice lines that are either mutated or knocked down for the JA pathway.

Nakano et al. (2006) indicate that the genome sequences of *Arabidopsis* and rice contain 122 and 139 ERF proteins, respectively, which are subdivided into I to X subgroups. Our sequence analysis indicates that the *OsAP2/ERF* studied here belongs to Xb subgroup of the ERF subfamily which is characterized by the presence of Cys repeats at its N-terminal region. We have treated rice leaves and roots with LBA4404/p*OsAP2/ERF* in order to assess the effect of transient transfer of *OsAP2/ERF* on induction of innate immune responses. Rice leaves infiltrated with LBA4404/p*OsAP2/ERF* showed a significant increase in callose deposition as compared to leaves treated with LBA4404/pSB11 (control). Similarly, rice roots treated with LBA4404/p*OsAP2/ERF* exhibit programmed cell death, which is absent in roots treated with LBA4404/pSB11. Prior injection of LBA4404/p*OsAP2/ERF* in rice mid-veins induces resistance against subsequent infection with *X. oryzae* pv. *oryzae*. Treatment with LBA4404/pSB11 also induces a certain degree of resistance against subsequent bacterial infection as evident from fewer leaves showing *X. oryzae* pv. *oryzae* induced disease lesions. Although this resistance is significantly lesser than that induced by LBA4404/p*OsAP2/ERF*, it suggests that Agrobacterium can also induce a certain amount of resistance. Overall, these results suggest that transient overexpression of *OsAP2/ERF* induces rice resistance responses. However, these results need to be confirmed by making stably transgenic rice lines that overexpress *OsAP2/ERF*, preferably under the control of an inducible promoter.

Besides the cell-wall-degrading enzymes secreted by *X. oryzae* pv. *oryzae*, several other molecules are known to be elicitors of innate immune responses in rice. The OsFLS2 protein, a receptor kinase, has been shown to be the receptor for the flg22 peptide (Takai et al. 2008); Xa21, a rice resistance protein and receptor kinase, is the receptor for AvrXa21 peptide (Lee et al. 2009), and the rice chitin-binding protein CeBiP, a transmembrane protein with particular extracellular motifs, is the receptor for chitin (Kaku et al. 2006). The small GTPase OsRAC1 is involved in the immune responses induced by *N*-acetylchitooligosaccharide and spingolipids (Nakashima et al. 2008). None of these above mentioned proteins were differentially expressed (2-fold level) in the microarray experiments described here. However, several putative receptor-like protein kinases are differentially expressed following ClsA treatment, and it remains to be determined whether these are involved either in elicitor perception or in further signal processing. *X. oryzae* pv. *oryzae* lipopolysaccharide is also an inducer of rice defense responses, but the receptor has not yet been characterized (Desaki et al. 2006).

In summary, we have performed microarray analysis of gene expression changes that are induced in rice by ClsA treatment. A number of rice functions, particularly transcription factors, are differentially expressed under these conditions. Initial studies using Agrobacterium-mediated transient gene transfers suggest that overexpression of one of the highly upregulated transcription factor genes, *OsAP2/ERF*, results in induction of callose deposition, cell death, and enhanced resistance against subsequent infection by *Xanthomonas oryzae* pv. *oryzae*. Future studies will be aimed at understanding the role of *OsAP2/ERF* and other differentially expressed genes in ClsA-induced innate immunity in rice.

Methods

RNA isolation

Fifteen-day-old seedlings of the susceptible rice cultivar TN-1 were grown in a greenhouse in the months of December/January and shifted to a growth chamber (28°C; 80% relative humidity; light intensity of 440 μmole/m^2/s; 12/12 h light/dark cycle) 48 h before the treatment. The adaxial surfaces of the leaves were syringe infiltrated, with approximately 10 μl of ClsA (500 μg/ml) purified from the culture supernatant of wild type *X. oryzae* pv. *oryzae* (strain BXO43; our laboratory wild type) or with buffer (10 mM potassium phosphate buffer pH 6.0) alone (as described in Jha et al. (2007)). In this procedure, approximately, 30–40 μl of either ClsA or buffer are taken into a needle less 1 ml syringe for infiltration, and we are able to infiltrate ~10 μl of the solution into the leaf while the rest of the solution either falls off the adaxial surface or passes right through to the abaxial surface. Twenty to 30 leaf pieces covering the infiltrated zone from each of the treatments were harvested 12 h after infiltration. At this time point, HR like symptoms are not observed in ClsA-treated leaves, and it would take a further 24 h for these symptoms to be visible (Supplementary Fig. S1). The leaf tissues were ground to a fine powder (in liquid nitrogen using a mortar and pestle) and subjected to total RNA isolation by using Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The quality of the isolated RNA was assessed by agarose gel electrophoresis and quantitated using a spectrophotometer.

Microarray analysis

Eight micrograms of total RNA isolated from ClsA- and buffer-treated leaves were converted to double-strand cDNA using an Affymetrix (Santa Clara, CA) one cycle labeling kit following the manufacturer's instructions. The synthesized double-stranded cDNA was column purified and was further subjected to RNA amplification using an Affymetrix IVT labeling kit. The amplified RNA (cRNA) generated in this manner was quantitated by using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The normalized cRNA was fragmented (into 50–200 bp fragments), hybridized to rice gene chips, washed, stained, and scanned as per Affymetrix protocols. The experiment was repeated with three different biological replicates using RNA isolated from three batches of rice leaves treated with either *X. oryzae* pv. *oryzae* ClsA or buffer.

Data analysis

The data were analyzed by using the GCOS of Affymetrix. The present calls ranged between 40% and 42% in ClsA-treated leaves and between 38% and 40% in the buffer-treated leaves. All the CEL (cell intensity) files generated by GCOS were uploaded to Avadis™ software version 4.3 (an Affymetrix approved software manufactured by Strand Life Sciences, Bangalore, India) for further analysis. The CEL files of the buffer-treated samples were grouped as control whereas the ClsA-treated sample files were grouped as treatment. The data were normalized using RMA (Irizarry et al. 2003a, b) and PLIER (Affymetrix Inc 2005) algorithms available in Avadis and subjected to differential expression analysis as per the manufacturer's instructions. The genes identified by both the algorithms as being either ≥2.0- or ≥1.5-fold differentially expressed with $p<0.05$ were selected. *Q*-value-based significance analysis was performed for rice genes that were commonly differentially expressed at fold change 1.5 and $p<0.05$. *Q* values were generated from p values for all 862 genes using the online tool (http://genomics.princeton.edu/storeylab/qvalue/index.html) with settings of interval 0.01 and smoother method with FDR 0.02 (Storey and Tibshirani 2003). GO enrichment analysis was performed to identify overrepresented GO terms amongst 1.5-fold change differentially expressed rice genes following ClsA treatment. The Affymetrix probe set IDs of the ≥1.5-fold upregulated and downregulated rice genes were subjected to Gene Ontology Enrichment Analysis Software Toolkit analysis using an online tool accessible at http://omicslab.genetics.ac.cn/GOEAST following default standard setting (Zheng and Wang 2008). The ≥2.0-fold differentially expressed genes were subjected to NCBI BLASTX analysis to manually curate their functions. A gene was assigned the function of its orthologs if it showed significant homology with expect value $\geq 1\times10^{-7}$. Based upon the assigned functions, the differentially expressed genes were further categorized into 14 different functional categories as per Bevan et al. (1998) with some minor changes. We did not have any differentially expressed genes that were predicted to be involved in protein destination and storage. Therefore, we dropped this category. Some of the differentially expressed genes were predicted to function in protein synthesis, while others appeared to encode functions involved in protein degradation. We formed a new category called protein synthesis/turnover into which we clubbed genes involved in protein synthesis as well as protein degradation. Genes related to abiotic stress were put into a new category called "stress" that is distinct from genes related to biotic stress which were placed under the category of "disease/defense." We did this because we wanted to separate genes related to biotic stress from those related to abiotic stress. The genes having unclear, unclassified or no significant similarity in the available databases were grouped together in a category called "others". In order to identify *Arabidopsis* orthologs of rice genes, BLASTX searches were performed through TAIR (The *Arabidopsis* Information Resource; www.arabidopsis.org).

For the ≥1.5-fold differentially expressed genes, the locus information corresponding to the Affymetrix probe set IDs was obtained from the National Science Foundation rice oligonucleotide array project (http://www.ricearray.org/matrix.search. shtml) through the rice genome server of The Institute for Genome Research, Rockville, MA (Yuan et al. 2005; Ouyang et al. 2007). These were subjected to pathway analysis using RiceCyc 1.2 software (Jaiswal et al. 2006) available at http://dev.gramene.org/pathway.

Data submission

All the six CEL files (generated by GCOS) and the Avadis™ processed (PLIER and RMA normalized) files are deposited at the NCBI microarray repository, Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE8216 with details provided about the experimental procedures that are minimum information about a microarray experiment compliant.

Real-time PCR

Two micrograms of freshly isolated RNA were converted into first-strand cDNA as per the recommended protocol by using Superscript™ II reverse transcriptase (Invitrogen) and oligo-dT primer. One microliter of the 1:100-fold diluted cDNAs were subjected to real-time PCR analysis using SYBR green PCR master mix (Applied Biosystem, Barrington, UK) following the manufacturer's instructions using gene specific primers designed to amplify 130–170 bp fragments of each gene of interest. After 10 min of initial denaturation at 95°C, the samples were subjected to the cycling parameters of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s (for 40 cycles) using a 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). The relative expression of the gene between ClsA and buffer-treated samples was calculated using the $2^{-\mathrm{ddC}}$T method with *OsGAPDH* as an endogenous control (Livak and Schmittgen 2001). The real-time PCR assays were performed at least three times with the same cDNA sample and repeated at least three times with independently isolated RNA samples.

Co-infiltration of ClsA and wild-type *X. oryzae* pv. *oryzae* into rice leaves

Three milliliters of wild-type *X. oryzae* pv. *oryzae* cultures were grown to saturation in Peptone Sucrose broth (pH 7.2) (Ray et al. 2000). The cells were pelleted and washed in double-distilled water following centrifugation. The pelleted cells were resuspended (at a concentration of approximately 3×10^{9} cells/ml) in 1 ml of purified *X. oryzae* pv. *oryzae* ClsA (final conc. 500 µg/ml) and syringe infiltrated into the leaves as described above. The total RNA isolated from the zone of infiltration in the leaves was subjected to quantitative real-time PCR analysis (as described above). A Student's *t* test was performed to check the level of significance for this suppression.

Sequence analysis of *OsAP2/ERF*

Gene sequence of *OsAP2/ERF* (LOC_Os08g36920) was downloaded from TIGR (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/), and BLASTX analysis was performed in Rice Genome Annotation Project BLAST Search (http://rice.Plantbiology.msu.edu/blast.shtml). Amino acid sequence of the gene was used to do a domain search (http://www.expasy.ch/prosite). Secondary structure prediction was performed with Jpred web server (http://www.compbio.dundee.ac.uk/www-jpred).

Cloning *OsAP2/ERF* in Agrobacterial T-DNA vector and transfer to Agrobacterium strain LBA4404

OsAP2/ERF full-length gene was PCR amplified by using a pair of primers, each containing a BamHI site and cloned into the BamHI site between CaMV35S promoter and PolyA on pSB11 binary vector (Ramesh et al. 2004) (Supplementary Fig. S3). This construct was transformed into *Escherichia coli* DH5α cells. Positive clones were screened using gene and vector-specific primers and confirmed by sequencing. Plasmids from positive clones were further electroporated into electro-competent cells of *Agrobacterium* LBA4404.

Callose deposition assay

Rice leaves were syringe infiltrated with one of the following: water, ClsA (~100 µg/ml), LBA4404/pSB11, and LBA4404/p*OsAP2/ERF* strains (approximately 10^{9} CFU/ml resuspended in water). After 12 to 15 h, the leaves were heated with lactophenol at 65°C to remove chlorophyll, stained with aniline blue for 2 to 3 h, washed with water, mounted on a slide in 50% glycerol, and analyzed by an Axioplan2 epifluorescence microscope, using a blue filter (excitation wavelength 365 nm and emission wavelength above 420 nm long pass [LP]) and ×10 objective (Hauck et al. 2003).

Cell death assay

Rice seeds were surface sterilized with sodium hypochlorite and germinated under sterile conditions on a filter paper placed on 0.5% agar in Petri dishes for 3 to 4 days. Root tips, 1 to 2 cm long, were treated with one of the following: water, ClsA (~500 µg/ml), LBA4404/pSB11, and LBA4404/p*OsAP2/ERF*. Rice roots were treated with LBA4404/pSB11 and LBA4404/p*OsAP2/ERF* for 2 h, and roots were subsequently transferred

to water for another 14 h. After incubation for a total of 16 h at 28°C, roots were washed in 1X PBS and stained with PI by vacuum infiltration for 10 to 15 min. The roots were mounted on a microscopic slide in 50% glycerol in 1X PBS; 0.3-μm thick longitudinal optical sections were acquired on a Zeiss LSM-510 Meta confocal microscope using 63X oil immersion (NA 1.4) and were further projected to obtain the image of 2 to 3 μm total thickness. HeNe laser at 543 nm excitation and emission above 560 nm (LP) was used to detect PI internalization. All images were analyzed using LSM software and further edited using Photoshop (Adobe, San Jose, CA, USA).

Rice resistance assay

The midveins of leaves of approximately 40 days old TN-1 rice plants were injected with 40 to 50 μl of water alone, LBA4404/pSB11 or LBA4404/p*OsAP2/ERF* cells (resuspended in water at ~10^9 cells/ml) using a 1-ml hypodermic syringe and needle. Six hours later, the midveins of the leaves were inoculated with *X. oryzae* pv. *oryzae*, 2 to 3 cm below the point of initial injection, by pricking with a needle that had been used to touch a fresh bacterial colony. After 10 days, the leaves were observed for the appearance of visible disease lesions (discoloration of midvein and surrounding regions).

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