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Expression and characteristics of rice xylanase inhibitor OsXIP, a member of a new class of antifungal proteins

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Abstract

It has been hypothesized that xylanase inhibitors play important roles in plant defense against microbial pathogens. Currently, there is little information available about xylanase inhibitor OsXIP in rice and its gene expression. We cloned a xylanase inhibitor gene *OsXIP* from rice (*Oryza sativa* L. cv. Nipponbare) genomic DNA. To determine the function of OsXIP, we generated *OsXIP*-overexpressing transgenic rice plants. The transgenic plants had significantly higher *OsXIP* expression and showed enhanced defense response to *Magnaporthe oryzae* compared to the wild-type plants. The results also showed that the increased *OsXIP* expression was accompanied by the up-regulation of pathogenesis-related genes. To clarify the *OsXIP* expression pattern, a *ProOsXIP::GUS* vector was constructed and transgenic plants were obtained. GUS staining results revealed that *OsXIP* showed organ-specific expressions in rice plants. *OsXIP* was primarily expressed in the roots and in the veins, but it was weakly expressed in the leaves. Analyses of the *OsXIP* expression in response to biotic and abiotic stresses indicated that it was drastically induced by biotic stresses and methyl jasmonate treatment. OsXIP, a member of a new class of antifungal proteins, may function as a barrier that prevents the cell wall degradation by xylanases excreted by fungal pathogens. The *OsXIP* was found to be a stress-responsive gene and it could take part in plant defense *via* a JA-mediated signaling pathway.

Additional key words: biotic stress, gene expression, *Magnaporthe oryzae*, methyl jasmonate, *Oryza sativa*.

Introduction

Xylanase inhibitors (XIP) inhibit endo-β-1,4-xylanase activity, which degrades plant cell walls. Plant cell wall is the first barrier against pathogen infection. When pathogens start degrading the plant cell wall components, plants are able to sense the loss of cell wall integrity and activate the defense signaling pathways (Bellincampi *et al.* 2014). Xylan is the major hemicellulose polymer in cell wall of cereals. To counteract xylan degradation by microbial endoxylanases, graminaceous monocots produce xylanase inhibitors (Moscetti *et al.* 2015). Previous reports have suggested that xylanase inhibitors protect plants from the unwanted hydrolytic effects of endogenous enzymes and from infecting micro-organisms (Beliën *et al.* 2007, Dornez *et al.* 2010). To date, three types of xylanase inhibitors have been identified in cereals: XIP (xylanase inhibitor protein)-type, TAXI (*Triticum aestivum* xylanase inhibitor)-type, and TLXI (thaumatin-like xylanase inhibitor)-type (Debyser *et al.* 1999, McLauchlan *et al.* 1999, Fierens *et al.* 2007). A number of studies have shown that different classes of xylanase inhibitors exhibit different effectiveness towards various xylanases (Goesaert *et al.* 2004, Fierens *et al.* 2007, Misas-Villamil and Van der Hoorn 2008, Tundo *et al.* 2015). Xylanases from *Aspergillus niger* and

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Abbreviations: GFP - green fluorescent protein; *GUS* - β-glucuronidase gene; IPTG - β-D-thiogalactoside; MeJA - methyl jasmonate; OsXIP - rice xylanase inhibitor; PR - pathogenesis-related; qPCR - quantitative polymerase chain reaction; WT - wild type..

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Bacillus subtilis could be inhibited by TAXI-I (Fierens *et al.* 2003). Wheat xylanase inhibitors XIP-R1 and TAXI-I are also expressed in *Escherichia coli*, and wheat xylanase inhibitor XIP-R1 inhibits xylanases from *Thermomyces lanuginosus*, *Trichoderma longibrachiatum*, *Trichoderma viride, Aureobasidium pullulans*, and *Aspergillus niger* (Takahashi-Ando *et al.* 2007). Vasconcelos *et al.* (2011) reported that a chitinase-like XIP from *Coffea arabica* inhibits the germination of soybean Asian rust (*Phakopsora pachyrhizi*) spores. Tokunaga and Esaka (2007) revealed the existence of rice *XIP*-family genes.

Large economic losses may occur when crops succumb to fungal pathogen invasion. However, very few antifungal proteins have been reported to be xylanase inhibitors (Peng *et al.* 2013). Xylanase inhibitors are of a special significance because they resist pathogenic fungi in crops or they may affect the efficiency of microbial xylanases in biotechnological applications, such as cereal processing. Xylanase inhibitors constitute a new class of antifungal proteins, but there is currently little available information about xylanase inhibitors and xylanase inhibitor gene expression.

The aim of this study was to elucidate expression and function of *OsXIP*. A xylanase inhibitor gene *OsXIP* from rice was cloned and expressed in *Escherichia coli*. The xylanase inhibition specificity of the recombinant *OsXIP* and the biochemical properties of this protein were also examined. The *OsXIP* overexpressing transgenic rice plants were generated and defense responses to *Magnaporthe oryzae* including expression of pathogenesis-related (*PR*) genes were determined in T_1 plants. Additionally, we constructed a *Pro OsXIP::GUS* vector and used transgenic rice lines to clarify the expression pattern of *OsXIP* and to investigate how OsXIP responded to stresses and stress-related phytohormones.

Materials and methods

Plasmids, chemicals, and strains: Cloning host strain *Escherichia coli* DH5α, expressing host strain *E. coli* BL21, and expressing vector pET-30a(+) were all purchased from *Invitrogen* (Carlsbad, USA); the pMD19T vector was obtained from *Takara* (Tokyo, Japan); birchwood xylan was purchased from *Sigma* (St. Louis, MO, USA); and *Thermomyces lanuginosus* and *Trichoderma longibrachiatum* xylanases were purchased from *Sigma-Aldrich* (St. Louis, MO, USA).

Plants, growth conditions, and treatments: The rice (*Oryza sativa* L. cv. Nipponbare) wild-type (WT) and its transgenic lines were used. The seeds were germinated in water and then grown in a culture solution according to Wang *et al.* (2009) for 14 d in a growth chamber at a 14-h photoperiod, an irradiance of 1000 μmol m^{-2} s^{-1}, day/night temperatures of 28/18 °C, and a relative humidity ranged between 76 and 94 %. For the expression pattern analysis, the various tissues of seedlings were collected at different stages during the growth period. These seedlings were also used for pathogen inoculation and methyl jasmonate (MeJA) treatments. The agronomic traits were measured at the plant maturity stage using plants that had been cultivated in an experimental field.

The fungal isolate used in the study was *Magnaporthe oryzae* strain KJ201, provided by the College of Agriculture and Biotechnology, Zhejiang University, which was virulent towards the rice *japonica* cultivars. Two-week-old rice seedlings were sprayed with *M. oryzae* at a concentration of 1.5×10^5 (spores) cm^{-3} containing 0.05 % (v/v) *Tween-20* or with sterile water containing 0.05 % *Tween-20*, which served as a control (Lee *et al.* 2001). The inoculated plants were moved to a dew chamber set at 28 °C for 36 h and then were returned to a growth chamber. The leaf tissues were harvested at 0, 1, 2, and 3 d post-inoculation.

To analyze the *OsXIP* expression in the response to biotic and abiotic stresses, the β-glucuronidase (GUS) activities in the roots and shoots were measured in two-week-old rice seedlings that had been subjected to 0.2 mM MeJA, *Nilaparvata lugens*, wounding, low temperature (8 °C), or distilled water (control).

Molecular cloning: The rice genomic DNA was extracted according to the manufacturer's instructions (*Invitrogen*). The *OsXIP* sequence was amplified by PCR using rice genomic DNA as a template with primers 5′-ATGGCGCTTCGACGCCT′-3 (forward) and 5′-TCACAGGTGAGCCGTGAA′-3 (reverse), which linked with the *EcoR* I and *Xho* I sites, respectively. The PCR program consisted of preheating at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by extension at 72 °C for 10 min. A DNA fragment of approximately 900 bp was generated and cloned into the vector pMD19T. The clone that contained the resulting PCR product was transformed into *E. coli* strain DH5α.

Expression of OsXIP protein in *Escherichia coli*: The DNA sequence was amplified with primers from the pMD19T-OsXIP plasmid to obtain recombinant OsXIP protein. The amplified PCR product was digested and inserted into the pET-30a (+) vector (*Novagen*, Gibbstown, USA) at the *Eco*RI and *Xho*I sites. This expression vector was named pET30a-*OsXIP*. Then, pET30a-*OsXIP* was introduced into *E. coli* BL21. The transformants were screened on Luria-Bertani (LB) plates with 100 μg cm^{-3} kanamycin. The positive transformants were cultured in a liquid LB medium with 50 µg cm^{-3} kanamycin at 37 °C for 12 h and then induced with β-D-thiogalactoside (IPTG; 0.1 mM final concentration) for 8 h. The cells were harvested by centrifugation (10 000 *g*, 4 °C, 10 min), resuspended in McIlvaine's buffer (0.1 M citric acid, 0.2 M Na_2HPO_4; pH 6.0), sonicated, and centrifuged (15 000 *g*, 4 °C, 10 min). Electrophoresis was performed on 12.5 % (m/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The OsXIP fusion protein expressed by *E. coli* was digested with enterokinase after it had been purified using an *Ni-NTA Spin* kit (*Qiagen*, Hilden, Germany), according to the manufacturer's instructions. Then, the xylanase inhibitor activity was measured. The protein content was determined according to Bradford (1976). N-terminal protein sequencing was performed on an 492cLC protein sequencer (*Applied Biosystems*, Foster City, USA).

Vector construction and rice transformation: The method used to construct the transgenic vector (pTCK303-*OsXIP*) is shown in Fig. 1. The complete *OsXIP* coding sequence was amplified from the pMD19T-*OsXIP* plasmid with a pair of specific primers containing *Kpn*I or *Sac*I restriction sites (forward primer: 5′-GGGGTACCTCGCCGACGTCCCAGAAC-3′; reverse primer: 5′-CGAGCTCTTACGCTCGCAGGTGAGC CGTGAAG-3′). Then the PCR fragment was ligated to the pTCK303 vector, which contains the UBi-1 promoter, the reporter gene *GUS* (encoding β-glucuronidase), and the resistance gene hygromycin. The sequence from the -2122 to -1 fragments upstream of *OsXIP* in the promoter:*GUS* construct was amplified from the rice genomic DNA by PCR. The fragments were then introduced in front of the *GUS* reporter gene in the PBI101GUS-plus vector (a derivative of PBI101.3 that carries the GUS plus sequence).

The *OsXIP*-over and *Pro OsXIP*::*GUS* constructs were introduced into *Agrobacterium tumefaciens* L. strain EHA105 by electroporation. Subsequent transformations were carried out using calli derived from the mature seeds of *Oryza sativa* cv. Nipponbare as described previously (Hiei *et al.* 1994, Weng *et al.* 2013). The transformed calli were selected on hygromycin-containing medium. The transgenic rice lines that had been transformed were recorded as T_0. Their seeds were harvested from the panicles and the second generation was recorded as T_1. The transgenic rice T_1 lines were then used for further physiological and biochemical research.

Xylanase inhibitor activity: Xylanase inhibitor activity was determined using the 3,5-dinitrosalicylic acid method (Miller 1959). Xylanase inhibitor activity was determined by measuring the activity of different xylanases in the presence and absence of recombinant OsXIP according to the method of Tokunaga and Esaka (2007). Xylanase inhibition activities were determined in triplicate. The *Thermomyces lanuginosus* and *Trichoderma longibrachiatum* xylanases were purchased from *Sigma-Aldrich*. The extraction of proteins and determination of xylanase inhibitor activity in rice followed the method as described by Elliott *et al*. (2003).

RNA isolation, cDNA synthesis, and real-time quantitative PCR: Total RNA was isolated from the shoots and roots of rice (WT and transgenic seedlings) that had been subjected to different treatments using the *miniBEST* universal RNA extraction kit (*Takara*) according to the manufacturer's protocol. The cDNA was prepared using the *PrimeScript* first-strand cDNA synthesis kit (*Takara*). The real time qPCR was performed on a *Light Cycler480* (*Roche*, Rotkreuz, Switzerland) using a *SYBR*® *Premix Ex TaqTM* kit (*Takara*). The rice actin gene *Osactin* (GenBank: AK101613.1) was selected as an internal standard. The qPCR assays for all the genes were performed as previously described (Weng *et al.* 2013). The protocol consisted of 35 cycles of 95 °C for 5 s and 60 °C for 30 s. The gene-specific primers are listed in Table 1 Suppl.

GUS staining and quantitative analysis: Plant tissues and organs were collected and stained in X-glucuronide dissolved in 10 mM EDTA, 1 mM potassium hexacyanoferrate, 0.1 % (m/v) *Triton X-100*, and 100 mM phosphate buffer at pH 7.0 (at 37 °C), followed by 95 % (v/v) ethanol cleaning. The various tissues were observed with a *Carl Zeiss LSM510* laser scanning system (http://www. zeiss.com/). Quantitative GUS activity was measured using the method described by Jefferson *et al.* (1987) with some modifications.

Subcellular localization: The 35S*Pro*::*OsXIP*:*GFP* expression vectors were constructed by subcloning the full-length ORFs without terminators from *OsXIP* into the pCAMBIA1300-sGFP vector (Zhou *et al.* 2009). The resulting construct was transformed into *Agrobacterium tumefaciens* strain EHA105 and then introduced into *Nicotiana benthamiana* Domin leaves (Wroblewski *et al.* 2005). The leaves were observed with a confocal laser scanning fluorescence microscope (*LSM 710*, *Carl Zeiss AG*, Germany).

Statistical analysis: The enzyme activities were determined from three independent experiments. Analysis of variance was performed on the data, and significant differences from the control values were calculated by Duncan's multiple range test.

Results

The gene encoding OsXIP localized on chromosome group 11 was isolated from rice genomic DNA. The *OsXIP* coding sequence was cloned into expression vector pET-30a(+) and placed in a frame with an N-terminal extension that included a His-tag. The expressed protein was a fusion protein with 71 additional amino acids at the N-terminus of the vector. Protein expression in *E. coli* BL21 was induced by IPTG after 8 h. A clear band with a molecular mass of about 35 kDa was determined by SDS-PAGE analysis of the obtained fraction. The xylanase inhibitor activity toward xylanases from *T. lanuginosus* and *T. longibrachiatum* (Fig. 2*A*,*B*) indicated that the recombinant OsXIP had been purified.

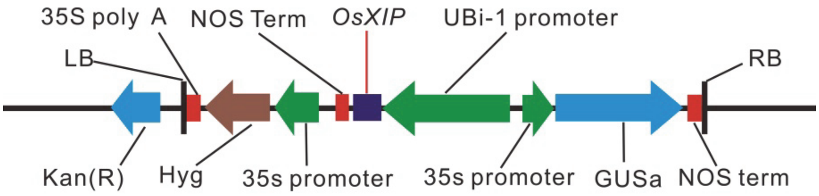

Fig. 1. The strategy for the construction of *OsXIP*-overexpressing vector.

Agrobacterium-mediated transformation produced 232 T_0 plants overexpressing *OsXIP* construct. The independent transgenic rice lines were selected by PCR analysis using gene-specific primers for the hygromycin resistance gene. Southern blot analysis was used to further confirm the positive transformants and estimate the copy number of the integrated transgene in the positive transformants (Fig. 1 Suppl.). The results showed that at least four independent lines had higher *OsXIP* mRNA induction than the WT plants. The *OsXIP* expression in T_1 plants (14-d-old seedlings) were analyzed by real time qPCR, and the *OsXIP* expressions in transgenic lines Os-2 and Os-8 (with single copy integration) were 52.8 and 56.8 times higher than in the WT plants, respectively (Fig. 3*B*). So the Os-2 and Os-8 lines were chosen for further physiological and biochemical research.

The pH and temperature optima for xylanase inhibitor activity in transgenic rice were analyzed using xylanases from *T. lanuginosus* as the target enzymes. The optimal pH for xylanase inhibitor activity in the transgenic lines was similar, with activity peaking at pH 5.0. Furthermore, there was 80 % activity between pH 4.0 and 6.0. The optimal temperature for OsXIP protein xylanase

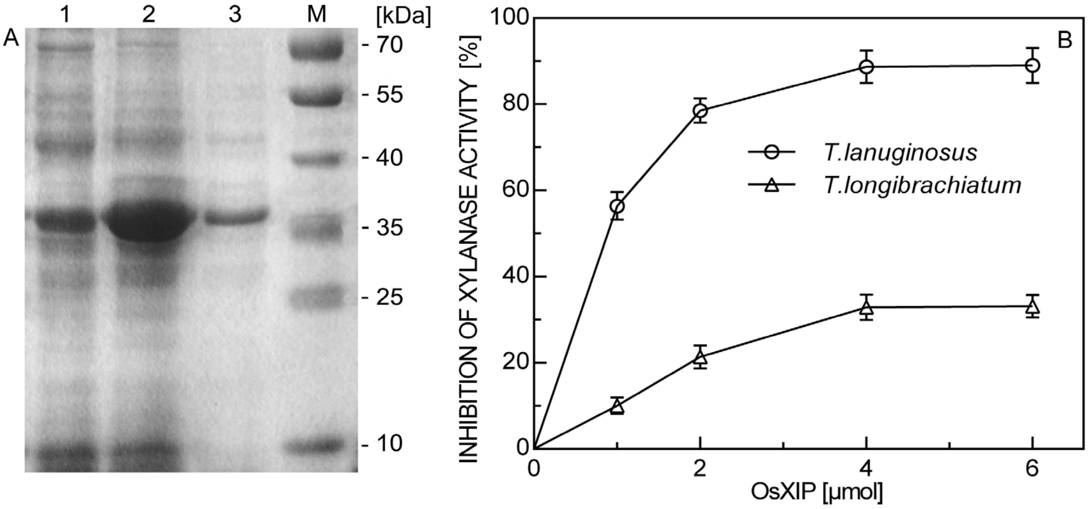

Fig. 2. SDS-PAGE and xylanase inhibitor activity of the recombinant *OsXIP* expressed in *Escherichia coli* BL21. *A* - SDS-PAGE of the protein fraction obtained from strains of *E. coli* BL21; the protein was stained with Coomassie brilliant blue. M - marker of proteins, *lane* 1 - crude protein extracts from control strain of *E. coli* containing pET30a-*OsXIP* vector, *lane* 2 - crude protein extracts from *E. coli* containing pET30a-*OsXIP* vector induced by IPTG after 8 h, *lane* 3 - purified recombinant OsXIP expressed in *Escherichia coli* BL21. *B* - Xylanase inhibitor activity of recombinant inhibitor protein OsXIP. Xylanases were from *Thermomyces lanuginosus* and *Trichoderma longibrachianum*. The data represent means ± SDs of three independent replicates.

inhibition activity in the overexpressing transgenic rice and recombinant *OsXIP* lines was measured (Fig. 3*A*). The results showed that their inhibition activities were similar. It was rather low at 20 - 40 °C, but then rapidly increased and reached a maximum at 60 °C.

The phenotype parameters were measured at plant maturity. The transgenic lines appeared to grow and develop normally. There were no significant differences in plant height, panicle length, and tiller and panicle numbers compared to non-transgenic (WT) plants (data not shown).

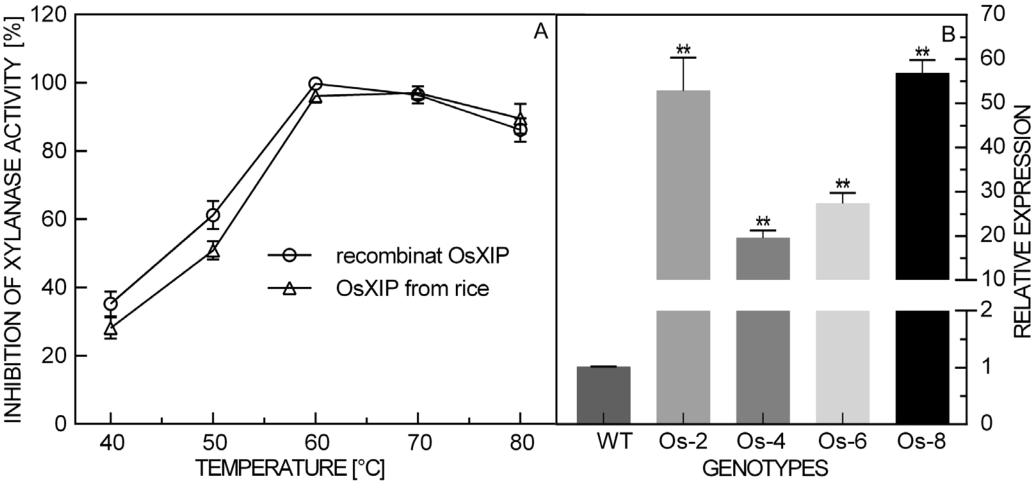

Fig. 3. Recombinant protein OsXIP and *OsXIP* gene expressed in transgenic plants. *A* - Effect of temperature on xylanase inhibitor activity. *B* - Relative expression of *OsXIP* mRNA in wild type (WT) and overexpressing transgenic plants (Os-2, Os-4, Os-6, and Os-8). The data represent means ± SDs of three independent replicates. In *B asterisks* indicate statistically significant differences compared with wild type (WT) at $P < 0.01$.

During pathogen infection, the transcription of the *OsXIP* gene in *OsXIP*-overexpressing transgenic plants increased, and the disease severity measured on the transgenic lines after infection with *Magnaporthe oryzae* KJ201 was significantly less than on the non-transgenic (WT) plants (Fig. 4*C*). The lesion number per leaf and the lesion size on the transgenic plants were significantly reduced (Fig. 4*A*,*B*). The transcription of the *Pyricularia oryzae* resistance-d2 (*Pid*2) gene was also measured. Compared with the WT, both the Os-2 and Os-8 plants showed an increase in the expression of *Pid*2 gene at 3 d after inoculation (Fig. 4*D*). The expressions of three pathogenesis-related protein (*PR*) genes in *OsXIP*-overexpressing plants (Os-2 and Os-8) were also examined. After infection with *M. oryzae* KJ201, the expression of *OsPR1* (PR protein1), *OsPR1b* (basic PR protein1), and *PR4* in the transgenic plants significantly increased compared to the WT plants (Fig. 4*D*).

The subcellular localization of the OsXIP protein was determined so that its role could be evaluated. We constructed a *35SPro*::*OsXIP*::*GFP* fusion gene and transiently expressed the constructs in *Nicotiana benthamiana* leaves. Confocal laser scanning fluorescence microscopy revealed that GFP fluorescence from the fusion protein was restricted to the outer parts of leaf cells (Fig. 5). The result strongly suggested that OsXIP might be located in the apoplastic space.

In order to investigate the expression pattern of the *OsXIP* gene, upstream DNA from the *OsXIP* gene (-2122 bp) in WT was PCR-amplified using specific primers and fused with the GUS reporter gene. This chimeric gene cassette was introduced into the WT plants *via Agrobacterium* mediated transformation. Histochemical staining for GUS activity in T_1 plants showed that *OsXIP* was expressed in the leaves of young seedlings, but was weakly expressed in leaves at the flowering stage (Fig. 6*E*,*F*). *OsXIP* was primarily expressed in the roots and the lemma, but not in the stamens (Fig. 6*A*,*B*,*C*,*D*). These results suggest that the *OsXIP* expression was tissue- and growth stage-specific.

Further, two-week-old seedlings were subjected to 0.2 mM MeJA, 200 mM NaCl, *Nilaparvata lugens* infection, wounding, low temperature (8 °C), or distilled water as control. Then, the GUS activities in the roots and shoots were measured (Fig. 7*A*,*B*). The responses of *OsXIP* to stress and stress-related phytohormones were studied by quantifying the GUS expression in the shoot and root tissues of the transgenic rice plants. The results showed that *OsXIP* expressions were considerably induced by *N. lugens*, wounding, and MeJA, and weakly induced by low temperature and NaCl. Furthermore, *OsXIP* expression in the roots was significantly higher than in the leaves. The *N. lugens* induction process was very similar to that caused by wounding. These results suggested that OsXIP protein induction by *N. lugens* might occur *via* a wounding-mediated pathway.

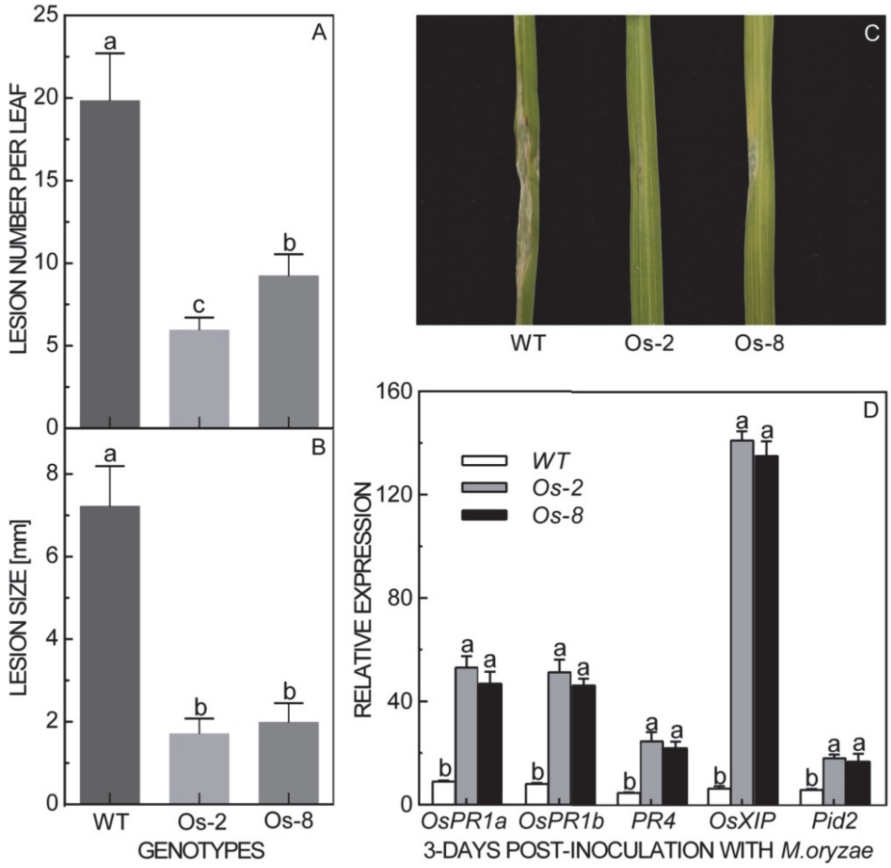

Fig. 4. The responses of *OsXIP*-overexpressing transgenic plants to *Magnaporthe oryzae* KJ201. *A* - Lesion number per leaf after inoculation with *M. oryzae* KJ201. *B* - Lesion size after inoculation with *M. oryzae*. *C* - Rice blast symptoms on WT and *OsXIP*-overexpression Os-2 and Os-8 plants infected with *M. oryzae*. *D* - Relative expression of *OsXIP* and *PR* genes (*OsPR1a*, *OsPR1b*, *PR4*, and *Pid2*) in WT, Os-2, and Os-8 plants at 3 d post-inoculation with *M. oryzae*. The data represent means ± SDs of three independent replicates; *bars* with different letters are significantly different at 5 % level.

Discussion

In this study, we cloned a xylanase inhibitor gene encoding OsXIP from rice genomic DNA and expressed it in *Escherichia coli* (Fig. 2*A*). Recombinant OsXIP protein was active against xylanases from *T. lanuginosus* and *T. longibrachiatum* (Fig. 2*B*), which was consistent with a previous report that suggested that XIP-type xylanase inhibitors inhibit fungal xylanases (Flatman *et al.* 2002). Xylanase inhibitors are a new class of antifungal proteins and may function as a barrier that prevents cell wall degradation by xylanases from fungal pathogens (Tokunaga and Esaka 2007, Moscetti *et al.* 2013). Because of high recognition specificity between the different xylanase inhibitors and microbial xylanases, XIP- and TAXI-type xylanase inhibitors exhibit different effectiveness towards various microbial xylanases (Goesaert *et al.* 2004, Dornez *et al.* 2010, Tundo *et al.* 2015). Xylanase inhibitors, reducing the activity of microbial xylanases produced by pathogens, could play an important role in plant defense. Tundo *et al.* (2016) reported that a combined expression of multiple inhibitors in a single wheat genotype could reinforce the cell-wall barrier. They also revealed that pyramiding polygalacturonase inhibiting protein and a xylanase inhibitor TAXI-III improve plant resistance to *Fusarium* head blight. This improved resistance is likely due to synergistic or additive effects of different types of inhibitors on activities of cell wall degrading enzymes.

A more direct evidence of their involvement in plant defense has been reported by using transgenic plants. Moscetti *et al.* (2013) reported wheat transgenic plants overexpressing xylanase inhibitor gene *TAXI-III* could limit *Fusarium graminearum* infection. By loss-of-function analysis Tokunaga *et al.* (2008) demonstrated that OsXIP functions in defense against phytopathogens, but it is not involved in rice development *per se*. In our experiment, *OsXIP*-overexpressing transgenic rice plants were generated and the disease severity measured on the transgenic lines was less than in the WT plants after

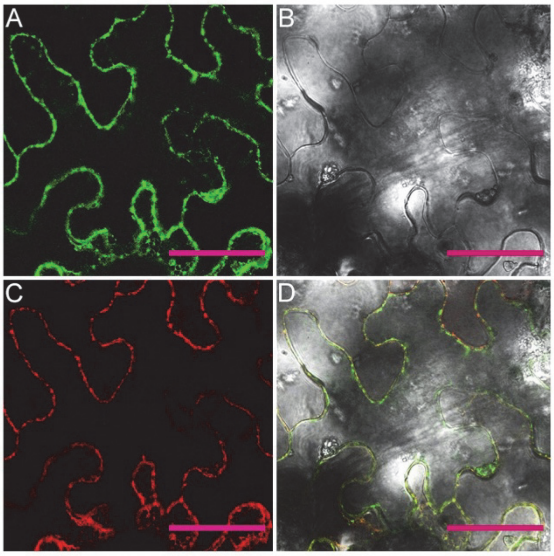

Fig. 5. Subcellular localization of OsXIP in *Nicotiana benthamiana* cells transformed with 35S*Pro*::*OsXIP*:*GFP*. After incubating for 48 h, the transformed cells were observed under a confocal microscope. The photographs were taken for detecting GFP fluorescence (*A*), under bright field (*B*), and in combination (merge; *C*), respectively. Empty vector (pCAMBIA1300-*GFP*) was used as a control (*D*). *Bars* 10 µm.

infection with *Magnaporthe oryzae* (Fig. 4). *OsXIP*-overexpressing transgenic plants displayed higher expressions of disease-resistant genes (*Pid2*), which may imply higher resistance. The *Pid2* gene is a disease-resistance gene with strong rice blast resistance and a broad resistance spectrum (Chen *et al*. 2006). We further investigated the mechanism of *OsXIP* mediated resistance by analyzing the expression of *PR* genes in the transgenic lines after *M. oryzae* infection. PR proteins were produced in response to pathogen attacks, as a part of systemic acquired resistance. Expressions of *PR* genes, such as *OsPR1a, OsPR1b*, and *PR4,* were significantly enhanced in the *OsXIP*-overexpressing plants (Fig. 4*D*), suggesting that rice xylanase inhibitor OsXIP might alleviate symptoms of blast disease by increasing the expressions of *PR* genes.

In cereals, xylanase inhibitors are encoded by a multigene family. Differential expression of individual genes has been demonstrated in various plants in response to environment cues. For example, the expressions of xylanase inhibitors Taxi-Ib/III and Taxi-IIb/IV in wheat leaves were induced after infection by the powdery mildew fungus *Blumeria graminis* (Igawa *et al.* 2004). Expressions of *OsXIP* and two other XIP-type xylanase inhibitors, *riceXIP* and *RIXI*, were

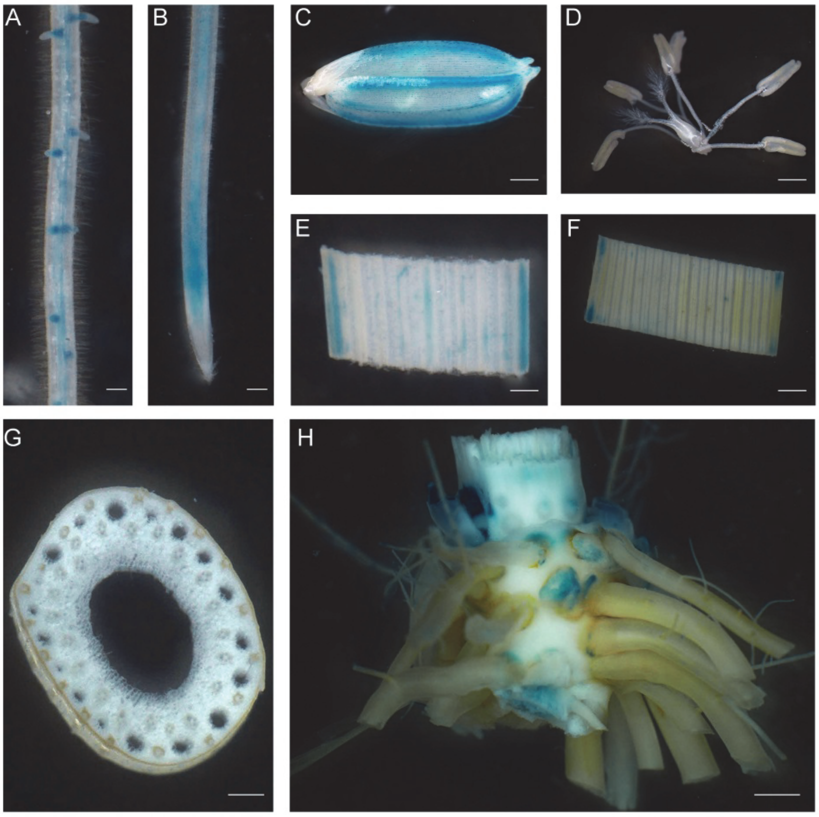

Fig. 6. Histochemical staining analysis of GUS activity in *ProRIXI*::*GUS* transgenic rice plants: *A* - lateral root, *B* - maturation, elongation, meristematic region, and root cap of primary root, *C* - glume, *D* - flower, *E* - young leaf, *F* - leaf in flowering stage, *G* - stem, *H* - root primordum. *Bars* - 100 µm (*A*, *B*, *E*, *F*, 500 µm (*C*, *D*, *G*), or 2 mm (*H*).

detected in any organ during germination of rice seeds (Tokunaga and Esaka 2007). By Northern blot analysis, *OsXIP* and *riceXIP* expression were drastically induced by wounding and MeJA treatment in the rice root (Tokunaga and Esaka 2007). Our previous study revealed that pathogens could induce the expression of *RIXI* (Hou *et al.* 2015). In this study, we showed that in transgenic lines, *Pro OsXIP::GUS* was expressed in grains and other parts (Fig. 6). The expression of *OsXIP* in healthy roots and leaves of young seedlings was consistent with its function as a pre-existing defense barrier in plant tissues that are vulnerable to pathogen attack (Croes *et al.* 2009).

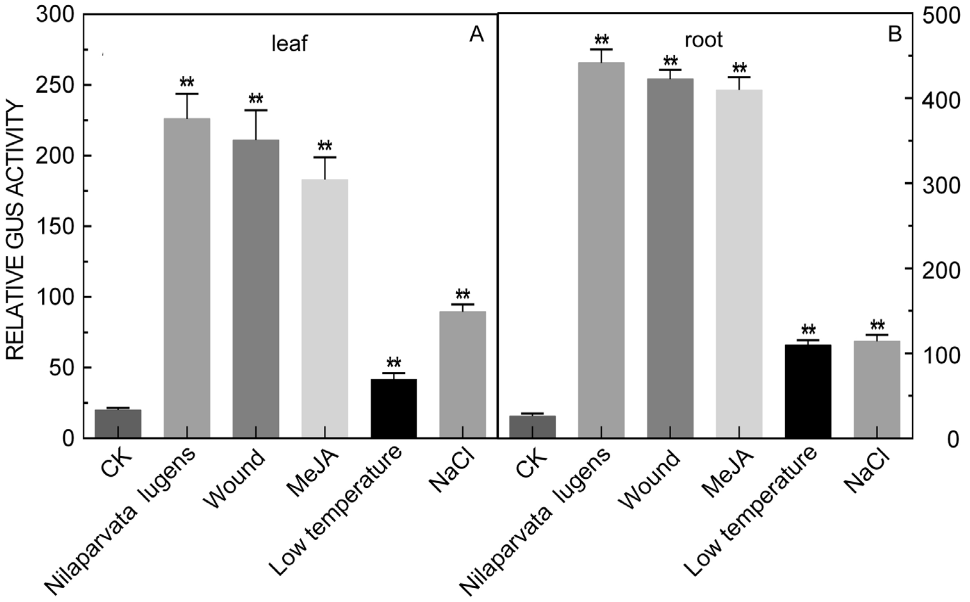

Fig. 7. GUS detection by quantitative fluorescence in leaf (*A*) and root (*B*) of *ProRIXI*::*GUS* transgenic rice plants. The data represent means ± SDs of three independent replicates. *Asterisks* indicate statistically significant differences between the treated and control plants at $P < 0.01$.

The promoter region of a gene could provide valuable information about the factors regulating its expression. Several biotic and abiotic stress-responsive elements have been identified by a comparative analysis of *cis*-elements in the xylanase inhibitor gene promoters. We identified consensus *cis*-acting elements implicated in pathogen- and wound-inducible gene expression, *i.e.*, GCC-box and W-box sequences in the 1.9-kb sequence upstream of the coding region of *OsXIP* gene.

We used *Pro OsXIP::GUS* transgenic rice lines to investigate how *OsXIP* responds to biotic and abiotic stresses. We revealed that *N. lugens* infestation, mechanical wounding, and MeJA treatment enhanced the expression of *OsXIP*, consistent with the results observed by Tokunaga and Esaka (2007) using Northern blot analysis. Furthermore, we showed that MeJA strongly induced the *OsXIP* expression in roots and mildly in leaves (Fig. 7). Meanwhile, the pattern of induction by *N. lugens* was very similar to that by wounding. It is well known that jasmonic acid (JA) plays a key role in the regulation of the defense signaling network, which is activated when an invader is first detected (Pieterse *et al.* 2012); wounding causes a marked accumulation of JA, followed by the induction of a number of pathogenesis-related genes (Kunkel and Brooks 2002, Turner *et al.* 2002). Besides reducing the activity of microbial xylanases produced by pathogens, OsXIP in cooperation with other pathogenesis-related proteins takes part in plant defense *via* a JA-mediated signaling pathway.

In conclusion, OsXIP as a member of a new class of antifungal proteins may prevent cell wall degradation by xylanases excreted by pathogens. *OsXIP* is a stress-responsive gene and may take part in plant defense *via* a JA-mediated signaling pathway. Overexpression of *OsXIP* results in increased PR gene expression as well as enhanced defense response to *M. oryzae*.

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