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Arabinogalactan biosynthesis: Implication of AtGALT29A enzyme activity regulated by phosphorylation and co-localized enzymes for nucleotide sugar metabolism in the compartments outside of the Golgi apparatus

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Arabinogalactan proteins are abundant cell surface proteoglycans in plants and are implicated to act as developmental markers during plant growth. We previously reported that AtGALT31A, AtGALT29A, and AtGLCAT14A-C, which are involved in the biosynthesis of arabinogalactan proteins, localize not only to the Golgi cisternae but also to smaller compartments, which may be a part of the unconventional protein secretory pathway in plants. In Poulsen et al.,[1] we have demonstrated increased targeting of AtGALT29A to small compartments when Y144 is substituted with another amino acid, and we implicated a role for Y144 in the subcellular targeting of AtGALT29A. In this paper, we are presenting another aspect of Y144 substitution in AtGALT29A; namely, Y144A construct demonstrated a 2.5-fold increase while Y144E construct demonstrated a 2-fold decrease in the galactosyltransferase activity of AtGALT29A. Therefore, the electrostatic status of Y144, which is regulated by an unknown kinase/phosphatase system, may regulate AtGALT29A enzyme activity. Moreover, we have identified additional proteins, apyrase 3 (APY3; At1g14240) and UDP-glucuronate epimerases 1 and 6 (GAE1, At4g30440; GAE6, At3g23820), from *Arabidopsis thaliana* that co-localize with AtGALT31A in the small compartments when expressed transiently in *Nicotiana benthamiana*. These proteins may play roles in nucleotide sugar metabolism in the small compartments together with arabinogalactan glycosyltransferases.

Cell surface arabinogalactan proteins (AGPs) change structure spatially and developmentally in a tightly regulated manner and are thus considered developmental markers.[2] However, little is known about the molecular mechanisms that control the synthesis and degradation of AGPs. AGPs are synthesized as post-translational modifications of proteins, which begin in the ER and continue in the Golgi apparatus.[3] We previously reported that at least some of the AGP glycosyltransferases (GTs) localize to an unknown subcellular compartment of ~0.5 μm in diameter in addition to the Golgi cisternae of ~1.0 μm in diameter when these proteins are expressed transiently in *N. benthamiana* and stably in an *Arabidopsis thaliana* mutant.[1] AtGALT31A was particularly highly localized to the small compartments and did not localize with the trans-Golgi network that was defined by SYP61, early endosomes stained by FM4-64, and prevacuolar compartments induced by Wortmannin treatment.[1] Instead, AtGALT31A-defined small compartments were partially co-localized with EXO70E2, a marker for the exocyst-positive organelle (EXPO[4]), which was recently described to mediate the unconventional protein secretory (UPS) pathway[5] in plants. *N*-glycosylation-processing enzymes were rarely found in the AtGALT31A-defined small compartments, indicating that the AtGALT31A-defined small compartments are mostly dedicated to AGP biosynthesis (protein *O*-glycosylation) and not the *N*-glycosylation of proteins.[1]

We previously presented that the site-directed mutagenesis of a phosphorylation site of AtGALT29A (Y144) that was identified by proteomics increased the localization frequency of the protein to the small compartments from 25% to 60–70% compared to the unmodified AtGALT29A when expressed in *N. benthamiana*.[1] The result indicated the importance of Y144 in the subcellular targeting of AtGALT29A; however, this targeting is most likely not regulated by the electrostatic status of Y144 as a result of phosphorylation since both Y144A and Y144E constructs

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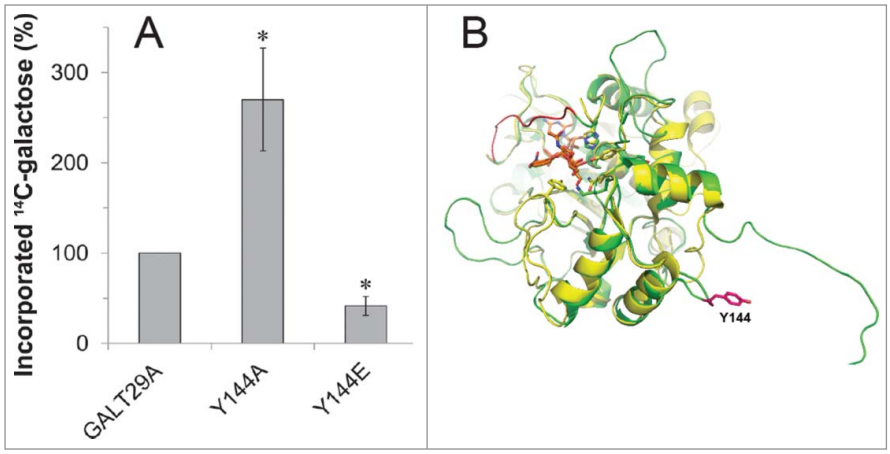

Figure 1. (**A**) Galactosyltransferase activity of AtGALT29A and site-directed mutants of AtGALT29A Y144A and Y144E. Affinity-purified recombinant proteins expressed in *N. benthamiana* leaves were incubated with UDP-^{14}[C]-Gal in the presence of a mixture of AGP acceptors (SP_{32}) as described previously.[7] Relative activity (%) is compared to the activity level of AtGALT29A, which represents 100% activity. The error bar indicates standard deviation (n = 3). * indicates significant differences compared to wild type AtGALT29A ($p < 0.05$, Student's t-test). (**B**) Predicted 3D structure of AtGALT29A (green) using the crystal structure elucidated from rat α-2,6-sialyltransferase[6] (2WNB, yellow). Y144 is located on the surface of a globular catalytic domain that is 35 Å from the predicted catalytic site (His319 of 2WNB); the substrate analog (orange) is indicated.

altered the localization in the same way. In this paper, we present the effect of Y144 mutagenesis on the enzyme activity of AtGALT29A. The recombinant AtGALT29A Y144A expressed in *N. benthamiana* showed 2.5-fold increased galactosyltransferase (GalT) activity, while the recombinant AtGALT29A Y144E showed 2-fold decreased GalT activity compared to wild type AtGALT29A (**Fig. 1A**). The results indicate that the charge status of Y144 likely affects the AtGALT29A GalT activity, which may be regulated by an unknown kinase/phosphatase system. The predicted 3D structure of AtGALT29A using rat α-2,6-sialyltransferase[6] as a template indicates that the Y144 site is located in the globular catalytic domain, far (35 Å) from the catalytic site, and on the surface of the globular domain (**Fig. 1B**). Surface charge status often affects protein conformation; thus, the regulatory mechanisms of AtGALT29A enzyme activity by the electrostatic status of Y144 might be due to conformational changes in the globular catalytic domain. Thus, change of the subcellular targeting of AtGALT29A by substitution of Y144 is not likely mediated by Y144 phosphorylation, while AtGALT29A enzyme activity seems to be regulated by Y144 phosphorylation status. We previously demonstrated that AtGALT29A and AtGALT31A form a heterodimer, and the enzyme complex exhibits increased GalT activity.[7]An interaction with AtGALT31A also occurs with AtGALT29A Y144A and Y144E.[1] Thus, how the 2 factors that enhance enzyme activity work with each other, namely whether the electrostatic status of Y144 and heterodimer formation with AtGALT31A act additively, synergistically, or in an antagonistic manner, in the regulation of AtGALT29A activity is an interesting question.

In this paper, we report proteins in addition to those analyzed previously that localize in the small compartments when expressed in *N. benthamiana*.[1] Under the same experimental condition as used for proteins analyzed previously, a nucleoside phosphatase family protein (NTPDase; apyrase; APY3), which is encoded by *At1g14240,* and 2 UDP-glucuronic acid 4-epimerases (GAE1 and 6 encoded by *At4g30440* and *At3g23820*, respectively) from *Arabidopsis thaliana* frequently co-localized with AtGALT31A in the small compartments (**Fig. 2**). In *A. thaliana*, 2 NTPDases (AtAPY1 or AtAPY2) have been characterized as functional NTPDases.[8] Both AtAPY1 and 2 are integral membrane proteins that localize to the Golgi membrane[9,10] and likely play a role in the metabolism of nucleotide diphosphate (NDP)-sugar, especially uridine diphosphate (UDP)-sugar, which is the substrate for the GTs reactions that occur in the Golgi lumen. Namely, the UDP produced after the GTs reaction is hydrolyzed to uridine monophosphate (UMP) by NTPDase, and UMP is transported to the cytosol by NDP-sugar transporters (antiporter) on the Golgi membrane. In the cytosol, UDP is used to generate new UDP-sugars that are transported by NDP-sugar transporters to the Golgi lumen where the UDP-sugars are then used for the GTs reactions. APY3 has not been biochemically characterized but might be a NTPDase dedicated to UDP metabolism in the small compartments. GAE1 and 6 have a topology similar to type II membrane proteins, and the recombinant proteins catalyze the epimerization of UDP-α-D-glucuronic acid (UDP-GlcA) to UDP-α-D-galacturonic acid (UDP-GalA).[11,12] UDP-GalA is a major precursor for the synthesis of pectin in plants, and pectin biosynthesis occurs in the Golgi apparatus;[13] however, our data indicate that GAE1 and 6 at least partially co-localize with AtGALT31A in the small compartments when expressed in *N. benthamiana*. A form of AGP that is covalently linked to pectin has recently been reported in APAP1 from *A. thaliana*,[14], thus GAE1 and 6 might provide UDP-GalA for the synthesis of a APAP1-type structure together with AGP GTs in the small compartments. .

Collectively, we present evidence that Y144 phosphorylation may regulate AtGALT29A enzyme activity in addition to Y144's involvement in determining the subcellular targeting of AtGALT29A, as reported previously.[7] Furthermore, we identified that *A. thaliana* APY3, GAE1, and GAE6 localize to the small compartments together with AtGALT31A when expressed transiently in *N. benthamiana*. Previously we have observed the dual localization of AtGALT31A in the Golgi and in the small compartments in both expression systems using *N. benthamiana* and *A. thaliana* mutant as hosts[1]. Also, we rarely observed localization of *N*-glycan processing enzymes in the small compartments when expressed in *N. benthamiana*,[1] which is consistent with the localization of the native enzymes detected by

antibodies. Therefore, we consider the dual localization observed for APY3, GAE1 and GAE6 when expressed in *N. benthamiana* is not likely an artifact of heterologous and transient expression using *N. benthamiana* as a host. However, we have previously observed a difference for the subcellular distribution for AtGALT31A expressed in *N. benthamiana* and *A. thaliana*.[1] We currently do not know the factors that caused the difference, but it might be due to different tissues, plant species, and/or expression methods of the proteins. Similar phenomenon might be true for APY3, GAE1 and GAE6, which should be investigated in future.

Nevertheless, at least partially APY3, GAE1 and GAE6 target to the small compartments together with AtGALT31A when expressed in *N. benthamiana* and therefore these enzymes may function together with AGP GTs in the small compartments. Further characterization of: (i) the proteins residing in the small compartments, e.g., protein kinases and phosphatases; (ii) targeting and retaining mechanism of those proteins in the small compartments; and (iii) biogenesis of the small compartments; are interesting questions to address to understand the function of the small compartments with respect to so-called the unconventional protein secretion in plants. Besides, the obtained knowledge may offer novel tools to make use of the unconventional protein secretion pathway of plants to produce useful recombinant products.[15-17]

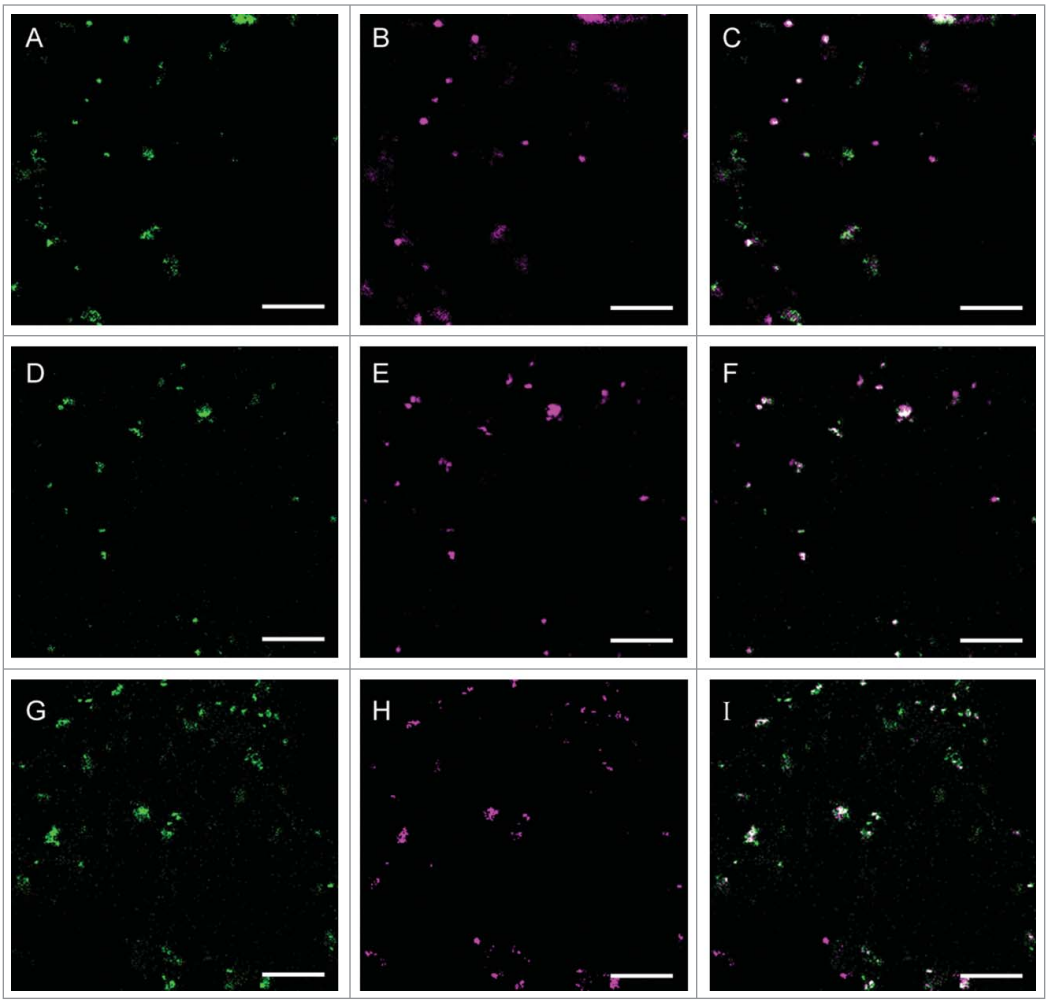

Figure 2. Co-localization of APY3, GAE1, and GAE6 with AtGALT31A in the small compartments. AtGALT31A-mCer3 (A-I, green) was co-expressed with APY3-YFP (A-C, magenta), GAE1-YFP (D-F, magenta), and GAE6-YFP (G-I, magenta) in *N. benthamiana* leaves. AtGALT31A-mCer3 frequently co-localized with APY3-YFP, GAE1-YFP, and GAE6-YFP in the small compartments, which are approximately 0.5 μm in diameter. Scale bars = 10 μm.

Methods

DNA cloning

DNA constructs of AtGALT31A-mCer3[7], AtGALT29A-mCer3[7], AtGALT29A Y144A[1] and Y144E[1] were described previously. Full-length cDNAs encoding APY3 (*At1g14240*), GAE1 (*At4g30440*), and GAE6 (*At3g23820*) were kindly provided by Dr. Joshua Heazlewood (Joint BioEnergy Institute, Lawrence Berkeley National Laboratory). Genes without stop codons were cloned into the Gateway vector, pDONR223, using following primer pairs: APY3-sense: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACACCGGAGACGGATG; APY3-antisense: GGGGACCACTTTGTACAAGAAAGCTGGGTGAAAGCCAAGATATTTTCTAG; GAE1-sense: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCTTCAATAGAAGATG; GAE1-antisense: GGGGACCACTTTGTACAAGAAAGCTGGGTGATGTACAAGCTTGGCTTTAG; GAE6-sense: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCCCTGTCGGCGAC; and GAE6-antisense: GGGGACCACTTTGTACAAGAAAGCTGGGTGAGCGGAATCTTCGGCGTG.

The inserts were further moved to the expression vectors (pEarleyGate mCer3 and pEarleyGate 101) and transformed into the *Agrobacterium tumefaciens* strain C58C1 pGV3850 according to the method described previously.[18]

Expression of recombinant proteins and enzyme assay

Transient expression of the constructs in *N. benthamiana* leaves, purification of recombinant proteins, and the enzyme assay were performed following the method described previously.[7] The relative protein concentrations were estimated by densitometric analysis of the Western blot using ImageJ,[19] and the enzyme activities were adjusted to protein concentration.

Co-localization study

Subcellular localization of recombinant proteins was studied in *N. benthamiana* leaves 2 days after infiltration of Agrobacterium harboring the respective construct using a Leica TCS SP5 inverted confocal laser scanning microscope (www.leica.com) as described previously.[7] For detecting the mCer3 and YFP signals, excitation was performed by argon laser at 458 and 514 nm, and emission was detected at 475–505 and 525–600 nm.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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