Identification and characterization of a UDP-D-glucuronate 4-epimerase in *Arabidopsis*

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Abstract **One of the major sugars present in the plant cell wall is D-galacturonate, the dominant monosaccharide in pectic polysaccharides. Previous work indicated that one of the activated precursors necessary for the synthesis of pectins is UDP-D-galacturonate, which is synthesized from UDP-D-glucuronate by a UDP-D-glucuronate 4-epimerase (GAE). Here, we report the identification, cloning and characterization of a GAE6 from *Arabidopsis thaliana*. Functional analysis revealed that this enzyme converts UDP-D-glucuronate to UDP-D-galacturonate in vitro. An expression analysis of this epimerase and its five homologs in the *Arabidopsis* genome by quantitative RT-PCR and promoter::GUS fusions indicated differential expression of the family members in plant tissues and expression of all isoforms in the developing pollen of *A. thaliana*.**

Keywords: Plant cell wall; Pectin; Nucleotide sugar; Epimerase

1. Introduction

The plant cell wall is a highly complex structure consisting of polysaccharides, structural proteins and various enzymes. Among the polysaccharides, pectic components make up a major portion of the primary cell walls of higher plants [1]. Considerable progress has been made in the structural elucidation of pectins, showing the existence of three major classes of pectic polysaccharides, the unbranched homogalacturonan and the branched rhamnogalacturonans I and II [2]. All pectin classes are characterized by a high content of galacturonate in their polysaccharide backbone.

Despite the detailed knowledge about pectin structure, far less is known about its biosynthesis [3]. It is generally believed that pectin is synthesized by glycosyltransferases which use activated sugar precursors as substrate [4]. Only recently two putative glycosyltransferases involved in pectin synthesis have been identified [5,6]. Furthermore, many genes encoding enzymes in precursor synthesis have been cloned and characterized (see [7] for review). However, for the conversion of UDP-D-glucose to UDP-L-rhamnose there is only sparse evidence on the enzyme coding regions [8,9], and for the conversion of UDP-D-glucuronate to UDP-D-galacturonate only the presence of an epimerase activity in plant extracts has been shown so far [10]. Here, we describe the cloning and characterization of such a UDP-D-glucuronate 4-epimerase (GAE) gene from *Arabidopsis thaliana*.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana cv. Columbia-0 plants were grown in the greenhouse for approximately 8 weeks with a 16 h light/8 h dark photoperiod at a temperature of 21 °C during the light phase and 17 °C during the dark phase.

2.2. Construction of expression vector and transformation of Pichia pastoris

To generate a *GAE6* expression vector, the full-length *GAE6* gene was PCR-amplified from genomic DNA with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) using the oligonucleotides 5′-ATTAGGAATTCATGCCCCTGTCGGCGACGGCGGATACAAGCAAGAC-3′ and 5′-ACTGGTCTAGAGCGGAATCTTCGGCGTGAGAAGTTTCCTTTTTTAC-3′ (*Eco*RI and *Xba*I sites engineered into the primers are underlined). The PCR product was cloned between the *Eco*RI and *Xba*I sites of the *P. pastoris* expression vector pPICZB (Invitrogen, San Diego, CA) leading to a translational fusion with the myc epitope and polyhistidine tags. The resulting construct was designated pPICZB-GAE6. The sequence of the construct was verified using the ABI Prism Big Dye Terminator Cycle Sequence Reaction Kit (Perkin–Elmer Applied Biosystems, Foster City, CA) and an ABI Prism 377 DNA sequencer. pPICZB-GAE6 and the empty vector pPICZB (control) were transformed into *P. pastoris* strain KM71 and 10 randomly selected pPICZB-GAE6 transformants were screened for expression of GAE6 as described previously [11]. One transformant was used for further analysis.

2.3. Enzyme assays on the recombinant GAE6

Crude *P. pastoris* extracts containing 20 μg protein were mixed with 25 nCi of UDP-D-[^{14}C]glucuronate (300 mCi/mmol), UDP-D-[^{14}C]galactols (300 mCi/mmol), UDP-D-[^{14}C]glucose (300 mCi/mmol) or UDP-D-[^{14}C]xylose (238 mCi/mmol) (American Radiolabeled Chemicals, St. Louis, MO) in 50 μl of 50 mM Tris–HCl, pH 7.9, and incubated at 25 °C overnight. Nucleotide-sugars were hydrolyzed by the addition of trifluoroacetic acid to 1.6 M final concentration and

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Abbreviations: GAE, UDP-D-glucuronate 4-epimerase

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incubated at 95 °C for 30 min. Samples were dried under vacuum and resuspended in 15 μl of 80% (v/v) ethanol. Resultant monosaccharides were separated by thin layer chromatography (TLC) on silica TLC plates (Aldrich, Milwaukee, WI) in a 6:2:1 (v/v/v) mixture of 1-propanol, saturated ammonia solution, and water. Radioactivity was visualized by phosphorimaging (Bio-Rad Laboratories, Hercules, CA) and quantified using Molecular Analyst Software (Bio-Rad Laboratories). Authentic sugar standards run in parallel were stained with aniline-hydrogen phthalate [12].

The recombinant MUR4 UDP-xylose 4-epimerase (EC 5.1.3.5) [11] and a recombinant UDP-glucose 4-epimerase (EC 5.1.3.2) were used in control reactions. All assays were repeated at least once. Measurements of protein concentrations were performed using a bicinchoninic acid kit (Sigma).

In an alternative approach, 100 nmoles of UDP-D-glucuronate (Sigma, St. Louis, MO) were incubated as described above and the resulting product was analyzed by high performance anion exchange chromatography (HPAEC) using a Carbopac PA 10 column with a UV detector as described earlier [13]. Authentic UDP-D-galacturonate and UDP-D-glucuronate (Sigma, St. Louis, MO) were run in parallel. The peaks were collected, mixed 1:1 (v/v) with 2,5-dihydroxybenzoic acid (10 mg/ml) and analyzed on a Voyager DE Pro MALDI-TOF instrument (Applied Biosystems, Darmstadt, Germany) using an acceleration voltage of 15 000 V with a delay time of 80 ns in negative reflectron mode.

For kinetic evaluation, the recombinant GAE6 was assayed using UDP-D-[^{14}C]glucuronate (<6 μM) and a mix of UDP-D-[^{14}C]glucuronate and unlabeled UDP-D-glucuronate at higher substrate concentrations (>6 μM). Linearity with respect to protein concentration and time was first established, and the concentration of UDP-D-glucuronate was then independently varied while the concentrations of all other reactants remained constant (20 μg extracted protein in 50 mM Tris–HCl, pH 7.9). The incubation time was 30 s. Results from the TLC assays were analyzed using GraphPad Prism Version 3.03 software, and the equation of the best fit was determined.

2.4. Generation of promoter::GUS plants

In order to localize *GAE* expression in *Arabidopsis*, promoter regions of all six members of the *GAE* gene family were amplified by PCR and inserted into pBI101.3 [14]. Primers were designed to give promoter fragments of about 2000 bp and provided additional *Xba*I and *Bam*HI sites (promoters for *GAE1–GAE5*), or *Xba*I and *Sma*I sites (promoter for *GAE6*). The following primers were used:

GAE1 5′-GCTCTAGAGCGATCTTAATTGCTTACTCCA-3′, 5′-CGGGATCCCGAATTTAATTAAACTCTCTTT-3′;

GAE2 5′-GCTCTAGAGCCCCTAAACCCCCAATTTCAT-3′, 5′-CGGGATCCCGCAAAGATTTGATTCCCAGA G-3′;

GAE3 5′-GCTCTAGAGCACAACTCCCAACCCTGATGA-3′, 5′-CGGGATCCCGATTTACCTCGACAGAGAGAA-3′;

GAE4 5′-GCTCTAGAGCTGTTGGAAAGACATGGTTCCT-3′, 5′-CGGGATCCCGTTTCTCTTCTTCTTCTTCTT-3′;

GAE5 5′-GCTCTAGAGCAATCAAGGAAACGACTACAAG-G-3′, 5′-CGGGATCCCCTTTCACCGGAGGAGGATTTA-3′;

GAE6 5′-GCTCTAGAGCTGTGACTTGTCCTTACGCGA-3′, 5-TCCCCCGGGGGATTTCGTCTATTTGATATAAA-3′

After digestion of the PCR products and the vector with the corresponding restriction enzymes, the promoter sequences were inserted upstream of the β-glucuronidase (GUS) (*uidA*) gene in pBI101.3. The constructs were introduced into *A. thaliana* cv. Columbia by *Agrobacterium tumefaciens* mediated transformation [15]. Transgenic lines were selected by addition of 60 μg/ml kanamycin to the MS growth medium. Selected plants were then transferred to soil and grown under long day conditions (16 h light/8 h dark).

GUS activity of at least six independent transformants per construct was visualized by incubation of plant tissue overnight at 37 °C in GUS-buffer (100 mM sodium phosphate buffer, pH 7.2, 0.1% Triton X-100, 2 mM $K_3[Fe(CN)_6]$; and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide). After development of the blue color, chlorophyll was extracted with 80% ethanol for 24 h at room temperature.

2.5. Quantitative RT-PCR

Total RNA was extracted from various tissues from greenhouse grown *A. thaliana* WT plants as reported earlier [16]. Residual genomic DNA was removed by treatment with RNAse–free DNAse (Roche, Mannheim, Germany) and approximately 2 μg of total RNA was reverse transcribed with MMLV reverse transcriptase (Qbiogene, Heidelberg, Gemany) using an oligo(dT) primer, to generate 21 μl of first strand cDNA. Real-time RT-PCR was performed using 9 μl of a 1/30 (v/v) dilution of the first strand cDNA reaction and SyberGreen master mix (Applied Biosystems, Darmstadt, Germany) in a reaction volume of 20 μl on a GeneAmp 5700 Sequence Detection System (PE-Applied Biosystems, Darmstadt, Germany). To accurately quantify relative expression levels, constitutive control genes were chosen with the help of the geNorm application [17]. The following oligonucleotides were used:

gae1 5′-CAAGCTTGGCTTTAGTATTGTAACCG-3′, 5′-CATG CCAATATTAGCTCAGCCCG-3′;

gae2 5′-TCAAGCAGCAGCAGCAACTTTC-3′, 5′-AAGCAC-GGATTTGCAGACTG-3′;

gae3 5′-GACCCGTCTGAAGATCGGTGGTG-3′, 5′-TCACCA-TTCTTGAGAGACTTC-3′;

gae4 5′-TAGCGGCGGCTTTTTTGTCGC-3′, 5′-ATGCTAAT-ATTAGTTTAGCTCAACG-3′;

gae5 5′-TCACCAAGAACTCTTCTTCTTCG-3′, 5′-ATGCCAA-TATCACGTTAGCGCAAGC-3′ and

gae6 5′-GGAATCTTCGGCGTGAGAAG-3′, 5′-GCATGCTAA-TGTGAGTTTAGCG-3′.

Control primers have been described previously [18].

3. Results and discussion

3.1. Bioinformatic characterization of the GAE family

Arabidopsis genes putatively involved in nucleotide sugar metabolism were identified using profile hidden Markov models from the Pfam site [19] and by homology to known bacterial genes. One candidate family comprising six members was most similar to bacterial genes involved in UDP-D-galacturonate synthesis [20] and was therefore tentatively named GAE [21].

The C-terminal part of all GAEs showed a strong similarity to the Pfam 1370 domain (NAD dependent epimerase/dehydratase). The similarity within the family was between 76.0% (GAE1 vs. GAE6) and 94.9% (GAE2 vs. GAE3) as determined by a local alignment [22]. For all GAEs, TMHMM [23] predicted the presence of at least one transmembrane domain in the N-terminal part, whereas the C-terminal part probably involved in catalysis was topologically always predicted to be non-cytoplasmic. SignalP analysis [24] predicted that all proteins have a signal anchor (>98% probability).

Searches for similar genes in other plant species in the TIGR gene index (The TIGR Gene Index Databases, The Institute for Genomic Research, Rockville, MD 20850 [25]) returned protein sequences from various plant species that had similar length as the *Arabidopsis* GAEs. They were aligned using CLUSTALW [26] and the resulting output was used to build a maximum likelihood family tree of the GAEs using the PHYLIP software package [27] (Fig. 1). Interestingly, the family tree indicates three major protein subgroups. Two branches of this tree are reliably occupied by GAE1 and GAE6, respectively, whereas GAE2 to GAE4 and with less confidence GAE5 are located together in one branch of the tree. This might indicate a more ancient role of GAE1 and GAE6, whereas the other GAEs might have evolved later.

3.2. Heterologous expression of recombinant AtGAE6 indicates UDP-D-glucuronate 4-epimerase activity

The intron-less GAE6 was cloned from genomic DNA and recombinantly expressed in *P. pastoris* for heterologous expression. Crude extracts from *P. pastoris* were incubated with radiolabeled UDP-sugars and the reaction products hydrolyzed to free monosaccharides followed by analysis through

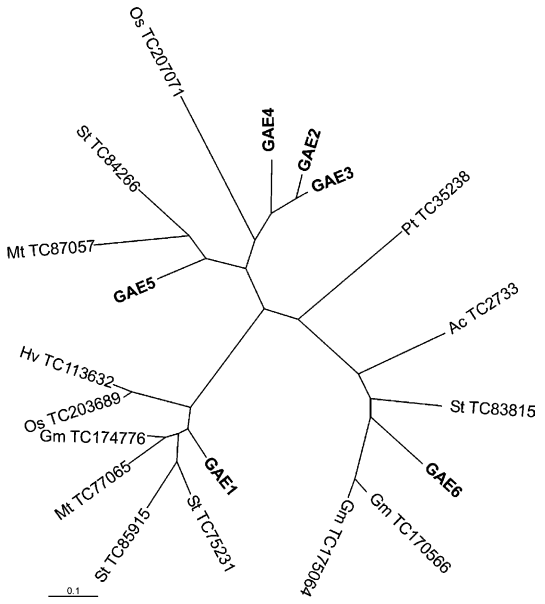

Fig. 1. Phylogenetic tree of the *A. thaliana* GAE family and homologous sequences from other plants.

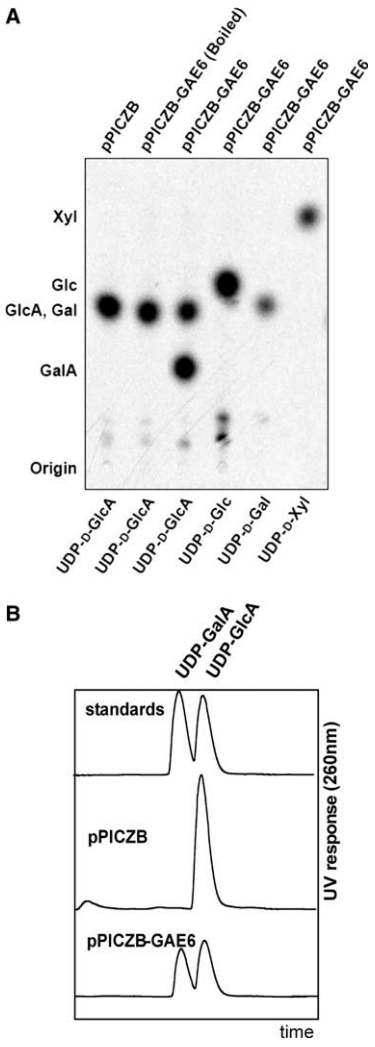

Fig. 2. GAE activity of GAE6. (A) Autoradiograph of a thin layer chromatogram of the ^{14}C-labeled reaction products from assays using crude extracts from *P. pastoris* and various UDP-D-[^{14}C]sugar substrates. The types of protein extracts used are indicated at the top and the nucleotide sugar substrates are indicated at the bottom. Reaction products were hydrolyzed to monosaccharides prior to TLC analysis. (B) HPAEC–UV trace of authentic UDP-D-glucuronate and UDP-D-galacturonate and reaction products from assays of crude extracts of *P. pastoris* incubated with UDP-D-glucuronate as substrate.

TLC. Incubation of extracts from *P. pastoris* expressing GAE6 with UDP-D-[^{14}C]glucuronate resulted in two products, with R_f values typical for D-glucuronate and D-galacturonate as determined by authentic sugar standards (Fig. 2A). Extracts from *P. pastoris* transformed with the empty vector pPICZB did not convert the UDP-D-[^{14}C]glucuronate substrate into any detectable products and boiled extracts from *P. pastoris* expressing GAE6 did not exhibit any detectable activity. Furthermore, the recombinant GAE6 did not convert UDP-D-[^{14}C]galactose, UDP-D-[^{14}C]glucose and UDP-D-[^{14}C]xylose (Fig. 2A). The identity of the product formed by incubation of GAE6 with UDP-D-glucuronate was further investigated. An incubation of the crude lysate containing the recombinant GAE6 with UDP-D-glucuronate was followed by analysis of the resulting reaction products by high performance anion exchange chromatography with a coupled UV detector. Two UV-absorbing peaks were observed having the same retention time as authentic UDP-D-galacturonate (22 ± 0.5 min) and UDP-D-glucuronate (23.5 ± 0.5 min), respectively (Fig. 2B). Furthermore, mass spectrometric analysis of fractions containing each of the observed peaks revealed the strongest intensity at an apparent mass of 579.4 in both peaks consistent with the mass of a UDP-hexuronic acid. Upon incubation with the empty vector control, only the peak corresponding to the substrate (UDP-D-glucuronate) was detected. In conclusion, the only interconversion reaction observed was the reversible 4-epimerization of UDP-D-glucuronate to UDP-D-galacturonate in the presence of the recombinant GAE6.

The influence of pH on the GAE activity of GAE6 was analyzed by assaying enzyme preparations in 50 mM potassium phosphate [pH 6.0–7.6] and 50 mM Tris–HCl [pH 7.4–8.9] using UDP-D-[^{14}C]glucuronate as substrate. The assays demonstrated a pH optimum of 7.9 (Fig. 3A). To analyze the effect of pyridine nucleotide co-factors, NAD^+, NADH, $NADP^+$ and NADPH were added to the GAE6 assays and the effects compared to assays without co-factors added. Up to 6 mM NAD^+, NADH, $NADP^+$ or NADPH did not affect the enzyme activity of the recombinant GAE6 (data not shown). Enzyme kinetics of recombinant GAE6 were established with a mixture of radiolabeled and unlabeled UDP-D-glucuronate. The data were analyzed by computerized non-linear regression fit and a Lineweaver–Burk plot (Fig. 3B). The apparent K_m of recombinant GAE6 was determined to be 0.23 mM for both plots ($R^2 = 0.99$).

3.3. Expression analysis by real-time RT-PCR and promoter::GUS fusions

The expression of the members of the AtGAE gene family was estimated by real-time RT-PCR (Table 1) and by querying the MPSS database (http://mpss.udel.edu). RT-PCR results indicated that both GAE1 and GAE6 are the isoforms expressed to the highest level in *A. thaliana*. This is in accordance with the data derived from the MPSS database where the

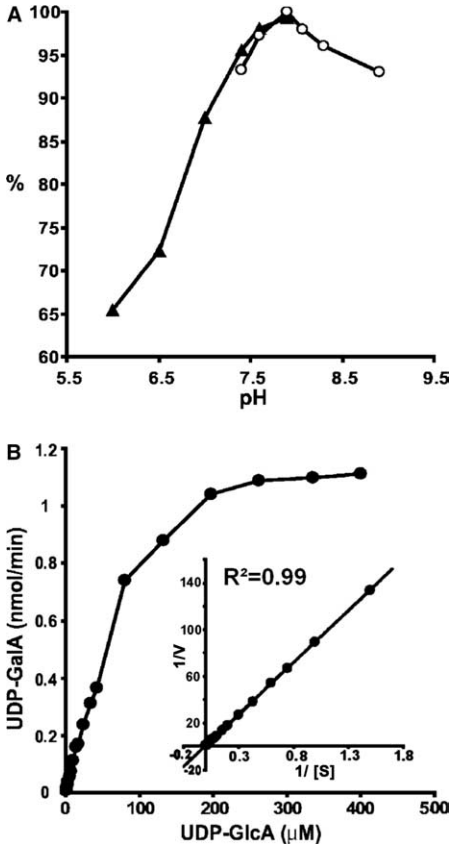

Fig. 3. Characterization of the recombinant GAE6. (A) pH profile obtained by assaying GAE6 in 50 mM potassium phosphate pH 6.0–7.6 (▲), and 50 mM Tris–HCl, pH 7.4–8.9 (○); (B) the effect of UDP-D-glucuronate concentration on the rate of UDP-D-galacturonate formation. Inset: Lineweaver–Burk plot of data.

normalized sum of tags in the classical dataset indicates an order of expression of GAE6 ~ GAE1 ≫ GAE2 > GAE3 > GAE5 (no class 1 tags were obtained for GAE4). Never a difference of more than fivefold between two organs for each gene was observed with real-time RT-PCR possibly due to the insufficient spatial resolution of RT-PCR.

Therefore, the tissue specific expression was further explored by promoter::GUS fusions of all six genes (Fig. 4). A rather high expression was detected for GAE1, GAE2, and GAE6 in all tissues in accordance with the RT-PCR data; however, GUS staining revealed that GAE6 expression in the leaves was confined to the leaf veins, whereas the other two genes seemed to be more ubiquitously expressed in the leaves. Contrary to the real-time RT-PCR results, GUS staining indicates that GAE2 seems to be the isoform expressed to the highest level. However, this discrepancy might be explained by the fact that for the GUS experiments only the immediate upstream promoter regions (~2 kb) were used and that potential enhancer elements, e.g., those that may be present in the 3′UTR region, were not investigated here.

Interestingly, all GAE family members seem to be expressed in the pollen of *Arabidopsis* (Fig. 4 flower panels). This could indicate that they are needed to provide precursors for pectic components, which are differentially expressed during microsporogenesis in *Arabidopsis* [28]. Specifically, GAE6 expression seems to occur in anther tissue, but expression in the pollen was only detected after pollen maturation. In addition, GUS staining suggests that GAE expression in the roots might be elaborately regulated. Both GAE1 and GAE2 seem to be expressed throughout the root, whereas GAE3 and GAE6 are expressed in the root hair zone. In contrast, GAE4 expression seems to be confined to the root tip.

Taken together, these results further indicate that some GAEs might be differently localized in the tissues of the considered organs. This could be corroborated in future experiments involving RNA in situ hybridizations that would give more insights into GAE expression on a tissue level. The absence of GUS staining in tissues, where transcript was detected with real-time RT-PCR, might be explained by the higher sensitivity of this method and the ''averaging'' of RNA based methods over the whole tissue.

Table 1
Transcript levels of the *AtGAEs*

Transcript	Stem	Root	Flower	Silique	Leaf	Cauline leaf
Actin2	20.69 ± 0.23	17.04 ± 0.03	17.78 ± 0.01	18.65 ± 0.04	19.14 ± 0.09	17.62 ± 0.21
Ubiquitin10	20.4 ± 0.6	16.82 ± 0.14	17 ± 0.09	17.37 ± 0.06	19.08 ± 0.05	16.24 ± 0.06
Aprt	23.6 ± 0.56	20.34 ± 0.18	20.82 ± 0.09	19.2 ± 0.16	20.36 ± 0.01	22.3 ± 0.35
β-6-Tubulin	23.79 ± 0.47	21.52 ± 0.44	22.91 ± 0.42	20.37 ± 0.14	20.54 ± 0.06	25.59 ± 0.08
GAE1	22.47 ± 0.53	19.42 ± 0.14	20.76 ± 0.69	19.36 ± 0.41	21.255 ± 0.32	20.14 ± 1.17
GAE2	24.06 ± 0.37	21.95 ± 0.19	20.41 ± 0.38	21.81 ± 0.24	23.37 ± 0.01	21.96 ± 0.21
GAE3	26.16 ± 0.04	23.44 ± 0.03	22.53 ± 0.11	23.10 ± 0.22	24.87 ± 0.44	23.39 ± 0.12
GAE4	26.27 ± 0.23	23.4 ± 0.20	22.85 ± 0.01	24.28 ± 0.30	25.34 ± 0.60	24.51 ± 0.31
GAE5	27.89 ± 1.00	25.63 ± 0.20	25.68 ± 0.40	25.63 ± 0.30	27.38 ± 0.73	26.49 ± 0.37
GAE6	22.29 ± 0.22	20.90 ± 0.14	19.5 ± 0.10	18.94 ± 0.10	21.97 ± 0.37	19.87 ± 0.23
GAE1 ratio	106.9	97.5	30.2	125	165	81.9
GAE2 ratio	35.5	16.9	38.7	22.9	38.1	23.2
GAE3 ratio	8.3	6	8.9	9.3	13.5	8.6
GAE4 ratio	7.7	6.2	7.2	4.1	9.7	3.9
GAE5 ratio	2.5	1.3	1.0	1.6	2.4	1.0
GAE6 ratio	121.5	34.8	72.7	166.7	100.5	98.7

Transcript levels for each gene were measured by real-time RT-PCR, normalized to the level of four different constitutive transcripts (actin2 (At3g18780), ubiquitin10 (At4g05320), tubulin (At5g12250) and adenine phosphoribosyltransferase (At1g27450)) in the same sample, and then expressed relative to the level of GAE5 transcript in cauline leaves (set at 1.0) similarly as described earlier [29].
C_T values (means ± S.D., $n = 2$) are presented above the relative gene expression level in each case.

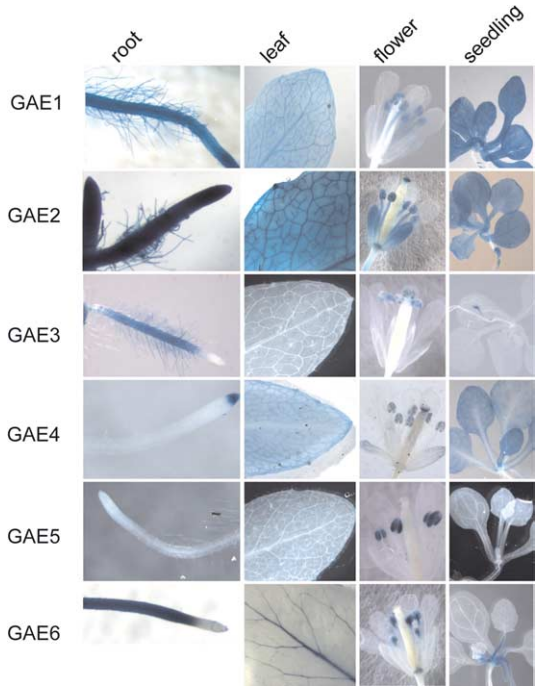

Fig. 4. Analysis of expression of the different *AtGAEs* using promoter::GUS fusions.

4. Conclusion

Here, we report the identification, biochemical characterization, and expression analysis of a plant GAE gene. Future work on knock-out plants is on the way to establish their role not only in pectin synthesis and its regulation but also in plant growth and development.

5. Accession numbers

The TC numbers shown in Fig. 1 are the accession numbers for the TIGR plant gene index and represent the following organisms: *Glycine max* (TC175064, TC175066 and TC174776), *Allium cepa* (TC2733), *Solanum tuberosum* (TC83815, TC84266, TC75231 and TC85915), *Pinus taeda* (TC35238), *Hordeum vulgare* subsp. *vulgare* (TC113632), *Oryza sativa* (TC203689 and TC207071) and *Medicago truncatula* (TC77065 and TC87075).

For GAE6, which had been submitted as AY056117, third party annotation has been entered under the submission number BN000504.

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