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Functions of rice beta-glucosidases and transglucosidases

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ABSTRACT: Continued improvement of yields of rice, a primary nutrient for much of humanity, in the future to feed a growing population will benefit from greater knowledge of the plant's function at the molecular level, including the roles of various enzymes and how their gene expression levels contribute to traits that can increase crop yields. One group of enzymes of interest are the β-glucosidases, which release single glucose residues from glucosides and oligosaccharides, due to their multiplicity and involvement in various functions. Known β-glucosidases fall in 4 sequence-similarity-based glycoside hydrolase (GH) families in plants, GH1, GH3, GH5 and GH116. Of these the GH1 family encodes the most known β-glucosidases and related enzymes, with 34–35 active genes in rice. These enzymes contribute to cell wall recycling, lignification, glycolipid recycling, release of phytohormones and their precursors, including response to biotic and abiotic stress, defense, and metabolic homeostasis, among other roles. In the past 20 years, numerous studies have teased out various aspects of rice β-glucosidase function using biochemical, cell biological and genetic approaches. Clear roles have been elucidated for certain isoenzymes, although most remain poorly understood. Efficient transglycosylation activities of certain enzymes affords production of novel glucoconjugates, including phytohormone glucosyl esters for identification of enzymes catalyzing related functions. Here, we provide an overview of what has been learned about rice β-glucosidase functions and what remains to be clearly understood.

KEYWORDS: rice enzymes, glycoside hydrolase families, enzyme functions, phytohormones, reverse genetics, functional studies

INTRODUCTION

Rice is a major source of caloric nutrition for over half the world population and an important part of the culture in much of Asia [1]. Southeast Asian countries like Thailand and Vietnam account for around 40% of rice exports, despite relatively low yields in these countries [2]. As the world population increases, yields will need to be improved by decreasing differences between theoretical and actual yields (yield gaps) through improved management and increasing achievable yields by genetic improvement of the rice plant itself. Although standard breeding and production of hybrid rice may improve yields [1], these approaches come with tradeoffs, such as increased seed prices and trading quality for quantity. Understanding of the enzymes and proteins that regulate rice growth, development and response to biotic and abiotic stress will allow a more educated approach to engineering improved rice plants for increased yields. Since the sequencing of the rice genome as the first monocot model [3], whole families of genes encoding enzymes of potential interest have been identified, including those for β-glucosidases [4].

β-Glucosidases (EC 3.2.1.21) hydrolyze glycosidic bonds at nonreducing terminal glucose moieties to release glucose from larger molecules, including glycosides and oligosaccharides. The release of glucose from oligosaccharides allows its utilization in metabolism, while the release of glucose from glycosides may be critical for activating the aglycone molecule for a biological function [5]. β-Glucosidases are found in all domains of living organisms. In archaea and bacteria, β-glucosidases primarily act to release glucose as a nutritional source, but in eukaryotes, they may have additional roles, particularly in plants. In addition to the glycolipid glucosylceramide, which is found in many eukaryotes, plants have a wide range of other glucosides, including those of various phenolic compounds and lipids, defense compounds like cyanogenic glucosides, storage forms of volatile compounds, and phytohormones. The glucosyl moiety can serve to solubilize the molecules and allow them to be sequestered on one side of a membrane or act as a blocking group to prevent undesired reactions or interactions until their proper time. Hydrolysis by β-glucosidases may be an activation step or a part of recycling, such as hydrolysis of gluco-oligosaccharides from the breakdown of cellulose and other β-glucans.

β-Glucosidases are a subgroup of glycoside hydrolases (GH), also called glycosidases. GH have been classified according to the reactions that they catalyze in the Enzyme Commission numbers (EC 3.2.-.-) and according to their amino acid sequence and corresponding structural relationships in the carbohydrate active enzyme [CAZy) database [6, 7]. The CAZy

database is accessible from the website www.cazy.org and currently contains 183 GH families. Many of these families are grouped into Clans, which are groups of families with conserved structures and catalytic residues, but with too diverse sequences to be identified as related based on sequence alone. Of the 183 families, GH 1, 2, 3, 5, 16, 30, 39, 116, 131, 175, and 180 are currently listed as containing β-glucosidases. Of these, the ones that have been characterized in rice or other plants include GH 1, 3, 5 and 116, with most characterized β-glucosidases falling in GH1.

All the GH families containing plant β-glucosidases are retaining enzymes, in which the glucose is released as the β anomer, thereby retaining the anomeric configuration of the β-glucoside substrate [8–10]. To do this, they use two acidic residues, one acting as a catalytic acid/base and the other as a nucleophile that forms a covalent intermediate with the substrate. In the first half reaction, the catalytic acid/base protonates the glycosidic bond oxygen of the nonreducing terminal glucosyl residue, while the nucleophile attacks the anomeric carbon (carbon 1) from the opposite side, allowing breakage of the glycoside bond and formation of the α-glucosyl-enzyme intermediate. In the second half reaction, the catalytic acid/base acts as a base to deprotonate an incoming water, to allow it to attack the anomeric carbon opposite the catalytic nucleophile and thereby displace it, releasing the β-glucose product.

The GH1 and GH5 families fall in GH Clan A, members of which have a $(\beta/\alpha)_8$-barrel (also called TIM barrel) structure (Fig. 1) with the catalytic acid/base and nucleophiles at the C-terminal ends of β-strands 4 and 7 of the barrel, respectively [11]. Plant GH3 enzymes have two domains contributing to the active site, with the catalytic nucleophile again on the end of strand 7 of a $(\beta/\alpha)_8$-barrel, but the catalytic acid/base is moved to a loop coming from a second domain with an $(\alpha/\beta)_6$ sandwich structure [12]. GH116 enzymes have an unrelated structure which is composed of an N-terminal β-sandwich domain and a C-terminal $(\alpha/\alpha)_6$ solenoid domain [13]. The catalytic nucleophile is on a long loop between α-helices 1 and 2 and the catalytic acid base on a long loop between helices 5 and 6 of the C-terminal solenoid domain.

Rice GH1 enzymes, including β-glucosidases, have been extensively studied, although the biological roles of many remain unclear [4, 5, 14–38]. Forty genes were originally identified in japonica and indica genome sequence data, but the last two appeared to be endophyte genes that contaminated the indica genome data [4]. A systematic naming system was proposed for these enzymes, where Os for *Oryza sativa* is followed by the chromosome number and then BGlu and a running number starting from the top of chromosome 1 and ending at the bottom of chromosome 12. For instance, *Os1BGlu1* is the first GH1 gene found starting from the top of chromosome 1 and *Os12BGlu38* is the last GH1 gene on chromosome 12. The NCBI database and later studies [31, 39] tend to drop the chromosome numbers for convenience, but we have retained these here, since they sometimes reflect clusters of nearby genes with similar functions [18, 26]. However, these are not all active genes, since *Os4BGlu15* and *Os4BGlu17* appeared to be two halves of a single gene on chromosome 4 into which a sequence including the entire *Os4BGlu16* gene is inserted, while *Os11BGlu35* and *Os11BGlu37* appeared to be pseudogenes in the japonica and indica genomes, respectively, although some studies showed expression of this gene [39]. Thus, there appear to be approximately 34–35 GH1 proteins produced in rice, indicating that they could have a wide range of functions.

The GH3 family is considerably smaller in rice than GH1 and is split into β-glucosidases on one side and β-xylosidases and α-L-arabinosidases on the other [40]. Most of the studies on GH3 in plants have been on the barley enzymes ExoI and ExoII, which are a type of β-glucosidases called β-D-glucan glucohydrolases, which cut glucose from the nonreducing end of β-glucan polysaccharides [12, 41, 42], as well as similar enzymes from corn [43]. However, GH3 β-glucosidases that release glucose from furastanol glycosides [44] and isoflavonoid glucosides [45] have also been described. So far, only two rice GH3 enzymes have been characterized, OsExoI and OsExoII, which had sequences and activities very similar to those of barley ExoI and ExoII [40, 46].

At present, only one plant β-glucosidase each has been characterized from GH5 and GH116 [47, 48]. The GH5BG β-glucosidase was a rice enzyme induced by stress. Both rice and Arabidopsis have four GH116 genes, but only one Arabidopsis β-glucosidase has been characterized for action on glucosylceramides, and designated AtGCD3, despite having a very low rate of hydrolysis of a fluorescent synthetic glucosylceramide [48].

POSSIBLE FUNCTIONS AND HOW THEY ARE STUDIED

Although many functions have been ascribed to plant β-glucosidases, which of these is appropriate to each of the rice β-glucosidases remains unclear. Functions postulated for plant β-glucosidases include release of glucose in cell wall recycling, recycling of glucolipids like glucosylceramide and sterol glucosides, activation of defense compounds and metabolic intermediates by removing a glucosyl blocking group, and release of phytohormones from inactive glucosyl conjugates [5]. Phytohormones that have been reported to be released by glucoconjugate hydrolysis include gibberellins (GA) [49], cytokinins [50], abscisic acid (ABA) [51], auxin (indole 3-acetic acid, IAA), the jasmonic acid derivative tuberonic acid (TA) [19], and salicylic acid (SA) [21]. Because of the appeal of signaling molecules, even the

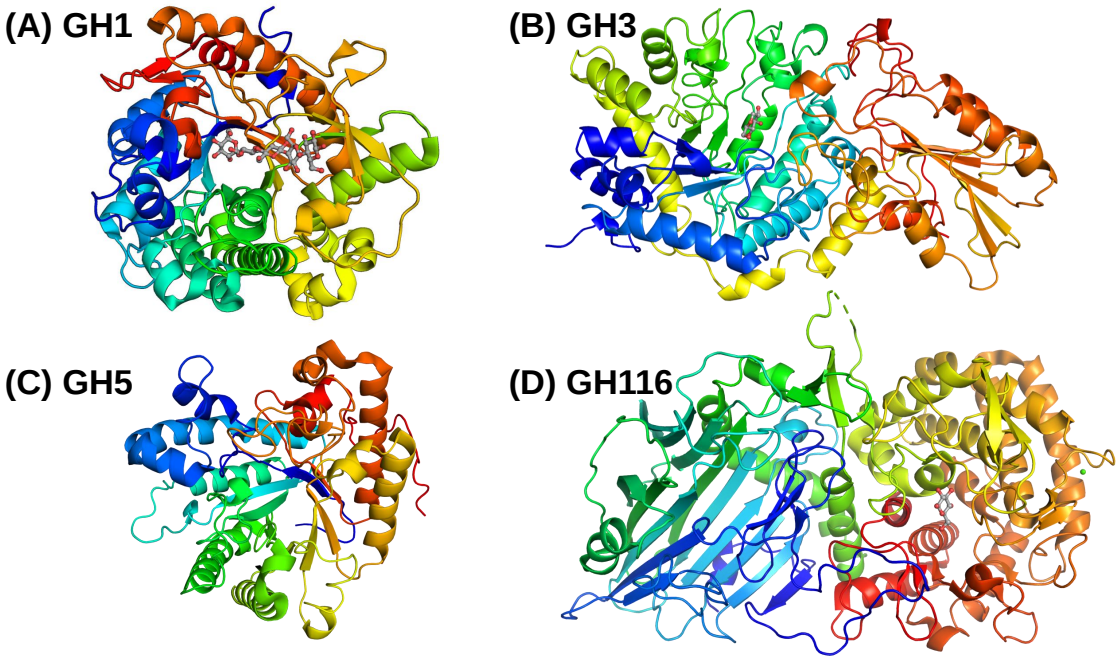

Fig. 1 Three-dimensional structures of β-glucosidase families found in rice. (A) GH1 rice Os3BGlu7 in complex with cellopentaose (PDB: 3F5K); (B) GH3 barley ExoI with retained glucose (PDB: 1EX1); (C) GH5 *Candida albicans* exo-1,3-β-glucanase (PDB: 1CZ1); (D) *Thermoanaerobacterium xylanolyticum* TxGH116 β-glucosidase in complex with glucose (PDB: 5BX5). Note that plant β-glucosidase structures are not yet available for GH5 and GH116.

weakest evidence for action on phytohormone conjugates is often touted as proof of this function, so one must be careful to evaluate the evidence, and the same could be said for other putative biological functions.

A variety of methodologies are used to elucidate rice enzyme function, each with its own strengths and weaknesses. Traditionally, scientists purified the protein from the plant based on its activity, as was done for rice cell wall β-glucosidase by Akiyama and colleagues [14]. This has the advantage of knowing a substrate for the enzyme, based on the substrate used to assay purification fractions, but it is difficult to get pure protein, particularly when there are many β-glucosidases with similar properties found in the plant [4]. An approach that we have used quite often is heterologous expression of β-glucosidases in microorganisms, followed by purification and kinetic analysis of hydrolysis of various glucosides and oligosaccharides. Aside from the hit and miss nature of this recombinant expression, this approach is critically dependent on the types of substrates that are tested, and a β-glucosidase that is very specific to a rare plant glycoside may be judged as inactive. Conversely, a slight activity toward a proposed substrate may be interpreted as evidence of function, if more rapidly hydrolyzed substrates are not tested [48]. One solution is a metabolomic approach, in which substrates and products are identified in plant extracts as compounds that decrease or increase upon enzyme treatment, based on LC-MS profiles. These compounds may not always be easy to identify, especially if one wishes to distinguish between isomers, which give the same mass. Another weakness to this approach is that generally all cell compartments are combined in these extracts, so we lose information on whether the enzyme is likely to have access to those substrates in the cell.

Complementary to the biochemical approach is a genetic or reverse genetic approach, in which the function is implied based on the phenotype of variant or knockout lines. In the past, this was largely done by generating sets of T-DNA [52] or transposon insertion lines [53], but these days CRISPR-Cas (Clustered regularly interspaced short palindromic repeats-CRISPR associated protein) gene editing technology allows the direct creation of mutations in the gene of interest [54]. When investigating a large family like GH1, some members of which may have redundant functions, we rarely found a clear function [29], but some others reported mild phenotypes for mutations in genes that had no obvious effects in our lines. So, it may depend on the rice line used for the mutations, as well as on the conditions tested and attention to details. The genetic approaches can be combined with metabolomics to identify the chemical phenotypes that may be clearer than physical appearance [55]. Further evidence can be provided by heterologous expression of the enzyme

of interest in the lines in which its gene is knocked out if this reverts the phenotype back to something more similar to wild type. Genome wide association (GWA) studies have also been used to identify genes that correlate to a particular phenotype [31]. These genetic approaches have the strength of associating the enzyme with a biological outcome, although they usually cannot identify the exact substrate for the enzyme and indirect effects can lead to misinterpretation in this respect.

Enzyme localization is another issue that affects enzyme function via its access to substrates and the effects of substrate hydrolysis. The tissue location and conditions of gene expression can be assessed from public microarray or RNA sequencing (RNA Seq) data or by conducting those or RT-PCR experiments in the lab [5, 39]. For instance, Cao et al [39] used RT-PCR to assess expression of Arabidopsis and rice GH1 genes in various tissues and under cold, osmotic and salt stress. The tissue expression of rice GH1 genes was similar to that derived from database analysis [5], validating the use of both approaches. Moreover, they showed that expression patterns for rice and Arabidopsis genes with closely related sequences were seldom similar, due in part to the apparent divergence of ancestral genes into clusters of genes more closely related to each other within each plant [39].

Gene expression studies may localize down to the cell for single cell studies, but not the localization of the protein within the cell, which is also critical to function. Localization in the cell can be assessed computationally by prediction of signal peptides for transport across membranes, such as by SignalP [56] and TargetP [57], or by programs that predict the final location of the protein, such as DeepLoc, etc [58]. However, some proteins targeted to the endoplasmic reticulum (ER) by a signal peptide have been shown to localize to the chloroplast [59], and once in the secretory pathway multiple destinations are possible, so experimental localization is advisable. Immunofluorescence localization may be most reliable, but the lack of appropriate antibodies and expertise, as well as signal issues, has made this approach less popular than fusing the protein of interest to fluorescent proteins like enhanced Green Fluorescent Protein (eGFP) [26, 28, 29, 34]. One must be careful in interpreting such studies, since fusion of a fluorescent tag in front of an N-terminal signal sequence may result in cytoplasmic localization of a secreted protein, while fusion of the fluorescent tag to the C-terminus can potentially mask C-terminal signals, such as some vacuolar localization signals [60]. Moreover, although chloroplast autofluorescence is measured in a red channel, there can be overlap with the GFP channel, leading to misinterpretation. The cells in which the localization of the protein is assessed also have effects, since protoplasts lack cell walls and cannot properly localize cell-wall-associated proteins, while many cells, including tobacco epithelial cells have large vacuoles pushing the cytoplasm, organelles and plasma membrane against the cell wall, making it hard to distinguish between cytoplasm, ER, Golgi, plasma membrane, and cell wall localization.

FUNCTIONS OF RICE β-GLUCOSIDASES

Given the caveats described above, we can consider the functions that have been ascribed to various β-glucosidases. The putative biological and biochemical functions are shown in Fig. 2, which indicates that sometimes only the biochemical substrate but not the biological effect is known but at other times only the biological effect is known but not the substrate or product.

Several rice β-glucosidases can hydrolyze cell-wall derived oligosaccharides, suggesting that they may play this role in cell wall recycling. For instance, Os3BGlu7 (originally designated rice BGlu1) has six subsites for binding β-1,4-linked glucosyl residues, as found in cello-oligosaccharides and can also hydrolyze β-1,3-linkages as may be found in mixed linkage β-glucan [15, 61]. Related β-glucosidases and β-mannosidases in the same sequence-based phylogenetic cluster, Os3BGlu8 and Os7BGlu26, have similar properties, although Os7BGlu26 prefers mannosides and mannooligosaccharides over glucosides and gluco-oligosaccharides [18]. These properties are like those of β-glucosidases/β-mannosidases purified from germinating barley, which are assumed to help hydrolyze cell wall glucans during endosperm breakdown and subsequent growth [41, 42]. Os3BGlu7 and Os3BGlu8 have been localized to the cell wall by cell wall proteomics and Os3BGlu8 appears to be linked to the membrane by a GPI lipid anchor [62]. Since all these enzymes also hydrolyze glycosides and have significant transglycosylation activities, roles other than oligosaccharide hydrolysis cannot be ruled out. It should also be noted that GH3 and GH5 enzymes have also been shown to break down oligosaccharides [42, 47, 63]. Indeed, barley GH3 enzymes ExoI and ExoII were identified as enzymes helping in barley germination when recycling of cell wall glucans takes place [42, 63].

Another GH1 enzyme that has strong activity on oligosaccharides is Os4BGlu12, which has the gene-derived protein sequence most similar to the sequence derived from cell wall associated β-glucosidase [4, 14]. Os4BGlu12 was shown to act on oligosaccharides released from cell wall by a stress-induced β-glucanase and to be induced by similar stresses [16]. However, it was later found to have similar activity on certain phytohormones [20, 21], as discussed below, so it is either multifunctional or its primary target substrate is unclear.

Os3BGlu6 is another enzyme that was found to have activity on β-1,3-linked gluco-oligosaccharides,

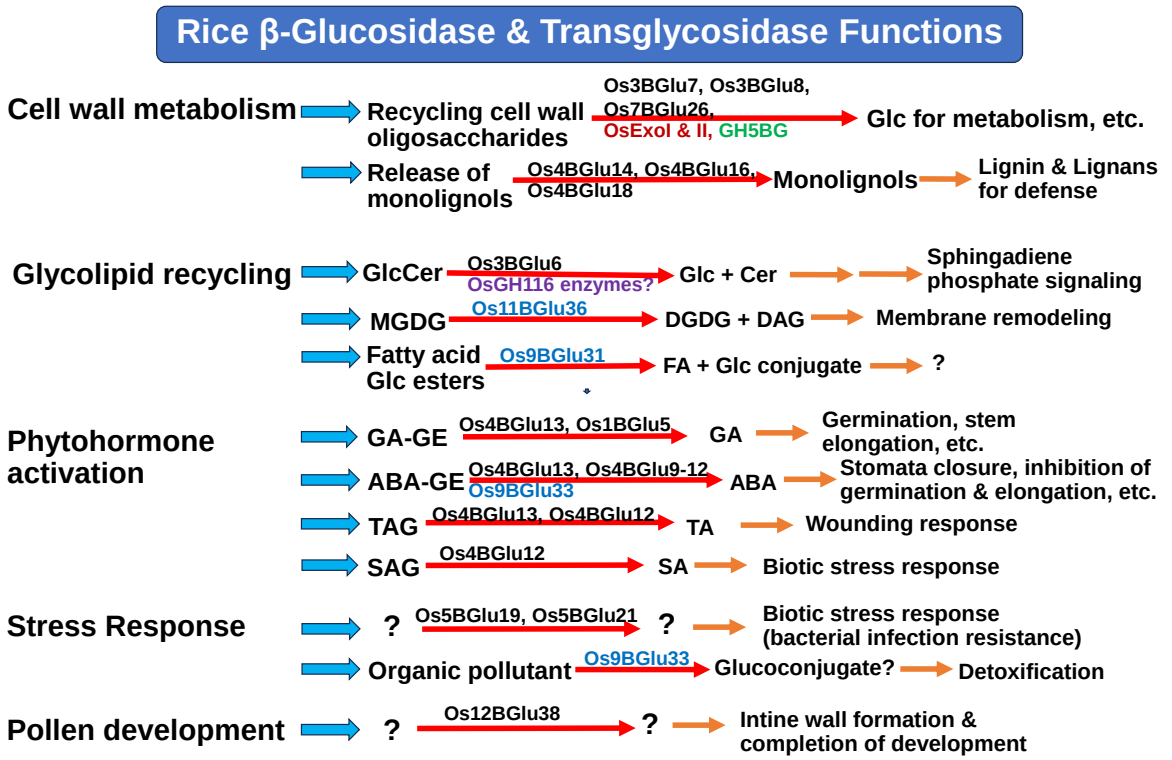

Fig. 2 Proposed biochemical and biological functions of rice beta-glucosidases. The general process to which the enzymes contribute is given on the left, followed by specific examples, including the enzymes with evidence of catalyzing the relevant reactions, the products and final effects. GH1 β-glucosidases are shown in black, GH1 transglycosidases in blue, GH3 enzymes in dark red, GH5 in green, and GH116 in purple. The abbreviations include ABA, abscisic acid; ABA-GE, ABA glucose ester; Cer, ceramide; DAG, diacylglyceride; DGDG, digalactosyl diacylglyceride; FA, fatty acid; GA, gibberellin; GA-GE, GA glucose ester; Glc, glucose; GlcCer, glucosylceramide; MGDG, monogalactosyl diacylglyceride; SA, salicylic acid; SAG, SA glucoside; TA, tuberonic acid; and TAG, TA glucoside.

but seemed to favor hydrolysis of hydrophobic glycosides and was later found to be a glucosylceramidase recycling membrane glycolipids [17, 35]. Os3BGlu6 was shown to account for nearly all the glucosylceramidase activity in certain rice tissues, based on its knockout mutation [35]. Before the identification of Os3BGlu6, Dai et al [48] had identified the GH116 enzyme from Arabidopsis, which they named AtGCD3 as a glucosylceramidase, based on the fact that it is related to the human glucosylceramidase GBA2 and could hydrolyze synthetic and plant-derived glucosylceramides. However, rice has similar GH116 genes, but the Os3BGlu6 knockout eliminated nearly all the detectable glucosylceramidase activity in rice leaf, stem, root, pistil and anther and decreased ceramide levels. In contrast, AtGCD3 knockout plant lines showed no disturbance in glucosylceramide levels, casting doubt on whether the plant GH116 enzyme's action on glucosylceramides is physiologically relevant. Moreover, AtGCD3 hydrolyzed synthetic C6-NDB-glucosylceramide with a k_{cat} of 0.008 s^{-1}, indicating a maximum turnover rate of less than 1 reaction per 2 min, which is too slow for biological relevance. In contrast, Os3BGlu6 rapidly hydrolyzed C6-NDB-glucosylceramide with a k_{cat} of 112 s^{-1} and a k_{cat}/K_m over 300 times higher than that of AtGCD3 and plant glucosylceramides with k_{cat}/K_m values 1.5–3 times higher than this [35]. This data is consistent with the previous gene expression data showing that Os3BGlu6 was induced by cold and osmotic stress, which may result in membrane remodeling [39]. Therefore, it seems likely that Os3BGlu6 is the main glucosylceramidase-type β-glucosidase in rice and its GH1 orthologues play this role in other plants.

Another aspect of cell wall construction is lignification, in which monolignols are polymerized by oxidative reactions to form the polyphenolic plastic that is lignin. The monolignols are stored in the plant cells as glucosides, although this does not appear to be necessary for the formation of lignin [64, 65]. A

β-glucosidase that hydrolyzes these monolignol glucosides was cloned from pine tree [66, 67], and its sequence served as the basis for identifying the genes for monolignol β-glucosidases in the Arabidopsis and rice genomes [4, 68, 69]. The Arabidopsis GH1 proteins AtBGLU45 and AtBGLU46 recombinantly expressed in *Pichia pastoris* showed the ability to hydrolyze monolignol glucosides, with AtBGLU45 showing highest activity on coniferin, although AtBGLU46 had higher activity toward salicin than monolignol glucosides [69]. Knock-out mutants of AtBGLU45 and AtBGLU46 have increased levels of the monolignol glucoside coniferin in stems, but little difference in lignin compared to wild-type Arabidopsis plants [65]. The most closely related rice enzymes are Os4BGlu14, Os4BGlu16 and Os4BGlu18 [4]. Recombinantly expressed Os4BGlu16 and Os4BGlu18 showed highest specificity constants for the hydrolysis of syringin and coniferin, with lower specificity for *p*-coumarol glucoside and non-monolignol glucosides [26]. Although Os4BGlu14 has its catalytic acid/base replaced with glutamine, heterologous expression of Os4BGlu14, Os4BGlu16 and Os4BGlu18 in the *atbglu45-2* knockout mutant Arabidopsis plants caused a decrease in the levels of coniferin, syringin, and *p*-coumarol glucoside, supporting the ability of all 3 enzymes to break down monolignol glucosides in the plant [27]. The action of Os4BGlu14 is further supported by the study of Ren et al [33] that showed its overexpression in rice plants increased lignin content and oxidative stress resistance, although it led to longer thinner grains that were less tolerant of accelerated aging. Similarly, rice with a frameshift mutation in the *Os4BGlu18* gene were found to have higher monolignol glucoside and lower monolignol levels than wildtype, although changes in expression of other monolignol metabolic enzyme genes may have influenced this [36]. This evidence supports the roles of Os4BGlu14, Os4BGlu16 and Os4BGlu18 in monolignol release, which might serve to increase lignification in abiotic or biotic stress responses, although monolignol glucosides are not necessary for normal lignification in development.

Precise roles for rice β-glucosidases in defense are yet to be elucidated. While maize, wheat and rye produce benzoxinoid glucosides and sorghum produces the cyanogenic glucoside dhurrin, which are stored in the vacuole and hydrolyzed by chloroplast-localized β-glucosidases to release their respective toxic compounds upon chewing of the plant tissue [70, 71], rice lacks these compounds and β-glucosidases orthologous to the chloroplastic enzymes [4]. Nonetheless, some GH1 β-glucosidases have been implicated in the response to biotic stress based on their expression patterns during such stress or the effect of their knockout mutations. For instance, Li et al [30] demonstrated that plants with knockout of the genes for Os5BGlu19 and Os5BGlu23 led to larger lesions and higher bacterial populations in the lesions in bacterial leaf streak caused by *Xanthomonas oryzae* pv. *oryzicola*.

The phytohormones jasmonic acid (JA) and salicylic acid (SA) are also involved in defense against herbivores and their derivatives include glucosides that may be activated by β-glucosidases. JA is activated by amidation with isoleucine [72], and it is generally hydroxylated at the 11 or 12 position to produce other signaling molecules that can be glucosylated. Tuberonic acid (12-O-jasmonic acid, TA) can be further modified by sulfation or glucosylation and each of these derivatives show different bioactivity [19, 73, 74]. Os4BGlu13 was purified from rice plants as an enzyme hydrolyzing tuberonic acid glucoside (TAG) and therefore named TAG glucosidase 1 (TAGG1) [19]. A year later, a second isoenzyme with this activity designated TAGG2 was purified and found to be Os4BGlu12 [20], which had previously been identified as a wounding-induced enzyme that hydrolyzes the oligosaccharide products of a wounding induced endoglucanase [4, 16]. Os4BGlu12 was subsequently found to have higher activity on salicylic acid glucoside (SAG) than TAG and oligosaccharides, suggesting its function may be to activate salicylic acid from its SAG storage form during plant defense responses [21].

One of the first set of phytohormone glucoconjugates to be identified as hydrolyzed by β-glucosidases in rice were gibberellins (gibberellic acids, GA) [49]. GAs are generally considered to promote germination of seeds and stem elongation, but they act in a wide range of processes in plants [75]. We tried two approaches to identify GA β-glucosidases, screening of recombinant enzymes and purification from rice plants [22, 24]. Comparison of the activities of Os3BGlu6, Os3BGlu7, Os4BGlu12, Os4BGlu18 and Os9BGlu31 showed that Os3BGlu6 had the highest activity toward GA_4 glucose ester (GA_4-GE) [22]. The later identification of Os3BGlu6 as a much more efficient and specific glucosylceramidase [35] indicated that GA_4-GE hydrolysis may be simply a result of the hydrophobic nature of GAs. Nonetheless, this work did at least show that glucose ester hydrolysis is qualitatively similar to that of simple glucosides, including formation of a covalent enzyme intermediate and dependence on a catalytic base to release glucose [22]. Subsequently Os4BGlu13 was purified from rice plants as an enzyme hydrolyzing GA_4-GE and had an approximately 25-fold higher specificity constant (k_{cat}/K_m) for GA_4-GE than Os3BGlu6, although Os4BGlu13's specificity constant for TAG was about 80% higher than for GA_4-GE. It is possible that Os4BGlu13 helps equilibrate the levels of multiple phytohormones and their glucoconjugates, but the physiological significance remains unclear.

Another rice β-glucosidase that has been suggested to act on GA is Os1BGlu5 [31]. Os1BGlu5 promoter variants were found to have the highest correlation

with a high chlorophyll a variable to maximum fluorescence ratio (F_v/F_m), which reflects photosystem II quantum yield, in rice accessions. The F_v/F_m ratio also showed a significant correlation to Os1BGlu5 expression levels. They proposed that this was an indirect effect of GA-GE hydrolysis, but unfortunately, they only reported changes in GA_3 levels, while GA_1 and GA_4 are the main bioactive GAs in rice [76, 77].

Another phytohormone glucose ester that has generated a lot of interest among those studying rice β-glucosidases is abscisic acid (ABA) glucose ester (ABA-GE). ABA promotes seed dormancy and stomata closure during water stress, among many other functions in plants [51, 78]. ABA-GE was shown to be hydrolyzed by β-glucosidase activity in barley seedling leaves and this activity was shown to be induced by salt stress [78]. The main inducement for this interest was the work by Lee et al [51] in which mutations in the gene for an Arabidopsis ER-localized GH1 β-glucosidase were shown to cause an ABA-deficient phenotype. They showed that its aggregation during water stress could lead to activation and release of ABA. Later, they showed another Arabidopsis GH1 enzyme, AtBGLU10, located in the vacuole had similar effects [79]. Since those papers, many investigators have ascribed ABA-GE hydrolysis functions to their enzymes.

Ren et al [32] studied Os4BGlu10, Os6BGlu24 and Os9BGlu33 knockout lines in rice variety Zhonghua 11, as well as the expression of the genes in wild-type plants and observed some phenotypes that could be related to ABA signaling. All the mutant lines had longer roots but poorer drought tolerance than wild type, with the *os9bglu33* knockout line showing the lowest tolerance. *Os9BGlu33* expression was induced by ABA, the auxin IAA, and osmotic stress, while *Os4BGlu10* expression was decreased by ABA but increased by osmotic stress, and *Os6BGlu24* expression exhibited variable responses to ABA and IAA and was reduced by osmotic stress. The authors concluded that Os9BGlu33 was the most likely to act to release ABA from ABA-GE, since the responses of its knockout line were consistent with having less ABA. This is consistent with Os9BGlu33 being closely related to Os9BGlu31 transglucosidase, which can move glucosyl groups between carboxylated phytohormones including ABA and other carboxylated compounds [23].

Wang et al [34] noted that *os3bglu6* knockout lines show a dwarf phenotype and drought sensitivity, which they interpreted as indicating its involvement in ABA-GE metabolism. They noted that ABA levels appeared to be lower in the *os3bglu6* knockout line and saw no difference in GA_3 levels but did not measure the major bioactive GAs in rice, GA_1 and GA_4, and ABA-GE. An overexpression line had about 20% higher ability to hydrolyze ABA-GE in leaf extracts compared to wild-type leaf extracts. They also reported that the knockout line had a significantly larger stomata aperture with and without drought stress and that an overexpression line had smaller stomata apertures than wildtype. Although these data are consistent with a role in ABA signaling, Koga et al [35] convincingly showed that Os3BGlu6 is a glucosylceramidase and showed no difference in ABA levels between wild type and two independent *os3bglu6* knockout lines. They noted that the ABA signaling pathway involves phosphorylation of sphingadienine, which is derived from ceramide released from glucosylceramide by glucosylceramidase, by sphingosine kinase to produce sphingadienine-1-phosphate. So, the knockout of Os3BGlu6 can lead to decreased ABA signaling in knockout lines and increased signaling in overexpression lines via its effects on sphingadienine levels.

To address the issue of which rice GH1 enzymes may be involved in ABA-GE release, we screened a set of recombinant enzymes in the lab, including Os1BGlu4, Os3BGlu6, Os3BGlu7, Os4BGlu12, Os4BGlu13, Os4BGlu18, Os7BGlu26, and Os9BGlu31 [28]. All these enzymes could hydrolyze ABA-GE to some extent, but Os4BGlu13 and Os4BGlu12 showed the highest specific activity, which was about 25–40% higher than that of Os1BGlu4, Os3BGlu7 and Os4BGlu18 and 4 times higher than that of Os3BGlu6. Os4BGlu13 had the highest specificity constant (k_{cat}/K_m) of 20 $mM^{-1}s^{-1}$ for ABA-GE, which is higher than its specificity constants for TAG and GA_4-GE. Since the genes for Os4BGlu12 and Os4BGlu13 exist in a cluster with those for the closely related enzymes O4BGlu9, Os4BGlu10 and Os4BGlu11, which were not successfully expressed in *E. coli* or *P. pastoris*, all 5 genes were heterologously expressed in *Arabidopsis thaliana*. Extracts of plants from 2 overexpression lines for each enzyme showed increased hydrolysis of ABA-GE and GA_4-GE, and the seedlings displayed increased tolerance to osmotic and salt stress. This suggests that Os4BGlu12 and its closely related homologues may regulate levels of ABA in rice, possibly in addition to their actions on other phytohormones.

Various rice genes have been identified based on increased or decreased resistance to abiotic stress in mutant lines. Seo et al [36] identified a line with a frameshift in the Os4BGlu18 gene, which was less sensitive to salt stress than wild-type rice. This would seem to indicate that Os4BGlu18 is involved in making rice more sensitive to salt stress, but many lignin metabolic genes were expressed differently than wild-type in this line and lignin levels were actually higher than wild-type. This led to the suggestion that the higher lignin and monolignol levels may aid in salt stress resistance. Expression analysis in salt-resistant rice cultivar Pokkali, suggested that Os3BGlu7 could be involved in rapid adaptation to salt-stress, since its gene was upregulated at 15 min and 3 h in Pokkali rice, but not in salt-sensitive rice [80]. At longer

time points, its expression rapidly decreased, however, and the exact role it would play in salt adaptation is unclear. Another stress that was shown to disturb the expression of GH1 genes is exposure to the antibiotic ciprofloxacin, an organic pollutant found in farm soils [37]. Interestingly, Os9BGlu33, which has not been characterized but has high sequence similarity with Os9BGlu31 transglucosidase, was the most highly induced GH1 gene at low ciprofloxacin concentrations. This suggests that the plant may use glycosylation via a transglucosidase to detoxify exogenous organic compounds, although known GH1 β-glucosidases, like Os4BGlu12 were induced at higher concentrations.

RELATED ACTIVITIES IN β-GLUCOSIDASE FAMILIES

Although GH1 predominantly contains β-glucosidases, some related activities have been identified. For instance, Os6BGlu26 was identified to be predominantly a β-mannosidase [18], while Os9BGlu31 is a transglucosidase, which mainly moves glucose from one organic acid or phenolic alcohol to another, at least *in vitro* [23]. Knockout of Os9BGlu31 in rice results in an increase in fatty acid glucosyl esters, suggesting that these compounds act as donor substrates *in vivo*, although their biological function remains unknown [55]. As noted above, Os9BGlu32 and Os9BGlu33 are closely related to Os9BGlu31, so Os9BGlu32 and Os9BGlu33 may also be transglycosidases. These 3 fall into the sequence-based phylogenetic cluster At/Os6 with AtBGLU10 [4], which was implicated in transglycosylation of anthocyanins in the vacuole, along with Os1BGlu2, Os1BGlu3, Os1BGlu5, and Os5BGlu19-23. So, all these enzymes may potentially transfer glucosyl moieties to other acceptors in rice, rather than simply hydrolyzing their substrates. Os11BGlu36 is also thought to be a galactolipid-galactolipid galactosyltransferase (GGGT), based on its similarity to the Arabidopsis GGGT Sensitive to freezing 2 (SFR2). Other activities are also possible, so GH1 enzymes cannot all be assumed to be β-glucosidases, although they tend to be given the short name BGLU or BGlu.

APPLICATIONS OF RICE β-GLUCOSIDASES

Potential applications of rice β-glucosidases can be in the use of their genes to improve plants or in biochemical applications of the enzymes themselves. In terms of the genes, the observations made of improved stress response on knockout or overexpression of rice β-glucosidases suggest that selecting rice accessions with the appropriate increase or decrease in expression of these genes could improve those properties. Moreover, with current CRISPR-Cas technology, it is convenient to modify the promoters of the genes to achieve these objectives to obtain rice with no foreign genes or other indications of genetic modification [54]. Another use that has been proposed is the use of the promoters themselves. Since the *Os12BGlu38* gene expression is nearly specific to late stage developing pollen, the *Os12BGlu38* promoter was used to drive expression of a toxic cysteine protease, in order to produce male-sterile rice for hybrid rice production. Later it was reported that Os12BGlu38 is critical for pollen development [29], so knockout of the gene itself would also be effective in generating male sterile rice for breeding hybrid rice.

Although β-glucosidases in general can be used for various industrial applications such as biomass conversion to glucose as a biorefinery feedstock and treatment of animal feed for improved nutrition, generalist microbial enzymes are usually used for these applications, rather than the more specialized rice enzymes. So far, production of transglycosylation products has been a more promising application of rice enzymes. For instance, Os9BGlu31 has been used to make phytohormone glycoconjugates that were used for screening and testing phytohormone β-glucosidases [28]. Upscaling of Os9BGlu31 glycoconjugate production will allow the production of sufficient amounts of these glucoconjugates for bioactivity testing in both plants and animal cells. Similarly, the GH3 enzyme OsExoI has been used to transglycosylate simple alcohols to make various glycosides, with the advantage that cellobiose, which can be derived from waste biomass, can be used as a glucosyl donor [46]. As we learn more about the specificities and abilities of rice β-glucosidases and transglucosidases, new applications will arise if we find ways to take advantage of their specificities and abilities.

CONCLUSIONS AND FUTURE PERSPECTIVES

Potential rice β-glucosidases have been identified in GH families GH1, GH3, GH5 and GH116, but only a few GH3 and one GH5 rice enzyme have been characterized, so most of our knowledge is about GH1 enzymes, as summarized in Fig. 2. Of the 34 or 35 potentially active rice GH1 enzymes, only 12 have been produced as purified recombinant enzymes, despite efforts to express, purify and characterize them all. The activities of several other enzymes were implied based on activities in crude extracts or changes in glucoside or aglycone levels upon heterologous expression in plants. Among the remainder, the action of Os11BGlu36 as a GGGT can be implied from its relationship to the Arabidopsis GGGT SFR2, but for most of the others, relationships with characterized enzymes are unclear. As we noted several years ago [5], development of a universal system to express, purify and characterize plant enzymes would be very useful and allow greater understanding for rice breeding and enzyme applications.

Nonetheless, much has been learned in the last 8 years, largely from genetic studies. As several new groups have moved into exploring rice GH1

genes, use of new cultivars that may show more obvious phenotypes and exploration of more conditions gives a greater chance of identifying biological effects. Further exploration at the chemical level via more metabolomic studies will be useful in identifying potential substrates and products of these enzymes in planta. The advances in CRISPR-Cas technology are allowing genes to be specifically modified to achieve both specific changes in promoters to drive expression and specific changes in encoded protein sequences [54]. This makes it an exciting time to explore the functions of enzyme families in rice, including β-glucosidases, although the knowledge gained may not have immediate application to generating new products. Nonetheless, this knowledge will eventually lead to improved rice varieties and biochemical applications of the enzymes, thereby contributing to increased rice production and innovation.

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