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Full Length Article

Genome-Wide Analysis of Chitinase Gene Family in Rice and *Arabidopsis* Reveal their Mechanisms and Diverse Roles in Defense Response

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Abstract

Members of plant *chitinase* (*CHI*) gene family are widely implicated in defense response. For rice (*Oryza sativa* L.), the important monocotyledonous model plant, a genome-wide overview of the *CHI* family members is not yet available. Here, 48 *O. sativa CHIs* (*OsCHIs*) were identified from rice genome. Then phylogenetic analysis of these OsCHIs as well as CHIs (*AtCHIs*) of *Arabidopsis thaliana*, the important dicotyledonous model plant, revealed eight distinct groups as strongly supported by exon/intron structure and motif organization. Further, it was revealed that expansion of *CHI* family has occurred largely via tandem duplication, while segmental duplication has a very limited role. Furthermore, analysis of oligonucleotide array data gained insights into diverse roles of *CHI* genes under various biotic stress conditions. Many *OsCHIs* were found to significantly respond to a parasite plant *Striga hermonthica*, suggested the new role of these *OsCHIs* in response to this stress. Most *AtCHIs* in the Group 7 and 8 were clearly up-regulated in response to three types of pathogens, indicating the function of the two groups in defense. This study provides comprehensive analysis on *CHI* gene family in rice and *Arabidopsis*, and potential candidates were indicated for improving resistance in plants through transgenic approach.
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Keywords: Plant chitinase; Gene duplication; Gene expression; Parasite plants; Pathogens

Introduction

Chitinases (CHIs) (EC 3.2.1.14) are lytic enzymes which catalyze the degradation of chitin (Henrissat 1991), a major component of cell walls of bacteria and fungi (Henrissat 1991; Chen *et al.* 2018; Mir *et al.* 2019). *CHIs* belong to a large gene family, and exist in microorganisms, plants and animals (Mishra *et al.* 2015; Xi *et al.* 2015; Xu *et al.* 2016; Chen *et al*. 2018; Filyushin *et al.* 2019). Based on the sequence similarity of the catalytic domains, CHIs have been classified into either the Glycosyl hydrolase 18 family (GH18) or GH19 (Henrissat 1991). The GH18 genes are found in various organisms, such as microorganisms, plants and animals, while the GH19 genes exist almost in plants (Jiang *et al.* 2013; Mir *et al*. 2019). According to the presence or absence of an N-terminal hevein domain and sequence similarity with an archetypal catalytic domain, traditionally plant CHIs are categorized into six classes (Neuhaus *et al*. 1996; Levorson and Chlan 1997). However, CHI members in each same class exhibit distinct enzyme activity, strongly suggesting that some other parts of CHI sequence also contribute to enzyme activity and thus should be considered in the classification of *CHI* gene families (Levorson and Chlan 1997; Sasaki *et al.* 2006).

Plant CHIs belong to a subgroup of pathogenesis-related proteins (PRs), and it is believed that plant CHIs can directly attack chitin from invading pathogens (Hamid *et al.* 2013; Chen *et al*. 2018). Also, the chitin fragments produced by plant CHIs can act as elicitors to activate defense responses in interactions of plant with various pathogens (Xu *et al*. 2016). The defense roles of some plant *CHIs* have been supported by many studies. For example, in some plant *CHIs* are up-regulated in response to infection with pathogenic bacteria and fungi (Mir *et al*. 2019). Further, some *CHIs* can confer resistance to plant disease. For example, *PnCHI1*, a *CHI* from *Panax notoginseng*, can confer tobacco resistance to *F. solani* (Bai *et al.* 2018). Overexpression of *NtPR-Q*, a member of the *PR3* family encoding chitinases, leads to enhanced resistance to *Ralstonia solanacearum* in *Nicotiana tabacum* (Tang *et al.* 2017). Dong *et al.* (2017) cloned a new *CHI EuCHIT2* from *Eucommia ulmoides* Oliver and found that expression of the *CHI* confers resistance to *Erysiphe cichoracearum* DC in tobacco plants. Thus, these data show that plant *CHIs* play an important role in defense against pathogen infections, and it is important to understand roles of this gene family in defense.

CHI gene families have been widely studied in various plant species. However, for rice (*Oryza sativa* ssp. *japonica*), the important monocotyledonous model plant, a genome-

wide overview of the *CHI* gene family members is not yet available. In fact, in earlier work, *CHI* gene family of this model plant has been investigated (Xu *et al.* 2007). However, the data in this work were provided with limited information for *CHIs*. For example, what was identified for *CHIs* in the work is their open reading frames (ORFs), consequently being lack of the important information about gene structure as well as chromosomal distribution of *CHIs*, and these ORFs is not available because their nomenclature was so out-of-date that these ORFs could be not retrieved from currently available databases, such as RAP, RGAP (the Rice Genome Annotation Project Database) and NCBI. Furthermore, gene structure, motif and duplication analysis of *CHIs* was not performed in *O. sativa CHIs* (*OsCHIs*) as well as *A. thaliana CHIs* (*AtCHIs*). In this study, *CHIs* from rice have been identified and detailed analysis, including gene structure, conserved motifs, gene chromosome location, gene birth and expression profiling, were performed on *OsCHIs* and *AtCHIs*. This genome wide analysis provides the framework for future studies to dissect functions of these genes.

Materials and Methods

Database screening and identification of *OsCHIs* and *AtCHIs*

A search for *A. thalinan CHIs* was performed using keyword 'CHITINASE' in The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/), and 26 *CHI* genes of A. thaliana were acquired. To identify *CHI* genes in rice, sequences of 26 *A. thaliana* CHI proteins were used in BLAST search of the Rice Annotation Project (RAP) database. Meanwhile, we also used keyword 'CHITINASE' to search for *CHIs* in the RAP database. After removing the redundant sequences, we used the NCBI-CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) database to investigate the conserved domain of remaining sequences (Marchler-Bauer *et al.* 2017). These protein sequences were analyzed in the SMART (https://smart.embl-heidelberg.de/) and Pfam (https://pfam.xfam.org/) databases to confirm the presence of CHI domain. Those proteins containing CHI domain (GH18 domain or GH19 domain) were defined to belong to the CHI family. GH18 domains include cd02876, cd02877, cd02879, smart00636, cd06544, cl10447 and pfam00704, and GH19 domains include cd00325, pfam00182 and cd06921. Protein subcellular localization was predicted by using WoLF PSORT program (https://wolfpsort.org) (Horton *et al.* 2007). The pI (isoelectric point), molecular mass and GRAVY (grand average hydropathy) values were determined by using the ExPASy-ProtParam tool (https://web.expasy.org/protparam/).

Protein sequence alignment and phylogenetic analysis

The ClustalX program was used to perform the multiple sequence alignments of protein sequences, and the alignments were corrected manually. The neighbor-joining method was used to construct the unrooted phylogenetic tree. Bootstrap analyses were performed using 1000 replicates. The phylogenetic trees were displayed using MEGA software version 6.0 (Tamura *et al.* 2013).

Gene structure of the *CHIs* and conserved motifs analyses

Gene structure information of *CHIs* was collected from the annotations from NCBI. The motifs in these CHI protein sequences were identified by using the program MEME (https://meme-suite.org/index.html) (Bailey *et al.* 2006).

Chromosomal localization and gene duplication

BLASTN was used to position the *CHIs* on the rice or *Arabidopsis* chromosomes. Based on close phylogenetic relationships, tandem duplications of *CHIs* tandemly arrayed at the same chromosomal location were identified. Segmental duplicates were recognized through comparing positions of *CHIs* in duplicated chromosomal blocks previously identified in the *Arabidopsis* genomes (https://bioinformatics.psb.ugent.be/beg/research/genome-duplication-polyploidy) or rice genomes (Blanc *et al.* 2003; Lin *et al.* 2006).

EST profiling and microarray analysis

For rice, the microarray data from GEO (Gene Expression Omnibus) database under the accession numbers GSE7256 (infection by *Magnaporthe grisea*) (Ribot *et al.* 2008) and GSE10373 (interaction with the parasitic plant *Striga hermonthica*) (Swarbrick *et al.* 2008) were used for expression analysis of *OsCHIs*. For *Arabidopsis*, the microarray data for various pathogen treatments were downloaded from GEO (series accession numbers GSE5684, GSE5685 and GSE5686). In addition, for genes with more than one probe sets, the median values represented their expression values. The genes which are up- or down-regulated more than 2-fold were considered to be differentially expressed significantly. Finally, the expression pattern images were based on the average log signal values and generated using the Genesis program (Sturn *et al.* 2002).

Results

Identification and phylogenetic analysis of *CHIs* in rice and *Arabidopsis*

A total of 48 *OsCHIs* were identified in the rice genome, according to multiple searches followed by confirming as encoding CHI proteins (Table 1). In these OsCHI proteins, there were 18 Xylanase inhibitor proteins (XIPs) which belong to GH18 protein because they contain typical GH18 domains and have sequence similarity to GH18.

Table 1: Chtinase genes identified in rice

	Gene symbol	Genomic position	PL[1]	Family	Description	Conserved domain	Mr	pI	GRAVY[2]	PSORT predictions[3]
1	Os01g0287600	chr01:10342026..10343570	290	GH19	Chitinase10	cd00325	31264.1	8.01	-0.229	E: 8.0, V: 3.0, C: 2.0
2	Os01g0303100	chr01:11208573..11209811	335	GH18	Acidic endochitinase SE2	cd02877, pfam00704	35004.4	6.86	0.041	C: 12.0, E: 2.0
3	Os01g0619800	chr01:24691392..24696311	260	GH18	Chitinase domain-containing protein 1	cd02876, smart00636	29659.6	6.12	-0.218	N: 7.0, M: 2.5, C_M: 2.5, CY: 2.0, C: 1.5
4	Os01g0660200	chr01:26885484..26886719	301	GH18	Acidic endochitinase	cd02877, pfam00704	31039.4	4.31	0.064	C: 6.0, V: 4.0, M: 2.0, E: 2.0
5	Os01g0687400	chr01:28354882..28356067	302	GH18	Acidic endochitinase	cd02877, pfam00704	31330.1	4.6	0.118	E: 8.0, C: 4.0, CY: 1.0
6	Os01g0691000	chr01:28534821..28536497	358	GH18	Acidic endochitinase SE2 isoform X2	cd02877, pfam00704	37583.4	5.14	0.152	C: 4.0, E: 4.0, E.R._plas: 2.8, E.R.: 2.5
7	Os01g0860400	chr01:37235781..37237206	297	GH18	Acidic endochitinase	cd02877, pfam00704	31494.6	6.3	-0.066	E: 8.0, C: 2.0, V: 2.0, N: 1.0
8	Os01g0860500	chr01:37239583..37240775	305	GH18	Hevamine-A[Includes: Chitinase; Lysozyme]	cd02877, pfam00704	32202.7	8.43	0.081	V: 5.0, E: 4.0, C: 3.0, M: 2.0
9	Os02g0605900	chr02:23743045..23744109	271	GH19	Chitinase 6	cd00325 pfam00182 pfam00187	28533.7	4.76	-0.032	E: 8.0, C: 3.0, V: 2.0
10	Os03g0132900	chr03:1860429..1861772	256	GH19	Chitinase 11	cd00325 pfam00182	27747.9	6.42	-0.453	E: 7.0, C: 3.0, V: 2.0, G: 2.0
11	Os03g0418000	chr03:17390820..17391862	326	GH19	Chitinase12	cd00325, pfam00182, pfam00187	33636.1	4.63	-0.107	C: 10.0, E: 2.0, M: 1.0
12	Os04g0347200	chr04:16519831..16521452	170	GH18	Acidic endochitinase	cd02877 pfam00704	18038.6	9.37	0.096	C: 14.0
13	Os04g0376400	chr04:18392642..18394466	479	GH18	Class V chitinase	cd02879, pfam00704	50277.3	9.53	0.227	C: 7.0, V: 3.0, N: 1.0, M: 1.0, E: 1.0
14	Os04g0493400	chr04:24687753..24689297	229	GH19	Chitinase 4	cd00325 pfam00182 pfam00187	25151.3	8.79	-0.322	C: 11.0, E: 2.0
15	Os04g0494100	chr04:24708801..24709910	288	GH19	Chitinase 5	cd00325 pfam00182 pfam00187	30486.9	8.31	-0.419	E: 12.0, C: 1.0
16	Os05g0138200	chr05:2217558..2218582	295	GH19	Chitinase 10-like	cd00325 pfam00182	32115.1	9.02	-0.267	C: 11.0, N: 1.0, CY: 1.0
17	Os05g0247100	chr05:8902501..8903789	297	GH18	Chitinase III protein	cd02877, pfam00704	32548.9	6.08	-0.08	C: 6.5, C_M: 6.0, M: 4.5, CY: 2.0
18	Os05g0247500	Chr5:8959285..8960426(+)	293	GH18	Chitinase-like protein	cd02877	31838.1	6.59	-0.032	E: 5.0, V: 4.0, C: 3.0, N: 1.0
19	Os05g0247800	chr05:8959416..8960566	293	GH18	Xylanase inhibitor protein 2-like, chitinase-like protein	cd02877 pfam00704	32435.8	8.76	-0.202	C: 4.0, V: 3.0, M: 2.0, E: 2.0, N: 1.0, E.R.: 1.0
20	Os05g0248200	chr05:8981788..8982953	297	GH18	Xylanase inhibitor protein 2-like, chitinase-like protein	cd02877 pfam00704	33039.7	9	-0.161	C: 7.0, M: 4.0, E: 2.0
21	Os05g0399300	chr05:19426767..19427874	338	GH19	Chitinase 2	cd00325 pfam00182 pfam00187	35388.5	7.39	-0.257	C: 12.0, E: 2.0
22	Os05g0399400	chr05:19435401..19436495	334	GH19	Chitinase 9	cd00325 pfam00182 pfam00187	34401.1	4.48	-0.121	E: 13.0
23	Os05g0399700	chr05:19445901..19447487	340	GH19	Chitinase 7	cd00325 pfam00182 pfam00187	35299.7	8.32	-0.081	C: 11.0, E: 2.0
24	Os06g0356800	chr06:14646987..14648089	248	GH18	Xylanase inhibitor protein 1 isoform X2, Chitinase-like protein	cd02877	27273	6.35	-0.008	M: 3.0, E: 3.0, V: 3.0, C: 2.0, N: 1.0
25	Os06g0726100	chr06:30887215..30888383	320	GH19	Chitinase 3	cd00325 pfam00182 pfam00187	33681.4	4.84	-0.292	E: 9.0, V: 3.0, C: 1.0
26	Os06g0726200	chr06:30890834..30892050	214	GH19	Chitinase 1	cd00325 pfam00182 pfam00187	22094.8	12.59	-0.86	N: 11.0, C: 3.0
27	Os07g0632000	chr07:26215095..26216256	316	GH18	Xylanase inhibitor protein 1, chitinase-like protein	cd02877	34194.7	8.49	-0.122	C: 6.0, E: 4.0, V: 2.0, N: 1.0
28	Os08g0518800	chr08:25758874..25759533	181	GH18	Xylanase inhibitor protein 2-like, Class III chitinase	cd02877 pfam00704	20410.7	6.2	-0.429	CY: 9.0, C: 2.0, N: 2.0
29	Os08g0518900	chr08:25762457..25763823	315	GH18	Xylanase inhibitor protein 1-like, Chitinase	cd02877 pfam00704	35285.7	8.22	-0.256	C: 8.0, M: 4.0, N: 1.0
30	Os08g0519300	chr08:25778245..25779466	283	GH18	Xylanase inhibitor protein 2-like, Chitinase-like protein	cd02877 pfam00704	31931.8	9.75	-0.195	C: 4.0, V: 4.0, M: 3.0, N: 1.0, E: 1.0
31	Os08g0522500	chr08:25975501..25977441	316	GH19	Chitinase-like protein 1	cd00325 pfam00182	34644.9	5.8	-0.286	E: 8.0, V: 5.0
32	Os09g0494200	chr09:19148042..19149871	326	GH19	Chitinase-like protein 1	cd00325 pfam00182	36519.6	7.07	-0.354	V: 4.0, M: 3.5, CY_M: 2.5, CY: 2.0, C: 1.0
33	Os10g0416100	chr10:14558964..14560173	307	GH18	Chitinase 2	cd06544	33679.2	5.08	0.128	C: 7.0, CY: 2.5, CY_N: 2.3, cysk_N: 1.3

Table 1: Continued

Table 1: Continued

34	Os10g0416500	chr10:14590045..14591113	286	GH18	Chitinase 1	cd06544, pfam00704	31089.9	5.17	0.021	M: 5.0, C: 4.0, CY: 3.0, N: 1.5, cysk_N: 1.5
35	Os10g0416800	chr10:14602523..14603656	288	GH18	Chitinase 2	cd06544 pfam00704	31212.8	4.48	-0.036	CY: 8.0, C: 3.0, M: 2.0
36	Os10g0542900	chr10:21205700..21207611	261	GH19	Chitinase 8	cd00325 pfam00182	27551.6	6.09	-0.118	C: 12.0, E: 2.0
37	Os10g0543400	chr10:21219598..21221819	296	GH19	Chitinase 8	cd00325	32168.8	5.64	-0.186	E: 6.0, V: 4.0, C: 2.0, N: 1.0
38	Os11g0462100	chr11:15764680..15766318	451	GH18	Class V chitinase	cl10447 cl15255 smart00636	49352.2	4.84	-0.055	P: 7.0, V: 5.0, C: 1.0
39	Os11g0700900	chr11:28722579..28724062	245	GH18	Xylanase inhibitor protein 1-like, Class III chitinase homologue	cd02877 pfam00704	27482.1	7	-0.244	C: 7.0, M: 3.0, V: 2.0, N: 1.0
40	Os11g0701000	chr11:28727508..28728821	312	GH18	Xylanase inhibitor protein 1-like, Class III chitinase homologue	cd02877 pfam00704	34988.5	9.21	-0.279	C: 11.0, N: 2.0
41	Os11g0701100	chr11:28730171..28731320	290	GH18	Xylanase inhibitor protein 2, Similar to Class III chitinase homologue	cd02877, pfam00704	31653.6	6.13	-0.162	E: 10.0, E.R.: 2.0, M: 1.0
42	Os11g0701200	chr11:28733145..28734249	292	GH18	xylanase inhibitor protein 2-like, Chitinase-like protein	cd02877, pfam00704	31523.7	6.44	0.045	E: 5.0, C: 4.0, V: 3.0, M: 2.0
43	Os11g0701400	chr11:28735986..28737061	289	GH18	Xylanase inhibitor protein 2-like, Chitinase III	cd02877, pfam00704	32238.6	9.28	-0.286	C: 8.0, M: 2.0, E: 2.0, CY: 1.0
44	Os11g0701500	chr11:28739616..28740634	284	GH18	Xylanase inhibitor protein 2-like, Class III chitinase homologue	cd02877, pfam00704	31196.2	5.87	-0.155	E: 6.0, C: 3.0, V: 3.0, CY: 1.0
45	Os11g0701600	chr11:28748102..28748484	125	GH18	Xylanase inhibitor protein 2-like, Chitinase-like protein	cd02877, pfam00704	13306.1	6.23	0.227	E: 6.0, V: 4.0, C: 2.0, M: 1.0
46	Os11g0701800	chr11:28753859..28755033	304	GH18	Xylanase inhibitor protein 1, Class III Chitinase homologue	cd02877	33946.8	9.33	-0.238	C: 7.0, V: 5.0, M: 1.0
47	Os11g0701900	chr11:28755994..28757153	300	GH18	Xylanase inhibitor protein 1-like, Chitinase-like protein	cd02877	32507.6	7.2	-0.126	E: 5.0, C: 4.0, M: 3.0, V: 2.0
48	Os11g0702100	chr11:28758837..28760003	301	GH18	Xylanase inhibitor protein 1-like, Similar to Class III chitinase homologue	cd02877, pfam00704	32996.2	7.07	-0.161	C: 10.0, M: 3.0
49	Os11g0702200	chr11:28760411..28761600	302	GH18	Xylanase inhibitor protein 1-like, Chitinase-like protein	cd02877 pfam00704	33516.1	8.23	-0.179	C: 10.0, M: 3.0

[1]PL means Protein Length; [2]GRAVY means Grand average of hydropathicity; [3] PSORT predictions: E (extracellular), P (plasma membrane), V (vacuolar membrane), CY (cytosol), C (chloroplast), N (nuclear), E.R. (endoplasmic reticulum), M (mitochondrion) and G (Golgi apparatus), Cysk (eytoskeleton)

In *A. thaliana*, 24 *AtCHI* genes have been annotated previously. Here, from TAIR database (https://www.arabidopsis.org/) we found 26 *AtCHIs* (Table 2). The extra two *AtCHIs* are *At4g01040* and *At3g47540-2*, an alternative form of *At3g47540*.

Most of these *CHIs* encode hydrophobic polypeptides (<0) ranged from 125 (Os11g0701600) to 430 (AT4G01040) amino acids residues, with pI values ranged from 4.41 to 12.59. Subcellular location prediction showed that most CHIs identified in this study are localized in the extracellular or Chloroplast. In addition, many CHIs were predicted to be localized to other organelles such as the mitochondrial, plasma or vacuolar membranes, nucleus, golgi apparatus or cytoplasmic. These subcellular localization predictions suggested that the *CHIs* would function in various aspects.

Phylogenetic analysis showed that all of OsCHIs and *AtCHIs* were divided into two distinct clades: the GH18 and GH19. Further, according to phylogenetic relationships, the GH18 class was divided into 5 subclasses designated as Groups 1–5, respectively. For the GH19 class, phylogenetic analysis revealed three subclasses designated as Groups 6–8 (Fig. 1a). Remarkably, Groups 1 and 3 do not include any *AtCHIs*, suggesting these were acquired for monocotyledonous rice but not for dicotyledonous *Arabidopsis*. Groups 2 and 7 mainly consist of OsCHIs and only one and three *AtCHIs* are, respectively, in the two groups, which suggested that these *ATCHIs* were actually clustered into about 5 groups. In contrast, Group 8, consisting of 13 members, ten of which are *AtCHIs*, and the other three are OsCHIs. Additionally, Group 4 consists of two CHIs, one from rice and another from *Arabidopsis*. Group 1 constitutes the largest clades in the CHI phylogeny, containing 21 members. These data indicated that some CHI groups could to some extent be specific for rice and others for *Arabidopsis*, suggesting that some CHIs have specialized roles in monocotyledons while others in dicotyledons.

Gene structure, conserved domains and motifs of the *CHI* family genes in rice and *Arabidopsis*

In order to understand the structural diversity of the *CHIs*, gene structure of each *CHI* was investigated. Firstly, we compared the exon/intron structure of each *CHI* and found that most members within the same groups shared very similar exon/intron structure on either intron numbers or exon lengths (Fig. 1b). Further, it was observed that 54% of *CHIs* in rice are intron less. For example, all but two members of Group 1 and all of Group 3 are intron less. In contrast, none of *CHIs* in *Arabidopsis* are intron less, although 75% of these genes only consist of one intron.

Table 2: Chitinase genes identified in *Arabidopsis*

	Gene	Genomic position	PL[1]	Family	Description	Conserved domain	Mr	pI	GRAVY[2]	PSORT predictions[3]
1	AT1G02360	Chr1:471990..473160(-)	272	GH19	Chitinase family protein	cd00325 pfam00182	30136.8	7.55	-0.301	E: 10.0, C: 1.0, N: 1.0, M: 1.0
2	AT1G05850	Chr1:1766503..1768695(-)	321	GH19	Endo chitinase-like protein	cd00325 pfam00182	35579.4	7.49	-0.202	E: 8.0, V: 3.0, G: 3.0
3	AT1G56680	Chr1:21250338..21251417(-)	280	GH19	Chitinase family protein	cd00325 pfam00182 pfam00187	31182.8	8.89	-0.195	E: 5.0, C: 3.0, V: 3.0, N: 1.0, M: 1.0
4	AT2G43570	Chr2:18076224..18077463(-)	277	GH19	Chitinase	cd00325 pfam00182 pfam00187	29775.4	5.78	-0.195	E: 8.0, C: 3.0, V: 2.0
5	AT2G43580	Chr2:18078649..18080028(-)	265	GH19	Chitinase family protein	cd00325 pfam00182 pfam00187	28780.7	8.31	0.002	E: 7.0, E.R.: 2.5, E.R._P: 2.5, C: 2.0, P: 1.5
6	AT2G43590	Chr2:18081331..18082767(-)	264	GH19	Chitinase family protein	cd00325 pfam00182 pfam00187	28352.9	8.43	-0.123	E: 12.0, G: 2.0
7	AT2G43600	Chr2:18086049..18087018(-)	273	GH19	Chitinase family protein	cd00325, pfam00182, pfam00187	30920.4	8.7	-0.299	E: 10.0, V: 2.0, G: 2.0
8	AT2G43610	Chr2:18087840..18089224(-)	281	GH19	Chitinase family protein	cd00325 pfam00182 pfam00187	29999.4	9.54	-0.171	C: 9.0, E: 2.0, V: 2.0
9	AT2G43620	Chr2:18093770..18095025(-)	283	GH19	Chitinase family protein	cd00325 pfam00182 pfam00187	30377.7	8.91	-0.084	C: 7.0, E: 4.0, M: 1.0, V: 1.0
10	AT3G12500	Chr3:3962382..3963984(-)	335	GH19	Basic chitinase	cd00325, cd06921 pfam00182	36183.7	7.81	-0.334	C: 9.0, C: 1.5, C_N: 1.5, P: 1.0, E: 1.0
11	AT3G16920	Chr3:5776486..5777889(-)	348	GH19	Endo chitinase-like protein	cd00325, pfam00182	38446.4	6.15	-0.358	C: 6.0, E.R.: 5.5, E.R._P: 3.5, M: 2.0
12	AT3G47540-1	Chr3:17521029..17522624(+)	214	GH19	Chitinase family protein	cd00325 pfam00182	23297.3	9.32	-0.285	C: 5.0, E: 4.0, V: 2.0, E.R.: 2.0
13	AT3G47540-2	Chr3:17521029..17522624(+)	283	GH19	Chitinase family protein	cd00325 pfam00182	31214.3	9.52	-0.365	C: 6.0, E: 4.0, V: 2.0, E.R.: 2.0
14	AT3G54420	Chr3:20145910..20147063(+)	273	GH19	Homolog of carrot EP3-3 chitinase	cd00325 pfam00182 pfam00187	29435.8	5.05	-0.134	E: 11.0, G: 3.0
15	AT4G01040	Chr4:453369..455548(+)	430	GH18	Stabilin-1 interacting chitinase-like protein	cd02876, smart00636	49142.4	9.08	-0.306	N: 5.0, P: 3.0, C: 2.0, G: 2.0, V: 1.0
16	AT4G01700	Chr4:732313..733510(-)	280	GH19	Chitinase family protein	cd00325 pfam00182	31464.7	9.04	-0.396	E: 11.0, C: 2.0
17	AT4G19720	Chr4:10730363..10731750(-)	363	GH18	Chitinase insertion domain-containing protein	smart00636, cl10447	40205.3	6.32	-0.189	N: 5.0, C: 5.0, cysk: 4.0
18	AT4G19730	Chr4:10733864..10734975(-)	332	GH18	Chitinase-like	smart00636, cl10447	36669.9	5.3	-0.252	C: 7.0, cysk: 5.0, N: 1.0
19	AT4G19740	Chr4:10739567..10740620(-)	211	GH18	Chitinase-like	smart00636, cl10447	23539.3	4.95	-0.211	C: 9.0, V: 2.5, M: 2.0, E.R._V: 2.0
20	AT4G19750	Chr4:10745682..10747127(-)	362	GH18	Chitinase insertion domain-containing protein	cd02879, smart00636	39731.3	4.86	-0.188	C: 8.0, N: 5.0
21	AT4G19760	Chr4:10750381..10752028(+)	369	GH18	Chitinase insertion domain-containing protein	cd02879, smart00636	40589.1	4.68	-0.278	N: 9.0, C: 3.0, C: 1.0
22	AT4G19770	Chr4:10753310..10754181(-)	261	GH18	Chitinase insertion domain-containing protein	smart00636, cl10447	28791	4.41	-0.17	C: 11.0, M: 2.0
23	AT4G19800	Chr4:10760830..10762104(-)	398	GH18	Chitinase insertion domain-containing protein	cd02879, smart00636	44357.2	4.48	-0.232	cysk: 14.0
24	AT4G19810	Chr4:10763934..10765753(-)	379	GH18	Chitinase insertion domain-containing protein	cd02879	41128	8.91	-0.099	E: 5.0, V: 3.0, C: 2.0, G: 2.0, M: 1.0
25	AT4G19820	Chr4:10767436..10768614(-)	366	GH18	Chitinase insertion domain-containing protein	pfam00704, smart00636	40873.4	9.35	-0.095	C: 9.0, C: 2.0, M: 2.0
26	AT5G24090	Chr5:8143699..8145252(-)	302	GH18	Chitinase A	cd02877 pfam00704	33096.5	9.17	-0.234	C: 10.0, N: 2.0, M: 1.0

*Notes of PL, GRAVY and PSORT are shown in Table 1

These observations indicated strikingly distinct *CHI* gene structural patterns between monocotyledonous rice and dicotyledonous *Arabidopsis*.

Intron phase was used to assess gene models of *OsCHIs* and *AtCHIs*. Introns phase means the position of an intron within a codon and assigned to three different phase classes: phase 0 (before the first base), phase 1 (after the first base) and phase 2 (after the second base). As shown in Fig. 1b, the majority of introns are within phase 1, for *OsCHIs* (54%) and *AtCHIs* (65%), while 29% and 25% of introns found in *OsCHIs* and *AtCHIs*, respectively, are within phase 2. Phase 0 introns represent only 18% of all *OsCHIs* introns and only 10% of all *AtCHIs* introns. Interestingly, most members of each group shared the same or similar intron phase. Further, it was found that within several pairs of putative paralogous genes (*At1g05850*/*At3g16920* in Group 6, *Os09g0494200*/*Os08g0522500* in Group 6, and *Os10g0542900*/*Os10g050543400* in Group 7), not only is phase of two adjacent introns shared, but length of exon between the two introns is highly conserved (Fig. 1b), indicating a close evolutionary relationship of these paralogous genes. In addition, it was observed that there are two alternative mRNA forms in the locus At3g47540

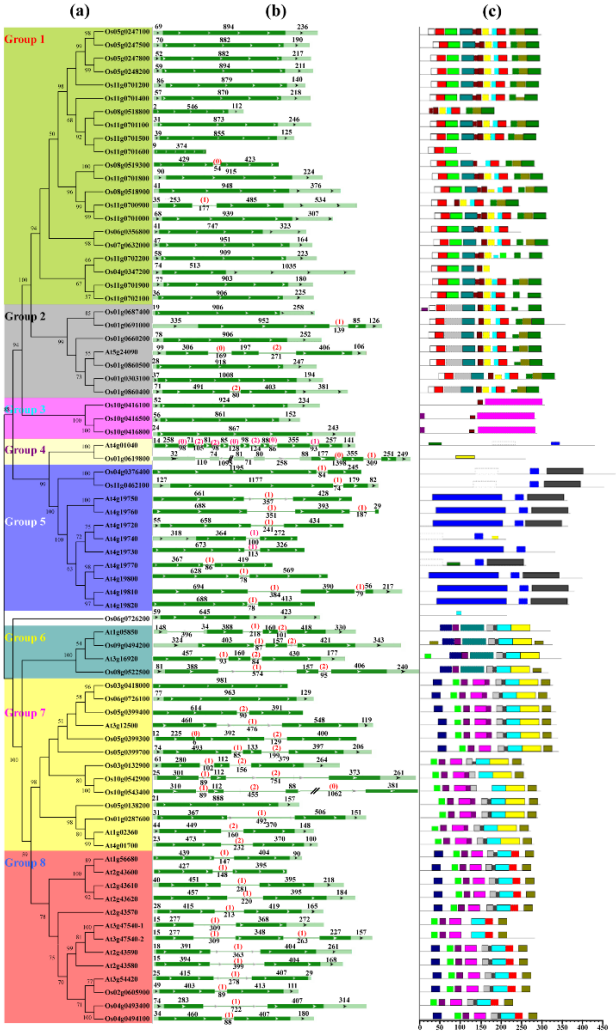

Fig. 1: Phylogenetic relationships, gene structure and motif composition of CHIs in *Arabidopsis* (*At*) and rice (*Os*). (**a**) The molecular phylogeny (left panel) was constructed by neighbor-joining method. The number at the nodes represent the bootstrap values (>50%) from 1000 replicates. The 8 major groups designated from 1 to 8 are marked with different color backgrounds. (**b**) The gene structure (5′-UTR/exon/intron/3′-UTR organization) of the *CHIs* is shown in the middle panel. Light green boxes represent 5′-UTR or 3′-UTR, dark green boxes represent exons and green lines represent introns. And their length in base pairs is also indicated, respectively. Numbers between brackets correspond to the intron phase. (**c**) A schematic representation of conserved motifs (obtained by MEME) in CHIs is displayed in the panel on the right. Different motifs are diaplayed by different colored boxes

wherein the 3’ end sequences were recruited to generate a new intron, this results in birth of two genes, named *At3g47540-1* and *At3g47540-2*, from the locus.

As CHI domain, GH18 or GH19 domain is essential for the chitin hydrolysis. The results showed that these identified CHIs possess only 1–3 CHI domain (s). In addition, some CHIs contain other domains, such as pfam00187 and cl15255. The domain pfam00187 is characteristic of chitin recognition protein, and cl15255 is Src homology2 (SH2) domain, a protein domain that plays important roles in the signal transduction of receptor tyrosine kinase pathways. To define more divergent patterns in the functional domain, the program MEME was used to examine smaller individual motifs in these CHI sequences. Thirty distinct motifs were identified in these CHI sequences. As shown in Fig. 1c, most members of GH18 class (Group 1–5) possess 19 motifs, while most members of GH19 class (Group 6–8) have 13 motifs. Four same motifs are shared by most of GH19 proteins, while different groups of the GH18 class share specifically different motifs. Importantly, most members in each group have common motif compositions, strongly supporting group-dividing results of the phylogenetic analyses. The motif composition conservation among members of the same groups indicated that members in the same group may be functionally conserved.

Genomic organization and expansion of the *CHI* gene family

First the genomic distribution of the *OsCHIs* and *AtCHIs* was examined and observed that the distribution of these *CHIs* is uneven throughout chromosomes of the *Arabidopsis* or rice genomes. As shown in Fig. 2a, for rice, chromosome 12 harbors no *CHIs*, whereas chromosome 11 harbors 12 *CHIs*, and each of chromosomes 1 and 5 harbors eight *CHIs*. Other chromosomes have 1–5 *CHIs* localized on them. For *Arabidopsis*, chromosome 5 harbors only one *CHI* gene, whereas chromosome 4 harbors 11 *CHIs*. Five *CHIs* were identified on each of chromosomes 2 and 3, and three on chromosomes 1 (Fig. 3a). Further, it was observed that there are some *CHIs* clusters at rice or *Arabidopsis* chromosomes (Fig. 2a and 3a), suggesting that these *CHIs* in the same clusters may be tandemly duplicated genes.

To gain insights into gene duplication in *CHIs* genes, separate phylogenetic trees were constructed exclusively using the full-length *CHI* sequences of rice and *Arabidopsis* (Fig. 2b and Fig. 3b). In rice, the obvious tandem repeats were *Os01g0687400*/*Os01g0691000* and *Os01g0860400*/*Os01g0860500* on chromosome 1 (Fig. 2a and 2b), *Os04g0493400*/*Os04g0494100* on chromosome 4 (Fig. 2a and 2d), *Os10g0542900*/*Os10g0543400* on chromosome 10 (Fig. 2a and 2d), *Os10g0416100*/*Os10g0416500*/*Os10g0416800* (Fig. 2a, 2b and 2c) and *Os05g0247100*/Os05g0246100/*Os05g0247800*/*Os05g0248200* on chromosome 5 (Fig. 2a and 2c). These clustered genes were generated by recent tandem duplication because terminal clades generated by them were well-supported in phylogenetic tree, respectively, and did not contain any *CHIs* on other chromosomes (Fig. 2b and 2d). Another example that may be the result of tandem duplication is *Os05g0399300*/*Os05g0399400*/*05g0399700* (Fig. 2a and 2d). The largest *CHI* gene cluster, located on chromosome 11, contains 11 tandemly arrayed members (Fig. 2a and 2c),

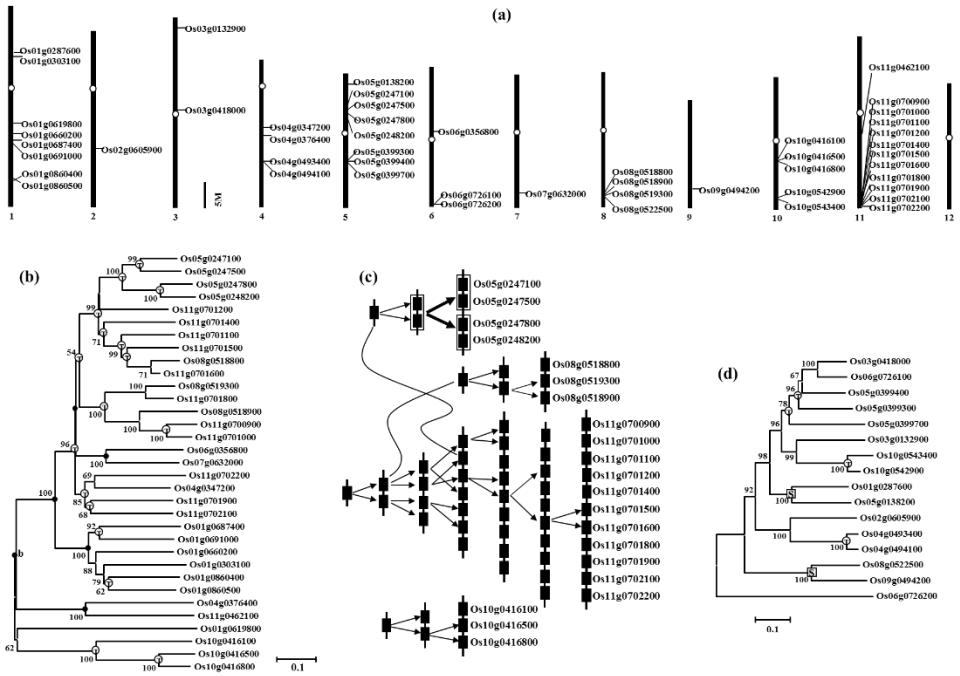

Fig. 2: Evolution of the *Oryza CHIs* (*OsCHIs*). (**a**) Chromosomal locations. (**b**) Phylogenetic relationships of the GH18 *OsCHIs*. The letters T and S on the nodes of the phylogenetic tree indicate the positions where tandem duplication and segmental duplication have occurred, respectively. (**c**) Hypothetical origins of 21 *OsCHIs* by tandem duplication and, most likely, retro position. (**d**) Phylogenetic relationships of the GH19 *OsCHIs*

and another cluster on chromosome 8 contains 3 tandemly arrayed members (Fig. 2a, 2b and 2c). Further, it was observed that the terminal clades containing the two clusters also contain *CHIs* located at other chromosomes (Fig. 2b and 2c), respectively, suggesting that the two clusters of genes were generated by more ancient tandem duplication. Interestingly, it was observed that the Os05g0247100/Os05g0247500/Os05g0247800/Os05g0248200 cluster may be formed by a single tandem duplication of a two-gene cluster (Fig. 2c).

For *Arabidopsis*, the largest *CHI* gene cluster is on chromosome 4 and contains nine tandemly arrayed genes including all *CHIs* identified on the chromosome but *At4g01040* and *At4g01700* (Fig. 3a). The clustered genes were also generated by recent tandem duplication because the terminal clade generated by them is a well-supported in phylogenetic tree and not contain any other chromosome *CHIs* (Fig. 3b and 3c). Another *CHI* gene cluster is located on chromosome 2. The cluster includes all *CHIs* identified on the chromosome (Fig. 3d and 3e). However, the clustered genes were generated by more ancient tandem duplication, because the terminal clade containing them also include other chromosome *CHIs*, such as *At1g56680* on chromosome 1, *At3g54420* and *At3g47540* on chromosome 3.

The locations of *CHIs* were also compared in duplicated chromosomal blocks previously identified in rice and *Arabidopsis*. It was found in the rice and *Arabidopsis* genomes for chromosomal segments (or duplicate blocks) that contain *CHIs*. In rice, one block contains *Os01g0287600*, while its duplicate block includes *Os05g0138200* at the same position (Table 3, Fig. 2d). This suggests that *Os01g0287600*/*Os05g0138200* might be the results of a segmental duplication event. Likewise, *Os08g0522500*/*Os09g0494200* might be the results of another segmental duplication. However, none of other *CHI* gene-containing blocks has a *CHI* gene in its duplicate block. Following the same procedures, however, we did not found any segmental duplication occurred in *AtCHIs*.

These data showed that tandem duplication explains the birth of relatively large proportion of the *CHI* family genes in rice and *Arabidopsis*, whereas segmental duplication plays a very limited role in increasing *CHI* number in the two plants.

Differential expression of *CHIs* in response to biotic stresses

It has been reported that plant *CHIs* exhibit basal expression level under normal conditions. So the change of gene expression caused by pathogen infection can provide important hints for the gene function. The hemi bio-trophic fungi *Magnaporthe grisea* causes severe loss to rice yield. The microarray data was used to analyze the expression response of *OsCHIs* against this pathogen. The data analysis revealed that only eight *OsCHIs* were significantly up-regulated (more than 2-fold) at 3 dpi or/and 4 dpi,

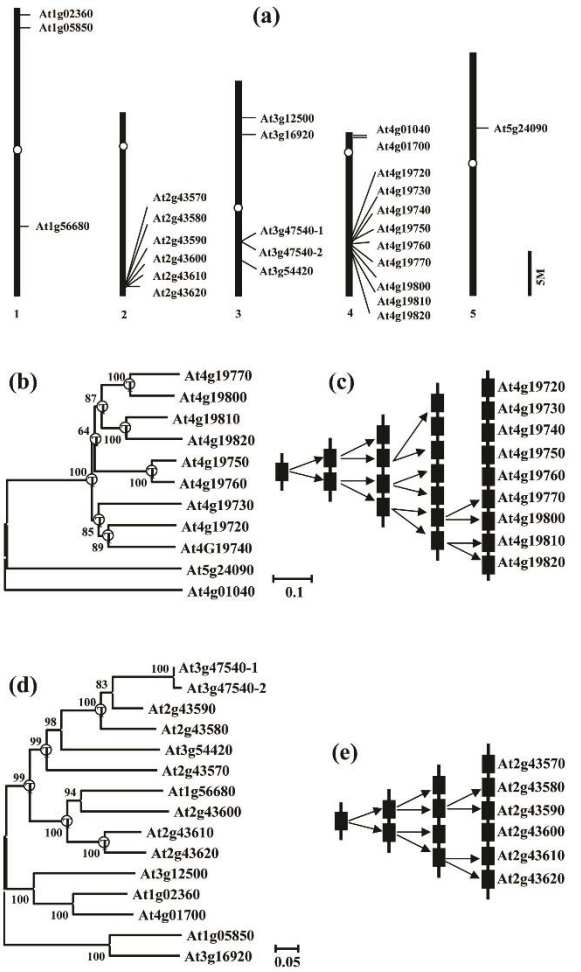

Fig. 3: Evolution of the *Arabidopsis CHIs* (*AtCHIs*). (**a**) Chromosomal locations, (**b**) phylogenetic relationships of the GH18 *AtCHIs*, (**c**) hypothetical origins of nine *AtCHIs* by tandem duplication, (**d**) phylogenetic relationships of the GH19 *AtCHIs*, and (**e**) hypothetical origins of six *AtCHIs* by tandem duplication

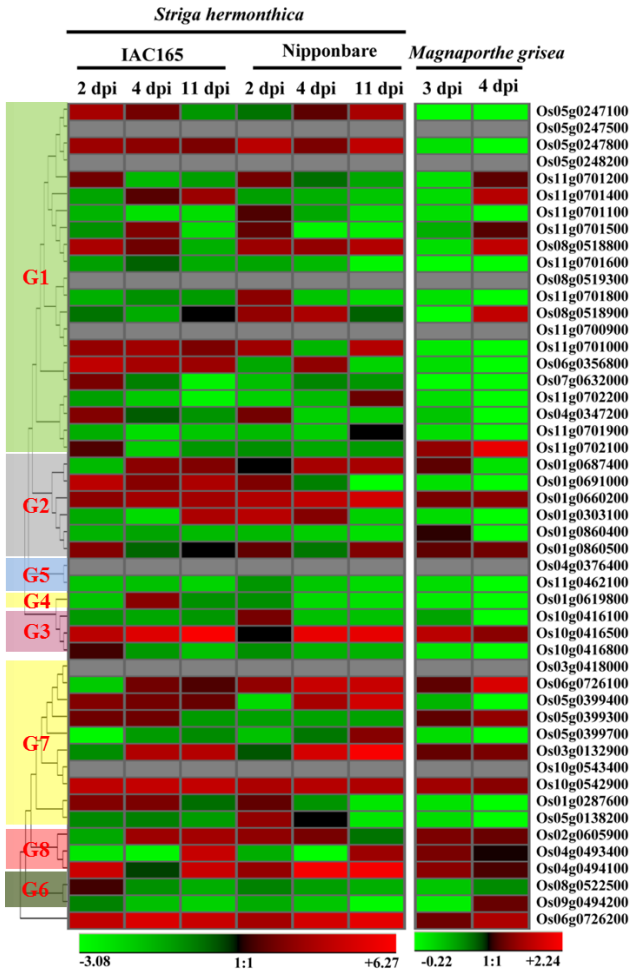

Fig. 4: Expression profiles of *OsCHIs* showing differential expression in response to various biotic stress treatments. The expression profile image is generated based on the fold-change log values in the treated sample when compared with its mock-treated control sample and is displayed according to the order in the corresponding phylogenetic tree. G1-G8 means Group1-Group8 in Fig. 1. The color scale for fold-change values is shown at the bottom

while none of genes were significantly down-regulated (Fig. 4), suggesting that a limited number of *OsCHIs* is involved in defense to the pathogen.

The obligate root hemi-parasite *S. hermonthica* also cause severe loss to rice yield, and so far there have been no reports investigating roles of plant *CHIs* in defense to any parasitic plants. Here, we tried to investigate the response of *OsCHIs* to *S. hermonthica* by using microarray data from a study in which the gene expression profiling was analysed in roots of susceptible (IAC165) and highly resistant (Nipponbare) cultivars after infection with this parasitic plant (Swarbrick *et al*. 2008). The results showed that most *OsCHI* were significantly differential expressed either in the susceptible cultivar or in the highly resistant one as compared to control (Fig. 4), indicating that many *OsCHIs* play roles in defense to infection by *S. hermonthica*.

In *Arabidopsis*, it was investigated that expression patterns of the *AtCHIs* in response to the obligate bio-trophs oomycte pathogen *Erysiphe orontii*, necrotrophs fungal pathogens *Botrytis cinerea* and the bacterial pathogen *Pseudomonas syringae*, a hemi-biotroph. The most members in the Group 7 and 8 are clearly up-regulated, indicating their roles in defense to these pathogens (Fig. 5). Interestingly, although *E. orontii*, *B. cinerea* and *P. syringae* were three different type of pathogen, expression patterns of these *CHIs* appear a similar trend in response to the three pathogens. For example, some genes (*At2G43590*, *At2G43580*, *At3G54420*, *At2G43570* and so on) in Group 8 were highly expressed in all of the three pathogen infection. Further, it was found that protein products of these *CHIs* were predicted to be located in the extracellular (Table 2), suggesting that these CHIs may function as secretory proteins to directly attack the pathogens as CHIs can degrade the cell wall of pathogens.

Table 3: Duplicate blocks in the rice (*Oryza sativa* ssp. *Japonica*) genome that contains Chitinase genes (*CHIs*)

Duplicate block I	*CHIs* in Duplicate block I	Duplicate block II	*CHIs* in Duplicate block II
Chr1:10199920-11169993	*Os01g0287600*	Chr5:2294268-1693885	*Os05g0138200*
Chr1:11208348-11958355	*Os01g0303100*	Chr5:16654659-17318420	/
Chr1:24256830-26782456	*Os01g0619800*	Chr5:29035554-28073724	/
Chr1:26994290-32799607	*Os01g0660200* *Os01g0687400* *Os01g0691000*	Chr5:28045369-24918365	/
Chr1:39876564-40552436	/	Chr8:25970032-26634296	*Os08g0522500*
Chr2:17791462-18491310	/	Chr4:18025021-19031997	*Os04g0376400*
Chr2:21862513-28892506	*Os02g0605900*	Chr4:22058834-29983886	/
Chr3:479951-84660	/	Chr10:20800647-21708303	*Os10g0542900* *Os10g0543400*
Chr3:1605705-2058832	*Os03g0132900* *Os03g0418000*	Chr10:20520249-20792948	/
Chr8:25929007-26306223	*Os08g0522500*	Chr9:19076622-19294993	*Os09g0494200*

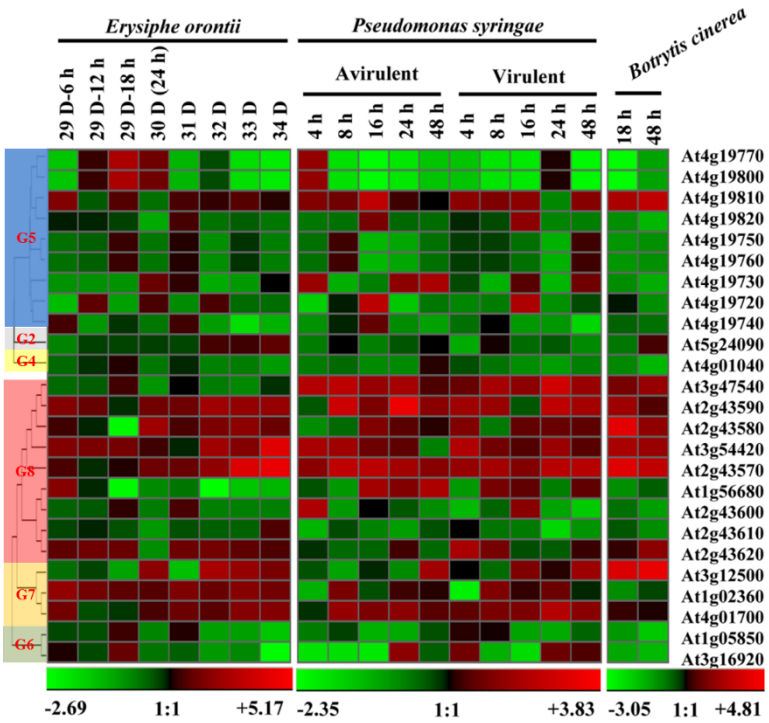

Fig. 5: Expression profiles of *AtCHIs* showing differential expression in response to various biotic stress treatments. The expression profile image is generated based on the fold-change log values in the treated sample when compared with its mock-treated control sample and is displayed according to the order in the corresponding phylogenetic tree. G1-G8 means Group1-Group8 in Fig. 1. The color scale for fold-change values is shown at the bottom

Thus, member of Group 7 and 8 may function actually as PR protein and play important roles in defense to pathogen infection. And these *CHIs* are selected as resistance candidate genes for further studying in future.

Discussion

Plant *CHIs* belong to large gene family, and some of these play essential roles in plant defense against pathogens, and thus it is important to unravel their function for application on genetic analysis and breeding. To date, genome-wide identification of *CHI* family has been performed in many plant species. However, for rice, a genome-wide overview of the *CHI* family members is not yet available. In fact, in an earlier work, 37 ORFs of *OsCHIs* has been investigated in this model plant (Xu *et al*. 2007). However, these ORFs cannot be retrieved from currently available databases. In the study, first a genome-wide survey was performed which provided new data that *O. sativa*, the important monocotyledonous model plant, have 48 *CHIs (OsCHIs)* in its genome.

Truong *et al.* (2003) indicated that the rice had at least 7 family of CHIs and only 19 OsCHIs were investigated. In this study, 47 OsCHIs, together with 26 *AtCHIs*, were clustered into 8 groups, and the group classification was strongly supported by gene structure and motif

compositions. In addition, Os06g0726200, being basal to a large clade be generated by Groups 6–8 in the phylogenetic tree (Fig. 1a), was not classified into any groups, which is strongly supported by the motif compositions because its motif compositions are distinctly different from those of any group members (Fig. 1c). The phylogenetic analysis also showed that the 26 *ATCHIs* were actually clustered into 5 groups (Fig. 1a), which was generally consistent with results of previous research (Passarinho and Vries 2002; Xu *et al*. 2007). Furthermore, it is noted that more number of *CHIs* were found in rice compared to *Arabidopsis*, which may be attributed due to large genome size of rice with 12 chromosomes whereas *Arabidopsis* has only 5 chromosomes. Moreover, *OsCHIs* or *AtCHI*s are unevenly distributed in rice or *Arabidopsis* chromosomes, respectively, and expansion of the *CHI* family is caused by tandem duplication, instead of segmental duplication, in genomes of the two model plants, which is similar to those reported in *Brassica rapa*, *B. juncea*, *Camelina sativa* and *P. trichocarpa* (Jiang *et al*. 2013; Chen *et al*. 2018; Mir *et al*. 2019).

CHI can serve as a defense-related enzyme that inhibits fungal growth due to its function in breaking down chitin. However, present study data showed that most members of *OsCHI* family exhibit down-regulating expression in response to the fungi *M. grisea*, suggesting these *OsCHIs* play limited role in the defense. This was supported by results from a RNA-sequencing analysis. In the analysis, 21 identified DEGs (differentially expressed genes) related to *CHI* were identified in Dongdao-4, a widely grown Japonica-type rice cultivar, after infection with *M. grisea*, but only 1 DEG was up-regulated while other 20 DEGs were down-regulated (Tian *et al.* 2018). Contrastly, most members of *OsCHI* gene family were up-regulated in response to *S. hermonthica*, a parasitic plant, indicating roles for these *OsCHIs* in the defense. The plant *CHI* has been receiving attention concerning its roles in defense to pathogens as well as insects, and to date there have been no reports investigating the roles of *CHIs* in interactions of host plants with parasitic plants. Thus, this is an unexpected finding and it is valuable to study furthermore.

For *AtCHIs*, *CHIs* in the Group 7 and 8 were highly up-regulated in response to the infection with three pathogens. Among these *CHIs*, *AT3G54420*, a Group 8 member, has been reported to be transcriptionally induced by infection with a hemibiotrophic pathogen (Gerhardt *et al.* 1997). Lin *et al*. (2008) showed that *AT5G24090* and *AT3G12500*, two other Group 8 *CHIs*, were induced in *Arabidopsis* plant leaves by the necrotrophs *B. cinerea* infection (Lin *et al.*, 2008). Similarly, in present investigation, *AT3G12500* was significantly induced by *B. cinerea*, and *At5g24090* was down-regulated in response to the hemibiotrophic pathogen *P. syringae* but up-regulated 48 h after infection by *B. cinerea*. In *Beta vulgaris* plants, one *CHI*, its protein with 53% identity to *At3g54420*-encoded protein (NP_191010.1), is up-regulated after the necrotrophic pathogen infection (Nielsen *et al.* 1994). Another *CHI*, whose protein sequence have 70% identity to NP_191010.1, is induced in *Phaseolus vulgaris* roots infected with a hemi-biotrophic pathogen (Lange *et al.* 1996). Further, data showed that the expression of *At3g54420* was remarkably up-regulated in response to all the three types of pathogen. Protein subcellular localization prediction showed that these *CHIs* in Group 7 and 8, which highly expressed in all of the three pathogen infection, are associated with the secretory pathway. It has been reported that apoplastic CHI proteins, which are induced by pathogen infection, can directly inhibit the pathogen growth in the intercellular space as CHIs can catalyze the degradation of chitin (De *et al*. 1997). Thus, members of Group 7 and 8 may function actually as PR protein and play important roles in defense to pathogen infection, which offer an insight into the defense role of specific *CHIs* in the large *CHI* family. And these *CHIs* were considered as potential resistance candidate genes, and should be further studied for improving plant resistance.

Conclusion

This study provided new insights that *O. sativa* is important monocotyledonous model plant, containing 48 *CHIs (OsCHIs)* in its genome. These identified OsCHIs as well as *A. thalinan* CHIs (*AtCHIs*) formed eight groups as supported by phylogeny, exon/intron structure and motif organization. Gene duplication analysis revealed that tandem duplication plays a dominant role in the birth of the *CHI* family genes in rice and *Arabidopsis*, while segmental duplication has a very limited role. Further, expression analysis gained insights into the expression of *CHIs* and provided useful information in selecting resistance candidate genes; for example, it was found that many *OsCHIs* expression significantly respond to *S. hermonthica*, a parasite plant, firstly indicating a possible role for *CHIs* in plant defense to parasite plants, and for *AtCHIs*, most members in the Group 7 and 8 were clearly up-regulated in response to three types of pathogens, indicating the potential function of the two groups in defense.

Acknowledgements

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