Localisation and Topogenesis Studies of Cytoplasmic and Vacuolar Homologs of the Galanthus nivalis Agglutinin

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The Galanthus nivalis agglutinin (GNA) is synthesized as a preproprotein. To corroborate the role of the different targeting peptides in the topogenesis of GNA and related proteins, different constructs were made whereby both the complete original GNA gene and different truncated sequences were coupled to the enhanced green fluorescent protein (EGFP). In addition, a GNA ortholog from rice that lacks the signal peptide and C-terminal propeptide sequence was fused to EGFP. These fusion constructs were expressed in tobacco BY-2 cells and their localization analyzed by confocal fluorescence microscopy. We observed that the processed preproprotein of GNA was directed towards the vacuolar compartment, whereas both the truncated forms of GNA corresponding to the mature lectin polypeptide and the rice ortholog of GNA were located in the nucleus and the cytoplasm. It can be concluded, therefore, that removal of the C-terminal propeptide and the signal peptide is sufficient to change the subcellular targeting of a normally vacuolar protein to the nuclear/cytoplasmic compartment of the BY-2 cells. These findings support the proposed hypothesis that cytoplasmic/nuclear GNA-like proteins and their vacuolar homologs are evolutionarily related and that the classical GNA-related lectins might have evolved from cytoplasmic orthologs through an evolutionary event involving the insertion of a signal peptide and a C-terminal propeptide.

Keywords: Cytoplasmic ortholog — Galanthus nivalis agglutinin — Lectin — Subcellular location.

Abbreviations: CTP, C-terminal propeptide; EGFP, enhanced GFP; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; GFP, green fluorescent protein; GNA, Galanthus nivalis agglutinin; PVC, prevacuolar compartments; SP, signal peptide; UTR, untranslated region.

Introduction

In 1987 the first plant lectin with an exclusive specificity towards mannose was isolated from Galanthus nivalis (snowdrop) bulbs (Van Damme et al. 1987). The so-called Galanthus nivalis agglutinin, or GNA, is a homotetramer of non-covalently linked 12 kDa monomers. Each monomer consists of three tandemly arrayed subdomains harboring a mannose-binding site. GNA is synthesized on the endoplasmic reticulum (ER) as a preproprotein (Van Damme and Peumans 1988, Van Damme et al. 1990, Van Damme et al. 1991). After co-translational removal of the signal peptide (SP), the proprotein undergoes a proteolytic cleavage of the C-terminal propeptide (CTP). Though it is evident that GNA follows the secretory pathway, neither the site of the post-translational processing nor the final destination of the mature protein have yet been identified (Van Damme and Peumans 1990). It is hypothesized that GNA localizes in the vacuole similarly to other plant lectins that are synthesized as preproproteins.

During the last two decades numerous plant proteins have been identified that are genuine orthologs of GNA and, like GNA, are synthesized as preproproteins with an N-terminal SP and a CTP (Van Damme et al. 1991, Van Damme et al. 1998). In the meantime, GNA-like proteins or corresponding genes were also identified outside the plant kingdom, namely in the slime mold Dictyostelium discoideum (Jung et al. 1996), the pufferfish Takifugu rubripes (Tsutsui et al. 2003, Tsutsui et al. 2006), the freshwater sponge Lubomirskia baicalensis (Wiens et al. 2006) and various fungi (Machida et al., 2005). Though no data have been reported about the sugar-binding activity of most of these non-plant GNA homologs, biochemical analyses of the so-called comitin from D. discoideum (Jung et al. 1996) and puffin from T. rubripes (Tsutsui et al. 2003, Tsutsui et al. 2006) provided firm evidence that these proteins contain a functional mannose-binding domain. Importantly, cloning of the corresponding genes revealed that comitin and puffin lack an SP and, accordingly, do not follow the...
secretory pathway but are synthesized on free ribosomes in the cytoplasm. Evidently, the identification of these presumed cytoplasmic GNA homologs was somewhat surprising because it implies that the same sugar-binding domain is located in functionally different cell compartments in plants, slime molds and fishes. The issue of the different subcellular locations became even more puzzling after recent transcriptome analyses revealed that some plant genes also encode primary translation products that share a high sequence identity with mature GNA but lack the SP and the CTP (Van Damme et al. 2004a). The identification of these non-vacuolar GNA-like plant proteins implies a new role for mannose-binding lectins in plants (Van Damme et al. 2004b). It was suggested that the newly identified cytoplasmic GNA homologs are regulatory/signaling plant proteins, functionally different from their defense/storage-related vacuolar counterparts. In addition, it was proposed that the cytoplasmic lectins may have served as templates for the development of their vacuolar homologs through the insertion of an SP and a CTP (Van Damme et al. 2004a).

To test this hypothesis, we have investigated the roles of the different targeting peptides in the topogenesis of GNA and related lectins. Confocal microscopy of enhanced green fluorescent protein (EGFP)-tagged fusion proteins expressed in tobacco BY-2 cells demonstrated that both the SP and the CTP are necessary and sufficient to target GNA to the vacuole. Removal of the CTP severely affected the trafficking of the fusion protein, which was retained in the ER. Expression of GNA lacking the CTP caused a dwarfing phenotype in transgenic Arabidopsis plants, suggesting that the CTP is required not only for vacuolar delivery but also to inactivate the lectin in the ER temporarily. In contrast to GNA, EGFP fused to either mature GNA or a presumed cytoplasmic GNA ortholog from rice was exclusively located in the nucleus and the cytoplasm. The impact of these findings on the understanding of the molecular and functional evolution of a vacuolar lectin from an existing cytoplasmic homolog is discussed.

**Results**

*Expression and localization of the GNA–EGFP fusion protein in BY-2 cells*

Previous biochemical and molecular studies have indicated that GNA is synthesized as a preproprotein with an N-terminal SP and a CTP (Van Damme and Peumans 1990, Van Damme et al. 1991). However, the exact subcellular location of the snowdrop lectin or any other related plant lectin remains to be determined. The recent identification of presumed cytoplasmic orthologs of GNA (Van Damme et al. 2004a) prompted us to address this issue. We therefore studied the subcellular location of GNA and the role of the SP and CTP in detail. EGFP-tagged fusion proteins (Fig. 1) were transiently expressed in tobacco Bright Yellow-2 (BY-2) cells and the expression analyzed at various time points after transformation using confocal fluorescence microscopy. As controls we expressed untargeted EGFP (free EGFP) and sweet potato sporamin C-terminally fused to EGFP. As expected, free EGFP was exclusively located in the cytoplasm and nucleus (Fig. 2m) whereas sporamin-EGFP accumulated in the central vacuole (Fig. 2n). Time course studies revealed no visible changes in the localization patterns of free EGFP and sporamin-EGFP as a function of the time elapsed after transformation (data not shown).

Expression analysis of the entire coding sequence of GNA (including a 23 amino acid SP, the mature 109 amino acid lectin polypeptide and the 24 amino acid CTP) fused at its C-terminus to EGFP (SP–GNA–CTP–EGFP) (Fig. 1a) demonstrated that 24 h after transformation of BY-2 cells intense EGFP punctate structures were visible around the nucleus and at the edge of the cell (Fig. 2a). A projection of different stacks in the Z-direction confirmed that the fluorescence appears as punctate stains distributed over the whole cell (Fig. 2a insert). No visible changes could be seen in the fluorescence pattern between 24 and 48 h after transformation.
The signal peptide and C-terminal propeptide of GNA are necessary and sufficient for targeting of GNA to the prevacuolar compartments (PVCs).

To confirm the role of the SP, a construct was made in which the SP of preproGNA was replaced by EGFP (EGFP–GNA–CTP) (Fig. 1b) and expressed in BY-2 cells. As shown in Fig. 2b, the corresponding fusion protein could be detected in the cytoplasm and the nucleus of the plant cells but not in the nucleolus. This distribution pattern is virtually the same as that of free EGFP (Fig. 2m) and, accordingly, confirms that the SP is necessary for GNA to enter the secretory pathway.
To corroborate the role of the CTP in intracellular trafficking, the entire coding sequence of GNA minus the 24 residues at the C-terminus was fused at its C-terminus to the EGFP marker (SP–GNA–EGFP) (Fig. 1c) and the corresponding protein expressed in BY-2 cells. As shown in Fig. 2c, fluorescence appeared in the ER and nuclear envelope. This pattern differs from the punctate staining pattern seen for SP–GNA–CTP–EGFP. No visible changes occurred as a function of time elapsed after transformation. Even 67 h after biolistic delivery, fluorescence was still detected around the nucleus, indicating that GNA lacking the CTP is retained in the ER. Therefore, it appears that the fusion protein encoded by SP–GNA–EGFP enters the secretory pathway but lacks an appropriate targeting sequence for further transport. These data indicate that the CTP is necessary to complete the intracellular trafficking of GNA.

To check whether the results obtained with SP–GNA–CTP–EGFP could have been influenced by the fact that the CTP was not located at the extreme C-terminus of the fusion protein but was followed by EGFP, we expressed a construct in which EGFP was inserted between the SP and the mature polypeptide of GNA (SP–EGFP–GNA–CTP) (Fig. 1d) in BY-2 cells. Microscopic analysis performed at different time points after transformation of tobacco BY-2 cells revealed three different localization patterns. Whereas shortly after transformation the fluorescent label was mainly concentrated around the nucleus (Fig. 2d), a punctate staining pattern similar to that observed for SP–GNA–CTP–EGFP was observed for SP–EGFP–GNA–CTP in the next phase (Fig. 2e). It seems that the fusion protein encoded by SP–EGFP–GNA–CTP enters the secretory pathway and might be transported into the PVCs since finally fluorescence was mainly observed in the central vacuole of transformed tobacco cells (Fig. 2f). The fluorescence pattern observed in the latter cells is similar to that for sporamin–EGFP (Fig. 2n) and was particularly observed at later time points. These localization patterns suggest that the final destination of GNA is the vacuole, and that GNA most probably reaches the vacuole via PVCs.

The results with SP–EGFP–GNA–CTP were confirmed by expression of a fusion protein consisting of EGFP flanked by the SP and CTP of GNA (SP–EGFP–CTP) (Figs. 1c, 2g). As shown in Fig. 2h, SP–EGFP–CTP is transported to the vacuole of the BY-2 cells. This implies that the CTP of GNA is able to direct EGFP to the same cell compartment as the full-length proGNA. From this we can conclude that—provided that the construct enters the secretory pathway—the CTP is sufficient for vacuolar targeting. It should be noted that fusion of the CTP of the GNA precursor to the C-terminus of free EGFP (EGFP–CTP) (Fig. 1f) did not affect the subcellular location of GFP. One day after particle bombardment, fluorescence was detected in the nucleus and the cytoplasm of plant cells. As shown in Fig. 2i, representing a BY-2 cell which is less vacuolated, the fluorescence is abundant in the cytoplasm and the nucleus. Between 48 and 60 h, the fusion protein was apparently more concentrated in the nucleus, but still occurred in the cytoplasm.

A rice GNA-like protein lacking a signal peptide and C-terminal propeptide resides in the cytoplasm and nucleus

Recently an expressed GNA homolog was identified in rice (GenBank accession No. CA755500) (further referred to as GNA_{rice}) that apparently lacks both an SP and a CTP. Though there is no guarantee that the deposited sequence starts with a 5’-untranslated region (UTR), it definitely includes a 3’-UTR [including a poly(A) tail]. Accordingly, there is no doubt that GNA_{rice} lacks the CTP found in GNA. Taking into account that all other identified GNA orthologs without a CTP (from Dictyostelium, fishes, fungi and the fresh water sponge L. baicalensis) are synthesized without an SP, one can reasonably assume that GNA_{rice} also does not include an SP. Therefore, GNA_{rice} can be considered a naturally occurring nucleocytoplasmic counterpart of the vacuolar GNA orthologs. Sequence alignment confirmed that GNA_{rice} corresponds to the mature GNA polypeptide and contains three fully active mannoside-binding sites (Fig. 3).

![Fig. 3](alignment.png) Alignment of the amino acid sequences of GNA and a cytoplasmic GNA-like protein from Oryza sativa (referred to as Orysa). The signal peptide and C-terminal propeptide of GNA are indicated in lower case letters. Orysa and mature GNA share 37.6% identity and 73.4% similarity, respectively (in the overlap covering the mature GNA polypeptide). Residues forming the conserved carbohydrate-binding sites are boxed in grey.
To check the predicted subcellular location, the coding sequence of GNA
rice was fused to the C-terminus of EGFP (EGFP–GNA
rice) and transiently expressed in tobacco BY-2 cells (Fig. 1g). Confocal microscopy revealed that the corresponding fusion protein was located in the nucleus and the cytoplasm (Fig. 2k). The fluorescence pattern was similar to that of free EGFP in that the protein was also absent from the nucleolus.

Removal of the signal peptide and the C-terminal propeptide converts vacuolar GNA into a nuclear/cytoplasmic protein

The results obtained with EGFP–GNA
rice raised the question of whether vacuolar GNA can be converted into a nuclear/cytoplasmic protein by removal of its SP and CTP. To address this question, a construct was made in which the SP and the CTP of GNA were replaced by a methionine residue and EGFP, respectively, and transiently expressed in BY-2 cells (Me-GNA–EGFP) (Fig. 1h). Localization studies confirmed that the corresponding fusion protein is, like the rice homolog, confined to the cytoplasm and nucleus of the plant cell (Fig. 2i). This localization pattern did not change at different time points after transformation.

Intracellular localization of SP–GNA–CTP–EYFP in tobacco leaf epidermal cells

It has been shown that the vacuolar sorting of a protein with a C-terminal sorting determinant can be disrupted by the addition of two glycine residues at the extreme C-terminus, resulting in secretion of the protein from the cell (Dombrowski et al. 1993). However, when we fused the entire sequence of EGFP to the C-terminus of GNA, it labeled punctate structures within the cell. This unexpected behavior led us to investigate further the nature of these punctate structures by co-expression with fluorescent markers for different organelles in tobacco leaf epidermal cells. To make sure that the results obtained in vitro conform to the in planta situation, we infiltrated whole tobacco leaves with the constructs. We replaced EGFP with enhanced yellow fluorescent protein (EYFP) to facilitate co-expression with mGFP3-tagged marker proteins (Haseloff et al. 1997); this did not appear to affect the localization of the protein within the cell, which was very similar to that seen in BY-2 cells (Fig. 4a, b). We then co-expressed SP–GNA–CTP–EYFP with the ER/Golgi marker ERD2–GFP3 (Boevink et al. 1998) to test whether the punctate structures labeled by SP–GNA–CTP–EYFP were Golgi bodies. Interestingly, there was no co-localization with the Golgi apparatus, but it appeared that in some cells the SP–GNA–CTP–EYFP-labeled structures were distributed along the ER membranes (Fig. 4c–e). The staining of the ER network is clearly visible in images with a higher brightness (Supplementary Fig. S1).

We then speculated that, as GNA is a vacuolar protein, perhaps the punctate structures corresponded to PVCs. We therefore co-expressed SP–GNA–CTP–EYFP with the PVC marker spGFP3–BP80 (Hanton and Brandizzi 2006). As shown in Fig. 4h, there was no co-localization of the punctate structures labeled by SP–GNA–CTP–EYFP with spGFP3–BP80, indicating that SP–GNA–CTP–EYFP is not transported to the PVCs. The nature of the punctate structures remains unclear, but our data suggest that the CTP of GNA does not behave in the same manner as other CTPs that are responsible for vacuolar sorting.

Expression of GNA without the C-terminal propeptide causes a dwarving phenotype in Arabidopsis

To elucidate the role of the CTP of GNA, we overexpressed constructs encoding preproGNA and GNA lacking the CTP in Arabidopsis thaliana. Insertion of the transgenes in the genome of Arabidopsis plants was confirmed by PCR, and lectin expression was shown by Western blot analyses (Fig. 5C, D). PCR analysis on the genomic DNA of 2-week old Arabidopsis plants expressing preproGNA or the GNA construct lacking the CTP yielded PCR fragments of approximately 405 and 327 bp, respectively. The size of the amplified fragments is in agreement with the expected length of the sequences. In wild-type plants, there was no amplification of a GNA-related sequence. Western blot analysis using polyclonal antibodies directed against GNA revealed immunoreactive bands of 12 kDa in crude extracts from most transgenic lines expressing the preproGNA construct. The size of these polypeptides is in perfect agreement with the molecular mass reported for the GNA polypeptide. However, similar Western blot analysis on crude extracts from Arabidopsis plants expressing the GNA construct lacking the CTP revealed bands with a molecular mass that was clearly smaller than 12 kDa, suggesting that the lectin peptides synthesized in these plants are cleaved/degraded soon after synthesis.

Upon germination of the transgenic seeds, it was clear that transgenic plants expressing the GNA gene without the C-terminal propeptide were growing more slowly than plants expressing the construct for preproGNA (Fig. 5A). In addition, many plants died (~70%) before setting seeds, and those that survived were dwarfed (Fig. 5B) and set fewer seeds. Transgenic A. thaliana plants expressing a gene encoding the complete GNA sequence showed no visible phenotype and yielded a normal seed set.

All these observations clearly suggest that GNA synthesized without the CTP is cytotoxic. Taking into account that mature GNA has a high affinity for high-mannose N-glycans, it might be the case that in the absence of the CTP the newly synthesized lectin interferes with the synthesis/sorting of N-glycosylated proteins in the ER,
and that accordingly the C-terminal extension possibly plays a role in the temporal inactivation of this high-mannose N-glycan-binding lectin in the ER. It is very difficult to correlate the expression levels with the severity of the mutant phenotype since most dwarf plants do not survive. Furthermore, studies are hampered by the fact that we are dealing with a phenotype that results in much lower biomass for the dwarf plants, and much lower seed set.

**Discussion**

The combined results of previous biochemical/molecular analyses and recent transcriptome sequencing programs provided compelling evidence for the occurrence of two subfamilies of GNA-related proteins with distinct subcellular locations in plants. Though the sequence data indicate that the classical ‘vacuolar’ GNA orthologs follow the secretory pathway while their ‘cytoplasmic’ counterparts are synthesized on free ribosomes in the cytoplasm, the notions ‘vacuolar’ and ‘cytoplasmic’ are still tentative because the localizations of these proteins have not been studied. Moreover, it still remains to be explained how plants manage to synthesize in the ER lectins that, by virtue of their strong affinity for high-mannose N-glycans, could interact with newly synthesized N-glycosylated proteins which are present in the same cell compartment.

Since a detailed knowledge of the subcellular location is a prerequisite for further functional studies, the localization and intracellular trafficking of GNA and of a presumed cytoplasmic homolog from rice were investigated using confocal microscopy of EGFP-tagged fusion proteins expressed in BY-2 cells. Preference was given to a GFP-based approach because GFP technology has proven to be a very useful tool for bioimaging of plant cells (Brandizzi et al. 2004). GFP expression is not only easily detectable but also has the advantage of not affecting cell viability or physiology. Moreover, appropriate control experiments with free EGFP, which localizes to the nucleus and cytoplasm, and EGFP fused to various targeting sequences can be included.

Confocal microscopy analysis of the expression of the fusion protein encoded by constructs comprising the complete GNA coding sequence and EGFP (construct SP–EGFP–GNA–CTP) confirmed that GNA is synthesized in the ER and follows the secretory pathway (Van Damme and Peumans 1988, Van Damme et al. 1991). In addition, it could be demonstrated that GNA first
accumulates in punctate structures, and later in the central vacuole.

Analysis of the localization patterns of the EGFP fusion proteins encoded by constructs lacking the CTP of GNA (construct SP–GNA–EGFP) demonstrated that the propeptide is necessary for targeting of GNA to the PVCs. In the absence of the CTP, we detected fluorescent signal in the ER/nuclear envelope. Although it is not possible to exclude partial secretion of SP–GNA, the ER retention of newly synthesized proteins has been ascribed to aggregation following inhibition of glycosylation (Sparvoli et al. 2000) or to misfolding or incomplete folding and assembly (Hammond and Helenius 2002). However, since there is no evidence for glycosylation of the CTP of GNA, it is questionable whether glycosylation inhibition is responsible for the observed ER retention of GNA. A more plausible explanation is that the carbohydrate-binding sites of GNA synthesized without a CTP are fully accessible so that the protein can interact in the lumen of the ER with glycoproteins carrying high-mannose N-glycans, resulting in the formation of aggregates that are excluded from the secretory pathway. Mature GNA has a very high affinity for high-mannose N-glycans (Shibuya et al. 1988) and accordingly is fully capable of interacting with all ER-located glycoproteins carrying such glycans. Taking into consideration the similarity in sugar binding activity/specificity between GNA on the one hand and calnexin/calreticulin on the other hand, plants must have developed a mechanism to inactivate GNA-like lectins temporarily, to ensure that they cannot interfere with glycoprotein quality control and sorting in the ER. A simple but efficient mechanism could consist of the attachment of a protease-cleavable propeptide that would physically protect the carbohydrate-binding sites from their potential interaction with ER-resident glycoproteins.

Fig. 5 Phenotype of transgenic Arabidopsis plants overexpressing constructs encoding SP–GNA–CTP and SP–GNA without the C-terminal propeptide. (A) Four-week-old Arabidopsis plants expressing the preproGNA sequence (upper panel) and GNA sequence without the C-terminal propeptide (lower panel). Petioles of plants expressing GNA without the C-terminal propeptide are smaller than those of plants expressing preproGNA. (B) Seven-week-old plants. Transgenic plants expressing GNA without the C-terminal propeptide (right) show stunted growth in comparison with wild-type plants (left) and transgenic plants expressing preproGNA (middle). (C) PCR amplification of the lectin transgene on genomic DNA isolated from 2-week-old transgenic plants transformed with SP–GNA–CTP (lane 2) or SP–GNA (lane 3). There was no amplification on genomic DNA of wild-type plants (lane 1). Lane M shows the GeneRuler™ 100 bp DNA Ladder Plus from Fermentas (arrowhead = 500 bp, filled circle = 300 bp; St. Leon-Rot, Germany). (D) Western blot analysis of crude extracts from leaves of different transgenic lines expressing constructs encoding preproGNA (upper panel) or GNA without the C-terminal propeptide (lower panel), using an anti-GNA antibody. Purified GNA was loaded in lane GNA (positive control, 12 kDa).
target glycans. Experiments with transgenic *Arabidopsis* plants yielded additional evidence for the importance of the CTP. Plants expressing preproGNA grew normally, whereas those expressing the same protein without the CTP had a stunted growth. This observation strongly suggests that the CTP of GNA does not act solely as a targeting signal, but is also required for a temporal inactivation of the lectin in the ER.

The targeting of the SP–EGFP–CTP fusion to the vacuole, taken together with the fact that it is generally accepted that GFP fused to a signal peptide (see GFP) follows the secretory pathway and is transported to the extracellular compartment of plant cells (Zheng et al. 2004), provides firm evidence that the CTP of GNA is not only necessary, but also sufficient for targeting to the vacuole. We therefore suggest that the CTP of GNA can be considered as a C-terminal vacuolar sorting determinant. Interestingly, fusion of EGFP to the C-terminus of preproGNA (SP–GNA–CTP–EGFP) did not result in secretion of the protein. Dombrowski et al. (1993) had shown that adding two glycine residues at the C-terminus of the targeting signal of barley lectin resulted in secretion of the protein into the extracellular medium. The fact that SP–GNA–CTP–EGFP labels punctate structures within the cell indicates that the C-terminal localization of the targeting signal may be coincidental to the manner of its function, although this aspect remains to be investigated more fully.

Confocal microscopy confirmed that the presumed cytoplasmic GNA homolog from rice remains in the cytoplasmic/nuclear compartment of BY-2 cells. This observation is important because one cannot a priori preclude that a protein synthesized in the cytoplasm is, as was demonstrated for some galectins, secreted by a non-classical pathway (Boulianne et al. 2000). Localization studies of a truncated form of GNA corresponding to an artificial cytoplasmic ortholog confirmed that the original vacuolar protein was converted into a cytoplasmic/nuclear form through the deletion of the SP and CTP. These findings clearly demonstrate that, depending on the presence/absence of appropriate targeting sequences, the same carbohydrate-binding domain can be directed towards functionally different compartments of the plant cell.

The high sequence identity/similarity [e.g. GNA<sub>rice</sub> and mature GNA share 37.6% sequence identity and 73.4% similarity, respectively (in the overlap covering the mature GNA polypeptide)] suggest that the cytoplasmic and vacuolar GNA homologs from plants are evolutionarily related. As previously proposed, it seems likely that cytoplasmic/nuclear GNA-like proteins served as a template for the development of the vacuolar homologs through the insertion of an SP and CTP (Van Damme et al. 2004a). This particular evolutionary scheme can explain why vacuolar GNA-related lectins were hitherto exclusively found in plants, whereas cytoplasmic forms were also identified in a slime mold (*D. discoideum*) (Jung et al. 1996), a fish (*T. rubripes*) (Tsutsui et al. 2003), a freshwater sponge *L. baicalensis* (Wiens et al. 2006) and various fungi (Machida et al. 2005). Phylogenetic analysis of the identified cytoplasmic GNA-like proteins from plants, fungi, fishes and *L. baicalensis*, and the vacuolar GNA orthologs, which as far as is known are confined to plants, does not indicate GNA<sub>rice</sub> as a direct ancestor of the vacuolar GNA orthologs (Fig. 6). However, it should be emphasized here that there is evidence for the occurrence in plants of another type of cytoplasmic GNA ortholog that is very closely related evolutionarily to some fungal GNA othologs (e.g. from *Gibberella zeae* and *G. moniliformis*) (Van Damme et al. 2004a), and might be a better candidate ancestor of the vacuolar GNA orthologs from plants. The reason why GNA<sub>rice</sub> was chosen for the present study is that the near identity between the cytoplasmic GNA orthologs from, for example, *Zea mays* and *G. moniliformis* raised the still unanswered question of a possible contamination of the plant material with the pathogenic fungus.

Additional support for the proposed generation in plants of vacuolar GNA homologs from a cytoplasmic form is provided by the documented occurrence of a similar evolutionary event in the family Moraceae in which a cytoplasmic lectin served as a template for a vacuolar homolog (Peumans et al. 2000, Van Damme et al. 2004a).

The finding that some GNA homologs are located in the cytoplasmic/nuclear compartment of the plant cell is of paramount interest for our understanding of the physiological role of plant lectins. Indeed, one can reasonably assume that these lectins are at least in principle capable of interacting with N-glycosylated proteins present in those cell compartments that play a determining role in the regulation of most cellular processes. Though still speculative, cytoplasmic GNA homologs might be considered regulatory/signaling plant proteins functionally different from the defense/storage-related vacuolar GNA homologs.

**Materials and Methods**

*Plant material*

Nicotiana tabacum L. cv.1 BY-2 cells (Nagata et al. 1992) were grown in 250 ml Erlenmeyer flasks in 40 ml of BY-2 medium supplemented with 40 μl of a 1,000-fold concentrated BY-2 vitamin/hormone stock [containing per ml: 0.4 mg 2,4-D (dissolved in ethanol), 1 mg thiamine and 100 mg myo-inositol]. The BY-2 medium consists of 4.3 g l<sup>–1</sup> Murashige and Skoog (MS) micro and macro nutrients (Duchefa, Haarlem, The Netherlands), 30 g l<sup>–1</sup> sucrose, 0.2 g l<sup>–1</sup> KH<sub>2</sub>PO<sub>4</sub>, and is adjusted to pH 5.8 with 1 M KOH. The cell suspension culture was maintained at 25°C on a rotary shaker at 150 rpm in total darkness. Plant cell cultures
were refreshed weekly by diluting 1 ml of a dense culture in 40 ml of fresh BY-2 medium.

*Nicotiana tabacum* (cv Petit Havana) plants were grown at 25°C in a greenhouse. *Arabidopsis thaliana* (ecotype Col-0) was grown at 22°C, under a 16 h light/8 h dark photoperiod.

**GFP fusion constructs**

Different EGFP fusion constructs with GNA fragments were generated using the Gateway™ cloning technology developed by Invitrogen (Carlsbad, CA, USA). An overview of all constructs is given in Fig. 1. Primers were synthesized by Invitrogen. All constructs were expressed under the control of the 35S promoter.

All GNA fragments were amplified by PCR using the cDNA clone encoding GNA (GenBank accession No. M55556, Van Damme et al. 1991) as a template. Control constructs contained EGFP alone or EGFP coupled to the C-terminus of sporamin. The *Ipomoea batatas* Tainong 57 preprosporamin (gSPOR5-31) gene (GenBank accession No. U12436, Wang et al. 1995) was amplified from the plasmid pUC-SPOA101 (kindly provided by Kai-Wun Yeh, Institute of Plant Biology, National Taiwan University of Taipei). To generate a fusion of EGFP with the GNA ortholog from rice (*EGFP–GNArice*), PCR was performed on the pBluescript II SK(+) vector, containing the expressed sequence tag coding for the GNA ortholog from rice. This plasmid was kindly provided by Hans Bohnert (Department of Plant Biology, University of Illinois, Urbana, IL, USA) (GenBank accession No. CA755500).

For N-terminal and C-terminal fusion with EGFP, the destination vectors pK7FWG2 and pK7WGF2 were used, respectively (Karimi et al. 2002). The vector pK7WG2 was employed to generate the constructs where GFP is fused between two GNA fragments.

All cloning was carried out according to standard molecular biology techniques.

**Transient transformation of BY-2 cells and tobacco plants**

The different GNA constructs were transiently expressed in tobacco BY-2 cells using particle bombardment essentially as described by Ratnayaka and Oard (1995). Four days before transformation, a full grown BY-2 cell culture was diluted 1 : 10 in 40 ml of fresh MS medium. After 4 d culturing at 25°C in the dark on a rotary shaker (150 rpm), the cells were in an early logarithmic phase. A 7 ml aliquot of the grown cells was spread in a thin layer on a Whatman filter paper using a Buchner filter. Subsequently the Whatman filter paper using a Buchner filter. Subsequently the Whatman filter was transferred to solid MS medium containing 0.2 M mannitol and 0.2 M sorbitol, and the cells were incubated for 4 h at 25°C in the dark.

DNA for the different GFP fusion constructs was purified using the Plasmid Midi Kit (Qiagen, Venlo, The Netherlands) and precipitated on gold particles (1 μm diameter) by mixing the microcarriers with 10 μl of DNA (1 μg μl⁻¹), 25 μl of CaCl₂ (2.5 M) and 10 μl of spermidine (0.1 M). The Bio-Rad model PDS-1000 He Biolistic Particle Delivery system was used to introduce the DNA into BY-2 cells. Therefore, the microcarriers coated with the DNA were accelerated at a pressure of 900 psi in a vacuum of 26 inch Hg towards the cells at a target distance of 6–9 cm. After biolistics, the plates were incubated at 25°C in the dark for 24–55 h before microscopic analysis.

For expression of proteins in leaf epidermis, 4-week-old N. tabacum (cv Petit Havana) greenhouse plants grown at 25°C were used. *Agrobacterium tumefaciens* (strain GV3101)
were infiltrated at a bacterial optical density (OD600) of 0.1, in a transient expression system (Batoko et al. 2000).

Table 1  List of cytoplasmic and vacuolar GNA homologs from plants, fungi, fishes and sponges: sequences are shown in the Supplementary material

<table>
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<th>Species</th>
<th>Code</th>
<th>Accession No.: Annotation</th>
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<td>ABF70332: leaf lectin</td>
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<td>Amoco</td>
<td>AAP04617: 3DAAK precursor</td>
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<td><em>Ananas comosus</em></td>
<td>Anaco</td>
<td>AAM28277: mannose-binding lectin</td>
</tr>
<tr>
<td><em>Chimonanthus praecox</em></td>
<td>Chipr</td>
<td>ABC72741: mannose-specific lectin</td>
</tr>
<tr>
<td><em>Clivia miniata</em></td>
<td>Climi</td>
<td>AAA19912: lectin</td>
</tr>
<tr>
<td><em>Dioscorea polyestachya</em></td>
<td>Diopo</td>
<td>BAD67183: mannose-specific lectin</td>
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<tr>
<td><em>Galanthus nivalis</em></td>
<td>Galni</td>
<td>AAA33346: GNA</td>
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<tr>
<td><em>Hernandia moerenhoutiana</em></td>
<td>Hermo</td>
<td>AAD45250: seed lectin</td>
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<tr>
<td><em>Hyacinthoides hispanica</em></td>
<td>Hyahi</td>
<td>AAD16403: lectin SCAMan</td>
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<tr>
<td><em>Listera ovata</em></td>
<td>Lisov</td>
<td>AAC37422: LOA</td>
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<tr>
<td></td>
<td>LisovM</td>
<td>AAC37423: monomeric mannose-binding protein</td>
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<td><strong>Marchantia polymorpha</strong></td>
<td>Marpo</td>
<td>AU081743: EST</td>
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<tr>
<td><strong>Narcissus hybrid</strong></td>
<td>Narhy</td>
<td>AAA33546: NPA</td>
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<tr>
<td><em>Polygonatum multiflorum</em></td>
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<td>AAC49413: mannose-specific lectin</td>
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<tr>
<td><em>Taxus x media</em></td>
<td>Taxme</td>
<td>AAT73201: mannose-binding lectin</td>
</tr>
<tr>
<td><em>Tulipa hybrid</em></td>
<td>Tulhy</td>
<td>AAC49386: mannose-binding lectin: ISOTYPE = MIL1</td>
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<td><strong>Zingiber officinale</strong></td>
<td>Zinof</td>
<td>AAV70492: mannose-binding lectin precursor</td>
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<td><strong>Cytoplasmic plant homologs</strong></td>
<td></td>
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<tr>
<td><em>Oryza sativa</em></td>
<td>Orysa</td>
<td>CA75500: similar to mannose-binding lectin SCAMan</td>
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<td><strong>Zea mays</strong></td>
<td>Zeama</td>
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<td><em>Aspergillus oryzae</em></td>
<td>AsporA</td>
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<td><em>Coccidioides immitis</em></td>
<td>Cocim</td>
<td>EAS27517: hypothetical protein CIMG_10122</td>
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<td><em>Gibberella moniliformis</em></td>
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<td>AAZ30384: mannose-binding lectin</td>
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<td><em>Gibberella zeae</em></td>
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<td>EAA71530: hypothetical protein FG03828.1</td>
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<td><strong>Cytoplasmic fish homologs</strong></td>
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<td><em>Leiognathus nuchalis</em></td>
<td>Leinu</td>
<td>BAE79275: lily-type lectin</td>
</tr>
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<td><em>Lophiomus setigerus</em></td>
<td>Lops1</td>
<td>BAD90685: skin mucus lectin</td>
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<tr>
<td><em>Onchorhynchus mykiss</em></td>
<td>Onemy</td>
<td>AAU14874: lectin</td>
</tr>
<tr>
<td><em>Platyccephalus indicus</em></td>
<td>Plain</td>
<td>BAE79274: lily-type lectin</td>
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<tr>
<td><em>Takifugu rubripes</em></td>
<td>Takru1</td>
<td>BAC57043: skin mucus lectin</td>
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<tr>
<td><em>Takifugu rubripes</em></td>
<td>Takru2</td>
<td>BAC57044: intestine mucus lectin</td>
</tr>
<tr>
<td><strong>Tetraodon nigroviridis</strong></td>
<td>Tetni</td>
<td>CAG10253: unnamed protein product</td>
</tr>
<tr>
<td><strong>Cytoplasmic sponge homolog</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>Lubomirskia baicalensis</em></td>
<td>Lubba</td>
<td>CAI91574: mannose-binding lectin</td>
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</tbody>
</table>

were infiltrated at a bacterial optical density (OD600) of 0.1, in a transient expression system (Batoko et al. 2000).

**Arabidopsis transformation using floral dip**

*Arabidopsis thaliana* ecotype Columbia plants were transformed using the floral dip method described by Clough and Bent (1998). Binary vectors with constructs for proGNA (SP-GNA–CTP) and GNA lacking the CTP (SP–GNA) under the control of the 35S promoter were introduced into *A. tumefaciens* GV3101 (A 07-77) using electroporation. Aerial plant parts of *Arabidopsis* at the initial flowering stage were dipped for 5 s in infiltration medium containing transformed *Agrobacterium* cells resuspended in 10% sucrose and 0.05% surfactant Silwet L-77 (Osi Specialties Inc., Danbury, CT, USA). After infection plants were covered with transparent plastic for 24 h to
maintain humidity. Afterwards, plants were grown and seeds collected.

Analytical techniques

Genomic DNA was isolated from Arabidopsis plants overexpressing the lectin constructs encoding the sequence of preproGNA or GNA without the CTP, using the Fast DNA Spin kit in a homogenizer (FastPrep Instrument, MP Biomedicals and Qbiogene, Irvine, CA, USA) following the manufacturer’s recommendations. PCR was performed on the genomic DNA to check if the lectin genes are incorporated in the genome. The preproGNA sequence was amplified using a forward primer complementary to the 5' end (5'-GACAATATTGTGTACCTC CGTGAG-3') of the CDS of GNA and a reverse primer complementary to the 3' end of the CTP (5'-TTACTTTGCGGT CACAAGCTTTAATCTT-3'). For amplification of the GNA sequence without the CTP, the same forward primer was used in combination with a reverse primer complementary to the 3' end of the CDS of the mature GNA sequence (5'-TCCGTTGTAGGTT CCAGTAGCCCAA-3'). PCRs were performed in a 50 µl reaction volume containing 200 ng of genomic DNA, 10× DNA polymerase buffer, 1.5 mM MgCl₂, 0.4 mM dNTPs, 0.2 µM of each primer and 1.25 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA). The PCR program consisted of 25 repetitive cycles with a denaturation step at 94°C for 15 s, an annealing step at 50°C for 30 s and an elongation step at 72°C for 1 min. The PCR cycles were preceded by an extra denaturation step of 2 min at 94°C and ended with an extra elongation step of 5 min at 72°C.

Crude extracts from leaves were analyzed by SDS-PAGE in 15% acrylamide gels as described by Laemmli (1970). Proteins were visualized by staining with Coomassie brilliant blue or blotted onto polyvinylidene fluoride (0.45 µm) transfer membranes (Biorad™ PVDF, PALL, Gelman Laboratory, USA). Western blot analysis was performed using a specific primary antibody against GNA (Van Damme and Peumans 1990) and a horseradish peroxidase-coupled goat anti-rabbit IgG (Dako A/S, Denmark) as the secondary antibody. Immunodetection was achieved by a colorimetric assay essentially as described by Wang et al. (2003).

Confocal laser scanning microscopy

Image analysis was carried out with a confocal laser scanning microscope (Radiance 2000, Bio-Rad, Hertfordshire, UK) mounted on an Eclipse 300 Nikon microscope (Japan) using a 10× Plan Apo objective lens (NA 0.45) or a 40× oil immersion lens (NA 1.40). EGFP was excited with a 488 nm line of an argon ion laser, and emission light was selected with a HQ 515/30 nm filter. Images were analyzed with ImageJ version 1.30v (http://rsb.info.nih.gov/ij).

For tobacco leaf tissue, transformed leaves were analyzed 48 h after infection of the lower epidermis. Confocal imaging was performed using an inverted Zeiss LSM 510 META confocal microscope and a 63× water immersion objective. For imaging expression of EYFP constructs, mGFP₃ constructs or both, we used imaging settings as described in Brandizi et al. (2002) with a 3 µm optical slice. Appropriate controls were done to exclude the possibility of energy transfer between fluorochromes and cross-talk. Post-acquisition image processing was done with CorelDraw 12 software.

Phylogenetic analysis

Protein sequences were aligned using the ClustalW program (Chenna et al. 2003). Parsimony analyses on the alignments were conducted with PAUP* version 4.0b10 (Swofford 2002). Non-parametric bootstrap support was obtained by resampling the data 1,000 times using parsimony. Heuristic searching used 10 random taxon addition replicates, holding 100 trees at each step, tree bisection-reconnection branch swapping, MulTrees, Collapse, and Steep Descent options, and no upper limit for trees held in memory. The phylogenetic tree is visualized using Treedilator (Trooskens et al. 2005).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

Acknowledgments

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References


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