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# Purification, Cloning and Functional Characterization of an Endogenous Beta-glucuronidase in *Arabidopsis Thaliana*

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**Beta-glucuronidase (GUS) activities have been extensively characterized in bacteria, fungi, and animals, and the bacterial enzyme GUSA from *Escherichia coli* is commonly used as a reporter for gene expression studies in plants. Although endogenous GUS activity has been observed in plants, the nature and function of the enzymes involved remain elusive. Here we report on tissue-specific localization, partial purification and identification of AtGUS2, a GUS active under acidic conditions from *Arabidopsis thaliana*. This enzyme belongs to the GH79 family in the Carbohydrate-Active Enzymes database, which also includes mammalian heparanases that degrade the carbohydrate moieties of cell surface proteoglycans, and fungal enzymes active on arabinogalactan proteins (AGPs). We characterized a knockout insertion line (*atgus2-1*) and transgenic lines overexpressing *AtGUS2* (*$Pro_{35S}$:AtGUS2*). Endogenous GUS activity assayed histochemically and biochemically was absent in *atgus2-1* tissues and four times higher in *$Pro_{35S}$:AtGUS2* lines. AGPs purified from *atgus2-1* and *$Pro_{35S}$:AtGUS2* seedlings showed higher and markedly lower glucuronic acid content, respectively. Our results suggest that endogenous GUS activity influences the sugar composition of the complex polysaccharide chains of AGPs. We also show that transgenics display hypocotyl and root growth defects compared to wild-type plants. Hypocotyl and root lengths are increased in *$Pro_{35S}$:AtGUS2* seedlings, whereas hypocoyl length is reduced in atgus2-1 seedlings. These data are consistent with a role for the carbohydrate moieties of AGPs in cell growth.**

**Keywords:** Arabidopsis— Arabinogalactan protein — Beta-glucuronidase — Family 79 glycoside hydrolase — Hypocotyl and root growth.

Abbreviations: AGPs, arabinogalactan proteins; ConA, concanavalin A; GH, glycoside hydrolase; GlcA, glucuronic acid; GUS, beta-glucuronidase; MALDI-TOF MS, matrix assisted laser-desorption ionization time-of-flight mass spectrometry; *p*NPGlcA, *p*-nitrophenyl-β-D-glucuronide; X-GlcA, 5-bromo-4-chloro-3-β-indolyl-glucuronide.

## Introduction

Glycoside hydrolases (GHs) cleave glycoside bonds between carbohydrates moieties or between a carbohydrate and a non-carbohydrate compound. They are ubiquitous in eukaryotes, archaea, and bacteria and are classified into several families based on amino acid sequence similarities (Henrissat 1991). In plants, GHs are involved in a number of fundamental processes, including the remodeling of cell-wall polysaccharides (Minic and Jouanin 2006), seed germination (Minic et al. 2006), chemical defenses (Barth and Jander 2006), lignification (Escamilla-Trevino et al. 2006), and regulation of active phytohormone levels (Lee et al. 2006).

Beta-glucuronidase (GUS) enzymes (EC 3.2.1.31) catalyzing the hydrolysis of *O*-linked glucuronic acids to aglycones (glucuronides) or glycosaminoglycans have been classified as GH1, GH2 and GH79. Found in the GH1 family, klotho is a type I membrane protein from mammals that hydrolyzes steroid beta-glucuronides (Tohyama et al. 2004). The GH2 family contains a large number of GUSs from mammals, bacteria, and fungi. The substrates of mammalian GUSs in this family are glucuronate-containing glycosaminoglycans (Ray et al. 1999) and flavonoid glucuronides (O'Leary et al. 2001). Bacteria and fungi have GH2 GUSs that hydrolyze glucuronides, thus, providing access to glucuronic acid as a carbon source (Wenzl et al. 2005). The GH79 family contains mammalian GUS enzymes (heparanases) that degrade heparan sulfate proteoglycans located on the cell surface (Sasaki et al. 2004), and GUSs from fungi that hydrolyze beta-glucuronosyl residues of AGPs (Konishi et al. 2008).

Several studies have reported endogenous GUS activities in plants under acidic conditions (Sood 1980, Plegt and Bino 1989, Hu et al. 1990, Alwen et al. 1992, Hodal et al. 1992, Sudan et al. 2006), although little is known about the molecular origin of such activities. To date, the Arabidopsis enzymes that belong to the GH1, GH2 and GH79 families (48, 2 and 3, respectively) have not been shown to exhibit

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GUS activity. Endogenous GUS activity is found in pollen and in the tapetal and sporogenous cells of anthers from potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*) (Plegt and Bino 1989), and in germinating pollen grains from *Portulaca grandiflora* (Sood 1980). The authors of these observations suggested that the corresponding hydrolytic enzymes were involved in the polysaccharide metabolism required for pollen tube growth, such as the hydrolysis of acidic carbohydrates or glucuronic acid-rich AGPs. Hu et al. (1990) detected GUS activity in 52 plant species, including gymnosperms and all the key groups of angiosperms. This activity was predominantly found in fruit walls, seed coats and endosperms or embryos. Vegetative organs of tobacco, potato, wheat and radish also exhibit GUS activity (Alwen et al. 1992). Physiologically, endogenous GUS activity is associated with cell elongation in Arabidopsis, maize (*Zea mays*), rice (*Oryza sativa*) and tobacco (*N. tabacum*), and is enhanced with the growth hormones 6-benzylaminopurine and gibberellic acid (Sudan et al. 2006). Moreover, an inhibitor of GUS (saccharo-1–4-lactone) was shown to retard stem, root and root hair elongation in tobacco seedlings (Sudan et al. 2006). Biochemical approaches showed two GUSs purified from rye leaves (*Secale cereale*) and Chinese skullcap (*Scutellaria baicalensis Georgi*), respectively (Schulz and Weissenbock 1987, Morimoto et al. 1995). These enzymes catalyze the hydrolysis of flavone glucuronides in vitro, and the cloned gene from Chinese skullcap encodes a protein (SbGUS) that belongs to the GH79 family (Sasaki et al. 2000).

The carbohydrate moieties of arabinogalactan proteins (AGPs) contain glucuronic acid (GlcA) residues (Fincher et al. 1983) and therefore represent a potential substrate for plant endogenous GUSs. Here we report the in situ localization of AtGUS2, an enzyme displaying GUS activity under acidic conditions in Arabidopsis. AtGUS2 was partially purified from stem tissues and the corresponding gene was subsequently cloned. AtGUS2 belongs to the GH79 family, known to contain two other similar enzymes in Arabidopsis (Woo et al. 2007). A knockout mutant and lines overexpressing *AtGUS2* showed modifications in the polysaccharide chains of AGPs. Using gas chromatography analysis, the estimated terminal-GlcA content in AGPs purified from these lines was found to be negatively correlated with the endogenous AtGUS2 activity level. Interestingly, AtGUS2 activity levels were positively correlated with hypocotyl and root growth in all transgenics, suggesting that GUS activity could contribute to the biological function of AGPs by modulating their GlcA content.

## Results

### *Endogenous GUS activity in Arabidopsis*

In-planta GUS activity was observed in wild-type Arabidopsis using a histochemical GUS assay in which X-GlcA in a pH 5.0 buffer was provided as the substrate. Intrinsic GUS activity was observed in all organs of the plant, including seeds and young seedlings (Fig. 1). At early stages of development, GUS activity was largely restricted to the hypocotyl, the crown, and the vascular system of the cotyledons (Fig. 1A–C). Trichomes also displayed GUS activity (Fig. 1D). No GUS activity was detected in the roots of seedlings grown under sterile conditions; however, roots from soil-grown plants had high GUS activity (Fig. 1E). Later in development (5-week-old plants), high

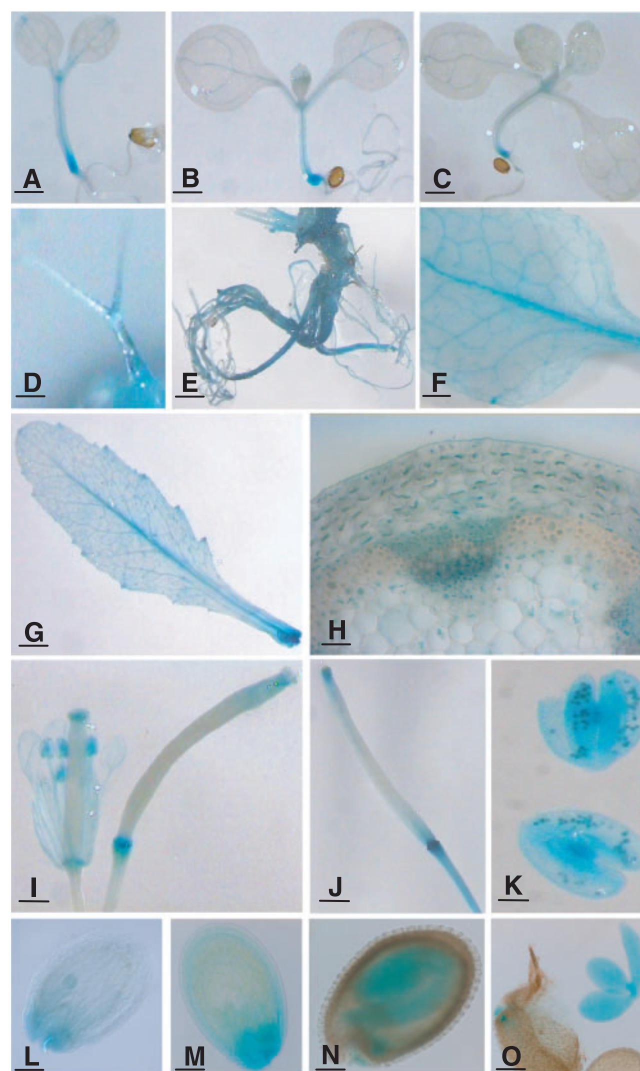


**Fig. 1** Endogenous GUS activity in wild-type Arabidopsis. Endogenous activity was detected in 5-d (A), 8-d (B), and 14-d-old seedlings (C), trichomes (D), roots from 3-week-old soil-grown plants (E), rosette and cauline leaves ([F] and [G]), stems from 5-week-old plants ([H] transverse section), flowers and siliques ([I] and [J]), immature pollen in anthers (K), fertilized ovules (L) and embryos at various stages ([M], [N], [O]). Bars = 0.1 cm except for (L) to (O) where they = 100 µm, and for (D), (H) and (K) where they = 50 µm.

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level of GUS activity was observed in the vascular system of rosette and cauline leaves (Fig. 1F, G). This activity was detected in young xylem vessels and phloem elements on transverse sections of stems as well as in tissues undergoing secondary cell wall thickening and lignification (i.e., mature xylem and interfascicular fibers) (Fig. 1H). Weak activity was detected in some parenchyma cells and in the epidermis (Fig. 1H). In flowers, GUS activity was detected in the sepals, stigmatal papillae, anthers, and pollen (Fig. 1I, K). The dehiscence zone in siliques (Fig. 1I, J), the micropyle-chalaza zone of fertilized ovules (6 d post-anthesis, Fig. 1L) and the embryo at various developmental stages (Fig. 1M, N, O) also displayed GUS activity. Endogenous GUS activity was detected only in the cotyledon hydatodes and flower stigmata after 12 h of incubation at pH 7.0.

*Purification of the enzyme displaying GUS activity*

The enzyme with GUS activity was partially purified from a crude protein extract of Arabidopsis stem tissue. Enzyme activity was monitored with *p*-nitrophenyl-β-D-glucuronide (*p*NPGlcA) as a substrate and tested in acidic conditions. The purification protocol involved three column chromatography steps: lectin concanavalin A (ConA) chromatography, cation-exchange chromatography and gel filtration. A single peak of GUS activity was eluted from the lectin ConA Sepharose column. The corresponding fractions were then subjected to cation-exchange chromatography on a CM-Sepharose column. One peak of activity corresponding to proteins eluted with 250 mM NaCl was detected. To increase specific GUS activity, the corresponding fractions were subjected to a final purification step (exclusion chromatography on Superdex 200) and a single peak of activity was eluted. Based on the elution of known markers, this peak corresponds to proteins of ~55 kDa. Enzyme yields and purification factors obtained after each purification step are summarized in Table 1. An optimum pH of 5.0 and temperature of 55°C were determined for this partially purified enzyme (Fig. 2A). This final extract exhibited no activity above the background when *p*-nitrophenyl-α-D-glucuronide was used as a substrate (data not shown), indicating a specificity of the partially purified enzyme for beta-linked glucuronosyl residues.

*Identification of AtGUS2 and amino acid sequence analysis*

The proteins isolated after the final gel filtration purification step were analyzed by SDS-PAGE. The fractions with GUS activity were pooled, concentrated and loaded on a 10% polyacrylamide gel. Staining with colloidal Coomassie blue G250 revealed a large band of several copurified proteins with apparent molecular masses around 55 kDa (Fig. 2B). This band was analyzed by MALDI-TOF MS after tryptic digestion, and five co-purified proteins were identified: At2g42800, At5g07830, At3g54400, At1g33590 and At1g33600. Only At5g07830 was found to have a GH domain typical of family GH79 (Pfam profile PF03662). At5g07830 was renamed AtGUS2 according to the nomenclature proposed by Woo et al. (2007).

We investigated the molecular properties and positions of putative catalytic amino acid residues in AtGUS2. First, the analysis of its sequence predicted three *N*-glycosylation sites (Fig. 3), accounting for the affinity of AtGUS2 for the ConA Sepharose matrix used in the first step of purification (Minic et al. 2007). Next, the AtGUS2 sequence was aligned with SbGUS, the only GH79 plant enzyme for which GUS activity has been reported. These sequences were 38% identical and alignment showed the conservation of crucial residues for GUS activity (Sasaki et al. 2000). In the active site, the acid-base catalyst ($Glu^{201}$ of AtGUS2 and $Glu^{212}$ of SbGUS) and the catalytic nucleophile ($Glu^{320}$ of AtGUS2 and $Glu^{329}$ of SbGUS) are conserved, as well as a tyrosine residue ($Tyr^{273}$ of AtGUS2 and $Tyr^{281}$ of SbGUS) which is implicated in GUS activity. In addition, an ATP/GTP-binding site and a leucine zipper motif, for which functions are unknown, are also conserved. PSORT and TargetP/SignalP programs predict a cleavage site between $Ala^{24}$ and $Gln^{25}$ in the protein sequence, suggesting that AtGUS2 is targeted to the secretory pathway.

*Characterization of an AtGUS2 mutant and* $Pro_{35S}$:AtGUS2 *overexpressing lines*

A reverse genetic approach was used to validate the role of AtGUS2 in the endogenous GUS activity observed. The T-DNA insertion line SALK_040732 for the *At5g07830* gene

**Table 1** Enzyme yields and purification factors

| Step of purification | Yield | | Specific activity | Recovery[a] | Purification factor[a] |
|---|---|---|---|---|---|
| | Protein | Activity | | | |
| | mg | nmol min–1 | nmol min–1 mg–1 | % | fold |
| Crude homogenate | 25 | 13 | 0.52 | 100 | 1 |
| Con A Sepharose | 2.5 | 10 | 4.00 | 77 | 7.7 |
| CM-Sepharose | 0.24 | 1.26 | 5.25 | 9.7 | 10.1 |
| Superdex 200 | 0.03 | 0.69 | 23.00 | 5.3 | 44.2 |

[a] Recoveries are expressed as percentage of initial activity and purification factors are calculated on the basis of specific activities.

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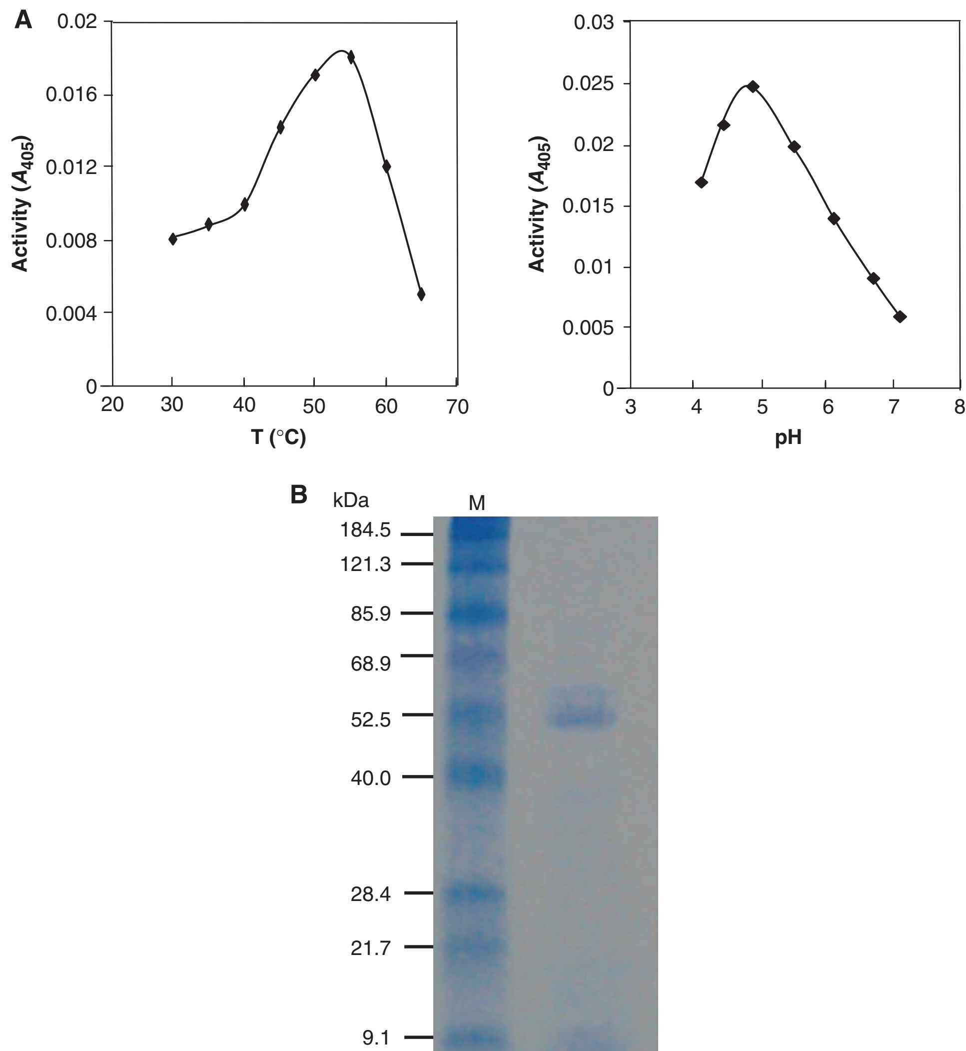


**Fig. 2** Properties of partially purified GUS enzyme from Arabidopsis. (A) Temperature (left) and pH (right) dependence of GUS activity. (B) SDS-PAGE of the partially purified enzyme (3 μg) displaying GUS activity. Lane M, marker proteins (the sizes are indicated).

was analyzed (Fig. 4A). This line (named *atgus2-1*) has a T-DNA inserted in the fourth exon of the coding sequence, resulting in the absence of *AtGUS2* transcripts in lines homozygous for the insertion (Fig. 4B). In addition, transgenic lines overexpressing *AtGUS2* under the control of a 35S cauliflower mosaic virus constitutive promoter ($Pro_{35S}$:*AtGUS2*) were generated. Overexpression of *AtGUS2* transcripts in $Pro_{35S}$:*AtGUS2* transformants was confirmed by semi-quantitative RT-PCR (Fig. 4B). We compared GUS activity in stem protein extracts from *atgus2-1*, $Pro_{35S}$:*AtGUS2*, and wild-type plants, following fractionation after a single cation exchange chromatography step. No activity was detected in any of the fractions obtained from the *atgus2-1* protein extract. However, the activity detected in fractions corresponding to proteins eluted with 250 mM NaCl was five times higher for the $Pro_{35S}$:*AtGUS2* extract compared to the wild type (Fig. 4C). GUS activity was also assayed histochemically under acidic conditions (pH 5.0) and for 12 h at 37°C. As compared to the wild type (Fig. 1), no GUS activity was detected in any organs of *atgus2-1* (data for flowers are shown in Fig. 4D as an example). In contrast, $Pro_{35S}$:*AtGUS2* plants accumulated the GUS staining earlier and in a more constitutive, less-localized pattern (Fig. 4E). All together, these data confirm that GUS activity detected in our purification procedure and histochemical assay is originating from AtGUS2.

### *Analysis of AGPs from* atgus2-1 *and* $Pro_{35S}$:AtGUS2 *lines*

To investigate whether altering AtGUS2 activity level in plants could modify AGPs, we purified AGPs from *atgus2-1*, $Pro_{35S}$:*AtGUS2* and wild-type seedlings grown

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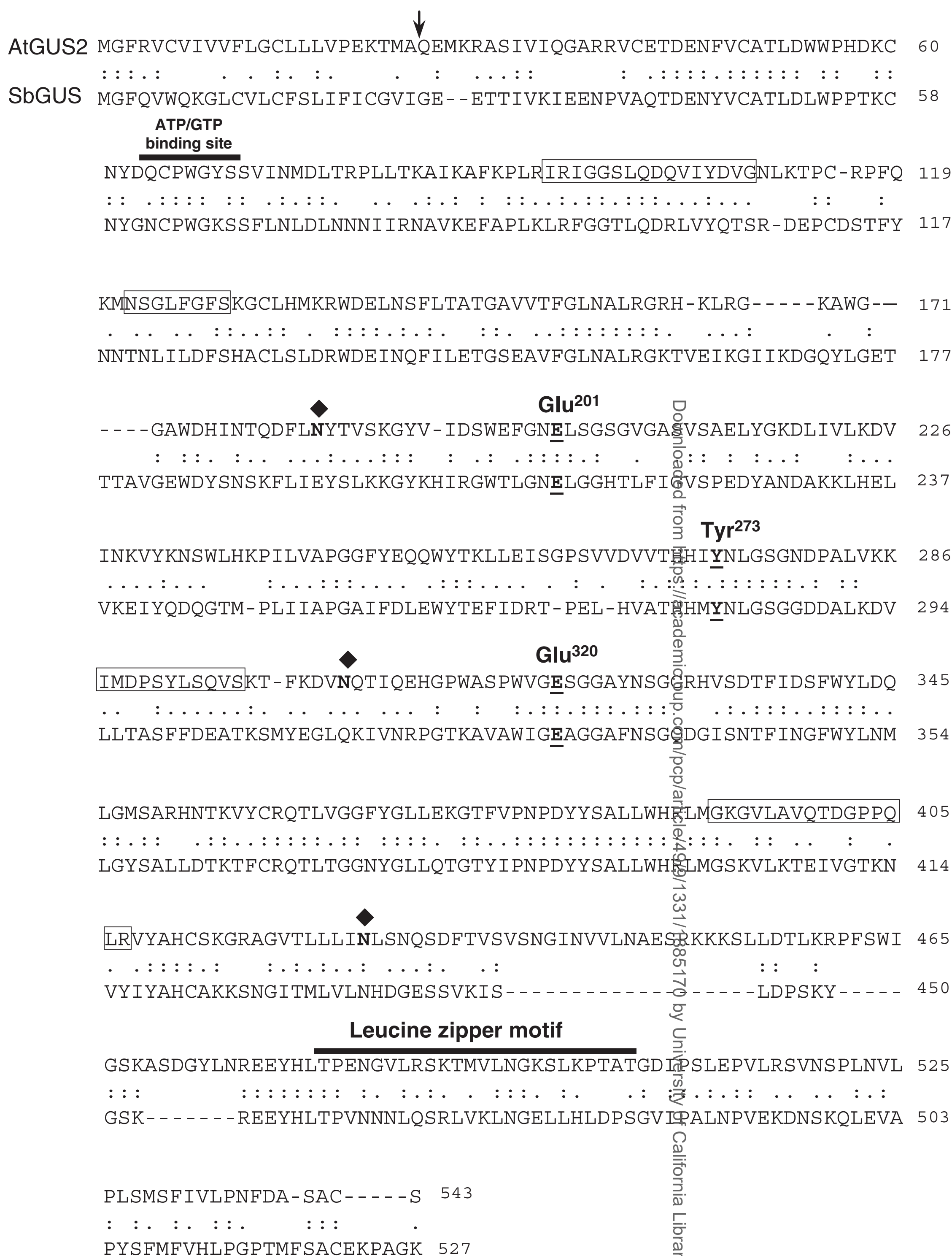


**Fig. 3** Conserved motifs and residues between AtGUS2 and SbGUS amino acid sequences. The acid-base and nucleophilic residues of the active site (Glu[201] and Glu[320]) and Tyr[273] are highlighted. An ATP/GTP binding site and a leucine zipper motif are shown. A predicted cleavage site (arrow) is also indicated in position 24–25. Diamonds represent putative sites of *N*-glycosylation. Boxed amino acids indicate peptide fragments obtained by MALDI-TOF MS after proteolytic cleavage by trypsin. Symbols underneath residues indicate identity (**:**) and similarity (**.**).

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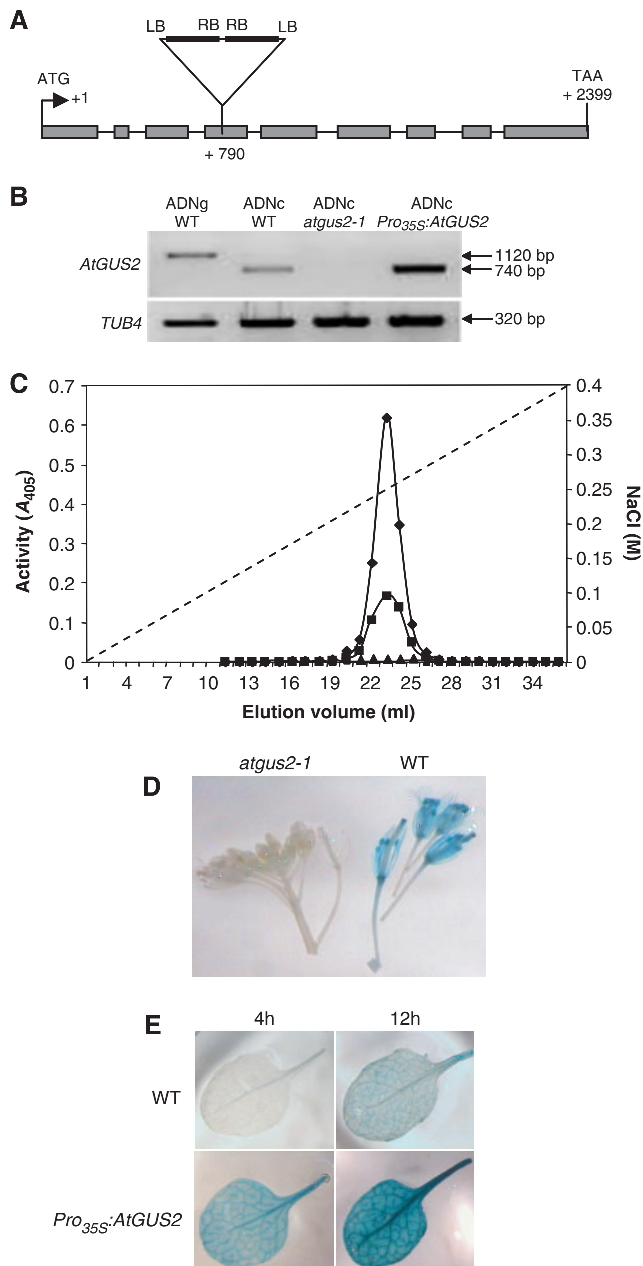


**Fig. 4** Characterization of the *atgus2-1* mutant and a *Pro$_{35S}$:AtGUS2* line. (A) Structure of the *AtGUS2* gene and schematic representation of the T-DNA insertion (line SALK_040732). Each gray rectangle represents an exon of *AtGUS2*. Black rectangles represent the T-DNA insertion. PCR analysis of flanking sequences of the T-DNA showed that the insertion occurred as shown in the fourth exon. RB: right border. LB: left border. Lengths are in base pairs. (B) Semi-quantitative RT-PCR to monitor *AtGUS2* expression level in the *atgus2-1* mutant and a selected *Pro$_{35S}$:AtGUS2* transformant. Amplifications were made using *AtGUS2*-specific primers (upper panel) and *TUB4*-specific primers (lower panel) which is considered as a constitutively expressed gene. (C) Elution profile of GUS activity in CM-Sepharose cation-exchange chromatography using stem protein extracts from wild-type (squares), *atgus2-1* (triangles) and *Pro$_{35S}$:AtGUS2* (diamonds). Two-ml dialyzed cell-free extracts (2 mg protein) were eluted as described for purification step 2 (see Material and Methods). The first 10 fractions were not analyzed for GUS activity since these fractions contained pigments which showed high absorbance at 405 nm. (D) Histochemical GUS assay on flowers from *atgus2-1* and wild type (WT). (E) Histochemical GUS assay on leaves from *Pro$_{35S}$:AtGUS2* and wild type (WT) after 4 and 12 h of incubation.

in vitro, using Yariv reagent (Schultz et al. 2000). Similar quantities of AGPs were obtained for the three lines (~60 µg AGP/g fresh weight). For each genotype, identical amounts of AGPs were permethylated with and without a preliminary reduction step of uronic acids (adapted from Kim and Carpita 1992). Permethylated polysaccharides were then hydrolyzed, reduced and derivatized prior to their separation by gas chromatography. The difference between the amounts of glucitol measured in the reduced and non reduced samples allowed us to quantify terminal-GlcA contents in AGPs. The data obtained shows that AGPs from the transgenics were modified compared to those purified from the wild type. Terminal-GlcA residues were not detectable in AGPs purified from *Pro$_{35S}$:AtGUS2* seedlings, and were more abundant in the case of *atgus2-1* compared to the wild type (Table 2). Additionally, AGPs purified from *atgus2-1* showed lower xylose and higher galactose contents. In contrast, AGPs purified from *Pro$_{35S}$:AtGUS2* showed higher xylose and lower galactose contents. The arabinose content was lower in both AGPs purified from *atgus2-1* and *Pro$_{35S}$:AtGUS2* (Table 2). These correlations between modulations of AtGUS2 activity and changes in GlcA content suggest that AtGUS2 hydrolyzes terminal-GlcA from AGPs in vivo. Furthermore, the modulation of AtGUS2 activity seems to have an impact on the overall sugar composition of AGPs since their arabinose, galactose, and xylose contents are also altered in transgenics.

### atgus2-1 *and* Pro$_{35S}$:AtGUS2 *lines show growth defects*

AGPs have been implicated in cell elongation and division (Showalter 2001). To investigate the effect of non- or mis-expressed *AtGUS2*, we measured dark-grown hypocotyls and light-grown roots of transgenic plants (Fig. 5A). Dark-grown hypocotyls and light-grown roots of *Pro$_{35S}$:AtGUS2* seedlings showed an ~15% and an ~30% increase in length respectively compared to the wild type. Dark-grown hypocotyls of *atgus2-1* seedlings showed an ~15% decrease compared to the wild type. Conversely, roots lengths from *atgus2-1* and wild-type seedlings did not differ significantly.

Thus, the higher level of endogenous GUS activity in *Pro$_{35S}$:AtGUS2* was correlated with an increase in hypocotyl and root growth, whereas the absence of endogenous GUS activity in *atgus2-1* resulted in a decrease in hypocotyl

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**Table 2** Sugar composition of seedling AGPs purified from wild-type (WT), *atgus2-1*, and $Pro_{35S}$:*AtGUS2* seedlings. Values are expressed in mol percentage.

| Sugar | WT | *atgus2-1* | $Pro_{35S}$:*AtGUS2* |
|---|---|---|---|
| Arabinose | 67.2 | 56.4 | 59.0 |
| Galactose | 24.7 | 38.0 | 17.2 |
| Xylose | 7.4 | 4.4 | 23.8 |
| t-GlcA | 0.7 | 1.2 | ND |

The mol percentage is based on the value for the individual sugar divided by the total of the four sugars. ND, not detected.

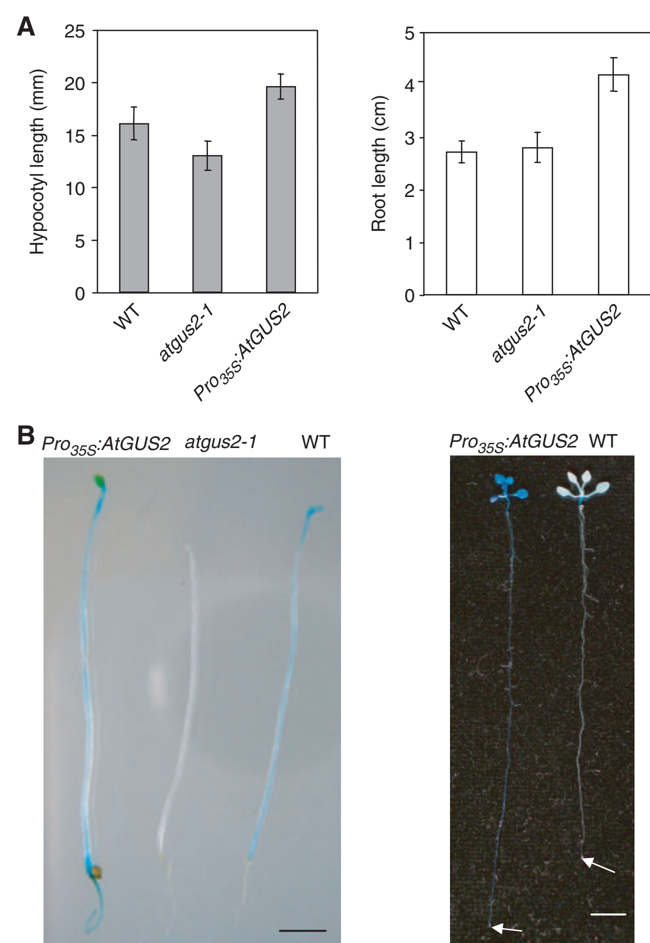


**Fig. 5** Hypocotyl and root growth in the *atgus2-1* mutant and $Pro_{35S}$:*AtGUS2* seedlings. (A) Length measurements of hypocotyls from 5-d-old dark-grown seedlings (gray rectangles) and of roots from 8-d-old light-grown seedlings (white rectangles) for the wild-type (WT), $Pro_{35S}$:*AtGUS2* and *atgus2-1*. Each error bar represents the average ± SD of 80 seedlings for hypocotyls and of 30 seedlings for roots. (B) Left: endogenous GUS activity in dark-grown hypocotyls of $Pro_{35S}$:*AtGUS2*, *atgus2-1* and wild-type (WT) seedlings. Bar = 2 mm. Right: endogenous GUS activity in roots of $Pro_{35S}$:*AtGUS2* and wild-type (WT) seedlings. Bar = 0.3 cm. *Arrows* indicate root tips.

growth (Fig. 5B). Absence of GUS activity in roots of *atgus2-1*, and a presumably very low GUS activity in roots of the wild type resulted in similar root lengths, respectively.

## Discussion

Endogenous GUS activity was first described in Arabidopsis by Jefferson et al. (1987). Its optimum pH (pH 5.0) was considered not to interfere with the *E. coli* GUSA (optimum pH 7.0) used as a reporter in genetic studies. We report here the characterization of AtGUS2 and substantiate its role for endogenous GUS activity in acidic conditions in Arabidopsis. The activity of partially purified AtGUS2 at pH 7.0 represents ~20% of its maximum activity (observed at pH 5.0) (Fig. 2A), which strongly suggests that AtGUS2 contributes to the GUS background activity occasionally observed by researchers using *uidA* as a reporter gene in Arabidopsis. Two other enzymes (AtGUS1 and AtGUS3) are similar to AtGUS2 in the GH79 family. However, they display no detectable GUS activity in the *atgus2-1* mutant grown under standard conditions (using X-GlcA and *p*NPGlcA as substrates). This might be explained by a different subcellular localization of these other AtGUS proteins or different optimal conditions for their activity. Recently, a study based on expression profiles suggested that the three AtGUS enzymes could operate in tandem with specific glucuronyltransferases to modulate the activity of common unknown substrates in dividing tissues (Woo et al. 2007). Whether these enzymes are active toward a common substrate or whether they exhibit substrate specificities is so far unknown.

Our data show that differential in-vivo AtGUS2 activity levels can modify GlcA content in the polysaccharidic side chains of AGPs. Several lines of evidence support the idea that AGPs are an in-vivo substrate for AtGUS2. First, in-situ localization patterns of AtGUS2 activity are indicative of those for AGPs (Showalter 2001). In addition, AtGUS2 was identified in proteomic studies that isolated cell-wall proteins in the apoplastic fluids of rosettes, cell suspension cultures and inflorescence stems (Boudart et al. 2005, Bayer et al. 2006, Minic et al. 2007). This subcellular localization for AtGUS2 matches the localization of AGPs which are found in the outer face of the plasma membrane and the cell wall (Showalter 2001). Second, the optimum pH for activity of our partially purified AtGUS2 enzyme (pH 5.0) is similar to the pH of the plant cell wall (Grignon and Sentenac 1991) where AGPs are detected. Finally, as shown with the quantification using the gas chromatographic method, AGPs purified from the *atgus2-1* mutant and from lines overexpressing *AtGUS2* display an increase and a decrease of terminal-GlcA residues, respectively. These transgenics show differences for roots and/or etiolated hypocotyls lengths compared to the wild type,

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suggesting a link between the sugar compositon of AGPs and their functions.

The carbohydrate moieties of AGPs (representing ~90% of the proteoglycan) are important for their biological activity during somatic embryogenesis (van Hengel et al. 2002), cell–cell interaction (Motose et al. 2004) and cell elongation (Seifert et al. 2002, van Hengel and Roberts 2002). Our data raise the question of how modified GlcA contents in AGPs lead to an altered hypocotyl and root growth. Xyloglucan transglycosylase activity, which is involved in cell elongation, is increased by certain anionic polymers containing uronic acid residues (e.g., AGPs) (Takeda and Fry 2004). However, AGPs account for ~1% of the cell-wall dry weight (Serpe and Nothnagel 1995) and GlcA residues represent less than 1% of the sugar mass of AGPs in Arabidopsis, implicating that drastic alterations in AtGUS2 activity would marginally influence the global GlcA content of the cell wall and the activity of cell wall modifying enzymes. A more plausible scenario is that AtGUS2 could indirectly modulate the action of other GHs exhibiting specificity for AGPs. Indeed, AGPs undergo an in-muro post-synthesis turnover of their polysaccharide chains, mediated by endogenous cell wall GHs (Takeuchi and Komamine 1980, Gibeaut and Carpita 1991). Such enzymes can release galactosyl and arabinofuranosyl residues from AGPs through a stepwise mechanism (Kotake et al. 2005, 2006). However, GlcA residues present at the non-reducing ends of the side chains of most AGPs potentially prevent the access for GHs to sugar residues located within these chains (Nothnagel 1997). Consequently, a reduced deglycosylation of AGPs in the *atgus2-1* mutant could be considered because of their high terminal-GlcA content. Similarly, a high GUS activity contributing to a decrease of terminal-GlcA in AGPs could favor the access for other degrading enzymes. Our data support this concept since we showed that altering AtGUS2 activity not only modifies GlcA content in AGPs, but also influences the content of other sugars such as galactose, arabinose, and xylose.

How this deglycosylation could contribute to cell growth is unclear and would require more investigations. An appealing hypothesis is that modified AGPs or oligosaccharides released after deglycosylation could represent a source of signaling molecules that promote cell growth (Etzler 1998). Such a mechanism was proposed to explain the enhancement of somatic embryogenesis when embryogenic cell cultures from carrot (*Daucus carota*) were incubated with AGPs and a chitinase (van Hengel et al. 2001, 2002). Alternatively, partial deglycosylation of some cell surface AGPs may be important in their role as co-receptors to mediate the regulation of growth factor signaling pathways. This process was described in animals for which degradation of heparan sulfate proteoglycans mediated by heparanases (GH79) is necessary to create a selective surface for the binding of ligands and for effective presentation to second specific receptors (Ma et al. 2006, Xu et al. 2007).

Finally, we propose here that deglycosylation of AGPs could contribute to hypocotyl growth, and that AtGUS2 is one of the enzymes involved in this process. The proposed mechanism probably requires GHs other than AtGUS2, especially in the primary root which does not show any GUS activity, but requires AGPs to elongate (Willats and Knox 1996). Interestingly, an AtGUS2 gain of function leads to an increased growth in this organ. The data presented here suggest that AtGUS2 is active towards AGPs. The substrates of the two other Arabidopsis enzymes in the GH79 family are still unknown, and the potential for AtGUS enzymes to hydrolyze flavonol glucuronides (although not identified yet in wild-type Arabidopsis) and the pectic sugar rhamnogalacturonan II which contains a beta-glucuronosyl residue in its side chain A remains to be investigated.

## Materials and Methods

*Chemicals*

*p*NPGlcA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and 5-bromo-4-chloro-3- β-indolyl-glucuronide (X-GlcA) purchased from Biosynth AG (Switzerland). Yariv reagent and *p*-nitrophenyl-α-D-glucuronide were kind gifts from Dr. Azzedine Driouch (University of Rouen, France) and Dr. Motomitsu Kitaoka (National Food Research Institute, Japan), respectively.

*Plant material*

Wild-type Arabidopsis (Col-0) was grown in the greenhouse at 22°C with a 16-h photoperiod at 150 μE $m^{-2} s^{-1}$. The *atgus2-1* (SALK_040732) allele was obtained from the Nottingham Arabidopsis Stock Centre. Stem tissues at the flowering stage were used for the analysis of GUS activity and protein purification. For growth in vitro, plants were grown on Petri dishes containing Estelle and Somerville medium (Estelle and Somerville 1987) without sucrose at 25°C. Plants were grown vertically for root length experiments. For dark growth, seeds were cold treated for 48 h and then exposed to fluorescent white light (200 μmol $m^{-2} s^{-1}$) for 4 h to synchronize germination. The plates were then wrapped in three layers of aluminum foil.

*Arabidopsis transformation*

Binary vectors were introduced into *Agrobacterium tumefaciens* strain C58pMP90 (Koncz and Schell 1986) by electroporation. Plants were transformed by flower infiltration (Bechtold and Pelletier 1998). T1 transgenic plants were selected on Estelle and Somerville medium (Estelle and Somerville 1987) containing hygromycin (50 mg $liter^{-1}$). T2 plants were used for the GUS bioassays and further analysis.

*Histochemical GUS assay*

For GUS assays on wild-type Arabidopsis, the *atgus2-1* mutant and $Pro_{35S}$:*AtGUS2* transgenic lines, plant organs were

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incubated for 4 h or 12 h at 37°C in a GUS buffer: 100 mM sodium acetate pH 5.0 containing 0.5 mg ml$^{-1}$ X-GlcA, 0.1% Triton X-100, 2.5 mM $K_3Fe(CN)_6$ and 2.5 mM $K_4Fe(CN)_6$. The samples were vacuum infiltrated for 5 min (ca. 200 mbar) to facilitate penetration of the assay buffer.

*Preparation of protein extracts from Arabidopsis stems*

Stem tissues (25 g) from Arabidopsis at the flowering stage were ground in a blender with 25 ml of ice-cold extraction buffer (25 mM BisTris, pH 7.0, 200 mM $CaCl_2$, 10% (v/v) glycerol, 4 μM sodium-cacodylate, and 1/200 (v/v) protease inhibitor cocktail). The ground and suspended material was centrifuged twice at 4°C for 5 min at 10,000 × *g*, and the supernatant was centrifuged again for 1 h at 17,000 × *g*. The resulting supernatant was concentrated by 'Ultrafree-CL' (10 kDa) (Sigma-Aldrich, St Louis, MO, USA) and used for chromatography.

*Purification of the enzyme displaying GUS activity*

The stem protein extract (10 ml, 25 mg protein) was used for purification of the GUS activity, using a three-step purification procedure, as described below.

*Step 1: Lectin chromatography*. A 0.5 × 3 cm column was filled with 1 ml of Con A Sepharose (Sigma-Aldrich, St Louis, MO, USA) and washed with 3 ml of wash buffer (20 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1mM $MnCl_2$, 0.5 M NaCl, pH 7.4). The soluble protein extract was added and the column was washed again with 10 ml of wash buffer at a flow rate of 5 ml h$^{-1}$. Proteins were eluted with 0.2 M methyl-α-glucopyranoside in the same buffer. The eluates were collected (0.5 ml per fraction), and 50 μl samples from each fraction were tested for GUS activity, as described below.

*Step 2: Cation-exchange chromatography*. The fractions from step 1 displaying GUS activity were equilibrated in 20 mM Tris–HCl (pH 7.4) containing 5% (v/v) glycerol and 0.015% (w/v) Triton X-100, and loaded on a CM-Sepharose (GE Healthcare, Piscataway, NJ, USA) cation-exchange column (1.5 × 5 cm; Sigma-Aldrich, St Louis, MO, USA). A loss of activity was observed in absence of detergent. Thus to prevent enzymatic inactivation by low ionic strength of the buffer, Triton X-100 (0.015%) was added to the solution. In this way, enzyme activities were stable for 1 week at 4°C. Proteins were eluted with a 0.0 to 0.4 M discontinuous gradient of NaCl in the same buffer, at a flow rate of 20 ml h$^{-1}$. One ml fractions were collected and assayed for GUS activity. Peak fractions with GUS activity were pooled and used for the third step of purification.

*Step 3: Gel-filtration chromatography*. The pooled and concentrated fractions from step 2 were fractionated by FPLC on a Superdex 200 HR10/30 column (GE Healthcare, Piscataway, NJ, USA) precalibrated with the following markers of known molecular mass: thyroglobuline (670 kDa), bovine gamma globuline (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa). Equilibration and elution were performed at room temperature, with 20 mM Tris–HCl buffer (pH 7.4), containing 150 mM NaCl and 0.015% (w/v) Triton X-100. Fractions of 0.4 ml were collected at a flow rate of 0.5 ml min$^{-1}$, and 50 μl of each fraction was used for GUS assays. Fractions displaying GUS activity were used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

*GUS activity*

The reaction mixture contained 2 mM *p*NPGlcA, 0.1 M acetate buffer (pH 5.0), 2 mM sodium azide, and 50 μl of protein extract in a total volume of 0.5 ml. The reaction was carried out at 45°C for 1 h and stopped by the addition of 0.5 ml of 0.4 M sodium bicarbonate to the assay mixture. The concentration of the resulting *p*NP solution was determined spectrophotometrically at 405 nm, using a calibration curve.

*pH and temperature profiles*

The partially purified enzyme obtained after the three purification steps was assayed with 2 mM *p*NPGlcA for 4 h for each point. Temperature dependence was assessed at pH 5.0, as described above for the GUS assay, except that the temperature ranged from 30°C to 65°C. Optimum pH was determined at a temperature of 37°C, with pH varying from 4.0 to 7.0 in 100 mM sodium acetate buffer.

*SDS-PAGE*

Protein concentration was determined according to the procedure of Bradford (1976) using bovine serum albumin as a standard. Denaturing SDS-PAGE was carried out using 10% polyacrylamide gels (Laemmli 1970). Standard markers (BenchMark, Invitrogen, Carlsbad, CA) were used to determine the approximate molecular masses of co-purified proteins. The gel was stained with colloidal Coomassie blue G250.

*Identification of proteins by mass spectrometry*

The protein band obtained in SDS-PAGE (*M*r ~55 kDa) was excised and digested in the gel with trypsin, according to the conditions for loading and elution described by Santoni et al. (2003). Tryptic peptides were analyzed by MALDI-TOF MS on a REFLEX III instrument (Bruker Instruments, Billerica, MA, USA), and proteins were identified using the MASCOT program (http://www.matrixscience.com/).

*Bioinformatics analyses*

The Conserved Domain Database (Marchler-Bauer et al. 2005) was used to identify GH domains in the Arabidopsis protein sequences. The MyHits web server (http://myhits.isb-sib.ch, Falquet et al. 2002) was used to predict *N*-glycosylation sites in the AtGUS2 amino acid sequence. The sequences of AtGUS2 and SbGUS were aligned using the FASTA program (http://fasta.bioch.virginia.edu/fasta/align.htm). The subcellular distribution and cleavage site of the enzyme were predicted with PSORT program (http://psort.nibb.ac.jp/form.html) and TargetP 1.1/SignalP 3.0 servers at http://www.cbs.dtu.dk/services/TargetP and http://www.cbs.dtu.dk/services/SignalP/respectively (Bendtsen et al. 2004, Emanuelsson et al. 2000).

*Cloning procedures*

For the overexpression of *AtGUS2*, the At5g07830 gene sequence was placed under the control of a dual *35S* promoter. The At5g07830 cDNA sequence (1632 bp) was amplified using the clone SALK_U67364 as a template. Pfu Ultra DNA polymerase (Stratagene) and the following oligonucleotides: 5′-ATGGGC TTCAGAGTTTGTGTT-3′ and 5′-TCATGAACAAGCAGAAG CATC-3′ were used for PCR. The purified PCR product was inserted into a blunt-ended *Sma*I-digested pMAGIC vector (Nesi et al. 2002), according to standard methods (Sambrook et al. 1989).

*mRNA expression*

Total RNA was extracted from wild-type, *atgus2-1* and $Pro_{35S}$:*AtGUS2* 8-d-old seedlings grown in vitro, using Trizol

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reagent (Qiagen USA, Valencia, CA, USA). Reverse transcriptase reaction was carried out using 5 μg of total RNA for each sample. Semi-quantitative reverse-transcription (RT)-PCR was performed using the following oligonucleotides to amplify the *AtGUS2* cDNA: 5′-ATGGGCTTCAGAGTTTGTGTT-3′ and 5′-GTGTA CCATTGTTGTTCATAGA-3′. A control RT-PCR was made using the same cDNA preparations and the following nucleotides specific for the *TUB4* cDNA (*At5g44340*) encoding for the tubulin beta-4: 5′-GTCCAGTGTCGTGATATTGCAC-3′ and 5′-GCTTA CGAATCCGAGGGTGCC-3′. Bands intensities were analyzed with the Quantity One software (Bio-Rad, Richmond, CA).

*Purification of AGPs and determination of their relative terminal-GlcA contents*

AGPs were purified from 400 light-grown Arabidopsis seedlings grown in vitro for 7 d, using Yariv reagent, as described in Schultz et al. (2000). For each genotype (wild type, *atgus2-1* and $Pro_{35S}$*:AtGUS2*), 100 μg of purified AGPs were permethylated with and without a preliminary reduction step of the uronic acids as described in Kim and Carpita (1992). Permethylated polysaccharides were then hydrolyzed, reduced and derivatized as in Burton et al. (2000). The derivatives were analyzed by gas chromatography (GC 8000 Thermo Finningan, Waltham, MA, USA) coupled to a mass spectrometer (MD800). A 25-m × 0.22-mm (i.d.) BPX 70 column (SGE Analytical Science) was used for all separations. Identification of the derivatives and deduction of the glycosidic linkages were based on both the elution order of standards and fragment ion signatures. The 1,5-di-*O*-acetyl-1-deuterio-2,3,4,6-tetra-*O*-methyl-D-glucitol corresponds to the terminal-glucose residues in the non-reduced samples and to the terminal-GlcA plus terminal-glucose residues in the reduced samples, respectively. Subtractions consequently allow the determination of terminal-GlcA contents.

*Hypocotyl and root lengths measurement*

Formaldehyde in aqueous solution (0.4%) was added to seedlings growing in vitro in Petri dishes. Hypocotyls and roots were spread on agar plates, and images were captured with a digital camera. Lengths were calculated with image analysis software (Optimas 5.2; IMASYS, Surennes, France), as described by Gendreau et al. (1997).

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