Discourse Journal of Agriculture and Food Sciences
www.resjournals.org/JAFS
ISSN: 2346-7002
Vol. 4(3): 31-38, October, 2016

Transcriptional and translational characterization of cysteine proteinase inhibitor genes in Rice (*Oryza sativa* L. *indica*)

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Abstract

Cysteine protease inhibitor (cystatin) genes exist in animals, plants and microorganisms. There are 12 cysteine protease inhibitors in rice (Oryzacystatin, OC) which associated with biotic/abiotic stresses responses and normal growth and development. Oryzacystatin belongs to pathogenesis-related gene family 6 (PR6), namely protease inhibitor class in rice genome. In this study, the domain architecture and subcellular localization of oryzacystatin was predicted, and transcription analysis was also carried out. Furthermore, oryzacystatin expression profiling was surveyed in different tissues during rice normal growth using western blot (WB) technology, most of the proteins were detected in late stage of rice growth but merely in the seedling stage.

Keywords: Rice; Oryzacystatin; Transcription; MPSS; Western blot

INTRODUCTION

Protease inhibitors widely exist in animals, plants and microorganisms and they are one of the most abundant protein species. Protease inhibitors, a kind of polypeptide or protein with small molecular weight, have suggested that these inhibitors act as defense proteins against pests and pathogens and as regulators of protein turnover. Pathogenic microorganism depends on their extracellular protease to degradate the host tissues in order to obtain the necessary amino acid for themselves during their growth and proliferation, meanwhile plants prevent pathogen from degradation through the protease inhibitors to make them malnutrition, to stunt their growth and to achieve the disease resistance at last. According to the different enzyme active sites, protease inhibitors are mainly divided into serine protease inhibitor, cysteine protease inhibitor, aspartic acid protease inhibitor and metal carboxypeptidase inhibitor.

So far, cysteine protease inhibitors have been identified in invertebrates, vertebrates and plants, such as rice (Abe *et al.*, 1987), *Arabidopsis* (Belenghi *et al.*, 2003), corn (Yamada *et al.*, 2000), pea (Misaka *et al*., 1996), barley (Gaddour *et al*., 2001), wheat (Corr-Menguy *et al*., 2002) and sesame (Shyu *et al*., 2004) and so on. Cysteine protease inhibitors in plants are a large family of evolutionarily closely related proteins, and they can form the compact compounds with the cysteine protease family C1A and C13, such as papain, cathepsin B/H/L/S, to regulate the endogenous cysteine protease activity and proteolysis (Martinez and Diaz, 2008). According to the sequence of amino acids and the particular space structure, cysteine protease inhibitors belong to inhibitors I25 family, which includes three subfamilies: I25A, B, C (Rawlings *et al*., 2004). Most cystatins have a molecular mass in the 12-16 kD and devoid both of disulfide bonds and putative glycosylation sites (Abe *et al.*, 1987; Turk and Bode, 1991). Cysteine protease inhibitors are involved in pests resistance (Chan *et al*., 2010), disease resistance (Yang *et al*., 2005), stress resistance (Van der Vyver *et al*., 2003) and the regulation of protein metabolism.

There are at least 7 cystatin genes in barley. The gene (*Hv-CPI*) was expressed in embryos, developing endosperms,

Project supported by National Natural Science Foundation of China (No.31400700) and Science and Technology Research for Youth Foundation of Colleges and universities in Hebei Province (QN2014110)
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leaves and roots as assessed by northern blot analysis. Western blot analysis detected a slightly retarded band in leaves that was not present in roots or seeds (Gaddour *et al*., 2001). *Arabidopsis* also has 7 cystatin genes. *AtCYS1* had been successfully cloned which induced by wounding in leaves and showed the resistance to pests and pathogens (Belenghi *et al*., 2003). There are 12 cystatin genes in rice genome. According to the whole genome sequence of rice, the structure and chromosomal localization of the 12 genes have been identified. Structural examination revealed that three of them (OC-I, OC-II and OC-III） one intron was present between the sequence encoding the conserved LARFAV and the active-site QxVxG. Eight cystatin sequences from rice (OC-IV to OC-XI) had no introns in their ORFs. The longest coding sequences OC-XII had three introns. The cystatin sequences were shown to be distributed on five of the 12 rice chromosomes. Tandemly arranged sequences indicative of recent duplication events were found on rice chromosomes 1 (OC-IV and OC-V) and 3 (OC-VI、OC-VII and OC-IX) (Martinez *et al*., 2005). In 1987, Abe reported the first cyteine protease inhibitors in rice and named as Oryzacystatin-I (OC-I) later which encoded 102-amino acid residues and located at the first chromosome (Abe *et al*., 1987). Heterologous expression of OC-I in tobacco, potato, alfalfa and banana plants could inhibit cysteine protease of pathogen and pests in order to improving the plant resistance to virus (Gutierrez-Campos *et al*., 1999), to nematodes (Atkinson *et al*., 1996; Samac and Smigocki, 2003), and to coleoptera pests (Kiggundu *et al*., 2010). Heterologous expression of rice OC-I gene in tobacco exhibited special growth phenotype and improved the resistance to chilling injury (Van der Vyver *et al*., 2003). Cysteine protease inhibitors from rice seed could inhibit the growth of rice blast hyphostoma (Zeng *et al*., 1996). In addition, Hiroto Kondo had identified a new type of cystatin (oryzacystatin-II, OC-II) in rice seeds which encodes 107 amino acid residues whose sequence is similar to that of oryzacystatin-I (approximately 55% of identity). OC-I and OC-II are remarkably distinct in two respects: (1) their inhibitor activity against cysteine proteinases (Kondo *et al*., 1993; Dou *et al*., 2011); and (2) the expression patterns of their mRNAs (Kondo *et al*., 1993). In fact, oryzacystatins have mutually different specificities toward target cysteine proteinases, somewhat different partial structures and dissimilar position which are all associated to the related but slightly different physiological role.

OC-I could be phosphorylated by XA21 and effectively inhibited cysteine proteases with the inhibition rate reached up to 50% (Dou *et al*., 2011). These results suggest that oryzacystatin may be involved in the innate immune response of rice. In this study, oryzacystatin belongs to rice PR6 family would be as the object. We should analyze the transcriptional and translational expression characteristics and try to provide clues to reveal their functional of cystatin subfamily.

MATERIALS AND METHODS

Plant material

Rice variety *93-11* was used.

Methods

Bioinformatic analysis

Protein sequences of oryzacystatin were obtained from rice genome database according to their Locus number. Protein domains were analyzed by using the software of SMART (http://smart.embl-heidelberg.de). From the rice genome annotation project (Rice Genome Annotation Project) database (http://rice.plantbiology.msu.edu/), we downloaded the RNA-Seq data of the target oryzacystatin genes to form FPKM (Reads per kilobase of exon model per million mapped reads) data. From the rice MPSS (Massively parallel signature sequencing) web site (http://mpss.udel.edu/rice/), we downloaded the transcriptional data, get the statistical information and compare different transcription signal intensity among different samples according to gene transcription. The chart were generated by Origin 9.0 software.

Rice samples

To examine expression of oryzacystatins under normal growth conditions, Leaf samples at seedling (1 cm, 2 cm, 5 cm, 10 cm, and 15 cm), tillering, booting, flowering and mature stage were collected, respectively, and then immediately frozen and stored in liquid nitrogen at -70℃.

Polyclonal antibody genearation

Synthetic polypeptides were conjugated with KLH and used as antigen to generate antibodies. To generate

Table 1. List of rice Oryzacystatin genes

Gene	Locus #	Brief annotation	MW （kD）
OC-Ⅰ	Os01g58890	cysteine proteinase inhibitor precursor protein, putative, expressed	15.4
OC-Ⅱ	Os05g41460	cysteine proteinase inhibitor precursor protein, putative, expressed	16.6
OC-Ⅲ	Os05g33880	cysteine proteinase inhibitor precursor protein, putative, expressed	20.0
OC-Ⅳ	Os01g68660	cysteine proteinase inhibitor precursor protein, putative, expressed	16.6
OC-Ⅴ	Os01g68670	cysteine proteinase inhibitor precursor, putative	15.7
OC-Ⅵ	Os03g11180	cysteine proteinase inhibitor 6 precursor, putative, expressed	12.4
OC-Ⅶ	Os03g11170	cysteine proteinase inhibitor precursor, putative	12.5
OC-Ⅷ	Os03g31510	cysteine proteinase inhibitor 8 precursor, putative, expressed	12.9
OC-Ⅸ	Os03g11160	cysteine proteinase inhibitor precursor, putative	12.4
OC-Ⅹ	Os04g28250	cysteine proteinase inhibitor precursor, putative	15.4
OC-Ⅻ	Os01g16430	cysteine proteinase inhibitor precursor protein, putative, expressed	12.9

oryzacystatin protein-specific antibodies, specific epitopes in the rice whole proteome were selected using PepDesign software. The generation of polyclonal antibodies was carried out by Beijing Protein Innovation Co., Ltd., Beijing, China, with KLH-conjugated synthetic polypeptides as immunogens.

Total protein extraction

Rice tissue was ground into a fine powder in liquid nitrogen. An 800µl aliquot of extraction buffer [62.5 mM TRIS-HCl (pH 7.4), 10% glycerol, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5% (v/v) β-mercaptoethanol] was added to each 300 mg powder sample. The mixture was vortexed and then chilled on ice for 10 min. Samples were centrifuged at 12 000 rpm for 10min at 4℃, and the supernatant was collected and stored at –70℃ (Li *et al*., 2011).

Western blot and signal quantification analysis

WB detection were performed as described previously (Li *et al*., 2011) and repeated at least three times. The images were scanned and the intensity of each band was captured using ImageJ software. The intensity of each band was standardized as a percentage of the total intensity and the results were referred to as a relative volume that represents the relative expression abundance of the gene in the samples tested.

Results

Rice cysteine proteinase inhibitor genes

There are 12 cysteine protease inhibitors in rice PR6 family members (Li *et al*., 2011). Their Loc number, annotation and molecular weight were listed in Table 1. The molecular weight of Oryzacystatin subfamily ranged from 12.4 to 27.3 kD.

Domain structure and subcellular localization prediction

With SMART software, the domains of oryzacystatin were predicted, the results were showed in Figure 1. The structure of oryzacystatin is similar and exists the individual differences. The main domain is a cysteine protease inhibitor-like

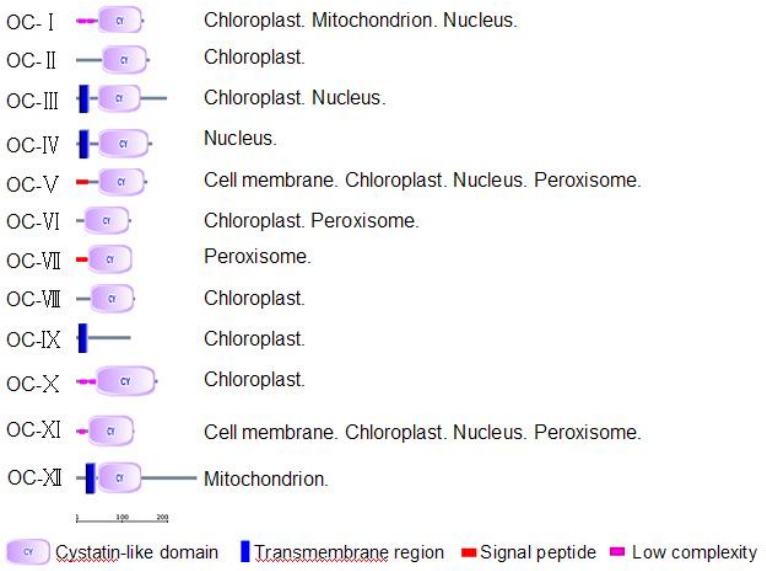

Figure 1. Predicted domains of rice oryzacystatin subfamily and CELL—Ploc subcellular localization

domain (Cystatin-like domain, CY), besides some transmembrane domain and signal peptide. In particular, there is no obvious cystatin-like domain in OC-IX and the gene expression had not been detected by subsequent bioinformatic analysis and Western blot. Combined with the structure characteristics of OC-IX, we can speculate that the gene has a certain specificity. OC-III, OC-IV, OC-IX and OC-XII all has a transmembrane structure. OC-V and OC-VII has signal peptide sequence.

Transcription and cluster analysis

According to RNA-Seq analysis, FPKM value of rice oryzacystin genes were obtained at different tissue/organs, including shoot, leaf, pre-emergence inflorescence, post-emergence inflorescence, anther, pistil, seed, embryo and endosperm (Table 2). Comparison the transcription signals of different tissues, the OC-I FPKM value is the highest, showed the highly intensity of transcription especially in shoot, leaf and post-emergence inflorescence but lower in other tissues. OC-I, OC-II, OC-III, OC-VIII and OC-XII have certain transcription signals in the tested tissues and transcribed constitutively. This result showed these genes may be connected with the normal growth and development of rice. OC-IV has no transcription signals in the shoot and leaf tissue but exists in seeds, presumably the relationship with seed maturation. OC-VII has the higher value in embryo tissue while OC-IX and OC-XI detected no any transcription signal in all tested materials. The transcription signal is not strong in OC-VI and OC-VII. According to the transcription information provided by RNA-Seq, cluster analysis (Figrue 2) illustrated that the close sequence similarity and evolution relationship, the similar transcription profiles. So, the similarity of transcription support the complementarily of its function.

Transcriptional analysis of oryzacystatin genes in the interactions between rice and *Xoo*

The MPSS data of oryzacystatin were obtained from rice leaves when inoculated with *Xoo* in compatible and incompatible materials by using Origin9.0 software (Figure 3). Transcription level of OC-I is the highest especially in compatible rice leaves after 6 h when inoculated with *Xoo*. OC-II is in the highest level after 24 h when inoculated with water. Transcription level of OC-III is the highest especially in resistance response after 12h when inoculated with *Xoo*, suggesting that OC-III may be participate in the resistance against blast disease. OC-VIII in compatible leaves is higher during 3-6 h when inoculated with *Xoo*. Transcription level of OC-X is the highest in resistance response after 3h when inoculated with *Xoo*, suggesting that OC-X may be participate in the resistance against blast disease. OC-XII has strong reaction when inoculated with water and *Xoo* in all compatible and incompatible materials. OC-IV, OC-V, OC-VI, OC-VII, OC-IX and OC-XI have no any transcription signals, suggesting may not be associated with blight disease. All the results above shows that oryzacyctatin have their own diverse physiological roles due to their different forms, different places and specificity interaction for different types of cysteine proteases.

Table 2. FPKM value of rice oryzacystatin genes at different tissue/organs

Gene	Leaf			Inflorescence				Seed				Total
	Shoot	4 leaf stage	20 d	Pre-E	Post-E	Anther	Pistil	5 DAP	10 DAP	25 DAP-Em	25 DAP-En	
OC-Ⅰ	559.7	171.5	119.4	21.6	130.2	61.0	48.8	65.3	2.8	22.7	2.2	1205.2
OC-Ⅱ	51.5	20.1	28.2	4.8	29.1	25.4	27.2	23.6	5.4	5.7	6.7	227.7
OC-Ⅲ	133.1	34.4	52.9	81.5	145.7	62.6	80.8	84.9	27.4	22.4	35.2	760.9
OC-Ⅳ	0	0	0	0	0	0.5	1.5	28.6	10.9	36.9	6.8	85.2
OC-Ⅴ	4.6	1.3	0	0	0	1.7	5.3	114.6	30.3	17.3	63.1	238.2
OC-Ⅵ	0	2.0	0	0	0	0	5.1	7.9	0	0	0	15.0
OC-Ⅶ	0	0.4	0	0	0	0	7.3	0	0	0	0	7.7
OC-Ⅷ	49.5	16.7	5.6	58.9	39.4	33.6	108.0	87.6	60.3	299.3	118.1	877.0
OC-Ⅸ	0	0	0	0	0	0	0	0	0	0	0	0
OC-Ⅹ	13.1	12.3	1.2	72.0	15.1	21.9	82.9	56.8	0	12.1	0	287.4
OC-Ⅺ	0	0	0	0	0	0	0	0	0	0	0	0
OC-Ⅻ	37.0	12.1	44.7	42.1	77.6	40.6	55.7	84.9	30.3	99.8	22.2	547.0
Sub-total	848.5	270.8	252	280.9	437.1	247.3	422.6	554.2	167.4	516.2	254.3	4251.3

FPKM: fragments per kilobase of exon per million fragments mapped; Pre-E: pre-emergence inflorescence; Post-E: post-emergence inflorescence; DAP: Days after pollination; Em: embryo; En: endosperm. Data derived from Libraries: Shoots: SRR042529;4-leaf: SRX016110;20 d: OSN_AA; Pre-E: OSN_AC; Post-E: OSN_AB; Anther: OSN_AD;Pistil:OSN_AE; 5DAP: OSN_AF; 10DAP: OSN_AK; Em: OSN_AG; En: OSN_AH.

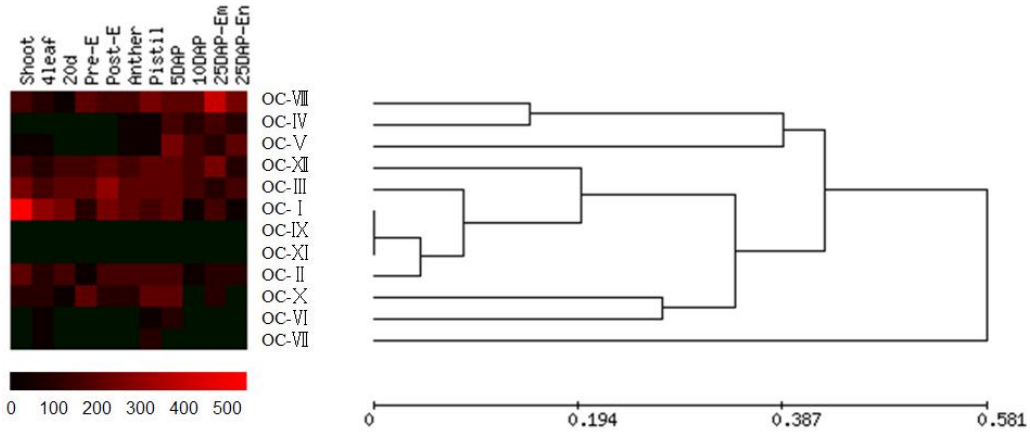

Figure 2. Cluster analysis of oryzacystatin genes based on transcription data
Pre-E: pre-emergence inflorescence; Post-E: pre-emergence inflorescence; DAP: days after pollination; Em: embryo; En: endosperm.

Expression profiling analysis of oryzacyctatin proteins

In order to understand the expression characteristics of oryzacystatin, we prepared polypeptide antibody to analyze the expression characteristics by western blot technique. Most of oryzacyctatin protein in the seeding stage was so low that could not be detected, while expressed in leaves during the late of normal growth (tillering stage, booting stage, blossom stage and mature stage, Figure 4). Among them, OC-V and OC-VIII were not detected any expression signal at different periods. There is no direct association between protein expression and RNA transcription, suggesting that complexity and relative independence of biological processes in transcriptional regulation, post-transcriptional splicing, post- transcriptional modification, translation post-translational

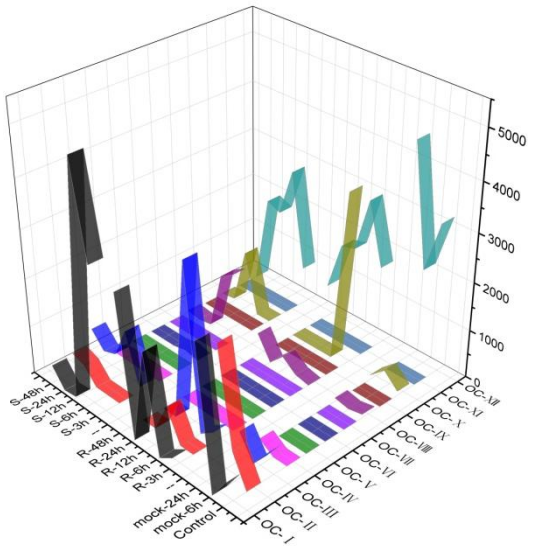

Figure 3. MPSS value analysis of oryzacystatin genes interactions among rice-*Xoo*

MPSS: Massively Parallel Sinature Sequencing (# of MPSS tag) ; M-6h: Mock 6 h; M-24h: Mock 24 h; R-3h: Incompatible reaction 3h; R-6h: Incompatible reaction 6h; R-12h: Incompatible reaction 12h; R-24h: Incompatible reaction 24h; R-48h: Incompatible reaction 48h; S-3h: Compatible reaction 3h; S-6h: Compatible reaction 6h; S-12h: Compatible reaction 12h; S-24h: Compatible reaction 24h; S-48h: Compatible reaction 48h

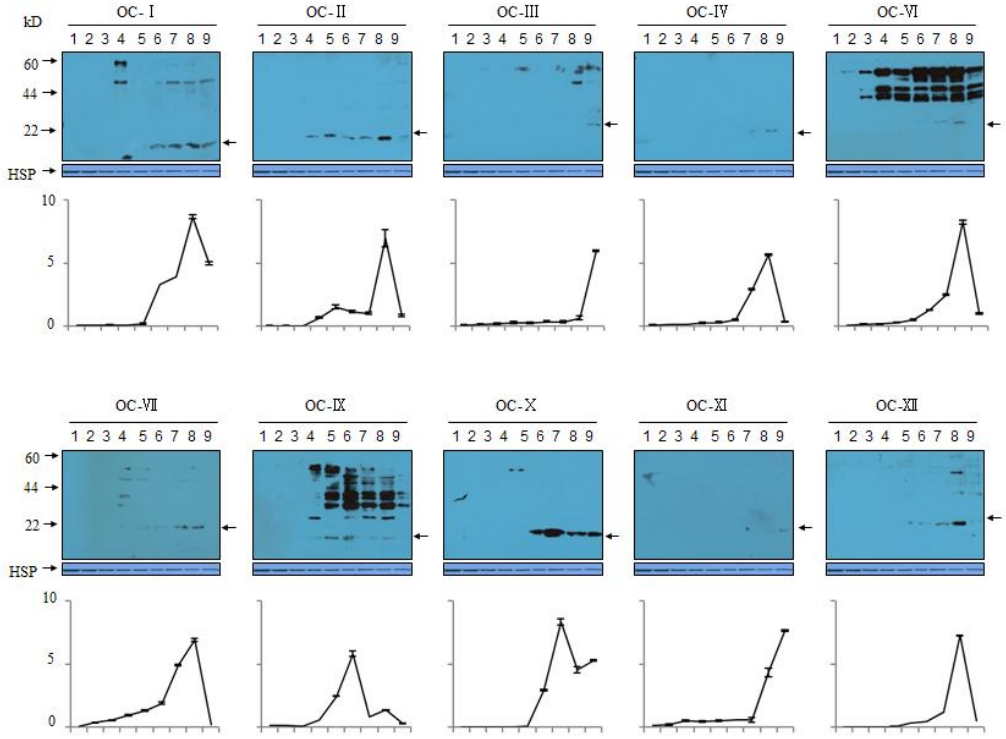

Figure 4 Expression patterns of oryzacystatin in normal growth leaves

Upper panels: WB detection of the expression of oryzacystatin at different time points in rice leaves; Middle panels: WB detection of HSP expression in rice leaves to demonstrate equal loading; Lower panels: Lanes 1–9: Protein samples isolated from normal rice leaves at seedling stage(1 cm, 2 cm, 5 cm, 10 cm, and 15 cm), tillering stage, booting stage, flowering stage and mature stage, respectively.

modification, activation and inhibition of protein. We tried to analyze the relevance of transcription and translation, but did not find a significant relevance between them. In fact, transcription and translation regulation mechanism are at different levels, a lot of data reveals that the independence of them.

DISCUSSIONS

Researches have showed that cysteine protease inhibitor involves in insect resistance, disease resistance, stress resistance and protein metabolism. It is possible for exploring the functions of the cysteine protease inhibitor family at the genome level system based on the analysis of the rice genome sequence.

Based on the analysis of the whole genome sequence and prediction and annotation of the genes, it can give us a more comprehensive understanding about different gene family. In rice genome, there are more than 900 pathogenesis-related (PR) proteins, accounting for about 2% of the whole genome encoding genes. Among them, proteinase inhibitors belongs to PR6 gene family, including 32 members divided three subfamily, trypsin inhibitor, cysteine proteinase inhibitor and proteinase inhibitor II subfamily.

There are 12 cysteine proteinase inhibitor genes based on the rice genome annotation. According to the amino acid sequence of oryzacystatin, the protein domain could be predicted. Most of the oryzacystatin has a single cysteine protease inhibitor-like domain (Cystatin-like domain, CY), and including three special motif, (1) the sequence QxVxG of inhibitor to the active site; (2) glycine near the N-terminal; (3) the conserved tryptophan in the rear part of the protein (Martinez *et al*., 2005), which interacts with the corresponding protease. With the progress of high-throughout sequencing technology and MPSS, transcription data could be obtained more sensitive and accurate. Our study shows that the transcription of oryzacystatin in rice tissues during normal growth stages may play a role in the normal development process. Cluster analysis based on the similarity of the expression profile shows members of the same family may be complementary of physiological functions. The more closer cluster relationship of the genes have, the more complementary functions they have. With the accumulation of the transcriptional data, the analysis of gene relativity on a larger scale may obtain the related cues of their functions. In this part of our research, we make a good try to cover the bioinformation and expression analysis of the cysteine protease inhibitor family.

Antibodies against the 12 oryzacystatin were prepared by using artificial peptides vaccinating rabbits. And we detected the protein expression pattern in different tissues of rice by western blot. The antibody-based rice proteomics (AbRP) strategy could study the target protein (Liu *et al*., 2011). And those antibodies will also be the important resources for research of subcellular localization and co-immunoprecipitation.

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