



Title	Identification of Rice α -Glucosidase with High Hydrolytic Activity towards Salicylic Acid β -D-Glucoside
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1 Running title: Characterization of Rice β -Glucosidase

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3 **Identification of Rice β -Glucosidase with High Hydrolytic Activity towards**
4 **Salicylic Acid β -D-Glucoside**

5

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21

22 *Abbreviations:* JA, jasmonic acid; TA, tuberonic acid; TAG, tuberonic acid

23 β -D-glucoside; rOsTAGG2E, recombinant OsTAGG2 produced in *Escherichia coli*;

24 SAG, salicylic acid β -D-glucoside; pNP, *p*-nitrophenyl; oNPG, *o*-nitrophenyl

25 β -D-glucoside; pNPG, pNP β -glucoside; *m*-SAG, *m*- β -D-glucopyranosyloxybenzoic

26 acid; *p*-SAG, *p*- β -D-glucopyranosyloxybenzoic acid; EDTA, ethylenediaminetetraacetic

27 acid; rOsTAGG2P, recombinant OsTAGG2 produced in *Pichia pastoris*; PVDF,

28 polyvinylidene difluoride; SA, salicylic acid

1 β -Glucosidases (EC 3.2.1.21) split β -glucosidic linkages at the non-reducing end of
2 glucosides and oligosaccharides to release β -D-glucose. One of the important functions
3 of plant β -glucosidase is deglucosylation of inactive glucosides of phytohormones to
4 regulate levels of active hormones. Tuberonic acid is a jasmonate-related compound that
5 shows tuber-inducing activity in the potato. We have identified two enzymes,
6 OsTAGG1 and OsTAGG2, that have hydrolytic activity towards tuberonic acid
7 β -D-glucoside in rice (*Oryza sativa* L.). The expression of *OsTAGG2* is upregulated by
8 wounding and by methyl jasmonate, suggesting that this isozyme is involved in
9 responses to biotic stresses and wounding, but the physiological substrate of OsTAGG2
10 remains ambiguous. In this study, we produced recombinant OsTAGG2 in *Pichia*
11 *pastoris* (rOsTAGG2P), and investigated its substrate specificity in detail. From 1 L of
12 culture medium, 2.1 mg of purified recombinant enzyme was obtained by ammonium
13 sulfate precipitation and Ni-chelating column chromatography. The specific activity of
14 rOsTAGG2P (182 U/mg) was close to that of the native enzyme (171 U/mg), unlike
15 recombinant OsTAGG2 produced in *Escherichia coli*, which had approximately 3-fold
16 lower specific activity than the native enzyme. The optimum pH and temperature for
17 rOsTAGG2P were pH 3.4 and 60°C. After pH and heat treatments, the enzyme retained
18 its original activity in a pH range of 3.4-9.8 and below 55°C. Native OsTAGG2 and
19 rOsTAGG2P showed 4.5-4.7-fold higher activities towards salicylic acid β -D-glucoside,
20 an inactive storage-form of salicylic acid, than towards tuberonic acid β -D-glucoside
21 (TAG), although OsTAGG2 was originally isolated from rice based on TAG-hydrolytic
22 activity.

23

24

25 **Key words:** β -glucosidase; rice; tuberonic acid; salicylic acid; substrate specificity

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1

2 β -Glucosidases (EC 3.2.1.21) split β -glucosidic linkages at the non-reducing end of
3 glucosides and oligosaccharides with net retention of anomeric configuration. They are
4 ubiquitous enzymes, found in all domains of living organisms, Eukaryota, Eubacteria,
5 and Archaea, and they have a variety of functions, including degradation of biomass,
6 hydrolysis of glycolipids, catabolism of cell-wall oligosaccharides, and activation of
7 phytohormones.¹⁾ They were categorized into glycoside hydrolase (GH) families 1, 3, 5,
8 9, 30, and 116 on the basis of a sequence-based classification of glycoside hydrolases.²⁾
9 Of these families, GH family 1 has the greatest variety of activities, including
10 β -galactosidase (EC 3.2.1.23), β -mannosidase (EC 3.2.1.25), β -glucuronidase (EC
11 3.2.1.31), β -fucosidase (EC 3.2.1.38), in addition to β -glucosidase. Plant β -glucosidases,
12 belonging to this family, are thought to be responsible for regulation of the
13 physiological activity of phytohormones, which are stored in inactive glucosylated
14 forms. It is believed that a specific enzyme hydrolyzes a phytohormone glucoside to
15 release an active phytohormone, although only a few enzymes have been demonstrated
16 to play this physiological role.³⁻⁶⁾ Maize β -glucosidase (Zm-p60.1) and *Arabidopsis*
17 enzymes (BG1 and BG2) have been found to have central roles in the hydrolysis of
18 cytokinin β -glucoside and abscisic acid 1-*O*- β -D-glucosyl ester, respectively, *in vivo*.⁴⁻⁶⁾

19 We have identified two rice (*Oryza sativa* L.) β -glucosidases (OsTAGG1 and
20 OsTAGG2),^{7,8)} hydrolyzing tuberonic acid β -glucoside, a glucosylated form of
21 tuberonic acid, a derivative of jasmonic acid regulating stress responses as to insect
22 herbivory and pathogen attack,^{9,10)} and plant growth.¹¹⁻¹³⁾ OsTAGG1 and OsTAGG2 are
23 encoded by Os4BGlu13 (Rice Genome Project locus *Os04g0474900*) and Os4BGlu12
24 (*Os04g0474800*), respectively, and their sequences fall into a phylogenetic cluster of
25 defense-related β -glucosidases, including white clover and cassava linamarinases.¹⁴⁾
26 OsTAGG2 was found to be identical to cell-wall bound β -glucosidase purified from
27 germinating rice seeds.¹⁵⁾ Expression of *OsTAGG2* is induced by wounding, methyl
28 jasmonate, and ethephon in 10-d-old rice seedlings.^{8,16)} Subtractive hybridization cDNA

1 library screening also revealed that the transcript levels of *OsTAGG2* increased in
2 response to brown hopper feeding.¹⁷⁾ These findings suggest that *OsTAGG2* is involved
3 in responses to wounding and biotic stress.

4 Since *OsTAGG2* is phylogenetically close to defense β -glucosidases, it might be
5 involved in the hydrolysis of glucosides of bioactive compounds other than TAG. In this
6 study, we produced recombinant *OsTAGG2* in the methylotrophic yeast *Pichia pastoris*,
7 and investigated its aglycone specificity for various β -D-glucosides, including salicylic
8 acid β -D-glucoside (SAG), an inactive form of salicylic acid activating disease
9 resistance,¹⁸⁾ and its derivatives.

10

11 **Materials and Methods**

12 *Materials.* The structures of the β -D-glucosides analyzed in this study, other than
13 *p*-nitrophenyl (pNP) β -D-glycosides, *o*-nitrophenyl β -D-glucoside (oNPG), and
14 oligosaccharides, are shown in Fig. 1. From Sigma (St. Louis, MO), pNP β -D-glucoside
15 (pNPG), oNPG, pNP β -D-fucoside, pNP β -D-galactoside, pNP β -D-xyloside, and pNP
16 β -D-mannoside were purchased. Cellobiose and gentiobiose were from Nacalai Tesque
17 (Kyoto, Japan). Cellotriose, cellotetraose, sophorose, and laminaribiose were from
18 Seikagaku (Tokyo). Helicin was from Tokyo Chemical Industries (Tokyo). TAG, SAG,
19 *m*- β -D-glucopyranosyloxybenzoic acid (*m*-SAG), and *p*- β -D-glucopyranosyloxybenzoic
20 acid (*p*-SAG) were synthesized as reported previously.^{19,20)} Native *OsTAGG2* and
21 r*OsTAGG2E* were prepared as reported previously.^{8,21)}

22

23 *Construction of an expression plasmid for OsTAGG2.* Total RNA was prepared from
24 leaf sheath of the rice plant (*O. sativa* L. cv. Kitaake) with an RNAqueous kit (Applied
25 Biosystems, Foster City, CA), and cDNA was synthesized with *BcaBEST* RNA PCR kit
26 Ver. 1.1 (Takara Bio, Otsu, Japan). *OsTAGG2* cDNA was amplified by PCR with this
27 cDNA as template, primers 5'-ATGGCGGCAGCAGGGGCAATG-3' (sense
28 orientation) and 5'-TTTAACTGGATTACTTCCATCTCTTGTAAC-3' (antisense

Fig. 1

1 orientation), and Primestar HS DNA polymerase (Takara Bio). The PCR product was
2 cloned into the *EcoRV* site of pBluescript II SK (+)(Stratagene, La Jolla, CA) and
3 sequenced with an ABI Prism 310 Genetic Analyzer DNA sequencer (Applied
4 Biosystems) and a Big Dye Terminator 1.1 Sequencing Kit (Applied Biosystems). The
5 resulting plasmid was used as template in PCR to construct an expression plasmid of
6 OsTAGG2. The PCR product, amplified with primers
7 5'-CGGCGGTACCATGGGCGCCGGC (sense, *KpnI* site underlined) and
8 5'-TTTCCGCGGTCAGGAGGAACTTC (antisense, *SacII* site underlined), and
9 pPICZ α A (Invitrogen, Carlsbad, CA) were digested with *KpnI* (Takara Bio) and *SacII*
10 (Takara Bio), and ligated with DNA Ligation Kit Mighty Mix (Takara Bio). The DNA
11 sequence of *OsTAGG2* in the expression plasmid was sequenced as described above.

12
13 *Optimization of production of recombinant OsTAGG2 in P. pastoris.* The expression
14 plasmid of OsTAGG2, linearized by *SacI* digestion, was introduced into *P. pastoris*
15 strain X-33 by electroporation in a Gene Pulser (Bio-Rad, Richmond, CA) following the
16 manufacturer's instructions. Nine colonies grown on a YPDSZ plate (10 mg/mL of yeast
17 extract, 20 mg/mL of peptone, 20 mg/mL of D-glucose, 1 M sorbitol, 20 mg/mL of agar,
18 and 100 μ g/mL of zeocin) were selected and incubated in 10 mL of BMGY medium (10
19 mg/mL of yeast extract, 20 mg/mL of peptone, 13.4 mg/mL of yeast nitrogen base, 4
20 μ g/mL of D-biotin, 10 mg/mL of glycerol, and 0.1 M potassium phosphate buffer, pH
21 6.0) at 30°C with vigorous shaking until A_{600} reached 2.0. Cells were collected by
22 centrifugation and suspended in 10 mL of BMMY medium (10 mg/mL of glycerol in
23 BMGY was changed to 0.5% v/v methanol). The cell suspension was further incubated
24 at 30°C for 96 h. Protein production was maintained by the addition of methanol at a
25 final concentration of 0.5% v/v every 24 h. The enzyme activity of the culture
26 supernatant of each transformant was measured as described below. The transformant
27 with the highest productivity of recombinant OsTAGG2 was selected for further
28 analysis.

1 This transformant was incubated in 300 mL of BMGY medium as described above.
2 The cells collected by centrifugation were suspended in 300 mL of BMMY medium,
3 and the cell suspension was dispensed with 25 mL to each 100-mL Erlenmeyer flask.
4 Culture induction was carried out at 20°C or 30°C in the presence of 20 mg/mL of
5 casamino acids and/or 5 mM ethylenediaminetetraacetic acid (EDTA). The activity of
6 recombinant OsTAGG2 (rOsTAGG2P) in the culture supernatant was measured, and the
7 recombinant protein was detected by SDS-PAGE, in which the protein was visualized
8 with Sil-Best-Stain One (Nacalai Tesque). The His₆-tag at the C-terminal of the
9 recombinant protein was confirmed by Western blotting.

10

11 *Purification of rOsTAGG2P.* The transformant of *P. pastoris* harboring the expression
12 cassette for *OsTAGG2* was cultured in 1 L of BMGY medium (500 mL per 2-L
13 Erlenmeyer flask), as described above. The medium was exchanged to 1 L of BMMY
14 medium supplemented with 5 mM EDTA, and induction culture was done at 20°C for 96
15 h. The culture supernatant was recovered by centrifugation, and solid ammonium sulfate
16 was added up to 80% saturation. After incubation at 4°C overnight, the protein
17 precipitated was collected by centrifugation and dissolved in 100 mL of 50 mM sodium
18 phosphate buffer (pH 7.0) containing 300 mM NaCl. The resulting sample was loaded
19 onto a Ni-chelating Sepharose column (ϕ1.5 x 9 cm, GE Healthcare, Uppsala, Sweden).
20 After thorough washing of the column with 50 mM sodium phosphate buffer (pH 6.0)
21 containing 300 mM NaCl, the adsorbed protein was eluted with 50 mM sodium acetate
22 buffer (pH 5.0) containing 300 mM NaCl. Fractions of the adsorbed protein with high
23 purity were collected and dialyzed against 50 mM sodium acetate buffer (pH 5.0). The
24 enzyme solution was concentrated by ultrafiltration using Vivaspin 20 filters (nominal
25 molecular weight limit 30,000; Sartorius, Göttingen, Germany) up to approximately 0.4
26 mg/mL, and stored at 4°C. The protein concentration of the enzyme purified was
27 determined based on the concentration of each amino acid liberated by complete
28 hydrolysis of the protein with 6 N HCl. Amino acids were measured by the ninhydrin

1 colorimetric method²²⁾ with an JLC-500/V automatic amino acid analyzer (Jeol, Tokyo).

2
3 *Western blotting.* One μg of protein was separated by SDS-PAGE, and transferred to a
4 polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Billerica, MA)
5 by electroblotting in a semi-dry blotting apparatus.²³⁾ The membrane was incubated with
6 anti His-Tag antibody (0.4 μg , Novagen, Darmstadt, Germany) in 20 mM Tris-HCl
7 buffer (pH 7.5) containing 0.15 M NaCl, 10 mg/mL skimmed milk, and 1 mg/mL Tween
8 20 at room temperature for 1 h. Proteins binding the antibody were detected by
9 incubating the membrane for 1 h with alkaline phosphatase anti-mouse IgG (H+L)
10 (Vector Laboratories, Burlingame, CA) and BCIP/NBT phosphatase substrate
11 (1-component) (KPL, Gaithersburg, MD).

12
13 *N-Terminal sequence analysis.* Ten μg of protein was separated by SDS-PAGE and
14 transferred to a PVDF membrane as described above. The bands of recombinant
15 TAGG2, detected by staining with Coomassie Brilliant Blue R-250, were cut off from
16 the membrane and subjected to N-terminal sequence analysis with a Procise 492 protein
17 sequencer (Perkin Elmer, Waltham, MA).

18
19 *Enzyme assay.* For the standard assay, pNPG hydrolyzing activity was measured. A
20 reaction mixture (100 μL) consisting of an appropriate concentration of the enzyme, 20
21 mM sodium acetate buffer (pH 5.0), and 2 mM pNPG was incubated at 37°C for 10 min.
22 The reaction was terminated by the addition of 200 μL of 1 M sodium carbonate, and
23 A_{405} was measured to determine the *p*-nitrophenol released. One U of β -glucosidase
24 activity was defined as the amount of enzyme that hydrolyzes 1 μmol of pNPG per min.

25 The substrate specificity of rOsTAGG2P was investigated by the measuring velocity
26 of hydrolysis of the following substrates (2 mM): pNPG, oNPG, pNP β -D-fucoside, pNP
27 β -D-galactoside, pNP β -D-xyloside, pNP β -D-mannoside, TAG, helicin, SAG, m-SAG,
28 p-SAG, cellobiose, cellotriose, cellotetraose, sophorose, laminaribiose, and gentiobiose.

1 Reaction velocities for the hydrolysis of pNP β -D-glycosides and oNPG were measured
2 as for pNPG. Liberated *o*-nitrophenol was measured based on A_{405} . In the reactions to
3 β -glucosides other than pNPG and oNPG, the enzyme reactions were stopped by adding
4 200 μ L of 2 M Tris-HCl buffer (pH 7.0), and D-glucose liberated was measured by the
5 glucose oxidase-peroxidase method²⁴) by Glucose CII Test (Wako Pure Chemical
6 Industries). The kinetic parameters of various substrates were determined by fitting the
7 initial velocities at various substrate concentrations to the Michaelis-Menten equation
8 with the Grafit version 7.0.2 computer program (Erithacus Software, West Sussex, UK).

9
10 *Effects of pH and temperature on the activity and stability of rOsTAGG2P.* Optimum
11 pH and temperature were determined by measuring enzyme activities at given pH
12 values and temperatures. The reaction pH was adjusted with 40 mM Britton-Robinson
13 buffer (pH 2.2-11.0), composed of a mixture of 40 mM acetate, phosphate, and glycine,
14 the pH of which was adjusted with 5 N NaOH. pH stability was determined on the basis
15 of residual activity after incubation of 7.8 μ g/ml of enzyme solution in 90 mM
16 Britton-Robinson buffer at various pH values at 4°C for 24 h. Temperature stability was
17 determined by measuring residual activity after incubation of the enzyme in 20 mM
18 sodium acetate buffer (pH 5.0) at given temperatures for 20 min. The ranges of pH and
19 temperature at which the enzyme retained more than 90% of its original activity were
20 considered stable.

21

22

23 **Results and Discussion**

24 *Optimization of the production of rOsTAGG2P*

25 The culture medium of the transformant of *P. pastoris* carrying the expression
26 cassette for *OsTAGG2* showed 0.5 U/mL after induction at 30°C for 96 h, but the
27 enzyme produced barely adsorbed onto a Ni-chelating Sepharose column (data not
28 shown). His₆-tag antibodies detected no band in the non-adsorbed fraction, suggesting

1 that the His₆-tag has been removed or destroyed during culture. Hence we tried to
2 reduce the deficiency of the tag by changing the induction conditions (Fig. 2).
3 Regardless of additive substances, more rOsTAGG2P was produced at 20°C than at
4 30°C. Western blotting revealed that the His₆-tag was protected only in the culture with
5 EDTA at 20°C, although the level of production of rOsTAGG2P in the presence of
6 EDTA was similar to that without EDTA. The addition of EDTA is thought to prevent
7 the His-tag of the recombinant protein from cleavage. Loss of the His₆-tag occurred at
8 30°C even in the presence of EDTA. The addition of casamino acid decreased the
9 production of rOsTAGG2P.

Fig. 2

10

11 *Production and purification of recombinant OsTAGG2*

12 On a 1-liter-scale of the production of rOsTAGG2P, 774 U of the enzyme was
13 obtained. The protein in the culture supernatant was collected by precipitation with 80%
14 saturation ammonium sulfate, and Ni-chelating Sepharose column chromatography was
15 carried out. No enzyme activity was detected in the non-adsorbed fraction. The enzyme
16 was recovered from the adsorbed fraction at high yield (37% of the starting material),
17 and 2.1 mg of rOsTAGG2P (182 U/mg) was obtained. The specific activity of
18 rOsTAGG2P was close to that of the native enzyme purified from rice (171 U/mg). On
19 the other hand, rOsTAGG2E had approximately 3-fold lower specific activity (60.7
20 U/mg) than the native enzyme and rOsTAGG2P. This difference in activity between
21 rOsTAGG2E and the others was presumably due to structural differences, as discussed
22 below.

23 On SDS-PAGE, rOsTAGG2P showed two bands of similar molecular masses (Fig. 3).
24 Both of these proteins reacted with His₆-tag specific antibody. The N-terminal
25 sequences of the proteins of high and low molecular masses were XFTWPSR and
26 GXXEPPVSXR, respectively. These sequences correspond to the N-terminal sequences
27 of the mature proteins when the precursor is cleaved at the Ste13 protease cleavage site
28 of the α -factor from the plasmid and at the signal sequence cleavage site of OsTAGG2,⁸⁾

1 respectively. These two derivatives could not be separated by other column
2 chromatographic procedures such as cation exchange and gel filtration column
3 chromatography (data not shown). Production of rOsTAGG2P with an expression
4 plasmid in which *OsTAGG2* was inserted into the *EcoRI* and *SacII* sites of the vector to
5 delete the N-terminal extra sequence, was unsuccessful, because, unaccountably, the
6 recombinant enzyme was digested during production. The N-terminal of OsTAGG2 is
7 apart from the catalytic site,²⁵⁾ and small difference in N-terminal sequence is not
8 thought to cause a large functional difference, thus this preparation was used in further
9 analysis. rOsTAGG2P is single peptide as rOsTAGG2E, as judged by the molecular
10 mass measured by SDS-PAGE. In contrast, the native enzyme purified from rice plants
11 is composed by two peptides, 40 and 26 kDa, encoded by a single gene, the N-terminals
12 of which are Gly28 and Gly359, respectively.⁸⁾ This cleavage might occur through
13 proteolysis catalyzed by a selective protease, because 11 amino acid residues around the
14 cleavage sites of OsTAGG1⁷⁾ and OsTAGG2⁸⁾ (LPPSNGLNNSY; peptide bond between
15 N and G is cleaved) are completely conserved.

Fig. 3

17 *Effects of pH and temperature on the activity and stability of rOsTAGG2P*

18 The optimum pH and temperature for rOsTAGG2P were pH 3.4 and 60°C. The
19 optimum pH of rOsTAGG2P was lower than those of the native enzyme (pH 4.5) and
20 rOsTAGG2E (pH 5.0).^{8,16)} After the pH and heat treatments, rOsTAGG2P retained its
21 original activity in a pH range of 3.4-9.8 and below 55°C. The stable ranges for pH and
22 temperature of rOsTAGG2P were similar to those for the native enzyme.⁸⁾

24 *Substrate specificities*

25 The substrate specificities of rOsTAGG2P, rOsTAGG2E, and the native enzyme were
26 investigated with pNP β -D-glycosides, oligosaccharides, and various β -D-glucosides,
27 including TAG and SAG at a concentration of 2 mM, and compared (Table 1).
28 Previously we measured reaction velocities towards various substrates at a low

1 concentration (50 μM) with a UPLC-MS/MS system,⁸⁾ but in this study we measured
2 them at a higher substrate concentration by the spectrophotometric method to determine
3 accurate reaction velocities. The substrate specificities of the native enzyme
4 investigated by the two methods were different from each other. The reaction velocity at
5 low concentration (close to the endogenous concentration) appears to be important
6 physiologically, but it is too difficult to quantify exactly very low amounts of reaction
7 product released from low concentrations of substrates. Hence the data produced in this
8 study are presumably more reliable than previous results.

9 All the enzymes showed similar substrate specificities. They showed high activities
10 towards pNPG and pNP β -D-fucoside, and low activities towards pNP β -D-mannoside
11 and pNP β -D-xyloside. The preferences of all the OsTAGG2 derivatives for
12 oligosaccharides were also similar to each other, although the hydrolytic velocities
13 towards oligosaccharides of rOsTAGG2P relative to that towards pNPG were lower
14 than the native enzyme and rOsTAGG2E. Laminaribiose was the best substrate for all
15 the enzymes among the β -glucobioses, followed by sophorose. Gentiobiose and
16 cellobiose were poor substrates for all the OsTAGG2 derivatives. Cellooligosaccharides
17 longer than cellobiose were much more rapidly hydrolyzed than cellobiose. Thus +2
18 subsite appears to contribute to the binding of oligosaccharides linked by the
19 β -1,4-glucosidic linkage.

Table 1

20 OsTAGG2 derivatives showed high hydrolytic activities towards β -glucosides with
21 various aglycon structures. The aglycon binding site of OsTAGG2 is formed mainly by
22 hydrophobic residues, including Trp181, Phe193, and Trp365,²⁵⁾ and this enzyme is
23 thought to recognize the aglycon parts of substrates loosely. Interestingly, rOsTAGG2P
24 and the native enzyme showed 4.5- and 4.7-fold higher activity towards SAG,
25 respectively, and rOsTAGG2P had a 2.8-fold higher $k_{\text{cat}}/K_{\text{m}}$ value for SAG than for TAG
26 (Table 2), although OsTAGG2 was originally isolated from rice based on
27 TAG-hydrolytic activity. Among the SAG derivatives, rOsTAGG2P showed the highest
28 $k_{\text{cat}}/K_{\text{m}}$ for *m*-SAG. SAG is thought to be an inactive storage form of salicylic acid (SA),

Table 2

1 which regulates various aspects of growth and development and serves as a critical
2 signal activating disease resistance, the induction of pathogenesis-related proteins, and
3 systemic acquired resistance in various plant species.²⁶⁾ SAG is synthesized in the
4 cytosol by glucosylation of SA by UDP-glucosyltransferases, which have been
5 identified in *Arabidopsis*²⁷⁻²⁹⁾ and rice,³⁰⁾ and is transported into the vacuoles.
6 Hydrolysis of SAG in the tobacco apoplast has been observed, and this reaction might
7 be catalyzed by cell-wall-associated β -glucosidase.³¹⁾ OsTAGG2 is identical to a cell
8 wall-bound β -glucosidase from germinated rice.¹⁵⁾ This suggests that OsTAGG2 is
9 responsible for the hydrolysis of SAG in the apoplasts. Vacuolar and plasma membranes
10 have been observed to fuse, releasing signal compounds that accumulate in the vacuoles
11 to the apoplasts when pathogenic bacteria proliferate.³²⁾ This process presumably
12 contributes to the transportation of SAG from the vacuoles to the apoplasts. In plants,
13 salicylic acid is not generally induced by mechanical wounding, but in the case of rice,
14 an increase in SA is observed at the initial stage of wounding stress (30 min after
15 wounding), along with a decrease in endogenous SAG.³³⁾ An increase in the expression
16 of *OsTAGG2* was observed 1 h after wounding, but OsTAGG2 was obtained from
17 non-wounded rice plants, and it could contribute to the hydrolysis of SAG.⁸⁾ Mechanical
18 wounding also induces TA and TAG in rice, as reported elsewhere.⁸⁾ In this stress
19 response, OsTAGG2 might also hydrolyze TAG to produce TA, but the physiological
20 functions of TA in wounding stress are controversial, and further analysis is needed,
21 because repression of wound-inducible genes by TA has been demonstrated at much
22 higher concentrations than the physiological.³⁴⁾

23 As described above, all OsTAGG2 derivatives showed higher hydrolytic activity
24 towards SAG and its derivatives than towards TAG, and hence we have concluded that
25 OsTAGG2 has high preference for SAG over TAG. But in the reactions to certain
26 substrates, including oNP β -D-glucoside, pNP β -D-galactoside, and cellooligosaccharide,
27 OsTAGG2 derivatives showed preferences significantly different from each other. This
28 might have been due to structural differences in the OsTAGG2 derivatives. Native

1 OsTAGG2 is separated to two peptides, of 40 and 26 kDa, as described above.⁸⁾ The
2 cleavage site is far from the active site,²⁵⁾ but this cleavage might affect substrate
3 specificity through slight changes in the overall structure. Another possibility as to
4 changes in the structure of enzyme is the presence of *N*-glycans attached to the
5 *N*-glycosylation sites of rOsTAGG2P and the native enzyme. Their molecular masses
6 are higher than the theoretical mass, and they have 5 putative *N*-glycosylation sites.
7 Removal of the *N*-glycans of rOsTAGG2P by endoglycosidase H did not reduce the
8 enzyme activity at all (data not shown), and it appears that the *N*-glycans hardly
9 contribute to the enzyme functions. *N*-Acetylglucosaminyl residues remained at the
10 *N*-glycosylation sites after treatment, and they might have caused structural differences,
11 which respect to the *N*-glycan-free OsTAGG2 like rOsTAGG2E. In the preparation of
12 rOsTAGG2E, a thioredoxin tag fused at the N-terminal was eliminated, but an extra
13 peptide comprised of 22 amino acid residues was attached before the mature enzyme.²¹⁾
14 The N-terminal of OsTAGG2 is far from the active site, as pointed out above, and this
15 extension probably does not affect substrate specificity.

16
17

18 **Conclusions**

19 In this study, rice OsTAGG2 was successfully produced in *P. pastoris* and the
20 recombinant enzyme was characterized in detail. Originally, this enzyme was purified
21 from rice based on TAG-hydrolyzing activity, but it had significantly higher activity
22 towards SAG than towards TAG. To our knowledge, this is the first report of an enzyme
23 with high activity towards SAG, although the physiological significance of OsTAGG2
24 in the hydrolysis of SAG should be analyzed.

25
26

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1 **Figure legends**

2 **Fig. 1.** Chemical Structures of the Natural Glucoside Substrates Used in This Study.

3

4 **Fig. 2.** Time Course of the Production of Recombinant OsTAGG2 in *P. pastoris*.

5 A, Enzyme activities of the culture supernatants of *P. pastoris* cultivated under
6 various conditions. Filled and open symbols show results at 30°C and 20°C,
7 respectively. Circles, triangles, squares, and diamonds indicate the culture in the
8 presence of no additive, casamino acid, EDTA, and both casamino acid and EDTA,
9 respectively. B, SDS-PAGE analysis of the culture supernatant. Induction culture was
10 carried out for 96 h without additive (lanes 1 and 5), and in the presence of casamino
11 acid (lanes 2 and 6), EDTA (lanes 3 and 7), and both casamino acid and EDTA (lanes 4
12 and 8). Lanes 1-4 and lanes 5-8 indicate the results of culture at 20°C and 30°C,
13 respectively. Molecular masses of the standard proteins are shown on the left. C,
14 Western blot analysis. Lane numbers correspond to those shown in panel B.

15

16 **Fig. 3.** SDS-PAGE and Western Blot Analyses of rOsTAGG2P.

17 A, SDS-PAGE: N-terminal sequences of two proteins (1 and 2) of slightly different
18 mobility are indicated on the right. Molecular masses of the standard proteins are shown
19 on the left. B, Western blot: Triangles indicate positions corresponding to proteins 1 and
20 2 shown in the panel A. C, Cleavage sites of the signal peptide of rOsTAGG2P,
21 indicating the starting positions of proteins 1 and 2 within the pre-protein sequence.

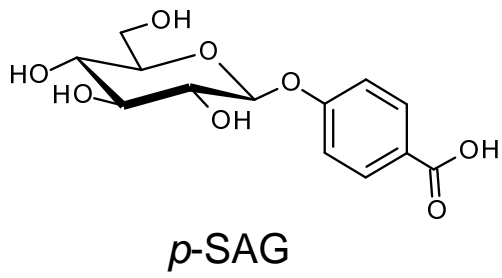
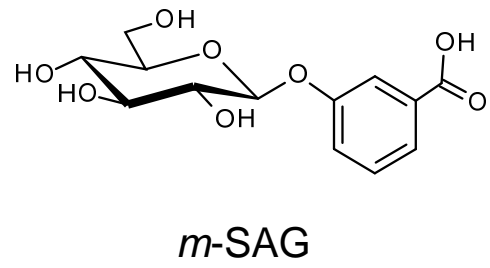
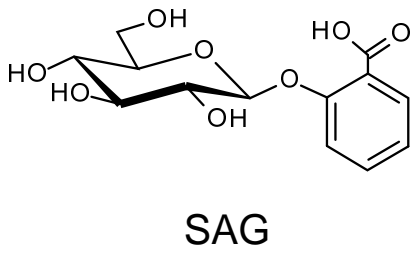
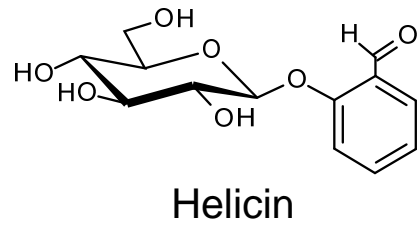
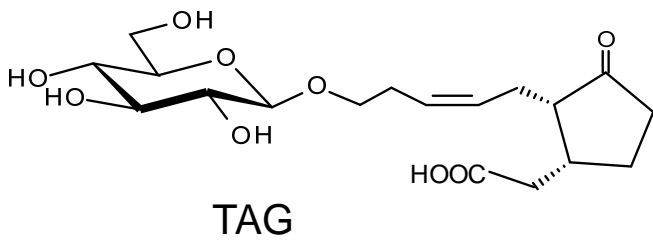


Fig. 1, Himeno, et al.

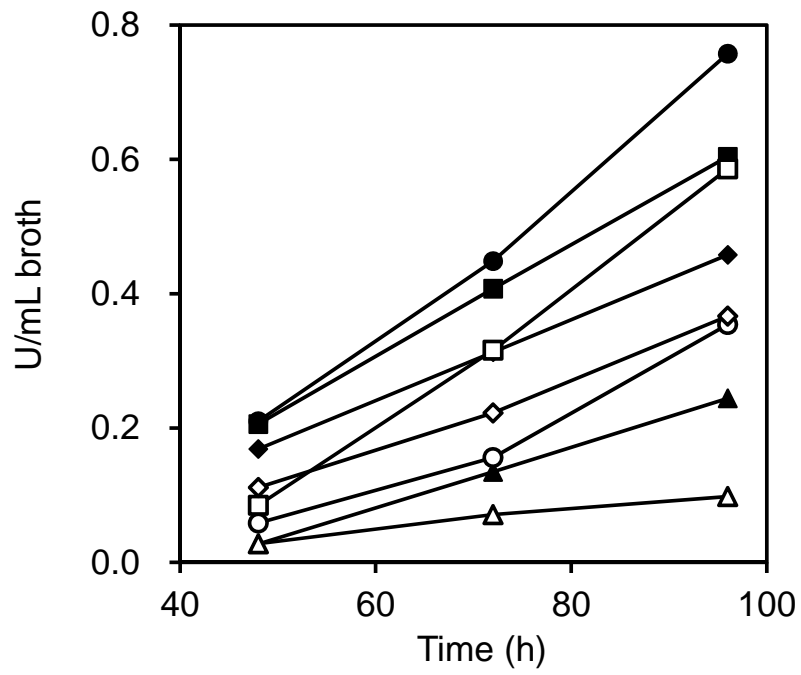
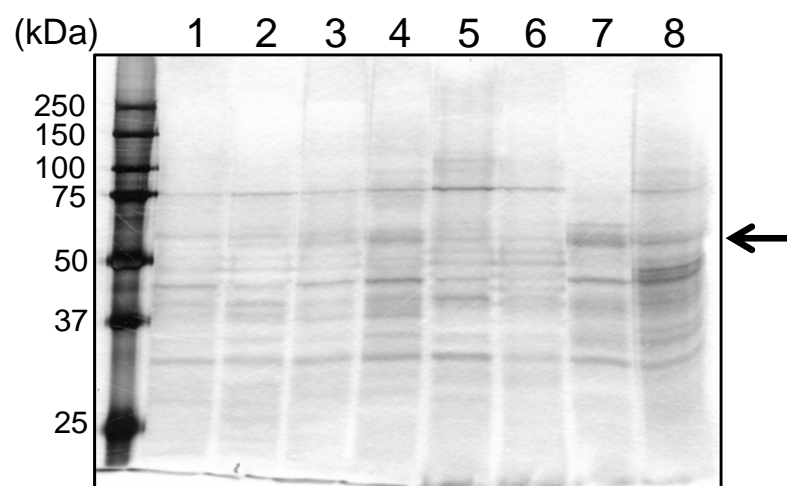
A**B****C**

Fig. 2, Himeno et al.

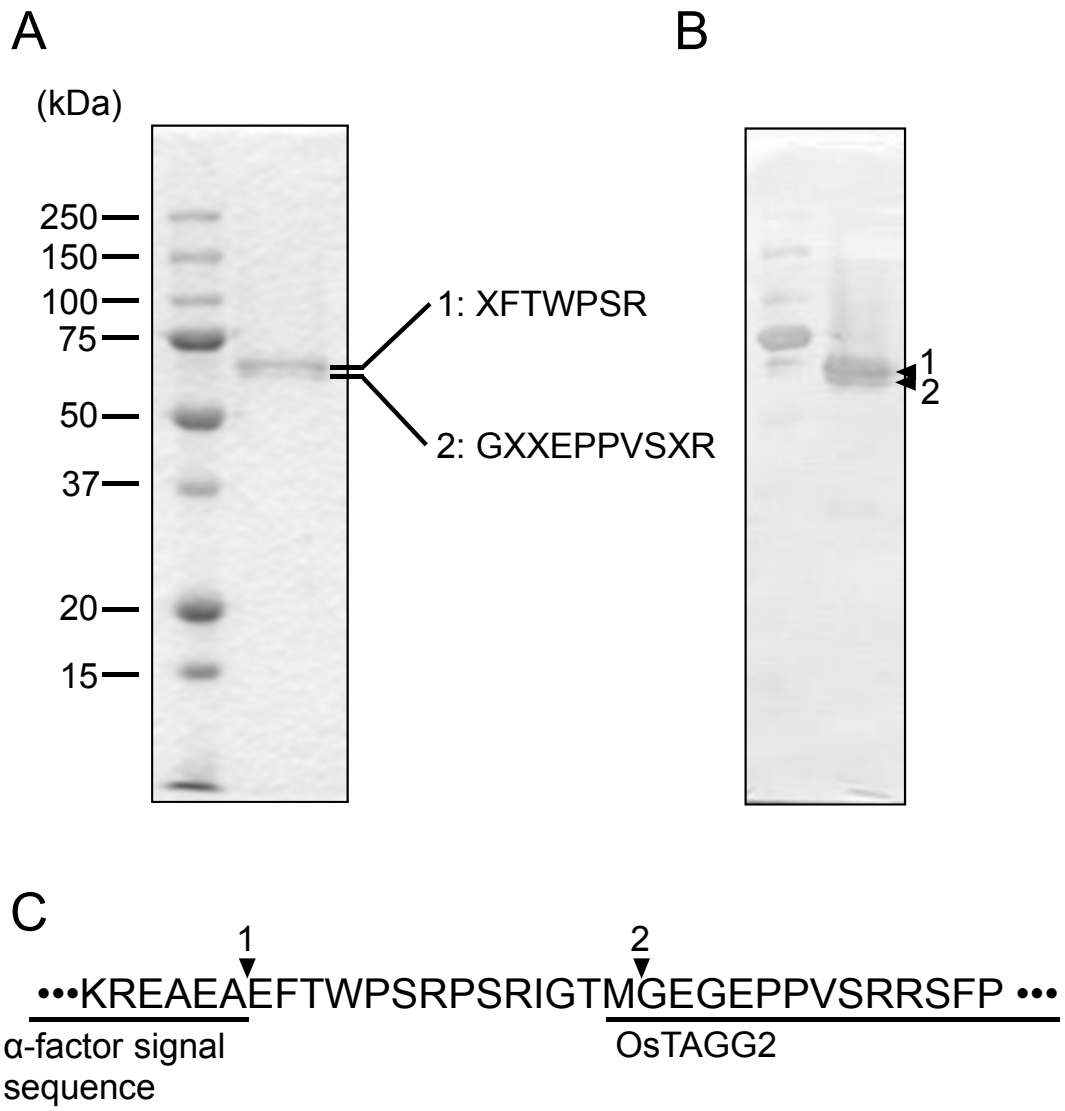


Fig. 3, Himeno et al.

Table 1. Hydrolytic Velocities of OsTAGG2 towards Various Substrates

Substrate	rOsTAGG2P		rOsTAGG2E		Native OsTAGG2	
	v ($\mu\text{mol}/\text{min}/\text{mg protein}$)	Relative v (%)	v ($\mu\text{mol}/\text{min}/\text{mg protein}$)	Relative v (%)	v ($\mu\text{mol}/\text{min}/\text{mg protein}$)	Relative v (%)
pNP β -D-Glucoside	182 \pm 6	100	60.7 \pm 1.1	100	171 \pm 7	100
oNP β -D-Glucoside	182 \pm 4	100	23.0 \pm 1.6	34.1	300 \pm 14	175
pNP β -D-Fucoside	300 \pm 6	165	96.7 \pm 9.9	159	138 \pm 6	80.7
pNP β -D-Galactoside	45.9 \pm 0.8	25.2	43.0 \pm 4.5	70.8	184 \pm 1	108
pNP β -D-Xyloside	6.77 \pm 0.38	3.72	13.4 \pm 0.32	22.1	15.3 \pm 0.2	8.95
pNP β -D-Mannoside	1.55 \pm 0.03	0.852	0.37 \pm 0.03	0.610	1.18 \pm 0.18	0.690
TAG	44.5 \pm 1.5	24.5	50.1 \pm 1.0	82.5	25.3 \pm 0.3	14.8
Helicin	179 \pm 4	98.4	186 \pm 3	306	274 \pm 5	160
SAG	201 \pm 8	110	60.3 \pm 1.4	99.3	118 \pm 4	69.0
<i>m</i> -SAG	215 \pm 6	118	61.0 \pm 3.3	100	130 \pm 10	76.0
<i>p</i> -SAG	164 \pm 8	90.1	105 \pm 5	173	157 \pm 4	91.8
Cellobiose	Trace	N.D.	0.520 \pm 0.003	0.857	N. H.	N. H.
Cellotriose	7.33 \pm 0.07	4.02	15.5 \pm 0.5	25.5	36.8 \pm 3.9	21.5
Cellotetraose	13.8 \pm 0.4	7.58	26.1 \pm 1.2	43.0	45.6 \pm 2.9	26.7
Sophorose	3.32 \pm 0.37	1.82	4.28 \pm 0.33	7.05	19.9 \pm 0.5	11.6
Laminaribiose	11.6 \pm 0.6	6.37	28.8 \pm 0.2	47.4	61.7 \pm 6.2	36.1
Gentiobiose	0.117 \pm 0.008	0.0643	0.450 \pm 0.006	0.741	0.859 \pm 0.145	0.502

Data are mean \pm SD for three independent experiments. N. D., not determined. N. H., not hydrolyzed. Trace, accurate activity could not be determined due to low activity.

Table 2. Kinetic Parameters of Recombinant OsTAGG2 Produced in *P. pastoris* for the Hydrolysis of Various Substrates

Substrate	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{s}^{-1}\text{mM}^{-1}$)
pNP β -D-Glucoside	466 \pm 4	2.71 \pm 0.11	172
oNP β -D-Glucoside	359 \pm 9	2.01 \pm 0.16	179
pNP β -D-Fucoside	499 \pm 9	1.85 \pm 0.19	270
pNP β -D-Galactoside	236 \pm 19	6.83 \pm 1.19	34.6
TAG	72.7 \pm 3.6	1.41 \pm 0.14	51.6
SAG	458 \pm 21	3.21 \pm 0.33	143
<i>m</i> -SAG	563 \pm 21	1.99 \pm 0.15	283
<i>p</i> -SAG	286 \pm 11	1.33 \pm 0.25	215
Helicin	497 \pm 48	2.65 \pm 0.51	188