A Plasmodesmal Glycosyltransferase-Like Protein

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Abstract

Plasmodesmata (Pd) are plant intercellular connections that represent cytoplasmic conduits for a wide spectrum of cellular transport cargoes, from ions to house-keeping proteins to transcription factors and RNA silencing signals; furthermore, Pd are also utilized by most plant viruses for their spread between host cells. Despite this central role of Pd in the plant life cycle, their structural and functional composition remains poorly characterized. In this study, we used a known Pd-associated calreticulin protein AtCRT1 as bait to isolate other Pd associated proteins in Arabidopsis thaliana. These experiments identified a beta-1,6-N-acetylglucosaminyl transferase-like enzyme (AtGnTL). Subcellular localization studies using confocal microscopy observed AtGnTL at Pd within living plant cells and demonstrated colocalization with a Pd callose-binding protein (AtPDCB1). That AtGnTL is resident in Pd was consistent with its localization within the plant cell wall following plasmolysis. Initial characterization of an Arabidopsis T-DNA insertional mutant in the AtGnTL gene revealed defects in seed germination and delayed plant growth.

Introduction

Perhaps one of the most intriguing, yet least studied, aspects of intercellular communication and transport in most higher eukaryotes, from mammals to plants, is traffic of macromolecular complexes through direct cytoplasmic bridges between the adjacent cells. These cytoplasmic intercellular connections, termed tunneling nanotubes (TNTs) [1,2] in mammals and plasmodesmata (Pd) [3–5] in plants, are involved in such major cellular events as transfer of organelles and membrane-bound vesicles between mammalian cells [1] and spread of regulatory molecules, such as different transcription factors and RNA silencing signals [6–10], between plant cells. Furthermore, these transport mechanisms are subverted by pathogens for their movement between the host cells [2,11]. Thus, the importance of Pd in plant physiology, development, morphogenesis, and interactions with biotic and abiotic environmental factors is impossible to overestimate, yet our understanding of their molecular composition is still incomplete.

Pd are lined with the plasma membrane, and their central region is occupied by the ER, which spans Pd and forms a continuum between the adjacent cells [4,12]. The space between the ER and the plasma membrane contains permeable channels [13], through which molecules move from cell to cell [14]. This space, as well the intra-Pd plasma membrane and trans-Pd ER, contain numerous proteins, the identity of which has begun unravel only in the course of the last two decades. To date, Pd have been shown to contain or associate with calreticulin [15–18], a beta-1,3-gluconase [19], type I membrane receptor-like proteins (PDLPs) [20], a protein kinase [21], Pd callose binding proteins (PDCBs) [22], class 1 reversibly glycosylated polypeptides (C1RGPs) [23], and actin/myosin filaments [24,25]. The hunt for additional Pd components continues, and this communication reports the discovery of a Pd-associated core 2/I branching beta-1,6-N-acetylglucosaminyl transferase-like protein (GnTL).

Results

Identification of AtGnTL

To define better the complement of the Pd-associated proteins, we searched our yeast two-hybrid (Y2H) cDNA library from Arabidopsis thaliana [26,27] for interactors with Arabidopsis calreticulin, AtCRT1, known to accumulate within Pd in several plant species, including Arabidopsis. To avoid non-specific interactions via calcium ion-binding domains of calreticulin, we used as bait a fragment of AtCRT1 that lacked these sequences. These experiments isolated a cDNA prey encoding a protein product that interacted with the AtCRT1-based bait (Fig. 1A). Amino acid sequence analysis of this interactor protein, broadly designated as a putative glucosaminyl transferase-like enzyme (AtGnTL, AGI code At3g52060, GenBank accession number NM_180350), revealed that it belongs to the annotated family of core 2/I branching beta-1,6-N-acetylglucosaminyl transferases, with members in more than 19 plant species, including such agronomically important and diverse crops as poplar (Populus trichocarpa, XP_002315417) and grape (Vitis vinifera, XP_00226454) (Fig. 1A). Fig. 1B shows that AtGnTL is a 346-residue protein, contains two distinct functional domains, an amino-terminal signal peptide, inherent to endoplasmic eukaryotic proteins [28,29], which is followed by a catalytic domain (GnT) of a Branch family/glycosyltransferase family 14 (Fig. 1A).
molecule to an acceptor via a glycosidic bond. Further protein domain analysis using the Pfam database (http://pfam.sanger.ac.uk/) demonstrated that AtGnTL contains a conserved glutamic acid residue (Glu-263 in AtGnTL) potentially important for the enzymatic activity (Fig. 1).

The interaction between AtGnTL and full-length AtCRT1 was demonstrated in yeast and in plant cells. Fig. 2A shows that AtGnTL interacted with AtCRT1 in the Y2H system, and that this interaction was specific because it did not occur with topoisomerase 1 (TOP1) or with lamin C, known non-specific Y2H activators best suited to eliminate false positive interactions [30,31]. Specifically, co-expression of AtGnTL with AtCRT1, but not with topoisomerase 1 or lamin C, activated the HIS3 reporter gene (Fig. 2A, left panel). Under the non-selective conditions, all combinations of the tested proteins resulted in the efficient cell growth (Fig. 2A, right panel).

The Y2H data were then confirmed by an independent assay using bimolecular fluorescence complementation (BiFC), in which protein interaction is monitored in vivo, directly within living plant cells; furthermore, this approach simultaneously determines

Figure 1. AtGnTL belongs to the acetylglucosaminyl transferase family. (A) Identification of an AtCRT1 interactor in the Y2H library screen. Cells expressing the indicated combinations of proteins were grown on leucine/tryptophan-deficient medium and analyzed for β-galactosidase activity. (B) Amino acid sequence alignment of the *A. thaliana* GnTL (CAA05894) with its homologs from *P. trichocarpa* and grape (*V. vinifera*). Symbol designations: "*" identical residues, "*:" conserved substitutions, ".." semi-conserved substitutions. The conserved glutamic acid residue likely involved in the enzymatic activity is indicated by box, and gaps introduced for alignment are indicated by dashes. Alignment was performed using the ClustalW algorithm (http://www.genebee.msu.su/clustal/advanced.html). (C) Schematic representation of the AtGnTL protein domains. Positions of amino acid residues delimiting each of the indicated domains are shown.

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The notion that AtGnTL is a plasmodesmal protein was examined in further detail. Fig. 3 shows that GFP- or mCherry-tagged AtGnTL expressed in N. benthamiana leaf epidermis either from a constitutive 35S CaMV promoter or from its native promoter, respectively, accumulated in the Pd-like puncta. This punctate accumulation pattern was not affected by the nature of the protein tag, i.e., GFP or mCherry, and it was not observed with a free tag (Fig. 3).

Another characteristic feature of Pd is that they are embedded within the cell wall. We demonstrated that the AtGnTL-containing puncta indeed reside in the cell wall using the plasmolysis assay. During plasmolysis, only cell wall components retain their original localization pattern, whereas the cell cytoplasm and the plasma membrane become displaced and relocated following physical shrinking of the plasmolysed cells [39]. Fig. 4 shows that, following plasmolysis, the cell cytoplasm, visualized by transiently-expressed free CFP, indeed detached from the cell wall, with the cell content becoming compressed in the center of the cell interior. This detachment was best visible when the fluorescence data were superimposed over the phase images of the whole cells. In contrast, AtGnTL-mCherry coexpressed in the same cell retained its punctate localization pattern at the cell periphery (Fig. 4, arrows), indicating that these puncta are situated within the cell wall; note however, that some of the AtGnTL-mCherry population remained associated with the cytoplasm, apparently in microaggregates. As expected (see Fig. 3), AtGnTL-mCherry accumulated in the Pd-like puncta in non-plasmolysed cells (Fig. 4).

Finally, we examined whether AtGnTL actually colocalizes with a Pd marker, a cellular protein known to reside within Pd. To this end, we selected an Arabidopsis GPI-anchor callose binding protein AtPDCB1 which specifically localizes to the neck region of Pd [22,40]. We transiently coexpressed in N. benthamiana leaves AtGnTL tagged with GFP and AtPDCB1 tagged with mCherry, and analyzed the distribution of the corresponding fluorescent signals. Fig. 5 shows that both proteins accumulated in the characteristic punctate patterns on the cell periphery, and that many, but not all, of such puncta overlapped each other (arrows), indicating colocalization. Note that, consistent with previous data [22,40], AtPDCB1 also exhibited some cell wall-associated signal between puncta; obviously, these areas represented the non-overlapping signal (Fig 5). Quantification of colocalization based on the number of individual signal puncta formed by each protein and on those of them that colocalized, indicated that 80% of AtGnTL-GFP colocalized with AtPDCB1-mCherry, and 49% of AtPDCB1-mCherry colocalized with AtGnTL-GFP.

Interestingly, AtGnTL practically did not colocalize with the Tobacco mosaic virus (TMV) cell-to-cell movement protein (MP) (Fig. 5), which also resides in Pd, forming distinct punctate patterns [41], but accumulates in the Pd inner areas, often within the central cavity [42]. Quantification of these data indicated only 6% of AtGnTL-mCherry/TMV MP-YFP colocalization and 3% of TMV MP-YFP/AtGnTL-mCherry colocalization. These observations suggest that AtGnTL and TMV MP may target different regions of Pd; this is unlike AtCRT which most likely accesses both Pd locations as it interacts with AtGnTL as well as with TMV MP [17]. These findings raise an interesting possibility of differential Pd targeting and/or localization patterns for different proteins.

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**Figure 2. AtGnTL interacts with AtCRT1.** (A) Interaction in the Y2H system. The indicated dilutions of cell cultures were grown on leucine/tryptophan-deficient medium either in the absence (left panel) or in the presence of histidine (right panel); in this assay system, cell growth without histidine represents the selective condition for protein-protein interaction. (B) Interaction in the BIFC assay in N. benthamiana. Inset: magnified image of the BIFC signal within the area indicated by dashed rectangle. YFP fluorescence is in green, plastid autofluorescence is in red. Images are single confocal sections. Bars = 20 μm.

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subcellular localization of the interacting proteins [32]. The AtGnTL-AtCRT1 interaction was tested in N. benthamiana, a choice plant for transient gene expression experiments. Fig. 2B shows that nYFP-tagged AtGnTL interacted with AtCRT1-cYFP in planta, resulting in the YFP fluorescence. This recognition of AtCRT1 was specific because it was not observed with cYFP-tagged unrelated Arabidopsis VIP1 and Agrobacterium VirF proteins (Fig. 2B). Importantly, most of the population of the interacting AtGnTL and AtCRT1 proteins accumulated at distinct punctate locations at the cell periphery (Fig. 2B, inset), which are diagnostic of plasmodesmata [Pd] [17,18,20,33–38], whereas some of the signal localized in cytoplasmic microbodies, possibly aggregates (Fig. 2B).

AtGnTL localizes to Pd

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Next, we partially uncoupled between the AtGnTL-AtCRT1 binding and Pd localization. Specifically, we identified a domain
within AtGnTL (AtGnTL1-91N), which comprises 91 amino-terminal residues of this protein, that still interacted with AtCRT1 in the BiFC assay in living plant cells (Fig. 6). The interaction between the nYFP-tagged AtGnTL1-91N mutant and AtCRT1-cYFP was specific because it was not observed with unrelated proteins, cYFP-VIP1 and cYFP-VirF (Fig. 6). Fig. 6 also shows that the AtGnTL1-91N-nYFP-AtCRT1-cYFP complexes accumulated at the cell periphery in a somewhat more diffused fashion than the AtGnTL1-91N-nYFP-AtCRT1-cYFP complexes (see Fig. 2B). These observations suggest that this amino-terminal domain of AtGnTL retains its ability to bind AtCRT1, but is at least partially compromised in its ability to traffic to and/or accumulate at Pd. The defect in Pd localization of AtGnTL1-91N was even more dramatic when this protein domain, tagged with mCherry, was expressed alone. Fig. 6 shows that AtGnTL1-91N-mCherry localized to large patches at the cell periphery, never forming the Pd-type puncta, which were clearly seen in parallel experiments with AtGnTL1-mCherry. Collectively, the data in Fig. 6 suggest that the AtGnTL1-91N has lost most of the ability of the full length AtGnTL to target to Pd, and that its interaction with AtCRT1 partially restored Pd targeting, potentially, by using the Pd localization activity of AtCRT1.

**AtGnTL affects plant growth and is expressed in the areal parts of the plant**

To better understand the biological role of AtGnTL, we isolated an Arabidopsis mutant, designated gnl-1, from the Salk collection [43] with a T-DNA insertion in the 5’UTR sequence of the AtGnTL gene (Fig. 7A, B). We then produced homozygous gnl-1 plants (Fig. 7C) and used RT-PCR to demonstrate that, unlike the wild-type plants, this gnl-1 line did not express the AtGnTL mRNA whereas both wild-type and mutant plants produced transcripts specific for a constitutively expressed TUBULIN gene (Fig. 7D). Interestingly, the absence of AtGnTL expression in the gnl-1 mutant did not detectibly alter the Pd-specific localization pattern of TMV MP or AtCRT1 (Fig. 8), indicating that AtGnTL is not required for transport of these proteins to Pd. Thus, that AtGnTL1-91N prevented Pd targeting of AtCRT1 (see Fig. 6) is most likely due to steric interference between these two proteins.

The major apparent phenotypic characteristic of the gnl-1 mutant was its reduced seed germination frequency and delayed...
plant growth (Fig. 9). Specifically, when seeds of the wild-type and *gntl-1* mutant plants were plated on agar media and germination frequencies of each population were measured, nearly 90% of the wild-type seeds germinated within six days, whereas only up to 60% of the *gntl-1* mutant seeds germinated even after 13 days (Fig. 9). Once germinated, the mutant seedlings exhibited significantly delayed growth until week 5 post germination. At

**Figure 4.** Subcellular localization of AtGnTL-mCherry in plasmolysed and non-plasmolysed tissues of *N. benthamiana*. mCherry fluorescence is in red, CFP fluorescence is in blue; plastid autofluorescence was filtered out. Punctate mCherry fluorescence pattern at the periphery of the plasmolysed cells visible in the merged phase/fluorescence images is indicated by arrows. Note that, because, without plasmolysis, the CFP signal outlines the cells, we did not include the phase images of non-plasmolysed cells. Images are single confocal sections. Bar = 20 μm. doi:10.1371/journal.pone.0058025.g004

**Figure 5.** Colocalization of AtGnTL-GFP with AtPDCB1-mCherry, but not with TMV MP-mCherry, in *N. benthamiana*.
that time, the mutant plants approached the size of their wild-type counterparts, albeit never reaching it completely (Fig. 10). At present, we do not have sufficiently detailed knowledge of the AtGnTL function in the plant to explain the mutant phenotype. Obviously it is likely related to potential defects in glycan synthesis either on the global plant scale or in specific tissues; for example, mutations in a different type of glucosyltransferase has been shown to cause defective seed phenotypes [44].

Finally, we analyzed the overall expression pattern of AtGnTL in Arabidopsis plantlets. To this end we produced three independent lines of Arabidopsis plants transgenic for AtGnTL tagged with the β-glucuronidase (GUS) reporter and driven by the AtGnTL native regulatory sequences. Fig. 11 shows that all three lines exhibited relatively strong GUS expression throughout the shoot, but not in the root areas. As expected no GUS activity was detected in control, wild-type plantlets (Fig. 11). Thus, the native AtGnTL gene expression is most likely restricted to the areal parts of the plant. The biological rationale for this expression pattern remains to be elucidated.

Discussion

The important role of Pd in many aspects of the plant life cycle combined with our relatively limited understanding of the mechanisms of Pd function call for identification of their full protein complement. One way to approach this task is by employing an already known Pd protein as a lead to isolate other, otherwise unpredictable, proteins that attach to it and, thus, are expected also to associate with Pd. Using this rationale, we identified an interactor of AtCRT1, an ER chaperone found in Pd [15–18]. Subcellular localization experiments showed that this protein, AtGnTL, an annotated member of the glycosyltransferase superfamily, indeed represents a novel type of Pd-associated enzymes. Beta-1,6-N-acetylglucosaminyl transferases play crucial roles in glycan synthesis [45] and are known to catalyze attachment of oligosaccharide side chains to glycoproteins [46]. That a beta-1,6-N-acetylglucosaminyl transferase is involved in Pd function makes biological sense. Increasing evidence indicates that sugar-containing molecules, such as glucans and glycans, take part in Pd biogenesis and regulation. For example, among other factors, Pd permeability is modulated by callose (a beta-1,3-glucan)
sphincters surrounding the Pd orifices [40,47–50], and glycosylation has been implicated in control of protein transport through Pd [51,52]. Although the specific role of AtGnTL in Pd function requires further experimentation, it is tempting to speculate that this putative enzyme may act to modify other Pd component(s) or even the transported proteins en route to the neighboring cells. The latter possibility is especially intriguing as it would suggest that Pd not only transport proteins between cells, but also participate in post translational modification of the transport substrates such that a molecule that enters the “recipient” cell is biochemically different from the same molecule that has left the “donor” cell. This cargo modification most likely would occur within trans-Pd ER, a strand of ER that traverses Pd [4] and in which AtGnTL and its interactor AtCRT1 most likely are located.

The ability of AtGnTL to interact with AtCRT1 suggests that AtCRT1 may play a role in the AtGnTL function at Pd, for example by acting as a chaperone, a known activity of calreticulin [53,54], in presenting the AtGnTL substrates. Indeed, both proteins likely reside in the ER, with AtCRT1 being a known ER chaperone [53,54] and AtGnTL containing a signal peptide (see Figure 7. Characterization of the gntl-1 Arabidopsis mutant line. (A) Location of the mutagenic T-DNA insertion in the 5’ UTR region of the AtGnTL gene. Positions of nucleotide residues delimiting the gene and its UTR regions are shown. (B) Mutagenic T-DNA insertion in the gntl-1 line. AtGnTL-T-DNA right border integration junction sequence is shown, in which the T-DNA sequence is shown in lower case and shaded; the nucleotide positions of the AtGnTL sequence are indicated. (C) PCR analysis of the wild-type and homozygous gntl-1 plants. Lanes M, molecular size markers; lanes 1, AtGnTL UTR-specific primers; lanes 2, AtGnTL UTR/T-DNA-specific primers. The wild-type AtGnTL gene and its gntl-1 allele containing the mutagenic T-DNA are represented by 1.0-kb and 1.1-kb PCR products, respectively. (D) RT-PCR analysis of the wild-type and homozygous gntl-1 plants. Constitutively expressed TUBULIN gene was used as internal control. Lane M, molecular size markers; lanes 1, 2, wild-type and homozygous gntl-1 plants, respectively, analyzed with AtGnTL mRNA-specific primers; lanes 3, 4, wild-type and homozygous gntl-1 plants, respectively, analyzed with TUBULIN mRNA-specific primers.

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Figure 8. Pd localization of TMV MP-YFP and AtCRT1-DsRed2 in the wild-type and homozygous gntl-1 Arabidopsis plants. YFP fluorescence is in green, DsRed2 fluorescence is in red; plastid autofluorescence was filtered out. Images are single confocal sections. Bars = 20 μm.

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Figure 9. Loss of AtGnTL function leads to reduced seed germination frequency. Red and blue bars represent the wild-type and homozygous gntl-1 Arabidopsis plants, respectively. Both types of seeds were collected simultaneously from the parental plants grown under identical conditions. The data were represent average values of three independent experiments with indicated standard deviations.

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Fig. 1B, C, one of the hallmarks of ER proteins [28,29]. Within the ER they may cooperate, potentially along with calnexin and other chaperones known to function together with glucosyltransferases, in glycoprotein folding and quality control [55]. Alternatively, AtCRT1 may simply chaperone the AtGnTL protein itself, helping it to fold properly.

Regardless of the exact mechanism of the AtGnTL action, the observation that a putative glycosyltransferase-like protein may reside and function at or around Pd suggests that plant intercellular connections are not just conduits for macromolecular transport but also represent sites for glycan synthesis and, potentially, post translational modification.

Materials and Methods

Plants

Wild type A. thaliana (Columbia-0 ecotype) and the gntl-1 T-DNA insertion line (SALK_012392.54.50.X, obtained from ABRC), and N. benthamiana plants were used in the experiments. All plants were grown under the same conditions in soil in an environment-controlled chamber at 22–24°C under long day conditions of 16 h white light (70–80 μmol photons m-2 s-1) and 8 h dark. At least 10 plants were used for each experiment condition, and all experiments were repeated three times. For PCR-based analyses, Arabidopsis genomic DNA was extracted using DNeasy Plant Kit (Qiagen). Then, to identify plants carrying the wild-type AtGnTL, we utilized the gene-specific forward and reverse primers 5'-AGGGGAATAATGACGTCAGCAAAA-3' and 5'-AGCTGAGATGTTGCCAGGAGAAGT-3', respectively, whereas plants homozygous for T-DNA insertion in this gene, the gntl-1 mutant, were identified using the gene-specific forward primer 5'-AGGGGAATAATGACGTCAGCAAAA-3' and the T-DNA left border-proximal reverse primer 5'-ATCAACAAG-GATTTTCGGCTGCT-3'.

Y2H assay

For bait construct, a AtCRT1 sequence (GenBank accession number NM_104513.2) fragment containing amino acid residues between positions 21 to 38, a CFP linker, and residues 415 to 425 was cloned into the BglII-SalI sites of a LexA plasmid pSTT91.
(TRP1+) [56]. Arabidopsis cDNA library in pGAD424 (LEU2+, Clontech), as well as human lamin C and topoisomerase 1 in pSST91 were described previously [26,27,57]. The A*cRT71 cDNA was cloned into the Smal-PstI sites of pSST91, and the AtGoTL cDNA was cloned into the EcoRI-Sall sites of pGAD424; to allow efficient Y2H transcriptional activation, both cDNA clones lacked their 25-bp and 30-bp 5’-terminal sequences, respectively, coding for the amino-terminal signal peptides. All plasmids were introduced into yeast cells using a standard lithium acetate protocol [58]. Protein interactions were selected in Saccharomyces cerevisiae strain L40 (MATa his3A200 tp1-301 leu2-3,112 ade2 lys2-801amas URA3::(lexAop)4-HIS3) [39] by growing cells for 3 days at 30°C on a leucine-, tryptophan- and histidine-deficient medium. All positive interactions were confirmed by a β-galactosidase assay as described [60].

**Agroinfiltration and microbombardment**

For agroinfiltration of N. benthamiana, binary plasmids were introduced into the Agrobacterium strain GV3101 [61] grown overnight at 25°C and infiltrated into intact leaves as described [62,63]. For biotic delivery of Arabidopsis, 100 µg DNA was absorbed onto 10 mg of 1-µm gold particles (Bio-Rad) and microbombarded into leaf epidermis at a pressure of 90–150 psi using a portable Helios gene gun system (Model PDS-1000/He, Bio-Rad) essentially as described [41]. After incubation for 36–48 h at 22–24°C, the agroinfiltrated or microbombarded tissues were viewed under a Zeiss LSM 5 Pascal confocal laser scanning microscope. All PCR reactions were performed using a high-fidelity Phu DNA polymerase (Stratagene) and products were verified by DNA sequencing. All experiments were repeated at least three times.

**Confocal microscopy**

Specimen preparation and imaging were performed as described [39]. Briefly, plant tissue samples were mounted in water between number 1 1/2 coverglasses, using silicon vacuum grease to create spacers between the glass surfaces. Images were collected with a Zeiss LSM 5 Pascal laser scanning confocal microscope system. In all cases, a high numerical aperture (1.2–1.3) water immersion objective (60–63x) was employed. A 458 nm, 488 nm, or 514 nm line from an argon ion laser was used to excite CFP, GFP, and YFP, respectively, and 543 nm or 587 nm line from a helium-neon ion laser was used to excite DsRed2 or mCherry, respectively. All image acquisition, i.e., laser intensity and photomultiplier tube (PMT), settings were preserved between different experiments. On average 100–120 cells were analyzed for each experiment.

**BiFC**

The full-length AtCRT71 cDNA was cloned into the BglII-Sall sites of pSATAc-eYFP-N1 GenBank accession number DQ169002). The full-length AtGoTL cDNA and the AtGoTLI-91N sequence coding for the first 91 amino acid residues of AtGnTL were cloned into the Xhol-KpnI sites of pSAT4A-eYFP-N1 (GenBank accession number DQ169003). Note that, in both of these tagged constructs, the fusion proteins retain their native amino-terminal signal peptide sequences. cYFP-AvIVP1 and cYFP-VIrF fusion constructs were described previously [64,65]. The tested construct pairs were mixed 1:1 (w/w) and microbombarded into N. benthamiana leaves, allowed to express for 36–48 h at 22–24°C, and analyzed by confocal microscopy.

**Protein localization**

The full-length AtGoTL cDNA was cloned into the Xhol-KpnI sites of pSAT6-EGFP-N1 (GenBank accession numberAY181382). The mCherry coding sequence (GenBank accession number JQ627840) was cloned into the KpnI-BamHI sites of pSAT3-MCS – which is identical to pSAT6-MCS (GenBank accession number AY181383), except that the entire expression cassette is flanked by I-CelI [66] – to produce pSAT3-mCherry-N1. The AtGoTLI-91N cDNA was then cloned into the Xhol-KpnI sites of pSAT5-mCherry-N1. For native promoter construct, we first replaced the CaMV 35S promoter in pSAT3-mCherry-N1 with the native AtGnTL promoter (John Innes Centre). The tested binary constructs, i.e., those expressing AtGnTL-GFP and AtPDCB1-mCherry or TMV MP-mCherry, were microinfiltrated into the Xhol-KpnI and KpnI-BamHI sites, respectively, of this vector. For microinfiltration, the expression cassettes were excised with AgeI or I-CeuI from their pSAT6- or pSAT3-based vectors, respectively, and each inserted into a separate pPZP-RCS2 binary vector [66,67]. For microbombardment, the proteins were expressed directly from the pSAT-based vectors. The construct expressing TMV MP-YFP was described previously [40]. The AtCRT71-DsRed2 expression construct based on the pSAT4 plasmid [66] was a generous gift from Dr. Ueki (Okayama University). The tested constructs were microinfiltrated into N. benthamiana or microbombarded into Arabidopsis, incubated for 36–48 h at 22–24°C, and observed by confocal microscopy. For quantification, we counted the total number of puncta in confocal images for each of the individual proteins (i.e., AtGnTL-GFP, AtPDCB1-mCherry, and TMV MP-YFP) as well as those puncta that show colocalization on merged images. We then calculated the percentage of the colocalized signal relative to total number of puncta for each protein.

**Plasmolysis**

The AtGoTL cDNA was inserted into the Xhol-KpnI sites of pSAT3-mCherry-N. CFP coding sequence was subcloned from pSET-CFP (Invitrogen) into the Ncol-Xhol sites of pSAT4-MCS (GenBank accession number DQ005466). The resulting AtGoTL-mCherry and CFP expression cassettes were excised with I-CeuI or I-SceI from their pSAT3- or pSAT4-based vectors, respectively, and each inserted into a separate pPZP-RCS2 binary vector. The resulting binary constructs were mixed at 1:1 (w/w) ratio and transiently expressed for 36–48 h at 22–24°C in N. benthamiana leaves following agroinfiltration. For plasmolysis, leaf sections were excised, incubated in 0.45 M mannitol as described [68] until epidermal cells were visibly plasmolysed, and examined by confocal microscopy.

**Protein colocalization**

The AtGoTL-GFP expression cassette was excised PI-PpII from pSAT6-EGFP-N1 and inserted into pPZP-RCS2. The AtPDCB1-mCherry binary construct [22] was kindly provided by Dr. Maule (John Innes Centre). The tested binary constructs, i.e., those expressing AtGoTL-GFP and AtPDCB1-mCherry or TMV MP-YFP and AtGoTL-mCherry, were mixed at 1:1 (w/w) ratio and transiently expressed for 36–48 h at 22–24°C in N. benthamiana leaves following agroinfiltration, and protein subcellular localization was analyzed by confocal microscopy.

**RT-PCR**

For RT-PCR, total RNA from two-week old seedlings was isolated with TRI-reagent (Molecular Research Center), treated with RNase-free DNase (DNA-free kit, Ambion), and 0.5 µg of purified DNA-free RNA was reverse-transcribed with Protoscript.
First Strand cDNA synthesis kit (New England Biolabs) and PCR-amplified for 20–32 cycles using AtGnTL 5′UTR- specific forward and reverse primers 5′-ATGGTACATCTTAGCCAGGATTGGTGT- TATTC-3′ and 5′-TCAATCCCTAAAGATACAGC- CAGGTCGTC-3′, respectively. The absence of contaminating genomic DNA was confirmed by PCR using TUBULIN-specific forward and reverse primers 5′-AGATTCCATCACAT- CAGGGTGTTGTC-3′ and 5′-TCTACCTACGCCCCGTTAA- CATCTC-3′, respectively. That an intron sequence to distinguish between PCR products derived from DNA and mRNA templates [69]; TUBULIN also served as an internal control of a constitutively expressed gene. These RT-PCR reactions amplified 1,041-bp and 1,141-bp products from the AtGnTL and TUBULIN transcripts, respectively.

Generation of transgenic Arabidopsis plants and GUS activity assay

For production of plants that express the AtGnTL-GUS fusion protein from the native AtGnTL regulatory elements, we utilized the 1-ko AtGnTL sequence upstream of the ATG codon, based on the size of the predicted AtGnTL intergenic region. This region was amplified from the wild-type Arabidopsis genomic DNA with the forward and reverse primers 5′-ACCGGTAGCTGA- and 5′-TCAATCCCTAAAGATACAGCAGG-GTGTTGTC-3′ and 5′-CCGGCTGACCGCTCTGCTT- TATACAACACAC-3′ and cloned into the AgeI-KpnI sites of the pSAT4-35SP-MCS-35ST-GUS vector, replacing the 35S promoter; pSAT4-35SP-MCS-35ST-GUS is based on the pSAT4-35SP-MCS-35ST vector [70] and was kindly provided by Dr. Adri Zaltsman (Stone Brook University). Next, the cDNA sequence of AtGnTL with its own translation initiation codon was inserted into the Xhol-KpnI sites downstream of the AtGnTL native promoter and in-frame to the GUS coding sequence. The resulting expression cassette was then transferred into the I-SceI site of the pPZP-RC82 binary vector [66,67], containing the bar gene for BASTA selection in its Xhol-BamHI sites [71]. This binary construct was introduced into the Agrobacterium EHA105 strain, used to transform the wild-type Arabidopsis plants by flower dipping [72], and transformants were obtained using BASTA selection.

To visualize GUS activity, transgenic Arabidopsis seedlings at the 4-leaf stage aseptically grown in baby food jars in MS agar with 3 mg/l BASTA [73] were assayed histochemically as described [73], and recorded under a Leica MZ FLIII stereoscope.

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Author Contributions

Conceived and designed the experiments: LZK VC. Performed the experiments: LZK. Analyzed the data: LZK VC. Contributed reagents/materials/analysis tools: LZK. Wrote the paper: LZK VC.

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