AtBXL1, a novel higher plant (Arabidopsis thaliana) putative beta-xylosidase gene, is involved in secondary cell wall metabolism and plant development

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Summary
To investigate mechanisms involved in cell wall development, an Arabidopsis T-DNA insertion mutant collection was screened to identify mutants with beta-glucuronidase fusion gene expression in tissues undergoing secondary cell wall thickening. This promoter-trapping strategy allowed the isolation of a transformant containing the GUS coding sequence inserted 700 bp upstream of the ATG of a putative beta-xylosidase gene. The transformant has no phenotype as the expression of the gene was not disrupted by the insertion. The analysis of the predicted protein, AtBXL1, suggests its targeting to the extracellular matrix and its involvement in cell wall metabolism through a putative activity towards xylans. The 2-kb promoter sequence of AtBXL1 was fused to the GUS coding sequence and introduced into wild-type Arabidopsis thaliana. GUS expression was shown to be restricted to tissues undergoing secondary cell wall formation. Beta-xylosidase activity was associated with the cell wall-enriched fraction of different organs of wild-type plants. The level of activity correlates with transcript accumulation of AtBXL1 and other AtBXL1-related genes. Transgenic plants expressing the AtBXL1 cDNA in antisense orientation were generated. Lines exhibiting the highest decrease in AtBXL1 transcript accumulation and beta-xylosidase activity had phenotypic alterations. This newly identified gene is proposed to be involved in secondary cell wall hemicellulose metabolism and plant development.

Keywords: Arabidopsis, beta-xylosidase, hemicelluloses, secondary cell wall, plant development.

Introduction
The cell wall is a dynamic structure that undergoes changes in its composition and structure throughout the cell’s development and specialization. It provides mechanical support to the plant cell and also plays a biological role as a source of signaling molecules (Miller et al., 1997). Local variations in wall thickness and composition are now considered as an integral part of the cell’s differentiation process in relation to its neighboring cells (Roberts, 2001). The plant cell wall is mostly composed of polysaccharides (cellulose, hemicelluloses, and pectins), proteins, and aromatic compounds (lignins and other wall-linked phenolic acids). Plant cells show two types of cell wall deposition: the primary cell wall is generally synthesized during cell expansion in the first stages of development. The secondary cell wall is deposited in fully expanded and specialized cells. The secondary thickening of the cell wall can involve polysaccharides (e.g. cellulose in cotton fibers, cellulose, and xylans in wood) and/or aromatic components (e.g. lignins in xylem vessels...
The hemicellulosic components of the primary cell wall of angiosperm dicots and especially of Arabidopsis thaliana have been well characterized and consist mainly of xyloglucans (Zablackis et al., 1995). The heterogeneity of xyloglucans results from differences in their molecular mass, distribution and levels of substituted xylosyl units with galactosyl and fucosyl residues (Vargas-Rechía et al., 1998). The metabolism of xyloglucans in the cellulose microfibril network is believed to be important for cell wall expansion. Support for this hypothesis comes from alterations to xyloglucans that contribute to wall extensibility. Endo-beta-1,4-D-glucanases, xyloglucanases, alpha-L-fucosidases, and/or endo-glycosyltransferases such as xyloglucan endotransglycosylases have been shown to be involved in the auxin- or acid-promoted breakdown of xyloglucan (Hayashi et al., 1984; Hetherington and Fry, 1993). In contrast, little information is available on the hemicellulosic fraction of secondary cell walls. Recent studies have shown that cultured tobacco cells, differentiating secondary cell walls, and accumulated xylans in the cell wall possess a highly enhanced xylan synthase activity (Blee et al., 2001). Xylans and the other hemicellulosic components of the cell wall are synthesized in the Golgi apparatus and exported to the cell wall (Bolwell, 2000; Bolwell and Northcote, 1983). Xylans consist of a backbone of beta-(1,4)-linked xylose residues associated with side-chains of 4-O-methylglucuronic acid and arabinose which are present in varying amounts (glucuronoxylan and arabinoxylan), and with acetyl groups. Glucuronoxylans are the most abundant hemicellulosic components of angiosperm wood (hardwood). They play a major role in the organization of lignified cell walls as they can cross link with lignins, particularly in grasses, via feruloylated bonds (Hatfield et al., 1999). They also interact with cellulose (Awano et al., 2001; Vian et al., 1986).

A powerful method for identifying genes involved in plant cell wall formation is the identification of cell wall mutants (Fagard et al., 2000; Reiter et al., 1997). Screens to identify cell wall mutants have been set up and a number of mutants affected in secondary cell wall deposition have been described (reviewed by Turner et al., 2001). Many genes involved in secondary cell wall formation have been cloned and characterized. The cellulose synthase catalytic subunits of A. thaliana encoded by AtCesA7 and AtCesA8 are involved in cellulose deposition in the secondary cell wall. The corresponding mutants (irx3 and irx1) were identified by a screen based on secondary cell wall alteration (Taylor et al., 1999, 2000; Turner and Somerville, 1997, 2001). Most of the genes of lignin monomer synthesis of the secondary cell wall has yet been cloned. Of the cell wall screens are based on chemically mutagenized plant libraries (Reiter et al., 1997; Turner and Somerville, 1997), which renders the cloning of the gene from the mutated line time-consuming and difficult. Screens based on promoter or gene trap strategies allow the isolation of mutants of potential interest depending upon the screen and, in addition, permit the cloning of the mutated genes directly (Babiychuk et al., 1997; Krysan et al., 1999; Lindsey et al., 1993; Sundaresan et al., 1995).

The Arabidopsis Versailles collection, which is based upon the use of a T-DNA containing the promoterless uidA (GUS) gene at the right border from the vector pGBK5 (Bechtold et al., 1993; Bouchez et al., 1993), was screened for GUS expression in tissues undergoing secondary cell wall thickening (xylem and/or interfascicular fibers). Such an approach should allow the identification of genes specifically involved in secondary cell wall thickening. We describe the use of this approach to isolate AtBXL1, a putative beta-xylosidase gene, potentially involved in secondary cell wall hemicellulose metabolism. The molecular characterization of this gene and the consequences of its downregulation on the overall phenotype and polysaccharide composition of the cell wall are evaluated. Its role in cell wall metabolism and its involvement in plant development are discussed as is the presence of high beta-xylosidase activity in the cell wall of wild-type plants.

While a number of beta-xylosidases have been isolated from a variety of fungal and mesophilic bacterial sources and their rate limiting role in xylan hydrolysis well documented (Van Peij et al., 1997), the presence and the role of such a gene has not been yet described in higher plants.

**Results**

**Isolation of the fgp1 transformant T-DNA tagged in a beta-xylosidase gene**

The T-DNA used to generate the A. thaliana Versailles insertion collection contains the promoterless uidA (GUS) coding sequence (Bouchez et al., 1993). A screen based on the detection of GUS activity in tissues of the mature stem showing extensive secondary cell wall formation (i.e. xylem and/or interfascicular fibers) was initiated. Thirty-three mutants out of 14 000 lines screened, displaying GUS activity in either the xylem or in the interfascicular fibers or both, were identified. Among them, the fgp1 transformant (for fiber gus positive 1) was characterized by the restriction of GUS expression to developing xylem and fibers throughout the stem development (Figure 1). Genetic analysis of the segregation of the fgp1 phenotype and the T-DNA insertion indicated tight linkage between the T-DNA insert and the histochemical GUS staining in the xylem and fibers, suggesting a single insertion. Southern analyses identified an inverted tandem T-DNA insertion in the genome (data not shown). Molecular cloning of the
genomic right border, using a PCR-based method (Balzer-
gue et al., 2001), revealed a T-DNA insertion in the promo-
ter sequence 700 bp upstream of the ATG of a putative beta-
xylosidase gene. Southern hybridization, using a genomic
probe of this putative gene (Figure 2), showed that the wild-
type plant and the fgp1 line displayed different patterns.
Southern analyses also showed that this gene is present as
a single copy in the genome of A. thaliana.

AtBXL1 belongs to a seven-member gene family

The EST H5E2T7, corresponding to this gene, was
sequenced and corresponds to a recently sequenced cDNA
clone (accession number: AY120767). The potential protein,
BAB09906, encoded by the tagged gene is a putative beta-
xylosidase (MW 83.5 kDa) with a 30-amino acid hydropho-
bic sequence located at the N-terminus and several poten-
tial N-glycosylation sites (data not shown). PSORT and
SIGNALP software (Nakai and Horton, 1999; Nielsen
et al., 1997) predict the cleavage of this signal peptide and a
targeting of the cleaved product to the extracellular matrix.

This predicted protein possesses the features of the gluco-
syl-hydrolase family (Figure 3a). Analysis of the com-
plete genome sequence of A. thaliana (Arabidopsis
genome initiative 2000) revealed that BAB09906 belongs
to a small gene family. A putative phylogenetic tree pre-
diction from sequence multiple alignment by cluster and
topological algorithms (Brodsky et al., 1995) was gener-
ated, including protein sequences of bona fide beta-xylo-
sidase from Aspergillus niger (Z84377) and Escherichia coli
(P07129) (Figure 3b). This phylogenetic analysis revealed
that these genes are divergent and only two groups were
found to be phylogenetically closely related as shown in
Figure 3. Beta-xylosidases of A. niger and E. coli form two
different isolated groups and the A. thaliana putative beta-
xylosidase family is distinct from any beta-xylosidase iso-
lated from fungi or bacteria. In addition, AtBXLs exhibited
different molecular evolutionary rates as shown by variable
branch lengths. These proteins are considered as xyloli-
dases or beta-xylosidases in GenBank because of their
high similarity with beta-xylosidases from fungi and bac-
teria and because of the presence of glucosyl-hydrolase

Figure 1. GUS expression in the stem section of
the fgp1 transformant. GUS activity is localized
in xylem (X) and fibers (F) of the transformant,
bar: 150 μm.

Figure 2. Characterization of the T-DNA insertion in fgp1 transformant.
(a) Schematic representation of the insertion of the inverted tandem T-DNA insertion in line fgp1. LB: Left border of the T-DNA; RB: Right border of the T-DNA; ATG: start codon; Stop: stop codon; bp: base pairs; kb: kilobase pairs.
(b) Southern analysis of wild-type (WS) and fgp1 lines in stringent conditions. One microgram DNA from each line was digested with 1: EcoRI, 2: EcoRV, 3: HindIII, 4: BamHI and 5: PstI, then run on an agarose gel, transferred onto nylon membrane and hybridized with a genomic probe as indicated in (a).
signature domains. For practical reasons, these putative proteins were renamed AtBXL1, 2, 3, 4, 5, 6 and 7 for BAB09906, AAG10624, BAB09531, BAB11424, BAB02547, T49983 and AAF17692 accession numbers, respectively. Bioinformatic analyses were used to determine the chromosomal location, the subcellular targeting, the percentage identity with AtBXL1, and the number of identified EST for each gene. These results are summarized in Table 1. AtBXL2, 4, and 5 were found to be targeted to the extracellular matrix as well as AtBXL1; whereas AtBXL3, 6, and 7 were found to be targeted to the plasma membrane. Forty-six ESTs in GenBank correspond to AtBXL1, suggesting that it is the most expressed beta-xylosidase-like gene in wild-type A. thaliana plants. AtBXL4 was also represented by ESTs although to a lesser extent (24 ESTs). In addition, ESTs corresponding to AtBXL5 were also identified in cDNA libraries from Medicago truncatula, Glycine max, cotton, tomato, ice-plant, potato and barley (data not shown).

Table 1 Bioinformatic analysis of the AtBXL gene family in Arabidopsis thaliana

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein accession number</th>
<th>Chromosomal location identity with AtBXL1</th>
<th>Percentage of amino acid</th>
<th>Number of Arabidopsis thaliana EST found in GenBank</th>
<th>Predicted location</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtBXL1</td>
<td>BAB09906</td>
<td>Chromosome 5, TAC clone K7J8</td>
<td>100</td>
<td>46</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>AtBXL2</td>
<td>AAG10624</td>
<td>Chromosome 1, BAC clone T14P4</td>
<td>66.4</td>
<td>12</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>AtBXL3</td>
<td>BAB09531</td>
<td>Chromosome 5, BAC clone F17114</td>
<td>56.4</td>
<td>1</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>AtBXL4</td>
<td>BAB11424</td>
<td>Chromosome 5, P1 clone MUB3</td>
<td>57.4</td>
<td>25</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>AtBXL5</td>
<td>BAB02547</td>
<td>Chromosome 3, P1 clone MMB12</td>
<td>47.3</td>
<td>0</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>AtBXL6</td>
<td>T49983</td>
<td>Chromosome 5, BAC clone F12B17</td>
<td>45.7</td>
<td>6</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>AtBXL7</td>
<td>AAF17692</td>
<td>Chromosome 1, BAC clone F28K19</td>
<td>42.1</td>
<td>6</td>
<td>Plasma membrane</td>
</tr>
</tbody>
</table>

Chromosomal location, amino acid percentage identity with AtBXL1, EST representation in cDNA libraries and putative protein targeting are indicated.
As a result, the GUS expression pattern in fgp1 might not correspond to the expression of the tagged gene because the insertion is located 700 bp upstream of the putative ATG start codon of the first exon. For this reason, a vector containing the uidA coding sequence (as reporter gene) under the control of the 2-kb promoter region of AtBXL1 was introduced into wild-type Arabidopsis plants. Histochemical analysis of GUS expression in the progeny of transformed plants showed that this 2-kb region drove the GUS expression in the vascular region of all organs (roots, leaves, flowers, and siliques). Stem sections revealed the GUS expression in protoxylem, metaxylem, intrafascicular cambium, and fibers. This restricted pattern of expression demonstrates that this gene is expressed in tissues undergoing secondary cell wall thickening (Figure 4).

**Figure 4.** Expression pattern of AtBXL1 promoter::GUS fusion in transgenic Arabidopsis.
(a) Schematic representation of the construct introduced into wild-type Arabidopsis thaliana plants to determine the expression of the 2-kb promoter region of AtBXL1. 35SP: 35S promoter of the cauliflower mosaic virus (CaMV); 35ST: 35S CaMV terminator sequence; hpt: coding sequence of the hygromycin phosphotransferase gene; uidA: beta-glucuronidase coding sequence; NosT: nopaline synthase gene terminator sequence; LB: T-DNA left border; RB: T-DNA right border.
(b) GUS expression patterns under the control of the AtBXL1 promoter in: (i) primary root (bar: 500 μm); (ii) flower (bar: 500 μm); (iii) silique (bar: 500 μm); (iv) leaf (bar: 50 mm); (v) stem section (bar: 500 μm); and (vi) 7-day-old seedling (bar: 500 μm). F: fiber; P: primordia; S: stele; V: vein; X: xylem.

The expression of AtBXL1 and related genes is developmentally regulated

Expression analysis was focused on the AtBXL genes whose products are supposed to be targeted to the extracellular matrix (Table 1). Northern analyses were performed on above-ground organs (7-day-old seedlings, leaves, basal part of the stem, apical part of the stem, flowers, and siliques) of wild-type Arabidopsis plants to determine the expression patterns of these AtBXL genes (Figure 5a). AtBXL1 transcripts accumulated in all organs at different levels. Transcript accumulation was very high in the basal and the apical part of the stems, tissues differentiating secondary cell wall thickenings (fibers and xylem vessels) and low in seedlings and leaves. AtBXL4 transcript accumulation was restricted to the apical part of the stem, the flowers and the siliques. No transcripts were detected for AtBXL2 and as no EST was available for AtBXL5, Northern experiment was not performed for this gene.

**Figure 5.** Expression pattern of AtBX at the mRNA and protein levels in different parts of wild-type Arabidopsis.
(a) Northern blot analysis of RNA from seedlings (Se), leaves (Le), basal part (Sb), and apical part (Sa) of the stem, flowers (Fl), and siliques (Si) hybridized with AtBXL1 and AtBXL4 cDNA probes. Ethidium bromide staining was used as a control for RNA loading.
(b) Beta-xylosidase activity (expressed in mU FI min⁻¹) performed on 5 μg proteins of the soluble (S) and cell wall (CW) fractions of the same plant parts than in (a).
Beta-xylosidase activity is highly associated with the cell wall in the wild type

Beta-xylosidase activity was investigated in both soluble and cell wall-enriched protein samples corresponding to those analyzed by Northern blot. The use of 4-methylumbelliferyl-β-D-xyloside (MeUX) allowed the detection of beta-xylosidase activity in the different protein extracts. Activity was high in the cell wall-enriched fractions whereas it was below detection level in all of the soluble fractions, except for the siliques (Figure 5b). Cell wall beta-xylosidase activity was high in stems, flowers, and siliques and low in seedlings and leaves (Figure 5a,b).

AtBXL1 downregulation in transgenic Arabidopsis

Two strategies were envisaged for functional analysis of AtBXL1 in planta. First, the Versailles T-DNA insertion library was screened by a PCR-based method to identify an AtBXL1 null mutant. As no null mutant of this gene was found, an antisense strategy was adopted. A 2.2-kb 5′ sequence (see Experimental procedures) of AtBXL1 cDNA, under the control of the CaMV 35S promoter with a double enhancer cassette, was cloned in a vector containing a kanamycin resistance gene to select transformed cells. The choice of the 35S promoter to drive the expression of the antisense RNA was motivated by several publications in which this promoter was used to downregulate genes involved in secondary cell wall metabolism, such as 4CL and CCR (Lee et al., 1997; Piquemal et al., 1998). Forty-seven independent transformed lines were obtained following transformation. Six lines segregating 3 : 1 for resistance to kanamycin were selected (ASBX4, 7, 12, 15, 42, 45). The corresponding homozygous lines used for further analyses were selected in the self-progenies of the primary transformed plants; AtBXL1 repression efficiency in the transformants was determined by Northern blot analyses (Figure 6b). To determine whether the AtBXL1 antisense strategy had an impact on other genes, Northern experiments were performed on total stem RNA of wild-type and ASBX plants using AtBXL2, AtBXL3 and AtBXL4 probes (Figure 6a). No signal could be detected for AtBXL2 and AtBXL3 in both the wild-type and the ASBX lines. The transcript accumulation level of AtBXL4 was not altered in the ASBX lines in comparison to the wild type. The lines presenting the greatest decrease of AtBXL1 transcript accumulation, ASBX7 and ASBX12, were selected for further analyses.

The most AtBXL1 downregulated lines have decreased beta-xylosidase activity

Beta-xylosidase activity was assayed in the ASBX7 and ASBX12 lines to determine whether the decrease of transcript level could be correlated with a decrease in beta-xylosidase activity. Leaves were first chosen to measure this activity because AtBXL4 transcripts were not detected in these organs of wild-type plants. Detectable beta-xylosidase activity in leaf cell wall protein fractions should therefore mainly reflect the activity of the AtBXL1 product. ASBX7 leaf cell wall fractions presented an overall beta-xylosidase activity corresponding to 40% of the wild-type plants, while ASBX12 showed higher residual activity (data not shown). In order to confirm that decreased beta-xylosidase activity was due to the alteration of a single enzyme, analyses of beta-xylosidase activity in the stem of wild-type and antisense Arabidopsis lines were performed after chromatographic fractionation. The stem extracts of wild-type, ASBX7, and ASBX12 lines were analyzed by Superdex 75 chromatography. The results obtained (Figure 6c) demonstrated that the three tested lines exhibited two peaks of beta-xylosidase activity. However, the profile of beta-xylosidase activities was different among the different lines. The major peak of beta-xylosidase activity was higher in the wild type than in ASBX7 and ASBX12 lines. Activity for this peak was measured for all three lines and expressed as a percentage of activity obtained for wild type (Table 2). The smallest beta-xylosidase activity was found in line ASBX7 (Figure 6c; Table 2). The major peak of these lines (named peak I) was subjected to a CM-Trisacryl chromatography which allowed to reveal a major peak of beta-xylosidase activity (Figure 6d). This peak (named peak II) which showed the highest activity in wild-type protein extract was strongly reduced in lines ASBX7 and ASBX12 to a lower extent (Table 2; Figure 6d). Reduction in beta-xylosidase activity of the peaks I and II in ASBX12 and ASBX7 was the consequence of the downregulation of AtBXL1 in these two antisense lines.

The AtBXL1 downregulated lines have a marked phenotype

ASBX7 and ASBX12 lines showed altered phenotypes, and more especially the ASBX7 line. In dry conditions, maximum plant height of the ASBX lines was lower than that of the wild type whereas no difference could be seen in wet conditions (data not shown). The leaves of mature ASBX7 plants were curled outwards, as were the leaves of ASBX12 plants but to a lower extent (Figure 7a). Even though germination did not appear to be affected, ASBX7 and ASBX12 lines produced significantly lower amounts of seeds per plant and the siliques were shorter than those of the wild type (Figure 7b,c).

Neutral sugar composition of the secondary cell wall hemicellulosic fraction in the AtBXL1 downregulated lines

Beta-xylosidase activity was decreased in the ASBX lines, therefore, we decided to determine the sugar composition...
of the cell wall in stem tissues for two reasons. First, as AtBXL1 is highly expressed in the stem, the impact on the phenotype should be easily detectable. Second, the only probable polysaccharide substrates for a beta-xylosidase in muro should be the glucuronoarabinoxylan (GAX) or arabinoxylan fractions, which are composed of beta-linked xyloses. Analysis of the total sugar composition of cell wall materials from stems of wild-type and ASBX lines were performed after acidic hydrolysis according to Blakeney et al. (1983) and no differences were detected (data not shown). TFA hydrolysis and gas chromatography analysis of the resulting sugars (York et al., 1985) were performed to complete this analysis. This method releases the sugars of the hemicellulose and pectic fractions of the cell wall, without hydrolyzing crystalline cellulose and lignins. No alteration in the overall sugar composition was observed (Table 3). In order to see whether other cell wall components were affected, the cellulose content in wild-type and
Plant N-linked glycans harbor a beta-(1,2)-xylose residue linked to the core beta-mannose (reviewed in Lerouge et al., 1998). As it has been previously shown that plant-secreted beta-xylosidases are able to release beta-xylose from N-linked glycans (Tezuka et al., 1993b), the N-glycosylation patterns of cell-wall glycoproteins were also investigated. Proteins were extracted from stems and their N-glycosylation was analyzed by immunodetection on blots using antibodies specific for the beta-(1,2)-xylose epitopes of plant N-glycans (Faye et al., 1993). No differences were detected (data not shown), thereby indicating that N-linked glycans do not appear to be altered in the ASBX lines.

**Discussion**

The promoter-trap screen approach based on GUS expression is a powerful tool to study cell wall-specific genes

Up to now, most of the mutants affected in cell walls have been isolated from chemically mutagenized collections (Reiter et al., 1997; Turner and Somerville, 1997; Turner et al., 2001) and cloning of corresponding genes accomplished after physiological characterization of the mutant. In this work, we demonstrate that gene- or promoter-trap based screens are suitable for characterizing novel genes specifically expressed in the target tissues. First, this approach allows us to tag genes performing redundant functions because the identification of the gene does not rely on a phenotype induced by gene disruption. Secondly, it permits us to tag essential genes by creating insertions that do not affect the gene function. Thirdly, the screening can be performed at the hemizygous stage of the potential mutant. Recently, a screen using vascular tissue GUS expression in roots has allowed the isolation of a null mutation. Recently, a screen using vascular tissue GUS expression in roots has allowed the isolation of a null mutant in AtOMT1, the gene encoding the enzyme responsible for the last methylation step of lignin monomers (Goujon et al., 2003). In this paper, while the insertion affecting the *AtBXL1* gene did not induce a deregulation of gene expression, further experiments demonstrated its involvement in secondary cell wall metabolism. The promoter-trapping approach based on a vascular GUS expression screen has also allowed the isolation of other transformants of interest for studies on secondary cell wall thickening. Characterization of these mutants and cloning of the corresponding genes are currently underway.

**AtBXL1 belongs to a small gene family**

Southern blot analysis showed that *AtBXL1* is present in the *Arabidopsis* genome as a single copy because hybridization with a specific probe under stringent conditions

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**Table 2** Activities of two peaks of beta-xylosidase obtained after chromatographic separations from lines WS, ASBX7 and ASBX12 (see Figure 6b,c)

<table>
<thead>
<tr>
<th></th>
<th>Peak I (%)</th>
<th>Peak II (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ASBX12</td>
<td>78</td>
<td>69</td>
</tr>
<tr>
<td>ASBX7</td>
<td>67</td>
<td>50</td>
</tr>
</tbody>
</table>

The control was the incubation mixture at time 0. Results are expressed as a percentage of activity obtained in wild type (WS). Activities determined after Superdex 75 had an error less than 3% and after CM-Trisacryl chromatography had an error less than 6% for three independent experiments.

**Table 3** Sugar composition (relative percentage) of the TFA fraction and cellulose percentage of the cell wall from the stem of wild-type (WS) and ASBX7 lines

<table>
<thead>
<tr>
<th></th>
<th>WS</th>
<th>ASBX7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>5.7 ± 0.2</td>
<td>6.9 ± 1.0</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>6.8 ± 0.3</td>
<td>6.8 ± 1.6</td>
</tr>
<tr>
<td>Fucose</td>
<td>1.8 ± 0.3</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>44.3 ± 1.7</td>
<td>41.3 ± 4.5</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>15.9 ± 1.4</td>
<td>19.3 ± 3.4</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>4.2 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>13.2 ± 0.9</td>
<td>13.6 ± 2.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.0 ± 1.3</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>Cellulose/cell wall</td>
<td>58.5 ± 1.5</td>
<td>57.9 ± 0.1</td>
</tr>
</tbody>
</table>

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**Figure 7.** Consequences of *AtBXL1* repression on ASBX lines. (a) Comparison between wild-type leaf (left) and ASBX7 leaf (right). (b) Comparison between wild-type siliques (left) and ASBX7 siliques (right). (c) Comparison between wild-type siliques (left) and ASBX12 siliques (right).

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transformed lines was determined. The TFA-insoluble residue, which is considered to consist of crystalline cellulose, was quantified after seaman hydrolysis. No differences between the wild-type and the ASBX lines could be detected (Table 3). Similarly, analysis of the lignin content and composition revealed no differences between the wild-type and the ASBX lines (data not shown).

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**The promoter-trap screen approach based on GUS expression is a powerful tool to study cell wall-specific genes**

Up to now, most of the mutants affected in cell walls have been isolated from chemically mutagenized collections (Reiter et al., 1997; Turner and Somerville, 1997; Turner et al., 2001) and cloning of corresponding genes accomplished after physiological characterization of the mutant. In this work, we demonstrate that gene- or promoter-trap based screens are suitable for characterizing novel genes specifically expressed in the target tissues. First, this approach allows us to tag genes performing redundant functions because the identification of the gene does not rely on a phenotype induced by gene disruption. Secondly, it permits us to tag essential genes by creating insertions that do not affect the gene function. Thirdly, the screening can be performed at the hemizygous stage of the potential mutation. Recently, a screen using vascular tissue GUS expression in roots has allowed the isolation of a null mutant in *AtOMT1*, the gene encoding the enzyme responsible for the last methylation step of lignin monomers (Goujon et al., 2003). In this paper, while the insertion affecting the *AtBXL1* gene did not induce a deregulation of gene expression, further experiments demonstrated its involvement in secondary cell wall metabolism. The promoter-trapping approach based on a vascular GUS expression screen has also allowed the isolation of other transformants of interest for studies on secondary cell wall thickening. Characterization of these mutants and cloning of the corresponding genes are currently underway.

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**Southern blot analysis** showed that *AtBXL1* is present in the *Arabidopsis* genome as a single copy because hybridization with a specific probe under stringent conditions

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showed a single band for genomic DNA. In contrast, analysis of the whole genome sequence (Arabidopsis Genome Initiative, 2000) allowed the identification of other genes related to AtBXL1. This gene family does not show highly conserved sequences. The genes are widespread in the genome and no recent duplication events were noticed. AtBXL products contain predicted transit peptides and seem to be targeted to the extracellular matrix (AtBXL1, 2, 4, and 5) or to the plasma membrane (AtBXL3, 6, and 7). The presence of several potential N-glycosylation sites in the AtBXL sequences is in accordance with the extracellular location. Therefore, the putative beta-xylosidases in planta seem to act outside or at the interface of the cell. They are enzymes that are commonly found in the stock of cell wall degrading enzymes of pathogens such as fungi or bacteria (de Vries et al., 2000). The observation that such plant enzymes are apparently targeted to the cell wall suggests that they are active towards cell wall components. In plants, beta-xylosidase activity has already been reported in the media of sycamore maple (Acer pseudoplatanus) cell suspension cultures (Tezuka et al., 1993a). This activity has also been observed in ripening avocado fruits (Ronen et al., 1991), and a thermostable beta-xylosidase has been isolated from sugarcane (Chinen et al., 1982). To our knowledge, the present study using A. thaliana provides the first molecular and physiological characterization of such a gene in plants. A search for ESTs corresponding to AtBXL1 in other species suggested that beta-xylosidases are widespread throughout the plant kingdom in both dicots and monocots.

**AtBXL1 is expressed in tissues undergoing secondary cell wall thickenings**

The GUS expression of the fgp1 line was restricted to tissues containing cells with extensive secondary cell walls. However, as the insertion was located 700 bp upstream of the ATG start codon, it was possible that the GUS expression pattern in the fgp1 line did not reflect AtBXL1 activity. In order to verify this point and to study the AtBXL1 promoter activity, a fusion of the 2-kb AtBXL1 promoter region with the GUS coding sequence allowed us to determine the tissue specificity of this gene’s expression. This experiment also enabled us to eliminate the possibility of cryptic promoter tagging, as has already been reported in some cases (Ökrez et al., 1998), and to precisely determine the expression pattern of this gene. AtBXL1 expression seems restricted to the vascular and interfascicular tissues and especially the xylem and cambium of the wild type. The GUS expression pattern was similar, although weaker, in the fgp1 line, probably due to the lack of upstream promoter region enhancing sequences. The strict spatial expression pattern of AtBXL1 suggests that the gene product has a specific activity in vasculature and fibers, and most probably in secondary cell wall formation.

The beta-xylosidase activity observed in the cell walls of A. thaliana can be correlated with the expression of at least two genes

In this paper, evidence is presented to show that putative beta-xylosidase activity is located in the plant cell wall. This activity is high in the stems, flowers, and siliques, and low in other above-ground organs. The transcript accumulation patterns of AtBXL1 and AtBXL4, whose products are probably targeted at the cell wall, were different in the six tested samples. These experiments showed that two putative beta-xylosidase-encoding genes could be expressed in the same organs (stem, flowers, and siliques), but at different expression levels, suggesting different regulation of these genes and, as a consequence, different functions in the cell wall. However, the overlapping expression patterns of these two genes in the stem, flowers, and siliques are probably responsible for the overall levels of beta-xylosidase activity found in these organs. Such a result suggests a biological functional redundancy between these two genes. Moreover, the presence of several beta-xylosidase activities was clearly demonstrated by the chromatography-based purification experiments.

**Downregulation of AtBXL1 induces alterations of cell wall composition and plant development**

The antisense strategy was adopted in order to study the function of AtBXL1 in planta. ASBX7 and ASBX12 lines were found to have the lowest AtBXL1 transcript accumulation and only the AtBXL1 gene was affected by the antisense strategy. The other AtBXL genes (AtBXL2, AtBXL3, and AtBXL4) were neither downregulated nor upregulated. This reduction in AtBXL1 transcripts could be correlated to the lower beta-xylosidase activity (mainly for ASBX7). Nevertheless, even if downregulation of AtBXL1 gene expression decreased beta-xylosidase activity, residual activity was still high. A null mutant would have a more marked phenotype than the antisense lines. However, downregulation of AtBXL1 induces alterations in plant development and the ASBX lines have a marked phenotype. The leaves of ASBX7, and to a lower extent, ASBX12 lines were found to be curled outwards, and the siliques were shorter with a reduced seed content. These traits show that AtBXL1 is involved in the normal development of both leaves and siliques. Sugar analyses have shown that silique polysaccharides are rich in xylose (A. Faik, personal communication). AtBXL1 deficiency in siliques could therefore have an effect on silique cell walls and affect normal silique development. The external phenotype of the stem was not noticeably altered in the ASBX lines. However, under dry conditions, the size of the plants was reduced when compared to that of the wild type.
It has been previously proposed that a beta-xylosidase could be involved in the degradation of N-linked oligosaccharides of excreted proteins (Tezuka et al., 1993b). Nevertheless, AtBXL1 downregulation did not induce any change in the pattern of N-linked oligosaccharides from cell wall proteins. The stem polysaccharide composition of the ASBX lines was not altered suggesting that the putative beta-xylosidase could be involved in the hemicellulose structure. Xylose represents up to 50% of the total sugars of stem cell walls in wild-type A. thaliana whereas it represents only 20–30% in other tissues (Richmond and Somerville, 2001). This is indicative of a high percentage of GAX or arabinoxylan in this tissue.

The loss of AtBXL1 activity was not compensated for by the upregulation of AtBXL4 nor by the expression of AtBXL2 (whose products are also potentially targeted to the cell wall), the latter being undetectable by Northern experiments in both wild-type and downregulated lines. This result indicated a biological functional redundancy between AtBXL1 and AtBXL4, but not a physiological functional redundancy in planta.

A role for AtBXL1 in the cell wall?

As mentioned above, beta-xylosidases are found in the stock of pathogen-degrading enzymes. One can therefore pose the question as to the role of an enzyme – apparently involved in the catabolism of cell wall polysaccharides – in cell wall formation and plant development. While a number of cell wall components degrading enzymes have previously been described, their role remains unclear. KOR is a beta-endoglucanase which should be involved in cellulose metabolism (Sato et al., 2001) and the corresponding mutant was previously identified as a result of impaired hypocotyl elongation in darkness (Nicol et al., 1998). This gene seems to be involved in both primary and secondary cell wall formations (H. Höfte, personal communication). However, even if its function has been determined, its real substrate in muro has not yet been clearly identified. Similarly, an alpha-xylosidase gene has been recently cloned in A. thaliana (AtXYL1, Sampedro et al., 2001). AtXYL1 encodes an apoplastic alpha-xylosidase active against xyloglucan oligosaccharides, especially in young leaves where xyloglucan turnover is high. Its role in the cell wall and its action towards xyloglucans remains to be elucidated.

AtBXL1 could be active against beta-D-xylose residues, and most likely against GAX. GAX have an important role in secondary cell wall fitness, as they can establish covalent bonds with lignins (Hatfield et al., 1999) and tight interactions with cellulose (Awano et al., 2001). AtBXL1 could be involved in the organization and loosening of GAX in the cell wall during cellulose deposition in the secondary cell wall so as to facilitate lignin polymerization in the polysaccharide matrix. If AtBXL1 acts as an important factor in secondary cell wall formation, other enzymes, like xylanases, could also be involved in the catabolism of GAX (Caspers et al., 2001; Slade et al., 1989). Xyloglucan endo-transglycosylases (XET) play a central role in the construction and modification of primary cell wall architecture where they cut and reassemble xyloglucan chains. To allow expansion of plant cells, cellulose microfibrils need to move past one another. It is believed that wall loosening happens when xyloglucans capable of tethering adjacent microfibrils are modified (Minorsky, 2002). In addition, in fungi, beta-xylosidase activity has been reported to be rate-limiting in xylan hydrolysis where it cleaves off the terminal xylose units from the non-reducing end of the xylose oligomers which results from endo-xylanase activity (Van Peij et al., 1997); the same kind of mechanism would be likely to happen in plant secondary cell wall.

It is also well known that many carbohydrate-active enzymes have dual functions. For example, an Agrobacterium tumefaciens beta-glucosidase also shows beta-xylosidase activity (Watt et al., 1998). In addition, beta-xylosidases from bacteria and fungus can also act as alpha-L-arabinofuranosidases in xylan degradation (Herrmann et al., 1997; Whitehead and Cotta, 2001). It can therefore be suggested that, as well as its beta-xylosidase function, AtBXL1 could also be active towards other polysaccharides in the cell wall and thereby have a broader range of activity.

The real substrate of AtBXL1 in muro remains to be characterized but recent and preliminary results indicate that purified AtBXL1 can metabolize xylobiose (Minic et al., in preparation). The AtBXL1 function in muro could also be investigated by isolation and comparison of the structure of xylans in both wild-type and ASBX lines. The determination of the mode of action of this enzyme will be essential to understand hemicellulose metabolism in the secondary cell wall and its role in secondary cell wall structure and plant development.

Experimental procedures

Plant material

Wild-type and transgenic A. thaliana plants, Wassilevskija background (WS), were grown in the greenhouse under standard conditions. The vector pGKB5 (Bouchez et al., 1993) was used to transform wild-type A. thaliana, ecotype WS and to establish the Versailles T-DNA insertion collection. This ecotype was used to generate AtBXL1 antisense transformants and AtBXL1 promoter-GUS fusion transformants.

Screening of the Versailles T-DNA insertion mutant library

Seeds from each line of the Versailles library were sown in greenhouse and grown until stem maturity. Five stem segments of

independent individuals from each transformed line were collected in 96-well microplates. Each plate was submitted to GUS assay and positives (i.e. stem segments displaying blue coloration) were selected. Hand-made sections with a razor blade of these stems were submitted to GUS assay. Positive tissues were identified using a binocular microscope. Lines displaying GUS activity in xylem and/or fibers were sown a second time to confirm GUS expression pattern in the stems.

**Bioinformatic analyses**

Sub-cellular targeting and cleavage sites of AtBXL-deduced proteins were performed using PSORT and SIGNALP software (Nakai and Horton, 1999; Nielsen et al., 1997) at http://psort.nibb.ac.jp/form.html and http://www.cbs.dtu.dk/services/SignalP/#submission, respectively. Putative phylogenetic tree from sequence multiple alignments by cluster and topological algorithms (Brodsky et al., 1995) was generated at http://www.genebee.msu.su/services/malign_reduced.html. Chromosomal location of the AtBXL genes, percentage identity and number of identified ESTs were determined using MapViewer and BLAST at The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org).

**DNA sequencing**

EST H5E2T7 (accession number: W43785) was sequenced using the standard T3 and T7 primers and an internal primer (5'-GAGACTTGGTATGTTTGATGG-3').

**DNA constructs**

AtBXL1 heterologous expression. AtBXL1 complete and truncated cDNAs were amplified with primers 1NdeI (5'-CATAT GTCTTTGTAATAAAGCAC3') and Stop BamHI (5'-GGATCCCT- AAAGTTGGCGGTTGG-3') for the complete cDNA and with primers 90NdeI (5'-CATATGCTTCGACCACTGTTTGC-3') and Stop BamHI for the truncated cDNA, using Pfu polymerase (Promega, Mannheim, Germany), according to manufacturer's instructions and H5E2T7 as template. The amplified products were digested with NdeI and BamHI and subsequently cloned into pET14b vector (Invitrogen, Carlsbad, CA, USA), digested with the same enzymes. The two constructs were transferred by electroporation into E. coli, strain BL21 DE-3. Transformed cells were induced by IPTG 0.5 mM and proteins were harvested and purified according to manufacturer's instructions.

**AtBXL1 promoter::GUS fusion**. Genomic DNA from A. thaliana, ecotype WS, was used to amplify the 2-kb AtBXL1 promoter region with primers P1 (5'-GGG CTCG GCC GTG GTA TTA TATAA CAA GAC CAT-3') and P2 (5'-CTGAAA TTGGCGGTTGG-3') using Pfu polymerase from Promega according to manufacturer's instructions. The PCR fragment was cloned into EcoRV-digested pBSKS. This intermediate construct was then digested with PstI and HindIII and cloned into pCAMBIA1391Xb in frame with the uidA coding sequence (Jefferson, 1983) in the PstI and HindIII sites. The latter construct was then introduced into A. tumefaciens strain C58MP90 (Koncz and Schell, 1986) for plant transformation.

**Plant transformation**

Arabidopsis thaliana plants ecotype WS were transformed by dipping the floral buds of 4-week-old wild-type plants into an Agrobacterium solution, according to Clough and Bent (1998). Plants were left to self-pollinate and seeds were harvested after complete drying of the stems. Primary transformants were selected on Arabidopsis medium (Estelle and Somerville, 1987) containing 50 mg l⁻¹ kanamycin. Primary transformants were then self-pollinated to obtain plants homozygous for the insertion. Only lines segregating 3 : 1 for the resistance to kanamycin were selected for further analyses. Analyses were performed on mono-insertional homozygous lines, checked by Southern blot experiments.

**RNA analysis**

Total RNAs were isolated from 1 g of frozen tissues (1-week-old seedlings, young leaf blades, apical and basal part of the stem, flowers, and immature green siliques harvested from 4-week-old plants), according to Verwoerd et al. (1989). RNA samples (20 µg) were separated on formaldehyde/agarose gels, transferred onto nylon filters (NEN, Boston, USA) and hybridized to randomly primed double stranded DNA probes. The AtBXL1 probe corresponds to the 300-bp EcoRI fragment of the 3' region of the cDNA clone H5E2T7. The AtBXL2, AtBXL3, and AtBXL4 probes correspond to the cDNA clones 203K11T7, SQ191a07, and APZL50c08, respectively (accession number: H78603, AV563651 and AV524052). These probes were synthesized by plasmid digestion and in-gel purification of the cloned cDNA. RNA quantification was estimated under UV light on gels stained with ethidium bromide.

**Soluble and cell wall fraction protein extraction**

Hundred milligrams of each tested tissue were ground in liquid nitrogen and transferred to a microfuge tube. Ground material was extracted with 500 µl 25 mM MOPS, pH 7, 0.5 mM Pefabloc and centrifuged 10 min at 1000 g. Supernatant was recovered and constituted the soluble fraction (SF). These steps were repeated three times. The cell wall residue was then extracted with 500 µl 25 mM MOPS, pH 7 and 200 mM CaCl₂ during 1 h under vigorous shaking at 4 °C. The tube was then centrifuged 10 min at 1000 g and the supernatant, the cell wall fraction (CWF), was recovered. The CWF was salt-purified by gel filtration using NAP5™ columns from Pharmacia, according to the supplier's instructions. Protein quantity was determined using protein dye reagent from Bio-Rad (Munich, Germany) according to Bradford (1976). Protein concentration was adjusted to 250 ng µl⁻¹ using a rotative evaporator.

**Beta-xylosidase activity assay**

One to five micrograms of total proteins of CWF or SF were incubated for 1 h at room temperature in 100 mM citrate buffer,
exhibited beta-xylosidase activities were pooled and dialyzed for beta-xylosidase activity at 45°C. One milliliter fractions were collected and 100 mM NaCl, in presence of 0.025% Triton X-100 (pH 5.0) at room temperature. Fractions of 0.4 ml were collected at a flow rate of 0.5 ml min⁻¹ and 100 µl of each fraction was assayed for beta-xylosidase activity at 37°C for 60 min. Fractions which exhibited major beta-xylosidase activity were further separated by second cation exchange chromatography.

**Size exclusion chromatography.** The stem protein extract in a volume of 0.5 ml (0.70 mg) was fractionated by FPLC (Pharmacia) on a Superdex 75 HR10/30 column (Amercham Pharmacia Biotech) pre-equilibrated in 20 mM Na-acetate buffer, 150 mM NaCl, in presence of 0.025% Triton X-100 (pH 5.0) at room temperature. Fractions of 0.4 ml were collected at a flow rate of 0.5 ml min⁻¹ and 100 µl of each fraction was assayed for beta-xylosidase activity at 37°C for 60 min. Fractions which exhibited beta-xylosidase activities were pooled and dialyzed against 20 mM Na-acetate buffer, pH 5.0, in presence 0.025% Triton X-100.

**Cation-exchange chromatography.** The pooled fractions were applied to a 1.5 cm x 3.0 cm column of CM-Trisacryl (Amercham Pharmacia Biotech) and eluted first with 20 mM Na-acetate buffer in presence 0.025% Triton X-100 (pH 5.0), and then with the same buffer in presence of 0.1–0.4 M NaCl discontinuous gradient. One milliliter fractions were collected and 100 µl assayed for beta-xylosidase activity at 45°C for 60 min.

**Assay for beta-xylosidase activity after fractionation**

The beta-xylosidase activity was measured by an assay adapted from Huang et al. (1991) and Ransom and Walton (1997). The reaction mixture contained 2 mM p-nitrophenyl beta-xylopyranoside (Sigma), 0.1 M acetate buffer (pH 5.0) and 100 µl chromatographic fraction in a total volume of 0.5 ml. The reaction was carried at 37 or 45°C for 60 min and stopped by the addition of 0.5 ml of 0.4 M sodium bicitarbate to the assay mixture. The amount of liberated p-nitrophenol was determined spectrophotometrically at 405 nm.

**GUS assays**

Beta-glucuronidase assays were performed on freshly harvested tissues with 0.5 mg ml⁻¹ XGluc (Biosynth AG) according to Jefferson et al. (1987) in 100 mM potassium phosphate buffer, pH 7, 0.1% Triton X-100, 10 mM K₃Fe(CN)₆ and 10 mM K₄Fe(CN)₆ to avoid diffusion of the intermediary reaction product.

**Southern experiments**

Plant DNA was extracted according to Doyle and Doyle (1990) and Southern experiments were performed according to Sambrook et al. (1989).

**Preparation of cell wall material and determination of the sugar composition**

Stems of wild-type and ASBX lines were allowed to dry in the greenhouse and ground to a fine powder using a Warring blender. Total neutral composition was determined according to Blakeney et al. (1983). For analysis of the hemicellulose fraction, the powder was washed twice with 70% hot ethanol. The remaining pellet, corresponding to the cell walls, was then freeze-dried. Cell wall material was hydrolyzed with 2 N trifluoro-acetic acid (TFA), and sugar composition was determined by gas chromatography analysis of their trimethylsilyl methyl ester methyl glycoside derivatives according to York et al. (1985) using inositol as internal standard.

**Analysis of the cellulose content**

Cell walls were hydrolyzed for 2 h at 110°C with 200 µl of 2 M TFA containing 10 µg of inositol. After centrifugation, the supernatant was collected, neutralized, and lyophilized. The TFA-insoluble cellulosic material was washed with water and then dissolved in 80 µl of 72% H₂SO₄ at room temperature for 1 h. After dilution to 3% H₂SO₄ with water and addition of 10 µg of inositol, the solution was heated at 110°C for 2 h. The sample was then neutralized with BaCO₃, centrifuged and the supernatant was lyophilized. Sugar in both cellulosic and the TFA-soluble fractions were quantified by gas chromatography using inositol as internal standard. Percentage of cellulose was expressed as the ratio between the amount of glucose in the cellulosic fractions and the amount of total sugar in the TFA-soluble and insoluble fractions.

**Mass spectrometry analysis of endoglucanase-generated xyloglucan fragments**

Xyloglucan fragments were generated by treating 1 mg of wall material with 5 U of endo-beta(1,4)-glucanase (Megazyme International Ireland, EC 3.2.1.4, cat. n E-CELTR) in 500 µl of 10 mM sodium acetate buffer pH 5 for 18 h. Matrix-assisted laser desorption/ionization-time of flight mass spectra (MALDI-TOF MS) of the resulting xyloglucan solubilized fragments were recorded on a Micromass Tof spec E MALDI-TOF mass spectrometer (Manchester, UK). Mass spectra were performed in the reflectron mode using 2,5-dihydroxybenzoic acid as matrix (Bakker et al., 2001).

**Lignin characterization**

The lignin content of ground extractive-free mature dry stems was estimated by the standard Klasson procedure (Dence, 1992). Lignin structure was investigated using the thioacidolysis technique as...
modified by Lapiere et al. (1995). The lignin-derived monomers were identified by GC-MS of their TMS derivatives.

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References


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