Carbohydrate-Binding Module of a Rice Endo-β-1,4-glycanase, *OsCel9A*, Expressed in Auxin-Induced Lateral Root Primordia, is Post-Translationally Truncated

Kouki Yoshida 1,*, Nobuyuki Imaizumi 2, Satoshi Kaneko 3, Yasushi Kawagoe 4, Akemi Tagiri 4, Hiroshi Tanaka 4, Kazuhiko Nishitani 5 and Kozo Komae 6

1 Hydraulic and Bio Engineering Research Section, Technology Center, Taisei Co., 344-1 Nase-cho, Totuka-ku, Yokohama, 245-0051 Japan  
2 Research Division, Japan Turfgrass Inc., 3-6-3 Akanehama, Narashino-shi, Chiba, 275-0024 Japan  
3 Biological Function Division, National Food Research Institute, 2-1-12 Kannondai, Tsukuba-shi, Ibaraki, 305-8642 Japan  
4 Department of Wheat and Barley Research, National Institute of Crop Science, 2-1-18 Kannondai, Tuskuba-shi, Ibaraki, 305-8518 Japan  
5 Department of Developmental Biology and Neurosciences, Tohoku University, Sendai, 980-8578 Japan  
6 Department of Wheat and Barley Research, National Institute of Crop Science, 2-1-18 Kannondai, Tuskuba-shi, Ibaraki, 305-8518 Japan

We report the cloning of a glycoside hydrolase family (GHF) 9 gene of rice (*Oryza sativa* L. cv. Sasanishiki), *OsCel9A*, corresponding to the auxin-induced 51 kDa endo-1,4-β-glucanase (EGase). This enzyme reveals a broad substrate specificity with respect to sugar backbones (glucose and xylose) in β-1,4-glycans of type II cell wall. *OsCel9A* encodes a 640 amino acid polypeptide and is an ortholog of *TomCel8*, a tomato EGase containing a carbohydrate-binding module (CBM) 2 sequence at its C-terminus. The expression of four rice EGase genes including *OsCel9A* showed different patterns of organ specificity and responses to auxin. *OsCel9A* was preferentially expressed during the initiation of lateral roots or subcultured root calli, but was hardly expressed during auxin-induced coleoptile elongation or in seed calli, in contrast to *OsCel9D*, a KORRIGAN (KOR) homolog. In situ localization of *OsCel9A* transcripts demonstrated that its expression was specifically up-regulated in lateral root primordia (LRP). Northern blotting analysis showed the presence of a single product of *OsCel9A*. In contrast, both mass spectrometric analyses of peptide fragments from purified 51 kDa EGase proteins and immuno blot analysis of EGase proteins in root extracts using two antibodies against internal peptide sequences of *OsCel9A* revealed that the entire CBM2 region was post-translationally truncated from the 67 kDa nascent protein to generate 51 kDa EGase isoforms. Analyses of auxin concentration and time course dependence of accumulation of two EGase isoforms suggested that the translation and post-translational CBM2 truncation of the *OsCel9A* gene may participate in lateral root development.

**Keywords:** Auxin — Carbohydrate-binding module family 2 — Glycoside hydrolase family 9 — Lateral root formation — Processing — Rice.

Abbreviations: ATCH, adrenocorticotropic hormone; CBM, carbohydrate-binding module; CTAB, cetyltrimethylammonium bromide; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EGase, endo-1,4-β-glucanase; GAX, glucuronarabinoxylan; GHF, glycoside hydrolase family; Glu-C, endoproteinase Glu-C; IEF, isoelectric focusing; KLH, keyhole limpet hemocyanin; KOR, KORRIGAN; LRP, lateral root primordia; Lys-C, endoproteinase Lys-C; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; m/z, mass-to-charge ratio; nano ESI MS/MS, nano electrospray ionization tandem mass spectrometry; PMF, peptide mass fingerprinting; RT–PCR, reverse transcription–PCR; TCA, trichloroacetic acid; UTR, untranslated region.

The nucleotide sequences of *OsCel9A* and *OsCel9D* have been submitted to the GenBank database with the accession numbers AB038510 and AB040817, respectively.

**Introduction**

Primary cell walls of higher plants are composed of cellulose microfibrils embedded in a matrix of xyloglucans, glucuronarabinoxylans (GAXs), 1,3-1,4-β-glucans, glucomannans, pectic polysaccharides and proteins (McNeil et al. 1984, Bacic et al. 1988, Carpita and Gibaeut 1993). Plant endo-1,4-β-glucanase (EGases) of glycoside hydrolase family (GHF) 9 generally cleave the internal β-1,4-linkages of the glucosyl backbones of amorphous cellulose, xyloglucan and 1,3,1,4-β-glucans (Brummell et al. 1994); however, it has recently been demonstrated that a few plant EGases can also hydrolyze xylan backbones (Ohmiya et al. 1995, Yoshida and Komae 2006) as do some microbial GHF9 enzymes (Eckert et al. 2002, Kurokawa et al. 2002). Xylan is a β-1,4-linked polymer of α-xylose, a saccharide unit structurally similar to glucose, and enzyme specificity for this polysaccharide probably arises from discrimination against the CH2OH substituent found on C-5 of glucose. Further discrimination could also be based on the planar nature of a cellobioigosaccharide chain compared with the more helical structure of xylan (Atkins 1992).
GHFs appear to accommodate a range of β-1,4-linked polymers (xylan and β-glucan) through the replacement of amino acids in the substrate-binding sites, as for β-glucosidase and β-xylosidase groups of GHF3 (Hrmova et al. 2002), or through subtle conformational changes in the substrate-binding sites such as microbial GHF10 (Notenboom et al. 1998). However, there is no molecular basis of broad substrate specificity with respect to sugar backbones in GHF9 enzymes.

Plant EGases belong to the E2 subclass of GHF9 (Béguin 1990). The E2 subclass of GHF9 enzymes is widely distributed among phyla, occurring in numerous bacteria, slime molds, fungi, and various plant and animal species. In general, microbial E2-type EGases typically possess a suite of a carbohydrate-binding module 3c (CBM3c), one or three fibronectin-domain-like 3 regions, a linker region and a CBM2 at the C-termini of their catalytic cores (Bayer et al. 2000, Kurokawa et al. 2002). CBMs have three general roles with respect to the function of their cognate catalytic modules of glycoside hydrolases: (i) a proximity effect; (ii) a targeting function; and (iii) a disruptive function (Linder and Terri 1997, Boraston et al. 2004), and there are recognition sites for CBMs in plant cell walls (McCartney et al. 2006). These modular types of microbial E2 enzymes are able to degrade crystalline cellulose. In contrast, most plant EGases studied consist solely of the catalytic core with or without a transmembrane domain (Ohmiya et al. 1995, Nicol et al. 1998). A new subclass of plant GHF9 enzymes including TomCel8, faEG3, Nicel8, PeEG2 and PpEG4 has been identified recently in dicots (Catala and Bennet 1998, Trainotti et al. 1999, Goellner et al. 2001, Hisawa et al. 2003, Trainotti et al. 2006). Rice and Arabidopsis also contain three orthologs of TomCel8 (Libertini et al. 2004, Yokoyama and Nishitani 2004, Hayashi et al. 2005). This subclass has an extra peptide at its C-terminus that is homologous to the eukaryotic CBM2 (Meinke et al. 1991, Wang et al. 2001), and the recombinant CBM2 of TomCel8 does indeed bind to crystalline cellulose in vitro (Urbanowicz et al. 2004). The catalytic core in the CBM2 type of plant GHF9 also appears to mimic those in the microbial E2 enzymes, since several aromatic platform residues (tryptophan, phenylalanine and tyrosine) at the substrate-binding subsites of the microbial E2 enzymes are conserved in this isozone (Master et al. 2004). However, the substrate range of the catalytic core isolated from the CBM2-type EGase of plants and the accumulation of this EGase protein remain to be demonstrated.

Auxin stimulates or mediates extensive lateral root formation (Himannen et al. 2002, Wang et al. 2003). Lateral root primordia (LRP) are derived from pericycle cells deep within the parental root tissue. Differentiated pericycle cells are highly vacuolated with thickened secondary walls (Sutherland and McCully 1976), while the cell wall of established LRP induced by 2,4-D shows a more amorphous structure (Nishimura and Maeda 1982). LRP pass through the parent cortex layer and penetrate the epidermis as it laterally elongates (Malamy and Benfey 1997). The retaining of intercellular walls by the pericycle and endodermal layer seems to be central to the restriction of lateral root penetration into the parental root tissue. As a consequence of the lack of plant cell movement, unlike animal systems, the local modification of cell walls assumes great importance (Knox 1992). This implies the localized activation of cell wall-related enzymes during the formation of LRP. Indeed, cell wall-modifying proteins such as expansin (Cho and Kende 1998), subtilisin-like protease (Neuteboom et al. 1999), GHF9 (Goellner et al. 2001), polygalacturonase (Roberts et al. 2002) and pectate lyase (Laskowski et al. 2006) are expressed in the LRP or during the emergence of lateral roots. The EGase gene and the expansin gene were mapped to intervals carrying quantitative trait loci for seminal root length and lateral root length, respectively, in rice varieties (Zheng et al. 2003). In the accompanying manuscript, we report the purification of three 51 kDa EGase isoforms whose activity is stimulated in primary roots of rice by an auxin analog, 2,4-D. The purified EGase of rice reveals a broader substrate range distinct from dicotyledonous and woody EGases. Its substrate-binding subsites can tolerate more hydrophobic xylose-based polymers, being compatible with the hemicellulosic components of type II cell wall in rice (Yoshida and Komae 2006).

Here we report the cloning of OsCel9A encoding the auxin-induced rice EGase (GHF9 isoyme) with this broad substrate specificity. OsCel9A is an ortholog of TomCel8. The entire CBM2 region was post-translationally truncated from the nascent form of OsCel9A (67 kDa) to generate a 51 kDa EGase. We describe the catalytic active site of OsCel9A based on homology modeling, auxin regulation, patterns of spatial localization of mRNAs of OsCel9A and the immunodetection of EGase isoforms with and without the CBM2 during lateral root formation.

**Results**

**Primary structure of OsCel9A protein**

To determine the complete amino acid sequence of the 51 kDa EGase of rice (cv. Sasanishiki) with broad substrate specificity, we isolated a 2.2 kb GHF9 clone designated OsCel9A from a cDNA library made from mRNA of primary roots treated with 2,4-D for 24 h. The 8.7 kb genomic clone of OsCel9A was also isolated from a genomic DNA library of rice cv. Sasanishiki (λ-DASH II) and sequenced. The deduced amino acid sequence of OsCel9A cDNA and genomic DNA completely matched...
the sequences previously obtained for the N-terminal peptide and 11 internal peptides of the purified 51 kDa EGase, which consist of a total of 216 amino acid residues (Supplementary Table S1 of Yoshida and Komae 2006). The isolation, structural characterization and chromosome position of the OsCel9A gene are described in the Supplementary data (Figs. S1, S2).

The complete amino acid sequence of OsCel9A was determined by a combination of sequencing peptides derived from the purified enzyme, and sequencing the corresponding cDNA and the genomic clone (Fig. 1A). In the accompanying manuscript, we describe the purification of three 51 kDa rice EGases (peaks I, II and III), whose 20 N-terminal amino acid sequences were identical, indicating that these proteins are isoforms cognate to one gene (Yoshida and Komae 2006). The N-terminal sequences of these 51 kDa EGases (GGGGHDYGMA... ) indicated that Gly35 is the N-terminal residue of the mature OsCel9A protein (shown by an open inverted triangle in Fig. 1A). Signal P 3.0 software, a predictor for signal peptides, indicated that the putative signal cleavage site is between Gly34 and Gly35 of OsCel9A, which is consistent with the sequencing data of the N-terminal peptides of the 51 kDa EGase forms (http://www.cbs.dtu.dk/services/SignalP/).

OsCel9A has an extra peptide of about 130 amino acid residues at the C-terminus when compared with other paralogs of GHF9 from rice. The C-terminal 90 amino acid residues of OsCel9A are homologous to those of the putative CBM of plant GHF9 enzymes. The putative CBM of OsCel9A contains the consensus pattern of CBM2 (W-N-[STAGR]-[STDN]-[LIVM]-X(2)-[GST]-X-[GSTM]-X(2)-[LIVMFT]-[GA]) (Prosite Documentation PDOC00485, http://www.expasy.org/cgi-bin/nicedoc/), while the consensus is not fully conserved and three amino acid residues are substituted (shown as a dotted line in Fig. 1A). The CBM of OsCel9A contains only three conserved tryptophan residues (star in Fig. 1A) upstream of the consensus pattern, and no cysteine, unlike the microbial CBM2 that is found at the C-terminus of bacterial GHF9 enzymes.

**Determination of the C-terminal residues of the 51 kDa isoforms of OsCel9A**

OsCel9A cDNA encodes a mature polypeptide of 606 amino acids, since the N-terminal signal sequence of OsCel9A is truncated as described above. The calculated molecular mass from the deduced amino acid sequence was 65,164 Da for OsCel9A, which has a calculated pI of 6.29 (Table 1). However, the calculated pI values do not correspond with the respective values of 5.75, 5.65 and 5.50 for peaks I, II and III of the 51 kDa EGase (Fig. 2A). In addition, the molecular mass deduced from the cDNA sequence is considerably higher than the values of 51,218, 51,391 and 51,605 obtained for the purified EGase using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) mass spectrometry (MS) (Table 1). The identities of the three 51 kDa EGase forms (peaks I, II and III) were confirmed by MALDI-TOF MS analysis of tryptic peptides produced from in-gel digests of the three purified forms of the 51 kDa EGase. Twenty-six fragments were present in mass spectra of each tryptic digest from the three 51 kDa isoforms. They corresponded to unique tryptic fragments of OsCel9A based on the cDNA sequence using the Mascot search program. This program revealed that the sequence coverage of observed fragments was 63–65% against the deduced amino acid sequences of OsCel9A after truncation of its signal peptide. We could not detect peptide fragments derived from the actual C-terminal regions of the 51 kDa EGase in MALDI-TOF mass spectra of tryptic digests.

Based on the previous determination of N-terminal residues (Gly35) and average molecular masses of the three isoforms, we predicted that the probable C-termini are Arg514 (peak I), Arg512 (peak II) and Ala511 (peak III) (shown by a downward-pointing arrow in Fig. 1A). Endoproteinasises Lys-C and Glu-C were chosen for analysis, since their recognition sites are not found around these probable C-termini (the nearest cleavage sites are shown by filled inverted triangles in Fig. 1A). A fragment of mass-to-charge ratio (m/z) of 12,037.48 was present in the mass spectra of Lys-C digests from peak III protein. This value agreed with the theoretical mass of 400SQV...HGAR513 (m/z 12,035.18) at the C-terminus of the 51 kDa protein of OsCel9A based on a Mascot search program. Two fragments of m/z 12,191.17 and 12,262.21 were observed in the mass spectra of Lys-C digests from peak II protein. A Mascot search revealed that two fragments from peak II protein also agreed with theoretical masses of 400SQV...HGAR512 (m/z 12,191.37) and 400SQV...HGAR513 (m/z 12,262.45). As shown in Fig. 2B, a fragment of m/z 2,153.15 (*2) was present in MALDI-TOF mass spectra of the peak III isoform digested by Glu-C. This fragment, as its trivalent ion (m/z 718.43), was sequenced with nano electrospray ionization tandem mass spectrometry (nano ESI MS/MS) using the PepSeq program and fitted to 400AAT...HGA511 at the C-terminus of the 51 kDa protein. Two fragments (*3, m/z 2,309.25) and *4, m/z 2,380.28) were found in Glu-C digests of the peak II isoform and were also sequenced using nano ESI MS/MS, fitted to 400AAT...HGA512 and 400AAT...HGAR513. Two fragments (*6, m/z 2,536.35) and *7, m/z 2,593.37) were present in the Glu-C digests of the peak I isoform in addition to the two fragments observed in the digest of peak II isoform (*3 and 4 signals in Fig. 2B). A Mascot search revealed that these larger fragments (*6 and *7 signals) corresponded...
Fig. 1

Post-translational truncation of rice E.Gase CBM2
to the calculated masses of \( ^{40}\text{AAT} \ldots \text{HGANR}^{514} \) (\( m/z \ 2,536.33 \)) and \( ^{40}\text{AAT} \ldots \text{HARARG}^{515} \) (\( m/z \ 2,593.35 \)), respectively.

A fragment of \( m/z \) 2,001.92–2,001.94 was present in all isoforms (peaks I, II, and III), corresponding to the calculated mass of \( ^{35}\text{GGG} \ldots \text{YFE}^{45} \) (\( m/z \ 2,001.94 \)) at the N-terminus (signal “1” in Fig. 2B). A fragment of \( m/z \) 2,520.22–2,520.27 was present in all isoforms, corresponding to the calculated mass of \( ^{97}\text{AIK} \ldots \text{YAE}^{118} \) (signal “5” in Fig. 2B). A fragment of \( m/z \) 2,268.08–2,269.15 was also observed in buffer blank with Glu-C, corresponding to the peptide fragment of Glu-C itself (signal “Glu-C” in Fig. 2B). These results are also summarized in Table 1. From these results, we concluded that the entire CBM is precisely removed from the 51 kDa forms of OsCel9A and that purified 51 kDa forms of OsCel9A contain multiple (at least five) C-terminal residues in the region between Ala511 and Gly515. The calculated pI values based on the amino acid residues are in accordance with the multiple pI values of the three purified 51 kDa EGase isoforms determined by isoelectric focusing (IEF) (Fig. 2 and Table 1).

**Immunodetection of OsCel9A polypeptides in primary root tissues of rice**

Two synthetic peptides comprising a 15 and a 13 amino acid sequence from OsCel9A were used to produce specific antibodies against OsCel9A. An antibody against the peptide \( ^{463}\text{GPDEHHDFADAERNNYY}^{478} \), which is upstream of the C-terminal cleavage site of OsCel9A, was designated ‘anti-celA’, while another antibody against the peptide \( ^{532}\text{TSPLHG}^{544} \), which is downstream of the C-terminal cleavage site of OsCel9A, was designated ‘anti-celB’ (Fig. 1A). The peptide sequences selected as epitopes were aligned with equivalent regions of the other rice GHF9 paralogs (Fig. 3A). Except for OsCel9B and OsCel9C, these sequences displayed relatively low identities of 40–67 and 0–38%, respectively, to equivalent regions of 20 rice GHF9 paralogs based on a Fasta search (http://www.dpbj.nig.ac.jp/search/fasta-j.html).

To characterize OsCel9A polypeptides, a protein extract isolated from root segments cultured with 2,4-D for 24 h was challenged with affinity-purified anti-celA and anti-celB antibodies. Fig. 3B shows that anti-celA reacted with three bands of 51, 67 and 110 kDa in total protein extract from acetone powder of root tissue with SDS–PAGE buffer at 100 °C (lane A in Fig. 3B), while another antibody against a peptide sequence downstream of the C-terminal cleavage site of OsCel9A reacted with a single band of 67 kDa (lane B in Fig. 3B). When the same extract was challenged with pre-immune IgG of rabbit, no significant signal was detected (lane N in Fig. 3B). The 51 and 67 kDa bands were consistent with the molecular masses of truncated and nascent forms of OsCel9A, respectively. In contrast, the 110 kDa protein did not correspond to the calculated molecular mass of any GHF9 isozyme in rice based on deduced amino acid sequences (54–68 kDa).

**Expression patterns of the OsCel9A gene in auxin-responsive tissues**

Nine cDNA clones of OsCel9A were isolated from a auxin-treated root cDNA library of rice in addition to seven cDNA clones classified into three different rice GHF9 paralogs (see Supplementary material and Fig. S2). This suggests that mRNA of OsCel9A may be comparatively abundant among the four GHF9 paralogs in auxin-treated root tissues, although we could not rule out the possibility that these clones were selectively enriched because a partial DNA fragment of OsCel9A was used to screen the cDNA library (probe 1 in Supplementary Fig. S1). To determine relative paralog abundance, the expression patterns of the four GHF9 paralogs were determined by Northern analysis.
Preferential expression of were seen among the four GHF9 paralogs (Fig. 4B). High levels of expression were found in coleoptile segments (lanes 6, 7 and 8) whereas little change was observed in root segments (lanes 1, 2 and 3). The expression patterns of OsCel9A showed a restricted expression pattern, with expression seen in 2,4-D-treated root segments (lane 3).

Real-time reverse transcription–PCR (RT–PCR) was used to confirm the transcript levels of OsCel9A and OsCel9D genes in primary roots, calli and coleoptiles of rice. The results obtained using RT–PCR experiments were consistent with those obtained by Northern analysis as seen by comparing the black (OsCel9A) vs. the white (OsCel9D) bars in Fig. 4C. mRNA of OsCel9A was hardly detected (<0.5 copies pg⁻¹ total RNA) in primary roots at the initial stage of culturing, when no primordia were detected (lane 1). After 24 h of culturing, when LRP were observed, mRNA of OsCel9A was substantially expressed in primary roots (approximately 57 copies, lanes 5–8). These results indicate that expression of OsCel9A is highly correlated with the formation of LRP, suggesting that the transcription level of OsCel9A may be developmentally regulated.

The expression patterns of a KORRIGAN (KOR) homolog of rice GHF9, OsCel9D, seemed to contrast with those of OsCel9A. OsCel9D has recently been identified as the Glu 1 gene (Zhou et al. 2006). A higher level expression of OsCel9D was seen in seed callus (lane 5 in Fig. 4B) but not in root callus (lane 4). Upon treatment with 2,4-D, the expression level of OsCel9D increased slightly during coleoptile elongation (lanes 6, 7 and 8) whereas little change was observed in root segments (lanes 1, 2 and 3). The expression of OsCel9D, a KOR homolog, appears to be associated with coleoptile elongation and the culturing of seed callus.

**In situ hybridization of OsCel9A in auxin-treated root tissues**

To determine the spatial expression pattern of the OsCel9A gene in auxin-treated root tissue, we conducted in situ mRNA hybridization on sections cut from rice primary root segments cultured with 15 μM 2,4-D for 24 h.

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**Table 1** Multiple isoforms of OsCel9A, a rice GHF9 enzyme with a putative CBM2

<table>
<thead>
<tr>
<th>Sequence of OsCel9A</th>
<th>Mol. wt</th>
<th>pI</th>
<th>Purified isoform</th>
<th>Mol. wt</th>
<th>pI</th>
<th>Immunodetection</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 kDa form&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>Peak III</td>
<td>51,216</td>
<td>5.50</td>
<td>Peak I</td>
</tr>
<tr>
<td>35–511 (GG...GA)</td>
<td>51,218</td>
<td>5.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35–512 (GG...GAR)</td>
<td>51,374</td>
<td>5.65</td>
<td>Peak II</td>
<td>51,391</td>
<td>5.65</td>
<td>peak II</td>
</tr>
<tr>
<td>35–513 (GG...GARA)</td>
<td>51,446</td>
<td>5.65</td>
<td>Peak II</td>
<td>51,391</td>
<td>5.65</td>
<td>peak II</td>
</tr>
<tr>
<td>35–514 (GG...GARAR)</td>
<td>51,602</td>
<td>5.75</td>
<td>Peak I</td>
<td>51,605</td>
<td>5.75</td>
<td>peak I</td>
</tr>
<tr>
<td>35–515 (GG...GARARG)</td>
<td>51,659</td>
<td>5.75</td>
<td>Peak I</td>
<td>51,605</td>
<td>5.75</td>
<td>peak I</td>
</tr>
<tr>
<td>From 35–511 to 35–515</td>
<td>51,218–51,659</td>
<td>5.65–5.75</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>51 kDa membrane-associated form</td>
</tr>
<tr>
<td>65 kDa form 35–606 (GG...YKLV)</td>
<td>65,164</td>
<td>6.29</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>67 kDa buffer-soluble form</td>
</tr>
<tr>
<td>68 kDa form 1–640 (MA...YKLV)</td>
<td>68,438</td>
<td>6.52</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>67 kDa membrane-associated form</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Molecular average mass determined with MALDI-TOF-MS.<br>
<sup>b</sup> pI values determined with an IEF glass column.<br>
<sup>c</sup> Summary of immunoblotting in Fig. 6A.<br>
<sup>d</sup> Summary of peptide sequencing and mass spectrometric analysis of purified 51 kDa endo-1,4-β-glucanase with broad substrate specificity.<br>
<sup>e</sup> Not purified or determined.
Longitudinal sections through 2,4-D-treated roots were treated with antisense or sense riboprobes corresponding to the 254 bp 3'-UTR of rice OsCel9A (probe 3 in Supplementary Fig. S1). Fig. 5 shows strong expression of OsCel9A in LRP at 24 h of culturing in the presence of 2,4-D in sections probed with an antisense (Fig. 5A, C) but not a sense (Fig. 5B, D) sequence. The OsCel9A gene is expressed in cells derived from the pericycle during early primordium development but not in the endodermal and epidermal layers (Fig. 5A) or the neighboring non-proliferative pericycle (Fig. 5C).

Concentration and time dependence of auxin effects on lateral root formation and the accumulation of 51 and 67 kDa polypeptides of OsCel9A

The concentration dependence and the time dependence of auxin effects on lateral root formation and OsCel9A protein accumulation were examined to investigate their
potential correlation and are described in the Supplementary material (Figs. S4, S5).

Multiple EGase isoforms of OsCel9A in buffer-soluble and microsomal fractions

The structure of the deduced OsCel9A with CBM2 suggested that the 67 kDa EGase protein would be bound to the cell wall, since the main function of microbial CBM2 is considered to be to attach the enzyme to the cellulose or xylan surface and thereby to increase the local concentration of substrate, leading to more efficient catalysis (Simpson et al. 2000). To test this possibility, immunodetection of OsCel9A protein in total extracts from primary root segments cultured with 2,4-D using the two polyclonal antibodies against the synthetic peptides (see Figs. 1 and 3A). Total protein from root explants cultured with 15 μM 2,4-D for 24 h (20 μg per lane) was separated by 7.5% SDS–PAGE, electroblotted to a HyBond-P membrane, probed with affinity-purified anti-cel_α antibody (lane A), affinity-purified anti-cel_β antibody (lane B) or purified pre-immune rabbit IgG (lane N), and detected with the ECL-plus system. Other experimental procedures are described in Materials and Methods.

In contrast to the soluble fraction, the anti-cel_α antibody reacted with a major 67 kDa band and two minor bands of 51 and 110 kDa in microsomes (lane 3 in Fig. 6A). A single band of protein was also detected in microsomal membranes with anti-cel_β, corresponding to 67 kDa (lane 3). Membrane-associated forms of rice EGases appear to be slightly larger than soluble EGases, as has been reported for bean EGases (del Campillo et al. 1988). The slightly larger form in microsomes could represent a pre-secretory form. The slight difference in the molecular weight could arise from post-translational processing by N-glycosylation, glycan trimming (Bennet and Christoffersen 1986) or truncation of the N-terminal charged and membrane-spanning regions of nascent protein (Brummell et al. 1997).

The cell wall (2 M NaCl-soluble), microsomal and supernatant fractions of auxin-treated rice root segments were analyzed for the distribution of EGase activity in the presence of 0.17% Triton X-100. As shown in Fig. 6B, the specific activity of membrane-associated EGase was very low even in the presence of Triton X-100, being 2% of total EGase activity in root tissues. This suggests that the membrane-associated EGases of 51 and 67 kDa may be minor components or inactivated forms.
Collectively, most of the OsCel9A form with CBM2 can be easily solubilized from the cell wall with low-salt buffer or by lipid extraction. In addition, the 67 kDa protein form may be mainly associated with microsomal membrane. The buffer-soluble 67 kDa form of OsCel9A appears to be a minor component.

**Discussion**

*CBM2 of OsCel9A is truncated during lateral root development*

In this report, we have described the accumulation of multiple OsCel9A proteins with and without a putative CBM2 associated with auxin-induced formation of LRP (Supplementary Figs. S4, S5; see Supplementary Results), the first such finding for a plant GHF9 enzyme. The 51 kDa form may accumulate predominantly, as previously characterized buffer-soluble 51 kDa EGases have broad substrate specificity (Yoshida and Komae 2006), while the buffer-soluble 67 kDa EGase may be a minor component (Fig. 6A). N- or C-terminal CBM (or propeptides with unknown functions) of several other cell wall-modifying proteins from higher plants are truncated at the post-translational level during seed germination (Simos et al. 1994, Lee et al. 2003, Simpson et al. 2003), fruit ripening (Chen and Paull 2003), maturation of microspores (Wu et al. 2002) or growth of cultured cells (Downes et al. 2001). In the case of OsCel9A, we could not isolate a cDNA encoding the 51 kDa EGase without CBM from the root cDNA library of Sasanishiki and retrieved no expressed sequence tag (EST) encoding the 51 kDa EGase cognate to OsCel9A from the genome database of Nipponbare (http://rapdb.lab.nig.ac.jp/). Also, multiple C-termini of the 51 kDa EGase (Ala511–Gly515) are inside exon VI of OsCel9A, encoding amino acids from Lys304 to His523.
Fig. 5  Localization of OsCel9A transcripts by in situ hybridization. The hybridized RNA probe (3’-UTR region of OsCel9A cDNA, see Materials and Methods) is shown by purple shading. Longitudinal sections of primary roots of rice cultured with 15 μM 2,4-D for 24 h are shown. The conditions for in situ hybridization are described in Materials and Methods. (A) Antisense probe; lower magnification; bar = 50 μm. (B) Sense probe; the section from the same sample as A, lower magnification. (C) Antisense probe; higher magnification; bar = 25 μm. (D) Sense probe; a section from the same sample as D, higher magnification. c, cortex; e, epidermis; LRP, lateral root primordium; p, pericycle; vc, vascular cylinder.

Fig. 6  (A) Immunodetection of multiple isoforms of OsCel9A protein in 2,4-D-treated rice root tissues. A 20 μg aliquot of protein was loaded in each lane except the cell wall fraction. The extract (20 μl) from 20 mg dry weight of cell wall was applied. Experimental procedures were as described in Materials and Methods. (B) Specific activity of EGases in the cell wall and the microsomal and supernatant fractions from root tissues treated with 2,4-D for 24 h. The EGase activity towards carboxymethyl-cellulose was determined viscometrically in the presence of 0.17% Triton X-100. Error bars show the standard error (n = 3).
C-termini. Variable sites of proteolytic cleavage may thus the slight difference in pIs among the three purified (Schlochtermeier et al. 1992). As shown in Table 1, like, cathepsin B-like (Arg
(Figs. 1A, 2B and Table 1). This implies that a trypsin-
N-terminal CBM4 and immunoglobulin-like domain
have also been reported in the truncation of the
trimming of OsCel9A occurs. Multiple cleavage sites
suggest that variable endolytic cleavage or additional
the multiple C-termini of the purified 51 kDa EGases
(Kanellis and Kalaitzis 1992). As shown in Fig. 2B,
while they reveal the same molecular masses of 54 kDa
separated by activity staining following IEF–PAGE;
1997). A ripe avocado fruit contains 11 forms of EGase
OsCel9A revealed multiple pI values of 5.5, 5.65 and 5.75
(Fig. 2A). Plant tissues contain multiple forms of EGase
separated by activity staining following IEF–PAGE;
the predominant forms have pIs of 5.6, 5.8, 5.95 and 6.2,
while they reveal the same molecular masses of 54 kDa
(Kanellis and Kalaizisis 1992). As shown in Fig. 2B,
the multiple C-termini of the purified 51 kDa EGases
suggest that variable endolytic cleavage or additional
trimming of OsCel9A occurs. Multiple cleavage sites
have also been reported in the truncation of the
N-terminal CBM4 and immunoglobulin-like domain from Streptomyces reticulii GHF9 enzyme in vivo
(Schlochtermeier et al. 1992). As shown in Table 1, the slight difference in pIs among the three purified EGases of rice may be mainly based on their heterogeneous
C-termini. Variable sites of proteolytic cleavage may thus be one of the mechanisms to generate multiple forms of plant EGases with variable pIs.

Catalytic machinery of OsCel9A
The purified 51 kDa EGase of OsCel9A hardly hydrolyzes crystalline cellulose but significantly depolymerizes phosphoric acid-swollen cellulose, 1,3-1,4-β-glucan,
arabinoxylan, xylan and glucomannan, which are comparable with the requirements for the modification of type II cell wall (Yoshida and Komae 2006). The purified enzyme also hydrolyzes a xylohexaose to produce two xylotrioses with minor formation of a xylobiose and a xylotriose, suggesting that the substrate-binding subsites may tolerate xylose-based polymers. As noted by Master et al. (2004), the tryptophan residues at positions 256, 209 and 313 in TiCel9A from Thermobifida fusca, the best-characterized cellulase of the microbial E2 subfamily, which are crucially involved in binding Glc-4, Glc-3 and Glc-2, respectively, of β-1,4-glucans are missing in membrane-anchored plant GHF9 enzymes (Supplementary Fig. S3). Rice OsCel9A and all other plant CBM-type enzymes contain a tryptophan corresponding to Trp313 (subsite –2) in TiCel9A (Supplementary Fig. S3). Trp209 of subsite –3 in TiCel9A is mimicked by Phe245 in rice OsCel9A, while a substitution to threonine or serine is conserved among the membrane-anchored GHF9 enzymes. The consensus pattern of two GHF9 catalytic signatures and a distinct stretch of four glycine residues around the invariant tryptophan (subsite –2) in the substrate-binding site of OsCel9A are described in the Supplementary material (Fig. S3).

With regard to Tyr318 of subsite –3 in TiCel9A, the substitution with phenylalanine is conserved in all CBM2 types of plant GHF9 enzymes. As shown in Fig. 1B, the modeled structure of OsCel9A reveals that this substitution might leave more room in the substrate-binding subsites of this enzyme. Intriguingly, the substrate-binding subsites of OsCel9A appear to be adapted to soluble substrates rather than a crystalline substrate on the grounds that the genetic replacement of Y318F in TiCel9A facilitates enzyme activity against carboxymethyl-cellulose (667% of wild-type enzyme) but lowers the activity on bacterial microcrystalline cellulose (15% of wild-type enzymes; Zhou et al. 2004). In addition, the amino acid residue corresponding to Ile388 (subsite –2) in TiCel9A is replaced with tyrosine or phenylalanine in plant CBM types including OsCel9A but not in membrane-anchored plant GHF9 enzymes (Fig. 1B and Supplementary Fig. S3).

Collectively, the substrate-binding clef of rice OsCel9A shows a primitive disposition of an aromatic platform residue (tyrosine, tryptophan or phenylalanine), which presumably interacts with the saccharide rings by hydrophobic stacking (Quiocho 1986), mimicking the
catalytic machinery of microbial E2 enzymes. OsCel9A probably contains five sugar-binding sites; three glycone (−1 to −3) and two aglycone (+1 to +2) subsites based on the constellation of aromatic platform residues (Davies et al. 1997). This appears consistent with the previous speculation regarding the substrate-binding sites of purified 51 kDa EGase, which was based on the observed hydrolytic pattern of the enzyme on cellooligosaccharides (Yoshida and Komae 2006). The distinct dispositions of aromatic and hydrophobic platform residues in the substrate-binding site of OsCel9A might be involved in the tolerance of this enzyme for relatively hydrophobic xyllose-based polymers, as described in the accompanying manuscript (Yoshida and Komae 2006).

Postulated roles of CBM2 truncation in OsCel9A

The EGase with the CBM of OsCel9A might participate in the establishment of LRP from differentiated pericycle cells, since 67 kDa proteins were significantly detected after 6 h (Supplementary Fig. S5B, +2,4-D) when the domed shape of auxin-induced LRP was not visible in roots (Supplementary Fig. S5A, +2,4-D). Immunolocalization studies have detected hemicellulosic polymers of type II walls, which are endogenous targets of the 51 kDa EGase cognate to OsCel9A, in the root tips, the endodermal layers and the stele of primary roots in gramineous plants (Suzuki et al. 2000, Trethewey and Harris 2002). Although the relationship between structural changes in type II wall polymers and the development of LRP in root tissues has not yet been studied, histochemical studies have revealed that the differentiated walls of pericycle cells become thinner and no longer stain green or blue-green with toluidine blue O during the initiation of lateral root meristems, suggesting the removal of secondary wall components (Sutherland and McCully 1976). Low-branched xylans are localized in lignified regions of maize cell walls (Suzuki et al. 2000). Small amounts of 1,3-1,4-β-glucan have also been detected in the secondary walls of gramineous plants (Chesson et al. 1985, Smith and Harris, 1999, Trethewey and Harris, 2002). Hence, the 67 kDa EGase of OsCel9A could participate in the removal of secondary walls of pericycle cells in which crystalline or insoluble polymers are abundant (Supplementary Fig. S5B, +2,4-D), since the CBM2 facilitates the hydrolysis of crystalline cellulose by the cognate catalytic core in the microbial E2 subgroup of GHF9 (Irwin et al. 1998, Zhou et al. 2004). A high salt-extractable EGase from 2,4-D-treated pea epicotyls undergoing lateral expansion and cell division around the vessels was purified >30 years ago (Fan and Machlaclan 1966, Byrne et al. 1975). The molecular mass of cell wall-bound pea EGase is 70 kDa, comparable with the 67 kDa form of OsCel9A. Intriguingly, the 70 kDa EGase does not appear to be immunologically related to the buffer-soluble EGase of 15 kDa isolated from the same tissue (Byrne et al. 1975). Regardless of their molecular masses, the pea EGases bind to pea cellulose but not crystalline cellulose (Hayashi et al. 1984), and hydrolyze Whatman cellulose powder and xyloglucan but not xylan (Wong et al. 1977, Hayashi et al. 1984). The substrate range of the 67 kDa EGase of rice remains to be inspected.

Alternatively, the buffer-soluble and membrane-associated 67 kDa EGases could be minor components or inactive forms of OsCel9A (Fig. 6). Indeed, only the 51 kDa EGase has been isolated in active form from auxin-treated root tissues (Yoshida and Komae 2006). The 67 kDa band of EGase was not detected in the cell wall fraction (Fig. 6A), suggesting that the CBM2 region of OsCel9A does not confer strong cell wall binding activity to the 67 kDa EGase in vivo (Fig. 6A). Nevertheless, we cannot rule out the possibility that it might be poorly extracted from cell wall with SDS-PAGE buffer, which has been used for in vitro binding assays of recombinant proteins containing CBM2 from bacteria and slime molds (Tomme et al. 1995, Ramalingam and Ennis 1997). This suggests that the biochemical properties of the putative CBM2 region in OsCel9A may be distinct from those of microbial CBM2 as a helper module for the catalytic domain. The CBM22 region of gramineous xylanases has been suggested to be important for normal holding of thezymogen of xylanases rather than for sugar binding activity (Caspers et al. 2001, Simpson et al. 2003). The truncation of the N-terminal CBM22 region and C-terminal propeptide may result in the activation of plant GHF10 xylanase (Wu et al. 2002). Hence, it is difficult to exclude the possibility that the 67 kDa form of OsCel9A with CBM2 is sorted to its target location as azymogenic form and that the truncation of CBM2 leads to the activation of the 51 kDa EGase at the proximal site of its target location such as endodermal walls. This can avoid active EGase being tangle up with a similar substrate in the LRP where OsCel9A mRNA exclusively accumulated (Fig. 5), as has been envisaged in the targeting of maize GHF10 xylanase from tapetum cells to pollen walls (Wu et al. 2002), or prohibit the active broad-substrate EGase from hydrolyzing newly synthesized hemicellulosic polymers in the Golgi and secretory vesicles before their integration into the mother cell walls of LRP.

In either event, the expression of OsCel9A may be more dependent on the stage of development or the cell origin rather than on exogenous hormone treatments (Figs. 4, 5), as has been shown for the other EGases with CBM2 such as LRP-associated NtCel8 in nematode-infected roots of tobacco (Goellner et al. 2001), strawberry FaEG3 (Trainotti et al. 1999), pear PC-Eg2 (Hisawa et al. 2003) and peach PpEG4 (Trainotti et al. 2006). Time course studies revealed that accumulation of both the 51 and 67 kDa EGases of OsCel9A coincided with the detection of
LRP in the parental tissue, albeit at low protein amounts in the absence of 2,4-D (see Supplementary Fig. S5, -2,4-D). Two isoforms of OsCel9A accumulated between 12 and 24 h, the initiation phase of LRP, and then gradually disappeared between 48 and 72 h, towards the completion of lateral root emergence in the absence of 2,4-D (Supplementary Fig. S5, -2,4-D). Hence, after the establishment of LRP, the extracellular truncation of CBM2 might lead to the generation of new EGases with different substrate specificity, as noticed in the processing of microbial cellulase (Nakayama et al. 1976, Moorman et al. 1993). At later stages of lateral root formation, when the LRP starts to penetrate into the endodermal layer (Supplementary Fig. S5A, B, -2,4-D), whose primary cell wall contains abundant hemicellulosic polymers including 1,3-1,4-β-glucan, GAX and glucomanann, the 51 kDa form of OsCel9A operating in the interstitial matrix may be preferred (Yoshida and Komae 2006) and brought about by specific proteolysis of the CBM2. Nearly all-out endolytic attack by the 51 kDa EGase on type II cell wall polymers might facilitate the subsequent expansion, division and penetration into the parent endodermal layer of LRP cells by creating a channel for LRP development.

Materials and Methods

Plant materials

Primary root segments of rice (Oryza sativa L.) cv. Sasanishiki were excised from 3-day-old seedlings as described in the accompanying manuscript (Yoshida and Komae 2006). Coleoptiles were excised at the node from aseptically cultured cv. Sasanishiki were excised from 3-day-old seedlings as described in the Supplementary Materials and Methods. Experimental procedures for sequence analysis are described in the Supplementary Materials and Methods.

Analytical IEF

The three isoforms of the purified 51 kDa EGase (Yoshida and Komae 2006) were analyzed for purity by separation on Ampholine PAGE gels (pH 4.0-6.5 for IEF, Pharmacia, Sweden). The Ampholine PAGE gels were run and stained with Coomassie brilliant blue R-250 (Wako, Japan) according to the manufacturer’s instructions.

Peptide mass fingerprinting (PMF) of purified rice EGase isoforms

Trypsin. Tryptic fragments were obtained by incubation of a gel fragment containing a purified EGase isoform (peak I, II or III) that had been cut from a 10% gel after SDS-PAGE and incubated with trypsin overnight at 35°C. Tryptic peptides were extracted and desalted using C18 ZipTip units (Millipore, USA) according to the manufacturer’s instructions and subjected to MALDI-TOF MS analysis.

Cloning of a rice GHF9-specific DNA probe

Degenerate oligonucleotides, 5’-gggtaacctcagacggg-3’ and 5’-cctgtcgc gaagctgaaagtnt-t3’, corresponding to two Lys-C peptide fragments of the purified rice EGase (LEP53 No. 3, 8GYYDAG92; and LEP56, 57-R41, 219QLFDFA226; primer 1 and primer 2 in Fig. 1) were synthesized and used as a primer set to amplify a GHF9-specific DNA fragment by RT-PCR. First-strand cDNAs were synthesized from total RNA of root tissues of rice cv. Sasanishiki, treated with 15 μM 2,4-D for 24 h, using a first-strand cDNA synthesis kit (Life Sciences, Inc., USA) and oligo(dT)_12-18 primers. First-strand cDNA samples (1 μl) were added to 24 μl of pre-mixed ExTaq reaction cocktail (TAKARA BIO INC.). PCR was performed for 95°C for 3 min, 30 repeated cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and 72°C for 5 min. The PCR fragment (436 bp) was cloned into pT7 Blue T-vector with the Perfectly Blunt cloning kit (Novagen, USA) and used as a DNA probe for cDNA screening (probe 1 in Supplementary Fig. S1).

Construction and screening of root cDNA libraries of rice cv. Sasanishiki

Poly(A)^+ RNA was enriched using the Biomag mRNA purification kit (PerSeptive Biosystems, USA) from total RNA of root tissues treated with 2,4-D (15 μM) for 24 h and used for synthesis of double-stranded cDNA with the ZAP-cDNA synthesis kit (Stratagene, USA). The cDNA products were cloned into the E.coli RI and XhoI sites of the λ vector Uni-ZAP XR using ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). The cDNA library was screened with a horseradish peroxidase-labeled GHF9-specific DNA probe (probe 1 in Supplementary Fig. S1) prepared with the ECL direct nucleic acid labeling system (Amersham Biosciences, UK). Plaque hybridization and detection of positive plaques were performed according to the manufacturer’s instructions. Sixteen independent clones were plaque-purified after the third screening, and the inserts were excised in vivo into pBluescript II SK+ (Stratagene). Experimental procedures for sequence analysis are described in the Supplementary Materials and Methods.

Total RNA isolation

Total RNA was extracted from the stored samples by grinding to a powder under liquid N2 using a guanidium thiocyanate/phenol/chloroform procedure (Sambrook and Russell 2001) or a modified cetyltrimethylammonium bromide (CTAB)/NaCl procedure (Chang et al., 1993) for tissues treated with or without 2,4-D. In the modified CTAB/NaCl procedure, polyvinylpyrrolidone and spermidine were omitted from the original extraction buffer. After the RNA precipitation step with LiCl in the original CTAB/NaCl procedure, the precipitate was suspended with diethyl pyrocarbonate (DEPC)-treated water containing 0.25 U/ml^{-1} DNase I (RNase-free, TAKARA BIO INC., Japan) and 8 mM MgSO4, gently shaken at 25°C for 1 h and centrifuged at 15,000 r.p.m. and 4°C for 20 min. The supernatant was successively extracted with equal volumes of 10 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA (saturated with phenol); chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). The aqueous phase was precipitated with LiCl (final concentration 2 M) at -20°C overnight, washed with 70% ethanol, dried, dissolved with DEPC-treated water and stored at -80°C.
reflector mode. Peptide data were collected after ionization in the 50–600 m/z range, and time to mass conversion was achieved using external calibration with 2,5-dihydroxybenzoic acid (m/z 273.0), des-Arg-bradykinin (m/z 904.5), angiotensin I (m/z 1,296.7), human adrenocorticotropic hormone (ACTH) (clip 1–17) (m/z 2,093.1) and ACTH (clip 7–38) (m/z 3,657.9). Obtained peptide masses were fitted to the deduced amino acid sequence of OsCel9A using the Mascot program (Matrix Science, UK).

Lys-C. The purified isoforms (peak II or peak III) in 10 mM HEPES (pH 7.0) containing 3 mM octyl-thio-glucoside (1 μl) were treated with 5 μl of 5 mM dithiothreitol (DTT) in 500 mM Tris–HCl buffer (pH 8.5) containing 6 M guanidine-HCl and 2 mM EDTA under N₂ at 37 °C for 2 h and then treated with 5 μl of 10.5 mM iodoacetamide in the same buffer under N₂ at room temperature for 30 min in the dark. The reduced alkylated protein solution was digested with Lys-C overnight at 35 °C. The Lys-C peptides were desalted using C18 ZipTip units (Millipore) and subjected to MALDI-TOF MS operated in linear mode. Peptide data were collected after ionization in the m/z range 4,000–20,000, and time to mass conversion was achieved using external calibration with insulin (m/z 5,743.6), thioredoxin (m/z 11,674.5) and apomyoglobin (m/z 16,952.6).

Glu-C. The reduced alkylated protein solution (peak I, II or III) was digested with Glu-C at 35 °C for 20 h. The Glu-C peptides were desalted using C18 ZipTip units (Millipore) and subjected to MALDI-TOF MS operated in reflector mode. Peptide data were collected after ionization in the m/z range 400–4,000, and time to mass conversion was achieved using external calibration with des-Arg¹-bradykinin (m/z 904.5), angiotensin I (m/z 1,296.7), ACTH (clip 1–17) (m/z 2,093.1) and ACTH (clip 7–38) (m/z 3,657.9). PMF and nano ESI MS/MS analyses were performed at APRO Life Science Institute (Tokushima, Japan).

Sequencing of Glu-C peptide fragments with nano ESI-MS/MS

The Glu-C peptides of purified protein (peak II or III, see above) were desalted to 50% acetonitrile containing 1% formic acid using C18 ZipTip units and subjected to a nano ESI MS/MS using a Q-ToF 2 tandem mass spectrometer (Waters Micromass, UK) equipped with a nano-flow ESI source. Mass spectra were collected in positive-ion mode. TOF-MS data of the peptide mixtures were acquired over the m/z range 250–3,000, and TOF-MS/MS data of the individual peptides were acquired over m/z 20–2,500 (peak III) or m/z 20–2,800 (peak II). Singly charged mass spectra were generated from multiply charged ESI data using the maximum entropy algorithm (MaxEnt-3 software, Waters Micromass). MS/MS data were analyzed by the PepSeq program in the BioLynx software package (Waters Micromass) for determination of peptide sequences.

Antibodies against OsCel9A gene products

The peptide of 15 amino acid residues (GGDPDEHDDFADEARNY) from the deduced amino acid sequence of OsCel9A was synthesized as an antigen (anti-celA in Figs. 1 and 3). The C-terminus of the antigen peptide was conjugated with keyhole limpet hemocyanin (KLH). The peptide of 15 amino acid residues (TSLPHGANHQHAS) was synthesized as another antigen peptide to enable recognition of only the nascent form of OsCel9A protein (anti-celB in Figs. 1 and 3). The N-terminus of the latter antigen peptide was conjugated with KLH. Polyclonal rabbit antibodies were raised against these peptide–KLH conjugates, and purified using affinity column chromatography on antigen peptide-conjugated Affi-Gel 102 resin (Bio-Rad, USA) after the precipitation of antigen with ammonium sulfate at 40% saturation and dialysis against phosphate-buffered saline. Pooled fractions of peptide-specific antibodies were frozen in aliquots and stored at −80 °C. Anti-celA and anti-celB antibodies were produced at the Peptide Institute (Osaka, Japan).

Northern blotting analysis

RNA samples (20 μg) were subjected to electrophoresis in denaturing agarose (1.2% w/v) gels containing 0.66 M formaldehyde, then transferred to HyBond-N membrane (Amerham Biosciences). Hybridization was carried out under stringent conditions in 50% formamide, 5× SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 1% (w/v) blocking reagent (Roche Diagnostics, Germany) dissolved in 10 mM maleic acid buffer containing 15 mM NaCl (pH 7.5) at 42 °C overnight with 32P-labeled PCR fragments of the 3'-UTRs of cDNAs for OsCel9A (254 bp), OsCel9D (240 bp), OsCel9E (322 bp) or OsCel9F (143 bp) as gene-specific probes. The sequences correspond to primers used for PCR of the 3'-UTR of the cDNAs are described in Supplementary Materials and Methods. After washing, membranes were exposed, and label on membranes was detected using a Fuji BAS 2000 Bio-imaging analyzer (Fuji Photo Film, Japan).

Quantitative real-time RT–PCR

The oligonucleotide primer sets used for real-time RT–PCR analysis were designed using Primer Express software (Applied Biosystems). The following sequences correspond to primers used for the RT–PCR analysis: OsCel9A, 5′-cagacaacctgaacacacct-3′ (forward), 5′-atagctctcgcacagggta-5′ (reverse), OsCel9B, 5′-caaggcgctctactc-3′ (forward), 5′-ctgtagctcactg-3′ (reverse), OsCel9D, 5′-caagggctttgcttc-3′ (forward), 5′-tsgaaagcttcc-3′ (TaQMan MGB probe); OsCel9E, 5′-caagggctttgcttc-3′ (forward), 5′-tsgaaagcttcc-3′ (TaQMan MGB probe). We conducted BLAST searches for each primer sequence to confirm that no other sequences in the rice genome or from rice cDNAs were similar to any primer. The experimental procedures including the RT–PCR conditions are described in detail in Supplementary Materials and Methods. The size and homogeneity of PCR products were examined after staining with ethidium bromide. RT–PCR experiments were conducted in triplicate, and the mean copy numbers of individual mRNA species were estimated using preparations of standard cDNAs cloned with pBluescript II SK− (Stratagene) of known molar concentrations. Transcript levels of individual mRNA species were normalized to the relative expression level of 18S rRNA for each sample (as endogenous control) and are shown as the number of mRNA molecules in 1 pg of total RNA.

In situ hybridization

Primary root tissues were cultured in the presence of 15 μM 2,4-D for 24 h, then fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2), dehydrated through a graded ethanol series and embedded in paraffin wax. Riboprobes for in situ hybridization were labeled with digoxigenin-11-rUTP using a DIG labeling kit (Roche Diagnostics, Germany) according to the manufacturer’s protocol. The PCR product of the 3′-UTR of OsCel9A, used as a gene-specific probe for Northern hybridization (see above), was cloned into pBluescript II SK+ with the PCR-Script Amp cloning kit (Stratagene). Antisense (NotI/T7 promoter) and sense (NotI/T7 promoter) probes were prepared from plasmids containing the 3′-UTR of OsCel9A (254 bp) in the reverse orientation. In situ hybridization was performed according to Kouchi and Hata (1993). Hybridization signals were visualized by anti-digoxigenin antibody conjugated to alkaline phosphatase.
Preparation of total protein extracts, SDS–PAGE and immunoblotting

The root segments were homogenized in liquid N2 and the fine powder was resuspended in a pre-cooled (−20 °C) solution of 10% trichloroacetic acid (TCA) in acetone with 0.07% 2-mercaptoethanol (2-ME) (Görg et al. 2000). Proteins were allowed to precipitate overnight at −20 °C. After centrifugation, the pellet was washed with ice-cold acetone containing 0.07% 2-ME. The supernatant was discarded and the pellet dried in vacuo. The acetone powder (100 mg fresh weight) was suspended in 100 μl of SDS–PAGE sample buffer (Bio-Rad) with 2-ME, and the pH adjusted with 1 M Tris base to approximately 7.0. After sonication on ice (3 × 10 s), the suspended sample was boiled for 10 min and centrifuged at 15,000 r.p.m. for 5 min at 4 °C (TX-300 centrifuge, Tomo Co., Japan). The supernatant was used for SDS–PAGE analysis. The procedure for the determination of protein content in the supernatant was as follows: supernatant (15 μl) was mixed with 60 μl of pre-cooled acetone: HCl: methanol (by vol. 120:0.01:120, −20 °C) and protein was allowed to precipitate overnight at −20 °C in order to remove SDS. After centrifugation at 15,000 r.p.m. at 4 °C for 10 min, the pellet was washed with ice-cold acetone. The supernatant was discarded and the pellet dried in vacuo. The pellet was washed in an ice-cold acetone containing 0.07% 2-ME (Go¨rg et al. 2000). Proteins were precipitated with 10% TCA on ice, resuspended in 10 mM HEPES-NaOH pH 7.0, 0.02% NaN3, 5 mM DTT) containing Complete mini protease inhibitor cocktail (Roche, Germany) and centrifuged at 1,000 g for 15 min. For immunodetection, the 1,000 g precipitate of 2,4-D-treated rice tissues of rice cv. Sasanishiki was constructed by Dr. Satoshi Sekiguchi of NIPPN Co. (Kanagawa, Japan). Primers for xylans. In Xylans and Xylanases Progress in Biotechology. Vol. 7, pp. 39–50. Elsevier, Amsterdam.

Post-translational truncation of rice EGase CBM2

For EGase assays, the 1,000×g precipitate of 2,4-D-treated root segments was isolated as described above, washed with 1.8 ml of homogenization buffer three times and extracted with homogenization buffer containing 2 M NaCl and the protease inhibitor cocktail at 4 °C by gently mixing for 18 h. After extraction, the mixture was immediately centrifuged at 13,000×g for 15 min at 4 °C. The supernatant was deionized and concentrated by ultrafiltration (Microcon YM-10 membrane, Amico-Millipore, USA) at 4 °C and designated as ‘cell wall fraction’. The microsome fraction was isolated from the 1,000×g supernatant as described above, with the microsome pellet suspended in the extraction buffer for the enzyme assays. Procedures for EGase assays in the presence of 0.17% Triton X-100 are described in Supplementary Materials and Methods.

Homology modeling of the catalytic domain of OsCel9A

Homology models of OsCel9A were generated by multiple alignment of the deposited and crystal coordinates obtained from the Protein Data Bank (PDB) (http://www.rcsb.org/) using Insight II/Homology software (Accelrys Inc., USA) for bacterial GHF9 members such as Thermobifida fusca Cel9A (PDB number 4TF4), Clostridium thermocellum Cel9A (PDB number 1CLC), Clostridium cellulolyticum Cel9G (PDB number 1K72), Clostridium cellulolyticum Cel9M (PDB number 1A7) and the animal GHF9 member Nasutitermes takasagoensis NiEgl (PDB number 1KSD). Then, the catalytic domain sequence of OsCel9A (Gly35-Ala511) was manually added to the alignment and the Insight II/Discovery program (Accelrys Inc., USA) was used for energy minimization of the resulting structural model. The picture in Fig. 1B was produced with the PyMOL program (DeLano 2002).

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References


**Note added in proof**

The ordered locus name of OsCel9A is OsOLg0220100 (http://rapdb.lab.nig.ac.jp/).

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