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Research article

Two short sequences from amaranth 11S globulin are sufficient to target green fluorescent protein and beta-glucuronidase to vacuoles in *Arabidopsis* cells

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Abstract

Vacuolar sorting of seed storage proteins is a very complex process since several sorting pathways and interactions among proteins of different classes have been reported. In addition, although the C-terminus of several 7S proteins is important for vacuolar delivery, other signals seem also to be involved in this process. In this work, the ability of two sequences of the *Amaranthus hypochondriacus* 11S globulin (amaranthin) to target reporter proteins to vacuoles was studied. We show that the C-terminal pentapeptide (KISIA) and the GNIFRGF internal sequence fused at the C terminal region of genes encoding secretory versions of green fluorescent protein (GFP) and GFP-β-glucuronidase (GFP-GUS) were sufficient to redirect these reporter proteins to the vacuole of *Arabidopsis* cells. According to the three-dimensional structure of 7S and 11S storage globulins, this internal vacuolar sorting sequence corresponds to the alpha helical region involved in trimer formation, and is conserved within these families. In addition, these sequences were able to interact *in vitro*, in a calcium dependent manner, with the sunflower vacuolar sorting receptor homolog to pea BP-80/AtVSR1/pumpkin PV72. This work shows for the first time the role of a short internal sequence conserved among 7S and 11S proteins in vacuolar sorting.

Keywords: Vacuolar sorting determinants; Amaranth 11S globulin; Seed storage proteins; Vacuolar sorting

1. Introduction

In plant cells, vacuoles are classified into lytic and storage vacuoles [1–5]. Nevertheless, it is not fully understood how the proper segregation of cargo molecules into the different types of vacuoles takes place. Proteins that are destined for these vacuolar compartments contain positive targeting information that causes them to be sorted away from the secretory default pathway. Three general types of vacuolar sorting signals (VSS) have been described, including sequence-specific (ssVSS) of sweet potato sporamin and barley aleurain, the C-terminal determinants of phaseolin, α and β subunits of β-conglycinin, tobacco chitinase and brazil nut 2S albumin (ctVSS), and the internal sorting determinant of phytohemagglutinin, barley phytepsin and castor bean ricin [6–14]. The ssVSSs are characterized by having the motif NPIXL/NPIR [8] and by exerting their effects regardless of their location; they are associated with Golgi-mediated traffic to the lytic vacuole (LV) and have been shown to interact with the vacuolar sorting receptor (VSR) family [15–18]. In contrast, C-terminal signals do not have similarity in amino acid composition and length; they must be accessible and are thought to be involved in targeting to storage vacuoles [6–8].

The sorting process of storage proteins is more complex than that of other vacuolar proteins. For example, 7S and 11S storage proteins form trimers in the lumen of the endoplasmic reticulum (ER) and are subsequently transported to the protein storage vacuole (PSV) either by precursor accumulating vesicles (PAC) that bud off directly from the ER, as occurs with pumpkin 11S proteins [19,20], or by Golgi-derived dense vesicles (DV), as happens with pea legumin [21,22]. In addition

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the polymorphism of these proteins also affects sorting; for example suppression of the expression β-conglycinin α and α′ subunits produced abnormal deposition of 11S proteins in soybean seeds [23]. Similar results are also observed for mutant soybean cultivars that are null for different subunits of 7S or 11S globulins [24]. Historically it was proposed that aggregation itself could be the signal for sorting of storage proteins (physical structure VSS or psVSS) because they do not have any conserved motif that could function as sorting determinant [6]. In addition, the first studies performed with legumin showed that the whole N-terminal domain was necessary for vacuolar sorting, and that its 76 amino acid C-terminus was sufficient to target the reporter protein invertase to vacuoles in tobacco [25]. In contrast, in the case of phaseolin, the C-terminal tetrapeptide AFVY has been shown to be sufficient for targeting to vacuoles [26]. It has been suggested that the difference in the length of the sequences required to modify the traffic of the reporter protein could relate to the size of the reporter protein used in the experiments [26]. Phaseolin lacking its C terminal tetrapeptide is secreted, showing that this sequence is necessary for vacuolar sorting [27]. In the presence of wild-type phaseolin, however, a proportion of trimers composed exclusively of ΔCt4 were also sorted to the vacuole in leaves of transgenic tobacco plants [28]. In the case of α′ and β subunits of β-conglycinin, the C-terminal decapeptides PLSSILRAFY and PFPSILGALY, respectively, were found to be sufficient to target GFP to the PSV matrix in maturing seeds, while proteins without these C-terminal decapeptides were targeted to the globoid compartment [9,10]. Consequently although the C-terminal sequence of phaseolin and β-conglycinin are absolutely necessary for sorting to the PSV matrix, other signals are also involved in the sorting process [9,10,28]. A recent report demonstrated that glycinins ($A_{1a}B_{1b}$ and A_3B_4) have a ssVSS located downstream of the disordered region 4, besides an additional sorting determinant that could be a psVSS [29].

In the present study we show that the amaranth 11S globulin (amaranthin) has two peptides (the C-terminus and an internal region) that are sufficient to efficiently redirect the reporter green fluorescent protein (GFP) to the vacuole in *Arabidopsis* cells. The internal vacuolar-sorting sequence identified is conserved among 7S and 11S globulins, and the three-dimensional structure of this family of proteins suggests that it is involved in trimer formation. In addition we show that these amaranthin targeting signals can mediate the sorting of reporter proteins of different size to vacuoles, and interact with the sunflower VSRs.

2. Materials and methods

2.1. Plasmid constructions

Putative amaranth 11S globulin vacuolar sorting signals were fused to genes coding for the green fluorescent protein (GFP) and β-glucuronidase (GUS). The GFP coding sequence was isolated from the plasmid pCD3-327 by polymerase chain reaction [30]. The forward primer (oligo 5′pRTGFP: GGT G*GG TAC C*GG CTA GCA CCA ATG AGT AAA GGA GAA GAA C, *KpnI* site in italic) was designed to place in frame the smRS-GFP coding sequence with the murine heavy chain immunoglobulin secretory signal peptide of pRTL202 [31]. The reverse oligonucleotide was designed to introduce the ER retention sequence HEKDEL and several restriction sites (oligo 3′GFPKDEL: AGC A*TC TAG A*TC ACA ACT CAT CCT TCT CAG AGA *GGA TCC C**GT CGA C*TT TGT ATA GTT CAT CCA TGC C, *XbaI*, *BamHI* and *SalI* sites in italic). The restriction site *XbaI* was introduced for cloning the PCR product into pRTL202. The *SalI* and *BamHI* restriction sites were introduced to subsequently fuse the *uid A* gene to GFP. The HEKDEL sequence was introduced between *XbaI* and *BamHI* sites. After the amplification of smRS-GFP with these oligonucleotides, the PCR product was digested with *KpnI*/*XbaI* and placed into pRTL202 between the secretory signal peptide and the CaMV 35S polyadenylation signal. This plasmid was named pER-G.

GFP was also fused to the putative vacuolar targeting sequences. To this end, PCRs were performed with different reverse primers: 3′AmhCt: CGG G*TC TAG A*TC AAG CAA TAG AAA TCT TGA *GGA TCC C**GT CGA C*TT TGT A to introduce the KISIA sequence; 3′AmhH1N: CGG G*TC TAG A*TC AGA ATC CAC GGA AAA TGT TGA *GGA TCC C**GT CGA C*TT TGT A to introduce the NIFRGF sequence. The plasmid pER-G was amplified with 5′pRTGFP and 3′AmhCt or 3′AmhH1N to obtain pG-AmhCt and pG-AmhH1N respectively. PCR products were cloned into pRTL202 as described above. To obtain the secretory GFP (pSec-G), the HEKDEL sequence in pER-G was removed by digestion with *XbaI* and *BamHI*, filling with Klenow polymerase I, and then the plasmid was self-ligated.

To analyze the ability of the different targeting signals to deliver much larger protein to vacuoles, the *uid A* gene coding for β-glucuronidase was introduced into these constructs. The *uid A* gene was amplified from pRTBV-E [32] with the oligonucleotides 5′Gus (ACG C*GT CGA C*AT GAT GTT ACG TCC TGT AGA A *SalI* site in italic) and 3′Gus (GCG *GGA TCC* CTT GTT TGC CTC CCT GCT G, *BamHI* site in italic). These oligonucleotides were designed to place the coding sequence of β-glucuronidase in frame with smRS-GFP and to remove the stop codon of the *uid A* gene to allow its fusion to the different targeting sequences.

2.2. Transient expression in Arabidopsis T87 protoplasts

Arabidopsis thaliana cells were cultured in suspension and protoplasts were isolated essentially as described by Axelos et al. [33]. Protoplasts were transformed with PEG using 10 μg of each GFP plasmid and 10 μg of sonicated sperm DNA as carrier [34]. Transfected protoplasts were incubated at 23 °C in the dark, and were observed by fluorescence microscopy at different times after transfection.

2.3. Fluorescent microscopy and laser-scanning confocal microscopy

The transformed *Arabidopsis* cells were inspected with a fluorescent microscope (Axioplan2 Carl Zeiss Inc.,

Thornwood, NY) using a GFP filter, a CCD camera (DXM1200, Nikon Instruments Inc., Melville, NY), and a light source (Arc HBO 100 W). Protoplasts were also observed with a laser-scanning confocal microscope (LSM510, Carl Zeiss Inc). GFP was excited with the 488 nm line of an argon laser and the emission was monitored using a 505–550 nm band-pass filter. The images were arranged using Adobe Photoshop (Adobe Systems Inc., Mountainview, CA).

2.4. Quantitative analysis of β-glucuronidase (GUS) activity

Protein samples from protoplasts were prepared using 50 mM phosphate pH 7.5, 10 mM EDTA, 0.1% Triton X-100, 10 mM 2-mercaptoethanol extraction buffer, and were quantified using a DC protein assay kit (BioRad Laboratories Inc., Richmond, CA). GUS activity was measured using 4-methylum-belliferyl-D-glucuronide (MUG) as substrate as described by Jefferson et al. [35].

2.5. Preparation of sunflower vacuolar sorting receptor protein extracts

Sunflower maturing cotyledons were homogenized in grinding buffer (150 mM Tricine, pH 7.5, 1 mM EDTA-Na, 10 mM KCl, 1 mM $MgCl_2$, 100 mM lactose). The resulting suspension was filtered through four layers of Miracloth (Biosciences, Inc, La Jolla, CA), and centrifuged at 10,000 × *g* for 15 min at 4 °C. The floating lipid layers were discarded and the supernatant from this step was further centrifuged at 120,000 × *g* for 1 h. Pellets were resuspended in CHAPS buffer (20 mM HEPES–KOH, pH 7.0, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% (w/v) 3,3-cholamidopropyl) dimethylammonio-1-propanesulfonate CHAPS and 10% (v/v) glycerol [17]; and incubated at room temperature for 30 min to solubilize integral membrane proteins. Residual membranes were removed by re-centrifugation, as in the first step, and the supernatant was carefully removed and transferred to a clean tube. These protein extracts were used in the binding assay.

2.6. Binding assay

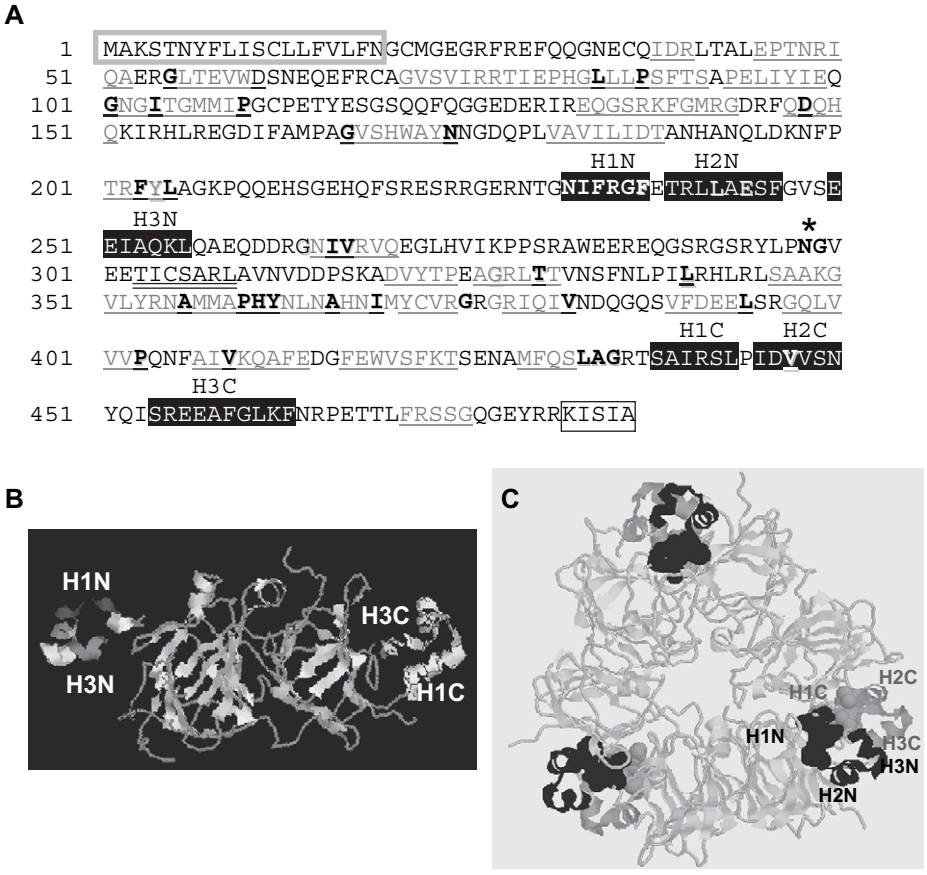
Peptides with the C-terminal sequence of the *Amaranthus hypochondriacus* 11S globulin (*C*GQGEYRRKISIA), H1N (*C*GERNTGNIFRGF) and AmhCt + 3Gly (*C*GQGEYRRKI SIA**GGG**), were commercially synthesized at Bio-Synthesis Inc (Lewisville, TX, USA). A cysteine residue was added at the N-terminal end of each peptide for subsequent chemical coupling to 2 ml of Sulfolink agarose beads (Pierce Chemical Co. Rockford, IL) according to the manufacturer's protocol. Three milliliters of CHAPS membrane protein extracts were applied to the affinity columns previously equilibrated with HEPES buffer (50 mM HEPES–KOH pH 7.0, 150 mM NaCl, 1 mM$CaCl_2$, 1% CHAPS), followed by incubation at 4 °C during 30 min. After that, the flow through fraction was collected and non-specific bound proteins were eluted by washing with HEPES buffer. Then, elution was performed with HEPES buffer containing 10 mM EGTA. Collected fractions were subjected to SDS-PAGE and subsequently to immunoblot analyzed using a rabbit polyclonal serum against the pumpkin vacuolar sorting receptor PV72 [15]. Next, the membranes were incubated with HRP conjugated anti-rabbit secondary antibody (BioRad Laboratories Inc., Richmond, CA). Immunoreactive signals were detected with Supersignal Chemiluminescent substrate (Pierce Biotechnology, Inc.).

The competition assay was performed as described above but proteins retained in the affinity columns were eluted using increasing concentrations of each peptide.

3. Results

3.1. Secretory reporter proteins fused to amaranth 11S protein sequences are sorted to vacuoles

The aim of the present work was to identify signals involved in vacuolar sorting of the amaranth 11S globulin. Although the C-terminus of phaseolin, α and β subunits of β conglycinin and glycinin $A_{1a}B_{1b}$ have been shown to be necessary and sufficient for vacuolar sorting, other signals also seem to be important in this process [9,10,26–29]. As can be observed in the protein sequence shown in Fig. 1A, amaranth 11S globulin has a sequence, called in this work H1N (helix 1 of the N terminus), that resembles the NPIXL/NPIR sequence present in proteins sorted to lytic vacuoles [8,36]. This sequence is part of the α helix domain, conserved among 7S and 11S storage globulins [37–40], that is surface exposed and involved in trimer formation (Fig. 1B and C). A similar sequence is also present in other 7S and 11S globulins, as shown in the alignment presented in Fig. 1D. In contrast, no conserved sequences were detected in helices 2 and 3 of the same proteins. To analyze the role of H1N and also of the amaranth 11S globulin C-terminal sequence in vacuolar sorting, different fusions to reporter genes were constructed (Fig. 2). The pentapeptide KISIA was taken as putative C-terminal VSS, because it follows the rules for C-terminal VSS; and resembles the KDEL sequence that was also shown to be involved in sorting to protein storage vacuoles, by ER-derived vesicles in germinating mung been seeds [41], and by Golgi-derived vesicles in tobacco seeds [42]. Both sequences were fused to genes encoding reporters of different sizes (GFP and GFP-GUS). As controls, secretory and reticulum endoplasmic versions of these reporters were also constructed. These constructs were transiently expressed in *Arabidopsis* protoplasts and expression of the *uidA* and GFP reporter gene was analyzed 36 h after electroporation. β-Glucuronidase activities of four independent transfection experiments are presented in Fig. 3. The activity of cells transfected with the secretory GFP-GUS construct was just above background level, which is consistent with secretion (Fig. 3, sec-GG). GUS-activity in the incubation medium was very low, consistent with the inhibition of GUS enzymatic catalytic activity when the protein is secreted [43]. The maximum GUS activity, in the transfected cells, was observed for the ER retained GFP-GUS fusion (Fig. 3, ER-GG). Amaranthin's peptides GFP-GUS fusions (GG-AmhCt and GG-AmhH1N) had



D

	H1N	H2N	H3N	aa
Amh 11S	G**NIFRGF**ETRLLAESF--GVSEEIAQKLQA			231-259
Ara 12S	K**NIFNGF**GPEVIAQA--LKIDLQTAQQLQN			213-240
Sun 11S	G**NIFNGF**TPELIAQSFNVDQETAQKLQGQN			225-251
Rice glut	Q**NIFSGF**STELLSEAFGISNQVARQLQCQN			226-252
Leg A2	N**NIFSGF**KRDFLEDAFNVNRHIVDRLQGRN			210-237
Gly	G**SILSGF**T*LEFL-EHAF*SV*DKQIAKNL*QGE			218-246
Pha	Q**SYLQEF**S*KHIL-EASFN*S*KFEEINRVLF*E			180-209
Congly-βsub	Q**SYLQGF**SHNILETSFHSEFEEINRVLFGE			170-199

Fig. 1. Amaranth 11S globulin sequence and structure. (A) Amino acid sequence of the amaranth 11S globulin as deduced from the cDNA sequence (GenBank accession no. X82121) including the signal peptide (highlighted in gray). Secondary structure elements were identified by alignment with soybean glycinin (GenBank accession no. M36686), and by comparison with its three-dimensional structure (1FXZ.pdb). β-Strands are shown in gray and underlined while α-helices are highlighted in black. Residues that are absolutely conserved among 7S and 11S globulins are in bold. α-Helices involved in subunit contacts both at the N- (H1N, H2N, H3N) and C- (H1C, H2C, H3C) terminal domains are numbered. The C terminal pentapeptide KISIA is boxed. Asn-Gly processing site is indicated in bold with an asterisk. The double underlined sequence corresponds to the disordered region downstream of the hypervariable region 4 of glycinin that has been proved to function as ssVSS [29]. (B) Three-dimensional structure of the monomeric polypeptide. H1N and H3N indicate N-terminal α-helices; and H1C and H3C, C-terminal α-helices. (C) Three-dimensional structure of the trimer. The N- and C-terminal helix are indicated. The structures in (B) and (C) were drawn from file 1FXZ.pdb using RasMol. (D) Amino acid sequence alignment of the N terminal helix region of amaranth 11S globulin (Amh 11S, GenBank accession no. X82121), *Arabidopsis* 12S globulin (Ara 12S, Swiss Prot accession no. P14555), sunflower 11S globulin (Sun 11S, Swiss Prot accession no. P19084), rice glutelin (Rice glut Swiss Prot accession no. P07730) and legumin A2 (Leg A2, Swiss Prot accession no. P15838), glycinin (Gly, $A_{1a}B_x$ Swiss Prot accession no. P04776), phaseolin (Pha, Swiss Prot accession no. P07219), β-subunit β-conglycinin (Congly β-sub, Swiss Prot accession no. P25974). The alignment was generated using DNAstar (Madison, WI). α-Helices at the N-terminal domain are indicated as H1N, H2N and H3N. Underlined letters indicate α helix of proteins whose structures have been resolved and are published [38,39]. Helix 1N sequence is shown in black.

higher levels of GUS activity than the secreted form but lower levels (approximately 50%) than the KDEL one, indicating that are retained inside the cell and that GUS protein folds correctly. The difference in activity between these constructs and the ER-retained one can be attributed both to a lower stability of GFP-GUS in the vacuole and to glycosylation of the active site of GUS that blocks enzymatic activity [44].

Arabidopsis protoplasts were also observed with confocal laser scanning microscope 12–48 h after transfection. As expected, the secreted forms (Sec-G and Sec-GG) did not

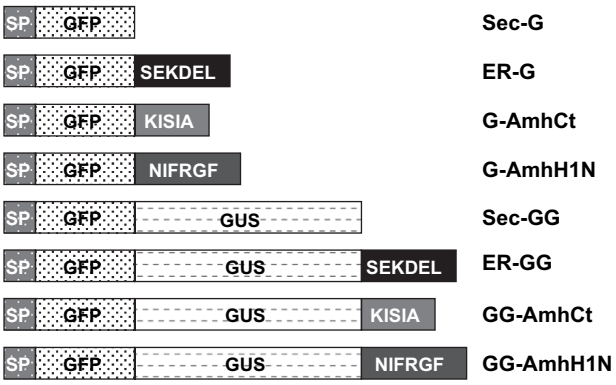


Fig. 2. Schematic representation of GFP and GFP-GUS fusion constructs. Sec-G and Sec-GG are secretory forms of GFP and GFP-GUS, ER-G and ER-GG are ER-retained version of these reporter proteins, and G-Amh Ct, GG-Amh Ct, G-AmhH1N and GG-Amh H1N are fusions to the putative vacuolar targeting signals of the amaranth 11S globulin.

accumulate inside cells and were not detected in the medium, where they were probably degraded or diluted (data not shown). This result is in concordance with GUS activity levels and it is similar to that informed by Di Sansebastiano et al. [4] but is different from that informed by Nishizawa et al. [9] who observed that although secretory GFP is predominantly secreted to the intercellular space, it is also partially delivered to vacuoles. The reticulum endoplasmic constructs (ER-G and ER-GG) showed a perinuclear distribution and formed a characteristic reticulate network, which is the typical ER pattern (Fig. 4A and B) [45]. GFP and GFP-GUS fused to the amaranthin C-terminus and to H1N were first detected in the ER (12 h after transfection). Between 18 and 24 h they were found in the vacuole of transfected cells; and this vacuolar localization pattern was predominant at 36 h (Fig. 4C–F). Similar results have been informed by Di Sansebastiano et al. [4] and

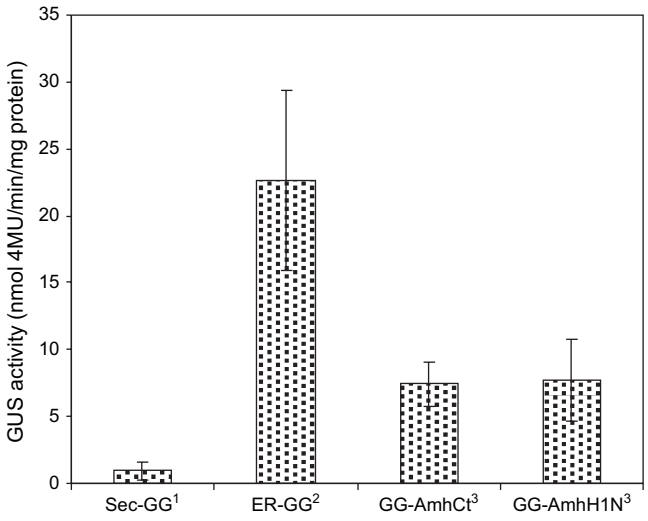


Fig. 3. GUS activity in *Arabidopsis* protoplasts transiently expressing the different sorting signal constructs. Electroporated protoplasts were analyzed for GUS activity after 36 h of culture. Bars represent the average of four independent experiments. Different numbers denote significant differences at $P < 0.05$.

Frigerio et al. [26] for GFP-fused tobacco chitinase A and phaseolin vacuolar sorting signals, respectively [4,26]. Therefore these experiments show that either the C terminal pentapeptide KISIA or the H1N peptide are able to redirect the reporter proteins (GFP and GFP fused with GUS) to vacuoles in transformed *Arabidopsis* cultured cells.

3.2. The vacuolar sorting signals of the amaranth 11S globulin are able to interact with sunflower VSRs in a calcium dependent manner

It has been reported that transport of storage proteins is saturable indicating the existence of a limiting factor, possibly the binding to a vacuolar sorting receptor [26]. The pea vacuolar sorting receptor BP-80 (VSR1) has been shown to be involved in the sorting process to lytic vacuoles. This receptor and its *Arabidopsis* homolog AtVSR1 (At-ELP), bind to the NPIRL sequences of barley aleurain and sweet potato prosporamin, and also to the carboxyl-terminal targeting determinant of brazil nut pro-2S albumin, but does not bind to the carboxy-terminal propeptides of barley lectin or tobacco chitinase [16,18]. AtVSR1 has been also shown to be involved in the targeting of storage proteins to PSV [46]. In order to analyze the binding of the amaranthin's vacuolar sorting signals here identified to a vacuolar sorting receptor, we prepared an extract containing the sunflower VSRs and analyzed its *in vitro* interaction with synthetic peptides. To this end, affinity columns containing the C-terminal peptide (GQGEYRRKISIA) and a peptide containing the helix 1N sequence (GERNTGNIFRGF) of the amaranthin, equilibrated at pH 7.0 in the presence of Ca^{2+}, were incubated during 30 min at 4 °C with sunflower detergent extract prepared from immature cotyledons. Then the flow through fraction was collected and non-specific bound proteins were eluted by washes with the same buffer until no signal was detected with antibodies specific to pumpkin vacuolar sorting receptor PV72 (Fig. 5A, lane wash). Subsequently, proteins interacting with the peptide affinity columns were eluted with EGTA solution. As shown in Fig. 5A, two proteins of molecular size 74 and 80 kDa were eluted with EGTA, indicating that both the C-terminal peptide and the peptide containing the helix 1N sequence of the amaranthin interacted with the sunflower VSRs in a calcium dependent manner. These two proteins could represent different isoforms of sunflower vacuolar sorting receptor or different posttranslational modifications of the same receptor.

A feature of ctVSS is that its function is disrupted by addition of one or more glycine residues at the C terminus [47,48]. Thus, to demonstrate that the C-terminus KISIA is a true ctVSS, three glycine residues were added at the carboxy terminus and the binding of the sunflower VSRs to an affinity column containing the AmhCt + 3Gly was analyzed. The result of this experiment was that binding of sunflowers VSRs was abolished (Fig. 5A), confirming our hypothesis that AmhCt is a ctVSS.

To further investigate the specificity of sunflower VSRs' interaction with the amaranthin's vacuolar sorting signals identified we carried out competition experiments with the two peptides. In these experiments, the AmhCt and AmhH1N

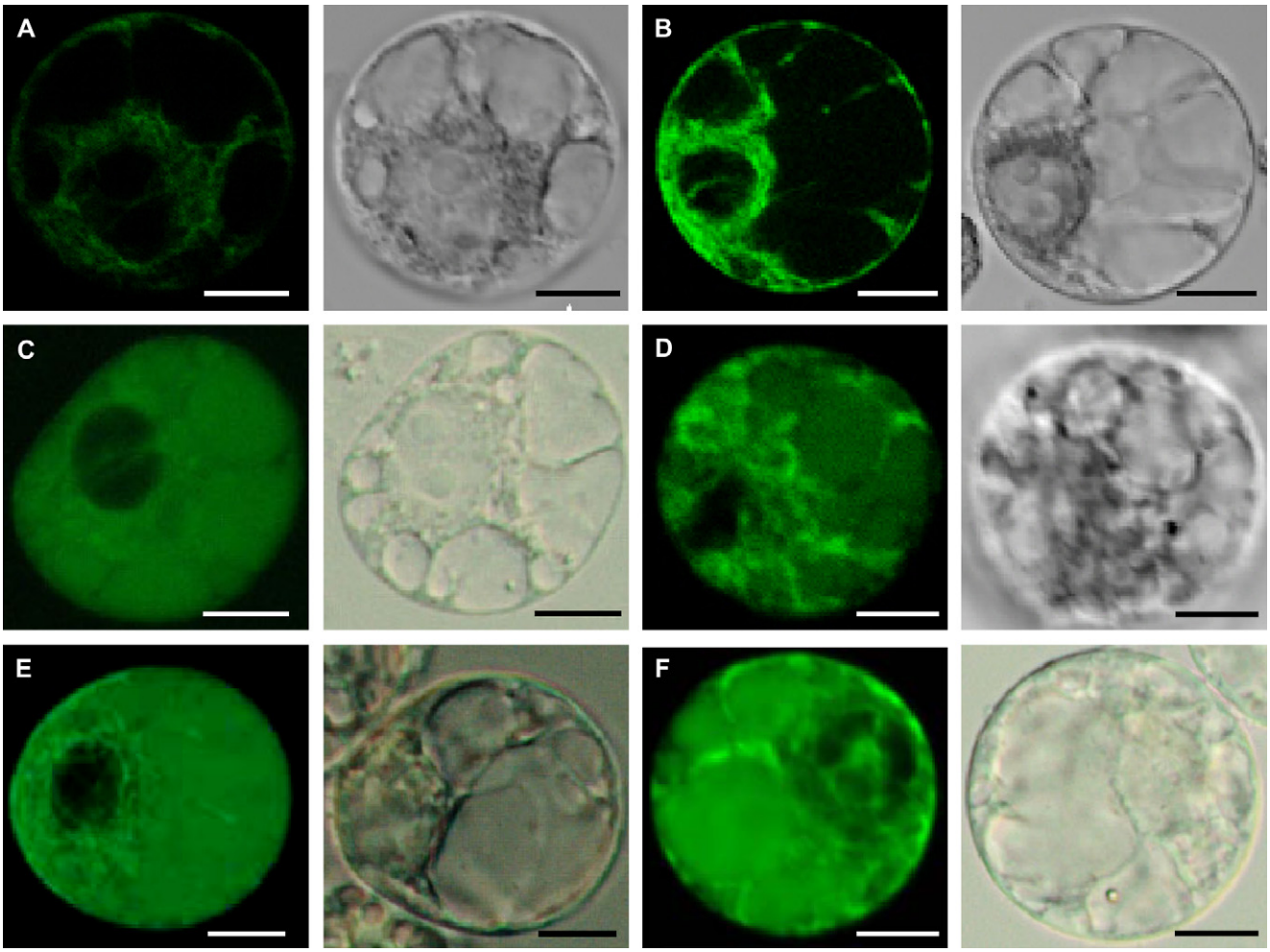


Fig. 4. Confocal fluorescence and differential interference contrast (DIC) images of *Arabidopsis* protoplasts expressing: (A) ER-G (B) ER-GG (C) G-AmhCt (D) GG-AmhCt (E) G-AmhH1N (F) GG-AmhH1N. Scale bar 10 μm.

peptides successfully competed for the binding of sunflower VSRs to the corresponding AmhCt and AmhH1N peptide columns at approximately 25 μM (Fig. 5B). Therefore both sequences bind to sunflowers VSR with high-affinity. In contrast, pea BP80 has greater affinity for ssVSS (K_d of 37 nM) than for ctVSS (K_d of 100 μM) [17]. Pumpkin PV72 binds in a calcium dependent manner to the internal peptide of the pumpkin pro2S albumin that lacks the NPIR motif with a K_d of 0.2 μM [49]. Interaction of Amh peptides with the sunflower VSRs in the concentration range showed here support their function as VSS at physiologic conditions.

A peptide concentration of 250 μM was required to elute sunflower VSRs bound to the affinity column containing the non-corresponding peptide (AmhCt vs AmhH1N) (Fig. 5B). The competition at very high peptide concentrations might suggest that two separate ligand binding sites may be involved in the interaction. It is known that pea BP-80 has two ligand binding sites (a NPIR specific site constituted by the interaction of the N-terminal/RMR homology domain and the central domain and a non-NPIR site defined by the central domain-EGF repeat domain structure) and that these domains can interact to keep the optimal conformation for ligand binding [50]. In this context, the competition of Amh peptides at 250 μM, could be explained by conformational changes in the central domain caused by the interaction of each amaranthin's peptide with its own binding site of the sunflower VSR. Competition between ctVSS and ssVSS for the binding to VSRs has been reported for the interaction of *Arabidopsis* 12S globulin C-terminal sequence to AtVSR1, which is successfully competed by *Arabidopsis* 12S globulin C-terminal sequence, *Arabidopsis* aleurain (NPIR) and brazil nut 2S albumin peptide (NLSP) [46]. In contrast, Ahmed et al. [18] found that the C-terminal targeting determinant of the barley lectin was not able to compete *in vitro*, for the binding of AtVSR1 to the barley aleurain and prosporamin NPIRL containing sequences affinity columns.

4. Discussion

In recent years, the C-termini of several 7S globulins such as common bean phaseolin (AFVY) and the α (PLSSILRAFY) and β (PFPSILGALY) subunits of soybean β-conglycinin were determined to be sufficient to target GFP to vacuoles. In contrast, the tetrapeptide IAGF, which is part of the vacuolar sorting determinant of brazil nut 2S albumin and resembles

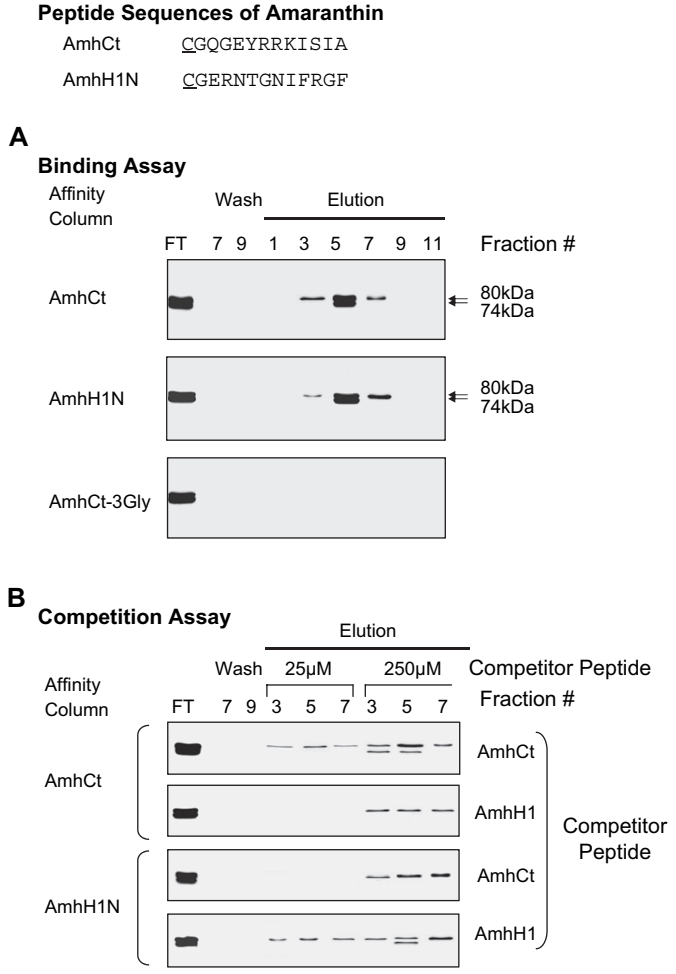


Fig. 5. Binding of sunflower VSRs to the peptides derived from the C-terminal and helix 1N sequence of the amaranth 11S storage protein. Peptides were immobilized in a Sulfolink column (Pierce Chemical Co.). Crude sunflower extract was applied to each column and after washing with HEPES buffer, bound proteins were eluted with 10 mM EGTA buffer (part A) or with the indicated peptides as competitors in the same binding buffer (part B). The flow through (FT) fraction (1,5 μl), wash fraction (15 μl) and eluted fractions (15 μl) were subjected to SDS-PAGE followed by immunoblot analysis with antibodies against pumpkin PV72 [15]. Sunflower VSRs are indicated by arrows.

AFVY, is not sufficient to deliver secretory invertase to vacuoles [47]. In addition, studies performed with field bean 11S globulin, which is structurally related to 7S globulins, showed that the complete α chain is necessary to target chloramphenicol acetyl transferase (CAT) to vacuoles in tobacco [25]. To explain these differences it has been proposed that the size of the reporter protein used in the experiment may be critical for C-terminal vacuolar sorting signals and/or that few amino acid residues at the end of reporter protein could also be part of the signal recognized by the receptor [27]. In order to address these hypotheses, two reporter proteins, GFP and GFP-GUS, were used in this work. We showed that the C-terminal pentapeptide (KISIA) and the helix 1N peptide (NIFRGF) of amaranthin were sufficient to target both GFP and GFP-GUS to vacuoles in *Arabidopsis* cells; therefore the size of the reporter protein does not modify protein sorting. Vacuolar sorted GFP-GUS fusions had values of GUS activity several fold above the background level indicating that GUS protein was correctly folded and that targeting to vacuoles was not due to misfolding. The differences in the activity between GFP-GUS-AmhVSS fusions and the ER-retained one can be explained both the lower stability of GFP-GUS in the vacuole and to loss catalytic activity caused by glycosylation of the enzyme active site [43–44]. We provided microscopical evidence that the AmhCt and AmhH1N are able to redirect the reporter proteins to the plant vacuole. Therefore amaranth 11S globulin has at least two targeting signals: C-terminal KISIA and H1N sequence. While previous studies have shown the importance of the C-terminus in the sorting process of storage proteins of the 7S family, and also of the 11S globulin glycinin $A_{1a}B_{1b}$ but not A_3B_4 [9,10,26,29]; here we demonstrate that the C-terminus of amaranth 11S globulin is sufficient for sorting to vacuoles. The KISIA sequence follows the rules for C-terminal sorting signals of chitinases and glucanases [8].

Recently, it has been reported that the C-terminal ten amino-acid sequences of α′ and β subunits of soybean β-conglycinin contain two types of vacuolar determinants: the SIL sequence that is present in several 7S globulins, and functions as ssVSS because it is able to target reporter proteins to vacuoles when it is placed at the N-terminus; and the ctVSS that can function only at the very C-termini of proteins [51]. Most 7S globulins contain hydrophobic amino acid residues similar to the ctVSS (AFY) of α′ at their C-termini [52]. In contrast, amaranth 11S globulin does not have the SIL sequence and although its C-terminus is hydrophobic it does not resemble the AFY sequence found in 7S globulins.

The NIFRGF helix 1N sequence of amaranthin resembles the NPIRL/P found in Kunitz type proteinase inhibitors or papain-like cysteine proteases [8]. A similar sequence is present in several 7S and 11S storage proteins (Fig. 1) and is a conserved motif within these families: [usually Asn or Ser]-[preferably Ile or Tyr]-[Leu or Phe][charged or hydrophilic]-[any amino acid]-[Phe]. This sequence could function as an internal sorting signal as has been reported for ricin. The latter contains a LIRP motif that also resembles the NPIR-like targeting signal for lytic vacuoles, even though ricin is normally a protein of the PSV [52]. This ricin internal sequence is also sufficient to direct secretory reporters to the vacuoles of tobacco protoplasts [12]. The role of this internal sequence in vacuolar sorting of storage proteins could explain why a proportion of trimers composed exclusively of phaseolin without its C terminal tetrapeptide are sorted to the vacuole in leaves of transgenic tobacco plants [28] and also the targeting α′ and β subunits of β-conglycinin without their C-terminal decapeptides to the globoid compartment [9,10]. A recent report studied candidates for ssVSS in soybean glycinin $A_{1a}B_{1b}$ by analyzing sequences containing Ile or Leu residues located on the molecular surface of this protein [29]. Using this approach the sequence ICTMRL located downstream of the disordered region 4 was chosen as putative VSS. This sequence was able to redirect secretory GFP to PSV in maturing soybean cotyledons when it was introduced in an internal position; consequently, it functions as a ssVSS. Amaranth 11S globulin has the sequence ICSARL at this position (see Fig. 1) and thus contains the Ile residue located on the molecular

surface that is critical for sorting. The studies of soybean 11S globulin sorting signals by Utsumi's group also showed that functional inhibition of $A_{1a}B_{1b}$ combined with the Ile297Gly mutation, did not abolish vacuolar sorting of $A_{1a}B_{1b}$, suggesting the presence of additional sorting determinants [29]. The H1N motif identified here can be this sorting signal.

Using affinity columns the two sorting signals identified were shown to bind sunflower VSRs in a calcium-dependent manner. The interaction of these sequences to the sunflower VSRs was strong enough to support their role *in vivo* as sorting signals. Although amaranthin's C terminal (GQGEYRRKISIA) and helix 1N (GERNTGNIFRGF) peptides have different amino acid sequences, they competed for the binding to sunflower VSR at high concentrations. Similar results have been reported for the interaction *in vitro* of AtVSR1 with *Arabidopsis* 12S globulin (ASYGRPRVAAA) sequence of this 12S globulin, *Arabidopsis* aleurain (A**NIGF**DES*NPIR*MVSDGLREV) and brazil nut 2S albumin (PSRC*NLSP*MRCPMGGSIAGF) [46]. *Arabidopsis* aleurain has a sequence that is more closely related to the H1N motif (sequence shown in bold) than to the NPIR motif. The contribution of this sequence to vacuolar sorting has not been studied, but a sequence in an equivalent position in barley aleurain propeptide has been shown to contribute to vacuolar sorting [6]. In addition the pumpkin 2S albumin also has an internal region containing an NPIR motif that has been shown to interact with pumpkin VSR (PV72) *in vitro*. However, mutations in this motif do not affect binding and it has been suggested that physicochemical properties of the peptides rather than their primary structure are important for recognition by the receptor [15]. In this context, the role of the helix 1N motif in sorting of 7S and 11S globulins would explain both the existence of additional sorting determinants informed for these proteins [28,29] and the low conservation of this sorting signal primary sequence. Additionally, it has been proposed that hydrophobic interactions between the side chain of Ile/Leu and a receptor would play a pivotal role in sorting [8,12,29,36].

Although the three-dimensional structure of the vacuolar sorting receptors is still unknown, biochemical studies performed with pea BP-80 showed the existence of two separate binding sites: a non-NPIR site (that contains the EGF motif) and an NPIR-specific site [50]. The EGF repeats are important for high-affinity binding to the NPIR motif although they are not part of the NPIR binding site; therefore both binding sites cooperate for ligand binding. These features of VSR1 explain our competition results. Multiple ligand binding sites that act cooperatively may determine the simultaneous recognition of several sorting determinants, insuring the proper targeting. Both 11S and 7S globulins are transport to vacuoles as trimers; consequently several molecules of the receptor should participate in the recognition of trimers and their sorting within maturing seed cells.

Which receptor(s) is involved in sorting to storage vacuoles is still a matter of controversy. AtVSR1 sorts vacuolar proteins such as aleurain and sporamin to the plant lytic vacuole [18] and because its pea homolog BP-80 localizes in clathrin coated vesicles in legume embryo cells vesicles [53], most researchers have assumed that the function of these receptors is to sort proteins to lytic vacuoles. However, AtVSR1 also mediates the transport of both 2S albumin and 12S globulin precursors to the PSV in *Arabidopsis* [46] and it is found in storage protein-containing vesicles and also in the internal vesicles of the storage protein multivesicular bodies [54]. Therefore AtVSR1 also plays a role in sorting of storage proteins to protein storage vacuoles in *Arabidopsis* embryos. In addition, another receptor called At RMR1 (for *Arabidopsis* receptor homology region transmembrane domain ring H2 motif protein 1) has been proposed to act as a possible receptor for protein storage vacuole destined proteins [55]. AtRMR1 interacts *in vivo* with the C-terminal propeptide of the transiently expressed 7S globulin phaseolin in *Arabidopsis* leaf protoplasts but not with proteins containing an NPIRL sequence [56,57]. Why several receptors seem to play a role in sorting of storage proteins and how proteins are properly sorted to their final destination is still unknown. If several receptors play a pivotal role in the sorting or some of them participate only in a complementary mechanism as occurs in other cellular processes needs to be investigated. For example, ER-located soluble proteins such as reticuloplasmins have redundant sorting information that favors their ER location independently of the HDEL/KDEL C-terminal tetrapeptide [58–60]; the KDEL receptor only participates from the capture of proteins that escape from the ER. In this context, some of the vacuolar sorting signals of storage proteins may be redundant, and be recognized by the sorting machinery when the main mechanism fails. This hypothesis is supported by the fact that in *Arabidopsis* plants in which VSR1 expression has been blocked by insertion of a T-DNA, 12S globulins and 2S albumins are found not only in the inter cellular space but also in protein storage vacuoles [46].

This is the first report to show that an internal region conserved among 7S and 11S proteins interacts with the vacuolar sorting receptor and is sufficient to redirect secretory reporter proteins to vacuoles. We are currently studying whether this sequence is necessary for sorting to vacuoles. Such experiments, however, are troublesome due to the fact that some amino acid residues of this motif are absolutely conserved within 7S and 11S storage protein families and their deletion can affect folding, as has been observed for the deletion of the whole helix region of phaseolin [61].

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