Functional characterization of a new pathogen induced xylanase inhibitor (RIXI) from rice

Chun-Xiao Hou · Yi-Hua Zhan · De-An Jiang · Xiao-Yan Weng

Abstract Rice xylanase inhibitor RIXI is a XIP-type inhibitor that belongs to the glycoside hydrolase family 18 (GH18), which includes plant class III chitinases (EC 3.2.1.14) known as PR-8 proteins. The aim of this study was to elucidate whether RIXI had any effect on rice endoxylanase and its role(s) in plant defence. *RIXI* encoding sequence was cloned from rice (*Oryza sativa* cv. Nipponbare), and was expressed in *Escherichia coli*. The activity of recombinant RIXI was investigated – among the tested xylanases, the GH11 xylanase from *Aspergillus niger* was the most inhibited, while rice endogenous xylanase OSX was least inhibited. Semi-quantitative real-time polymerase chain reaction (PCR) and quantitative real-time PCR showed that the xylanase inhibitor gene *RIXI* was up-regulated in rice seedlings infected by *Magnaporthe grisea*. The increased *RIXI* expression was also accompanied by significantly elevated expression of pathogenesis-related protein gene (*PR-1*) and class III chitinase gene (*Cht-3*). This suggested that RIXI might be involved in environmental responses such as defense against phytopathogens.

Keywords Xylanase inhibitor proteins · Xylanase · Gene expression · Plant defence

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Abbreviations

RIXI	Rice xylanase inhibitor
Cht-3	Class III chitinase gene
GH	Glycoside hydrolase family
PR-1	Pathogenesis-related protein gene
qRT-PCR	quantitative real-time polymerase chain reaction

Introduction

The plant cell wall, protecting the cell from its surrounding environment, is the first barrier to pathogenic attack. Plant-pathogenic microorganisms use a large amount of cell wall degrading enzymes to break this barrier to invade plants or to feed on the released nutrients (Misas-Villamil and Hoorn 2008). Endo-β-(1,4)-xylanases (EC 3.2.1.8, hereafter referred to as xylanases) are enzymes that degrade xylan, one of the most abundant polysaccharides in the cell wall of higher plants (Collins et al. 2002). Xylanase inhibitors in cereals have been reported to play a role in resistance against pathogens.

Three types of xylanase inhibitors, XIP (xylanase inhibitor protein)-type, TAXI (*Triticum aestivum* xylanase inhibitor)-type and TLXI (thaumatin-like xylanase inhibitor)-type have been identified in cereals (Debyser et al. 1999; Mclauchlan et al. 1999; Fierens et al. 2007). Among these three types, only XIP-type

proteins can interact with glycoside hydrolase family (GH) 10 xylanases, the family to which plant xylanases belong (Goesaert et al. 2004; Fierens et al. 2007). Xylanase inhibitors may play a dual role in plants, namely in the regulation of endogenous arabinoxylan hydrolysis, as well as the inhibition of exogenous enzymes produced by microorganisms (Simpson et al. 2003). Most studies have indicated that xylanase inhibitors may play a role in plant defence rather than in the regulation of endogenous xylanase activity (Belien et al. 2006; Tokunaga et al. 2008).

XIP-type xylanase inhibitors have also been isolated from rye, durum wheat, barley and maize (Goesaert et al. 2004). Tokunaga and Esaka (2007) reported at least eight candidates for XIP genes in rice by analysis using the database of full-length cDNA clones from japonica rice. Among rice XIP family members, RIXI is a novel XIP-type inhibitor and named 'Rice xylanase inhibitor' (Durand et al. 2005). The protein sequence of RIXI has been published in GenBank and the function of other rice xylanase inhibitors have been examined in some detail (Goesaert et al. 2005; Tokunaga and Esaka 2007). However, reports on the interaction mechanism between RIXI and endogenous xylanase of rice and the role(s) of plant xylanase inhibitor proteins in plant metabolism as a whole are scarce.

This study described the cloning of xylanase inhibitor gene *RIXI* from rice and its expression in *Escherichia coli*. Additionally, xylanase inhibition specificity of the recombinant RIXI and the response of RIXI to pathogens were investigated. To our knowledge, this has not previously been reported.

Materials and methods

Plasmids, chemicals and strains

The cloning host strain *E. coli* TOP10, the expressing host strain *E. coli* BL21 and the expressing vector pET-30a(+) were all purchased from Invitrogen. Primers and pUCm-T vector were obtained from Sangon. Standard protein molecular weights were obtained from MBI. Birchwood xylan was purchased from Sigma (St Louis, MO, USA). *Aspergillus niger* xylanase (ANX, molecular weight of 20 kDa), *Bacillus subtilis* xylanase (BSX, molecular weight of 20 kDa), *Thermomonospora fusca* xylanase (TFX, molecular weight of 31 kDa) and *Oryza*

sativa xylanase (OSX, molecular weight of 41 kDa) were preserved by our laboratory (Sun 2003; Weng and Sun 2005; Liu et al. 2006; Sun et al. 2007; Ma 2011). *Magnaporthe grisea* KJ201 was provided by the College of Agriculture and Biotechnology, Zhejiang University.

Molecular cloning

The genomic DNA of rice (*Oryza sativa* cv. Nipponbare) was extracted according to the protocol provided by the manufacturer (Invitrogen) and served as a template for the following amplification. DNA concentration and purity were determined by OD260/OD280 absorbance. Polymerase chain reaction (PCR) on genomic DNA was conducted with primers 5′-ACTA TACATTATTAAGGCTAACCAG-3′ and 5′-TTAACG CATAGTAGCAGTAAATAGT-3′ for amplification of the complete *RIXI* coding sequence. PCR was conducted with preheating at 94 °C for 2 min, and with 35 cycles of 94 °C for 50 s, 56 °C for 50 s and 72 °C for 2 min; and then 72 °C for 10 min. A DNA fragment of approximately 1,000 bp was generated and cloned into the vector pUCm-T. The clone that contained the resulting PCR product was verified by restriction enzyme digestion and sequencing and transformed into *E. coli* TOP 10.

Expression of RIXI protein in *E. coli*

To obtain the recombinant RIXI protein, the DNA sequence was amplified with primers 5′-ACG<u>GAAT TC</u>ATGGTGGCGCTCGG-3′ (forward, *Eco*RI site underlined) and 5′-GTT<u>CTCGAG</u>TTAAGCCCAGTA CTTG-3′ (reverse, *Xho*I site underlined) from pUCm-T+RIXI plasmid. The amplified PCR product was digested and inserted into the vector pET-30a (+) (Novagen) at *Eco*RI and *Xho*I sites. This expression vector was named pET-30(a)+RIXI. Then pET-30(a)+ RIXI was introduced into *E. coli* BL21. Transformants were screened on Luria–Bertani (LB) plates with 100 μg ml^{-1} kanamycin. Positive transformants were cultured in a liquid LB-kanamycin (50 μg ml^{-1}) medium at 37 °C for 12 h, and then induced with β-D-thiogalactoside (IPTG) (0.1 mM final concentration) for 6 h. Cells were harvested by centrifugation (10 000×g, 10 min, 4 °C), resuspended in McIlvaine's buffer (0.1 M citric acid, 0.2 M Na$_2$HPO$_4$ at pH 6.0), sonicated and centrifuged (15 000×g, 10 min, 4 °C).

Electrophoresis was performed on 12.5 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) as described by Laemmli (1976). After the fusion protein expressed by *E. coli*, RIXI was purified using Ni-NTA Spin Kit (Qiagen, Hilden, Germany) according to its manual, it was digested with enterokinase, and then xylanase inhibitor activity was measured. The concentration of protein was determined by the method of Bradford (1976). The N-terminal protein sequencing was performed on an Applied Biosystems 492cLC Protein Sequencer System.

Measurement of xylanase inhibition activity

Xylanase inhibitor activity was determined by comparing the reduction in xylanase activity in the presence of recombinant RIXI with appropriate controls, which were incubated in the absence of recombinant RIXI. A known concentration of ANX, BSX, TFX and OSX was incubated with birchwood (1,4)-β-xylan in the presence or absence of recombinant RIXI using the dinitrosalicylic acid (DNS) assay described by Miller et al. (1959). Xylanase inhibition activities were determined in triplicate. ANX, BSX, TFX are GH11 xylanases from *A. niger*, *B. subtilis*, *T. fusca*, respectively, and OSX is GH10 xylanase from rice (*Oryza sativa*). We cloned *ANX*, *BSX*, *TFX* and *OSX* and expressed them in *E. coli*, then obtained the recombinant ANX, BSX, TFX and OSX proteins. The characterization of recombinant ANX, BSX, TFX and OSX have been demonstrated in previous paper of our lab (Sun 2003; Liu et al. 2006; Sun et al. 2007; Ma 2011). The xylanase activity was measured with 1 % birchwood xylan (*w/v*) as substrate using the method described by Bailey et al. (1992). Xylanase preparations (25 μl) were added to 1 % (*w/v*) birchwood (1,4)-b-xylan (Sigma) (50 μl) solubilized in McIlvaine's buffer (pH 5.0) and incubated at 50 °C for 10 min. The liberation of reducing sugars was estimated by the DNS method, with D-xylose as a standard. After 10 min at room temperature, the suspension was shaken and absorbance at 540 nm of the filtrate was measured. One unit of xylanase activity was defined as the amount of protein that released 1 μmol xylanase min^{-1} at 50 °C and pH 5.0. Reactions containing ANX (25 μl), BSX (25 μl), TFX (25 μl) and OSX (25 μl) pre-incubated (5 min, 50 °C) with purified recombinant RIXI (30 μl, 0.9 μg) from transformed *E. coli*, were assayed for xylanase activity.

Treatment of rice seedlings by inoculating with *M. grisea*

Fourteen-day-old rice plants were inoculated with spore suspensions (1.5×10^5 spores ml^{-1} containing 0.05 % Tween 20) of *M. grisea* KJ201 for treatment, or with sterile water containing 0.05 % Tween 20 for control. The inoculated plants were moved to a dew chamber at 28 °C for 36 h and were then shifted back to a growth chamber.

RNA isolation

Total RNA was isolated from leaves of rice plants at 72 h after *M. grisea* KJ201 treatment using RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instruction. First-strand cDNA was then synthesized from 1 μg of total RNA by reverse transcription using the PrimeScript® RT reagent Kit (Perfect Real Time) (Takara, Shiga, Japan). Quantitative real-time PCR (qRT-PCR) for all genes was done using the same pool of cDNA for each treated sample to ensure uniformity of results.

Semi-quantitative (sq) RT-PCR and qRT-PCR

The cDNAs of *β-actin*, *RIXI*, pathogenesis-related protein gene (*PR-1*) (GenBank: U89895.1) and Class III chitinase gene (*Cht-3*) (GenBank: HC733649.1) were then detected by sqRT-PCR and qRT-PCR with specific primer pairs (Table 1), and a primer set 5′-TTATGGTT GGGATGGGACA-3′ and 5′-AGCACGGCTTGAAT AGCG-3′ for *β-actin* (GenBank: AK101613.1), 5′-ACGCCACCTGCTCCTACAACC-3′ and 5′-GATT CCGCCATAGTTCTTCGCC-3′ for *RIXI*, 5′-AATGCC AAAGATGCG-3′ and 5′-GTTTAGACAAAGAGGG ACA-3′ for *Cht-3*, and 5′-TGAAAAGTTGTGCT TAA-3′ and 5′-GACCAGATGTTGTATGC-3′ for *PR-1*. A pair of gene-specific primers was chosen for each gene and their specificities were confirmed in an agarose gel before they were used in the sqRT-PCR and qRT-PCR analysis. In experiments, *β-actin* was used as an endogenous reference gene. The PCR reactions were conducted according to the protocol of the SYBR® Premix Ex TaqTM kit (Takara) containing TaKaRa Ex Taq HS, SYBR Green, optimized PCR buffer, MgCl$_2$ and dNTP mix. qRT-PCR was performed on an ABI Prism 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) and the size of each

Table 1 Primers and PCR conditions for real time PCR

Gene	GenBank accession No.	Primers sequence (from 5′ to 3′)	Ta (°C)	Amplicon size (bp)
β-actin	AK101613.1	TTATGGTTGGGGATGGGACA AGCACGGCTTGAATAGCG	60	292
RIXI	D55712.1	ACGCCACCTGCTCCTACAACC GATTCCGCCATAGTTCTTCGCC	60	203
Cht-3	HC733649	AATGCCAAAGATGCG GTTTAGACAAAGAGGGACA	60	293
PR-1	U89895.1	TGAAAAGTTGTGCTTAA GACCAGATGTTGTATGC	60	307

Ta annealing temperature, *RIXI* xylanase inhibitor gene, *Cht-3* class IIIchitinase gene, *PR-1* pathogenesis related (PR) protein 1 gene, *β-actin* internal control

sqRT-PCR product was confirmed by gel electrophoresis on standard 1 % agarose gels stained with ethidium bromide and visualized by exposure to ultraviolet light. The PCR program was performed as follows: denaturation (95 °C for 30 s), amplification and quantification repeated 35 times (95 °C for 5 s, 60 °C for 30 s), melting curve (65–95 °C, with a heating rate of 0.5 °C s^{-1} and a continuous fluorescence measurement) and finally a cooling step to 4 °C. Melting curve analysis was used to corroborate the specificity of the PCR products.

The threshold cycle (Ct) value, defined as the threshold cycle number of PCR at which the sample fluorescent signal passes a fixed threshold above the baseline (Johnson et al. 2000), was fixed arbitrarily and a common value determined for all the genes. For each cDNA sample, qRT-PCR was performed in triplicate, and an average Ct value calculated from these three qRT-PCR reactions. The relative quantification of gene expression was analyzed by the comparative method ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001) with some modifications. Using the $2^{-\Delta\Delta Ct}$ method, data were presented as the fold-change in mRNA expression normalized to the endogenous reference gene (*β-actin*) and relative to the control.

Results and discussion

Cloning and expression of recombinant RIXI in *Escherichia coli*

The gene encoding RIXI is localized on chromosome group 11 and contains no introns, so we isolated the DNA fragment encoded RIXI from rice genomic DNA.

Nucleotide sequence analysis revealed a sequence of 1, 020 bp in length with an open reading frame (ORF) of 915 bp, from 55 to 969 bp; RIXI encoded a predicted polypeptide of 304 amino acids with a calculated molecular weight of 34 kDa and pI of 9.33. The alignment of RIXI with the reported sequence (GenPept databank; AN: BAA23810.1) (Durand et al. 2005), revealed 100 and 99.7 % amino acid sequence and nucleotide sequence identities, respectively. The base composition of the RIXI coding sequence was G/C-rich (65.03 %).

The ORF was cloned into expression vector pET-30a (+) arranged in frame with an N-terminal extension including a His-tag to give the expression construct pET-30(a)+RIXI. The expressed protein was a fusion protein with 71 additional amino acids at the N-terminus from the vector. The protein expression in *E. coli* induced by IPTG was secreted in the supernatant after 6 h induction, the concentration of soluble protein extracts was about 230 mg/l. Using His-tagged Ni–NTA Spin Kit, the concentration of purified protein was about 30 mg/L. SDS-polyacrylamide electrophoresis analysis showed that the purified proteins from *E. coli* containing pET30a+RIXI plasmid exhibited a clear band with a molecular weight of about 40 kDa (Fig. 1, Lane 2), in total agreement with the predicted calculated mass including the extra 71 amino acids and a His-tag in the N-terminal from the vector. No band was observed in the extract from the control strain of *E. coli* containing pET-30a(+) vector (Fig. 1, Lane 1). After digested by enterokinase, the N-terminal amino acid sequence AMADIGSEFMVALGRRS for purified protein was determined, thus in complete agreement with the predicted peptide.
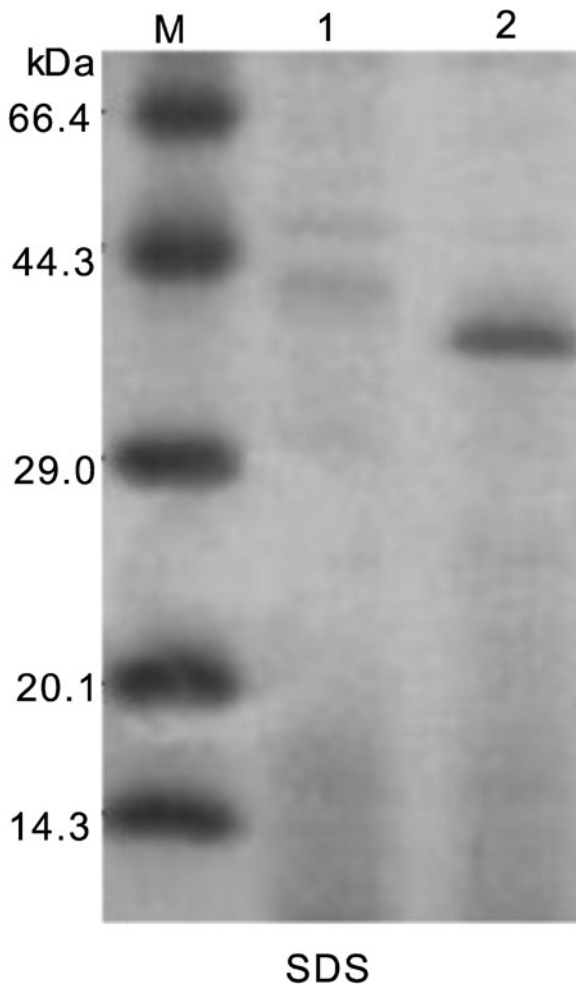
Fig. 1 SDS-PAGE of the purified recombinant RIXI expressed in *Escherichia coli* BL21. *M* - marker of proteins, *Lane 1* - cell extracts from control strain of *E. coli* containing pET-30a(+) vector and *Lane 2* - cell extracts from *E. coli* containing pET-30a+*RIXI* vector induced by IPTG after 6 h. The sizes of the markers are indicated at the left of each gel

Comparison of RIXI with other xylanase inhibitors

RIXI belongs to the GH18 family as well as class Ш chitinases, despite its lack of enzymatic activity (Durand et al. 2005). The phylogenetic tree for plant members of GH18 showed that RIXI belongs to the XIP-type xylanase inhibitor subfamily (Tokunaga and Esaka 2007). The alignment of RIXI (GenBank: BAA23810.1) with other xylanase inhibitors was performed by Mega 5.0 and Clustalx (Fig. 2a, b), and the signal sequences of all sequences (RIXI and other xylanase inhibitors) for the alignment was ruled out. According to the alignment, these proteins all show

the (β/α)8 barrel fold, and regions encompassing the former chitinase catalytic moiety and the predicted sites of interaction with xylanases GH10 and GH11 active sites are highlighted in Fig. 2b. On the protein level, RIXI shared more sequence identity with XIP-П (65 %, GenBank: CAC87260.1) from *Triticum durum* than with OsXIP (48 %, GenBank: BAG93671.1) and riceXIP (57 %, GenBank: BAG89082.1) from rice. In contrast, lower identities were shared with the other inhibitor sequences; i.e. 51, 50 and 3 % with XIP-I (GenBank: CAD19479), XIP-Ш (GenBank: BAD99103.1), TLXI (GenBank: AFI49341.1) from wheat, respectively, 51 % with a putative *Zea mays* XIP (GenBank: DQ245902) and *Sorghum* XIP (GenBank: EER98073.1), 41 % with basic chitinase (GenBank: BAA22266.1), 36 % with hevamine (GenBank: CAA07608) which is a class Ш chitinase from rubber tree (*Hevea brasiliensis*), and 30 % with *Coffea arabica* XIP (GenBank: ADZ48381). Figure 2a shows the relationship between these sequences.

The enzymes of the GH family 18 showing chitinase activity have a conserved aspartic acid and a catalytic glutamic acid residue in the active site, corresponding to Asp^{125} and Glu^{127} in hevamine. However, XIP-type xylanase inhibitors are devoid of chitinase activity. In the structures of RIXI, *T. Durum* XIP-П, OsXIP and riceXIP, Asp^{125} and Glu^{127} are replaced by Phe and Asp at positions 124–126, 122–124, 115–117 and 130–132, respectively (Fig. 2b), which mostly account for the lack of chitinase activity reported for these proteins. The fact that RIXI is closer to the *T. durum* xylanase inhibitor sequence than to other rice and wheat xylanase inhibitor proteins suggests that the different members of the XIP gene family underwent sequence conservation and/or divergence during evolution. The profile of organ-specific expression of *RIXI* differs from other rice XIP genes, *OsXIP* and *riceXIP. RIXI* was constitutively expressed in shoot and not induced by defence-related phytohormones, while *OsXIP* and *riceXIP* mRNAs were not detected in basal conditions and were induced in root by wounding and methyl jasmonate (Tokunaga and Esaka 2007). Thus we speculate that the defence mechanism against pathogens of RIXI may differ from other rice XIPs.

Xylanase inhibition specificity of RIXI

RIXI's inhibition varied among xylanases. Xylanase inhibition activity of the recombinant RIXI was
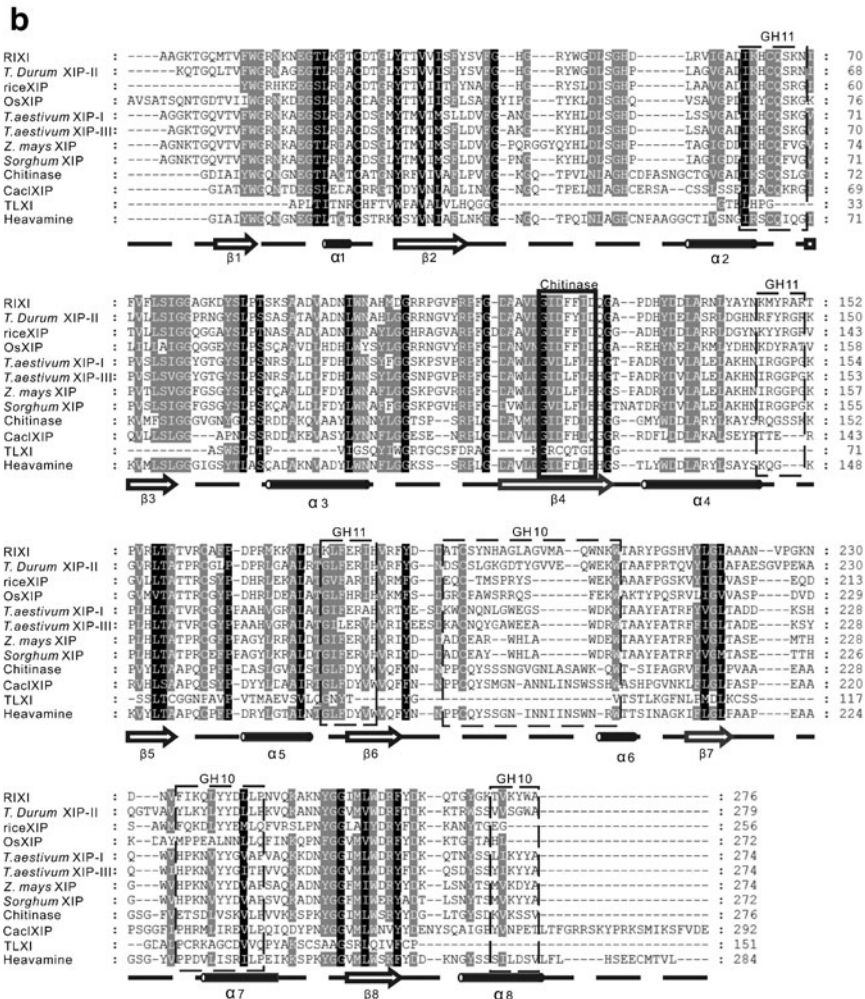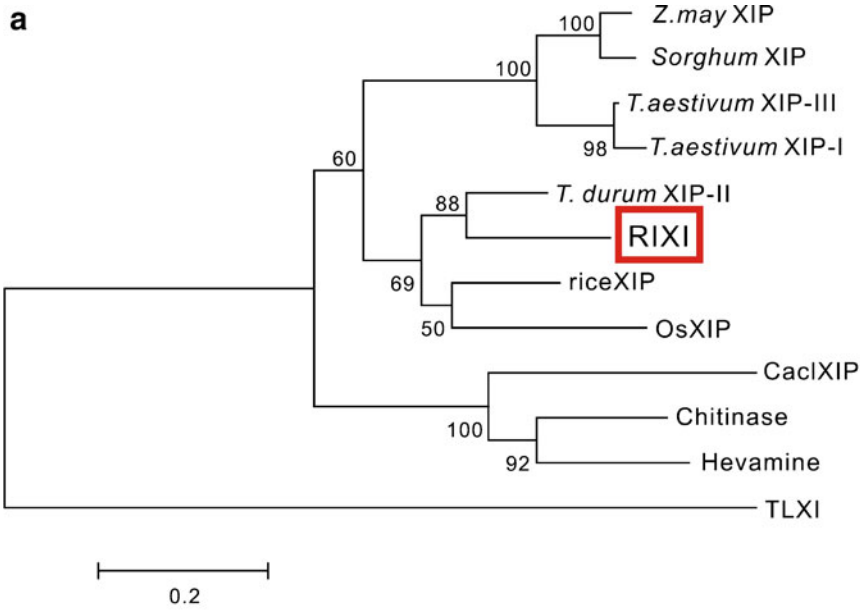
◀ **Fig. 2** The analysis of the deduced amino acid sequence of RIXI. **a** Dendrogram showing the relationship between different xylanase inhibitors. The tree was derived from the alignment of the complete deduced amino acid sequences (ruling out signal sequence) produced using the Mega 5.0 program. The tree shows the relationship between sequences of RIXI, *T. durum* XIP-II (CAC87260.1), rice XIP proteins [OsXIP (BAG93671.1) and riceXIP (BAG89082.1)], *Sorghum* XIP (EER98073.1), a putative XIP of *Z. mays* (DQ245902), and xylanase inhibitor proteins from *T. aestivum* [XIP-I (CAD19479), XIP-III (BAD99103.1) and TLXI (AFI49341.1)], and basic chitinase (BAA22266.1), hevamine (CAA07608) which is a class III chitinase from rubber tree (*H. brasiliensis*) and *C. arabica* XIP (GenBank: ADZ48381). **b** Amino acid sequence alignment of selected different xylanase inhibitors and secondary structure prediction. Region encompassing the catalytic domain of class III chitinases are marked with a *continuous box*. Sequences in the *dotted box* show residues involved in complexing with GH10 or GH11 xylanases in RIXI and its counterparts in the other proteins. Secondary structure alignment was performed with Jalview software and improved by hand

Fig. 3 Xylanase inhibitor activity of RIXI. The inhibition of purified recombinant RIXI to xylanases using birchwood (1,4)-b-xylan as substrate, and three GH11 xylanases from *Aspergillus niger* (ANX), *Bacillus subtilis* (BSX) and *Thermomonospora fusca* (TFX) and GH10 xylanases from *Oryza sativa* (OSX) as the target enzyme. A 0.9-μg aliquot of recombinant RIXI was used. The residual xylanase activity is shown. 'RIXI+' and 'RIXI–' represent in the presence and absence of recombinant RIXI, respectively. Error bars indicate $\pm$ SD (n=3). Statistical differences between 'RIXI+' and 'RIXI–' are marked with two *asterisks* when $p<0.01$ according to Student's t test

measured after digested by enterokinase. *A. niger* is a fungal pathogen, and *B. subtilis* and *T. fusca* are bacterial soil pathogens. The results showed that RIXI inhibited ANX and BSX, and also weakly inhibited TFX and OSX. These four xylanases were previously expressed in *E.coli* or *Pichia pastoris* by our laboratory, and the characterization of them have been described previously (Sun 2003; Liu et al. 2006; Sun et al. 2007; Ma 2011). Under the experimental conditions with the same inhibitor concentration, xylanase activities of ANX, BSX, TFX and OSX remained at 29.8, 47.4, 93.2 and 95.3 %, respectively (Fig. 3), indicating that *A. niger* xylanase was the most inhibited xylanase of these, while *O. sativa* endoxylanase (a plant GH10 family xylanase) was the least inhibited. These results verified the previous observation that XIP-type xylanase inhibitors were active against GH11 xylanases from both bacterial and fungal origins, and were inactive towards GH10 xylanases from plants (Fierens et al. 2003; Gebruers et al. 2004). Durand et al. demonstrated that none of the GH10 xylanases from *A. niger, A. aculeatus* and *A. nidulans* (fungal) or from *Cellvibrio japonicus* (bacterial) were inhibited by RIXI (Durand et al. 2005).

E. coli is often the first-choice microorganism for recombinant protein production. *E. coli* has been widely used for primary cloning, genetic modification and small-scale production for research purposes (Ferrer-Miralles et al. 2009). In our lab, we expressed the xylanase inhibitor protein RIXI both in *E. coli* and
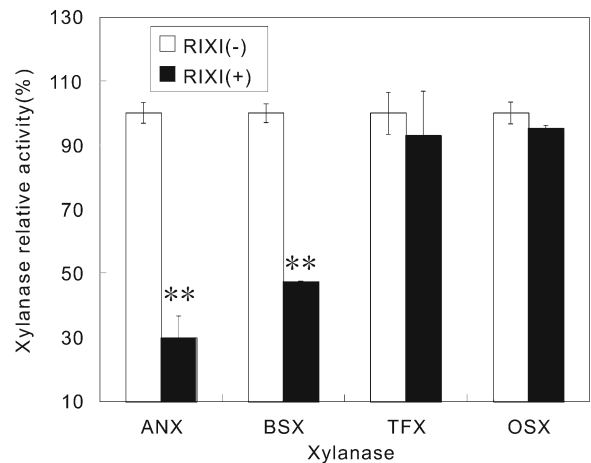
Pichia pastoris, respectively. Both recombinant proteins showed xylanase inhibitor activity, and the molecular weight was similar (data not shown). The level of xylanase inhibitor activity of the recombinant RIXI expressed in *E. coli* was similar to that in *Pichia pastoris*. Our results indicated the recombinant protein expressed in the correct way as wild-type, and retained the natural characteristics. Several research groups also reported the expression of xylanase inhibitors in *E. coli*. Examples include OsXIP from rice (Tokunaga and Esaka 2007), Xip-R1 and TAXI-I from wheat (Fierens et al. 2003; Takahashi-Ando et al. 2007). Using *E. coli* for recombinant protein production is much faster and less expensive.

The recombinant OsXIP expressed in *E. coli* could inhibit GH11 xylanase from *Trichoderma viride* and *Trichoderma longibrachiatum*, and the activity of GH11 xylanase from *A. niger* was not influenced by recombinant OsXIP (Tokunaga and Esaka 2007). Wheat xylanase inhibitors XIP-R1 and TAXI-I were also expressed in *E. coli*, xylanase inhibitor activity of XIP-R1 was assayed using xylanases from *Thermomyces lanuginosus*, *T. longibrachiatum*, *T. viride*, *Aureobasidium pullulans* and *A. niger* (Takahashi-Ando

et al. 2007). Xylanases from *A. niger* and *B. subtilis* were assayed for xylanase inhibitor activity of TAXI-I (Fierens et al. 2003). Numerous researches reveal different classes of xylanase inhibitors exhibit different effectiveness towards various xylanases (Gebruers et al. 2004; Goesaert et al. 2004; Fierens et al. 2007; Misas-Villamil and Hoorn 2008). Plant xylanases are structurally similar to microbial GH10 xylanases (Simpson et al. 2003), and in this experiment RIXI probably weakly inhibited rice endogenous xylanases. Therefore, it has been speculated that RIXI involved in plant defence against fungal and bacteria pathogens.

Characteristics in plant defence

To gain further insight into the plant defence mechanisms of xylanase inhibitor RIXI, 14-d-old rice plants were inoculated with *M. grisea* KJ201 and the expression levels of *RIXI*, *Cht-3* and *PR-1* were then determined. *M. grisea*, also known as rice blast fungus, is a plant-pathogenic fungus that causes an important disease affecting rice. KJ201, a strain of *M. grisea*, can infect leaf tissue of *O. sativa* cv. Nipponbare. The qRT-PCR and sqRT-PCR indicated that *RIXI* was up-regulated in rice seedlings infected by *M. grisea* KJ201 (Fig. 4a, b). This was in accordance with the conclusions of Vasconcelos et al. (2011). During infection, plant cells produce a group of proteins, named PR proteins, which accumulate to increase the resistance of the whole plant against a pathogenic attack (Sexton and Howlett 2006). PR protein families have been identified from cell-wall/-membrane degrading enzymes, protease

inhibitors and proteins related to oxidative metabolism (Sels et al. 2008). PR-1 protein is the most widely used marker of acquired resistance after pathogen infection. XIP-type inhibitors can be classified as PR proteins of classes PR-8 based on their homology with chitinases. In the present study, the increased *RIXI* expression was accompanied by significantly elevated expression of *PR-1* and *Cht-3* after pathogen infection (Fig. 4a, b). This suggested that xylanase inhibitors could be expressed in combination with other defensive proteins, and used to develop resistance to pathogens.

Igawa et al. (2005) proposed that wheat *XIP-I* transcription can be induced by pathogens as well as by abiotic stress, such as wounding and methyl jasmonate treatment. In maize cells, XIP synthesis and secretion into the extracellular matrix is up-regulated in response to *Fusarium* elicitor molecules (Chivasa et al. 2005). XIP-like genes are also expressed in tissues during growth and development of healthy wheat plants (Croes et al. 2009). In rice, three different XIP inhibitors were also induced by phytohormones and wounding in different tissues (Tokunaga and Esaka 2007). Cacl XIP, a chitinase-like XIP from coffee (*C. arabica*) was able to inhibit the germination of spores of *Phakopsora pachyrhizi* (Vasconcelos et al. 2011). XIP and TAXI from wheat directly inhibited the growth of *Fusarium graminearum* and *Rhizoctonia solani*, respectively, on medium containing glucose (Fierens et al. 2007). TLXI-type xylanase inhibitor proteins from wheat share 60 % sequence similarity with TLPs (Fierens 2007), and PpTLPs, thaumatin-like proteins (TLPs) from *Pyrus pyrifolia* Nakai cv Huobali can inhibit the hyphal
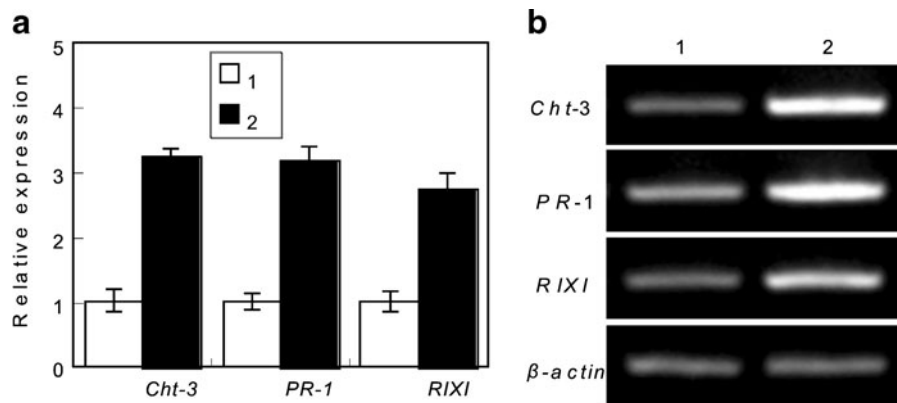

Fig. 4 Expression of *RIXI*, *Cht-3* and *PR-1* in rice seedlings after *Magnaporthe grisea* treatment by qRT-PCR (**a**) and sqRT-PCR (**b**). 1 - Uninfected seedlings and 2 - infected seedlings. Total RNA for expression analysis was isolated from shoots of 14-d-old seedlings. The relative expression is the mean of three technical replicates. *Error bars* denote standard errors

growth of *Sclerotinia sclerotiorum*, *Phomopsis* sp., *Phytophthora parasitica* var *nicotianae* and *Alternaria* sp. in different degrees (Liu et al. 2012). The expression of exogenous xylanase gene *ATX* in rice can up-regulate the expression of *RIXI* (Weng et al. 2013). These suggested that inhibition of xylanases was not the crucial factor for antifungal activity of xylanase inhibitor proteins due to the absence of xylan in the assay medium, but may be caused by the binding of xylanase inhibitor proteins and fungal β-D-glucans (Fierens et al. 2008), leading to disturbance of proper cell wall synthesis and/or modulation (Fierens et al. 2007). These results demonstrated that the role(s) of plant xylanase inhibitor proteins in plant metabolism as a whole remain largely obscure.

Conclusions

Recombinant protein RIXI was active against GH11 family xylanases of both bacterial and fungal origin and was inactive towards GH10 xylanases from rice. The *RIXI* gene was up-regulated in rice seedlings infected by *M. grisea* KJ201, and was accompanied by significantly elevated expression of *PR-1* and *Cht-3*. We suggest that xylanase inhibitors play a role in plant defence, rather than in plant regulation.

References

Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology, 23*, 257–270.

Belien, T., Van Campenhout, S., Robben, J., & Volckaert, G. (2006). Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Molecular Plant-Microbe Interactions, 19*, 1072–1081.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry, 72*, 248–254.

Chivasa, S., Simon, W. J., Yu, X. L., Yalpani, N., & Slabas, A. R. (2005). Pathogen elicitor-induced changes in the maize extracellular matrix proteome. *Proteomics, 5*, 4894–4904.

Collins, T., Meuwis, M. A., Stals, I., Claeyssens, M., Feller, G., & Gerday, C. (2002). A novel family 8 xylanase, functional and physicochemical characterization. *The Journal of Biological Chemistry, 277*, 35133–35139.

Croes, E., Gebruers, K., Luyten, N., Delcour, J. A., & Courtin, C. M. (2009). The three classes of wheat xylanase-inhibiting proteins accumulate in an analogous way during wheat ear development and germination. *Journal of Plant Physiology, 166*, 1253–1262.

Debyser, W., Peumans, W. J., Van Damme, E. J. M., & Delcour, J. A. (1999). TAXI, a new class of enzyme inhibitors affecting bread volume. *Journal of Cereal Science, 30*, 39–43.

Durand, A., Hughes, R., Roussel, A., Flatman, R., Henrissat, B., & Juge, N. (2005). Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18. *The FEBS Journal, 272*, 1745–1755.

Ferrer-Miralles, N., Domingo-Espin, J., Corchero, J. L., Vazquez, E., & Villaverde, A. (2009). Microbial factories for recombinant pharmaceuticals. *Microbial Factories For Recombinant Pharmaceuticals, 8*, 17.

Fierens, E. (2007). TLXI, a thaumatin-like xylanase inhibitor: isolation, characterisation and comparison with other wheat (*Triticum aestivum* L.) xylanase inhibiting proteins. PhD Dissertation, K.U. Leuven, Belgium.

Fierens, E., Gebruers, K., Courtin, C. M., & Delcour, J. A. (2008). Xylanase inhibitors bind to nonstarch polysaccharides. *Journal of Agricultural and Food Chemistry Journal, 56*, 564–570.

Fierens, E., Rombouts, S., Gebruers, K., Goesaert, H., Brijs, K., Beaugrand, J., et al. (2007). TLXI, a novel type of xylanase inhibitor from wheat (Triticum aestivum) belonging to the thaumatin family. *Biochemical Journal, 403*, 583–591.

Fierens, K., Brijs, K., Courtin, C. M., Gebruers, K., Goesaert, H., Raedschelders, G., et al. (2003). Molecular identification of wheat endoxylanase inhibitor TAXI, member of a new class of plant proteins. *FEBS Letters, 540*, 259–263.

Gebruers, K., Brijs, K., Courtin, C. M., Fierens, K., Goesaert, H., Rabijns, A., et al. (2004). Properties of TAXI-type endoxylanase inhibitors. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1696*, 213-221.

Goesaert, H., Elliott, G.O., Kroon, P.A., Gebruers, K., Courtin, C. M., Robben, J., et al. (2004). Occurrence of proteinaceous endoxylanase inhibitor in cereals. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1696*, 193–202.

Goesaert, H., Gebruers, K., Courtin, C. M., & Delcour, J. A. (2005). Purification and characterization of a XIP-type endoxylanase inhibitor from Rice (Oryza sativa). *Journal of Enzyme Inhibition and Medicinal Chemistry, 20*, 95–101.

Igawa, T., Tokai, T., Kudo, T., Yamaguchi, I., & Kimura, M. (2005). A wheat xylanase inhibitor gene, Xip-I, but not Taxi-I, significantly induced by biotic and abiotic signals that trigger plant defense. *Bioscience, Biotechnology, and Biochemistry, 69*, 1058–1063.

Johnson, M. R., Wang, K., Smith, J. B., Heslin, M. J., & Diasio, R. B. (2000). Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Analytical Biochemistry, 278*, 175–184.

Laemmli, U. K. (1976). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Liu, D. Q., He, X., Li, W. X., Chen, C. Y., & Ge, F. (2012). Molecular cloning of a thaumatin-like protein gene from Pyrus pyrifolia and overexpression of this gene in tobacco increased resistance to pathogenic fungi. *Plant Cell, Tissue and Organ Culture, 111*, 29–39.

Liu, M. Q., Weng, X. Y., & Sun, J. Y. (2006). Expression of recombinant Aspergillus niger xylanase A in Pichia pastoris and its action on xylan. *Protein Expression and Purification, 48*, 292–299.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using Real-Time Quantitative PCR and the 2$^{-\Delta\Delta Ct}$ method. *Methods, 25*, 402–408.

Ma, M.Z. (2011) Cloning and expression of endoxylanase gene *osx* from *Oryza sativa* and research on inhibition of the inhibitor RIXI. M.S. Dissertation, Zhejiang University, China (English abstract).

Mclauchlan, W. R., Garcia-Conesa, M. T., Williamson, G., Roza, M., Ravestein, P., & Maat, J. (1999). A novel class of protein from wheat which inhibits xylanases. *Biochemical Journal, 338*, 441–446.

Miller, G. L., Blum, R., Glennom, W. E., & Burton, A. L. (1959). Measurement of methods for assay of xylanase activity. *Analytical Biochemistry, 2*, 127–132.

Misas-Villamil, J. C., & van der Hoorn, R. A. L. (2008). Enzyme-inhibitor interactions at the plant-pathogen interface. *Current Opinion in Plant Biology, 11*, 380–388.

Sels, J., Mathys, J., De Coninck, B. M., Cammue, B. P., & De Bolle, M. F. (2008). Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiology and Biochemistry, 46*, 941–950.

Sexton, A. C., & Howlett, B. J. (2006). Parallels in fungal pathogenesis on plant and animal hosts. *Eukaryotic Cell, 5*, 1941–1949.

Simpson, D. J., Fincher, G. B., Huang, A. H. C., & Cameron-Mills, V. (2003). Structure and function of cereal and related higher plant (1→4)-β-xylan endohydrolases. *Journal of Cereal Science, 37*, 111–127.

Sun, J.Y. (2003) Construction and expression of hybrid gene encoding thermostable xylanase and property of hybrid enzyme. PhD Dissertation, Zhejiang University, China (English abstract).

Sun, J. Y., Liu, M. Q., Weng, X. Y., Qian, L. C., & Gu, S. H. (2007). Expression of recombinant Thermomonospora fusca xylanase A in Pichia pastoris and xylooligosaccharides released from xylans by it. *Food Chemistry, 104*, 1055–1064.

Takahashi-Ando, N., Inaba, M., Ohsato, S., Igawa, T., Usami, R., & Kimura, M. (2007). Identification of multiple highly similar XIP-type xylanase inhibitor genes in hexaploid wheat. *Biochemical and Biophysical Research Communications, 360*, 880–884.

Tokunaga, T., & Esaka, M. (2007). Induction of a novel XIP-type xylanase inhibitor by external ascorbic acid treatment and differential expression of XIP-family genes in rice. *Plant and Cell Physiology, 48*, 700–714.

Tokunaga, T., Miyata, Y., Fujikawa, Y., & Esaka, M. (2008). RNAi-mediated knockdown of the XIP-type endoxylanase inhibitor gene, OsXIP, has no effect on grain development and germination in rice. *Plant and Cell Physiology, 49*, 1122–1127.

Vasconcelos, E. A., Santana, C. G., Godoy, C. V., Seixas, C. D., Silva, M. S., Moreira, L. R., et al. (2011). A new chitinase-like xylanase inhibitor protein (XIP) from coffee (Coffea arabica) affects Soybean Asian rust (Phakopsora pachyrhizi) spore germination. *BMC Biotechnology, 11*, 14.

Weng, X. Y., Huang, Y. Y., Hou, C. X., & Jiang, D. A. (2013). Effects of an exogenous xylanase gene expression on the growth of transgenic rice and the expression level of endogenous xylanase inhibitor gene RIXI. *Journal of the Science of Food and Agriculture, 93*, 173–179.

Weng, X. Y., & Sun, J. Y. (2005). Construction, expression, and characterization of a thermostable xylanase. *Current Microbiology, 51*, 188–192.