



Title	Identification of a $\beta$ -glucosidase hydrolyzing tuberonic acid glucoside in rice ( <i>Oryza sativa</i> L.)
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3 **Identification of a  $\beta$ -glucosidase hydrolyzing tuberonic acid glucoside**  
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5 **in rice (*Oryza sativa* L.)**  
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*Abbreviations:* JA, jasmonic acid; TA, tuberonic acid; TAG, tuberonic acid glucoside;  
MS/MS, tandem mass spectrometry; UPLC, ultra-performance liquid chromatography;  
MRM, multiple reaction monitoring; pNPG, *p*-nitrophenyl  $\beta$ -glucoside

## Abstract

Tuberonic acid (TA) and its glucoside (TAG) have been isolated from potato (*Solanum tuberosum* L.) leaflets and shown to exhibit tuber-inducing properties. These compounds were reported to be biosynthesized from jasmonic acid (JA) by hydroxylation and subsequent glycosylation, and to be contained in various plant species. Here we describe the *in vivo* hydrolytic activity of TAG in rice. In this study, the TA resulting from TAG was not converted into JA. Tuberonic acid glucoside (TAG)-hydrolyzing  $\beta$ -glucosidase, designated OsTAGG1, was purified from rice by six purification steps with 4300-fold purification. The purified enzyme migrated as a single band on native PAGE, but as two bands with molecular masses of 42 and 26 kDa on SDS-PAGE. The results from N-terminal sequencing and peptide mass fingerprinting of both polypeptides suggested that the two bands were derived from a single polypeptide, which is a member of glycosyl hydrolase family 1. In the native enzyme, the  $K_m$  and  $V_{max}$  values of TAG were 31.7  $\mu$ M and 14.7  $\mu$ mol/min/mg, respectively. OsTAGG1 preferentially hydrolyzed TAG and methyl TAG. Here we report that OsTAGG1 is a specific  $\beta$ -glucosidase hydrolyzing TAG, which releases physiologically active TA.

**Keywords:** *Oryza sativa* L. cv. Kitaake; glycoprotein;  $\beta$ -glucosidase; tuberonic acid glucoside; tuberonic acid; enzyme purification

## 1. Introduction

Jasmonic acid (JA), which is a member of oxylipins, is an essential compound involved in regulating responses to stresses such as insect herbivory and pathogenic attack in plants (McConn et al., 1997; Vijayan et al., 1998). In addition, JA is involved in plant growth and development, including root growth, fruit ripening, and pollen maturation (Staswick et al., 1992; Fan et al., 1998; Stintzi and Browse, 2000; Wasternack, 2007). Numerous genes have been previously identified that are inducible or down-regulated by JA (Sasaki et al., 2001; Wierstra and Kloppstech, 2000), suggesting that JA might be an important signaling molecule for plants in response to environmental changes and development.

JA is synthesized from the  $\alpha$ -linolenic acid released by lipase activity, followed by sequential reactions of peroxidation, dehydration, and cyclization to 12-oxo-phytodienoic acid (OPDA) (Song and Brash, 1991; Ishiguro et al., 2001; Schaller, 2001; Stenzel et al., 2003). OPDA is converted to JA by reduction and three  $\beta$ -oxidation steps (Vick and Zimmerman, 1984). JA is then metabolized by five basic reactions: (1) methylation at carbon 1 (Seo et al., 2001); (2) hydroxylation at carbon 11 or 12 (Helder et al., 1993; Yoshihara et al., 1989); (3) conjugation at carbon 1 with an amino acid and its analog (Sembdner et al., 1994); (4) reduction of the keto group at carbon 6 to form cucurbitic acid (CA) (Dathe et al., 1991); and (5) degradation of the carboxylic acid side chain to form cis-jasmone (Koch et al., 1997). Hydroxylated JA is further modified by *O*-glucosylation or sulfation (Gidda et al., 2003; Seto et al., 2009). 12-OH-JA-Ile, 12-OH-JA-Phe (combination of (2) and (3) above), and 12-OH-CA

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3 (combination of (2) and (4) above) have also been identified (Guranowski et al., 2007).

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5 The existence of various JA analogs has made it difficult to determine the specific role  
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7 of each compound in plant immunity and growth.  
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11 The genes involved in the JA biosynthetic pathway have been identified and  
12 well-studied. Analysis of *Arabidopsis* mutants showed that the genes *DAD1*, *LOX2*,  
13 *AOS*, and *OPR3* produce intermediates in the JA biosynthetic pathway (Bell et al., 1995;  
14 Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002). However, the  
15 enzymes that metabolize JA are still not known, although a few studies have been  
16 recently performed on corresponding mutants. JAR1 catalyzes the conversion of JA into  
17 JA-Ile, which promotes the interaction between SCF<sup>COII</sup> and JAZ1; this leads to the  
18 ubiquitination of JAZ1 and its degradation by the 26S proteasome (Chini et al., 2007;  
19 Thines et al., 2007). JMT catalyzes the formation of MeJA from JA. This enzyme is  
20 essential for JA-regulated plant responses, and MeJA acts as a diffusible intercellular  
21 transducer (Seo et al., 2001). ST2a is able to sulfonate hydroxylated JA (Gidda et al.,  
22 2003). Thus, plants containing mutations in these enzymes should be used to provide  
23 information on the molecular function of compounds related to JA.  
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46 Tuberonic acid (TA), 12-hydroxyjasmonic acid (Figure 1), was isolated as a  
47 tuber-inducing compound from potato (*Solanum tuberosum* L.), and the glucoside of TA  
48 (TAG) was also found to be a tuberization compound (Koda et al., 1988; Yoshihara et  
49 al., 1989). In a previous study, it was suggested that TAG generated from JA transmits a  
50 signal from the damaged parts to the undamaged parts of the plant (Seto et al., 2009)  
51 and demonstrated that TA contributes to the down-regulation of the expression of JA  
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3 biosynthetic genes, such as *LOX*, *AOS*, and *OPR*, which are JA-inducible. Furthermore,  
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5 they demonstrated that some house-keeping genes, such as *RBCS*, *CAB8*, and *TUB*, are  
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7 also strongly down-regulated (Miersch et al., 2007). These data suggest the importance  
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9 of TAG glucosidase as a regulator of TA levels according to environmental conditions.  
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12 However, the TAG hydrolyzing enzyme has not been identified.  
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17 In this study, we describe the *in vivo* hydrolysis of TAG into TA in rice plants,  
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19 purification and properties of an enzyme that catalyzes this hydrolysis, and  
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21 identification of its gene.  
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## 2. Results

### 2.1. *In vivo* hydrolysis of TAG to TA

We previously reported the synthesis of TAG from TA by glucosyltransferase in rice and tobacco plants (Seto et al., 2009). However, the mechanism of conversion of TAG into TA in plants is unclear (Figure 1). Since both TA and TAG are found in rice plants (Seto et al., 2009), we investigated the TAG hydrolyzing activity in rice during grain filling. Rice panicles and leaves were harvested, and then incubated in H<sub>2</sub>O (as a control) or 0.1 mM TAG-*d5* for 2 h. The extracts from the treated materials were analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) as described in the Experimental section. Figure 2 shows the trace chromatograms in multiple reaction monitoring (MRM) mode of standard TAG-*d5* detected by the daughter ion *m/z* 210.8 derived from *m/z* 392.03 and TA-*d5* detected by the daughter ion *m/z* 96.6 derived from *m/z* 229.97. The retention times of TAG-*d5* and TA-*d5* were determined to be 1.95 and 2.14 min, respectively (Figure 2A and 2B). In the chromatograms of treated rice leaves, abundant TA-*d5* derived from hydrolyzing TAG-*d5* and the absorbed original TAG-*d5* were detected (Figure 2C). However, the corresponding peaks were not discernable from the background noise in the chromatograms of the control plants (Figure 2D). These data indicate the existence of a TAG hydrolyzing enzyme(s) that generates TA in rice plants.

### 2.2. Purification of *OsTAGG1* from immature rice seeds

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3 OsTAGG1 was purified from frozen rice panicles by ammonium sulfate fractionation  
4 and five chromatographic steps as shown in Table 1. Two peaks of activity for TAG  
5 glycosidase, designated OsTAGG1 and OsTAGG2, were detected by CM-Sepharose  
6 chromatography (data not shown), indicating that at least two isoforms of OsTAGG  
7 exist in rice panicles. OsTAGG1, which was detected as the major peak primarily eluted  
8 by CM-Sepharose chromatography, was further purified. After six purification steps,  
9 0.065 mg of OsTAGG1 was obtained from about 2 kg of rice panicles with 4300-fold  
10 purification (Table 1). The purified enzyme migrated as a single band on native PAGE,  
11 but as two polypeptides with molecular masses of 42 and 26 kDa on SDS-PAGE (Figure  
12 3A and 3B, left panel). The sum of the molecular masses of these polypeptides was  
13 roughly consistent with the molecular mass estimated by gel filtration chromatography  
14 (ca. 65 kDa, data not shown). Previous studies have shown that many  $\beta$ -glucosidases are  
15 glycoproteins (Hughes et al., 1992; Suzuki et al., 2006). Thus, the purified protein was  
16 also treated with a glycoprotein-staining reagent (Pro-Q Emerald 300 Gel Stain Kit),  
17 and the results indicated that both polypeptides constituting OsTAGG1 possessed sugar  
18 chains (Figure 3B, right panel), which was consistent with the binding of OsTAGG1 to  
19 ConA-Sepharose.  
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### 46 *2.3. Properties of OsTAGG1*

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51 The enzymatic properties of the purified OsTAGG1 were investigated. OsTAGG1 was  
52 most active at around pH 4 and 50°C, and was fairly stable between pH 3.3–10.3, since  
53 more than 90% of original activity was retained after incubation at 4°C for 16 h. When  
54 the enzyme was maintained for 15 min at various temperatures ranging from 30°C to  
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3 70°C, the original activity was retained up to 50°C (Supplemental figure S1). When  
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5 TAG was used as a substrate in the assay, the specific activity was 3.7  $\mu\text{mol}/\text{min}/\text{mg}$ ,  
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7 and the Lineweaver–Burk plots of the reaction catalyzed by OsTAGG1 yielded  $V_{\text{max}}$  and  
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9  $K_{\text{m}}$  values of 14.7  $\mu\text{mol}/\text{min}/\text{mg}$  and 31.7  $\mu\text{M}$ , respectively. The substrate specificity of  
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11 the purified OsTAGG1 was studied in comparison with TAG and related compounds  
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13 (Table 2). The relative activities for the hydrolysis of methyl TAG and  
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15 jasmonyl-1- $\beta$ -glucose were 80% and 51%, respectively, of the value for TAG.  
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17 OsTAGG1 efficiently hydrolyzed the synthetic substrate *p*-nitrophenyl  $\beta$ -D-glucoside  
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19 (ca. 1.8-fold greater activity than that for TAG) but not *p*-nitrophenyl  $\beta$ -D-mannoside,  
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21 *p*-nitrophenyl  $\beta$ -D-galactoside, *p*-nitrophenyl  $\beta$ -D-cellobioside, and *p*-nitrophenyl  
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23  $\alpha$ -D-glucoside. These data indicate that OsTAGG1 specifically recognizes the  
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25  $\beta$ -D-glucosidic linkage. Furthermore, when some natural glucosides, such as salicylic  
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27 acid glucoside, linamarin, and zeatin-*O*-glucoside, were used as substrates, OsTAGG1  
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29 hydrolyzed only salicylic acid glucoside (25% of the value for TAG) but did not show  
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31 any activity against linamarin and zeatin-*O*-glucoside. It was qualitatively confirmed  
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33 that OsTAGG1 was able to hydrolyze to laminaribiose and celotriose (data not shown).  
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#### 44 *2.4. Identification of the OsTAGG1 gene and sequence comparison studies*

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49 The purified OsTAGG1 showed two bands on SDS-PAGE with molecular masses of 42  
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51 and 26 kDa. The N-terminal amino acid sequences of both polypeptides were  
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53 determined to be EPPISRSSF and GLXNSYSTDA (X indicates an unidentified amino  
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55 acid), respectively. These sequences were found in the internal amino acid sequences of  
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57 AK070962 (Os04g0474900) (Kikuchi et al., 2003), which was deposited as a cDNA of  
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3 1853 bp containing a 1521-bp open reading frame encoding a 57-kDa polypeptide with  
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5 506 amino acid residues. The EPPPISRSSF sequence coincided with the N-terminal  
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7 sequence generated by the cleavage of a signal sequence comprising the first 25  
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9 residues of the precursor protein as predicted by SignalP 3.0 (Bendtsen et al., 2004) for  
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11 the AK070962 cDNA gene product. The tryptic digestion of both bands was analyzed  
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13 by MALDI-TOF MS. The data obtained were entered into the MASCOT database and  
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15 the same  $\beta$ -glucosidase showed a high score (data not shown). These results strongly  
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17 suggest that both 42- and 26-kDa polypeptides are encoded by a single gene  
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19 (Os04g0474900). The molecular mass of the mature enzyme is predicted from the  
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21 sequence to be ca. 55 kDa, but is inconsistent with the sum of the molecular masses of  
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23 the two polypeptides (68 kDa). This discrepancy may be explained by the addition of  
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25 sugar chains. Indeed, there are five potential N-glycosylation sites at positions 118, 225,  
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27 357, 367, and 421 in the amino acid sequence of the precursor protein (Figure 4).  
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37 The predicted amino acid sequence of the OsTAGG1 precursor contained conserved  
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39 amino acid residues among the GH1 members, such as Asn-Glu-Pro (residues 198–200)  
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41 and Ile-Thr-Glu-Asn-Gly (residues 411–415). The glutamate residues positioned at 199  
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43 and 413 were predicted to be an acid–base catalyst and a catalytic nucleophile,  
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45 respectively, based on previous studies (Withers et al., 1990; Wang et al., 1995).  
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49 Residues involved in the binding of the sugar moiety of the substrate, which are highly  
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51 conserved in all glycosyl hydrolase (GH) 1 members, were also found in the amino acid  
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53 sequence of OsTAGG1; i.e., Gln-49, His-153, Asn-198, Glu-199, Glu-413, Trp-462,  
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55 Glu-469, and Trp-470 (Figure 4) based on previous studies (Czjzek et al., 2000;  
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57 Verdoucq et al., 2004). These results support the conclusion that OsTAGG1 is a  
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3 member of the GH 1 family. High amino acid sequence identities were also found with  
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5 rice Os4BGlu12 (87%) (Opassiri et al., 2006), two hypothetical proteins from sorghum  
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7 (*Sorghum bicolor*), accession numbers EES12355 (86%) and EES12357 (78%), and the  
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9 putative rice  $\beta$ -glucosidases Os4BGlu9 (78%), Os4BGlu10 (77%), and Os4BGlu11  
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11 (79%). Other enzymes exhibiting high amino acid sequence identities included the  
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13 white clover cyanogenic  $\beta$ -glucosidase (ABV54716) (64%) (Barrett et al., 1995),  
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15  $\beta$ -glucosidase D2 (*Lotus japonicus*) (ACD65510), which hydrolyzes hydroxynitrile  
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17 glucosides (67%) (Morant et al., 2008), an unnamed protein product from *Vitis vinifera*  
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19 (CAO21978) (67%),  $\beta$ -glucosidase (*Rosa hybrid cultivar*) (BAG13451) (65%),  
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21 isoflavone conjugate-hydrolyzing  $\beta$ -glucosidase from soybeans (*Glycine max*)  
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23 (BAF34333) (62%) (Suzuki et al., 2006), prunasin hydrolase (*Prunus serotina*)  
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25 (AAF34650) (60%) (Zhou et al., 2002), amygdalin hydrolase (*P. serotina*) (AAA93234)  
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27 (58%) (Zhou et al., 2002), all of which are known members of the GH1 family in plants  
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34 (Figure 5) except for the uncharacterized enzymes.  
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### 3. Discussion

The purified OsTAGG1 migrated as a single polypeptide on native PAGE, but as two polypeptides with molecular masses of 42 kDa and 26 kDa under denaturing conditions. Similar observations were reported in a previous study that the purified preparation of  $\beta$ -glucosidase from lodgepole pine xylem showed 28 kDa and 24 kDa proteins on SDS-PAGE, and it was concluded that this  $\beta$ -glucosidase should be a 55-kDa monomeric enzyme (Dharmawardhana et al., 1995, 1999). The molecular mass of the native protein calculated by gel filtration was coincident with the sum of the two OsTAGG1 subunit bands on SDS-PAGE. Therefore, OsTAGG1 also appears to be a monomeric enzyme composed of two polypeptides produced by cleavage within a molecule *in vivo* or during purification similar to  $\beta$ -glucosidase from the lodgepole pine xylem.

TAG has an acidic group (carboxyl) in its aglycone moiety. Typical plant  $\beta$ -glucosidase substrates do not contain such an acidic aglycone moiety except glucosinolates, which are substrates for myrosinase. We performed primary sequence alignment between OsTAGG1 and myrosinase to predict the binding sites for negatively charged substrates (data not shown). The results showed that all amino acid residues involved in the interaction with the glucose moiety were conserved in OsTAGG1. However, two arginine sites, known as aglycone binding sites in a myrosinase (Burmeister et al., 1997), were not found in OsTAGG1. Furthermore, comparison with sorghum  $\beta$ -glucosidase, in which the crystal structure and aglycone binding sites have been analyzed, predicted almost no binding sites with an aglycone moiety, except S471 in OsTAGG1 according to S462 sorghum  $\beta$ -glucosidase. Therefore, we believe that other residues are involved

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3 in the interaction with the aglycone moiety of TAG.  
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7 TA is synthesized by hydroxylation of JA and then metabolized into TAG as evidenced  
8 by a time-dependent accumulation in a JA-dependent manner after wounding tomato  
9 leaves (Miersch et al., 2007). Furthermore, TA and TAG were not detected in a  
10 JA-deficient mutant (Miersch et al., 2007). A previous study showed that  
11 glucosyltransferase activity occurred towards TA in several plants and identified a TA  
12 glucosyltransferase in rice (Seto et al., 2009). The function of glycosylation is thought  
13 to be storage and/or transport of the bioactive aglycone in order to prepare for its release  
14 by  $\beta$ -glucosidase depending on a specific environmental cue such as wounding.  
15 However, the hydrolytic activity of TAG to generate TA has not been confirmed. In  
16 order to investigate the conversion of TAG to TA *in vivo*, TAG-*d5* was synthesized.  
17 TA-*d5* was qualitatively detected in the rice plants fed with TAG-*d5*, suggesting the  
18 existence of a specific  $\beta$ -glucosidase hydrolyzing TAG glucoside. We examined the  
19 possibility that TA was converted into JA in the same plant by feeding with TAG-*d5*, but  
20 deuterium-labeled JA was undetectable. This result was completely consistent with a  
21 previous study in which labeled JA and 12-HSO<sub>4</sub>-JA were undetectable after feeding  
22 with labeled TA (Gidda et al., 2003). These observations suggest that TAG has specific  
23 storage or transport forms for TA *in vivo*. Among JA metabolites, four glycoside  
24 conjugates have been reported apart from TAG: 11-hydroxyjasmonic acid glucoside  
25 (Matsuura et al., 2001), jasmonyl-1- $\beta$ -glucose, jasmonyl-1- $\beta$ -gentiobiose, and  
26 hydroxyjasmonyl-1- $\beta$ -glucose (Swiatek et al., 2004). These compounds are classified as  
27 hydroxylated JA or JA after glucoside hydrolyzation, suggesting that glycoside  
28 conjugates may be creatively used to produce different bioactive hormones *in vivo*.  
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5 While JA has been well studied as a plant hormone that protects against insect herbivory  
6 and pathogenic infections, the physiological functions and mechanisms of TA have been  
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8 rarely reported. TA and TAG were reported to play a physiological role in inducing  
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10 tubers (Yoshihara et al., 1989). Recent studies have indicated that TA is a  
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12 physiologically active compound. For example, AtST2a, which is a hydroxyjasmonate  
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14 sulfotransferase from *Arabidopsis thaliana*, was induced following TA treatment (Gidda  
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16 et al., 2003). Wound-induced metabolic conversion of JA/MeJA into TA alters the  
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18 expression pattern of genes including switching off JA signaling for the expression of a  
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20 subset of genes (Miersch et al., 2007). In previous studies, TA and TAG were increased  
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22 by mechanical wounding in rice and tobacco (Seto et al., 2009), supporting similar  
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24 results found in tomato plants (Miersch et al., 2007). These results indicate the  
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26 relationship between the wounding response in plants and TA and TAG accumulation.  
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28 Furthermore, GH1 enzymes are involved in defense against herbivores and pathogens  
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30 (Bjarnholt and Moller, 2008; Halkier and Gershenzon, 2006). Thus, OsTAGG1 may  
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32 also be a specific  $\beta$ -glucosidase releasing TA, thereby providing a defense mechanism  
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34 in plants. In future, analysis of the deletion variant will allow a better understanding of  
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36 the function of OsTAGG1 and hydrolysis of TAG in plant defense responses.  
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49 Phytohormones such as abscisic acid, gibberellic acid, auxin, cytokinin, jasmonate, and  
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51 brassinosteroids are metabolized to glycosylated conjugates (Sembdner et al., 1994).  
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53 However, few studies have attempted to identify  $\beta$ -glucosidase involved in hydrolyzing  
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55 these glycosylated phytohormones. Only TAG has been used as a substrate for two  
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57  $\beta$ -glucosidases, PYK10 (BGLU23) and BGLU18, which accumulate in constitutive and  
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3 inducible ER bodies of *A. thaliana* (Ogasawara et al., 2009). A recent study showed that  
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inducible ER bodies of *A. thaliana* (Ogasawara et al., 2009). A recent study showed that  
PYK10 and its close paralogs (BGLU21 and BGLU22) specifically hydrolyzed the  
natural substrate scopolin (Ahn et al., 2010). However, OsTAGG1 lies in a different  
cluster compared with PYK10 and BGLU18 (Figure 5), indicating that OsTAGG1 may  
have different functions compared with PYK10 and BGLU18 even TAG is a possible  
substrate for these enzymes. A previous study on the GH family 1 from rice showed that  
Os4bglu12  $\beta$ -glucosidase also has high exoglucanase activity and is therefore predicted  
to play a role in cell wall metabolism (Opassiri et al., 2006). OsTAGG1 had the highest  
similarity to Os4bglu12  $\beta$ -glucosidase and displayed hydrolytic activity towards  
laminaribiose and celotriose (data not shown), indicating the possibility that OsTAGG1  
is also involved in cell wall metabolism. Furthermore, the expression patterns of genes  
belonging to the rice GH family 1 showed that transcripts of OsTAGG1/Os4bglu13  
accumulate in wounded tissues (Opassiri et al., 2006). These observations together with  
the remarkably high efficiency of TAG hydrolysis suggest that OsTAGG1 may be  
involved in the signal transduction of JA analogs via TAG to wound and/or disease  
damage.

#### 4. Conclusions

In this study, the conversion of TAG to TA by  $\beta$ -glucosidase was demonstrated in rice,  
and rice TAG glucosidase (OsTAGG1) was purified. OsTAGG1 was shown to be a  
glycoprotein consisting of two polypeptides derived from a single gene

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3 (Os04g0474900) (AK070962). The enzyme showed high specificity for TAG compared  
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5 to other glucosidic plant hormones. In order to investigate in more detail the function of  
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7 OsTAGG1 *in vivo*, overexpressed and/or deficient mutants must be analyzed.  
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## 5. Experimental

### 5.1. Plant materials and chemicals

Plants (*O. sativa* L. cv. Kitaake) to be used for enzyme purification and the *in vivo* TAG hydrolyzing assay were grown in an experimental field at Hokkaido University, Sapporo, Japan, and in a growth chamber, respectively. The chamber conditions were set at 30°C, 60% moisture, and 530  $\mu\text{mol}/\text{m}^2/\text{s}$  illumination under a 10.5-h light period. TA, TAG, deuterium-labeled TA (TA-*d5*), deuterium-labeled TAG (TAG-*d5*), and jasmonyl-1- $\beta$ -glucoside were prepared as described previously (Matsuura et al., 2000). Linamarin, zeatin-*O*-glucoside, salicylic acid glucoside, and pNPG were obtained from Sigma Chemicals (St. Louis, MO). D-glucose- $^{13}\text{C}_6$  was purchased from Taiyo Nissan (Tokyo, Japan).

### 5.2. Purification of TAG glucosidase (*OsTAGG*)

Plant materials from rice, including the panicles, were harvested during grain filling (ca. 2 kg, fr. wt). These materials were frozen in liquid nitrogen and powdered using a blender (Nihonseiki Kaisha Ltd., Tokyo, Japan). The powdered materials were suspended in 4 l extraction buffer (20 mM phosphate buffer, pH 7.0, containing 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride) and homogenized using a Polytron (Kinematica GmbH, Switzerland). A crude extract was obtained by filtering through two layers of gauze and centrifugation at 12,000  $\times g$  for 20 min at 4°C. *OsTAGG* protein was purified from the crude extract by the following procedure:

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3 The protein fraction that was precipitated in 40%–80% saturation of  $(\text{NH}_4)_2\text{SO}_4$  was  
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5 collected by centrifugation at  $12,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The pellet was dissolved in  
6  
7 buffer A (MacIlvaine, pH 5.0) and then dialyzed against buffer A. The dialyzed sample  
8  
9 was loaded on a CM-Sepharose Fast Flow column ( $30 \times 280$  mm, GE Healthcare  
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11 Bio-Sciences, Piscataway, NJ) equilibrated with buffer A. After the column was washed  
12  
13 with buffer A, proteins were eluted with a linear gradient of 0–0.5 M NaCl in buffer A.  
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15 The active fractions were pooled and dialyzed against buffer B (20 mM phosphate  
16  
17 buffer, pH 7.0, containing 0.5 M NaCl). The retained fraction was applied to a Con  
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19 A-Sepharose 4B column ( $16 \times 100$  mm, GE Healthcare) equilibrated with buffer B, and  
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21 proteins were eluted with 0.5 M mannose. The active fractions were pooled,  
22  
23 concentrated, and then fractionated onto a HiLoad 16/60 Superdex 200 pg column ( $16 \times$   
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25  $600$  mm, GE Healthcare) equilibrated with buffer A containing 0.2 M NaCl. Proteins  
26  
27 were eluted at a flow rate of 0.8 ml/min using an ÄKTA system (GE Healthcare). The  
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29 active fractions were pooled and dialyzed against buffer A and then fractionated on  
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31 Resource S (1 ml; GE Healthcare) equilibrated with the same buffer. The protein was  
32  
33 eluted with a linear gradient of 0–0.2 M NaCl in buffer A. Finally, the active fractions  
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35 were pooled and dialyzed against buffer C (10 mM phosphate buffer, pH 7.0) and  
36  
37 resolved on hydroxyapatite (25 ml; Seikagaku Kougyou, Tokyo, Japan) equilibrated  
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39 with buffer C. The enzyme was eluted with a linear gradient of 10–200 mM phosphate  
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41 buffer. The purified OsTAGG was pooled, concentrated using a Vivaspin centrifugal  
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43 filtration device with a molecular weight cut-off (Vivascience, Hanover, Germany), and  
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45 then stored at  $-20^\circ\text{C}$  after adding glycerol (final concentration 40%).  
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### 58 *5.3. UPLC and MS conditions*

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5 The analytical procedures for TA and TAG were according to a previous study  
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7 (Matsuura et al., 2009). In the analysis for glucose, UPLC separation was performed on  
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9 a Waters Acquity ethylene-bridged (BEH) C<sub>18</sub> column (1.7 μm, 2.1 × 100 mm) at 38°C.  
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11 The analytes were eluted from the column with a solvent of MeOH–H<sub>2</sub>O (1:4, v/v;  
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13 solvent A) at an isocratic mode. MS optimization experiments and all quantifications  
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15 were performed in the MS scan and MRM modes, respectively. The tune page  
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17 parameters and conditions for each MRM transition were optimized by infusing the 100  
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19 ppm standard solution into the mass spectrometer. To ensure that the tune page  
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21 parameters were compatible with the UPLC flow during tuning, a UPLC flow of 0.3  
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23 ml/min for solvent A was introduced into the mass spectrometer at the same time by  
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25 utilizing a *T* unit (Upchurch Scientific, Oak Harbor, WA, USA). To collect the MRM  
26  
27 data during the UPLC experiments, the capillary voltage was 3.0 kV, source temperature  
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29 was 120°C, desolation temperature was 350°C, desolation gas flow was 800 l/h, and  
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31 cone gas flow was 50 l/h. During each UPLC injection, the mass spectrometer was set  
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33 to collect data in the MRM mode with electrospray ionization in the negative ion mode.  
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35 The MRM transitions for the tested compounds were as follows: glucose {[M-H]<sup>-</sup>:  
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37 178.80, transition ion (*m/z*): 88.4, cone voltage: 25.5 V, collision energy: 9.00 eV} and  
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39 D-glucose-<sup>13</sup>C<sub>6</sub> {[M-H]<sup>-</sup>: 184.87, transition ion (*m/z*): 91.4, cone voltage: 25.0 V,  
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41 collision energy: 10.00 eV}.  
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#### 54 *5.4. Enzyme assays*

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58 The reaction mixture (final volume, 100 μl) consisting of 10 μM TAG and 20 mM  
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3 NaOAc buffer (pH 4.0) was preincubated at 37°C for 3 min. The reaction was initiated  
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5 by adding the enzyme, incubating at 37°C for 10 min, and then terminating the reaction  
6  
7 by adding 90 µl of 100% MeOH. After adding 2.5 µM (final concentration) TA-*d5* as an  
8  
9 internal standard, the mixture was analyzed using the UPLC-MS/MS system. The  
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11 amount of the reaction product was calculated by comparing the peak areas of TA-*d5*.  
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13 When the optimum pH was determined, McIlvaine's buffer (pH 3.0–7.0) was used as a  
14  
15 reaction buffer instead of NaOAc. To determine the optimum temperature, the purified  
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17 enzymes were reacted at different temperatures (20°C–65°C) in the abovementioned  
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19 standard conditions. After pH stability was analyzed, the purified proteins were treated  
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21 for 16 h at different pHs in Britton-Robinson's buffer (pH 2.3–11.3). Then the treated  
22  
23 protein was diluted 10-fold with 0.2 M NaOAc buffer (pH 4.0) for reaction. The  
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25 purified proteins were heat-treated at different temperatures (30°C–70°C) for 15 min,  
26  
27 and then immediately cooled on ice to analyze the residual activity. The kinetic  
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29 parameters were derived from the Lineweaver–Burk plots. The effect of substrate  
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31 concentration on reaction velocity was examined at various concentrations (7, 10, 15, 25,  
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33 40, and 70 µM) of TAG. The enzyme activity showed a constant velocity for 10 min  
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35 even in the lowest TAG concentration (7 µM). For each substrate specificity analysis,  
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37 the enzyme was incubated with 50 µM of each glycosidic compound (Table 2) and then  
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39 the released glucose, mannose, or galactose was quantified with 2.5 µM glucose-<sup>13</sup>C<sub>6</sub> as  
40  
41 an internal standard using the UPLC-MS/MS system. These standard curves (glucose,  
42  
43 mannose, or galactose) were measured by the methods described in the section on the  
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45 UPLC and MS conditions and showed linearity for 0–50 µM concentrations.  
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### 5.5. Determination of molecular mass

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5 The molecular mass was determined by gel filtration on a Superose 12 10/300 GL (GE  
6 Healthcare) column and by SDS-PAGE. The Superose column (10 × 300 mm) was  
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8 equilibrated with buffer A containing 0.2 M NaCl and then the purified enzyme (6 µg)  
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10 was run. The molecular mass calibration was performed by eluting standard proteins in  
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12 the range of 1.35–670 kDa (Bio-Rad, Hercules, CA). SDS-PAGE was performed with a  
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14 15% (w/v) acrylamide separation gel. Protein mass markers in the range of 14.4–97.4  
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16 kDa (Bio-Rad) were used. Protein bands were visualized by staining with Coomassie  
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18 brilliant blue R-250 (CBB).  
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#### 27 *5.6. MALDI-TOF MS and N-terminal amino acid sequence analyses*

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31 About 1 µg of purified enzyme was resolved by electrophoresis on a 15% SDS-PAGE  
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33 gel (Figure 3B). After staining with CBB, the stained gel band was excised from the gel.  
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35 The gel pieces were thoroughly destained with 25 mM NH<sub>4</sub>HCO<sub>3</sub> in 50% acetonitrile  
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37 and dried under vacuum. A total of 20 µl of 10 µg/ml Trypsin Gold solution (mass  
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39 spectrometry grade; Promega, Madison, WI) was added, and the tube was placed on ice  
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41 for 30 min and then placed at 37°C for 12 h for tryptic digestion. The digested peptides  
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43 were eluted twice with 100 µl of 75% acetonitrile containing 0.1% TFA. After  
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45 concentration, the sample was desalted using a ZipTip C18 tip (Millipore, Billerica,  
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47 MA) and analyzed on a Voyager MALDI-TOF mass spectrometer (Applied Biosystems,  
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49 Foster City, CA). 2,5-Dihydroxy benzoic acid (10 mg/ml) was used as the matrix, and  
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51 the laser power was set at 2500 in reflect mode. The MASCOT database  
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53 (<http://www.matrixscience.com/>) was searched to identify the proteins found. The  
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3 N-terminal amino acid sequence was determined using a protein sequencer (Procise  
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5 491; Applied Biosystems) after the peptides of the purified enzyme were transferred  
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7 onto a polyvinylidene difluoride membrane from SDS-PAGE using a Trans-Blot  
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9 Semi-Dry Transfer Cell (Bio-Rad).  
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3 **Figure legends**  
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8 Fig. 1. Metabolism of jasmonic acid focusing on TA and TAG. The narrow arrows  
9 indicate TAG biosynthesis. JA is metabolized to TA by hydroxylation at the C-12  
10 position and then glucosylated to produce TAG. Bold arrows show the reverse pathway  
11 generating TA from TAG as described in this study.  
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20 Fig. 2. UPLC-MS/MS MRM chromatograms for TAG-*d5* and TA-*d5*. The trace  
21 chromatogram in the MRM mode shows the ion peaks  $m/z$  392.03/210.8 for TAG-*d5*  
22 and 229.97/96.6 for TA-*d5*. (A) and (B), Synthesized TAG-*d5* and TA-*d5* as standards;  
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27 (C) Crude extraction of rice leaves treated with 0.1 mM TAG-*d5*; (D) Crude extraction  
28 of control plant. The open and closed arrows indicate the detection peaks for TAG-*d5*  
29 and TA-*d5*, respectively.  
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37 Fig. 3. SDS-PAGE and native PAGE of purified OsTAGG1. (A) OsTAGG1 was loaded  
38 on 10% polyacrylamide and CBB-stained native PAGE gels. (B) Total protein was  
39 stained with CBB (left panel) and Pro-Q emerald for glycoprotein detection (right  
40 panel). Lane M was loaded with standards: myosin (200 kDa),  $\beta$ -galactosidase (116  
41 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa),  
42 bovine carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (22 kDa). The arrow  
43 shows the 42- and 26-kDa polypeptides that match with  $\beta$ -glucosidase (AK070962)  
44 according to N-terminal amino acid and MALDI-TOF MS analyses.  
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59 Fig. 4. Nucleotide and deduced amino acid sequences of the cDNA encoding OsTAGG1.  
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3 The circles and closed triangles denote the catalytic and sugar-binding site residues that  
4 are highly conserved in glycoside hydrolase family 1, respectively. The open triangle  
5 denotes the serine that is identical to the corresponding aglycone binding site in  
6 sorghum dhurrinase-1. The sequences corresponding to the N-terminal sequences of  
7 large and small subunits of purified OsTAGG1 on SDS-PAGE are underlined (see also  
8 Figure 3B). The circled highlighted residues indicate predictive N-glycosylation sites.  
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22 Fig. 5. A phylogenetic tree of the amino acid sequences of the glycoside hydrolase  
23 family 1 from various plants. The tree was constructed using ClustalX (version 2.0.11)  
24 (neighbor-joining method) and visualized using TreeView. Numbers indicate bootstrap  
25 values greater than 500. The position of OsTAGG1 is boxed. Proteins used for  
26 alignment without OsTAGG1 are as follows: hypothetical protein (*Sorghum bicolor*),  
27 EES12355; hypothetical protein (*S. bicolor*), EES12357; unnamed protein product (*Vitis*  
28 *vinifera*), CAO21978;  $\beta$ -glucosidase (*Rosa hybrid cultivar*), BAG13451; dalcochinin  
29 8'-O- $\beta$ -glucosidase (*Dalbergia cochinchinensis*), AAF04007; prunasin hydrolase  
30 (*Prunus serotina*), AAF34650; amygdalin hydrolase (*P. serotina*), AAA93234;  
31 cynogenic  $\beta$ -glucosidase (*Trifolium repens*), ABV54716; isoflavone  
32 conjugate-specific- $\beta$ -glucosidase (*Glycine max*), BAF34333; furostanol glycoside  
33 26-O- $\beta$ -glucosidase (*Costus speciosus*), BAA11831;  $\beta$ -glucosidase D2 (*Lotus*  
34 *japonicus*), ACD65510; coniferin  $\beta$ -glucosidase (*Pinus contorta*), AAC69619;  
35 linamarase (*Manihot esculenta*), AAB22162;  $\beta$ -primeverosidase (*Camellia sinensis*),  
36 BAC78656;  $\beta$ -mannosidase (*Lycopersicon esculentum*), AAL37714; strictosidine  
37  $\beta$ -glucosidase (*Catharanthus roseus*), AAF28800; zeatin-O-glucoside degrading  
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3  $\beta$ -glucosidase (*Brassica napus*), CAA57913;  $\beta$ -glucosidase (*Zea mays*), AAB03266;  
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5 raucaffricine-O- $\beta$ -glucosidase (*Rauwolfia serpentina*), AAF03675; vicianin hydrolase  
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7 (*Vicia sativa*), ABD03937; furcatin hydrolase (*Viburnum furcatum*), BAD14925;  
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9  
10 cardenolide 16-O-glucohydrolase (*Digitalis lanata*), CAB38854; dhurrinase (*Sorghum*  
11  
12 *bicolor*), AAC49177; myrosinase (*Sinapis alba*), CAA42534; BGLU18 (*Arabidopsis*  
13  
14 *thaliana*), AAF22295; PYK10 (*A. thaliana*), NP\_187537. Other rice  $\beta$ -glycosidases  
15  
16 similar to OsTAGG1 are shown as Os4bglu9 (B7ECS8), Os4bglu10 (Q7F9K4),  
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18 Os4bglu11 (Q7XKV5), and Os4bglu12 (Q7XKV4).  
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Figure 1

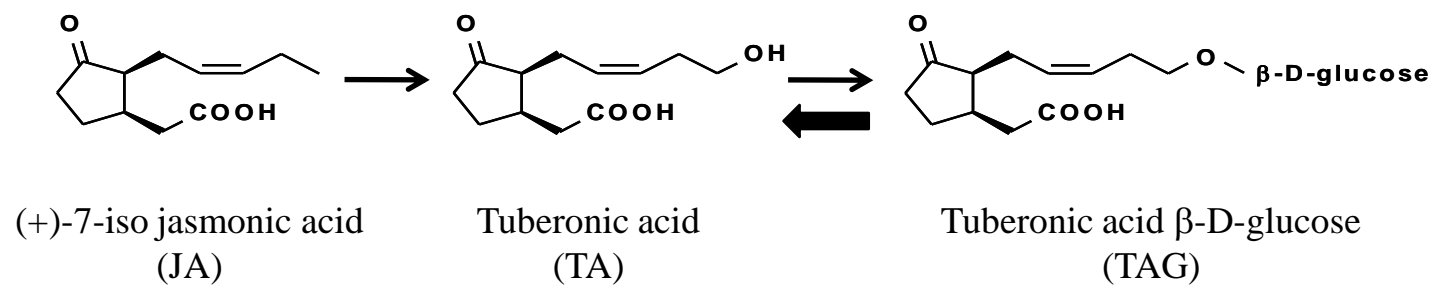


Figure 1. Wakuta et al.

Figure 2

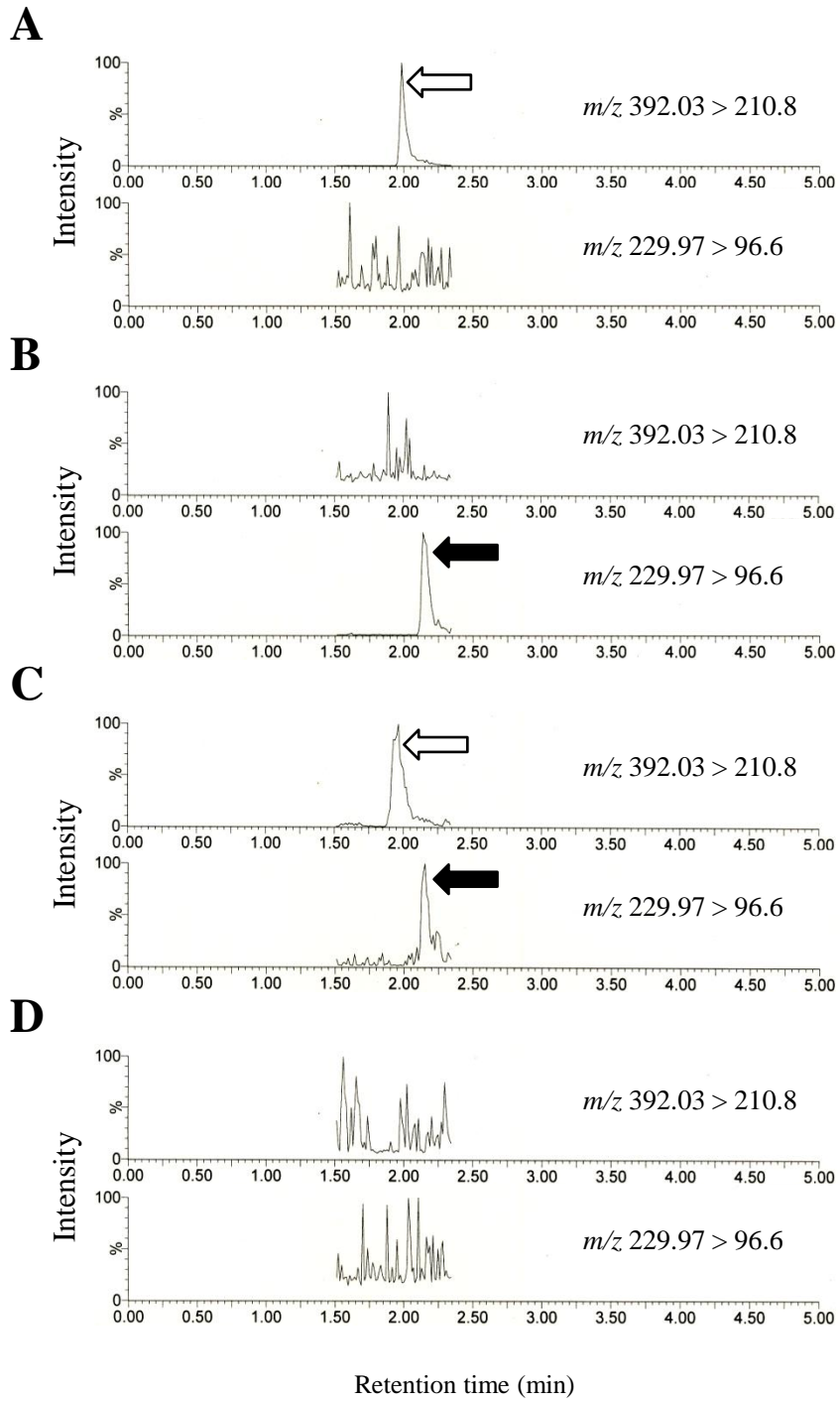


Figure 2. Wakuta et al.

Figure 3

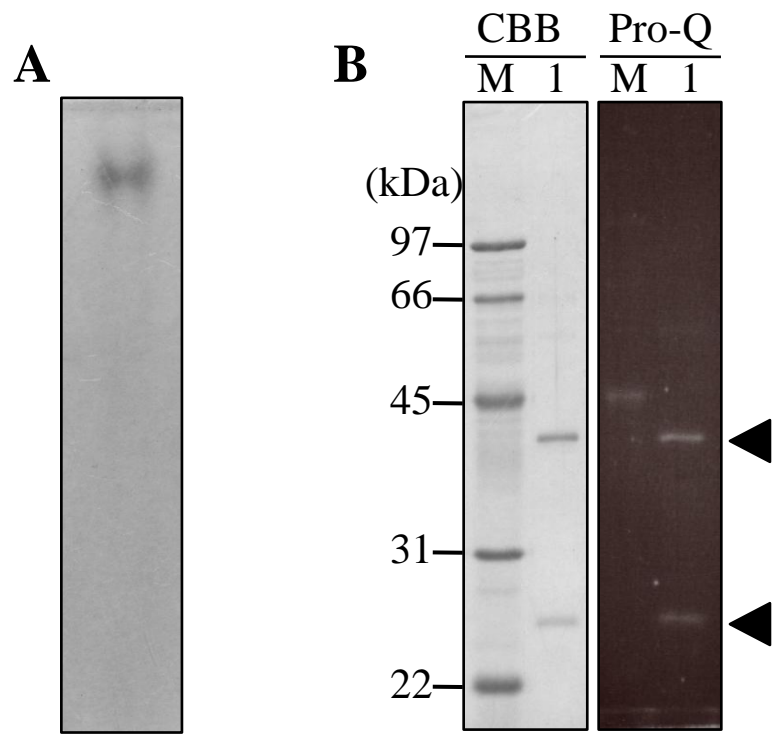


Figure 3. Wakuta et al.

Figure 4

1 ATGGCAGCTGCAGGGGAAGTGGTGATGCTTGGTGGCATTCTCCTCCCTCCTCCTGGTTGTCGCCGTCTCCGGTGAGCCGCCGCCGATC  
1 M A A A G E V V M L G G I L L P L L L V V A V S G E P P P I

91 AGCCGGAGGAGCTTCCCCGAGGGGTTTCATCTTCGGGACGGCCTCGTCGTGATCAGTATGAGGGTGGGCCAGAGAGGGGGCAGAGGA  
31 S R R S F P E G F I F G T A S S S Y Q Y E G G A R E G G R G

181 CCAAGCATCTGGGACACATTACACACCAGCACCAGATAAGATTGCTGACAAAAGCAATGGGACGTGGCTGCAGACTCCTACCATCTA  
61 P S I W D T F T H Q H P D K I A D K S N G D V A A D S Y H L

271 TACAAGGAAGATGTGCGCATCATGAAGGATATGGGAGTGGATGCATATAGGTTCTCCATCTCATGGACAAGAATCTTCCAAATGGAAGT  
91 Y K E D V R I M K D M G V D A Y R F S I S W T R I L P N G S

361 CTGAGCGGTGGAATCAACAGAGAAGGCATCAGTACTACAACAATTTGATCAATGAAGTATTACTGAAAGGGGTGCAACCATTTGTTACC  
121 L S G G I N R E G I S Y Y N N L I N E L L L K G V Q P F V T

451 CTTTTCCTACTGGGACTCGCCACAGGCATTAGAAGATAAATAATGGATTTCTTAGCCCTAATATCATAAATGACTATAAGGAGTACGCT  
151 L F H W D S P Q A L E D K Y N G F L S P N I I N D Y K E Y A

541 GAAACCTGCTTCAAAGAGTTGGTGACAGAGTGAAACATTGGATCACCTTCAATGAGCCTTTGAGCTTCTGTGTGGGGATATGCATCA  
181 E T C F K E F G D R V K H W I T F N E P L S F C V A G Y A S

631 GGTGGCATGTTTGACACCAGCCGCTGTTTCGCCTTGGGAGGAAATTCAGTGTGGCGATTGAGGAGGGGACCTTACACCGCATGCCAT  
211 G G M F A P G R C S P W E G N C S A G D S G R E P Y T A C H

721 CATCAACTACTTGCTCATGCGGAAACTGTTTCGGTTGTACAAGAGAAATATCAGGTCTTACAAGGGGAAGATGGAATAACTTTGGTC  
241 H Q L L A H A E T V R L Y K E K Y Q V L Q K G K I G I T L V

811 TCGAAGTGGTTTGTCCCTTCTCCCGCTCCAATCCAACATCGATGTGCAAGCGTGCTTTAGACTTCATGCTTGGATGGTTTATGGAT  
271 S N W F V P F S R S K S N I D A A R R A L D F M L G W F M D

901 CCCCTAATTAGAGCGAGTACCCCTAAGCATGAGAGAATGGTTCGGAATCGCTTGCCCTCAGTTCCTACTAAAGAACAATCTGAGTTGATC  
301 P L I R G E Y P L S M R E L V R N R L P Q F T K E Q S E L I

991 AAGGGTTCATTGATTTTATGGACTTAATTACTACACTTCAAATTTATGCTGGTAGCCTTCTCCATCAAATGGCCTCAATAACAGCTAT  
331 K G S F D F I G L N Y Y T S N Y A G S L P P S N G L N N S Y

1081 AGTACTGATGCTCGAGCTAATCTTACTGCTGTTGCAACGGCATTCCCATAGGTCCTCAGGCTGCTTCGCCTTGCTTTACATCTATCCT  
361 S T D A R A N L T A V R N G I P I G P Q A A S P W L Y I Y P

1171 CAAGGGTCCGTGAATTGGTGTCTTTATGTTAAGGAAACTATGGCAATCCTACCATCTACATCACCGAAAATGGTGTGATGAATCAAC  
391 Q G F R E L V L Y V K E N Y G N P T I Y I T E N G V D E F N

1261 AATAAGACCTTACCCTCCAGGAAGCCTTGAAGGATGACACTAGAATAGATTACTACCACAAGCACCTCCTTTCCTACTGCTAAGTGAATA  
421 N K T L P L Q E A L K D D T R I D Y Y H K H L L S L L S A I

1351 AGGGACGGAGCAAATGTGAAGGATACTTTGCATGGTCGCTGCTTGATAACTTCGAGTGGTCAAGCGCTATACTGTTCCGCTTTGGGATA  
451 R D G A N V K G Y F A W S L L D N F E W S N G Y T V R F G I

1441 AACTTTGTGGATTACAATGACGGAGCGAAGAGATACCCCAAAATGTCTGCCCATTTGGTTCAAGGAGTTCCTCCAGAAGTGA  
481 N F V D Y N D G A K R Y P K M S A H W F K E F L Q K \*

Figure 4. Wakuta et al.

Figure 5

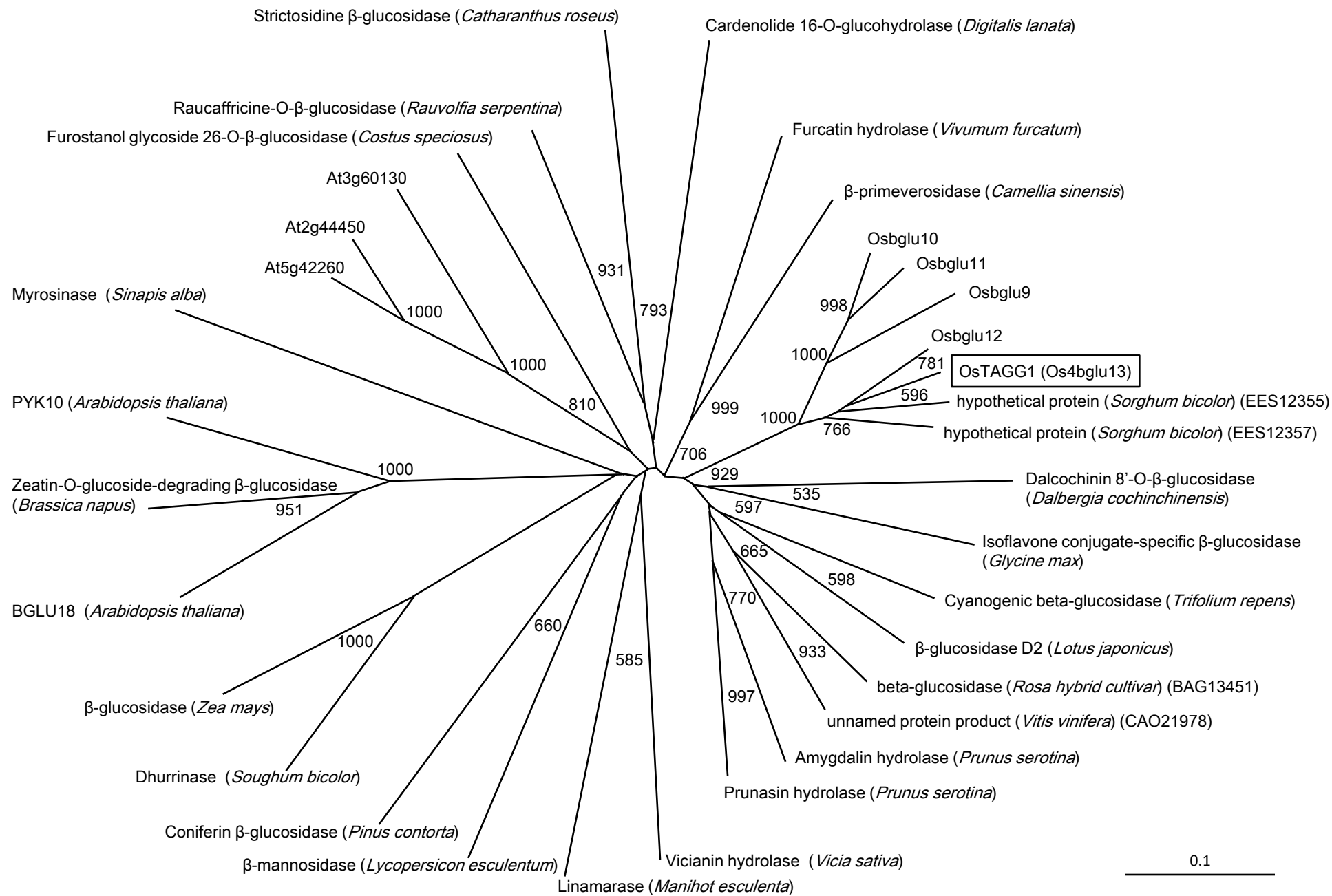


Figure 5. Wakuta et al.

**Table 1**

Summary of the purification of OsTAGG1 from rice

Purification step	Total activity (units)	Protein (mg)	Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ )	Yield (%)	Purification (-fold)
Crude extract	22	25,000	0.00088	100	1
$(\text{NH}_4)_2\text{SO}_4$ fractionation	18	3,100	0.0058	82	7
CM-Sepharose	7.6	290	0.026	35	30
Con A-Sepharose	3.0	17	0.18	14	200
Superdex 200	2.6	2.4	1.1	12	1,200
Resource S	0.54	0.23	2.3	2.5	2,700
Hydroxyapatite	0.24	0.065	3.7	1.1	4,300

**Table 2**

## Substrate specificity

Substrate	Relative activity (%)
Tuberonic acid glucoside	100
Methyl tuberonic acid glucoside	80
Jasmonoyl-1- $\beta$ -glucoside	51
Salicylic acid glucoside	25
Zeatin glucoside	0
Linamarin	0
pNP- $\beta$ -D-glucoside	178
pNP- $\beta$ -D-mannoside	1.3
pNP- $\beta$ -D-galactoside	3.2
pNP- $\beta$ -D-cellobioside	1.0
pNP- $\alpha$ -D-glucoside	0.45

**Supplementary figure 1**

[Click here to download Supplementary Information: suppl. fig.1.pptx](#)

**Supplementary figure 2**

[Click here to download Supplementary Information: suppl. fig.2.pptx](#)

## Identification of a $\beta$ -glucosidase hydrolyzing tuberonic acid glucoside in rice (*Oryza sativa* L.)

Shinji Wakuta<sup>a</sup>, Shigeki Hamada<sup>a,c\*</sup>, Hiroyuki Ito<sup>b</sup>, Hideyuki Matsuura<sup>a</sup>, Kensuke Nabeta<sup>a</sup>, and Hirokazu Matsui<sup>a</sup>

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Rice tuberonic acid glucoside-hydrolyzing  $\beta$ -glucosidase (OsTAGG1) was purified and examined its property. OsTAGG1 is a glycoprotein consisting of two polypeptides produced by cleavage within a molecule, and showed high specificity for TAG.

