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Targeting of proConA to the Plant Vacuole depends on its Nine Amino-acid C-terminal Propeptide

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Concanavalin A (ConA) is a well characterized and extensively used lectin accumulated in the protein bodies of jack bean cotyledons. ConA is synthesized as an inactive precursor proConA. The maturation of inactive proConA into biologically active ConA is a complex process including the removal of an internal glycopeptide and a C-terminal propeptide (CTPP), followed by a head-to-tail ligation of the two largest polypeptides. The cDNA encoding proConA was cloned and expressed in tobacco BY-2 cells. ProConA was slowly transported to the vacuole where its maturation into ConA was similar to that in jack bean cotyledons, apart from an incomplete final ligation. To investigate the role of the nine amino acid CTPP, a truncated form lacking the propeptide (proConAΔ9) was expressed in BY-2 cells. In contrast to proConA, proConAΔ9 was rapidly chased out of the endoplasmic reticulum (ER) and secreted into the culture medium. The CTPP was then fused to the C-terminal end of a secreted form of green fluorescent protein (secGFP). When expressed in tobacco BY-2 cells and leaf protoplasts, the chimaeric protein was located in the vacuole whereas secGFP was located in the culture medium and in the vacuole. Altogether, our results show we have isolated a new C-terminal vacuolar sorting determinant.

Keywords: BY-2 cells — Concanavalin A — GFP — Targeting — Vacuolar sorting determinant — Vacuole.

Abbreviations: 3D, three-dimensional; CE, cell extract; ConA, concanavalin A; CTPP, C-terminal propeptide; ctVSD, C-terminal VSD; ER, endoplasmic reticulum; EM, extracellular medium; GFP, green fluorescent protein; GFPChit, secGFP fused to chitinase vacuolar sorting signal; GFPConA, secGFP fused to ConA sorting signal; Mi, microsomal fraction; PEG, polyethylene glycol; PSV, protein storage vacuole; psVSD, physical structure VSD; S, soluble fraction; secGFP, secreted GFP; ssVSD, sequence specific VSD; VSD, vacuolar sorting determinant.

The nucleotide sequence of the proConA cDNA reported in this paper has been submitted to GenBank under the accession No. AF308777.

Introduction

In plant cells, secreted proteins enter the secretory system in the endoplasmic reticulum (ER) and are transported either to the cell surface or to specific subcellular compartments: the Golgi apparatus, the cell wall, the plasma membrane or the vacuole (reviewed by Vitale and Denecke 1999). Plant secreted proteins lacking specific targeting information follow a default pathway and are transported to the cell surface (Denecke et al. 1990, Hunt and Chrispeels 1991). In contrast, the delivery or retention of soluble proteins within the secretory organelles requires specific targeting information. Most proteins targeted to the plant vacuoles pass through the Golgi apparatus. However, a direct ER to vacuole pathway, by-passing the Golgi complex, has also been described for transport of seed storage proteins to the protein storage vacuole (PSV) (Hara-Nishimura et al. 1998, Herman and Larkins 1999, Chrispeels and Herman 2000). The diversity of vacuole delivery pathways for soluble proteins parallels a range of vacuolar sorting determinants (VSDs). Specific VSDs have been identified in the sequence of several precursors of vacuolar proteins. Deletion of these vacuolar targeting peptides causes the secretion of the proteins. Furthermore, the addition of vacuolar sorting sequences to secreted proteins is often sufficient to redirect them to the vacuole. In this way, different types of VSDs have been identified and they are classified in three categories: sequence specific VSDs (ssVSDs), C-terminal VSDs (ctVSDs), and physical structure VSDs (psVSDs) (reviewed in Matsuoka and Neuhaus 1999).

The first category, ssVSDs, are located within the N-terminal propeptide of sweet potato sporamin (Matsuoka and Nakamura 1991) and barley aleurain (Holwerda et al. 1992). These two model ssVSDs contain the consensus sequence NPIRL/P. Similar motifs, always containing an essential Ile or Leu, have been identified at the N-terminus and/or, the C-terminus of numerous vacuolar protein precursors (Matsuoka and Neuhaus 1999) and also in the internal propeptide of ricin (Frigerio et al. 2001). In addition, it was also shown that the sporamin N-terminal propeptide can redirect a secreted protein to the vacuole even when added at its C-terminal end (Matsuoka et al. 1995). The function of a ssVSD is consequently independent of its position.

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In the second group, the different ctVSDs identified to date do not share any consensus sequence and are variable in size (Matsuoka and Neuhaus 1999). However, these determinants are enriched in hydrophobic amino acids organized in patches. Despite of the differences in their sequences, ctVSDs are all inactivated when either two glycine residues or a N-glycan are added at their C-terminus end (Dombrowski et al. 1993, Koide et al. 1999).

The third class of determinant, psVSD, is related to internal folded domains in the case of phytohemagglutinin (von Schaewen and Chrispeels 1993) or to a saposin-like insert in the case of barley phytepsin (Törmäkangas et al. 2001).

Concanavalin A (ConA) is a lectin accumulated in the PSV of jack bean (*Canavalia ensiformis L.*). ConA is synthesized as a glycosylated precursor, proConA. ProConA has no lectin activity in contrast to ConA and the post-translational processing events that impart biological activity to mature ConA occur upon arrival or after storage in the PSV (Faye and Chrispeels 1987). The processing of proConA has been extensively described. It involves a complex sequence of post-translational modifications that is unique to ConA when compared to other seed storage protein maturation events (reviewed by Müntz 1998). First, proConA is N-glycosylated in the ER and deglycosylated probably after proper folding of the proprotein. Secondly, deglycosylation is followed by the excision of a 15 amino acid internal linker bearing the N-glycosylation site and located at the centre of the proConA sequence, which generates two large polypeptides, indicated as P1 and P2 in Fig. 1A. A nonapeptide is then proteolytically removed from the original C-terminal of proConA. Finally, the last step of proConA maturation is a head-to-tail ligation of the two cleavage products P1 and P2 leading to an amino acid sequence permutation (Carrington et al. 1985, Herman et al. 1985, Chrispeels et al. 1986, Faye and Chrispeels 1987, Ramis et al. 2001). Despite several maturation steps, conversion of proConA into a biologically active lectin does not seem to require a major modification of its three-dimensional (3D) structure as illustrated by 3D reconstruction data (Bowles and Pappin 1988) and our recent results show that deglycosylation and acidification are sufficient for in vitro conversion of the inactive precursor into an active lectin (Ramis et al. 2001). In addition, proConA can be deglycosylated without preliminary denaturation of the protein (Bowles and Pappin 1988, Ramis et al. 2001) supporting a high accessibility of the prolectin N-glycan, consistent with the structural 3D model proposed for proConA, where the internal glycopeptide and the C-terminal propeptide are exposed at the surface of the prolectin. In contrast to a lot of works carried out in order to study the lectin activity of ConA, much less is known regarding the targeting mechanisms of this protein in planta. In particular, the function of the nine amino acid C-terminal propeptide (CTPP) of proConA has never been investigated. However, the post-translational cleavage and accessibility of this CTPP suggest its possible implication in the sorting of the prolectin to the CtPSV.

In the present study we report the expression of recombinant ConA in a heterologous plant system and analyse the role of CTPP in the transport and vacuolar targeting of recombinant proConA. Expression of proConA and a CTPP-deleted proConA form in suspension-cultured tobacco BY-2 cells shows that proConA CTPP is necessary for proConA delivery to the plant vacuole. Furthermore, proConA CTTP is also sufficient to redirect green fluorescent protein (GFP) to the vacuole in BY-2 cells and tobacco leaf protoplasts. Consequently, our results show that the C-terminal cleavable propeptide of proConA is a CtVSD.

Results

Post-translational maturation of proConA and proConAΔ9 in BY-2 cells

In jack bean developing cotyledons, the glycosylated precursor proConA is processed and stored in the PSV as a deglycosylated and biologically active mature lectin. The different post-translational maturation events affecting proConA in jack bean seeds are illustrated in Fig. 1A. To study proConA processing and to investigate the potential role of cleavable CTPP in the intracellular transport of proConA, a cDNA for proConA was cloned from a Venezuelan cultivar of *C. ensiformis* (cv U-02) and expressed in an heterologous plant system. This cDNA (accession number AF308777) encodes a polypeptide containing a N-terminal 29-amino acid signal peptide and a 290-amino acid proprotein. Compared to an earlier sequence published by Carrington et al. (1985), the four key amino acids Tyr12, Asn14, Asp215 and Arg235 implicated in the lectin activity and the N-glycosylation site (Asn152) are conserved. However, there are seven nucleotide modifications that cause the substitution of two amino acids in the mature protein: Glu151→Asp and Arg155→Glu. These differences were attributed to the use of a different cultivar. To study the role of the CTPP, site-directed mutagenesis was performed and the sequence encoding the nine C-terminal amino acids constitutive of proConA CTPP was deleted (Fig. 1B). The full length cDNA and the truncated cDNAs were used to transform tobacco BY-2 cells via *Agrobacterium tumefaciens* according to Gomord et al. (1998). The constructs as well as the kanamycin-resistant tobacco transformants were named *ConA* when containing the entire cDNA and *ConAΔ9* when containing the truncated cDNA lacking the CTPP (Fig. 1B). The processing of recombinant proConA and proConAΔ9 was first analysed in BY-2 calli (Fig. 1C).

Four main polypeptides antigenically related to ConA were immunodetected from protein extracts obtained from tobacco calli expressing ConA. These polypeptides have molecular weights of 34, 30, 19, and 12.5 kDa, respectively (Fig. 1C, lane 3) while *ConAΔ9* calli accumulate three major ConA-related products of 33, 19 and 12.5 kDa, respectively (Fig. 1C, lane 4). These polypeptides are not detected in non

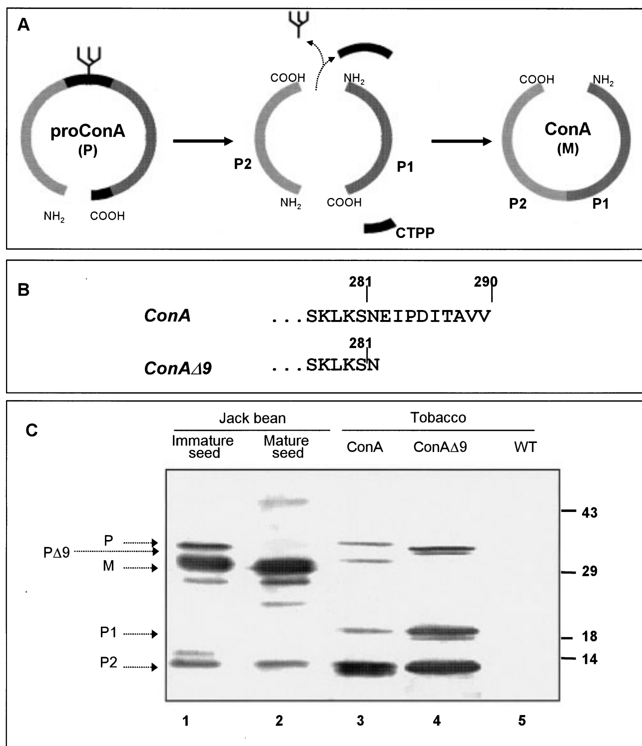

Fig. 1 Comparison of the maturation of native and recombinant proConA. (A) Schematic representation of proConA maturation in jack bean cotyledons. ProConA (P) is a polypeptide of 290 amini acid (aa) residues and carries a N-linked high mannose glycan. ProConA is processed into a 237 aa mature ConA (M) after elimination of a 15 aa internal glycopeptide and a 9 aa CTPP and a head to tail ligation of two polypeptides P1 (C-terminal) and P2 (N-terminal). (B) Comparison of the C-terminal sequences of the wild type (*ConA*) and the CTPP deleted precursors (*ConAΔ9*) expressed in this study. The numbers on top identify the positions of the residues, starting from the first residue of proConA (signal peptide not included). (C) Proteins extracted from immature (lane 1) and mature (lane 2) seeds of jack bean, from BY-2 tobacco cells transformed with the *ConA* construct (lane 3) or the *ConAΔ9* construct (lane 4), and from non-transformed BY-2 cells (lane 5) were electrophoretically separated and blotted onto nitrocellulose for immunodetection with a specific anti-ConA serum. The positions of proConA (P), proConAΔ9 (PΔ9), mature ConA (M) and the main processed polypeptides (P1 and P2) are indicated. Numbers on the right correspond to molecular weight markers in kilodaltons.

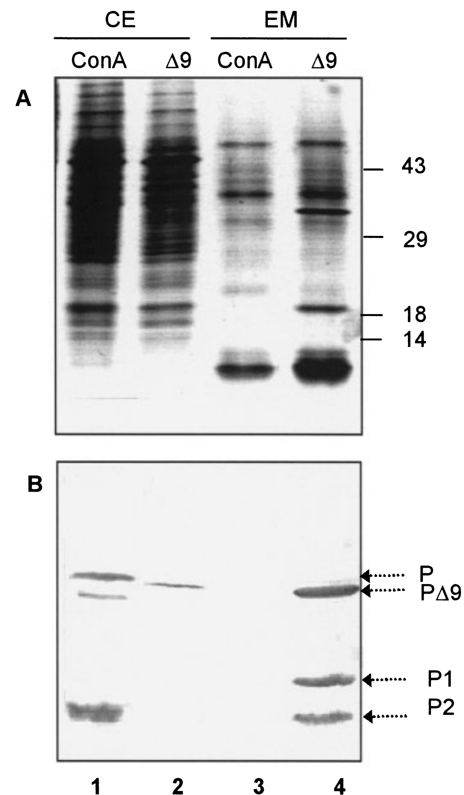

Fig. 2 The deletion of the CTPP induces the secretion of proConA. Proteins from the cell extract (CE) and extracellular medium (EM) of the transformants *ConA* (lanes 1 and 3) and *ConAΔ9* (lanes 2 and 4) were separated under denaturating conditions by SDS-PAGE and either silver stained in the gel (A) or transferred onto nitrocellulose for immunoblotting using a specific antiConA serum (B). The position of proConA (P), proConAΔ9 (PΔ9) and processed polypeptides (P1 and P2) are noted. Numbers on the right of panel A indicate molecular weight markers in kilodaltons.

transformed BY-2 cells (Fig. 1C, lane 5) illustrating the specificity of the immunserum used in this study.

Polypeptide patterns observed from *ConA* and *ConAΔ9* calli were compared to the patterns obtained for ConA-related products in immature (Fig. 1C, lane 1) and mature (Fig. 1C, lane 2) jack bean seeds. The 34 kDa polypeptide detected in *ConA* calli corresponds to the lectin precursor proConA (P) that is abundant in immature seeds but absent from mature seeds as previously described by Faye and Chrispeels (1987), and the 33 kDa polypeptide (PΔ9 in Fig. 1C, lane 4) corresponds to the prolectin lacking its nine C-terminal amino acids. Mature ConA, described in the literature as a 30 kDa polypeptide, is abundant in both mature and immature jack bean seed extracts but is present in very low amounts only in *ConA* calli extracts and is completely absent in protein samples obtained from *ConAΔ9* calli. Affinity detection using the ConA/peroxidase method (Faye and Chrispeels 1987) has shown the presence of an high-mannose type glycan N-linked onto the 34 and 33 kDa polypeptides (P and PΔ9, respectively) (data not shown). These data and the results illustrated in Fig. 4 indicate that proConA or proConAΔ9 bear a high mannose type N-glycan in BY-2 cells as in jack bean. In addition, a polypeptide of 32 kDa appearing exclusively in *ConAΔ9* cells as a doublet of the 33 kDa form is not detected after affinity detection and more probably corresponds to the deglycosylated form of proConAΔ9.

Smaller peptides of 19 and 12.5 kDa are detected in both transgenic BY-2 calli extracts (Fig. 1C, lanes 3 and 4). They correspond to the ultimate products of proteolytic cleavage P1 and P2 (Fig. 1A), and their abundance in BY-2 extracts compared to jack bean extracts is probably related to the low capac-

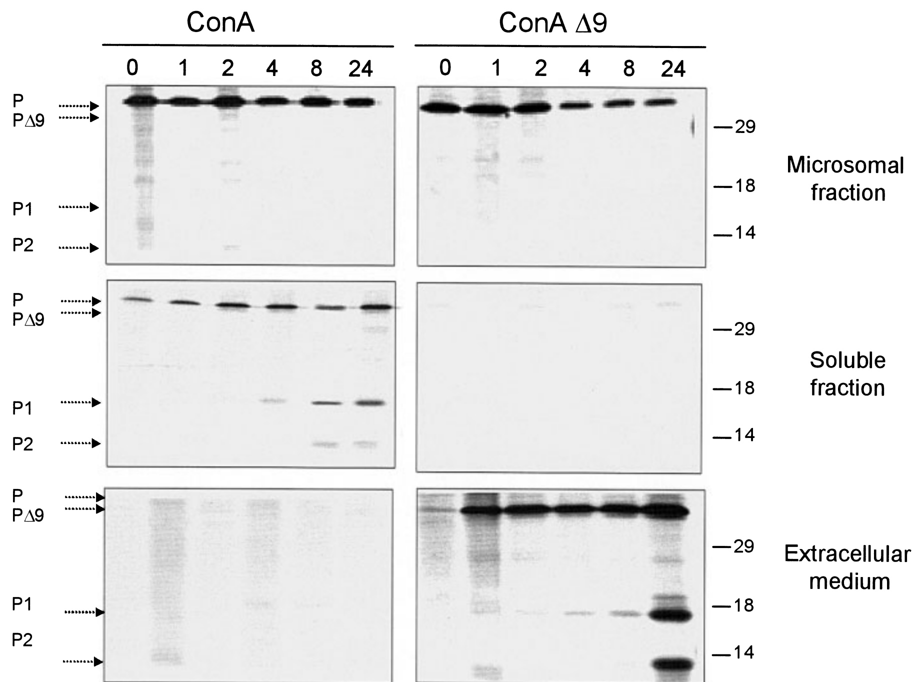

Fig. 3 Transport and maturation of proConA and proConAΔ9. *ConA* and *ConAΔ9* expressing cells were pulse-labelled for 1h with ^{35}[S]-methionine and ^{35}[S]-cysteine and chased for the indicated time periods. The extracellular medium was then collected and the intracellular proteins were subfractionated by ultracentrifugation to isolate the soluble protein fraction from the microsomal fraction. ConA related polypeptides were immunoprecipitated with a ConA-specific immune serum and directly analyzed by SDS-PAGE and fluorography. Numbers on the right indicate molecular weight markers in kilodaltons.

ity of the heterologous system to ligate these polypeptides to form mature ConA. In conclusion, our results show that in BY-2 cells (1) all the steps required to transform proConA into the intermediate polypeptides P1 and P2 are completed and (2) the ligation step leading to mature ConA is ensured, but with very low efficiency.

Deletion of CTPP induces the secretion of proConA

To investigate the role of the nine amino acid CTPP on proConA transport in tobacco BY-2 cells, we compared the localization of ConA-related polypeptides in suspension-cultured tobacco BY-2 cells initiated from the *ConA* and *ConAΔ9* calli analyzed above. Proteins from cell extract (CE, Fig. 2, lanes 1 and 2) and extracellular media (EM, Fig. 2, lanes 3 and 4) were analysed by SDS-PAGE and either gel silver staining (panel A) or immunoblotting using ConA-specific antibodies (panel B). In *ConA* expressing cells, polypeptides reacting with the antibodies were detected exclusively in the intracellular fraction (Fig. 2B, compare lanes 1 and 3), whereas, only trace amounts of the precursor proConAΔ9 were revealed in the cell extract of *ConAΔ9* expressing cells (Fig. 2B, lane 2). In contrast, the precursor proConAΔ9 is very abundant in the extracellular medium together with maturation polypeptides of lower molecular weight, P1 and P2 (Fig. 2B, lane 4). Taken together, these data indicate that removal of CTTP from proConA induces secretion of the truncated prolectin in the culture medium where maturation products accumulate.

The CTPP is responsible for proConA targeting to the plant vacuole

ProConA and proConAΔ9 transport and processing in tobacco BY-2 cells were further characterized in a detailed pulse-chase experiment. In *ConA* and *ConAΔ9* suspension-cultured BY-2 cells, proteins were pulse-labeled for one hour and chased for 0, 1, 2, 4, 8, and 24 h. The extracellular medium was then collected by filtration of the cell culture. The cell extracts were fractionated by ultracentrifugation into a microsomal fraction and a soluble fraction, gathering soluble cytoplasmic and vacuolar proteins (Faye and Chrispeels 1987). Then, polypeptides antigenically related to ConA were immunoprecipitated and analyzed by SDS-PAGE and fluorography (Fig. 3). After one hour of pulse, proConA (P) was already detected both in the microsomal and soluble fractions. The amount of proConA and of maturation polypeptides P1 and P2 slowly increased in the cell extract during the chase. Moreover, no ConA-related polypeptide was found in the extracellular medium during the pulse chase. In contrast to proConA, proConAΔ9 was chased out of the microsomal fraction and rapidly accumulated along the chase in the extracellular medium. As suggested in Fig. 2 (compare lanes 2 and 4), proConAΔ9 was processed into two polypeptides P1 and P2 after secretion into the extracellular medium. Even though the processing is slower in the extracellular medium than in the vacuole, this result illustrates that proteolytic activities required for proConA maturation events are present in both compartments.

N-glycosylation is a post-translational maturation of proConA that is necessary for an efficient transport of the prolectin through the secretory pathway in jack bean (Faye and Chrispeels 1987). In order to determine whether or not an inef-

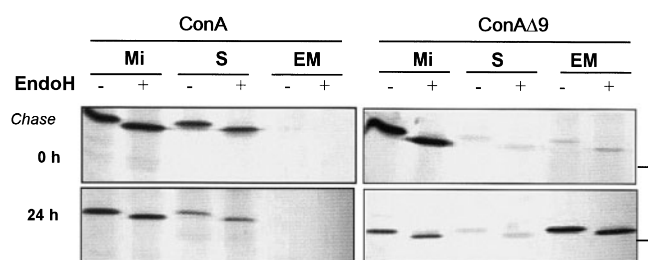

Fig. 4 ProConA and proConAΔ9 are glycosylated with high-mannose Endo-H sensitive N-glycans. *ConA* and *ConAΔ9* transformant cells were pulse-labelled for 1 h pulse with ^{35}[S]-methionine and ^{35}[S]-cysteine and chased for 0 or 24 h. After removal of the extracellular medium (EM), the proteins of the different subcellular compartments, i.e. the microsomal fraction (Mi) and the soluble fraction (S), were isolated by ultracentrifugation. ConA-related polypeptides present in Mi, S and EM were immunoprecipitated. For the EndoH digestion, duplicate samples were incubated at 37°C for 24 h in the absence (–) or presence (+) of EndoH. The proteins were then analyzed by fluorography after SDS-PAGE. The bar at the right hand margin of each panel indicates the position of the 29 kDa molecular mass marker.

ficient N-glycosylation could be the reason for the slow chase of proConA out of the microsomal fraction, we have checked for the presence and nature of the N-glycan. When expressed in tobacco BY-2 cells (Fig. 4), both proConA and proConAΔ9 are glycosylated with an Endo-H sensitive high-mannose N-glycan at the end of a pulse. This glycan remains in a high mannose form even after a 24 h chase, and the glycosylation is similar to the one previously described for proConA in jack bean seeds. Interestingly, the glycan is not matured into a complex oligosaccharide structure even when proConAΔ9 is secreted into the extracellular medium of BY-2 cells.

These data obtained from pulse-chase analysis show that proConA is slowly transported out of the ER and accumulates in the vacuole, while proConAΔ9 is detected mostly in the culture medium. These results strongly suggest that proConA CTPP is necessary for proConA targeting in the plant vacuole.

ProConA CTPP is sufficient to target GFP to the vacuole

We used GFP as a reporter to further investigate the role of proConA CTPP in the targeting of a soluble protein. For this purpose, the sequence encoding the nine C-terminal amino acids of proConA CTPP was fused at the C-terminal end of the secreted-GFP cDNA (*secGFP)* and designated secGFP fused to ConA sorting signal (*GFPConA*). As a positive control for vacuolar targeting, the sequence encoding the C-terminal VSD of tobacco chitinase was fused to *secGFP* to yield secGFP fused to chitinase vacuolar sorting signal (*GFPChit*) (Neuhaus et al. 1991). *GFPConA*, *GFPChit* and *secGFP* constructs were used to generate *Agrobacterium*-mediated BY-2 cell stable transformants (Fig. 5A). GFP localization was firstly determined for each GFP construct using fluorescence microscopy (Fig. 5B). Instead of being exclusively secreted, secGFP was transported into the vacuoles of BY-2 cells (Fig. 5B, left panel). This phenomenon was previously observed in some other plant expression systems (Frigerio et al. 2001, Nishizawa et al. 2003). We therefore analyzed secGFP targeting in BY-2 cells using a pulse-chase approach followed by immunoprecipitation using a specific anti-GFP antiserum (Fig. 5C, left panel). Consistent with the observation of secGFP in the vacuole using microscopy (Fig. 5B), and in complete agreement with the characterization of a VSD at the C-terminal end of GFP (Nishizawa et al. 2003), secGFP is mainly immunopurified from the cell extract even after 24 hours of chase (Fig. 5C, lanes 1–3). Nevertheless, a significant amount of the reporter protein is also secreted into the extracellular medium during the chase period.

In cells expressing GFPConA (Fig. 5B, middle) and GFPChit (Fig. 5B, right), fluorescence was found in the vacuole as observed for secGFP. However, when performing the same pulse-chase experiment as above, GFPConA and GFPChit were not detected in the extracellular medium (Fig. 5C, lanes 10 to 12 and 16 to 18, respectively) whereas secGFP was secreted (lanes 4 to 6). These experiments show that proConA CTPP prevents secGFP secretion in BY-2 cells. Moreover, proConA CTPP is almost as efficient as the C-terminal VSD of tobacco chitinase in targeting the reporter protein secGFP to the central vacuole in tobacco BY-2 cells (compare the middle and right panels in Fig. 5C).

Since we obtained a high background of secGFP in the vacuole of tobacco BY-2 cells, we used another expression system, tobacco leaf protoplasts, where secGFP is normally secreted (Di Sansebastiano et al. 1998; Batoko et al. 2000) to further validate the role of ConA CTPP as a vacuolar targeting determinant. As shown in Fig. 6A, GFPConA accumulates in the large central vacuole of tobacco leaf protoplasts whereas secGFP is not detectable in this compartment (Fig. 6C). The weak ER labelling occasionally detected in this transient expression system, in addition to the strong fluorescence of the vacuole, corresponds to a reporter on the route to the vacuole as already described using tobacco chitinase VSD (Di Sansebastiano et al. 1998).

These results clearly illustrate that when fused to GFP, proConA CTPP is sufficient to target the reporter protein to the vacuole in tobacco protoplasts and in tobacco BY-2 cells.

Discussion

ProConA is matured in tobacco cells but very little mature lectin is made in the heterologous expression system

Several vacuolar storage proteins and lectins have been successfully expressed and properly matured in heterologous systems (Matsuoka et al. 1995, Frigerio et al. 1998, Does et al. 1999, Frigerio et al. 2001). In the present study, proConA, the precursor of jack bean concanavalin A, was expressed in tobacco BY-2 cells in order to study (1) its processing in an heterologous system and (2) the role of the nine amino acids of the prolectin CTPP on its transport and maturation. In BY-2

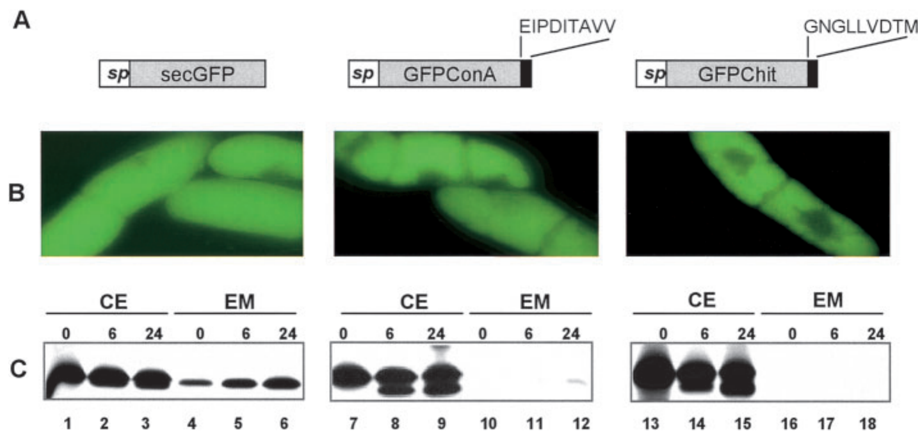

Fig. 5 The C-terminal cleavable propeptide of proConA is sufficient for vacuolar targeting in tobacco BY-2 cells. (A) Schematic representation of the different GFP fusions used in this study. GFP, *mgfp5* gene; *sp*, tobacco chitinase signal peptide; black boxes correspond to the nine C-terminal residues of proConA or the tobacco chitinase precursor. (B) Observation of 5-day-old tobacco BY-2 cells expressing the different *GFP* constructs by fluorescence microscopy. (C) Kinetic analysis of the transport of the different GFP fusion proteins in tobacco BY-2 cells. Cells were pulse labelled for 1 h as described in Fig. 3 and GFP was immunopurified from the cell extract (CE) and the extracellular medium (EM) after 0, 6 or 24 h of chase. GFP in the different samples was analyzed by fluorography after SDS-PAGE.

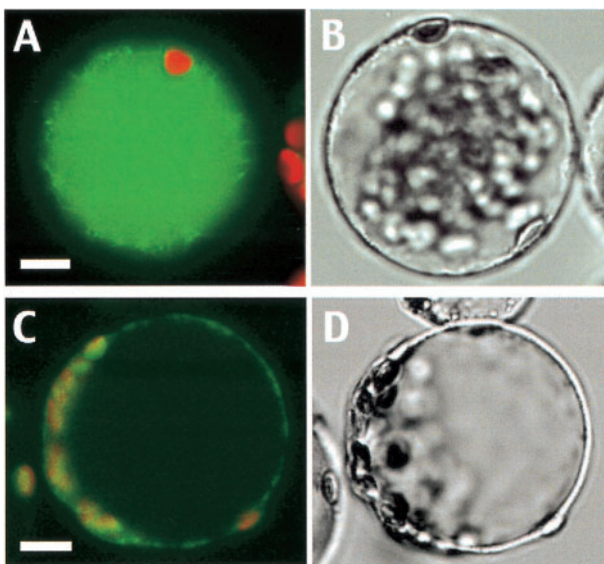

Fig. 6 The nine C-terminal amino acids of proConA are sufficient to target GFP to the vacuole in tobacco protoplasts. Tobacco leaf protoplasts were transiently transformed with GFPConA (A,B) or secGFP (C,D) and were observed 48 h after transformation using a confocal microscope. GFP fluorescence is shown in green and chloroplast autofluorescence in red. (A and B) A protoplast expressing GFPConA shows labeling in the central vacuole in the fluorescence mode (A). Transmitted light image of the same protoplast (B). (C and D) Protoplasts expressing secGFP. In fluorescence mode, no GFP is detected in the vacuole (C). Transmitted light image of the same protoplast (D). Bar = 10 µm.

cells, proConA and proConAΔ9 are synthesized as glycosylated proteins of 34 kDa and 33 kDa, respectively. Once proConA and proConAΔ9 reach the vacuole and the extracellular compartment respectively, they are both cleaved into two major non glycosylated polypeptides. These polypeptides show the same electrophoretic mobility as the ConA-related polypeptides P1 and P2, identified in jack bean cotyledons (19 kDa and 12.5 kDa, respectively), which indicates that the CTPP is removed from proConA in the heterologous expression system BY-2 cells as in jack bean cells. These data show that the first steps in proConA post-translational processing, including deglycosylation and proteolytic cleavage, are similar in tobacco and in jack bean cells. Deletion of the CTPP does not prevent these processing events, as they look identical for proConA and proConAΔ9. Some other vacuolar proteins have also been shown to be processed after secretion into the extracellular medium (Matsuoka et al. 1995, Frigerio et al. 1998). As an example, maturation of a vacuolar lectin, stinging nettle agglutinin, was also observed after secretion of the C-terminal truncated form of the prolectin in the apoplast of transgenic tobacco plants (Does et al. 1999). Here, our data suggest that endoglycosidases and endopeptidases involved in proConA maturation are present both in the vacuole and in the extracellular medium of tobacco suspension cultured cells.

In calli or in BY-2 suspension-cultured cells expressing proConA, a small amount of a polypeptide showing the same electrophoretic mobility as mature ConA was detected. This polypeptide was co-purified with proteins from the soluble fraction, which suggest that ligation of P1 and P2 polypeptides had occurred in the vacuole of BY-2 cells. Therefore, we hypothesized that the weak efficiency of ligation was due to overexpression of P1 and P2 in BY2-cells compared to a low amount of endogenous ligase. Interestingly, mature ConA was undetected in BY-2 cells expressing proConAΔ9 which indicates either that proConA CTPP is necessary to fulfil the complete maturation of the precursor into a mature lectin form or that, perhaps more likely, the ligation enzyme is not present in the extracellular medium. These results illustrate that endoglycosidase and endopeptidase activities required to cleave pro-

ConA and the enzymatic activity responsible for ligation of cleavage products are not specific to *C. ensiformis* cells and are also present in BY-2 cells.

ProConA is slowly transported to the vacuole, while proConAΔ9 is rapidly secreted into the culture medium

ConA is a lectin accumulated in the PSV of jack bean, and the localization of this lectin in colyledon cells has been shown by immunolabeling all along the secretory pathway, particularly in the ER, the Golgi apparatus and PSVs (Chrispeels et al. 1986). In the present study, as in jack bean cells, proConA expressed in suspension-cultured tobacco cells is detected exclusively intracellularly and, more precisely after immunolocalization, polypeptides antigenically related to ConA are detected both in the ER, the Golgi and the vacuoles. These results are consistent with our pulse chase analysis illustrating that, as previously observed in jack bean, proConA is slowly chased out of the microsomal fraction to finally accumulate in the plant vacuole. In contrast, when the nine amino acid C-terminal propeptide of proConA is deleted, the resulting truncated form, proConAΔ9, is rapidly chased out of the microsomal fraction and accumulates in the extracellular compartment when expressed in tobacco BY-2 cells. These results clearly illustrate that not only is proConA CTPP necessary to target proConA to the plant vacuole, but it also slows down the transport of proConA from the ER to the vacuole. Various mechanisms for slow down secretion or ER retention of newly synthesized proteins have been documented, such as overexpression or non native conformation (Sparvoli et al. 2000). Interestingly, in the present study, the chase of proConA out of the microsomal fraction is extremely slow even in suspension-cultured cells expressing proConA at a very low level (data not shown), an observation which is not consistent with ER retention resulting from of a high degree of overexpression. While proConA is slowly chased out of the microsomal fraction to the vacuole, proConAΔ9 is more rapidly chased out of the ER and secreted into the culture medium. The slow chase of proConA out of the microsomal fraction could not result from ER retention based on proConA misfolding because this would also be observed for proConAΔ9.

When glycosylation is prevented by tunicamycin, bean phaseolin forms aggregates in the ER because it does not fold properly (Sparvoli et al. 2000). In comparison, it has also been shown that glycosylation is necessary for the delivery of proConA to the vacuole. Indeed, when glycosylation of prolectin is inhibited after treatment of jack bean seeds with tunicamycin, unglycosylated proConA is retained in the ER/Golgi compartment (Faye and Chrispeels 1987). In BY-2 cells, both proConAΔ9 and proConA harbour an EndoH-sensitive high-mannose type N-glycan (Fig. 4), and consequently differences in the glycosylation of these forms do not account for the differences in transport of proConA *vs.* proConAΔ9 out of the ER. Based on these results, we propose that the slow transport of proConA out of the microsomal fraction depends at least in part on information contained in the prolectin C-terminal sequence. Therefore, proConA CTTP is not only necessary for transport to the vacuole but could also participate in an early selection (i.e. ER retention/concentration) of proteins targeted to the PSV.

The C-terminus of proConA acts as a C-terminal vacuolar sorting determinant

Although the glycan N-linked to proConA is not modified by Golgi enzymes and consequently does not provide information on the intracellular transport of the prolectin (Gomord and Faye 2000), previous studies have clearly shown that ConA reaches the vacuole via the Golgi apparatus (Faye and Chrispeels 1987). Cleavage and accessibility of the proConA C-terminal propeptide was the first indication that this sequence could be a C-terminal VSD. These results clearly illustrate that proConA CTTP is necessary for proConA targeting to the plant vacuole and that the deleted form proConAΔ9 lacking the targeting information contained in the C-terminal extention of the prolectin follows the default pathway to be secreted to the cell surface (Denecke et al. 1990, Hunt and Chrispeels 1991).

However, further controls were required to conclude that a VSD was associated with this C-terminal extension. GFP was used as a reporter protein because it is generally accepted that GFP fused with a signal peptide (secGFP) follows the secretory pathway and is transported to the extracellular compartment of plant cells (Zheng et al. 2004). We confirmed these results with secGFP expressed in tobacco protoplasts (Fig. 6). Nevertheless, we also observed a strong fluorescence in the vacuoles in tobacco BY-2 cells expressing secGFP. As illustrated in Fig. 5, it is impossible to distinguish BY-2 cells expressing secGFP from BY-2 cells expressing the vacuolar marker GFPChit using epifluorescence microscopy. Vacuolar delivery of a recombinant protein expected to be secreted in plant cells has been previously reported, in the case of secGFP when expressed in *Arabidopsis* (Frigerio et al. 1998, Nishizawa et al. 2003). In the present study, pulse-chase experiments performed in tobacco BY-2 cells expressing secGFP (Fig. 5) show that the VSD identified at the C-terminal end of GFP (Nishizawa et al. 2003) is recognized by the vacuolar sorting machinery in BY-2 cells but with a relatively low efficiency compared to ConA CTPP or Chit CTPP. However, using the same pulse-chase approach, a comparison of GFP distribution between the vacuole and the extracellular compartment clearly illustrates that proConA CTPP redirects GFP to the vacuole almost as efficiently as the C-terminal vacuolar determinant of tobacco chitinase. Further analysis using tobacco leaf protoplasts support this conclusion. Indeed, in this system no fluorescence is observed in the vacuole of secGFP-expressing protoplasts whereas GFPConA is targeted to the vacuole. In conclusion, these results indicate that a VSD is located in the nine amino acid C-terminal propeptide of proConA. This new ctVSD is enriched in hydrophobic amino acids (EIPDIATVV,

underlining indicates hydrophobic residues) and is sufficient for vacuolar targeting of GFP in transgenic tobacco. Interestingly, this ctVSD has no conserved amino acid sequence with the ctVSDs previously described in the literature (Neuhaus and Rogers 1998).

Materials and Methods

Materials

Restriction enzymes were obtained from Promega (Charbonnières, France). Protein A-Sepharose was purchased from Amersham Pharmacia Biotech (Orsay, France). Secondary peroxydase-conjugated antibody was purchased from Biorad (Marnes La Coquette, France).

RNA extraction and cloning of ConA cDNA

The RNAs were extracted with the 'RNeasy Plant' kit (Qiagen, Hilden, Germany) from immature seeds of *Canavalia ensiformis* (cv U-02) following the manufacturer's protocol. The specific *proConA* cDNA was generated by reverse transcriptase PCR (RT-PCR) using primers synthesized according to the sequence of *ConA* (GenBank accession number: X01632) published by Carrington et al. (1985).

The forward primer 5′-CGGGTACCTATACCACCATGGCAATCTC-3′ hybridized to the 5′ initiation codon and introduced a *Kpn*I endonuclease restriction site. The reverse primer 5′-CCGGATCCGGATGAGAATCAAACCACGG-3′ hybridized to the 3′ stop codon and created a *Bam*HI site. The single-stranded cDNAs and the PCR amplifications were performed using the Access RT-PCR system (Promega) from the total RNAs extracted as described above. The PCR product was cloned into the pBLTI221 vector (Pagny et al. 2000) using the *Kpn*I and *Bam*HI restriction sites.

Three independent PCR products were characterized by restriction mapping. The sequence of *proConA* cDNA was registered under the GenBank accession number AF308777.

Constructs and obtention of stable BY-2 transformants

The different constructs were generated by PCR to mediate site-directed mutagenesis at the 3′ end of *ConA* or *GFP* cDNAs. For *ConA*, the deletion of the nine C-terminal amino acids (ConAΔ9) was realized by PCR using the forward primer defined previously for the cDNA cloning: 5′-CGGGTACCTATACCACCATGGCAATCTC-3′ and the reverse primer 5′-CGGGATCCTCAATTGCTCTTCAACTTAGAAG-3′ with the cDNA of *ConA* cloned into pBLTI221. The forward and reverse primers introduced a *Kpn*I and a *Bam*HI restriction site, respectively, at each extremity of the PCR product.

The *mgfp5* gene (accession number: U87973) was subcloned into the pBLTI121 vector using the *Bam*HI and the *Sal*I restriction sites. For the *GFPConA* and *GFPChit* constructs, the addition of the nine C-terminal amino acids of ConA (EIPDITAVV) or of the tobacco chitinase (GNGLLVDTM) was achieved by using forward primer hybridyzing with the 35S promoter of the pBLTI121 plasmid: 5′-ATCCCACTATCCTTCGC-3′, and reverse primers: 5′-GGACTAGTGCGGCCGCTCAAACCACGGTAGCAATGTCCGGGATCTCTTTGTATAGTTCATCCAT-3′ or 5′-GGACTAGTGCGGCCGCTACATAGTATCGACTAAAAGTCCGTTTCCTTTGTATAGTTCATCCAT-3′, respectively, and the plasmid pBLTI121-*mgfp5* described before. The primers hybridyzed before the 5′ end of the *mgfp5* gene and conserved the *Bam*HI site and introduced a *Spe*I site at the 3′ end of the PCR products.

All PCR products were cloned into the pBLTI221 vector (Pagny et al. 2000) using *Kpn*I and *Bam*HI sites for *ConA* and *ConAΔ9* or *Bam* HI and *Spe* I sites for *secGFP*, *GFPConA* and *GFPchit* to be checked for sequence. Then all the constructs were subcloned into the binary plant expression vector pBLTI121 using the same respective digestion enzymes (Pagny et al. 2000) and transferred into *A. tumefaciens* (LBA4404) (Höfgen and Willmitzer 1988). Transgenic *Nicotiana tabacum* BY-2 cells were obtained as described in Gomord et al. (1998). Transgenic suspension cultured cells were initiated from calli and were subcultured weekly (Matsuoka and Nakamura 1991). Both the constructs and kanamycin-resistant tobacco transformants were designated *ConA* when containing the entire cDNA encoding sequence, *ConAΔ9* when containing the deleted mutant lacking the CTPP, or *secGFP, GFPChit* and *GFPConA* when containing the different forms of GFP. Three- to four-day-old cells were harvested by filtration.

Antibody production

The specific anti-ConA immunserum was prepared from commercial ConA (Sigma, St Quentin Fallavier, France) as described in Ramis et al. (2001). The specific anti-GFP immunserum was prepared using commercial GFP (Qbiogen, Illkirsh, France). For each immunization, 50 µg of purified protein were injected subcutaneously into New Zealand male rabbits. After a first injection with complete Freund's adjuvant, three more injections with incomplete Freund's adjuvant were given on day 14, 28 and 48. Bleeding was performed 10 d after the last injection.

Protein extracts

Protein seed extracts were prepared from jack bean seeds obtained from the Central University of Venezuela, as described in Ramis et al. (2001). For suspension-cultured cells, cell extract (CE) and extracellullar (EM) protein extracts were prepared as described in Gomord et al. (1997). For cell fractionation, tobacco BY-2 cells were first washed with ice-cold 0.5 M NaCl for 30 min to remove cell wall bound proteins and all subsequent manipulations were performed on ice. The EM was collected and the cells were homogenized with a pestle and mortar in ice-cold extraction buffer containing 100 mM Tris-HCl, pH 7.8, 12% (wt/vol) sucrose, and 1 mM EDTA as described in Faye and Chrispeels (1987). The homogenate was centrifuged (2,000×*g*, 10 min, 4°C) to remove cell debris. The supernatant was then centrifuged (150,000×*g*, 90 min, 4°C). The final supernatant constituted the soluble fraction (S) and contained the cytosolic and vacuolar proteins. The pellet was enriched in ER/Golgi organelles and was resuspended in extraction buffer containing 0.5% SDS and 0.5% Triton X100 and constituted the microsomal fraction (Mi).

Pulse-chase analysis

After filtration, tobacco BY-2 cells (1 g) were resuspended in 3.6 ml fresh culture medium. For each experiment, suspension-cultured cells were pulse-labelled in the presence of 4 MBq of [^{35}S] protein labelling mix (NEN Life Sciences Products, Inc., Boston, MA, USA) at 25°C with gentle shaking (140 rpm) for 1 h. After the pulse, cells were chased for various periods of time by adding 0.4 ml of a chase solution containing 50 mM methionine and 50 mM cysteine to the culture medium. Finally, the protein extracts were prepared as described above, in extraction buffer.

Immunoprecipitation of ConA-relative or GFP-related peptides was performed as described in Gomord et al. (1997) using 5 µl of the antiConA or antiGFP immunserum, respectively.

SDS-PAGE, fluorography and immunoblotting

Polypeptides were separated by SDS-PAGE on 15% polyacrylamide gels under reducing conditions according to Laemmli (1970). For the immunodetection on blots, after separation by SDS-PAGE, polypeptides were electrophoretically transferred onto a nitrocellulose membrane. The immunodetections were performed as described in Faye and Chrispeels (1987).

Protoplast transient expression

Tobacco protoplasts were prepared from young leaves of *Nicotiana tabacum* cv. SR1 as described previously (Di Sansebastiano et al. 1998). Transformation was performed using a polyethylene glycol (PEG) protocol (Negrutiu et al. 1987, Freydl et al. 1995) on 600,000 protoplasts with 10 μg of cesium chloride purified plasmid.

Microscopy

BY-2 cells expressing GFP were observed with a Leica DMRB epifluorescence microscope (Leica Microsystems, Rueil-Malmaison, France) and specimen were excited with blue light and imaged with a CCD camera (Hamamatsu Photonics, Massy, France).

Protoplasts were observed either 24 h or 48 h after transformation using a confocal laser microscope (DMR, Leica Microsystems, Wetzlar, Germany) with the Leica TCS 4D operating system. Internal controls were included for proper image collection. We used protoplasts transformed with GFPChit as a positive vacuolar control and secGFP as a negative control for secreted soluble GFP (Di Sansebastiano et al. 1998). Images were collected using a filter set for fluorescein that is suitable for GFP5 excitation and emission. A second image at the same confocal plan of a chosen protoplast was taken using a rhodamine filter set to collect the red autofluorescence emitted from the chloroplast. The laser power used to obtain specific GFP labeling was determined using the negative control cells transformed in parallel. This was to ensure that the intracellular signal was due to the additional sequence fused to the GFP and not to the reporter itself; the latter is mainly secreted but can still be detected in the vacuole if enough laser power is provided. Digital images were then pseudocolored green for GFP and red for chlorophyll autofluorescence.

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