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Induction of a Novel XIP-Type Xylanase Inhibitor by External Ascorbic Acid Treatment and Differential Expression of XIP-Family Genes in Rice

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Rice microarray analysis showed that a number of stress-related genes are induced by external addition of L-ascorbic acid (AsA). The gene designated as *AK073843* which is homologous to class III chitinase was found to exhibit the highest induction among these genes. However, its crucial residues within the chitinase active site are substituted with other residues, suggesting that the protein has no chitinase activity. The recombinant protein which is encoded by the *AK073843* gene produced in *Escherichia coli* has xylanase inhibitor activity, indicating that the gene encodes a novel rice XIP-type xylanase inhibitor protein (OsXIP). The expression of *OsXIP* was enhanced not only by exogenous AsA treatment but also by various stresses such as citrate and sodium chloride treatments, and wounding; however, it was not influenced by increasing endogenous AsA content. External AsA treatment caused a significant increase in electrolyte leakage from rice root. These results suggested that *OsXIP* was induced by stress which is caused by external AsA treatment. Rice XIP-family genes, *OsXIP*, *riceXIP* and *RIXI*, showed differential organ-specific expression. Also, these genes were differentially induced by stress and stress-related phytohormones. The transcripts of *OsXIP* and *riceXIP* were undetectable under normal conditions, and were drastically induced by wounding and methyl jasmonate (MeJA) treatment in the root. *RIXI* was constitutively expressed in the shoot but not induced by wounding and stress-related phytohormones. Thus, XIP-type xylanase inhibitors were suggested to be specialized in their function and involved in defense mechanisms in rice.

Keywords: Ascorbic acid — DNA microarray — *Oryza sativa* — Stress response — Xylanase inhibitor.

Abbreviations: AsA, ascorbic acid; DHA, dehydroascorbate; DIG, digoxigenin; GH, glycoside hydrolase family; GSH, reduced glutathione; L-Gal, L-galactono-1,4-lactone; IPTG, isopropyl-β-D-thiogalactopyranoside; MeJA, methyl jasmonate; SA, salicylic acid; TAXI, *Triticum aestivum* xylanase inhibitor; TBARS, thiobarbituric acid-reactive substances; XIP, xylanase inhibitor protein.

Introduction

L-Ascorbic acid (AsA) is a multifunctional compound in plant. For example, it is well known that AsA has important roles in protection against oxidative stress, photosynthetic energy partitioning and in various enzyme reactions as an essential cofactor (Conklin, 2001). We have previously generated the AsA-overproducing transgenic tobacco cells by overexpressing L-galactono-1,4-lactone dehydrogenese, the terminal enzyme in the AsA biosynthetic pathway of plants. These AsA-rich transgenic tobacco cells had a higher rate of mitotic division and exhibited late senescence (Tokunaga et al. 2005). Moreover, it was recently reported that an AsA-deficient *Arabidopsis* mutant showed higher pathogen resistance (Barth et al. 2004, Pavet et al. 2005). Thus, AsA may probably be involved in various plant physiological events other than those mentioned above. In this study, at first, we tried to determine AsA-responsive genes with rice microarray analysis. We found that various stress-related genes were induced by external AsA treatment in rice seedlings. A class III chitinase-homologous gene (*AK073843*) was found as a gene that exhibited the highest induction among these genes. However, we suggested that at least some of these genes including *AK073843* are not AsA-responsive genes, because their expression is not influenced by increasing endogenous AsA content. Furthermore, the *AK073843* gene was induced by various stresses, suggesting that exogenous AsA imparted some stress to rice seedlings. Therefore, we investigated what kind of stress would be caused by exogenous AsA on rice seedlings in the first stage of this report.

Secondly, we demonstrated that *AK073843* encodes a novel XIP-type xylanase inhibitor protein (OsXIP) in rice. So far, two distinct classes of proteinaceous xylanase inhibitors, namely xylanase inhibitor protein (XIP)-type and *Triticum aestivum* xylanase-inhibitor (TAXI)-type inhibitors, have been reported (Debyser et al. 1999, McLauchlan et al. 1999). XIP-type inhibitors are basic, monomeric proteins of approximately 30 kDa. A wheat

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XIP-type inhibitor, namely XIP-I, specifically and competitively inhibits fungal xylanase, but cannot inhibit the bacterial enzyme (Flatman et al. 2002. Juge et al. 2004). Meanwhile, there are two molecular forms of TAXI-type inhibitors, basic, monomeric proteins of approximately 40 kDa and dimeric proteins of 30 and 10 kDa subunits (Gebruers et al. 2001). They can inhibit xylanases of the glycoside hydrolase family 11 (GH11), but cannot inhibit those of glycoside hydrolase family 10 (GH10) (Gebruers et al. 2004). To date, these two types of xylanase inhibitors have been identified in various cereals such as durum wheat, rye, barley and maize (Elliott et al. 2003, Goesaert et al. 2003, Goesaert et al. 2004). Although xylanase inhibitors have been believed to be absent in rice (Goesaert et al. 2004), two XIP-type xylanase inhibitors, riceXIP and RIXI, were recently isolated from rice (Durand et al. 2005, Goesaert et al. 2005). The biochemical properties and structure of xylanase inhibitors have been analyzed, because they may affect the efficiency of microbial xylanases in biotechnological applications involving cereal processing. However, the physiological function and the gene regulation of xylanase inhibitors are not well known to date. Here, we found that *OsXIP* is a stress-responsive gene, and rice XIP-family genes, including *riceXIP* and *RIXI*, show differential expression in organs under stress and phytohormone treatment. The physiological function of XIP-type xylanase inhibitors in plants is discussed.

Results

Analysis of AsA-responsive genes in rice seedlings using microarray

To identify AsA-responsive genes in rice, we performed microarray analysis using the Rice 22K Custom Oligo DNA Microarray (Agilent Technologies, Palo Alto, CA, USA) containing approximately 22,000 probes. The rice seedlings treated with 20 mM sodium ascorbate (AsA-Na) and treated with distilled water as control were used for a microarray analysis. Cyanine 3 (Cy3)- and cyanine 5 (Cy5)-labeled cRNAs were prepared from total RNA isolated from these rice seedlings treated with AsA-Na and distilled water, respectively. These labeled cRNAs were hybridized with the probes on the microarray, and the expression profiles of the approximately 22,000 genes were analyzed. To assess the reproducibility, we performed another experiment for RNA samples using different labels, Cy3 for distilled water and Cy5 for AsA-Na. We selected candidates of AsA-responsive genes according to three criteria as follows: (i) the fold change value is ≥ 5 for both microarray experiments; (ii) the *P*-value is <0.01 for both microarray experiments; and (iii) the expression signal of control and/or AsA-treated rice is $\geq 1{,}000$. The 63 putative AsA-responsive genes identified in these two experiments are shown in Table 1. Of these genes, 60 were up-regulated while three were down-regulated (Fig. 1A). Up-regulated genes were classified according to their putative functions (Fig. 1B). Presumably, many up-regulated genes are involved in defense mechanisms (18.3%) and metabolism (16.7%), including lignin or flavonoid synthesis (i.e. cinnamoyl CoA reductase and chalcone synthase) required for plant defense (Richard et al. 2000, Fan et al. 2006, Kawasaki et al. 2006) (Table 1).

The gene represented by DDBJ accession No. AK073843 showed the highest fold change, namely the transcript for this gene (*AK073843*) was increased about 41-fold by AsA-Na treatment (Table 1, Fig.1A). To validate the microarray analysis, the mRNA for *AK073843* was analyzed by Northern blotting (Fig. 1C). In order to exclude the possibility that sodium ions affect this induction, treatment was also carried out with 20 mM AsA neutralized with potassium hydroxide (AsA-K). The transcript of *AK073843* was markedly increased by both treatments (AsA-Na and AsA-K), indicating that the *AK073843* gene was induced by external AsA treatment but not by sodium ions.

Effects of external AsA treatment on rice seedlings

We thought it interesting to find out whether or not *AK073843* is specifically induced by AsA. Thus, we examined the expression of *AK073843* in root and shoot by adding L-galactono-1,4-lactone (L-Gal) as a precursor of AsA and citrate as an organic acid (Fig. 2). We also investigated whether this gene is induced by salt stress (NaCl), one of the oxidative stresses, because there are some reports that exogenous AsA plays a role as a pro-oxidant that induces oxidative stress in cultured cells (Arakawa et al. 1994, Sakagami and Satoh 1997, Song et al. 2001a, Song et al. 2001b). The *AK073843* transcript was induced by AsA in root. However, it was hardly detected despite a marked increase in endogenous AsA content when L-Gal was added. On the other hand, the addition of citrate and sodium chloride induced *AK073843* expression in root, although AsA content did not increase in rice seedlings. These results suggest that *AK073843* was induced by some stress caused by exogenous AsA.

In order to clarify whether induction of *AK073843* expression was caused by a stress that arises from the pro-oxidant effect of AsA, we estimated lipid peroxidation, which has been used as a marker for oxidative stress, and the amount of hydrogen peroxide in rice root after AsA treatment (Fig. 3A, B). In both cases, significant changes were not exhibited, even though they tended to be slightly restrained at the late and early stage after AsA addition, respectively. We also investigated whether *AK073843* expression was induced by AsA via dehydroascorbate (DHA), because it is known that when AsA is added

Table 1 Indeced/repressed genes in rice seedlings with external AsA treatment

Probe name[a]	Accession[b]	Blast hit[c]	Putative function[d]	Fold change[e]	
Defense					
J033068H05	AK073843[f]	AAV32103	chitinase	39.1	44.7
001-116-H03	AK063517	AAX96129	dehydrin rab 16b	21.8	24.2
J023096D05	AK071366	P22912	dehydrin RAB 16C	13.6	12.8
001-125-H02	AK064074	NP_916529	WSI18 protein	12.3	10.6
J033095E15	AK102505	AAX95337	chitinase III	12.3	18.3
J033115J22	AK102970	XP_469148	antifungal thaumatin-like protein	9.5	11.7
001-104-D03	AK062520	NP_908901	mannose-binding rice lectin (SALT)	9.1	10.0
001-118-H06	AK063645	NP_913440	late embryogenesis abundant protein LEA14-A	8.3	9.2
002-121-E05	AK107065	NP_910394	WSI76 protein induced by water stress	8.3	9.3
J033136O07	AK103707	BAB85659	ribosome inactivating protein 2 (RIP2)	9.9	6.5
006-303-H08	AK060033	NP_921483	class III chitinase	5.7	5.7
001-110-E07	AK105219	ABA99661	metallothionein	−8.7	−7.3
Metabolism					
J023113E15	AK100678	AAT93945	pyruvate decarboxylase	24.4	31.3
J033148H10	AK103839	NP_920428	phosphoenolpyruvate carboxykinase	14.1	9.9
J013039L05	AK065739	XP_468806	cytosolic pyruvate orthophosphate dikinase	12.7	18.3
001-123-D09	AK063935	XP_468348	cinnamoyl CoA reductase	11.6	10.4
002-108-H04	AK064401	XP_468316	cinnamoyl CoA reductase	10.6	6.9
002-173-D05	AK110924	XP_478252	chalcone synthase	9.1	13.5
002-107-F12	AK104985	XP_467869	UDP-glucose glucosyltransferase1	8.8	7.7
J013159K10	AK068710	ABA96021	carboxyvinyl-carboxyphosphonate phosphorylmutase	8.3	7.1
001-113-B04	AK105239	BAD31135	sulfotransferase (STF-1)	7.6	5.7
J023099J05	AK100501	AAT93946	pyruvate decarboxylase	5.4	5.9
Signal transduction					
001-114-A05	AK063334	XP_450535	protein phosphatase 2C-like	7.4	8.0
J023101G03	AK071637	BAD54464	protein phosphatase 2C	6.0	5.5
J023012K18	AK069274	NP_912371	serine/threonine phosphatases	5.5	6.9
Transcription					
006-206-D03	AK059839	XP_468836	C2H2-type zinc finger protein ZFP36	5.9	8.8
J013170H07	AK068861	AAP42461	zinc finger protein ZFP14	5.5	5.7
Transporter					
001-120-C03	AK063714	AAV84280	dehydration up-regulated putative membrane pore protein	9.9	8.9
001-120-E03	AK063729	XP_475855	lipid transfer protein	6.6	5.9
Translation					
002-112-G01	AK064587	XP_475154	peptide chain release factor subunit 1 (eRF1)	15.3	22.0

(continued)

Table 1 Continued

Probe name[a]	Accession[b]	Blast hit[c]	Putative function[d]	Fold change[e]	
Others					
002-135-E05	AK107980	BAD45887	phytocyanin protein, PUP2	16.7	21.0
J013029M23	AK065631	ABA95364	von Willebrand factor type A domain	12.1	11.5
J033081F22	AK102039	XP_475170	plasma membrane associated protein	11.2	14.4
J023050F01	AK070289	ABA95365	von Willebrand factor type A domain	6.6	5.6
J033150D17	AK103890	BAD38019	ubiquitin/ribosomal protein CEP52	6.4	6.1
001-118-F12	AK063634	AAS55470	little protein 1	6.0	5.3
Unknown					
001-120-G06	AK063747		unknown	18.6	19.1
001-119-E08	AK063682	XP_473712	unknown	18.0	17.1
001-113-H09	AK063328		unknown	17.8	26.6
001-118-C12	AK063608		unknown	16.2	15.9
002-126-A05	AK107276	XP_469580	unknown	13.0	16.4
002-101-C06	AK106302	XP_474840	unknown	12.7	10.0
002-143-C01	AK108443		unknown	12.0	15.3
J023071K01	AK070872	NP_908456	unknown	11.3	10.4
001-101-B06	AK062310	NP_918640	unknown	10.4	9.0
002-181-F02	AK111335	XP_467649	unknown	9.2	10.0
002-150-B08	AK108716	XP_482384	unknown	8.0	6.5
002-103-G09	AK064163	XP_468042	unknown	7.7	6.6
002-104-G04	AK064224	XP_479676	unknown	7.7	8.2
001-009-E09	AK058934		unknown	7.6	7.0
J023021I18	AK069495	XP_470625	unknown	7.5	8.2
J033022D23	AK073109	XP_473850	unknown	7.5	8.1
001-107-B06	AK062784		unknown	7.2	8.0
J033089O13	AK102303	XP_470607	unknown	7.1	6.3
J033130P11	AK103494	BAD27962	unknown	6.9	8.0
002-145-F08	AK109702	AAU43986	unknown	6.5	5.1
001-100-G05	AK062588	AAX95726	unknown	6.4	6.7
002-188-H07	AK109325	XP_475741	unknown	6.2	5.2
002-102-A06	AK106356	XP_482383	unknown	5.7	5.4
J033130I02	AK103482	BAD88010	unknown	5.6	5.3
002-134-E10	AK107893		unknown	5.6	5.3
002-151-B01	AK108786	XP_465944	unknown	−6.6	−5.4
002-154-H05	AK109082		unknown	−8.6	−6.7

[a] Probe names are full-length cDNA clones (Kikuchi et al. 2003).
[b] The DDBJ accession no. given after registration of the cDNA clone with the DDBJ database.
[c] The accession no. of a protein registered on GenBank that showed more than 98% identity using BLASTP.
[d] Definition of a protein on GenBank that showed highest amino acid sequence identity using BLASTP.
[e] The fluctuation ratio of the transcript level. The results of two microarray experiments were shown respectively. Those down regulated were represented by minus.
[f] The gene focused on in this study.

to cell culture medium or hydroponic medium in which seedlings are growing, it is promptly oxidized to DHA. The *AK073843* transcript was beginning to increase at 6 h after AsA treatment and gradually increased in root, while its induction by DHA was later and weaker than that by AsA, even though DHA also caused a significant increase in *AK073843* transcript (Fig. 3C). Also, *AK073843* expression did not decrease when rice seedlings were treated

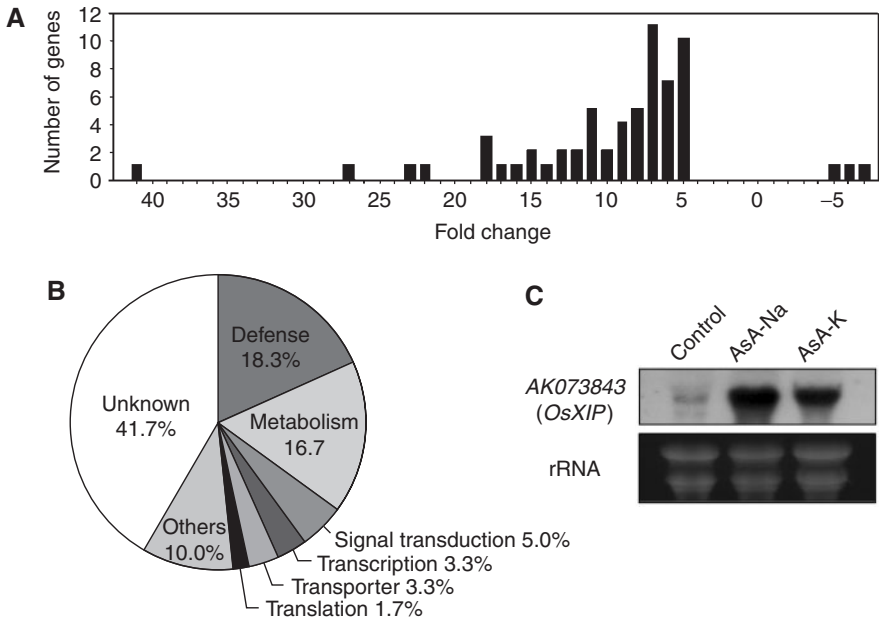

Fig. 1 The analysis of induced/repressed genes by 20 mM AsA-Na treatment for 3 d in rice seedlings. (A) Numbers of induced/repressed genes. The histogram shows the number of genes that had average fold change values 5.0 above and −5.0 below. (B) Classification of 60 up-regulated genes. The identified genes were categorized according to their putative functions. (C) Expression of the *AK073843* (*OsXIP*) gene with AsA treatment in rice seedlings. Total RNA was isolated from rice seedlings treated with 20 mM sodium ascorbate (AsA-Na), 20 mM AsA neutralized with potassium hydroxide (AsA-K) or distilled water (Control) for 3 d. A 5 μg aliquot of total RNA were loaded in each lane and analyzed by Northern blotting.

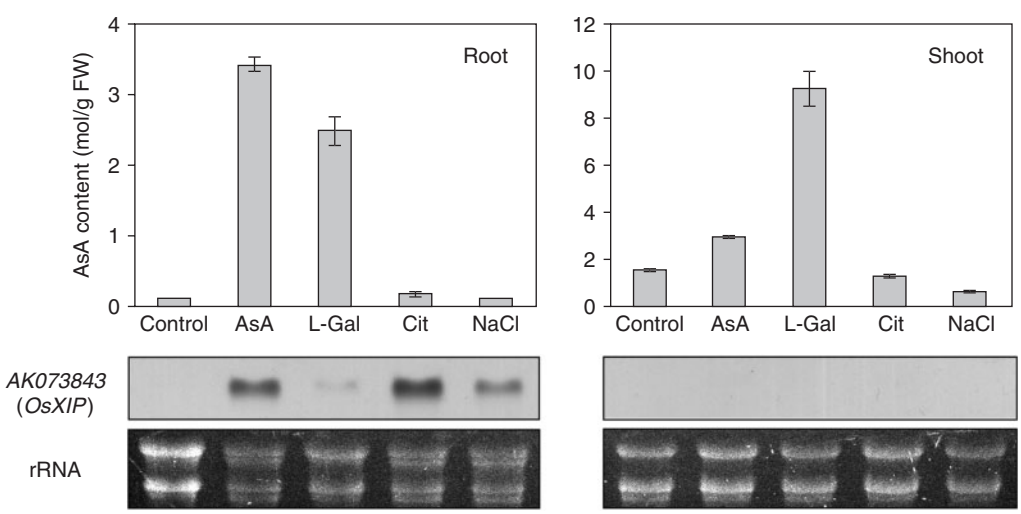

Fig. 2 Effects of various chemical treatments on AsA content and *AK073843* (*OsXIP*) expression in rice seedlings. Rice seedlings were treated with 20 mM AsA (AsA), 20 mM L-galactono-1,4-lactone (L-Gal), 20 mM citrate (Cit), 100 mM sodium chloride (NaCl) and distilled water (Control) for 3 d. AsA contents in root and shoot were measured. Values are means ± SE ($n = 3$). Total RNA was also isolated from root and shoot. A 2 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting.

with AsA solution containing reduced glutathione (GSH) which restrains oxidation of AsA in the medium, suggesting that exogenous AsA directly triggered *AK073843* expression (Fig. 3D). In addition, we confirmed that *AK073843* expression is not induced by addition of GSH (data not shown). Taken together, pro-oxidant actions of AsA may not be involved in induction of *AK073843* expression.

To investigate whether exogenous AsA and DHA cause membrane damage in rice root tissue, we measured electrolyte leakage from the roots (Fig. 3E). The electrolyte leakage significantly increased up to 73% within 24 h due to AsA treatment. In the case of DHA addition, there was an increase from 0 h up to 6 h before hitting a steady-state level of about 50% up to 24 h after the treatment, and then it rose again from 24 h. Rice seedlings died by 72 h after DHA treatment. Song et al. (2001b) reported that the uptake of DHA induces apoptotic death via oxidative stress in animal cells; therefore, the death of rice seedlings may have been caused by a similar phenomenon. The additions of citrate and sodium chloride also caused

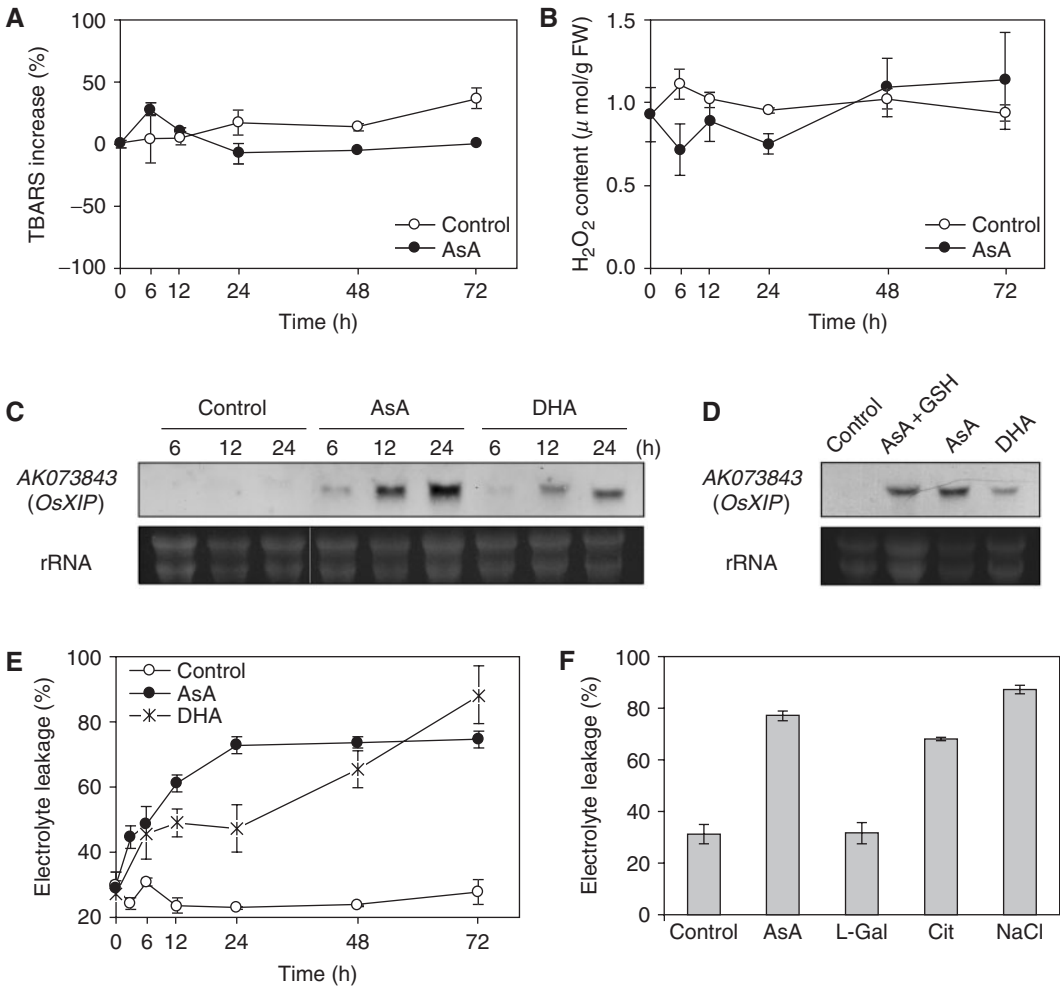

Fig. 3 Effects of AsA and other chemical treatments on rice root. (A, B) Time course of lipid peroxidation and hydrogen peroxide content in rice root after 20 mM AsA treatment. Values are means ± SE ($n = 3$). (C) Time course of *AK073843* (*OsXIP*) expression in rice root after 20 mM AsA or 20 mM DHA treatment. A 4 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting. (D) Expression of *AK073843* (*OsXIP*) in rice root treated with 20 mM AsA, 20 mM DHA or 20 mM AsA plus 20 mM GSH (AsA + GSH) for 12 h. A 4 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting. (E) Time course of electrolyte leakage from rice root after 20 mM AsA or 20 mM DHA treatment. Values are means ± SE ($n = 3$–6). (F) Electrolyte leakage from rice root treated with 20 mM AsA (AsA), 20 mM L-galactono-1,4-lactone (L-Gal), 20 mM citrate (Cit) and 100 mM sodium chloride (NaCl) for 3 d. Values are means ± SE ($n = 3$). Distilled water was used as control in all experiments.

marked increases in electrolyte leakage to about 68 and 87%, respectively, after 72 h when compared with the control (Fig. 3F). These results suggest that wound-like stress caused by AsA addition may contribute to the induction of the *AK073843* gene. This hypothesis was supported by the observation that *AK073843* expression was drastically induced by wounding to rice seedlings (Fig. 7A, B).

Sequence analysis of AK073843

It is interesting in terms of the mechanisms of plant defense that *AK073843* is significantly induced by stress. To elucidate the physiological function of AK073843 in rice, we carried out sequence analysis of this gene. The homology search of the deduced amino acid sequence of this gene using the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html/) showed that AK073843 is homologous to class III chitinase designated 'putative chitinase' in GenBank. According to the InterProScan program (http://www.ebi.ac.uk/InterProScan/), it was estimated that AK073843 belongs to glycoside hydrolase family 18 (GH18) as well as class III chitinases, but does not have the catalytic domain GxDxDxE which is highly conserved in all class III chitinases of plants, bacteria and fungi (Levorson and Chlan 1997), i.e. the second aspartate and last glutamate are substituted with phenylanine and

aspartate, respectively (Fig. 4B). Mutational studies with *Altermonas* chitinase (Tsujibo et al. 1993) and *Bacillus circulans* chitinase (Watanabe et al. 1993) have shown that, when the catalytic glutamate is changed to either aspartate or glutamine, chitinase activity is lost. These findings suggest that AK073843 would have no chitinase activity. From the phylogenetic tree for plant members of GH18, it was found that AK073843 belongs to the XIP-type xylanase inhibitor subfamily (Fig. 4A). Thus, we designated this gene *OsXIP* as a putative XIP-type xylanase inhibitor gene in rice. *OsXIP* is expected to encode a protein of 293 amino acid residues with a predicted relative molecular mass of 32,435 Da and a theoretical pI of 8.7. Sequence analysis using the pSORT program (http://psort.ims.u-tokyo.ac.jp/) predicted that OsXIP has a potential N-terminal signal peptide. The predicted cleavage site of the signal peptide lies between the 21st and 22nd alanine, suggesting that the mature protein of OsXIP has 272 amino acids with a predicted relative molecular mass of 30,222 Da. The alignment of OsXIP with other XIP-type xylanase inhibitors, riceXIP from rice (GenBank accession No. BAA77780), RIXI from rice (GenBank accession No. BAA23810) and XIP-I from wheat (GenBank accession No. CAD19479), and hevamine (GenBank accession No. CAA07608) which is a class III chitinase from rubber tree (*Hevea brasiliensis*), revealed 57, 47, 44 and 31% amino acid sequence identities, respectively, ruling out signal sequence (Fig. 4B). XIP-I has been previously reported to possess two disulfide bridges formed between Cys25 and Cys66, and between Cys164 and Cys195 (Payan et al. 2003). OsXIP also has four cysteine residues, Cys29, Cys71, Cys168 and Cys197. The structure involved in complex with a GH10 or GH11 xylanase is considered to be important for xylanase-inhibiting ability, i.e. XIP-I can inhibit GH10 and GH11 xylanases because two parts of the interacting site (residues 193–205 and 148–153) are shorter and longer than typical class III chitinases, respectively (Durand et al. 2005). In the case of OsXIP, the corresponding site complex with GH10 (residues 195–209) is five residues shorter, and the corresponding site complex with GH11 (residues 152–157) is two residues longer than hevamine. This information suggests that OsXIP may act as xylanase inhibitor, but not as a chitinase.

Xylanase inhibitor activity of the recombinant OsXIP produced in E. coli

In order to clarify whether OsXIP can function as a xylanase inhibitor protein, we attempted to measure the xylanase inhibitor activity of recombinant OsXIP produced in *Escherichia coli*. A full-length *OsXIP* cDNA in which the signal peptide-encoded region was removed was cloned into expression vector pET19b to give the expression construct pETOsXIP. The expressed protein lacked the signal peptide and was a non-fusion protein with no additional amino acids at the N- and C-termini. An approximately 30 kDa protein induced by isopropyl-β-D-thiogalactopyranoside (IPTG) specifically was detected in soluble protein extract from a culture of *E. coli* transformed with pETOsXIP, indicating that recombinant OsXIP protein was expressed in the transformed *E. coli* (Fig. 5A). The activity of GH11 β-xylanase from *Trichoderma viride* and *Trichoderma longibrachiatum* was reduced by approximately 35 and 25%, respectively, when the soluble protein extract containing recombinant OsXIP was added to the xylanase preparations (Fig. 5B). Thus, OsXIP was shown to be a novel xylanase inhibitor in rice. However, the activity of GH11 β-xylanase from *Aspergillus niger* was not influenced by recombinant OsXIP (data not shown).

Expression of XIP-family genes during germination of rice seed

The physiological function of xylanase inhibitors in plants remains unclear. Northern blot analysis was carried out for *OsXIP* and two other XIP-type xylanase inhibitors, *riceXIP* and *RIXI*, to investigate their expression profiles and estimate their functions in rice plant (Fig. 6). In order to exclude the possibility of cross-hybridization, we used the 3′-untranslated region of each gene as a probe for Northern blotting. No transcripts for *OsXIP* and *riceXIP* were detected in any organ during germination. *RIXI* mRNA was expressed in shoot within 4 d after the imbibition, but was not detected in root. No gene was expressed in grain throughout 3 d after the imbibition (data not shown).

Wound-responsive expression of XIP-family genes in rice seedlings

As described above, we found that *OsXIP* is markedly induced by wounding. It is interesting to determine whether other XIP-family genes are also induced by wounding. We examined expression of XIP-family genes after cutting of rice seedlings (Fig. 7A). *OxXIP* and *riceXIP* transcripts increased in root at 12 h, and then decreased at 24 and 48 h. The *OsXIP* and *riceXIP* genes were little or not expressed in shoot. In contrast, *RIXI* was constitutively expressed in shoot but not in root. No induction of *RIXI* by wounding was found. The detailed expression pattern of *OsXIP* and *riceXIP* after cutting was investigated in root (Fig. 7B). *riceXIP* transcript rapidly increased within 2 h after cutting and reached a maximum level at 6 h. *OsXIP* transcript gradually increased and reached a maximum level at 12 h.

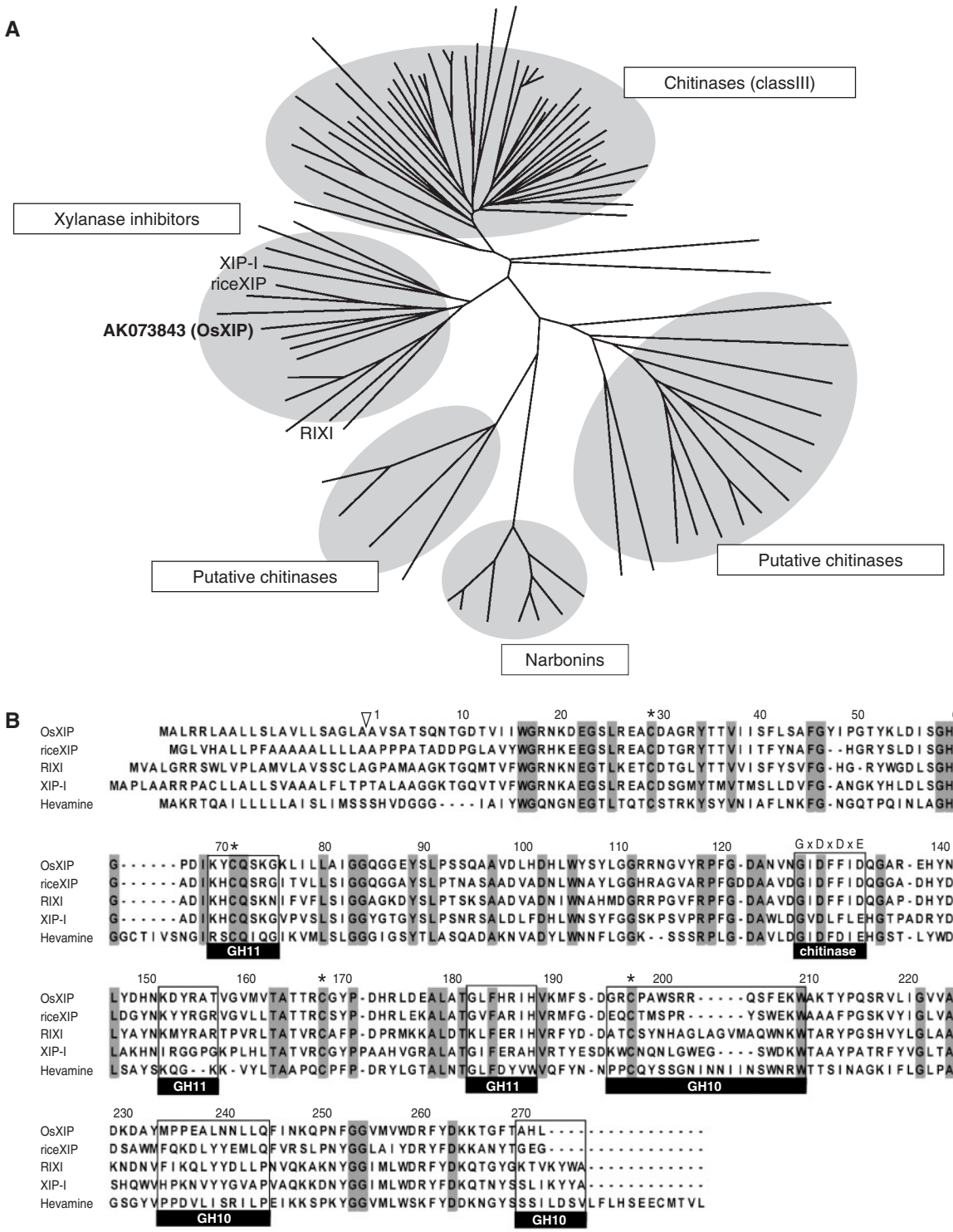

Fig. 4 The analysis of the deduced amino acid sequence of *AK073843* (*OsXIP*). (A) Phylogenetic tree for plant members of GH18. A phylogenetic tree is presented for the known plant members of the GH18 family. Ninety-one complete amino acid sequences were retrieved from the carbohydrate-active enzymes database, CAZy (http://afmb.cnrs-mrs.fr/CAZY/index.html). (B) Amino acid sequence alignment of GH18 plant members, OsXIP, riceXIP, RIXI, XIP-I and hevamine. Residue numbering is given for the OsXIP sequence. Gray shading shows residues conserved in all sequences. The putative cleavage site for the removal of N-terminal signal peptides in OsXIP is indicated by an open triangle. Four cysteine residues are indicated by asterisks. Sequences in the box show consensus residues involved in chitinase activity (GxDxDxE) and involved in complexing with GH10 or GH11 xylanase.

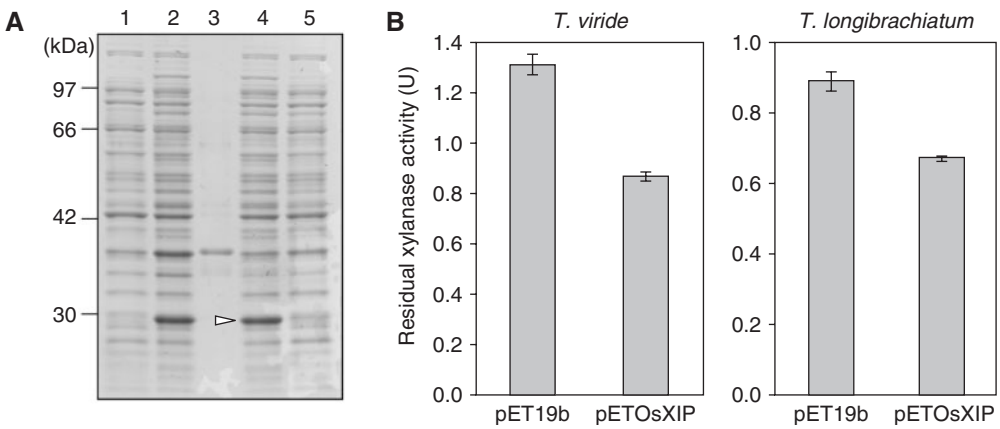

Fig. 5 SDS–PAGE and xylanase inhibitor activity of recombinant OsXIP expressed in *E. coli*. (A) Crude protein extracts obtained from a culture of *E. coli* transformed with pETOsXIP (Origami(DE3)[pETOsXIP]) and pET19b (Origami(DE3)[pET19b]) were assayed by SDS–PAGE. Gels were stained with Coomassie brilliant blue. The size of the molecular markers is indicated to the left. Lane 1, total protein extracts from Origami(DE3)[pETOsXIP] prior to addition of IPTG; lane 2, total protein extracts from Origami(DE3)[pETOsXIP] after addition of IPTG; lane 3, insoluble protein extracts from Origami(DE3)[pETOsXIP] after addition of IPTG; lane 4, soluble protein extracts from Origami(DE3)[pETOsXIP] after addon of IPTG; lane 5, soluble protein extracts from Origami(DE3)[pET19b] after addition of IPTG. The open triangle indicates the recombinant OsXIP. (B) Crude soluble protein extracts were prepared from Origami(DE3)[pETOsXIP] and Origami(DE3)[pET19b] cultures and assayed for xylanase inhibitor activity using birchwood (1,4)-β-xylan as substrate and *T. viride* or *T. longibrachiatum* xylanase as the target enzyme. The residual xylanase activity is shown. Values are means ± SE ($n = 3$).

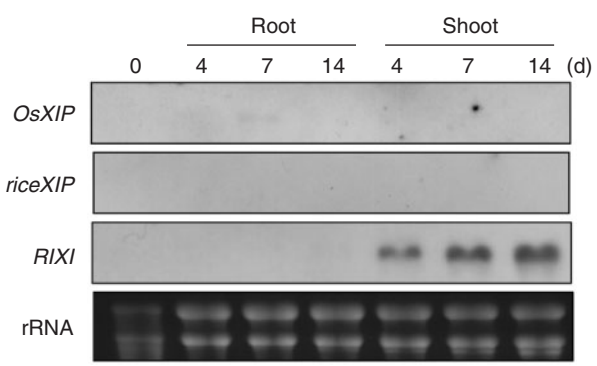

Fig. 6 Expression of XIP-family genes during germination of rice seed. Total RNA was isolated from grain, shoot and root for the indicated times. A 5 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting.

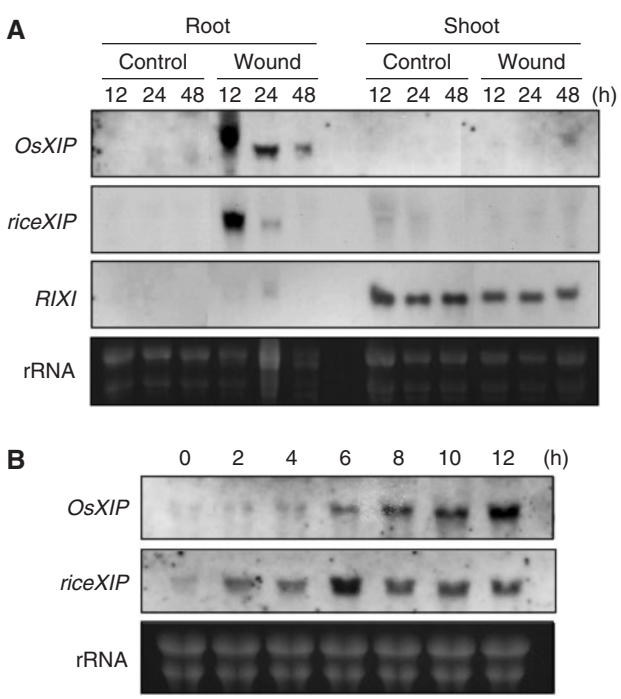

Fig. 7 Expression of XIP-family genes in rice seedlings after wounding. Rice seedlings were wounded by cutting into pieces, and floated on distilled water. The wounded organs were harvested at the indicated time intervals. (A) Expression of *OsXIP, riceXIP* and *RIXI* in root and shoot. A 3 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting. (B) The detailed expression pattern of *OsXIP* and *riceXIP* in cut rice root. A 5 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting.

Effects of plant stress hormones on XIP-family gene expression

The involvement of plant stress hormones, such as ABA, jasmonic acid (JA) and salicylic acid (SA), in signaling of stress-responsive expression has been reported, and a signaling cascade has been proposed (Zeevaart and Creelman 1988, Reymond and Farmer 1998, Turner et al. 2002). To estimate whether these phytohormones were involved in induction of xylanase inhibitors in rice, *OsXIP*, *riceXIP* and *RIXI* expression levels were investigated in rice seedlings treated with ABA (200 μM), methyl jasmonate (MeJA; 200 μM) and SA

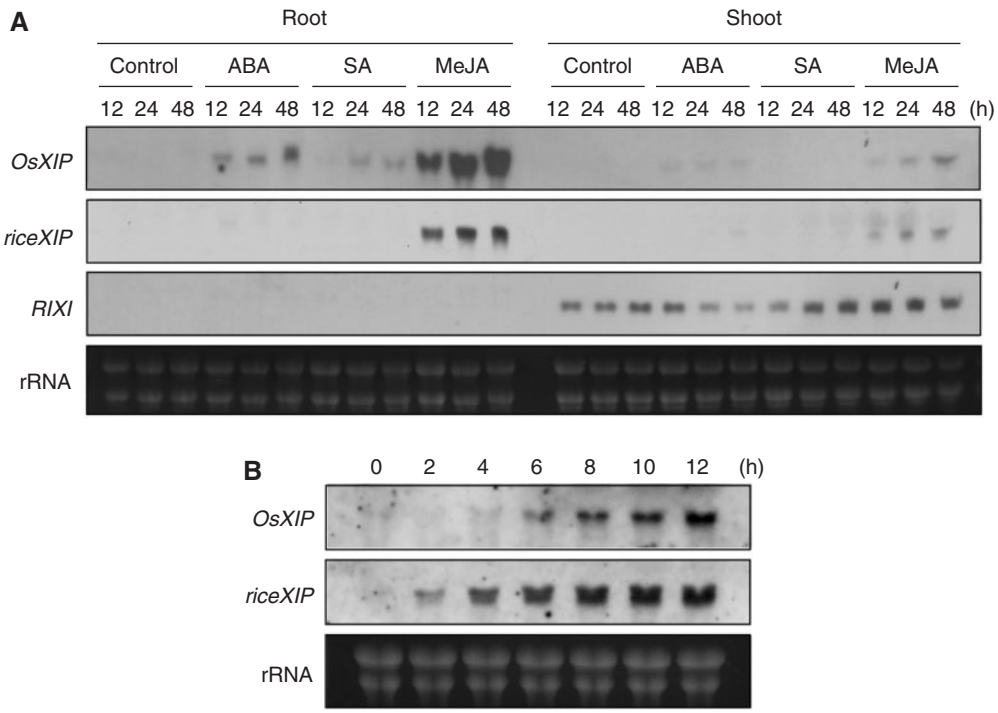

Fig. 8 Expression of XIP-family genes in rice seedlings after various phytohormone treatments. Rice seedlings were treated with 200 μM ABA, 5 mM SA, 200 μM MeJA or distilled water as control for the indicated time. (A) Expression of *OsXIP, riceXIP* and *RIXI* in shoot and root. A 3 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting. (B) The detailed expression pattern of *OsXIP* and *riceXIP* in rice root treated with 200 μM MeJA. A 5 μg aliquot of total RNA was loaded in each lane and analyzed by Northern blotting.

(5 mM) (Fig. 8A). *OxXIP* and *riceXIP* expression was drastically induced in root, and slightly increased in shoot when treated with MeJA. A slight effect of ABA on *OsXIP* expression was found in root. In addition, a small amount of *riceXIP* transcript could be detected after ABA treatment in root. The *RIXI* gene was constitutively expressed in shoot, with no significant induction by any phytohormones. The detailed analysis of *OsXIP* and *riceXIP* expression patterns after MeJA treatment was performed in roots (Fig. 8B). The induction of the *riceXIP* gene by MeJA was faster than that of *OsXIP*. This manner of induction by MeJA is very similar to that by wounding. These results suggest that the induction of *OsXIP* and *riceXIP* expression by wounding may occur via a JA-mediated signaling pathway.

Discussion

In rice seedlings, using microarray analysis, we found a number of genes induced by external AsA treatment. These genes were probably involved in plant defense mechanisms, especially in relation to pathogens (Table 1). Chitinases, thaumatin, mannose-binding lectin, ribosome-inactivating protein (RIP) and lipid transfer protein are considered as pathogenesis-related proteins (van Loon et al. 1999, Veronese et al., 2003, Kim et al. 2004). However, at least a some of these genes, including *AK073843*, were not influenced by increasing endogenous AsA content but by adding citrate and sodium chloride (Fig. 2), indicating that some stress is caused by exogenous AsA in rice seedlings, and the microarray data may also be affected by the stress caused by exogenous AsA.

We first suspected that these genes were induced by the pro-oxidant action of AsA. So far, several hypotheses have been proposed for the mechanism of oxidative stress occurring due to external AsA treatment. The first possibility is the generation of reactive oxygen species with AsA oxidation. It is known that when AsA is added to the cell culture medium, it is oxidized automatically and also in a heavy metal ion-dependent manner both inside and outside the cells, thus resulting in generation of reactive oxygen species such as hydrogen peroxide, which imparts oxidative damage to cells (Arakawa et al. 1994, Inai et al. 2005). The second perspective is the enhancement of oxidative stress caused by recycling of AsA. It was previously reported that DHA generated by oxidation of AsA in cell culture medium enters into animal PC12 cells and is rapidly reduced to AsA using the intracellular reducing power, resulting in serious oxidative stress (Song et al. 2001a, Song et al. 2001b). Also, when AsA

was added to the culture medium in which tobacco BY-2 protoplasts were being incubated, it was oxidized and taken up as DHA by the cells, where the DHA was immediately reduced to AsA (Horemans et al. 1998). However, we could find no evidence that exogenous AsA acts as pro-oxidant in this study, because it did not induce lipid peroxidation and did not increase hydrogen peroxide content in rice root (Fig. 3A, B). Furthermore, there is the possibility that the transcript of *AK07384* was directly induced by external AsA (Fig. 3C, D). Finally, we propose that expression of *AK073843* induced by exogenous AsA is not caused by a pro-oxidant effect of external AsA or by oxidative stress which occurred during recycling of AsA.

It is well known that many pathogenesis-related genes are induced by wounding via JA and ethylene signaling pathways (Ryan et al. 2000, Schilmiller and Howe 2005). Furthermore, the phenylpropanoid pathway involving lignin and flavonoid metabolism is activated by wounding (Richard et al. 2000, Matsuda et al. 2003). This information led us to speculate that exogenous AsA may induce wounding-related or -like stress in rice seedlings. We confirmed that membrane damage was actually caused by external AsA treatment, and it positively correlated with *AK073843* expression (Figs. 2, Fig. 3C, E, F). Although the detailed mechanism is as yet unclear, considering that the membrane damage is also caused by citrate treatment, the acidic property of AsA may contribute to this phenomenon. In the case of DHA, electrolyte leakage showed a two-step increase after the treatment. The second induction which occurs from 24 h may be incidental rather than a direct action of DHA, because rice seedlings were beginning to die after 24 h (Fig. 3E).

AsA and L-Gal have often been applied to tissues, culture medium or cultivating soil to increase the AsA content in plants. Several reports in the literature have previously shown different effects on plants due to external L-Gal and AsA treatment. For example, when AsA was added to culture medium in which onion roots were growing, the AsA redox status in the apoplast changed to the oxidized form; in contrast, L-Gal application caused the redox status to shift to the reduced form (Cordoba-Pedregosa et al. 2005). Also, it has been reported that the resistance of rice seedlings to chilling and water stress was increased by L-Gal treatment, but not by treatment with a high concentration of AsA (Guo et al. 2005). The present study suggested that external AsA addition imparted significant membrane damage to the root, while L-Gal had little effect on membranes. Thus, great care should be taken in choosing the strategy for enriching plants with AsA and in interpreting the results obtained. In summary, we found with microarray analysis that exogenous AsA produced wounding-like stress in rice roots, and the possibility was raised that various stress-responsive genes containing *AK073843* could be induced by this stress.

In this study, we confirmed, from analysis with the recombinant protein produced in *E. coli*, that *AK073843*, a stress-responsive gene, encodes a novel XIP-type xylanase inhibitor protein in rice (OsXIP). The recombinant OsXIP reduced the activity of GH11 xylanase from *T. viride* and *T. longibrachiatum*, although it could not inhibit the activity of *A. niger* xylanase (Fig. 5B). This observation can be explained by the specificities of xylanase inhibitors for xylanases. It is known that both XIP- and TAXI-type xylanase inhibitors exhibit different effectiveness towards various xylanases (Goesaert et al. 2004, Durand et al. 2005, Goesaert et al. 2005). In this study, we tested the inhibitor activity of OsXIP only against *T. longibrachiatum*, *T. viride* and *A. niger* GH11 xylanases using crude protein extracts containing recombinant OsXIP. Further study is still required to clarify whether OsXIP inhibits the activities of other xylanases, and to determine its inhibition kinetics.

Recently, the profile of XIP-I gene expression in wheat was investigated (Igawa et al. 2005). The present study revealed some similar and different expression properties between wheat and rice XIP genes. The common feature is that both XIP genes are drastically induced by wounding and MeJA (Figs. 7, 8), suggesting that the regulation of XIP-type inhibitor expression via the JA-mediated signaling pathway is common in monocots. It is well known that 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). This suggests that XIP-type xylanase inhibitors may be generally involved in plant defense. This speculation is supported by the observation that *OsXIP* expression was not affected by growth-related phytohormones such as auxin, cytokinin and gibberellin (data not shown). Recently, Brito et al. (2006) reported that xylanase excreted by phytopathogens would be required for pathogenesis. Therefore, the XIP-type xylanase inhibitor may be functional as a barrier which prevents the cell wall from degradation by xylanase excreted by pathogens.

On the other hand, the profile of organ-specific expression of XIP-family genes in rice is different from that of XIP-I in wheat, i.e. *OsXIP* and *riceXIP* mRNAs were not detected in basal conditions and were greatly induced in root by wounding and MeJA. *RIXI* was constitutively expressed in shoot and not induced by defense-related phytohormones (Figs. 6, 7, 8). Meanwhile, it has been reported that *XIP-I* expression is detected in root and shoot during germination. This difference may be explained by the number of XIP genes in plants. We found at least eight candidates for XIP genes in rice by analysis using the database of full-length cDNA clones from japonica rice (Knowledge-based Oryza Molecular

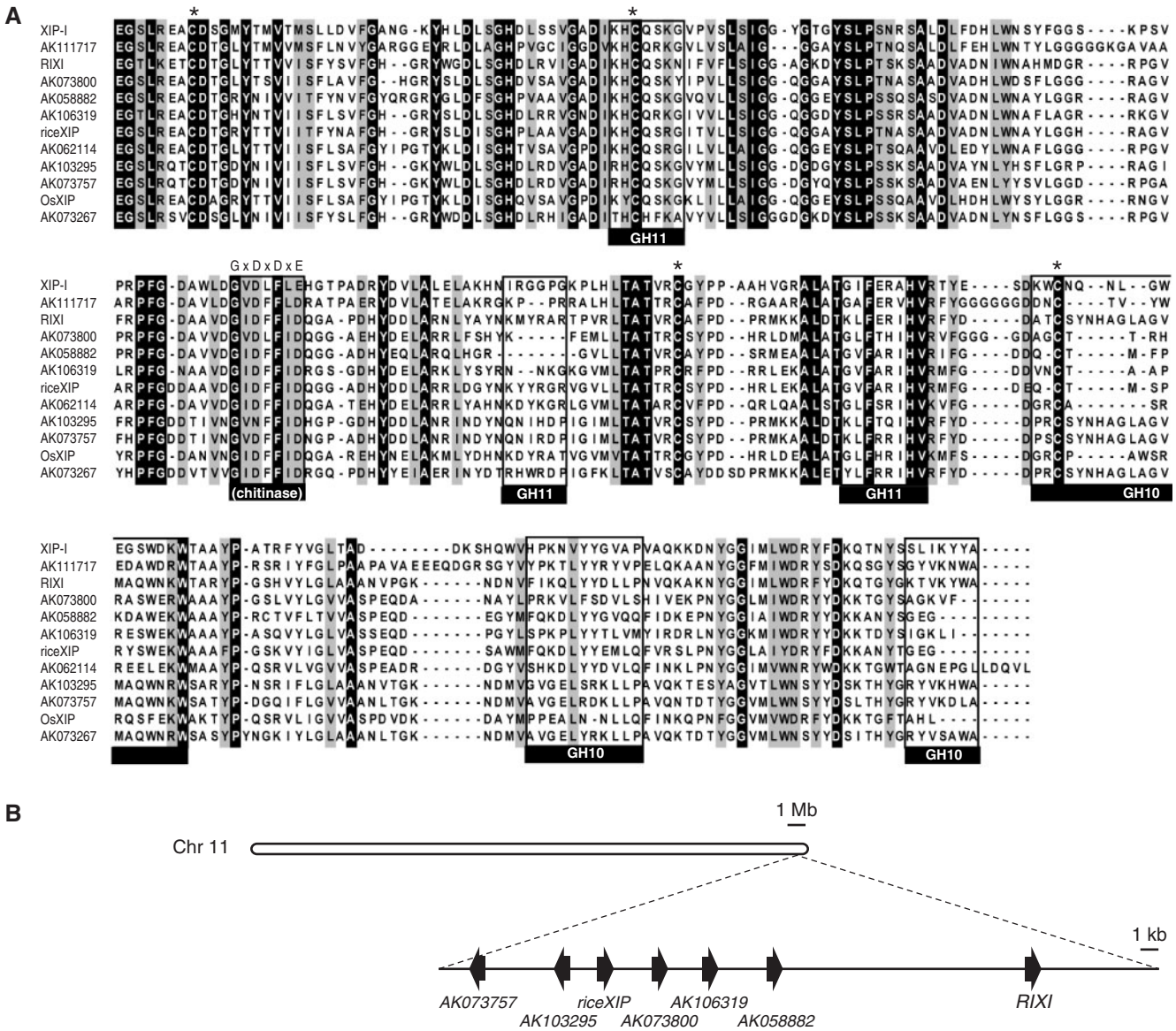

Fig. 9 The analysis of putative XIP-type xylanase inhibitor members in rice. (A) Amino acid sequence alignment of XIP-I and putative XIP-type xylanase inhibitor members in rice. Identical amino acids are shaded black and similar ones are shaded gray. Four cysteine residues are indicated by asterisks. Sequences in the box show residues corresponding to the chitinase active site and involved in complexing with a GH10 or GH11 xylanase. DDBJ database accession numbers are indicated on the left in order of identity with XIP-I. (B) Composition of the XIP-family gene cluster on chromosome 11. The arrowhead indicates the directions of the open reading frames. Bars indicate 1 Mb and 1 kb for the overview and detail of chromosomes, respectively.

biological Encyclopedia; KOME) (http://cdna01.dna.affrc.go.jp/cDNA/CDNA_main_front.html) (Fig. 9A). These genes share 42–56% amino acid sequence identity with the mature XIP-I and have structural similarities to the typical XIP-type xylanase inhibitor, indicating that a large number of XIP-family genes may exist in rice. In contrast, it was proposed that wheat has few XIP genes, and XIP-I actually plays a major role (Igawa et al. 2005). Thus, we speculate that a large number of genes may be attributable to the specialized features of XIP-type xylanase inhibitors in rice, i.e. OsXIP and riceXIP may specialize in defense towards pathogens in rice root, whereas RIXI may contribute to a basal pre-existing defense mechanism against pathogens in shoot.

We also found that seven of the rice XIP-family genes were clustered in tandem on chromosome 11 (Fig. 9B). This gene clustering is recognized in some pathogenesis-related genes such as protease inhibitor genes and disease resistance genes (Song et al. 1997, Qu et al. 2003), and has been assumed to contribute to produce diversity of genes

with recombination between chromosomes. A large number of xylanase inhibitor genes and the types of specificity toward xylanases may exist in order to address the various xylanases of external origin as also shown for protease inhibitors (Qu et al. 2003). Thus, it may be important for pathogen resistance for these genes to be expressed cooperatively. Considering that the expression patterns of *OsXIP* and *riceXIP* resemble each other, the expression of some XIP-family genes could be regulated by the same signal transduction component. The clarification of the molecular mechanism of XIP-family gene expression may lead to the generation of pathogen-resistant plants.

Materials and Methods

Plant materials and chemical treatment

Rice (*Oryza sativa* cv. Nipponbare) seeds were sown in water and grown at 30°C using a 16 h light and 8 h dark regime for 10–14 d. Rice seedlings were incubated with 20 mM AsA, 20 mM L-Gal, 20 mM citrate, 100 mM sodium chloride and 20 mM DHA solution for 3 d at 30°C. Each solution was adjusted to pH 5.4–5.6 with KOH. Distilled water was used as control. For wound stress, rice seedlings were cut into 5 mm width and floated on distilled water. Phytohormone treatment was carried out by the submerged method; rice seedlings were submerged in 200 μM ABA, 5 mM SA or 200 μM MeJA solution for 12, 24 and 48 h, and then shoots and roots were harvested separately. All phytohormones were dissolved in 50 μl of dimethylsulfoxide (DMSO) and added to the culture water. Distilled water containing 50 μl of DMSO was used for control seedlings. Each solution was adjusted to pH 5.4–5.6 with KOH.

Microarray analysis

A Rice 22K Custom Oligo DNA Microarray kit was used which contains approximately 22,000 oligonucleotides synthesized based on the sequence data of the rice full-length cDNA project (Kikuchi et al. 2003). Total RNA was purified from rice seedlings incubated with 20 mM sodium ascorbate solution or distilled water for 3 d using a Total RNA Mini kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA) and the yield and RNA purity determined spectrophotometrically. Integrity was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (200 ng) was labeled with Cy3 or Cy5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Fluorescently labeled targets were hybridized to Agilent Rice 22K Custom Oligo DNA Microarrays. Hybridization and wash processes were performed according to the manufacturer's instructions and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for the image analysis and data extraction processes.

RNA preparation and Northern blot analysis

Total RNAs were isolated from rice seedlings by the guanidine thiocyanate (GTC) method for Northern blot analysis. Digoxigenin (DIG)-labeled RNA probes were prepared as follows. First, PCRs were performed with cDNA clones (clones J033068H05 and 002-108-B03) obtained from the Rice Genome Resource Center as the template. The following oligonucleotide pairs were used for PCR: 5′-AGCCCAACTTCGGAGGCGTCAT-3′ (forward) and 5′-CTCAAAGCTCTTTATTATTTCACCCGAGG-3′ (reverse) for amplification of *OsXIP* (*AK073843*); and 5′-TTAGCTAGTGATCATCGTCTTTGC-3′ (forward) and 5′-ATTACAGCCAGTGATGATTAAT-3′ (reverse) for *riceXIP*. The approximately 300 and 250 bp PCR fragments covering the *OsXIP* and *riceXIP* 3′-untranslated region, respectively, were inserted into pGEM-T Easy Vector (Promega, Madison, WI, USA) and used as template for the RNA probe. In the case of *RIXI*, a cDNA clone (J033111J19) was digested with *Sca*I, and approximately 170 bp covering the *RIXI* 3′-untranslated region was used as template. The synthesis of DIG-labeled RNA probe was performed according to the DIG RNA Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA) protocol. An aliquot of 5 μg of total RNA was denatured and electrophoresed in RNA gel containing 1% agarose before blotting on to a nylon membrane (Hybond N^+; Amersham Biosciences, Buckinghamshire, UK). The membrane was UV cross-linked and pre-hybridized in hybridization buffer (DIG Easy Hyb; Roche Diagnostics) for 1 h at 68°C. Hybridization was performed in hybridization buffer for 12 h at 65°C. The membrane was washed with 2 × SSC containing 0.1% (v/v) SDS at room temperature for 10 min, and twice with 0.1 × SSC containing 0.1% SDS at 68°C for 15 min. The detection was carried out using the DIG detection method (Roche Diagnostics).

Measurement of ascorbic acid content

Frozen and homogenized rice seedlings (0.2 g fresh weight) were suspended in 1 ml of cold 6.0% (v/v) $HClO_4$ and centrifuged at 19,000 × *g* for 10 min at 4°C. An aliquot of 50 μl of the obtained extract was added to 445 μl of 200 mM succinate buffer (pH 12.7, adjusted with KOH) in the spectrophotometer. The absorbance of the solution was recorded immediately and again 5 min after the addition of 2.5 U of ascorbate oxidase from *Cucurbita* sp. (Wako, Japan) at 532 nm. The AsA concentration was calculated by comparison with a standard curve.

Measurement of electrolyte leakage

The rice roots were rinsed with deionized water and incubated in a tube containing 15 ml of deionized water. After incubation for 24 h, the electrical conductivity of the bathing solution was measured using an electrical conductivity meter (CM-21P; DKK·TOA, Japan). Data are expressed as a percentage of total ions, which was determined after killing roots by autoclaving.

Estimate of lipid peroxidation

The comparative rates of lipid peroxidation were assayed by determining the levels of thiobarbituric acid-reactive substances (TBARS) which are a product of lipid peroxidation in root tissue. TBARS were assayed by the thiobarbituric acid reaction. Frozen and homogenized rice roots (0.1 g) were suspended in 200 μl of 0.1% (w/v) trichloroacetic acid (Nacalai Tesque, Kyoto, Japan). An 800 μl aliquot of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid (Nacalai Tesque) was added to the sample. The mixture was boiled for 30 min, cooled and centrifuged at 19,000×*g* for 10 min at 4°C. The absorbance of the supernatant was read at 532 nm.

Measurement of hydrogen peroxide content

Hydrogen peroxide was determined according to the method of Patterson et al. (1984). Frozen and homogenized rice roots

(0.17 g) were added to a microcentrifuge tube containing 0.07 g of activated charcoal and 800 µl of 5% (w/v) trichloroacetic acid. The homogenate was adjusted to pH 8.4 with ammonia solution and centrifuged at 19,000×*g* for 5 min at 4°C. The supernatant was divided into aliquots of 250 µl. To one of these, 20 µg of catalase was added as a blank. The blank was kept at 25°C for 10 min, together with the other aliquots without catalase. A 250 µl aliquot of colorimetric reagent was added to both series. The colorimetric reagent was made daily by mixing equal volumes of 4-(2-pyridylazo) resorcinol (disodium salt) (Wako, Japan) and titanium potassium oxalate dehydrate (Wako, Japan) solutions, both 0.6 mM in water. The reaction solution was incubated for 1 h at 45°C. Absorbance was determined at 508 nm using a spectrophotometer. The hydrogen peroxide concentration was calculated by comparison with a standard curve.

Expression of OsXIP proteins in E. coli

To obtain the recombinant OsXIP protein without the signal peptide (mature form), a cDNA clone (J033068H05) was amplified with the oligonucleotides 5′-CATGCCATGGCCGTGTCGG CGACGTC-3′ (forward) and 5′-CCGCTCGAGCAGGTGAGC CGTGAAGC-3′ (reverse) linking *Nco*I and *Xho*I sites, respectively, and ligated to pET19b with the His tag removed. This expression vector was named pETOsXIP. pETOsXIP and pET19b were introduced into Origami(DE3) (Novagen, Darmstadt, Germany). A 50 ml aliquot of Luria–Bertani (LB) medium was inoculated with 0.5 ml of overnight culture derived from a single colony of Origami(DE3)[pETOsXIP] or Origami(DE3)[pETOs19b]. The cultures were grown at 37°C (180 r.p.m.) until an A_{600nm} 0.5, and then transferred to 25°C. After addition of IPTG (0.1 mM final concentration) and incubation for 12 h at 220 r.p.m., cells were harvested by centrifugation (10,000×*g*, 5 min, 4°C), resuspended in McIlvaine's buffer (0.1 M citric acid/0.2 M Na_2HPO_4, pH 6.0), sonicated (10 × 21 s), and centrifuged (20,000×*g*, 10 min, 4°C). The soluble protein extracts were concentrated by Ultrafree-MC 10,000 NMWL Filter Unit (Millipore, Bedford, MA, USA). Their concentration was determined by protein assay (Bio-Rad Laboratories) and adjusted to 3.4 µg µl^{-1}. The same quantity of soluble protein extracts was tested for xylanase inhibitor activity.

Meaturement of (1,4)-β-xylanase activity

Xylanase activity was determined according to the 3,5-dinitrosalicylic acid method of Bailey et al. (1992). Xylanase preparations (20 µl) were added to 1% (w/v) birchwood (1,4)-β-xylan (Fluka, Neu-Ulm, Germany) (180 µl) solubilized in McIlvaine's buffer (pH 6.0) and incubated at 30°C for 5 min. The relationship between xylanase concentration and activity was checked to ensure linearity of the reaction. One unit of xylanase activity was defined as the amount of protein that released 1 µmol xylanase per min at 30°C and pH 6.0. Xylanase inhibitor activity was determined by measuring the activity of GH11 xylanase from *T. viride* (Sigma-Aldrich, St Louis, MO, USA), *T. longibrachiatum* (XYLANASE M3; Megazyme, Bray, Ireland) and *A. niger* (XYLANASE M4; Megazyme) in the presence and absence of recombinant OsXIP. Reactions containing *T. viride* xylanase (5 µl, 39 ng) or *T. longibrachiatum* xylanase (5 µl, 667 ng), pre-incubated (5 min, 30°C) with soluble protein extracts (15 µl, 51 µg) from transformed *E. coli*, were assayed for xylanase activity.

Acknowledgments

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