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RESEARCH PAPER

Molecular cloning of a bifunctional β-xylosidase/α-L-arabinosidase from alfalfa roots: heterologous expression in *Medicago truncatula* and substrate specificity of the purified enzyme

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Abstract

Glycoside hydrolases are often members of a multigene family, suggesting individual roles for each isoenzyme. Various extracellular glycoside hydrolases have an important but poorly understood function in remodelling the cell wall during plant growth. Here, MsXyl1, a concanavalin A-binding protein from alfalfa (*Medicago sativa* L.) belonging to the glycoside hydrolase family 3 (β-D-xylosidase branch) is characterized. Transcripts of *MsXyl1* were detected in roots (particularly root tips), root nodules, and flowers. *MsXyl1* under the control of the CaMV 35S promoter was expressed in the model legume *Medicago truncatula* (Gaertner). Concanavalin A-binding proteins from the transgenic plants exhibited 5–8-fold increased activities towards three *p*-nitrophenyl (PNP) glycosides, namely PNP-β-D-xyloside, PNP-α-L-arabinofuranoside, and PNP-α-L-arabinopyranoside. An antiserum raised against a synthetic peptide recognized MsXyl1, which was processed to a 65 kDa form. To characterize the substrate specificity of MsXyl1, the recombinant protein was purified from transgenic *M. truncatula* leaves by concanavalin A and anion chromatography. MsXyl1 cleaved β-1,4-linked D-xylo-oligosaccharides and α-1,5-linked L-arabino-oligosaccharides. Arabinoxylan (from wheat) and arabinan (from sugar beet) were substrates for MsXyl1, whereas xylan (from oat spelts) was resistant to degradation. Furthermore, MsXyl1 released xylose and arabinose from cell wall polysaccharides isolated from alfalfa roots. These data suggest that MsXyl1 is a multifunctional β-xylosidase/α-L-arabinofuranosidase/α-L-arabinopyranosidase implicated in cell wall turnover of arabinose and xylose, particularly in rapidly growing root tips. Moreover, the findings of this study demonstrate that stable transgenic *M. truncatula* plants serve as an excellent expression system for purification and characterization of proteins.

Key words: Alfalfa (*Medicago sativa*), α-L-arabinofuranosidase, α-L-arabinopyranosidase, glycoside hydrolase family 3, heterologous expression, *Medicago truncatula*, β-xylosidase.

Introduction

The most abundant and technically useful biopolymers on the earth derive from plant cell walls. Polysaccharides such as cellulose, acidic polysaccharides (pectin), xyloglucans, and arabinoxylans are major structural components of the primary plant cell wall. Plant growth and plant developmental processes (e.g. fruit ripening and cell

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Abbreviations: CaMV, cauliflower mosaic virus; ConA, concanavalin A; DAP, diphenylamine-aniline-phosphoric acid; GC-MS, gas chromatography–mass spectrometry; GH, glycoside hydrolase; PNP, *p*-nitrophenyl; TLC, thin-layer chromatography; TMS, per-*O*-(trimethylsilyl).

elongation) require a co-ordination of complex processes regulating synthesis and degradation of these polysaccharides at high turnover rates. Extracellular glycosyltransferases, glycoside hydrolases (GHs), and nonenzymatic expansin proteins have an important but poorly understood function in these processes (Fry, 2004; Cosgrove, 2005; Farrokhi *et al*., 2006). In pea plants, for example, a role for xyloglucan endotransglycosylases and xyloglucan oligosaccharides in cell elongation has been proposed (Takeda *et al*., 2002). Remodelling of the cell wall is also an important step in the infection process of rhizobia, nitrogen-fixing bacteria that infect roots and induce the formation of root nodules on legumes. Many host proteins synthesized in response to rhizobia are glycosylated cell wall proteins. These 'early nodulins' are candidates to play a symbiotic role in the interface between rhizobia and the host legume (Brewin, 2004).

Cell walls of plants contain a large set of GHs, such as cellulases, glucanases, xylanases, xylosidases, arabinofuranosidases, galactosidases, and glucuronidases (Minic and Jouanin, 2006). Interestingly, pathogenic, saprophytic, and herbivorous organisms secrete related enzymes with similar activities, and most of these GHs play a crucial role in biomass breakdown and microbial fermentation processes (Faure, 2002; Shallom and Shoham, 2003). So far, the majority of these cell wall-related GHs has been isolated from bacteria and fungi, whereas precise biochemical properties of many cell wall-degrading plant enzymes remain largely undefined, particularly those of legumes. This is, at least in part, due to the fact that heterologous expression of plant GHs in prokaryotes and yeast failed in many cases, making it necessary to use plants as an expression system. Recently, the legume *Medicago truncatula* has become a popular research instrument for investigating various aspects of legume biology (http://medicago.org/). Its genome is now being sequenced by an international consortium. For certain lines of the model legume *Medicago truncatula*, efficient *Agrobacterium*-mediated transformation protocols have been developed. Stable transgenic *M. truncatula* plants are obtained after somatic embryogenesis-mediated plant regeneration (Hoffmann *et al*., 1997; Trinh *et al*., 1998; Cosson *et al*., 2006).

GHs are grouped into more than 100 families based on the nomenclature of the CAZy database, which describes families of structurally related catalytic and carbohydrate-binding modules (http://afmb.cnrs-mrs.fr/CAZY/; Davies *et al*., 2005). In this classification, plant enzymes with β-xylosidase (EC 3.2.1.37) or α-L-arabinofuranosidase (EC 3.2.1.55) activities belong to GH families 3, 43, and 51. Family 3 enzymes from the β-xylosidase branch are exo-type glycosidases that release xylose and arabinose from oligomeric or polymeric substrates. Structurally related enzymes from the same GH family may have different substrate specificities, whereas a given enzyme may hydrolyse a set of diverse substrates. Moreover, the complexity is further increased by the fact that GHs in plant genomes often belong to a multigene family. The diversity of these enzymes may reflect their importance in specific biological processes during different life stages of a plant, but this diversity makes it difficult to attribute a given enzyme activity to a protein encoded by a specific cDNA.

Medicago sativa, commonly known as alfalfa or lucerne, is the world's major forage legume. The cell wall composition and degradability of alfalfa fibres has been studied in the context of fodder quality (Sheaffer *et al*., 2000; Grabber *et al*., 2002; Jung and Engels, 2002). Recently, various cell wall-localized alfalfa proteins have been identified by a proteomic approach (Watson *et al*., 2004). Previous work on GHs from alfalfa indicated that *N*-glycosylated root proteins binding to concanavalin A (ConA) hydrolysed and inactivated lipo-oligosaccharide signals (Nod factors) from *Sinorhizobium meliloti* (Staehelin *et al*., 1994, 1995). The observation was made then that ConA-binding root proteins exhibited various activities towards *p*-nitrophenyl (PNP) glycosides. In an attempt to purify Nod factor-cleaving enzymes from alfalfa, a ConA-binding 65 kDa protein was purified from the roots and the corresponding cDNA cloned. The encoded protein, called MsXyl1, is an enzyme belonging to the GH family 3. The *MsXyl1* gene is strongly expressed in roots of germinating seedlings, especially in root tips. The enzyme exhibits bifunctional β-xylosidase/α-L-arabinosidase activity, suggesting a function in cell wall turnover of growing roots.

Materials and methods

Plant material

Seeds of alfalfa (*Medicago sativa* L. cv. Sitel) (Tourneur, Montauban, France) were surface-sterilized and allowed to germinate on inverted agar plates as described (Staehelin *et al*., 1994). For production of young alfalfa roots, surface-sterilized seeds (incubated for 2 d in the dark at 4 °C) were dispersed over a metal grid (1.6 mm mesh), which covered plastic Petri dishes filled with deposit-free Jensen medium (van Brussel *et al*., 1982). Plants were left to germinate in the dark at 24 °C and young roots which had grown through the metal grid were harvested after 2–3 d. *Medicago truncatula* (Gaertner) R108-1 plants were used for plant transformation (Hoffmann *et al*., 1997). For germination, seeds were scarified and surface-sterilized by treatment with diluted commercial bleach (≈0.35% active chlorine). After thorough washing with sterile water, seeds were left to germinate on water agar plates in the dark.

Alfalfa and *M. truncatula* seedlings were transferred to pots and cultivated either in a growth chamber at 24 °C (light period 16 h d^{-1}, photon flux ≈300 mol m^{-2} s^{-1}) or in a temperature-controlled greenhouse. Nitrogen-fixing nodules from *M. sativa* cv. Sitel were obtained after inoculation of roots with a culture of *Sinorhizobium meliloti* strain Rm41. Nodulated plants were cultivated in an aeroponic system with a nutrient solution containing 0.25 mM KNO_3.

Protein purification

Young alfalfa (cv. Sitel) roots, varying in length from 2 cm to 3 cm, were ground in a mortar with a pestle using quartz sand and 4 ml g^{-1} root FW buffer A [5 mM MES (K^+), pH 6, 0.1% (v/v) mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 1% (v/v) Triton X-100, 50 mM NaCl, and 0.1 mM each $MgCl_2$, $MnCl_2$, and $CaCl_2$]. After filtration through nylon gaze and centrifugation (4000 *g* for 20 min), the supernatant was dialysed against 5.0 l of buffer B (=buffer A without Triton X-100) for 2 h and loaded on a 4 ml column of ConA insolubilized on 4% beaded agarose, typ III-AS (Sigma-Aldrich, St Louis, MO, USA) equilibrated with buffer B. After washing the column with buffer B, glycoproteins binding on ConA were eluted with 15 ml of buffer C [5 mM phosphate (Na^+) buffer, pH 6.8, 0.2 M methyl-α-D-mannopyranoside]. A 65 kDa protein was further purified on a 1-ml hydroxyapatite column (Bio-Gel HTP Gel, Bio-Rad, Hercules, CA, USA) equilibrated with buffer D [5 mM phosphate (Na^+) buffer, pH 6.8]. After washing with 5 ml of buffer D, the 65 kDa glycoprotein was eluted with 15 ml of buffer E [50 mM phosphate (Na^+) buffer, pH 6.8]. The sample was then loaded on a HITRAP Q column (Amersham Biosciences, Uppsala, Sweden), which was equilibrated with buffer E. The protein fraction that passed through the column was then equilibrated with buffer F [50 mM TRIS (Cl^-) buffer, pH 7.6] using a Centricon 30 concentrator (Amicon, Millipore Corporation, Bedford, MA, USA). The sample was then applied to a new HITRAP Q column that had been rinsed with buffer F. The column was washed with the same buffer and the 65 kDa glycoprotein was finally eluted with 5 ml of buffer G [50 mM TRIS (Cl^-) buffer, pH 7.6, 50 mM NaCl]. Highest yields of the 65 kDa glycoprotein were obtained when buffers were supplemented with 10 μg ml^{-1} purified cytochrome *c* from horse heart (Sigma-Aldrich). The 65 kDa glycoprotein was concentrated with a Microcon YM-50 centrifugal filter device (Amicon, Millipore) and used for peptide sequencing. All purification steps were carried out at 4 °C.

Purification of MsXyl1 from *M. truncatula* was achieved with a homozygous line strongly expressing *MsXyl1* (T3 descendants of line L1). Leaves were harvested when plants were 5–7-weeks-old, freeze-dried, and stored at –20 °C until they were used. The protein was purified as described above with the following modifications: (i) the purification step on the hydroxyapatite column was omitted; (ii) buffers were adjusted by dialysis (10 kDa cut-off) instead of using Centricon concentrators; (iii) buffers were not supplemented with cytochrome *c*.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and protein content

Protein denaturating SDS-PAGE was performed on 10% or 12% polyacrylamide gels. Gels were stained either with Coomassie Brilliant Blue R-250 or with silver nitrate (Ausubel *et al*., 1991). Protein concentrations were determined by the method of Bradford (1976).

Peptide sequencing

After 10% SDS-PAGE, the purified 65 kDa protein from alfalfa was electroblotted on a polyvinylidene difluoride membrane and then incubated in 200 μl 100 mM TRIS (Cl^-) buffer, pH 8.8, containing 0.4 μg endolysin C and 0.03% SDS at 35 °C for 18 h. The peptides formed were separated by HPLC (DEAE-C18 column, gradient acetonitrile/TFA 0.1%). Two peptides were re-purified by HPLC and 15 amino acids sequenced by the automated Edman degradation method (model 610A sequencer; Applied Biosystems, Foster City, CA, USA).

Isolation of cDNAs

Two DNA fragments with a size of about 1500 bp were amplified from genomic DNA (cv. Nagyszénási) by a PCR reaction with the primers 5′-CARCGITAYACITTYGAYGC-3′ and 5′-GGNACRAAIGCIGTIARNCCYTG-3′ (I=inosine, Y=C/T, R=A/G, N=C/T/A/G). Fragments from the amplified DNA were then used as probes to screen a young nodule λZAP phage cDNA library of *M. sativa* ssp. *varia* line A2. A DNA fragment from an isolated clone served as a probe for a second screen. Sequence analysis of these clones (and corresponding subclones) indicated that two related cDNAs had been isolated. The cDNA of the *MsXyl1* gene (GenBank accession number EF569968) was deduced from a nearly full-length clone lacking the terminal ATG. The cDNA of the related *MsXyl2* gene (GenBank accession number EF569969) corresponded to the nucleotide sequence of the various full-length clones isolated.

Expression of MsXyl1 in M. truncatula

An intron (≈1 kb) was inserted at position 438 of the *MsXyl1* cDNA and a sequence coding for six histidine residues (6His-tag) introduced by PCR at the COOH-terminal end. The construct was then cloned into the multiple cloning site of pISV2678. The binary vector pISV2678 is a derivative of pGPTV-BAR (Becker *et al*., 1992) with a double cauliflower mosaic virus (CaMV) 35S promoter and a translational enhancer sequence from pBI-426 (Datla *et al*., 1991). *Agrobacterium*-mediated transformation of *M. truncatula* was performed according to previously published protocols (Hoffmann *et al*., 1997; Trinh *et al*., 1998). Briefly, leaf discs of *M. truncatula* R108-1 were infiltrated with a suspension of *A. tumefaciens* strain EHA 105 carrying the binary vector. The explants were then cultivated on SH3a medium supplemented with 400 mg l^{-1} augmentin and 3 mg l^{-1} glufosinate (phosphinothricin). The calli formed were further cultivated on SH9 medium. Regenerated plantlets were transferred into sand and cultivated in a greenhouse. The seeds obtained were left to germinate and transformed plants were selected after spraying with 80 mg l^{-1} glufosinate. Transgenic plants expressing *MsXyl1* and producing MsXyl1 protein were further propagated.

RNA and DNA blot analysis

Plant RNA from alfalfa and *M. truncatula* was isolated using the RNeasy kit (Quiagen, Hilden, Germany) and used for northern blot analysis. The membranes were hybridized with a ^{32}P-labelled probe derived from the sequenced *MsXyl1* cDNA clone (*Sst*I–*Hin*dIII fragment). To standardize RNA loading, blots were also hybridized with DNA from the constitutively expressed *Msc27* gene (accession number X98618). For Southern blot analysis, *Eco*R1-digested genomic DNA was hybridized with a ^{32}P-labelled DNA probe derived from *MsXyl1*.

Antiserum against MsXyl1 and immune blots

The peptide CDSVEVLYKDQHYTK (Fig. 1B) was chemically synthesized and conjugated to the carrier keyhole limpet haemocyanin (Syntem, Nîmes, France). The conjugated peptide was then used to immunize a New Zealand rabbit. For immune blots, proteins were separated by SDS-PAGE and then blotted onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). To detect MsXyl1 protein, the membranes were incubated with the antiserum raised against the conjugated peptide (1:800 dilution) and then with a horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum (Boster, Wuhan, China). Immune blots were developed with 3,3′-diamino-benzidine (Boster) as the chromogen.

Expression of MsXyl1 in vitro

In vitro expression of *MsXyl1* was achieved with the T3–TNT coupled reticulocyte lysate system according to the manufacturer's instructions (Promega France). [^{35}S]Methionine-labelled proteins

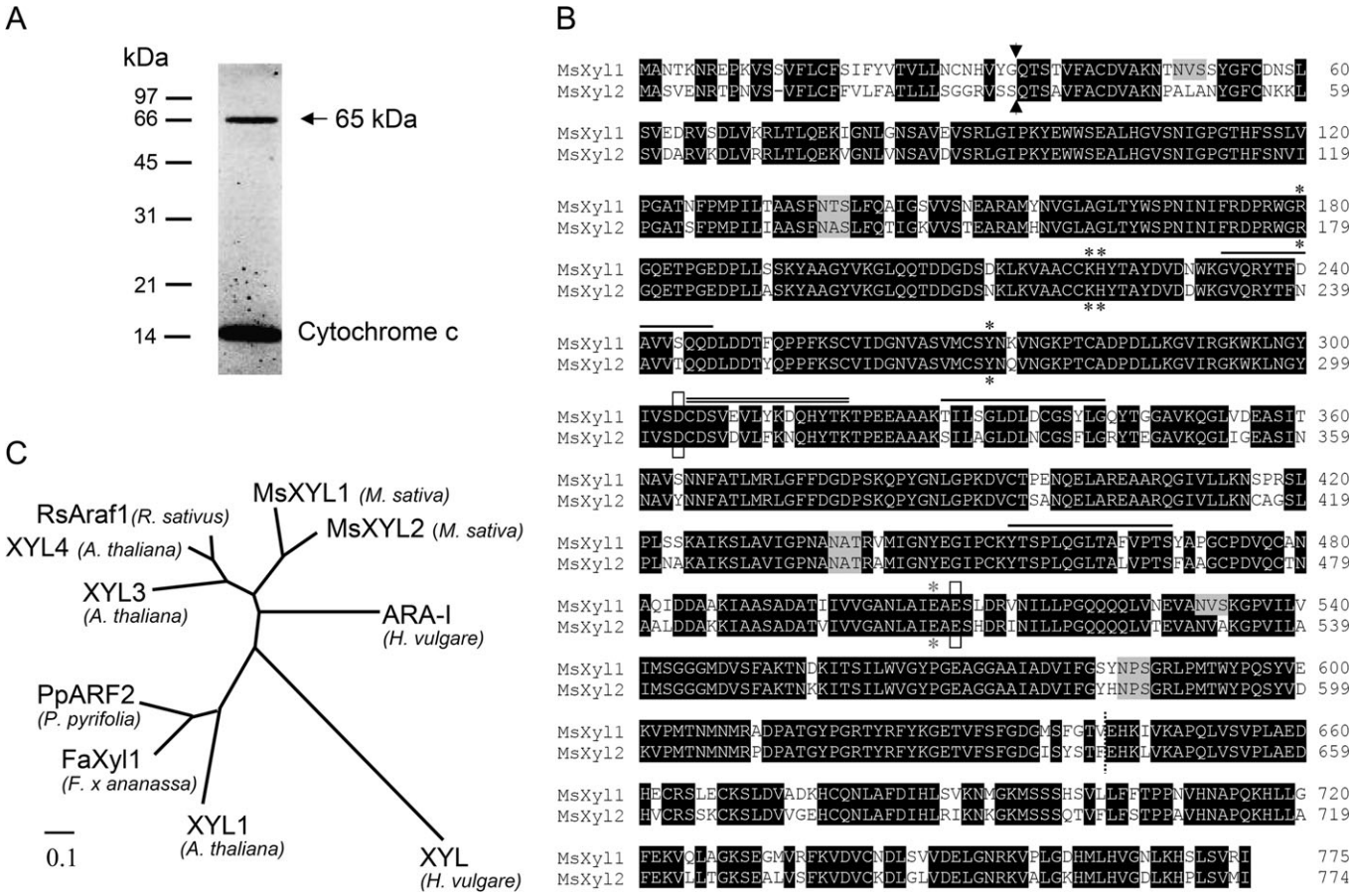

Fig. 1. Purification of the 65 kDa protein from alfalfa and cloning of the corresponding cDNA. (A) SDS-PAGE of the 65 kDa protein (MsXyl1) purified from roots of *M. sativa* (cv. Sitel). Proteins were stained with Coomassie Brilliant Blue R-250. Cytochrome *c* from horse heart was added to increase the purification yield. (B) Amino acid sequences of MsXyl1 and MsXyl2 deduced from cDNA clones of an *M. sativa* (ssp. *varia*) library. Amino acid residues are numbered from the first methionine. Identical amino acids are highlighted in black. The three peptides sequenced from the 65 kDa protein completely matched the corresponding sequences of the MsXyl1 protein (solid line above amino acid residues). Arrowheads indicate the putative cleavage site between the predicted signal peptide and the NH_2-terminus of the processed protein. The dotted vertical line marks a putative post-translational processing site in the COOH-terminal region. Lightly shaded amino acid residues indicate potential *N*-glycosylation sites. The catalytic nucleophile (Asp-304) and the putative catalytic acid/base (Glu-509) are boxed. Grey asterisks mark an alternative catalytic acid/base (Glu-507). Black asterisks indicate conserved amino acids likely to be involved in substrate binding. A double line indicates the sequence of the chemically synthesized peptide used for raising a rabbit antiserum against MsXyl1. (C) Unrooted phylogenic tree of plant family 3 GHs (β-D-xylosidase branch). Amino acid sequences were aligned with the Clustal W algorithm and a tree was then constructed with the Fitch–Margoliash method. The horizontal bar represents a distance of 0.1 substitutions per site. Accession numbers of proteins: BAE44362, RsAraf1 from *Raphanus sativus* (Kotake *et al*., 2006); BAB11424, XYL4 from *Arabidopsis thaliana* (Minic *et al*., 2004); BAB09531, XYL3 from *A. thaliana* (Minic *et al*., 2006); AAK38481, ARA-I from *Hordeum vulgare* (Lee *et al*., 2003); BAD98523, PpARF2 from *Pyrus pyrifolia* (Tateishi *et al*., 2005); AAS17751, FaXyl1 from *Fragaria×ananassa* (Bustamante *et al*., 2006); BAB09906, XYL1 (encoded by the *AtBXL1* gene) from *A. thaliana* (Minic *et al*., 2004); AAK38482, XYL from *Hordeum vulgare* (Lee *et al*., 2003).

were separated by SDS-PAGE. The gel was then exposed to an X-ray film and autoradiographed.

Substrates for hydrolytic assays

The aryl glycosides PNP-β-D-xyloside, PNP-α-L-arabinofuranoside, PNP-α-L-arabinopyranoside, PNP-β-D-glucopyranoside, PNP *N*-acetyl-β-D-glucosaminide, and xylan from oat spelts were purchased from Sigma-Aldrich (St Louis, MO, USA). Ortho-nitrophenyl-β-D-galactopyranoside was obtained from Serva (Heidelberg, Germany). 1,4-β-D-Xylotetraose, 1,5-α-L-arabinotetraose, arabinoxylan from wheat (medium viscosity), and arabinan from sugar beet were provided by Megazyme International (Bray, Ireland).

Polysaccharides from alfalfa cell walls were isolated according to Fagard *et al*. (2000). Briefly, roots of alfalfa (3-d-old) were incubated in 90% ethanol at 65 °C for 30 min and then ground with a mortar and pestle. The homogenate was centrifuged at 2300 *g* for 15 min. The pellet was washed with ethanol several times, incubated overnight in methanol/chloroform (2:3, v/v) on a shaker, and finally washed again with ethanol. The remaining pellet was dried at 80 °C and defined as cell wall material.

Enzyme assays with aryl glycosides

The reaction mixture contained 1 mM of a given aryl glycoside, 20 mM sodium acetate buffer (pH 5.0), and 20 µl of enzyme in a total volume of 150 µl. The reaction was carried out at 37 °C for 1 h and stopped with 850 µl of 0.2 M sodium carbonate. Samples were photometrically analysed at 405 nm and the degree of hydrolysis estimated from a calibration curve. For kinetic studies,

the concentration of aryl glycosides varied between 0.1 mM and 4 mM. Michaelis–Menten constants (K_m) and catalytic rate constants (k_{cat}) were calculated from Lineweaver–Burk plots.

Reactions with oligosaccharidic and polysaccharidic substrates were performed in a similar way. Where indicated, xylanase from *Thermomyces lanuginosus* (Sigma-Aldrich) was added to the reaction mixture. Thin-layer chromatography (TLC) was used to analyse the cleavage products released. After incubation, samples were centrifuged (15 000 *g* for 15 min) and the supernatants were supplemented with ethanol to reach a final concentration of 80% (v/v). After an additional centrifugation step (15 000 *g* for 15 min), supernatants were dried in a speed-vac evaporator and dissolved in 10 µl of water. Samples (2 µl) were fractionated on TLC plates (Silica gel 60 F_{254}; Merck, Darmstadt, Germany) with *n*-propanol:water (85:15, v/v) as mobile phase (Tateishi *et al*., 2005). Ethyl acetate: acetic acid:water (3:2:1, by vol.) (Lee *et al*., 2003) was used for analysis of cleavage products released from xylan and 1,4-β-D-xylotetraose. Sugars were visualized with diphenylamine-aniline-phosphoric acid (DAP) reagent (0.4 g diphenylamine, 0.4 ml aniline, and 2 ml phosphoric acid dissolved in 20 ml acetone). After spraying, plates were incubated at 85 °C for 10 min. Standards of 1,4-β-D-xylobiose and 1,4-β-D-xylotriose were obtained by incubation of 1,4-β-D-xylotetraose with xylanase and MsXyl1, respectively. Standards were then purified by TLC and the yield estimated with the DAP reagent using D-xylose as a reference.

Analysis of monosaccharides by gas chromatography–mass spectrometry (GC-MS)

Monosaccharides released from alfalfa cell walls were analysed as per-*O*-(trimethylsilyl) (TMS) methyl glycoside derivatives by GC-MS analysis. Samples were fractioned on TLC plates as described above, eluted from the silica gel with water and then dried with a speed-vac evaporator. TMS methyl glycosides were obtained according to Starke *et al*. (2000) and then analysed by GC-MS on a 30 m DB-5 fused silica capillary column equipped with a mass selective detector (gas flow rate, 1 ml min^{-1}; split ratio, 1:30; injection temperature: 230 °C; column temperature, (i) 100 °C for 3 min, (ii) gradient of 10 °C min^{-1} to 160 °C, (iii) gradient of 15 °C min^{-1} to 230 °C, and (iv) 230 °C for 3 min; ion source temperature, 220 °C; analysed mass range, m/z=28 to 497).

Bioinformatic analysis

Signal peptides were predicted with the SignalP 3.0 program (Bendtsen *et al*., 2004) and putative *N*-gycosylation sites with the NetNGlyc 1.0 program from the CBS prediction servers (http://www.cbs.dtu.dk/services/). Calculation of the theoretical isoelectric point and molecular weight were performed with the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html). For phylogenetic analysis, the amino acid sequences (without putative signal peptide) were aligned with the Clustal W algorithm and the unrooted radial tree was constructed by the Fitch–Margoliash method using the TreeView program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences belonging to the glycosyl hydrolase family 3 are listed in the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/). Sequence comparisons with databases were performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Molecular cloning of the MsXyl1 *gene*

During work aimed at the purification of Nod factor-degrading enzymes, a GH enzyme from young alfalfa roots was purified on ConA, hydroxyapatite, and HITRAP Q columns. When the sample was subjected to SDS-PAGE, the protein had an apparent molecular mass of approximately 65 kDa (Fig. 1A). N-terminal amino acid sequencing by the Edman degradation method was not conclusive. The protein was therefore digested with endolysin C to obtain internal peptides, and three of them were partially sequenced. Degenerated primers deduced from the sequenced peptides were used to amplify corresponding DNA, which served as a probe to screen a cDNA library of *M. sativa* ssp. *varia* line A2. Isolated cDNA clones were used for a second screen of the library. Two related cDNAs had been cloned. The deduced amino acid sequences are shown in Fig. 1B.

Sequence comparisons with protein databases (Blast algorithm) indicated similarities (identities in the range of 50–72%) with enzymes belonging to the β-D-xylosidase branch of GH family 3. The cloned cDNAs were therefore named *MsXyl1* and *MsXyl2* (*Medicago sativa* β-D-xylosidase 1 and 2). MsXyl1 and MsXyl2 are predicted to have signal peptides preceding the N-terminal sequence of the native protein, suggesting an extracellular localization. The three peptides sequenced from the purified 65 kDa protein completely matched the corresponding sequences of MsXyl1, indicating that a cDNA clone encoding the 65 kDa protein has been identified. The *MsXyl1* gene, however, appears to encode a protein of 775 amino acids with a calculated molecular mass of 83.858 kDa. Removal of the signal peptide would result in a protein with a molecular mass of 79.981 kDa. This discrepancy between the predicted mass from the cDNA and the purified 65 kDa protein suggests that a post-translational processing event occurred. The dotted vertical line in Fig. 1B marks a putative post-translational processing site in the COOH-terminal region as deduced from sequence alignment with a barley β-D-xylosidase, for which COOH-terminal processing has been described (Lee *et al*., 2003). The processed MsXyl1 protein (from Gln-35 to Val-642) has a calculated pI value of 5.0 and its predicted molecular mass is 65.233 kDa. Furthermore, asparagines predicted to be *N*-glycosylation sites are present in both sequences. The finding of these putative *N*-glycosylation sites is in agreement with the ability of the 65 kDa protein to bind to ConA. Amino acid sequence alignments with GH family 3 enzymes from barley (Varghese *et al*., 1999; Lee *et al*., 2003) suggest that Asp-304 of MsXyl1 is likely to be the catalytic nucleophile and Glu-509 (or Glu-507) the catalytic acid/base. As highlighted in Fig. 1B, the MsXyl1 and MsXyl2 sequences also contain several conserved amino acids, which are candidates to play a role in substrate binding (Hrmova *et al*., 2002).

Amino acid sequence alignments and phylogenetic analyses indicated that plant enzymes of the GH family 3 form two major branches. One branch seems to contain enzymes with β-D-glucan glucohydrolase activity.

The β-D-xylosidase branch consists of enzymes with proven or predicted β-D-xylosidase and/or α-L-arabinofuranosidase activity (Hrmova *et al*., 2002). Figure 1C shows an unrooted phylogenic tree of the β-D-xylosidase branch with MsXyl1, MsXyl2, and plant proteins with experimentally proven β-D-xylosidase and/or α-L-arabinosidase activity.

Characterization of MsXyl1

DNA blot analyses with genomic DNA and ^{32}P-labelled fragments of *MsXyl1* indicated that the alfalfa genome has several related sequences (Fig. 2A; data not shown). A screen of a genomic library of alfalfa (cv. Nagyszénási) resulted in isolation of various clones homologous to *MsXyl1* and *MsXyl2*. These genomic DNA sequences contained up to six introns of variable length (data not shown). Taken together, these findings suggest that tetraploid alfalfa plants possess various allelic variations of *MsXyl1* and *MsXyl2*.

To analyse the expression profile of *MsXyl1*, RNA was isolated from different tissues and used for northern blot analyses. As shown in Fig. 2B, *MsXyl1* was strongly expressed in young alfalfa roots. RNA from older roots showed significantly lower hybridization signals (not shown). High transcript levels were found in root tips, whereas RNA from roots with root tips removed only weakly hybridized with the *MsXyl1* probe. The gene was also expressed in root nodules containing symbiotic *S. meliloti* bacteria. Interestingly, transcripts of *MsXyl1* were found in flowers, but not in other aerial parts of the plant such as stems, hypocotyls, and leaves (Fig. 2B). Hybridization of RNA blots with an *MsXyl2* probe indicated high transcript levels in roots. By contrast to *MsXyl1*, transcripts of *MsXyl2* were detected in aerial parts of the plant, indicating a different expression pattern (not shown).

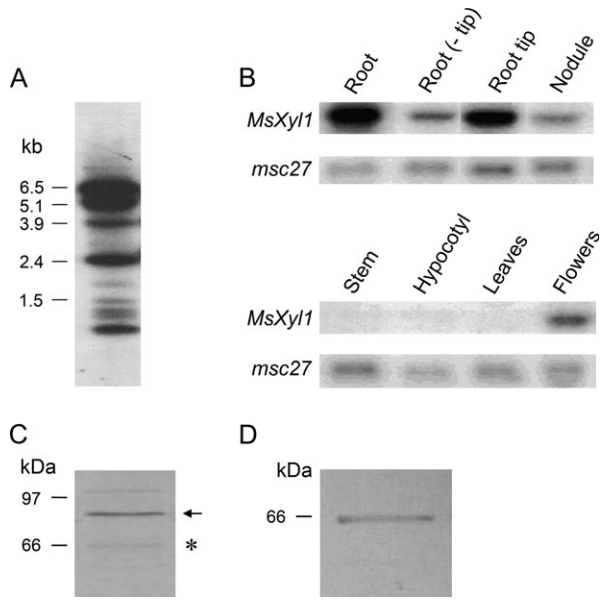

Fig. 2. Characterization of *MsXyl1* from alfalfa. (A) DNA blot with *Eco*R1-digested genomic DNA and a ^{32}P-labelled *MsXyl1* probe. (B) Expression of *MsXyl1* in different tissues. The RNA on the blot was hybridized with a ^{32}P-labelled *MsXyl1* probe. A second hybridization was performed with DNA from *Msc27*. Expression of *MsXyl1* was found in roots (3-d-old), nodules (30 d post-infection with *S. meliloti* strain Rm41), and flowers. Expression levels in root tips (zone up to emerging root hairs) were significantly higher than in roots with root tips removed [Root (–tip)]. (C) *In vitro* expression of *MsXyl1* with the T3–TNT-coupled reticulocyte lysate system. After SDS-PAGE, [^{35}S]methionine-labelled proteins were detected by autoradiography. (D) Immune blot with proteins from 3-d-old roots (corresponding to 45 mg FW) and an antiserum raised against a synthetic peptide of MsXyl1.

An *in vitro* expression experiment with a T3–TNT-coupled reticulocyte lysate system showed that the open reading frame of *MsXyl1* encodes a protein with a molecular weight of ≈84 kDa, indicating a non-processed form of the protein (Fig. 2C). Two additional faint bands were also seen, the lower protein (marked with an asterisk) was presumably formed by proteolytic degradation. Immune blot analyses provided evidence that MsXyl1 undergoes post-translational proteolysis in alfalfa roots. Figure 2D shows an immune blot with root proteins directly extracted in SDS sample buffer and an antiserum, which has been raised against a synthetic peptide sequence of MsXyl1. The antiserum recognized a protein with an apparent molecular weight of 65 kDa, whereas non-processed forms with a higher molecular weight were not detected. When alfalfa root tips were ground in MES buffer (pH 6) without Triton X-100 and then centrifuged, the 65 kDa protein was found in the pellet containing cell wall debris (immune blot not shown).

Expression of MsXyl1 in M. truncatula

To demonstrate an enzymatic activity for MsXyl1, *M. truncatula* plants were used as an expression system. *Agrobacterium*-mediated transformation of leaf discs followed by regeneration of calli to whole plants resulted in several stable transformants expressing MsXyl1 under the control of the CaMV 35S promoter. Northern blot analysis of wild-type *M. truncatula* indicated high levels of MsXyl1-related transcripts in roots, whereas hybridization signals with RNA from leaves were very faint (not shown). RNA isolated from leaves was therefore used to characterize transgenic *M. truncatula* plants expressing *MsXyl1*. Northern analysis with nine independent lines of the T1 generation showed high transgene expression in six lines (Fig. 3A). After selfing and further propagation, two homozygous lines (L1 and L3) were obtained. Immune blot analyses showed that plants from the T3 generation of L1 and L3 synthesized a protein of the expected molecular weight of ≈65 kDa (marked by an asterisk in Fig. 3B). Strongest detection signals were obtained for protein extracts from L1 leaves. In addition, a protein with a slightly lower apparent molecular weight of ≈64 kDa was recognized by the antiserum. This protein was also present in wild-type plants and is likely to be a GH family 3

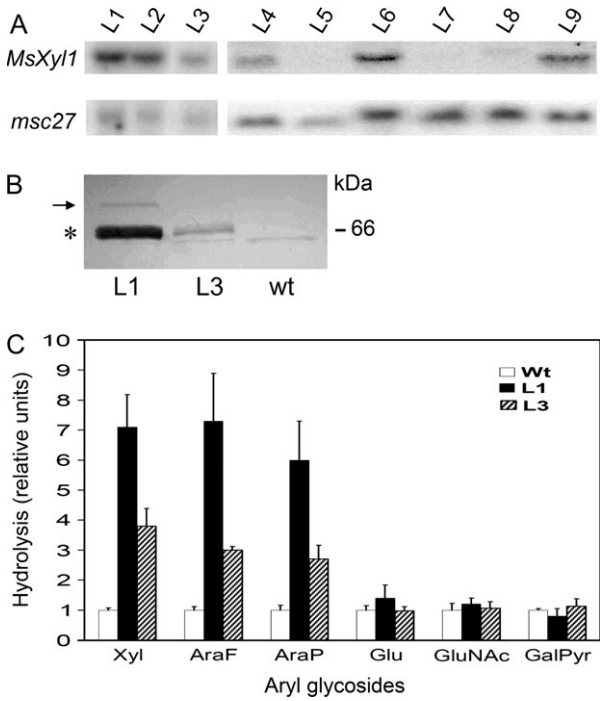

Fig. 3. Trangenic *M. truncatula* plants expressing *MsXyl1*. (A) RNA blots. Leaf RNA from nine independent lines (L1 to L9) was hybridized with a ^{32}P-labelled *MsXyl1* probe followed by a second hybridization with *Msc27*. (B) Immune blot analysis with the antiserum raised against the peptide CDSVEVLYKDQHYTK. Leaf proteins (corresponding to 32 mg FW) were isolated from the T3 generation of homozygous lines (L1 and L3) and from wild-type plants (wt). The processed 65 kDa protein synthesized in transgenic plants is marked by an asterisk. The antiserum also recognized a slower migrating protein from line L1 (marked by an arrow), a putative non-processed form of Xyl1. (C) Hydrolytic activities of ConA-binding proteins from L1, L3, and wild-type plants towards aryl glycosides. Data indicate means ±SE from at least four independent extractions with equal amounts (FW) of leaf material. Abbreviations: Xyl, PNP-β-D-xyloside; AraF, PNP-α-L-arabinofuranoside; AraP, PNP-α-L-arabinopyranoside; Glu, PNP-β-D-glucopyranoside; GluNAc, PNP *N*-acetyl-β-D-glucosaminide; GalPyr, ortho-nitrophenyl-β-D-galactopyranoside.

enzyme of *M. truncatula* related to MsXyl1. Interestingly, a third protein (marked by an arrow in Fig 3B) was seen in samples from L1, presumably a non-processed form of MsXyl1.

MsXyl1 from alfalfa binds to ConA and can be eluted from the ConA–agarose column with methyl-α-D-mannopyranoside. Using the antiserum, it was found that the recombinant protein from *M. truncatula* expressing MsXyl1 displayed similar properties. The ConA-binding protein fractions were then assayed with various aryl glycosides. Compared with wild-type plants, samples from L1 and L3 leaves showed significantly higher activities toward the substrates PNP-β-D-xyloside, PNP-α-L-arabinofuranoside, and PNP-α-L-arabinopyranoside. No increase in hydrolytic activity was found with PNP-β-D-glucopyranoside, PNP-*N*-acetyl-β-D-glucosaminide, ortho-nitrophenyl-β-D-galactopyranoside (Fig. 3C), or rhizobial Nod factors (data not shown). Similar results were also obtained with Con-A-binding root proteins, indicating that recombinant MsXyl1 is also active in the root tissue. Hydrolytic activities from transgenic control plants (from transformations with a vector containing a nonsense DNA sequence) were not different from those in wild-type *M. truncatula* (data not shown). Taken together, these findings indicate that the elevated β-xylosidase/α-L-arabinosidase activity in transgenic *M. truncatula* plants can be attributed to expression of *MsXyl1* and that leaf material is a suitable source for purification of recombinant MsXyl1.

Compared with wild-type plants, *M. truncatula* expressing *MsXyl1* grew normally. When inoculated with *S. meliloti*, all transgenic plants tested formed nodules, indicating that *MsXyl1* expression did not affect symbiosis (data not shown). Seed coats from the homozygous line L1 were brownish, suggesting a possible effect of MsXyl1 on seed coat coloration in plants with high *MsXyl1* expression levels.

Substrate specificity of purified MsXyl1

Leaf material from the transgenic line L1 (T3 generation) was used to purify MsXyl1 to near homogeneity. After elution from the ConA column, MsXyl1 was further purified by two additional chromatographic steps on a HITRAP Q column. Enzyme yields, specific activity measured with PNP-β-D-xyloside, and purification factors are given in Table 1. The final MsXyl1 preparation appeared as a single 65 kDa protein on SDS gels stained with Coomassie Brilliant Blue. An additional faint band was seen when gels were stained with silver nitrate (Fig. 4A). When control leaves from *M. truncatula* wild-type plants were subjected to the purification procedure, the 65 kDa protein did not appear on the stained gels (not shown). The antiserum raised against a synthetic peptide sequence of MsXyl1 recognized the purified 65 kDa protein, confirming that a processed form of MsXyl1 has been purified (Fig. 4B). Anti-6His antibodies did not recognize the 65 kDa protein purified, providing additional evidence for COOH-terminal processing of the protein (not shown).

As expected from the activity of ConA-binding proteins (Fig. 3C), MsXyl1 purified from transgenic *M. truncatula* cleaved PNP-β-D-xyloside, PNP-α-L-arabinofuranoside, and PNP-α-L-arabinopyranoside. Using different concentrations of the aryl glycosides in the enzyme assay, the Michaelis–Menten constant K_m, the catalytic rate constant k_{cat}, and the catalytic efficiency (k_{cat}/K_m) values were determined (Table 2). The K_m values for the three aryl glycosides were similar. Due to a high k_{cat} value, the catalytic efficiency was highest with PNP-α-L-arabinofuranoside as substrate. These kinetic data confirm that MsXyl1 is a bifunctional β-xylosidase/α-L-arabinosidase.

The activity of purified MsXyl1 was further assayed against various oligo- and polysaccharidic substrates. Low molecular weight products were separated by TLC and

Table 1. *Purification of MsXyl1 protein from* M. truncatula *leaves*

Purification step	Yield[a]		Specific activity (nmol min^{-1} mg^{-1})	Recovery[d] (%)	Purification factor[e]
	Protein[b] (mg)	Activity[c] (nmol min^{-1})			
Crude homogenate	57.19	20.13	0.35	100	1
ConA agarose	2.039	4.59	2.26	22.8	6.4
HiTrap Q	0.136	1.14	8.40	5.67	23.9

[a] Data indicate values from a typical purification with 7-week-old leaves (50 g FW) from transgenic line L1.
[b] Measured by the method of Bradford (1976).
[c] Xylosidase activity was determined with PNP-β-D-xyloside as substrate.
[d] Expressed as percentage of initial specific activity in the crude homogenate.
[e] Increase of specific activity compared with the crude homogenate.

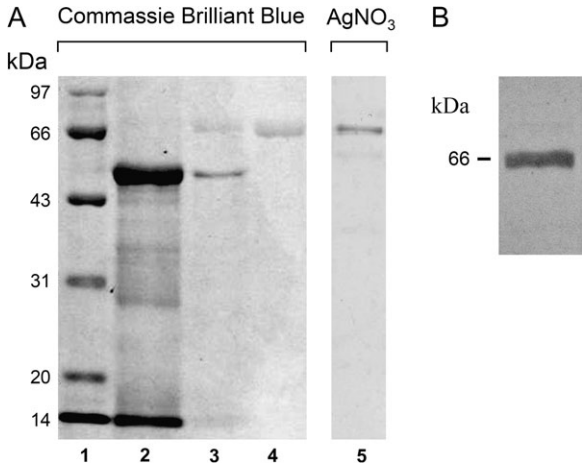

Fig. 4. Purification of MsXyl1 protein from *M. truncatula* leaves expressing *MsXyl1*. (A) Proteins from the various purification steps were separated by SDS-PAGE and stained with Commassie Brilliant Blue R-250 or silver nitrate. Lane 1, molecular weight markers; lane 2, soluble proteins extracted from leaves; lane 3, ConA-binding proteins after elution with methyl-α-D-mannopyranoside; lane 4, purified protein eluted from the HITRAP Q column; lane 5, purified protein stained with silver nitrate. (B) Immune blot with the purified MsXyl1 protein and the antiserum raised against the peptide CDSVEVLYKDQHYTK.

Table 2. *Kinetic parameters (37 °C) of purified MsXyl1 with aryl glycosides*

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
PNP-β-D-xyloside	0.59	0.96	1.63
PNP-α-L-arabinofuranoside	0.94	4.70	5.00
PNP-α-L-arabinopyranoside	1.20	1.75	1.46

visualized by DAP reagent. The substrate 1,4-β-D-xylotetraose was completely degraded to the monosaccharide with xylotriose and xylobiose as intermediates (Fig. 5A). MsXyl1 also rapidly hydrolysed 1,5-α-L-arabinotetraose (Fig. 5B). In addition to these oligosaccharidic substrates, MsXyl1 released monosaccharides from polymeric substrates, such as arabinan from sugar beet and arabinoxylan from wheat (Fig. 5C). Cleavage products were not seen when MsXyl1 was omitted from the reaction mixture (data not shown). Interestingly, xylan from oat spelts was resistant against degradation by MsXyl1. An endoxylanase from the fungus *Thermomyces lanuginosus* released oligosaccharides (mainly xylobiose) from the xylan. Further degradation to monosaccharides was observed when xylan was incubated with a mixture of xylanase and MsXyll (Fig. 5D).

The activity of MsXyl1 was further assayed on cell wall material from young alfalfa roots, which may reflect the *in vivo* function of the enzyme. Analysis of carbohydrate monomers after chemical hydrolysis with trifluoroacetic acid indicated that the cell wall polysaccharides consisted mainly of glucose, mannose, arabinose, xylose, and galactose (data not shown). Purified MsXyl1 was able to release carbohydrates from the cell wall material as visualized on TLC plates after staining with the DAP reagent. These degradation products were not observed in control reactions with heat-inactivated MsXyl1 (Fig. 6A). To identify the cleavage products, they were purified by TLC, and the TMS methyl glycosides were analysed by GC-MS. As shown in Fig. 6B, both arabinose and xylose were identified in approximately equal amounts.

Discussion

In this study, MsXyl1 was characterized from young alfalfa roots. The purified enzyme released xylose and/or arabinose from aryl glycosides, xylo-oligosaccharides, arabino-oligosaccharides, arabinan from sugar beet, and arabinoxylan from wheat. MsXyl1 did not cleave xylan from oat spelts, however. This is reminiscent of a recently characterized α-L-arabinofuranosidase isolated from Japanese pear, which was unable to cleave xylan from birchwood (Tateishi *et al*., 2005). In combination with xylanase, MsXyl1 degraded xylan from oat spelts to xylose, indicating that MsXyl1 is an exo-enzyme that acts synergistically with endohydrolases. MsXyl1 released xylose and arabinose from alfalfa cell walls, suggesting that the enzyme is a bifunctional β-xylosidase/α-L-arabinosidase implicated in the cell wall

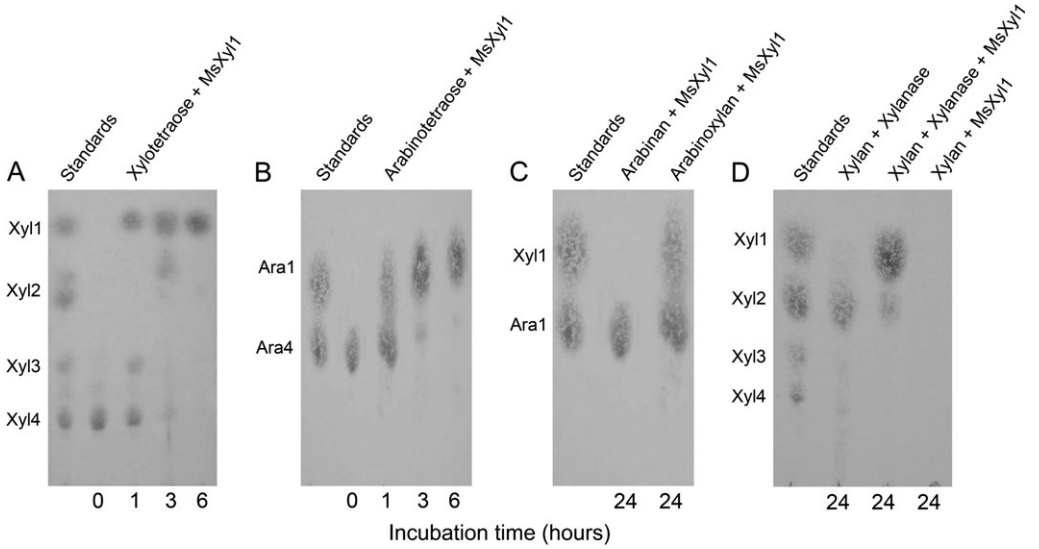

Fig. 5. Substrate specificity of purified MsXyl1 toward oligo- and polysaccharides. Substrates (0.1 mg) were incubated at 37 °C with 2 μg of protein (purified MsXyl1; xylanase from *T. lanuginosus*) for the indicated time. TLC analysis was then performed as described in the Materials and methods with standards on the left side of each plate (Xyl1, D-xylose; Xyl2, 1,4-β-D-xylobiose; Xyl3, 1,4-β-D-xylotriose; Xyl4, 1,4-β-D-xylotetraose; Ara1, L-arabinose; Ara4, 1,5-α-L-arabinotetraose). (A) Hydrolysis of 1,4-β-D-xylotetraose by MsXyl1. (B) Hydrolysis of 1,5-α-L-arabinotetraose by MsXyl1. (C) Cleavage products from sugar beet arabinan and wheat arabinoxylan released by MsXyl1. (D) Hydrolysis of xylan from oat spelts by xylanase and by a mixture of xylanase and MsXyl1. Incubation of xylan with MsXyl1 alone did not result in any cleavage product.

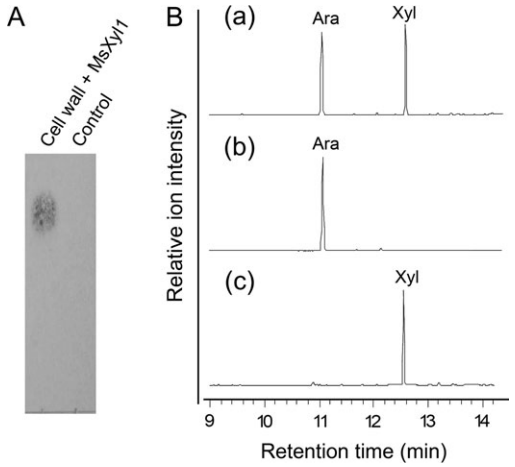

Fig. 6. Purified MsXyl1 releases arabinose and xylose from afalfa cell walls. (A) TLC analysis of monosaccharides released from cell walls from alfalfa roots. Cell wall polysaccharides (0.1 mg) were digested with MsXyl1 (2 μg) for 24 h. A control reaction was performed with heat-inactivated MsXyl1. (B) GC-MS analysis: total-ion chromatogram of TMS methyl glycosides: chromatogram (a), arabinose and xylose from cell wall polysaccharides released by MsXyl1; chromatogram (b), L-arabinose standard; chromatogram (c), D-xylose standard.

turnover of xylose and arabinose. In addition to cell wall polysaccharides, arabinogalactan proteins might represent alternative substrates for MsXyl1, as recently demonstrated *in vitro* for RsAraf1 from radish (Kotake *et al*., 2006).

MsXyl1 is a member of GH family 3 with sequence similarities to enzymes of the β-D-xylosidase branch (Fig. 1C; Hrmova *et al*., 2002). Some of these hydrolases efficiently release β-xylosyl residues from substrates and appear in the literature as β-xylosidases [XYL from barley (Lee *et al*., 2003); XYL1, XYL3, and XYL4 from *Arabidopsis* (Minic *et al*., 2004, 2006); FaXyl1 from *Fragaria×ananassa* (Bustamante *et al*., 2006)]. Closely related enzymes have a substrate preference towards α-L-arabinofuranosyl residues and were called α-L-arabinofuranosidases [ARA-I from barley (Lee *et al*., 2003), PpARF2 (Tateishi *et al*., 2005), and RsAraf1 (Kotake *et al*., 2006)]. The distinct substrate specificity of these enzymes might be caused by differences in particular amino acid residues, which remain to be identified. Conserved amino acid residues putatively involved in substrate binding have been proposed by Hrmova *et al*. (2002) based on the known three-dimensional structure of a β-D-glucan glucohydrolase from barley belonging to the β-D-glucan glucohydrolase branch of GH family 3 (Varghese *et al*., 1999).

MsXyl1 showed high activity towards PNP-α-L-arabinopyranoside and PNP-α-L-arabinofuranoside. These findings indicate that MsXyl1 displays activity towards substrates with L-arabinose in the α-pyranose and α-furanose conformation. By contrast with α-L-arabinofuranosidases, plant enzymes with α-L-arabinopyranosidase activity have not been characterized in detail. The β-xylosidases XYL1 and XYL4 from *Arabidopsis* displayed weak activities towards various aryl glycosides, including PNP-α-L-arabinopyranoside, but these associated activities were not further investigated (Minic *et al*., 2004). Similarly, α-L-arabinopyranosidase activity was co-purified with a β-galactosidase enzyme from apple fruit (Dick *et al*., 1990). By contrast with this limited knowledge on α-L-arabinopyranosidases from plants, certain bacteria and

fungi possess well-characterized enzymes with α-L-arabinopyranosidase activity. For example, a GH family 42 enzyme from *Clostridium cellulovorans* displayed bifunctional L-arabinopyranosidase/β-galactosidase activity (Kosugi *et al*., 2002). BXL1 (accession number Z69257), a recombinant β-xylosidase from the filamentous fungus *Trichoderma reesei* expressed in yeast, efficiently cleaved substrates with α-L-arabinose in the pyranose conformation (Margolles-Clark *et al*., 1996). This enzyme belongs to GH family 3 and its amino acid sequence exhibits homology with MsXyl1 (37% identity). It is worth noting in this context that most L-arabinose units in plant cell wall polysaccharides assume the α-furanose conformation. However, α-L-arabinose residues in the pyranose conformation are quantitatively minor components of arabinans in various plants, including legumes. Arabinan II from pigeon pea (*Cajanus cajan*) consists partially of arabinopyranose residues (Swamy and Salimath, 1991), and pectic arabinogalactan from soybean bears a side chain with arabinopyranose residues at the non-reducing terminal end (Huisman *et al*., 2001). Such terminal arabinopyranose residues might protect the legume cell wall against degradation by microbial α-L-arabinofuranosidases with a substrate preference for terminal arabinofuranose residues. Further work is required to test whether the occurrence of arabinopyranose residues in cell wall polymers implicates plant enzymes with α-L-arabinopyranosidase activity.

The native protein purified from alfalfa and the recombinant protein derived from transgenic *M. truncatula* leaves exhibited similar properties. In both cases, the ConA-binding proteins seem to be *N*-glycosylated. It cannot be excluded, however, that a fraction of the synthesized MsXyl1 in transgenic *M. truncatula* was not *N*-glycosylated. Immune blots indicated that approximately 20% of the MsXyl1 protein from transgenic *M. truncatula* L1 did not bind to the ConA column. Furthermore, the native protein in alfalfa and the recombinant protein in transgenic *M. truncatula* were both processed to a 65 kDa form, most likely in the COOH-terminal region as reported previously for barley β-D-xylosidase (Lee *et al*., 2003). Perhaps due to the high levels of synthesized MsXyl1 protein, processing in the COOH-terminal region seems to be incomplete in *M. truncatula* L1 leaves. The antibodies against the synthetic peptide recognized an additional protein of higher molecular weight (marked with an arrow in Fig. 3B)—this band is interpreted as a non-processed form of MsXyl1. These findings suggest that the capacity for post-translational modification is limited in *M. truncatula* leaves.

Purification of a protein to apparent homogeneity does not exclude the possibility that cryptic activities remain in the final protein fraction or that two related isoforms were characterized together. For example, the β-xylosidases and α-L-arabinofuranosidases from *Arabidopsis* purified by Minic *et al*. (2004) exhibited activities towards various aryl glycosides, and incubation of Nod factors from *S. meliloti* with the 65 kDa protein preparation from alfalfa in the present study caused hydrolysis at low rates (data not shown). Hence, it remains open whether a measured enzymatic activity can be attributed to a purified enzyme. Heterologous expression systems have the advantage of characterizing a specific enzyme activity of a protein encoded by a single gene. In the present study, the ConA-binding proteins from *M. truncatula* expressing *MsXyl1* exhibited increased β-xylosidase/α-L-arabinosidase activity and provided experimental evidence that MsXyl1 does not cleave Nod factors (data not shown). Using a similar approach, *RsAraf1* (a GH family 3 gene from radish) has been recently expressed in *Arabidopsis*. The cell wall protein fraction of transgenic plants exhibited increased α-L-arabinofuranosidase and slightly elevated β-xylosidase activity (Kotake *et al*., 2006). Taken together, these findings indicate that heterologous expression of GHs in *M. truncatula* is a promising approach for characterizing the substrate specificity of a given enzyme. As far as is known, heterologous expression and purification of GHs from transgenic legumes has not been reported so far.

MsXyl1 transcripts were detected in young roots and root nodules, but not in aerial parts of the plant, except in flowers. This tissue-specific expression profile indicates that MsXyl1 is developmentally regulated. Most of the genes with sequence similarities to *MsXyl1* seem to be implicated in developmental processes required for flowering and seed production. Transcripts of *MsXyl1*-related genes have been found in flowers and siliques of *Arabidopsis* (Goujon *et al*., 2003, Minic *et al*., 2006), in pollen of tobacco and *Arabidopsis* (Hrubá *et al*., 2005), in immature seeds of radish (Kotake *et al*., 2006), and in ripening fruits of various plants [tomato (Itai *et al*., 2003), Japanese pear (Tateishi *et al*., 2005), and strawberry (Bustamante *et al*., 2006)]. The genes induced in ripening fruits are likely to play a role in alterating the cell wall texture during softening. On the other hand, *AtBXL1* (encoding XYL1) from *Arabidopsis* (Goujon *et al*., 2003) and XYL from barley (Lee *et al*., 2003) showed a broad expression pattern, and their transcripts were detected in various aerial tissues and in roots as well. *AtBXL1* in roots from *Arabidopsis* is expressed in the central cylinder and might play a function in tissues undergoing secondary cell wall thickening. By contrast, the strong expression of *MsXyl1* in root tips of alfalfa rather suggests a function in modification of the primary cell wall. The present biochemical data indicated that MsXyl1 efficiently released arabinose and xylose from alfalfa cell walls. Thus, it is proposed that MsXyl1 plays a role in loosening and remodelling of the cell walls in rapidly growing roots, particularly in the root tip.

Recently, microbial β-xylosidases and α-L-arabinosidases have gained increasing industrial applications such

as synthesis of oligosaccharidic chemicals, improvement of wine flavours, clarification of fruit juices, digestion enhancement of animal feedstuffs, and delignification of pulp in the paper industry and fibre engineering (Shallom and Shoham, 2003; Numan and Bhosle, 2006; Trincone and Giordano, 2006). The GH characterized in this study might serve as a useful tool for these biotechnological applications. Advances in enzyme technology and the use of transgenic legumes as an expression system open the possibility for large-scale production of cell wall degrading GHs from plants. From the economic and ecological point of view, legumes with nitrogen-fixing root nodules are of particular interest as possible biofactories since they can accumulate high amounts of proteins and require less nitrogen fertilizer for growth. The model legume *M. truncatula* seems to offer interesting properties for molecular farming. Staining of proteins separated on SDS gels and immune blot analyses suggest that approximately 1% of the extracted soluble proteins from the transgenic *M. truncatula* L1 can be attributed to the MsXyl1 protein expressed (data not shown). Similar high yields of recombinant protein were recently obtained for transgenic *M. truncatula* expressing a fungal phytase gene. The comparative study showed that expression of the protein in *M. truncatula* leaves was significantly higher than in corresponding leaves from transgenic tobacco (Abranches *et al*., 2005). *Medicago truncatula* has favourable agronomic characteristics (e.g. with respect to biomass production) and an array of genetic tools has been established recently. Furthermore, seeds from the diploid *M. truncatula* can be rapidly multiplied and vegetative propagation is possible by leaf cuttings. By contrast to tobacco, leaves from *M. truncatula* do not contain poisonous nicotine. Hence, *M. truncatula* plants seem to be particularly suitable for the production of food-processing enzymes as well as for the synthesis of pharmaceutical proteins (Stoger *et al*., 2005).

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