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Targeting and post-translational processing of human α_1-antichymotrypsin in BY-2 tobacco cultured cells†

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Summary

The post-translational processing of human α_1-antichymotrypsin (AACT) in Bright Yellow-2 (BY-2) tobacco cells was assessed in relation to the cellular compartment targeted for accumulation. As determined by pulse-chase labelling experiments and immunofluorescence microscopy, AACT sent to the vacuole or the endoplasmic reticulum (ER) was found mainly in the culture medium, similar to a secreted form targeted to the apoplast. Unexpectedly, AACT expressed in the cytosol was found in the nucleus under a stable, non-glycosylated form, in contrast with secreted variants undergoing multiple post-translational modifications during their transit through the secretory pathway. All secreted forms of AACT were *N*-glycosylated, with the presence of complex glycans as observed naturally on human AACT. Proteolytic trimming was also observed for all secreted variants, both during their intracellular transit and after their secretion in the culture medium. Overall, the targeting of human AACT to different compartments of BY-2 tobacco cells led to the production of two protein products: (i) a stable, non-glycosylated protein accumulated in the nucleus; and (ii) a heterogeneous mixture of secreted variants resulting from post-translational *N*-glycosylation and proteolytic processing. Overall, these data suggest that AACT is sensitive to resident proteases in the ER, the Golgi and/or the apoplast, and that the production of intact AACT in the plant secretory pathway will require innovative approaches to protect its structural integrity *in vivo*. Studies are now needed to assess the activity of the different AACT variants, and to identify the molecular determinants for the nuclear localization of AACT expressed in the cytosol.

Introduction

Heterologous protein expression systems involving different eukaryotic organisms have been devised for the production of recombinant proteins subject to complex post-translational modifications (Yin *et al*., 2007). Although each system allows for the production of proteins with suitable stability and biological activity, specific post-translational modifications, leading to the accumulation of distinct structural variants, are usually observed from one system to another (Walsh and Jefferis, 2006). In practice, a close resemblance between the recombinant protein and its natural counterpart may represent an advantage, as the rapid acceptance of recombinant therapeutic proteins by health authorities relies in part on product equivalence. However, emphasis must be placed above all on the activity and pharmacokinetics of the recombinant protein, which suggests that each production platform will have its own advantages and drawbacks depending on each particular protein being expressed (Sethuraman and Stadheim, 2006; Saint-Jore Dupas *et al*., 2007). Glycosylated interferon-β produced in Chinese hamster ovary cells, for instance, was 10 times more active than a non-glycosylated form produced in *Escherichia coli* (Runkel *et al*., 1998), whereas a recombinant, non-glycosylated form

of human erythropoietin expressed in bacteria showed high specific activity *in vitro* relative to its glycosylated natural counterpart (Higuchi *et al*., 1992).

From a clinical perspective, plants show potential as protein expression hosts for a number of reasons, including low production costs, the absence of human pathogenic contaminants in protein preparations, and their well-documented ability to perform post-translational modifications typical of mammalian proteins (Daniell *et al*., 2001; Ma *et al*., 2003; Gomord and Faye, 2004; Saint-Jore Dupas *et al*., 2007). Current major challenges to further support the potential of plants as useful protein biofactories include a significant improvement of final yields and a thorough understanding of biochemical and cellular processes, which sometimes lead to non-optimal post-translational maturation and/or structural instability (Ma *et al*., 2003; Faye *et al*., 2005). Disappointing yields have been obtained for several plant-made proteins in recent years, often correlated with a deficient stability of the recombinant protein product during or shortly after biosynthesis and cellular migration (Doran, 2006; Goulet and Michaud, 2006; Benchabane *et al*., 2008a). *N*-Glycosylation and proteolytic processing, in particular, are two post-translational processes that require special attention, as they dramatically influence the structure and activity of several recombinant proteins expressed in plant systems (Faye *et al*., 2005).

N-Glycosylation is a universal protein post-translational process in eukaryotic cells, starting in the endoplasmic reticulum (ER) by the co-translational transfer of an oligosaccharide precursor on the nascent protein backbone at specific asparagine (Asn) residues of the consensus sequence Asn–X–Thr/Ser (where X is any amino acid, except proline or aspartic acid; Ser, serine; Thr, threonine). As the protein migrates through the secretory pathway, the *N*-glycan is processed by the addition or removal of sugar residues by ER and Golgi processing enzymes. Although precursor oligosaccharides and initial maturation steps in the ER are common to all eukaryotes, later steps of *N*-glycan maturation in the Golgi are species specific and give rise to complex glycans (and glycoprotein products) that diverge among species. In practice, these differences represent a potential limitation for clinically useful glycoproteins produced in plants, as plant glycoepitopes are immunogenic in most animal models and elicit the production of glycan-specific antibodies in humans (van Ree *et al*., 2000; Bardor *et al*., 2003; Gomord *et al*., 2005).

Proteolysis also represents a hurdle for the successful expression of recombinant proteins in plants (Doran, 2006; Benchabane *et al*., 2008a). Whereas the proteolytic removal of pre- and pro-regions is essential for the correct maturation of secreted proteins, unintended proteolysis may affect the yield and stability of several heterologous proteins (Faye *et al*., 2005). A number of recent studies have focused on devising strategies to minimize unintended proteolysis in plant systems (Goulet and Michaud, 2006; Rivard *et al*., 2006; Streatfield, 2007; Benchabane *et al*., 2008a,b), but empirical data with structurally complex proteins are still needed for the fine-tuning of expression strategies to allow stable and fully active proteins to be isolated. In this study, we targeted the human form of α_1-antichymotrypsin (AACT) to different subcellular compartments of Bright Yellow-2 (BY-2) tobacco cultured cells (Hellwig *et al*., 2004), with the aim of further understanding glycosylation and proteolytic processes influencing the fate of complex mammalian proteins in plant cell factories.

AACT is a member of the serpin protein superfamily, which includes inhibitors of serine proteases involved in diverse physiological, cellular and biochemical processes, such as coagulation, inflammation, fibrinolysis, apoptosis, neoplasia, viral pathogenesis, complement activation and protein folding (Silverman *et al*., 2001). Because dysfunctional serpins have been associated with severe diseases in humans (Stein and Carrell, 1995; Chang and Lomas, 1998), recombinant serpins have been readily proposed as useful candidates for replacement therapies (Rubin, 1992). AACT is a glycoprotein with six putative *N*-glycosylation sites and no disulphide bond, showing the typical tertiary structure of serpins with nine α-helices surrounding three β-sheets and a mobile inhibitory loop, referred to as the reactive centre loop (RCL) (Rubin *et al*., 1990; Wei *et al*., 1994). Glycosylation is not essential for AACT protease inhibitory activity (Rubin *et al*., 1990), in contrast with the tertiary fold of the polypeptide backbone, which is critical for both activity and stability. From a practical perspective, AACT is an interesting model to study the potential of plants or plant cells as expression platforms for the production of structurally complex human proteins in both their glycosylated and unglycosylated forms. In this study, we monitored the maturation, structural stability and overall homogeneity of human AACT expressed in the cytosol and secretory pathway of BY-2 tobacco cells.

Results

Human AACT was transiently expressed in *Nicotiana tabacum* leaves, and then stably expressed in *N. tabacum* cv. BY-2 cultured cells, to confirm the effectiveness of plant cells as a suitable expression platform for this protein. The transgenic material was generated by *Agrobacterium tumefaciens*-mediated transformation using different pCambia vectors containing a cDNA coding sequence for AACT and appropriate retrieval/targeting signals for differential subcellular targeting

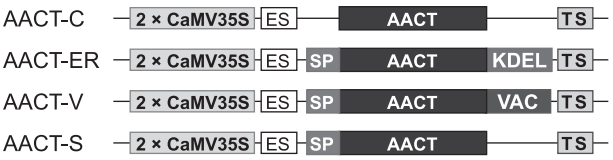

Figure 1 Gene constructs for the targeted expression of human α_1-antichymotrypsin (AACT) in tobacco cells. Gene constructs were devised for the expression of AACT in the cytosol (AACT-C construct), the endoplasmic reticulum (ER) (AACT-ER), the vacuole (AACT-V) or the apoplast (AACT-S). Each construct included a duplicated version of the cauliflower mosaic virus (CaMV) 35S constitutive promoter (2 × CaMV35S), the tobacco etch virus enhancer sequence (ES), the human AACT-encoding sequence (AACT) and the pA35S terminator sequence (TS). Constructs for AACT secretion also included the coding sequence of alfalfa protein disulphide isomerase signal peptide (SP). The tetrapeptide sequence KDEL for protein retention in the ER (KDEL) and the tobacco chitinase A vacuolar sorting sequence (VAC) were also added in the AACT-ER and AACT-V constructs, respectively.

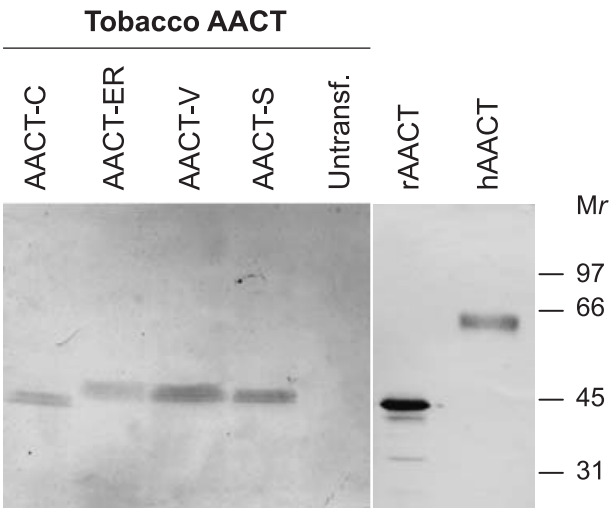

Figure 2 Transient expression of α_1-antichymotrypsin (AACT) in tobacco leaves agroinfiltrated with the AACT transgene constructs. Crude protein extracts from tobacco leaves expressing AACT in the cytosol (AACT-C), the endoplasmic reticulum (AACT-ER), the vacuole (AACT-V) or the apoplast (AACT-S) were resolved by 12% (w/v) sodium dodecylsulphate-polyacrylamide gel electrophoresis and immunodetected with anti-AACT monoclonal antibodies. A protein extract from untransformed tobacco leaves (Untransf.) was used as negative control. Recombinant AACT expressed in *Escherichia coli* (rAACT) and human glycosylated AACT (hAACT) were used as positive controls for immunodetection.

(Figure 1). Stable cell lines and transfected plants were produced for the experiments, targeting AACT to the cytosol (C), the ER, the vacuole (V) or the apoplast (S, for secreted). The signal peptide of an alfalfa protein disulphide isomerase was fused at the N-terminus of AACT to direct its co-translational import into the secretory pathway via the ER membrane. A C-terminal KDEL tetrapeptide was used for retention and accumulation of the protein into the ER. AACT delivery to the vacuole was achieved by fusing the vacuolar sorting signal of tobacco chitinase A at the C-terminus of the protein.

Expression and subcellular distribution of human AACT in tobacco cells

AACT expression was first monitored following transient expression in *Agrobacterium*-infiltrated tobacco leaves. Variants of the inhibitor were immunodetected as ~45-kDa polypeptides in crude protein extracts from tobacco leaves transfected with the AACT constructs (Figure 2). Recombinant forms of the protein targeted to the secretory pathway (i.e. AACT-ER, AACT-S and AACT-V) showed, overall, an apparent molecular mass slightly higher than that observed for unglycosylated AACT translated in the cytosol (AACT-C) or expressed in *E. coli* (rAACT), thereby pointing to the possible addition of glycan chains along the cell secretory pathway.

To test this possibility, BY-2 cells stably transformed with the different constructs were generated as described previously (Gomord *et al*., 1998). Transgenic calli were first screened for AACT expression by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunodetection with AACT-specific antibodies to confirm the presence of AACT in cells. Briefly, AACT protein patterns for the different BY-2 cell lines were similar to the patterns described above for transient expression in tobacco leaves (data not shown). High-expression calli for each transformation construct were selected to initiate suspension cultures, and employed for immunolocalization studies by confocal microscopy using anti-AACT antibodies incubated on fixed, permeabilized transgenic BY-2 cells. As illustrated in Figure 3, all transgenic cell lines harbouring an AACT-encoding construct expressed AACT, in contrast with wild-type cells (wt), which gave no fluorescence signal. A typical ER labelling pattern was observed for AACT-ER and, to a lesser extent, for AACT-S, similar to the pattern observed for the BiP protein, an ER-resident chaperone used as a specific marker for the ER. These observations, suggesting the presence of AACT in the ER as a result of KDEL-dependent accumulation (AACT-ER) or during its migration towards the apoplast (AACT-S), confirmed the correct transfer of the protein into the secretory pathway for gene constructs bearing the alfalfa protein disulphide isomerase signal peptide. Likewise, vacuolar immunostaining in cells harbouring the AACT-V construct confirmed the inclusion of AACT in the secretory pathway, whilst also illustrating the efficiency of the chitinase A vacuolar sorting

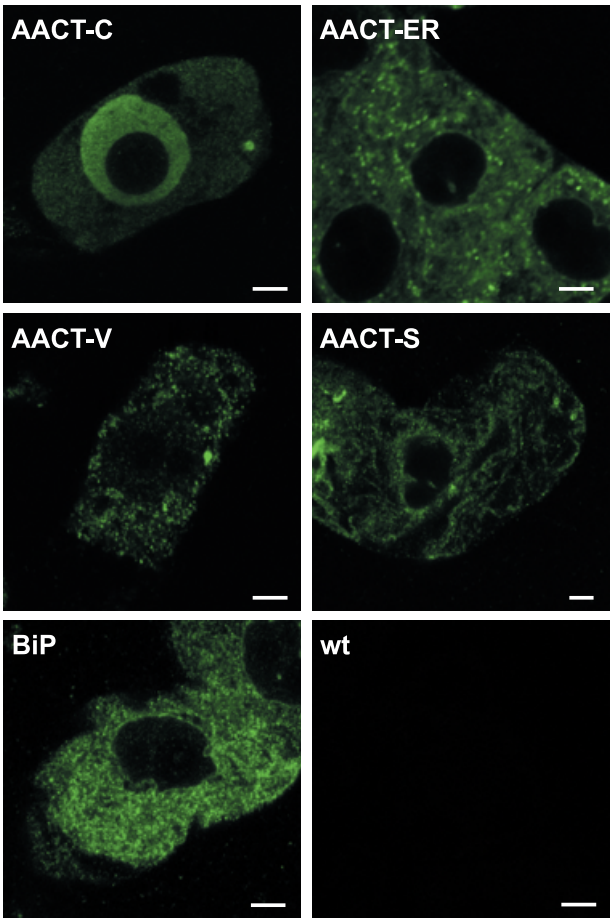

Figure 3 Immunolocalization of α_1-antichymotrypsin (AACT) targeted to different subcellular compartments of Bright Yellow-2 (BY-2) tobacco cultured cells. Retention and targeting signals were used to target AACT to the cytosol (AACT-C), the endoplasmic reticulum (ER) (AACT-ER), the vacuole (AACT-V) or the apoplast (AACT-S). Immunolocalization was performed on 3-day-old BY-2 cells using anti-AACT polyclonal antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. The ER-resident chaperone BiP was immunolocalized in wild-type cells as an ER-positive control, using anti-BiP antibodies. Wild-type cells (wt) treated with anti-AACT and FITC-conjugated antibodies were used as a negative control. Bars, 8 μm.

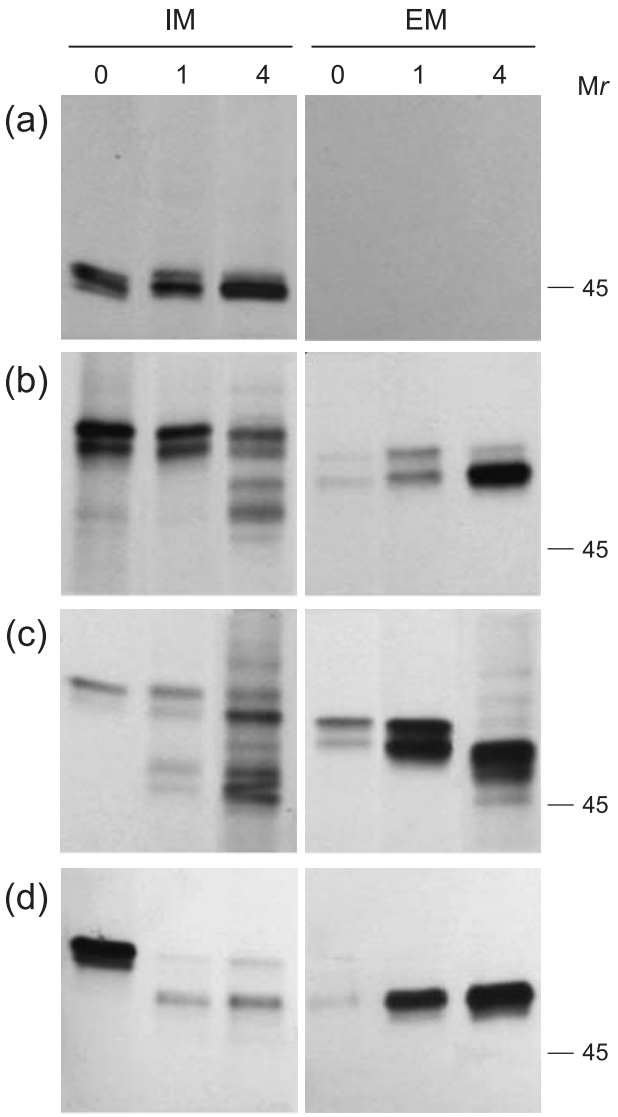

Figure 4 Post-translational processing of α_1-antichymotrypsin (AACT) in Bright Yellow-2 (BY-2) tobacco cultured cells. Pulse-chase labelling experiments were conducted for AACT targeted to the cytosol (AACT-C cell line) (a), the endoplasmic reticulum (ER) (AACT-ER) (b), the vacuole (AACT-V) (c) and the apoplast (AACT-S) (d). The cells were subjected to metabolic pulse radiolabelling with [^{35}S]-methionine and [^{35}S]-cysteine (60 min) and varying chase periods (0, 1 and 4 h). Recombinant AACT variants were immunoprecipitated from the intracellular (IM) and extracelluar (EM) media, and detected by fluorography following sodium dodecylsulphate-polyacrylamide gel electrophoresis.

signal in BY-2 cells. Unexpectedly, AACT expressed with no signal peptide for cellular secretion (AACT-C) was mainly localized in the nucleus, suggesting the occurrence of an intrinsic nuclear localization signal in the primary structure of recombinant AACT.

Secreted AACT is extensively processed along the cell secretory pathway

Pulse-chase labelling experiments involving the expression of radiolabelled AACT were conducted to describe more precisely the distribution and post-translational maturation of AACT at the cellular level, and to detect the protein in the culture medium following cellular secretion (Figure 4). Tobacco cells were pulse labelled for 1 h with [^{35}S]-methionine and [^{35}S]-cysteine, and then chased for 0, 1 or 4 h. Polypeptides antigenically related to AACT were then immunoprecipitated from the intracellular (IM) and extracellular (EM) media (or fractions), and analysed by fluorography following sodium

dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A stable, ~45-kDa polypeptide was detected in the IM of AACT-C cells at the end of the labelling period (Figure 4a) or after a 24-h chase period (not shown). No AACT was detected in the EM for these cells, confirming that the EM was not contaminated by IM proteins during the IM/EM fractionation process.

By contrast, AACT variants targeted to the secretory pathway were detected as ~54-kDa polypeptides rapidly processed in the transformed cells (Figure 4b–d). For instance, AACT in the IM of AACT-S cells was processed to a single form of ~48 kDa, and then secreted almost completely in the EM after 4 h (Figure 4d). The IM maturation of AACT targeted to the vacuole or the ER was even more complex, with several variants appearing successively in the IM and two intermediates detected in the EM (Figure 4b,c). Unexpectedly, all AACT variants harbouring a signal peptide for co-translational insertion in the ER lumen were detected mainly in the EM at the end of the chase period, independent of the presence or absence of additional targeting signals (Figure 4b–d). As suggested by its high relative abundance in the EM after only 1 h, AACT was rapidly secreted in the culture medium by AACT-S cells (Figure 4d), compared with a slower secretion rate for the AACT-ER line (Figure 4b). AACT was also accumulated in the EM of AACT-V cells (Figure 4c), thereby suggesting partial or complete proteolytic processing of the C-terminal targeting signal in the secretory pathway.

Major forms of AACT with similar electrophoretic mobility were detected at the end of the chase period in the EM of AACT-V, AACT-ER and AACT-S cell lines (Figure 4b–d). A rapid proteolytic processing of high-molecular-weight AACT precursors secreted in the EM probably explains the absence of AACT variants well above 45 kDa on immunoblots carried out with total protein extracts from tobacco leaves transiently expressing AACT (see Figure 2). Supporting this hypothesis, an ~50-kDa form readily detected in the EM of AACT-ER and AACT-V cell lines completely disappeared after a few hours, together with the accumulation of an ~48-kDa polypeptide probably corresponding to a stable proteolytic intermediate of glycosylated AACT (Figure 4b,c).

A common maturation pattern for AACT was also observed in the IM of AACT-V and AACT-ER cell lines (Figure 4b, c). Delayed transit along the secretory pathway, partial targeting to the vacuole in the AACT-V cell line and/or recycling of the protein towards the ER in AACT-ER cells possibly accounted for the multiple AACT forms detected in these cells. Although a significant amount of AACT was directed towards the vacuole (AACT-V cells) or accumulated in the ER (AACT-ER cells) (see Figure 3), AACT was detected mainly in the EM in both cases (Figure 4), which suggests either inefficient targeting processes or, more likely, complete or partial processing of C-terminal targeting signals.

Secreted forms of AACT harbour complex, plant-specific *N*-glycans

As a result of the heterogeneous distribution and complex nature of AACT variants in AACT-V and AACT-ER cell lines, we focused on AACT-S cells to characterize further the plant-made recombinant inhibitor. Pulse-chase labelling experiments suggested that AACT in this cell line was post-translationally modified by a combination of *N*-glycosylation and proteolytic trimming (see Figure 4d). Because little is known about the glycan moiety of human AACT, we first assessed the glycosylation status of native AACT purified from human plasma (Figure 5). AACT includes six potential consensus sequences for *N*-glycosylation (Figure 5b), with three or four of these sites presumably occupied by *N*-glycans (Baumann *et al*., 1991; Gettins *et al*., 1996; Zhang *et al*., 2003). As shown in Figure 5a, human AACT exhibits an apparent molecular mass of ~58 kDa following SDS-PAGE, compared with an apparent mass of ~45 kDa for the non-glycosylated form produced in *E. coli*, suggesting that *N*-glycans account for about 25% of this mass. Accordingly, enzymatic deglycosylation of the purified protein with peptide *N*-glycosidase F (PNGase F) gave an ~45-kDa, aglycosylated polypeptide similar in size to AACT produced in *E. coli* (Figure 5a, left panel). As inferred from specific labelling with *Sambucus nigra* lectin I, some glycan(s) on AACT bear terminal α-2,6-*N*-acetylneuraminic acid (Neu5Ac) residues linked to β-1,4-galactose (Gal) residues (Figure 5a, right panel). Supporting this hypothesis, neuraminidase digestion, eliminating terminal Neu5Ac residues, exposed β-1,4-Gal residues to generate *N*-glycans interacting with *Ricinus communis* lectin A (Figure 5a, central panel). Together, these results confirm the presence of complex *N*-glycans typical of mammalian glycoproteins on human AACT, accounting for 25% (i.e. 13–14 kDa) of its molecular mass. Assuming that *N*-glycans on this protein are distributed among three or four glycosylation sites, these observations suggest, overall, that *N*-linked oligosaccharides on native AACT present a minimum triantennary structure (Figure 5b).

Although smaller than native hAACT, AACT produced in the secretory pathway of BY-2 cells showed a molecular mass higher than that observed for unglycosylated (cytoplasmic) AACT (Figures 2 and 4), which could be explained by the addition of glycan residues on the polypeptide chain. In theory, the higher mass of secreted AACT could have resulted from a miscleavage of the signal peptide (Sojikul *et al*., 2003), but

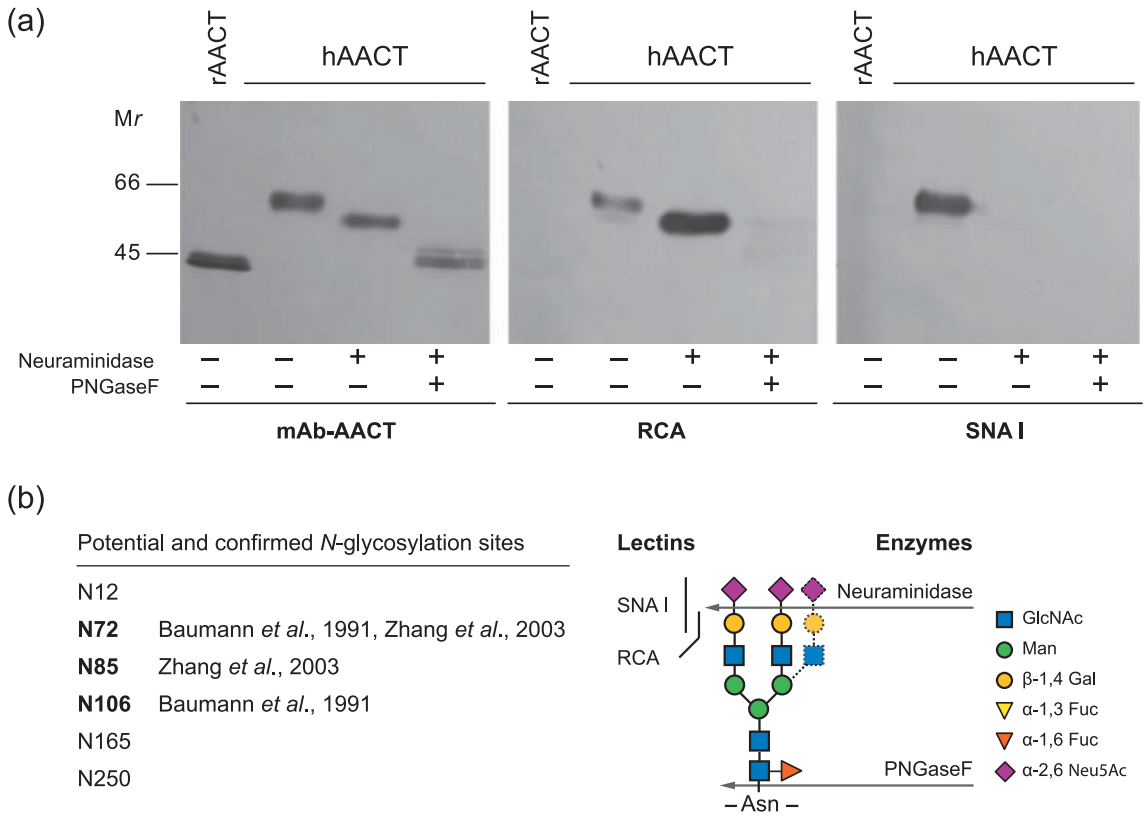

Figure 5 Human α_1-antichymotrypsin (AACT) is highly glycosylated with complex *N*-glycans containing sialic acids. (a) Human AACT (hAACT) digested with neuraminidase and peptide *N*-glycosidase F (PNGase F). All samples were analysed by immuno- or affinodetection with anti-AACT monoclonal antibodies (mAb-AACT), the *Ricinus communis* lectin A (RCA) or the *Sambucus nigra* lectin I (SNA I). Recombinant AACT expressed in *Escherichia coli* (rAACT) was used as a non-glycosylated control. (b) Potential *N*-glycosylation sites on hAACT and putative *N*-glycan structure of the protein, as inferred from exoglycosidase digestion and lectin-binding assays.

this is unlikely as the same signal peptide was correctly processed when fused to bovine aprotinin in transgenic potato (Badri *et al.*, 2008a). Pulse-chase labelling experiments with tunicamycin-treated AACT-S cells were carried out to investigate the glycosylation status of AACT variants (Figure 6). Tunicamycin is an inhibitor of *N*-glycosylation that prevents the biosynthesis of oligosaccharide precursors. Following this treatment, AACT variants of smaller size were detected, including a major form in the IM with a molecular mass of ~45 kDa, similar to the protein backbone (Figure 6a). Smaller polypeptides were also observed in both the IM and the EM, strongly supporting the hypothesis of unintended proteolytic processing along the secretory pathway.

AACT from AACT-S cells was then submitted to enzymatic deglycosylation with PNGase F, which releases *N*-glycans devoid of the plant-specific glycoepitope α-1,3-fucose from proximal *N*-acetylglucosamine (GlcNAc) residues (Lerouge *et al.*, 1998). On treatment with PNGase F, only the initial ~50-kDa form retained in the IM of AACT-S cells could be effectively deglycosylated (Figure 6b), suggesting the occurrence of oligomannosidic-type *N*-glycans on this protein variant. By contrast, AACT variants detected in the EM were not susceptible to PNGase F (Figure 6b), suggesting that *N*-linked oligosaccharides had been processed by the addition of α-1,3-fucose in the Golgi apparatus whilst transiting along the secretory pathway. To define more precisely the structure of *N*-glycans on mature AACT, the protein was enriched from the culture medium and its *N*-glycans were analysed either by affinodetection with the lectin concanavalin A (Con A) or by immunodetection with antibodies specific to β-1,2-xylose- and α-1,3-fucose-containing glycoepitopes (Figure 6c). Consistent with PNGase F assays (Figure 6b), no glycans were detected with Con A, specific to high-mannose-type glycans. By contrast, the glycans were recognized by antibodies specific to β-1,2-xylose- and α-1,3-fucose-containing glycoepitopes, characteristic of plant complex *N*-glycans. These results further demonstrate that recombinant AACT forms *in planta* are synthesized as glycoproteins in tobacco cells, with attached glycans matured to plant-specific complex *N*-glycans during their transit through the secretory pathway.

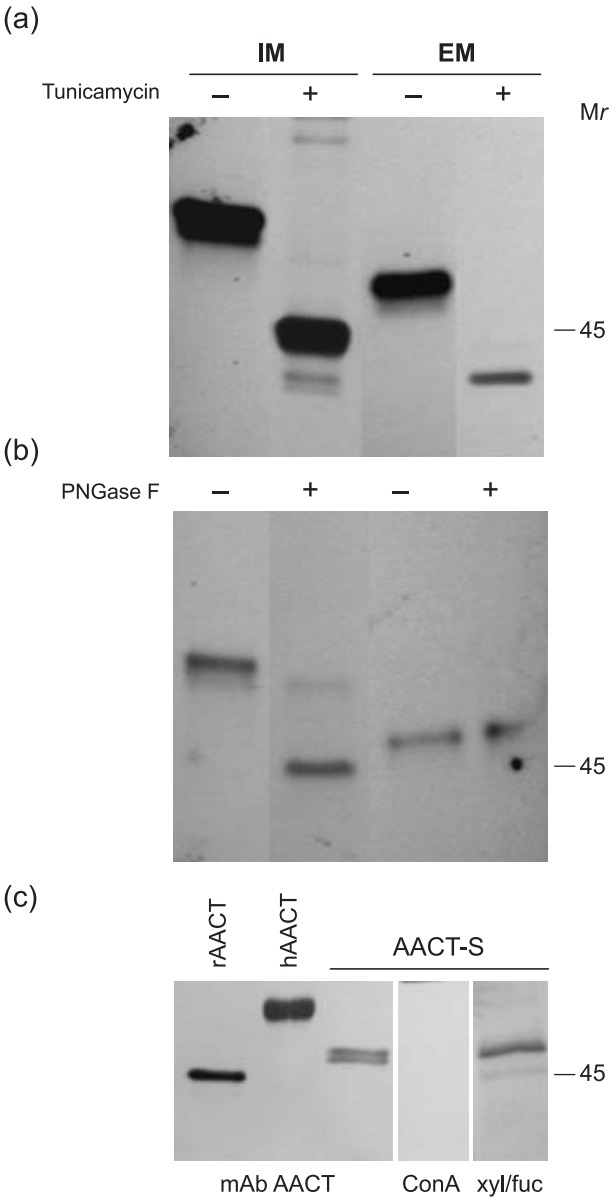

Figure 6 Recombinant α_1-antichymotrypsin (AACT) secreted from AACT-S tobacco cells is *N*-glycosylated and harbours sugar residues typical of plant complex *N*-glycans. (a) AACT-S cells were incubated with the *N*-glycosylation inhibitor tunicamycin (+) [or left untreated (–)] for 90 min, prior to pulse-chase labelling. AACT was immunoprecipitated from intracellular (IM) or extracellular (EM) protein extracts, and analysed by fluorography following sodium dodecylsulphate-polyacrylamide gel electrophoresis. (b) Peptide *N*-glycosidase F (PNGase F) digestion of AACT-S. AACT-S cells were radiolabelled for 60 min and chased for 2 h. AACT was immunoprecipitated from IM and EM protein extracts and submitted (+) or not (–) to PNGase F digestion. (c) Bacterial recombinant AACT (rAACT), human AACT (hAACT) and tobacco secreted AACT (AACT-S) were immunodetected with anti-AACT antibodies (mAb-AACT). Polymannosidic *N*-glycans were affinodetected with the concanavalin A lectin, and complex plant *N*-glycans harbouring β-1,2-xylose and α-1,3-fucose residues were detected with anti-β-1,2-xylose and anti-α-1,3-fucose antibodies (xyl/fuc).

AACT is cleaved in the RCL by serine proteases secreted in the EM

Together with *N*-glycosylation, pulse-chase labelling experiments pointed to the possible onset of proteolytic processing involving multiple cleavage steps along the secretory pathway (Figure 4), consistent with the context of a protease-rich environment in plant cells (Benchabane *et al*., 2008a). As AACT variants targeted to the secretory pathway were found mostly in the EM, spiking experiments were conducted with recombinant AACT produced in *E. coli* (rAACT) to detect eventual effects of the host plant EM proteases on the AACT polypeptide backbone. rAACT was incubated overnight at room temperature in the culture medium of a 4-day-old BY-2 cell culture to mimic the conditions under which the protein is accumulated in this plant expression platform. A stable truncation product shorter by 4–5 kDa than the complete protein was generated during this period (Figure 7a,b), in contrast with rAACT spiked in fresh culture medium and incubated under the same conditions which showed unchanged electrophoretic mobility. Diagnostic protease inhibitors were used to identify major EM protease activities involved in AACT cleavage. The inhibitors *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), pepstatin and ethylenediaminetetraacetic acid (EDTA), specific to cysteine, aspartate and metalloproteases, respectively, had a negligible effect on AACT stability in the culture medium, in sharp contrast with the broad-spectrum inhibitor of serine proteases, phenylmethylsulphonylfluoride (PMSF), that clearly prevented AACT cleavage.

The inhibitory reactive site of serpins is part of a protruding loop, the RCL, which acts as a bait to trap target proteases. The RCL of inhibitory serpins is very accessible to target (sensitive) proteases, but also inherently susceptible to degradation by non-target proteases, making it potentially labile in complex biological media containing proteases from different mechanistic classes. As the RCL of AACT is located in the C-terminal region, proteolysis on this side of the protein may dramatically affect its inhibitory activity (Potempa *et al*., 1991). Supporting this possibility, N-terminal sequencing of rAACT variants detected after treatment with the EM proteases exhibited either an intact N-terminal extremity, or a truncated N-terminal end short of eight amino acids (GIPNSPLD†E) corresponding to a molecular mass loss of only 812 Da (Figure 7c). The molecular mass of plant-cleaved rAACT was visually compared with that of rAACT treated with human neutrophil elastase (Figure 7a,b), known to process this serpin at positions P1-P1′, P4-P3 and P6-P5 of the RCL, according to the nomenclature of Schechter and Berger (1967), i.e. at the P1–P1′ interface and two or four residues upstream of P1

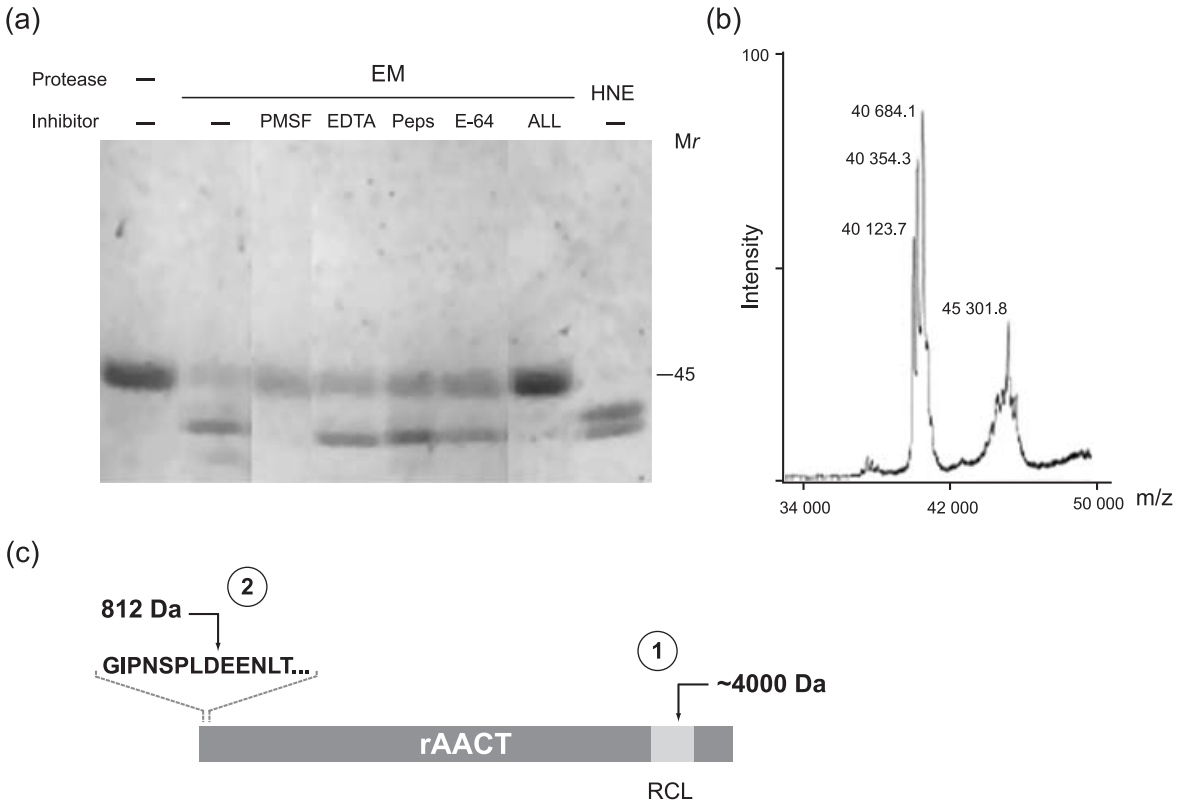

Figure 7 Proteolytic processing of α_1-antichymotrypsin (AACT) by Bright Yellow-2 (BY-2) tobacco cell extracellular proteases. (a) Bacterial recombinant AACT (rAACT) is cleaved to lower molecular mass forms by proteases secreted in the culture medium. rAACT was incubated in 4-day-old filtered BY-2 culture medium (EM) for 16 h at room temperature. As controls, rAACT was incubated in fresh culture medium (left lane) or with human neutrophil elastase (HNE) known to cleave AACT within the reactive centre loop (RCL). The addition of diagnostic protease inhibitors [phenylmethylsulphonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), pepstatin (Peps), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), or a combination of these inhibitors (All)] had different inhibiting effects on AACT processing by the extracellular proteases. (b) Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrum illustrating the cleavage of rAACT by HNE in the RCL. Molecular *m/z* values of 45 302 and ~40 000–41 000 were observed for the complete and truncated forms of the serpin, respectively. (c) Putative N- and C-terminal cleavage of rAACT by tobacco cell EM proteases, as inferred by comparison with HNE-generated fragments following sodium dodecylsulphate-polyacrylamide gel electrophoresis (panels a and b) (1) and N-terminal sequencing of the major truncation products (2).

(Rubin *et al*., 1994). As observed in Figure 7a, EM proteases and human neutrophil elastase generated AACT polypeptides of similar electrophoretic mobility, about 5 kDa smaller than the complete inhibitor (Figure 7b), which suggests a common cleavage zone for these enzymes, probably located within or near the solvent-exposed RCL.

Discussion

The production of recombinant serpins in plants has been limited, until now, to the antitrypsin inhibitor α_1-proteinase inhibitor (Terashima *et al*., 1999b; Huang *et al*., 2001). In this study, we assessed the potential of plant cells for the expression of AACT, another serpin of medical significance. The sequestration of recombinant proteins in different organelles using targeting/retrieval signals has been proposed by several authors to achieve high yields in various eukaryotic systems, including plants and plant cells. The neutral pH, oxidizing redox potential and low proteolytic content of the ER, together with the abundance of molecular chaperones and enzymes facilitating protein folding, often make this organelle an interesting location for the accumulation of stable and correctly assembled recombinant proteins. ER retention using the K/HDEL C-terminal tetrapeptide, in particular, has been largely exploited in plant systems, notably for the expression of clinically useful antibodies (Conrad and Fiedler, 1998; Gomord and Faye, 2004).

Despite the use of targeting signals on a routine basis in most protein expression systems, the actual IM location of foreign proteins is rarely assessed. A number of recent studies have suggested that signals for the subcellular targeting of recombinant proteins are usually functional in plant cell heterologous environments, but unexpected protein targeting may arise in some cases. This was illustrated, for instance,

with Guy's 13 secretory immunoglobulin A light and heavy chains in rice seeds, which were accumulated in ER-derived protein bodies and protein storage vacuoles in the endosperm, despite the absence of ER retention or vacuolar sorting peptides (Nicholson *et al*., 2005). In a similar way, the heat-labile enterotoxin antigen B from *E. coli* was accumulated in starch granules of transgenic maize kernels, despite an expected apoplastic secretion (Chikwamba *et al*., 2003), whereas, more recently, a KDEL-tagged scFv-Fc fusion antibody expressed in *Arabidospis thaliana* seeds was partially localized in the periplasmic space (Van Droogenbroeck *et al*., 2007). In the present work, immunolocalization observations showed, overall, a correct subcellular location for ER- and vacuolar-tagged AACT variants, confirming that heterologous targeting signals included in gene constructs were functional in tobacco cells. However, pulse-chase labelling experiments clearly showed that both AACT-V and AACT-ER cell lines were also accumulating AACT in the culture medium.

Most documented examples of recombinant proteins in unexpected cell compartments have been associated with expression in seeds, perhaps suggesting a tissue-specific efficacy for different protein targeting signals (Chikwamba *et al*., 2003; Arcalis *et al*., 2004; Nicholson *et al*., 2005; Drakakaki *et al*., 2006; Petruccelli *et al*., 2006; Van Droogenbroeck *et al*., 2007). By comparison, the unexpected targeting of AACT in BY-2 tobacco cells was probably the result of intrinsic structural features and modifications of recombinant AACT *in vivo*, rather than an atypical sorting mechanism in cultured cells. KDEL-tagged AACT found in the EM could have resulted from the leakage of K/HDEL-tagged proteins from the ER retrieval machinery, as documented previously for both endogenous and recombinant proteins (Henderson *et al*., 1997; Okamoto *et al*., 1999; Ko *et al*., 2003). Alternatively, the KDEL tag could be cleaved off as a result of protease-susceptible sites at the C-terminus of the protein (Badri *et al*., 2008b). In addition, an effective retrograde translocation of recombinant proteins from the Golgi back to the ER has been shown to depend on the steric accessibility of the K/HDEL sequence to specific ERD2 receptors in the *cis*-Golgi (Sriraman *et al*., 2004). The C-terminal structure of recombinant AACT may not be optimally exposing the KDEL signal to the Golgi retrieval machinery, thereby accounting for the (partial) EM localization of the protein. Similarly, AACT in AACT-V cells was mainly secreted in the EM, again suggesting that the C-terminal vacuole targeting signal was either cleaved off or not accessible for appropriate receptor-mediated sorting to the vacuole.

AACT expressed in the cytosol also showed an unexpected subcellular localization, with most fluorescence labelling confined to the nucleus (Figure 3). This intriguing observation, however, was consistent with previous studies reporting the accumulation of human plasma AACT in the nucleus of carcinoma cells (Takada *et al*., 1982, 1986), and with the natural ability of this protein to bind DNA (Naidoo *et al*., 1995). Small proteins, i.e. proteins smaller than ~40 kDa, may diffuse freely from the cytosol towards the nucleus through the nuclear pore complex, whereas the nucleocytoplasmic transit of larger proteins generally involves ATP-dependent processes, implicating specialized transport proteins after the recognition of specific nuclear localization signals (Merkle, 2004; Pemberton and Paschal, 2005). Because AACT is probably too large to easily diffuse into the nucleus, the transit of AACT towards the nucleus of tobacco cells remains to be understood, and the possible existence of still unidentified, cryptic localization signals for nuclear import needs to be confirmed (see accompanying paper: Benchabane *et al*., 2008c).

One advantage offered by plants for the production of recombinant proteins is their ability to operate complex post-translational modifications required for the activity and stability of several proteins (Gomord and Faye, 2004; Faye *et al*., 2005). As illustrated with the different variants detected during pulse-chase labelling experiments (Figure 4), recombinant AACT underwent several post-translational modifications in BY-2 tobacco cells. Like its human counterpart, plant-made AACT targeted to the secretory pathway was *N*-glycosylated and, by extrapolation from the size of AACT-glycosylated precursors at the end of the pulse period (~50 kDa) (Figure 4), the inhibitor would bear at least four *N*-glycan chains in plant cells, as also assumed for AACT purified from human plasma (Baumann *et al*., 1991; Gettins *et al*., 1996; Zhang *et al*., 2003). Because human AACT probably harbours large multi-antennary *N*-glycans with terminal sialic acid residues (Figure 5), the smaller mass of plant-made AACT variants compared with human AACT is probably the result of less extended glycan structures, rather than a smaller number of sites occupied by *N*-glycans. Unlike human AACT, plant-made AACT contains *N*-glycans rapidly processed to PNGase F-resistant complex glycans with α-1,3-fucose residues on proximal GlcNAcs. To substantiate these results, EM AACT was shown to react with α-1,3-fucose- and β-1,2-xylose-specific antibodies. α-1,3-Fucose and β-1,2-xylose residues, typical of plant *N*-glycans (Faye *et al*., 1993; Lerouge *et al*., 1998), have been reported to increase the immunogenicity and allergenicity of plant-derived glycoproteins in mammals (van Ree *et al*., 2000; Bardor *et al*., 2003; Gomord *et al*., 2005). Retaining AACT in the ER using a KDEL C-terminal signal represents a possible strategy to prevent the apposition of immunogenic epitopes in Golgi cisternae (Sriraman *et al*.,

2004; Gomord *et al*., 2005; Petruccelli *et al*., 2006); however, this proved to be unsuccessful here to obtain non-immunogenic AACT because of the low efficiency of the ER retrieval signal. Indeed, a large fraction of AACT in AACT-ER cells escaped the ER and migrated through the Golgi, where *N*-glycans were matured before secretion of the protein out of the cell.

This aberrant migration of KDEL-tagged AACT was probably the result of unintended proteolytic processing along the secretory pathway (Figures 4 and 7). Plant cells contain a complex array of proteases that are essential to ensure important metabolic and cellular functions (Beers *et al*., 2004; Schaller, 2004), but may also impair recombinant protein production and cellular translocation by directly hydrolysing nascent or mature polypeptide chains (Faye *et al*., 2005). Several strategies have been proposed to prevent unwanted proteolysis in plant hosts, including the targeting of recombinant proteins to specific organelles using appropriate sorting signals (Doran, 2006; Benchabane *et al*., 2008a). Protein sequestration in the ER has led, notably, to improved stability and higher yields for a number of proteins (Stoger *et al*., 2002; Pagny *et al*., 2003; Gomord *et al*., 2004; Peng *et al*., 2006; Petruccelli *et al*., 2006), but this compartment may not be ideal for all proteins, given the presence of proteases as early as in the ER along the endomembrane system, presumably involved in the degradation of misfolded proteins and the processing of pre- and pro-regions (Bass *et al*., 2000; Schmitz and Herzog, 2004). The bovine plasma protein aprotinin, for instance, showed greater stability when secreted in the apoplast of potato leaves than when retained in the ER using the KDEL signal, presumably as a result of proteolytic events at the C- and N-termini (Badri *et al*., 2008b). Likewise, the apoplastic milieu may represent an interesting compartment for recombinant protein expression (Liénard *et al*., 2007), but plant cells also secrete proteases that may significantly compromise protein accumulation *in vivo* (Hellwig *et al*., 2004; Schiermeyer *et al*., 2005). For instance, the expression of α_1-protease inhibitor in the EM medium of transgenic rice suspension cultured cells led to the accumulation of a truncated protein product with impaired inhibitory activity against target proteases (Terashima *et al*., 1999b), presumably resulting from the release of sulphydryl proteases in the cell culture medium (Terashima *et al*., 1999a). More recently, a recombinant form of α_1-plasminogen activator from *Desmodus rotundus* in BY-2 tobacco cells was altered in the EM medium by metalloproteases secreted by growing cells (Schiermeyer *et al*., 2005). In the present study, recombinant AACT was susceptible to PMSF-sensitive serine proteases secreted in the EM by tobacco cells, again illustrating the susceptibility of recombinant proteins to specific proteases in plant platforms, and stressing the importance of empirically assessing the stability of each protein to express on a case-by-case basis.

As shown by spiking experiments, tobacco cultured cells secrete proteases able to use AACT as a substrate, whereas pulse-chase labelling experiments suggest the proteolytic trimming of this protein within the secretory pathway before its secretion in the EM. Overall, our data suggest that AACT is sensitive to proteases in the ER and/or the Golgi, as well as in the apoplast, and that the production of intact AACT in the plant secretory pathway will require innovative expression schemes aimed at protecting the integrity of the protein backbone (Goulet and Michaud, 2006). The co-secretion of a companion protease inhibitor altering endogenous proteolytic activities along the secretory pathway could represent an effective strategy (Benchabane *et al*., 2008b), as illustrated recently for recombinant antibodies secreted by transgenic tobacco roots, which were stabilized by the co-secretion of a Bowman–Birk trypsin inhibitor from soybean (Komarnytsky *et al*., 2006). As observed here, simply expressing AACT with no targeting peptide could also provide an effective way to produce this protein in a stable form in plants (Figures 2 and 4). Many serpins are glycoproteins, but the inhibitory activity of these proteins may not be impaired in the absence of *N*-glycans (Rubin *et al*., 1990; Kwon *et al*., 1995a; Lamark *et al*., 2001). In practice, this observation underlines the potential usefulness of the cytosol for the production of AACT in a non-glycosylated form, as also suggested previously for *E. coli* (Rubin *et al*., 1990; Bird *et al*., 2004). Aglycosylated serpins, however, may exhibit a high plasma clearance rate (Minta, 1981; Travis *et al*., 1985) and an increased susceptibility to denaturants and thermal denaturation relative to glycosylated AACT (Powell and Pain, 1992; Kwon *et al*., 1995b; Kwon and Yu, 1997), which suggests a stabilizing role for glycan chains *in vivo*. Studies are now required to assess the inhibitory potency of non-glycosylated AACT in terms of specific activity compared with human AACT and *in vivo* stability in a therapeutic context. Work is also needed to understand why cytosol-targeted AACT is found in the nucleus of plant cells, and to assess the impact of such unexpected targeting on protein accumulation and stability *in planta* (Benchabane *et al*., 2008c).

Experimental procedures

Gene constructs

Four expression cassettes were devised to target recombinant AACT to the cytosol (AACT-C construct), the ER (AACT-ER), the vacuole (AACT-V) and the apoplast (AACT-S) of tobacco cells (see Figure 1). The coding sequence of AACT [National Center for Biotechnology

Information (NCBI) Accession Number J05176] was amplified by RT-PCR from a hepatocyte mRNA library using the following primers: 5′-AACAGCCCACTTGACGAGG-3′ and 5′-TTACTGAGAGCCCCACTGCTTG-3′. The resulting cDNA was subcloned in the PCR-blunt cloning vector (Invitrogen, La Jolla, CA, USA) and confirmed by automatic DNA sequencing (Service de Séquençage de l'Université Laval, Québec, QC, Canada). For targeting to the secretory pathway, the N-terminal signalling peptide of alfalfa protein disulphide isomerase (Shorrosh and Dixon, 1991) was introduced at the 5′ end of the AACT coding sequence. The ER retention signal KDEL and the tobacco chitinase A vacuolar sorting signal (Neuhaus *et al*., 1991) were added to the 3′ end of the coding sequence by PCR amplification using the following 3′ end primers: 5′-TATCTAGACTAAAGTTCATCCTTGGCTTGCTTGGGATTGG-3′ (for ER retention) and 5′-TAGACTACATAGTATCGACTAAAAGGTCGGCTTGCTTGGGATTGG-3′ (for vacuolar sorting). The four *aact* transgenes were cloned into a modified pUC-19 vector (Fermentas Life Science, Burlington ON, USA) including the double constitutive cauliflower mosaic virus (CaMV) 35S promoter, a tobacco etch virus (TEV) enhancer sequence and the CaMV PA35S terminator sequence. The resulting constructs were transferred into the commercial vector pCambia 2300 (CAMBIA, Canberra, Australia) and introduced in *Agrobacterium tumefaciens* LBA4404 for tobacco transformation.

For expression in *E. coli*, an AACT/pGEX-3X vector was constructed, allowing for the expression of AACT fused to glutathione *S*-transferase (GST), used as a tag for affinity purification. The *aact* sequence was amplified by PCR with the following DNA primers: 5′-AA*GGATCC*AAAACAGCCCACTTGACG-3′ and 5′-TT*GAATTC*TAGGCTTGCTTGGGATTG-3′. Unique *Bam*HI and *Eco*RI restriction sites were included in the primer sequences (italic) to allow ligation in the expression vector pGEX-3X (Amersham Biosciences, Baie d'Urfé, QC, Canada). Following PCR, the hybrid genes were controlled by automatic sequencing and subcloned into pGEX-3X, in frame with the GST coding sequence. This vector was introduced in *E. coli* strain BL21 for heterologous expression and purification.

Stable transformation of BY-2 tobacco cells

Stable transformation of tobacco (*N. tabacum*) cv. BY-2 cells was performed as described previously (Gomord *et al*., 1998), using *Agrobacterium* cells transformed with the pCambia vectors (see above). Transformants were selected in the presence of kanamycin (100 μg/mL), analysed for *aact* transcription by RT-PCR, and immunodetected with anti-AACT monoclonal antibodies (US Biologicals, Swampscott MA, USA). Microcalli exhibiting high levels of AACT were screened and used to initiate suspension cultures for recombinant protein expression. Tobacco cell cultures were maintained in Murashige and Skoog medium supplemented with 3% (w/v) sucrose, 1 mg/L thiamine, 50 mg/L myo-inositol, 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 mg/L KH_2PO_4, on an orbital shaker at 140 r.p.m. in the dark at 20 °C. The cells were transferred to a fresh medium every 2 weeks with 5% (v/v) inoculum.

Transient expression in *N. tabacum*

For transient expression in tobacco leaf cells, 2-day-old *Agrobacterium* cultures were washed and resuspended in fresh infiltration medium [50 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.6, 0.5% glucose (w/v), 2 mM Na_3PO_4, 100 mM acetosyringone]. The bacterial suspensions were infiltrated in the abaxial epidermis of *N. tabacum* cv. Xanthi leaves using a syringe. The plants were grown for 2 days prior to the extraction of total soluble proteins. Leaf-infiltrated tissues were cut out, frozen in liquid nitrogen, and ground with a mortar and a pestle in protein extraction buffer [75 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 3% (v/v) β-mercaptoethanol, 1 mM EDTA, 1 mM PMSF]. The soluble proteins were recovered by centrifugation for subsequent immunodetection.

Heterologous expression in *E. coli*

Heterologous expression and purification of recombinant AACT in *E. coli* were carried out using the GST gene fusion system (Smith and Johnson, 1988), as described previously for other protease inhibitors (Michaud *et al*., 1994). In brief, 1.5 mL of an overnight *E. coli* culture was inoculated into 150 mL of Luria–Bertani (LB) medium containing 100 μg/mL ampicillin. The culture was incubated at 37 °C with vigorous agitation until reaching an optical density at 600 nm (OD_{600}) of 0.4–0.6, prior to the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. The culture was incubated for an additional 16 h at 37 °C, before harvesting the bacteria by centrifugation for 10 min at 14 000 ***g***. After discarding the supernatant, the bacterial pellet was submitted to several freeze–thaw cycles, and then suspended in 50 mM Tris lysis buffer, pH 8.0, containing 5% (w/v) sucrose, 50 mM EDTA and 5% (v/v) Triton X-100. The bacterial lysate was incubated on ice for 5 min and centrifuged at 20 000 ***g*** for 10 min at 4 °C; the supernatant containing soluble proteins was saved for further analysis. Purification of the GST fusions was performed by affinity chromatography with reduced glutathione-Sepharose beads (Amersham Biosciences) as described previously (Kiggundu *et al*., 2006). The GST moiety was removed from the inhibitor using human Factor X_a (Novagen, La Jolla, CA, USA), according to the supplier's instructions. The purity of the inhibitors was visualized by SDS-PAGE (Laemmli, 1970) and immunodetection with anti-AACT antibodies. Protein concentrations were determined by densitometry of Coomassie blue-stained gels after SDS-PAGE, using the PHORETIX two-dimensional image analysis software (Nonlinear Dynamics, Durham, NC, USA) and bovine serum albumin (BSA) (Sigma-Aldrich, Oakville, ON, Canada) as a protein standard.

Metabolic pulse-chase labelling and immunoprecipitation

For pulse-chase labelling time course analyses, filtered BY-2 suspension cultured cells (300 mg) were pulse labelled by resuspension in 900 μL of fresh culture medium supplemented with 4 MBq of [^{35}S]-protein labelling mix (NEN Life Science Products, Boston, MA, USA). After pulse labelling for 60 min at 20 °C with gentle shaking (140 r.p.m.), the cells were incubated for various chase periods by adding 110 μL of chase medium containing 50 mM methionine and 50 mM L-cysteine. EM and IM proteins were isolated as described previously (Gomord *et al*., 1997). Briefly, EM proteins were collected by centrifugation for 3 min at 50 ***g***. The cell pellet was washed with TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) prior to harvesting by the addition of 500 μL of lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 1% (w/v) sodium ascorbate] containing the Complete™ protease inhibitor mixture (Roche Diagnostics, Laval, QC, Canada), and sonicated (2 × 20 s; 40% amplitude). The cell slurry was centrifuged for 15 min at 12 000 ***g***,

and the supernatant containing IM proteins was recovered. Supernatants from both cell medium and cell lysis mixture were immunoprecipitated with anti-AACT polyclonal antibodies (US Biologicals) (dilution 1 : 500) for 2 h at room temperature on an end-over-end shaker, before adding 50 μg of protein A Sepharose (Amersham Biosciences) and incubating for an additional hour. Immunocomplexes were washed four times in washing buffer [TBS containing 1% (v/v) Triton X-100 and 0.2% (w/v) SDS], twice in TBS containing 0.1% (v/v) Triton X-100 and, finally, twice in TBS. Radiolabelled proteins were recovered by heating at 80 °C for 10 min in SDS-PAGE loading buffer, fractionated by SDS-PAGE and detected by fluorography. Inhibition of co-translational *N*-glycosylation was carried out by incubating the cells for 90 min at room temperature in fresh culture medium supplemented with 18 μM tunicamycin (Sigma-Aldrich) prior to pulse labelling performed as described above.

Enzymatic deglycosylation of human (native) AACT

Human AACT (Sigma-Aldrich) was treated successively with neuraminidase and PNGase F (Roche Diagnostics), and a sample was put aside for gel analysis following each treatment. The protein (5 μg) was diluted in neuraminidase digestion buffer (90 mM sodium phosphate, pH 5.0), and incubated with 0.5 U of neuraminidase from *Arthrobacter ureafaciens* (E.C.3.2.1.18) (EY Laboratories, San Mateo, CA, USA) for 3 h at 37 °C. The neuraminidase-treated sample was further digested with PNGase F by diluting the mixture first in one volume of PNGase F digestion buffer [90 mM sodium phosphate, pH 8.0, 0.2% (w/v) SDS], and then heating for 5 min at 100 °C. The sample was left to stand for 2 min, prior to the addition of one volume of buffer [90 mM sodium phosphate, pH 8.0, 0.2% (v/v) Nonidet-P40] and PNGase F (0.4 U). The vial was incubated for 16 h at 37 °C. AACT samples after simple or double digestion were resolved by SDS-PAGE, electrotransferred on to a nitrocellulose sheet and analysed by affino- and immunodetection.

Enzymatic deglycosylation of plant-made AACT

Treatment with PNGase F (Roche Diagnostics) was performed on pulse radiolabelled AACT–protein A immunocomplexes by heating for 10 min in 60 μL of digestion buffer [50 mM Tris-HCl, pH 7.6, 0.2% (w/v) SDS]. The proteins recovered were left to stand for 2 min at room temperature and diluted in 60 μL of dilution buffer [50 mM Tris-HCl, pH 7.6, 0.02% (v/v) Nonidet P40]. Half of the sample was treated with 1 μL of PNGaseF (1 mU/mL) for 16 h at 37 °C. The proteins were resolved by SDS-PAGE and detected by fluorography.

Affino- and immunodetection

EM proteins from BY-2 tobacco cells were prepared from 3–4-day-old suspension cultures. The cells were harvested by filtration and the culture medium containing apoplastic proteins was put aside. To isolate cell wall proteins and peripheral proteins ionically bound to cell membranes, the cell pellet was washed in fresh medium containing 0.5 M NaCl, incubated for 20 min at 4 °C and filtered. The washing fluid was pooled with the culture medium, and the proteins were precipitated by two subsequent cycles of ammonium sulphate precipitation at 40% (w/v) and 90% (w/v) saturation. Protein extracts were fractionated by 12% (w/v) SDS-PAGE and electrotransferred on to a nitrocellulose sheet. AACT was detected using anti-AACT monoclonal antibodies at a dilution of 1 : 5000 in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 1% (w/v) skimmed milk, or using anti-β-1,2-xylose and anti-α-1,3-fucose antibodies (Faye *et al*., 1993; Gomord *et al*., 1997) diluted 1 : 1000 in the same buffer. Appropriate secondary antibodies (Sigma-Aldrich) coupled to alkaline phosphatase were diluted 1 : 10 000. *N*-Glycans were affinodetected using the lectins Con A (Sigma-Aldrich), *Sambucus nigra* agglutinin I (SNA I) (EY Laboratories) and *Ricinus communis* agglutinin I (RCA) (Vector Laboratories, Burlingame, CA, USA), specific to polymannosidic glycans, terminal α-2,6-*N*-acetyl-neuraminic acid β-1,4-galactose *N*-acetylglucosamine (α-2,6-Neu5Ac β-1,4-Gal) and terminal β-Gal residues, respectively. The membranes were saturated for 1 h in TTBS [TBS supplemented with 0.1% (v/v) Tween 20] prior to incubation with lectins for 90 min at room temperature on an orbital shaker. Con A (25 μg/mL) was diluted in Con A buffer (TTBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Biotin-SNA I (2 mg/mL) and biotin-RCA (20 μg/mL) were diluted in TTBS. The membranes were washed in the same buffer, and incubated for 1 h at room temperature with peroxidase (50 μg/mL) diluted in Con A buffer for the Con A assay, or streptavidin peroxidase for the biotinylated lectin assays. The proteins were revealed using an appropriate colorimetric peroxidase substrate.

Immunocytolocalization by confocal microscopy

For immunocytolocalization, 4-day-old BY-2 tobacco cells were harvested by filtration, fixed and permeabilized before staining with anti-AACT antibodies for confocal microscopy. Unless otherwise indicated, all steps were conducted at room temperature on an end-over-end shaker in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (PIPES) buffer supplemented with specific reactants. Cell fixation was performed by a 1-h incubation in 4% (w/v) paraformaldehyde. Permeabilization was realized by a 20-min incubation in 1% (w/v) cellulase + 0.1% (w/v) pectinase + 1% (w/v) BSA, followed by a 10-min incubation in 0.5% (v/v) Triton X-100. The cells were saturated with 1% (w/v) BSA + 1% (w/v) fish gelatin to eliminate nonspecific staining, probed with a 1 : 100 dilution of anti-AACT or anti-BiP (Gomord *et al*., 1997) polyclonal antibodies overnight at 4 °C, without agitation, and then washed three times in 1% (w/v) fish gelatin prior to a final incubation for 1 h in a 1 : 100 dilution of anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC). The cells were washed three times in PIPES, twice in water and mounted on a microscopy slide. Fluorescence images were captured on a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Rueil-Malmaison, France). Images were generated using a 488-nm argon ion laser source, and fluorescence was recorded with a photomultiplier set-up in the 493–538-nm range. Image patterns were processed using LCS Lite software version 2.64 (Leica Microsystems).

AACT stability assay

A stability assay was conducted with bacterially expressed AACT (rAACT; see above) incubated in BY-2 cell culture medium. Briefly, 4 μg of purified rAACT was added to filtered EM medium of 4-day-old wild-type BY-2 suspension cells. The protein was also incubated in fresh culture medium as a negative control. All samples were incubated overnight at room temperature on an end-over-end shaker. The following diagnostic protease inhibitors were included in

the culture medium to identify the protease classes involved in AACT hydrolysis: 1 mM PMSF, 1 μg/mL E-64, 5 mM EDTA and/or 1 μg/mL pepstatin A (Sigma-Aldrich). Partial digestion with human neutrophil elastase (E.C.3.4.21.37) (Sigma-Aldrich) was also performed to generate AACT variants cleaved in the RCL (Rubin *et al*., 1990). The cleavage was performed with an inhibitor to enzyme ratio of 20 : 1 in Tris buffer [100 mM Tris-HCl, pH 8.3, 0.025% (v/v) Triton X-100]. The reaction was carried out for 2 h at 37 °C, and protein hydrolysis was stopped by the addition of 2 mM PMSF. The proteins recovered were resolved by SDS-PAGE, and AACT was immunodetected as described above.

Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analyses

For MALDI-TOF-MS, 500 μmol of rAACT partially digested with human neutrophil elastase was desalted on a Centricon 10 column (Millipore, Molsheim, France) following the supplier's instructions, and then lyophilized. The protein was solubilized in 5 μL of 30% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA), prior to spectrometric analysis on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems, Foster City, CA, USA) equipped with a 337-nm nitrogen laser. Mass spectra were acquired in the linear delayed extraction mode using sinapinic acid (Sigma-Aldrich) as a matrix. The matrix solution was freshly prepared by dissolving sinapinic acid at 5 mg/mL in 30% (v/v) acetonitrile containing 0.1% (v/v) TFA, and mixed with solubilized protein samples at a 1 : 1 ratio. The spectra were recorded in the positive mode at an acceleration voltage of 25 000 V, with a delay time of 750 ns, a grid voltage of 92%, a 0.15% guide wire and a laser intensity of 50%. Up to 3000 laser shots were used to obtain an optimal signal-to-noise ratio, and the spectra were smoothed once. External calibrations were carried out using the commercial protein standards cytochrome B (12 360 Da), apomyoglobin (16 952 Da) and BSA (66 430 Da) (Applied Biosystems).

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