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BRIEF COMMUNICATION

Characterization of a xylanase inhibitor TAXI-I from wheat

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Abstract

Xylanase inhibitor *TAXI-I* gene was cloned from wheat (*Triticum aestivum* L.) and then *TAXI-I* encoding sequence was expressed in *Escherichia coli*. The recombinant TAXI-I protein inhibited glycoside hydrolase (GH) family 11 xylanases in *Aspergillus niger* (Anx; a fungal xylanase), and *Thermomonospora fusca* (Tfx; a bacterial xylanase), and also inhibited hybrid xylanases Atx (a hybrid xylanase whose parents are *T. fusca* and *A. niger*) and Btx (a hybrid xylanase whose parents are *T. fusca* and *Bacillus subtilis*). Among the tested xylanases, *A. niger* xylanase was the most inhibited one by wheat xylanase inhibitor TAXI-I, while *T. fusca* xylanase was the least inhibited one. The profile of *TAXI-I* gene expression in wheat in response to phytohormones was also investigated. TAXI-I gene expression was drastically induced by methyl jasmonate (MeJa), and hardly detected in gibberellic acid (GA) treatment. Therefore, TAXI-I might be involved in plant defense against fungal and bacteria xylanases.

Additional key words: *Aspergillus niger, Bacillus subtilis,* plant defense, stress response, *Triticum aestivum*.

Endo-β-(1,4)-xylanases (EC 3.2.1.8, further referred to as xylanase) are key plant or microbial enzymes in the degradation of xylan, which is one of the most abundant polysaccharides in the cell wall of higher plants (Thomson 1993, Cazemier *et al.* 1999, Collins *et al.* 2002). Plant xylanases play important physiological roles in seed germination and fruit ripening (Simpson *et al.* 2003). Recently, xylanase inhibitor protein have been identified in various plants such as durum wheat, rye, barley, maize and rice (Durand *et al.* 2005, Elliott *et al.* 2003, Goesaert *et al.* 2003, 2004, 2005). 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). Two distinct classes of xylanase inhibitors, with different structures and specificities, have been reported, the TAXI-type and XIP-type inhibitors (Gebruers *et al.* 2004, Juge *et al.* 2004). The XIP-type xylanase inhibitors specifically inhibit fungal xylanase and can not inhibit xylanases of bacterial origin (Flatman *et al.* 2002, Juge *et al.* 2004, Tokunaga and Esaka 2007). The TAXI-type xylanase inhibitors can inhibit xylanases of glycoside hydrolase family 11 (GH11), but have no activity towards glycoside hydrolase family 10 (GH10) (Gebruers *et al.* 2004). Studies have revealed that a number of *TAXI*-type genes may exist in wheat (Igawa *et al.* 2005). Most studies are consistent that xylanase inhibitors may play a role in plant defense rather than in the regulation of endogenous xylanase activity. But the interaction mechanism between xylanases and TAXI-type inhibitors and the function of TAXI-type inhibitors in plant are far from being understood.

This study described the cloning of xylanase inhibitor *TAXI-I* gene from wheat and expression it in *Escherichia coli.* Additionally, xylanase inhibition specificity of the recombinant *TAXI-I* and its expression in response to phytohormones were determined.

Genomic DNA was isolated from young leaves of wheat (*Triticum aestivum* L.) cv. Zhemai 1 grown at a 14-h photoperiod, irradiance of 800 μmol m^{-2} s^{-1}, day/night temperature of 25/18 °C and relative humidity 60 - 80 %. Polymerase chain reaction (PCR) on genomic DNA was conducted with primers 5TAXI01E: (5'-CACACC CACAAACAATTCCACGCT-3') and 3TAXI01E: 5'-GGACGAATCCACCTGTCGTTAAACA-3' for amplification of the complete TAXI-I coding sequence.

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Abbreviations: GA - gibberellic acid; MeJa - methyljasmonate; PCR - polymerase chain reaction.
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PCR was conducted with preheating at 94 °C for 2 min, and with 35 cycles of 94 °C for 50 s, 63 °C for 50 s, and 72 °C for 2 min; then following at 72 °C for 10 min. PCR products were purified using the *QIAquick* PCR purification kit (*Qiagen*, Valentia, USA) and sequenced on a 377 DNA sequencer (*ABI 377-01*, *Perkin-Elmer*, USA). The nucleotides and deduced amino acid sequence homology searches were performed on *NCBI* databases by *BLAST* search. After identification, the PCR products were purified from an agarose gel and cloned into the pUCm-T Vector with the T4DNA ligase. A DNA fragment of approximately 1.2 kb 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.

The DNA sequence encoding the mature peptide of TAXI-I was amplified by using primers of A1 (5-CG^AATTCCTTCCGGTGCTCGCTCCGGTCACCA AGGACCC-3, *Eco*RI recognition site underlined) and A2 (5-CC^TCGAGAGGCCGCCGCAACCCGTAAA-3, *Xho*I recognition site underlined) from recombinant pUCm-T TAXI-I plasmid. The amplified PCR product was digested and inserted into the vector pET-30a (+) (*Novagen*, San Diego, USA) at *Eco*RI and *Xho*I sites. The pET-30(a)+TAXI-I vector was obtained and transformed into *E. coli* TOP 10 according to Sambrook *et al.* (1989). Transformants were screened on Luria-Bertani (LB) plates with 10 μg cm^{-3} kanamycin. Positive transformants were cultured in liquid LB-kanamycin (10 μg cm^{-3}) medium at 37 °C for 12 h, and then induced with isopropyl-β-D-thiogalactoside (IPTG) 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_2HPO_4, pH 6.0), sonicated, and centrifuged (15 000 *g*, 10 min, 4 °C). The soluble protein extracts were concentrated. Their concentration was determined by protein assay (*Bio-Rad Laboratories*, Hercules, USA) and adjusted to 3.4 g dm^{-3}. The same quantity of soluble protein extracts was tested for xylanase inhibitor activity. Electrophoresis was performed on 12.5 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) as described by Laemmli (1976).

The xylanase activity was measured with 1 % birch wood xylan (m/v) as substrate using the method described by Bailey *et al.* (1992). The reaction mixture (0.1 cm^3), containing 0.01 cm^3 of the diluted enzyme solution and 0.09 cm^3 of 1% birch wood xylan in McIlvaine's buffer (pH 5.0), was incubated at 30 °C for 5 min. The liberation of reducing sugars was estimated by the dinitrosalicylic acid (DNS) method, and *D*-xylose was used as a standard (Miller *et al.* 1959). One unit of xylanase activity was defined as the amount of protein that released 1 mmol xylan per min at 30 °C and pH 5.0. Xylanase inhibitor activity was determined by measuring the activity of GH11 xylanase from *Aspergillus niger* in the presence and absence of recombinant xylanase inhibitor TAXI-I. The activity of recombinant xylanase inhibitor was defined as the relative percentage of endo-β-1,4-xylanase activity after addition of inhibitor. Xylanase inhibition was determined in triplicate.

Eight-day-old wheat plants were steeped in methyl jasmonate (MeJA, 100 μM), gibberellin (GA, 100 μM), and in water as a control. After 48 h treatment, total RNA of leaves was isolated using *Trizol*™ reagent (*Gibco BRL*, *Life Technologies*, Gaithersburg, MD, USA) according to the manufacturer s instruction. cDNA (Genebank No. AJ438880) was prepared as described by Igawa *et al.* (2004). The plasmid DNA was introduced into *E. coli*, and isolated with high pure kit. TAXI-I fragment was released by enzyme digestion and labeled with α-^{32}P-dCTP. Total RNA was separated on formaldehyde agarose gel, blotted on nylon membrane. Pre-hybridization and hybridization were performed with the standard procedure (Sambrook *et al.* 1989). The blots were exposed to X-ray film at -70 °C for 24 h.

The complete nucleotide coding sequence from wheat TAXI-I is shown in Fig. 1. Nucleotide sequence analysis showed its nucleotide sequence consisted of 1284 bp, which contained an open reading frame (ORF) from 42 to 1250 bp and encoded a peptide of 403 amino acids (Fig. 1). The base composition of the TAXI-I coding sequence was G/C-rich (68 %). The open reading frame encoded a signal peptide of 21 amino acids followed by a mature protein of 381 amino acids (http://www.cbs.dtu.dk/services/SignalP/). In addition, a potential N-glycosylation site was situated at Asn-105 (http://www.cbs.dtu.dk/services/NetNGlyc/).

In order to clarify characteristics of TAXI-I, we attempted to measure the xylanase inhibitor activity of recombinant TAXI-I expressed in *E. coli*. A full-length TAXI-I DNA, in which the signal peptide-encoded region was removed, was amplified using primers A1 and A2, and cloned into expression vector pET-30a (+) to give the expression construct pET-30a (+) TAXI-I. Protein expression in *E. coli* was induced by IPTG. The expressed protein lacked the signal peptide and was a fusion protein with 71 amino acid residues from pET30a (+) vector. An approximately 45 kDa protein was specifically detected in soluble protein extract from a culture of *E. coli* transformed with pET-30a (+)TAXI-I, consistent with the size of the recombinant TAXI-I protein containing several additional amino acid residues from pET30a (+)vector (Fig. 2). This indicated that recombinant TAXI-I protein was expressed in the transformed *E. coli.*

Soluble protein extracts were concentrated, purified, and used to evaluate its properties. To determine the xylanase specificity of TAXI-I, its inhibition activities against several microbial xylanases of GH11 and GH10 were analyzed. The results showed that TAXI-I inhibited GH family 11 xylanases of *A. niger* (Anx), *T. fusca* (Tfx), and also inhibited hybrid xylanases Atx (Atx; a hybrid xylanase whose parents are *T. fusca* xylanase and *A. niger* xylanase) and Btx (Btx; a hybrid xylanase whose parents are *T. fusca* xylanase and *B. subtilis* xylanase), which were constructed by our laboratory (Sun *et al.* 2005, 2007, Weng *et al.* 2005); while GH family 10 xylanases of *A. aculeatus* were not inhibited. Under the experimental

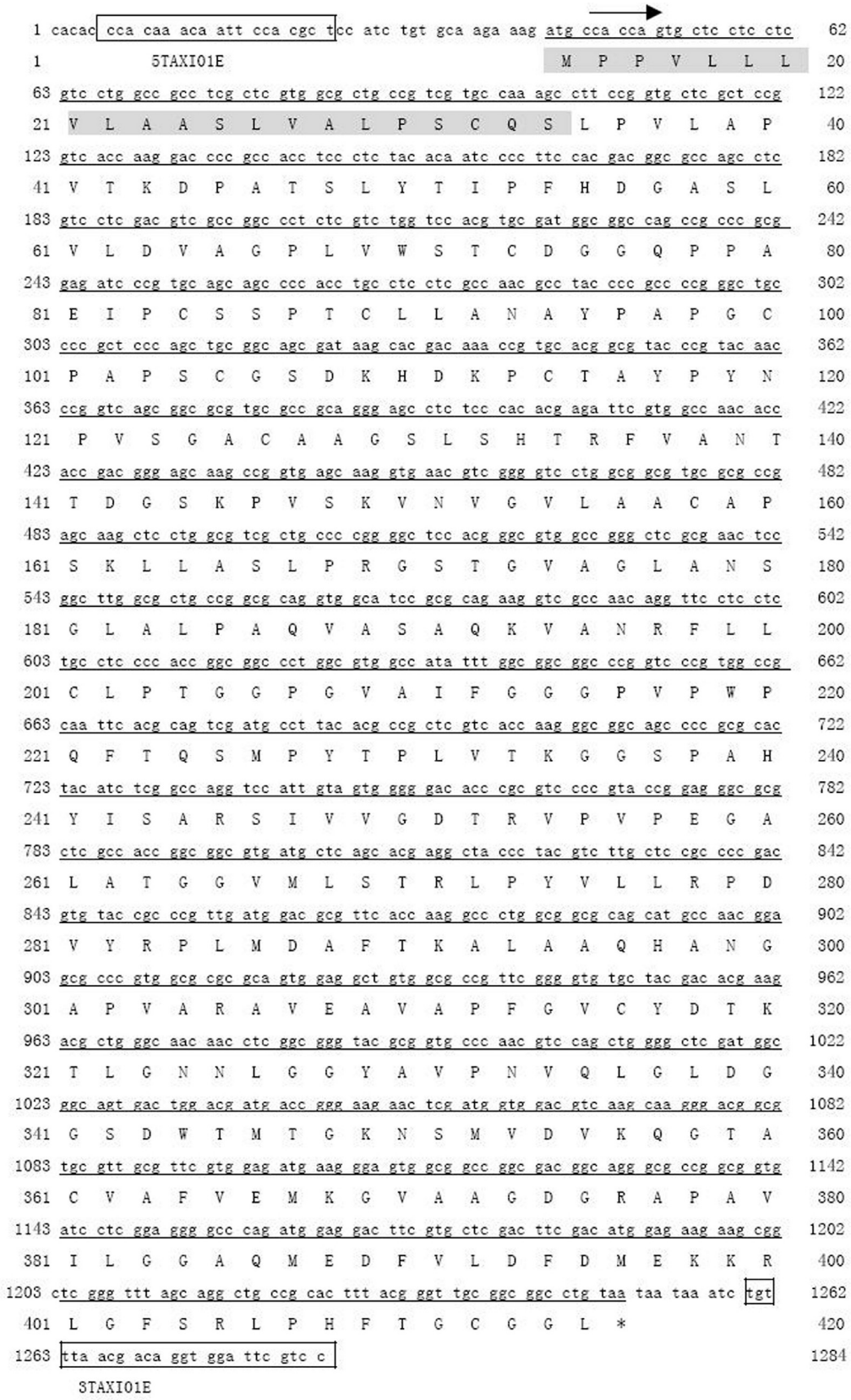

Fig. 1. Nucleotide and amino acid sequences of wheat xylanase inhibitor TAXI-I.

conditions with the same inhibitor concentration, xylanase activities of Anx, Tfx, Atx and Btx remained 25.1, 87.8, 52.9 and 73.3 %, respectively. This indicated that *A. niger* xylanase (a fungal xylanase) was the most inhibited xylanase among the tested ones, while *T. fusca* xylanase (a bacterial xylanase) was the least inhibited one. These results verified the previous observation that TAXI-type xylanase inhibitors were active against GH family 11 xylanases from both bacterial and fungal origin, and were inactive towards GH10 xylanases (Gebruers *et al.* 2004, Fierens *et al.* 2003). Since plant xylanases are structurally similar to the microbial GH10 xylanases (Simpson *et al.* 2003), TAXI probably does not have the ability to inhibit wheat endogenous xylanases. Thus TAXI-I might be involved in plant defense against fungal and bacteria xylanases.

TAXI type xylanase inhibitors were found in barley, maize, rye, and wheat (Simpson *et al.* 2003, Gebruers *et al.* 2004), and the genes were expressed in different plant tissues at different stages of plant development and under stress conditions (Fierens *et al.* 2003, Igawa *et al.* 2005). To gain further insight into the plant defense mechanisms, we studied the regulation of TAXI-I inhibitor expression by stress response phytohormone (methyl jasmonate) and growth-related phytohormone (gibberellin). Eight-day-old wheat plants were dipped in MeJA, GA, and in water for 48 h. TAXI-I gene expression was induced by MeJA, while TAXI-I gene transcript was hardly detected in GA treatment (Fig. 3). This was in accordance with the conclusion of Igawa *et al.* (2005). MeJA is a stress response phytohormone, and acts as an essential signal involved in the defense/stress pathway (Bentebibel *et al.* 2007). Therefore, expression of TAXI-I inhibitor was regulated *via* the JA-mediated signaling pathway, and its function was to prevent the plant cell wall from degradation by xylanases excreted by pathogens. So far, a number of TAXI-type xylanase inhibitor genes exist in order to address the various xylanases of external origin, and it may be important for pathogen resistance for these genes to be expressed cooperatively. The clarification of the molecular mechanism of TAXI-type gene expression may lead to the generation of pathogen-resistant plants.

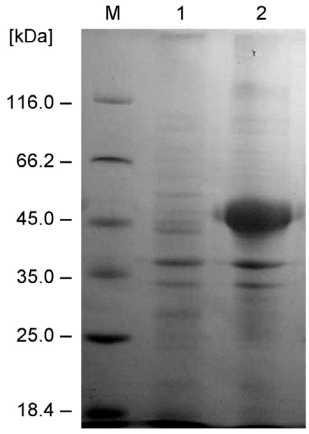

Fig. 2. The SDS-PAGE of xylanase inhibitor. M - marker of proteins, *lane 1* - *E. coli* BL21, *lane 2* - xylanase inhibitor induced by IPTG after 6 h.

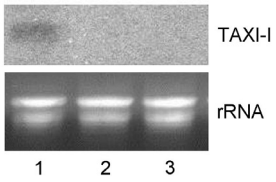

Fig. 3. Analysis of mRNA levels of wheat xylanase inhibitor TAXI-I in response to phytohormones. *Lane 1* - MeJA (100 μM) treatment, *lane 2* - GA (100μM) treatment, *lane 3* - water as a control.

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