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ORIGINAL PAPER

High-level expression of active human alpha1-antitrypsin in transgenic tobacco chloroplasts

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Abstract We have produced human alpha1-antitrypsin (A1AT), a major therapeutic protein, in genetically engineered tobacco plastids. Four different expression vectors have been evaluated which encode A1AT under the control of various 5′ and 3′ plastid expression elements. The use of heterologous promoter and terminator sequences derived from the corn and soybean plastid genomes leads to simpler and predictable recombinant genome patterns, avoiding unwanted recombination products between introduced and resident tobacco sequences. High level expression of unglycosylated A1AT, representing up to 2% of total soluble proteins, has been measured in leaves of transgenic tobacco lines. Some heterogeneity in the recombinant A1AT is detected after 2D protein separation, but the chloroplast-made protease inhibitors are fully active and bind to porcine pancreatic elastase.

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Introduction

Human alpha1-antitrypsin (A1AT) is an abundant blood circulating serine-protease inhibitor whose primary function is to inhibit neutrophil elastase, and which protects the pulmonary extracellular matrix from destruction (Brantly et al. 1988). A1AT deficiency is one of the most prevalent and potentially lethal hereditary diseases, resulting in lung problems such as emphysema or liver disorders (Wood and Stockley 2007). For treatment, an augmentation therapy is proposed, consisting normally in the intravenous injection of a few grams of A1AT, once a week (Heresi and Stoller 2008). The marketed product is purified from pooled human plasma. Although this has not caused important safety problems so far, the supply of purified human A1AT is limited, and other options such as recombinant production systems are being considered Karnakhova et al. 2006). Expression of human A1AT has been reported in diverse organisms such as *Escherichia coli* (Courtney et al. 1985), yeasts (Kwon et al. 1995; Kang et al. 1996; Hasannia et al. 2006), *Aspergillus* (Karnakhova et al. 2007), insect cells (Sandoval et al. 2002), mouse (Archibald et al. 1990; Zbikowska et al. 2002), sheep (Wright et al. 1991), and rice (Terashima et al. 1999; Huang

et al. 2001). Production in plants is another interesting option which offers the potential for very large-scale production of pharmaceuticals and reduced safety concerns about contamination with human or animal pathogens (Goldstein and Thomas 2004). One complication though, is that mature A1AT is a glycosylated protein containing three N-linked carbohydrate side-chains. The composition of these chains are known to differ between plants, fungi and mammals, raising the issue of immunogenicity of the product (Gomord et al. 2005). Glycosylation is important for the half-life of A1AT in the plasma (Kwon et al. 1995), but is not required for the binding to elastase. As a consequence, the production of an active unglycosylated version in plants can be envisaged. Also, new therapeutic concepts, such as the supply of A1AT by inhalation, which can be repeated more frequently (Brown 2006), or the stabilisation of the protein by conjugation to polyethylene glycol (Brantly 2002) enable this approach. There are different possibilities to achieve this goal by nuclear genome engineering, either by avoiding the secretion pathway or by expressing A1AT variants which have been mutated in their consensus glycosylation sites. A simpler strategy is based on the successful expression of active A1AT in *E. coli*, and consists in analyzing the potential for production by another prokaryotic system, the chloroplasts.

The engineering of the plastid genome is a technology which has been developed in higher plants more than 15 years ago (Svab et al. 1990; Svab and Maliga 1993). Until recently, biotechnological applications were exemplified essentially in tobacco (Daniell et al. 2004; Maliga 2004), but agronomic traits such as insect resistance, herbicide and salt tolerance have now also been generated in canola (Hou et al. 2003), soybean (Dufourmantel et al. 2005, 2007) and carrot (Kumar et al. 2004a). The transformation of a few other species has been described, such as *Arabidopsis thaliana* (Sikdar et al. 1998); potato (Sidorov et al. 1999; Nguyen et al. 2005); tomato (Ruf et al. 2001; Nugent et al. 2005), *Lesquerella fendleri* (Skarjinskaia et al. 2003), cotton (Kumar et al. 2004b), lettuce (Lelivelt et al. 2005; Kanamoto et al. 2006), petunia (Zubko et al. 2004), poplar (Okumura et al. 2006) and possibly rice (Lee et al. 2006). The main attractive feature of this technology is the potential for high-level expression of the transgene, up to 46% of total soluble proteins (De Cosa et al. 2001). The high protein synthesis capacity of chloroplasts makes them indeed ideal bioreactors to produce molecules of medical interest (Nugent and Joyce 2005), and a growing number of therapeutic proteins or antigens have already been produced (Daniell 2006; Bock 2007; Dubald et al. 2008). For such applications, tobacco is considered as a promising vehicle because of its high biomass and its absence from the food and feed chains.

Materials and methods

Plastid transformation vectors

The sequences of the tobacco plastid recombination regions (*rbcL-accD* region), the sequences of the expression cassettes, as well as their annotation, are available in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) with the following accession numbers for vector pAPR23 (EU497666), pAPR36 (EU497667), pAPR37 (EU497668) and pAPR52 (EU497669). The backbone of these vectors is identical and essentially derived from the high-copy ampicillin-resistant pUC19 plasmid (New England Biolabs, Beverly, MA, USA).

Generation of tobacco plastid transformants

Nicotiana tabacum (cv. PBD6) plastid transformants were selected as described by Svab and Maliga (1993). In vitro conditions were 24°C, 16 h photoperiod, and a light intensity of 50 μE m^{-2} s^{-1} provided by Fluora tubes L36 W/77 (Osram, München, Germany). Briefly, the abaxial side of leaves measuring 3–5 cm were bombarded with DNA-coated gold particles using a helium-driven particle gun built in the laboratory according to the model described by Finer et al. (1992). After 2 days, the treated leaves were then cut into squares of in average 1 cm length, and the selection of the transformants performed with 500 mg/l of spectinomycin hydrochloride. Explants were subcultured on fresh selection medium every 10 days. After 4–6 weeks, green calli or plantlets appearing on the bleached explants were isolated and transferred to hormone-free medium for regeneration and rooting, before transfer to the greenhouse.

Southern blot analysis

Total plant DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). PCR reactions were performed in a MU Research thermocycler (MU Research, Ramsey, MN, USA) using ReadyMix Taq PCR Reaction Mix (Sigma, St. Louis, MO, USA). The probes used for the Southern blot analysis (Fig. 1) were amplified from vector pAPR23 using primer pairs (i) 5′-gtagagctgtttatgaatgtcttcg-3′ and 5′-aaggatgtcctaaagt tcctccacc-3′ for *rbcL*, (ii) 5′-agacagatacaagtcaccatgat cagg-3′ and 5′-atggtcaaaacagctttatgtacgg-3′ for *A1AT*, and (iii) 5′-gatcgctagattatttgccgacta-3′ and 5′-tat-ggatcccgaagcggtgatc-3′ for *aadA*. The size of the expected amplified products is 675 bp for *rbcL*, 990 bp for *A1AT*, and 805 bp for *aadA*. PCR fragments were separated on 1% agarose gel and purified using a PCR purification kit (Qiagen), and radiolabelled with ^{32}P by random priming (Megaprime kit, Qiagen). Southern blot analyses were performed with 5 µg of total DNA extracted from tobacco, according essentially to Sam-brook et al. (1989). Plant DNA was digested overnight with either *HindIII* or *NcoI*, separated on a 0.8% agarose gel and transferred to a nylon membrane (Hybond N+, Bio-Rad, Hercules, CA, USA). The last most stringent wash was performed with a 0.1 × SSC and 1% SDS solution at 65°C. Autoradiograms were revealed after 2 h of exposure at −80°C, using an intensification screen. The autoradiograms obtained with the different probes are independent blots which have not been stripped and reprobed.

Protein extraction and western blot analyses

Total soluble proteins were extracted from mature fully developed leaves (T0 generation plants) ground in liquid nitrogen using as extraction buffer Tris–HCl 25 mM, NaCl 100 mM, glycerol 10% (w/v), pH 8. After centrifugation, protein quantification was performed on the supernatant according to Bradford (1976) using the Protein Assay Reagent kit from Bio-Rad. Samples were then combined with Laemmli buffer (Laemmli 1970) supplemented with 10% v/v β-mercaptoethanol and boiled for 5 min before separation by SDS-PAGE (12%). The A1AT human standard was from Sigma (A9024). After separation, proteins were transferred on a PVDF membrane (Bio-Rad) using a liquid electroblotting apparatus

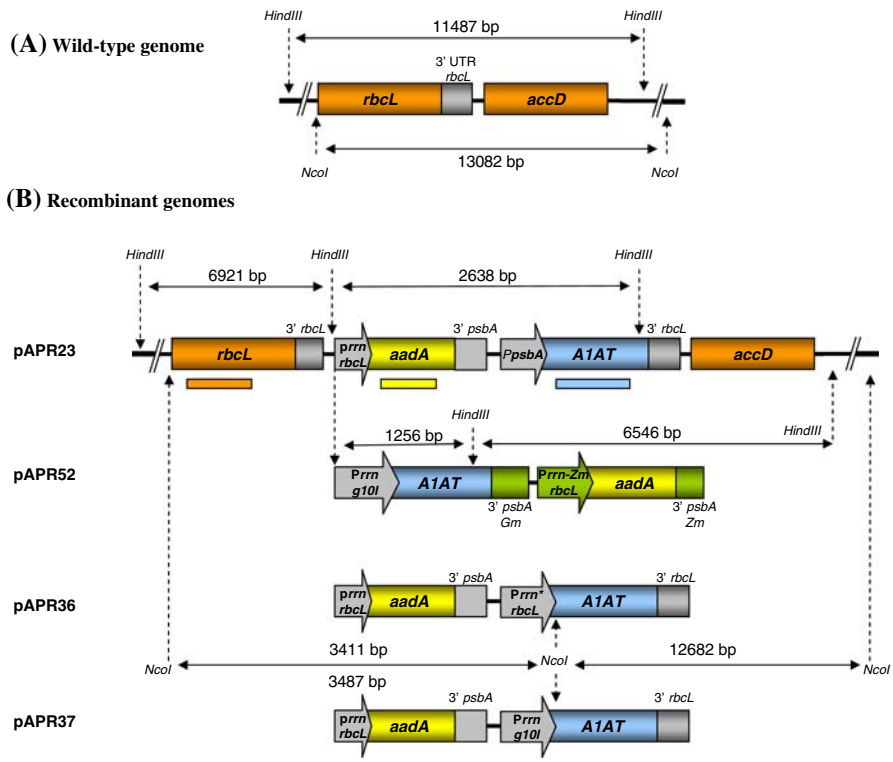

Fig. 1 Map of wild-type and recombinant plastid genomes at the insertion site Wild-type plastid genome (**a**) and recombinant plastid genomes (**b**) after targeted integration of vectors pAPR23, pAPR52, pAPR36, and pAPR37. The three probes used for the Southern blot analysis are indicated below the corresponding regions of pAPR23. Lower case p*rrn* short promoter, unlike P*rrn*, lacks the nuclear-encoded polymerase (nep) recognition site. P*rrn** contains the nep transcription start region from gene *ClpP*-53. The ribosome-binding sites are indicated (g10l or *rbcL*). Plastid expression elements which are not of tobacco origin are depicted in green and derive from either soybean (*Gm*) or corn (*Zm*)

(Mini-Protean 3 Cell, Bio-Rad). After transfer, the membrane was blocked in TTBS buffer (TBS from Bio-Rad, 0.1% v/v Tween 20, pH 7.5) with Blocking Reagent from Roche Diagnostics (Basel, Switzerland), then incubated overnight at 4°C in TTBS buffer with a mouse monoclonal antibody (Abcam, ab9399, Cambridge, UK) directed against A1AT. After three washes, the membrane was incubated for two hours at room temperature in TTBS buffer with a second monoclonal antibody directed against mouse immunoglobulins and coupled to alkaline phosphatase (Sigma A3562). Detection was performed after three washes in TTBS buffer and one wash in TBS buffer with Immun-Star AP substrate (Bio-Rad) and the generated chemoluminescence recorded on Hyperfilm ECL (Amersham, Buckinghamshire, UK).

Two dimensional gel electrophoresis and mass spectrometry analysis

Proteins were analyzed by 2D gel electrophoresis as described previously (Görg et al. 1987; Job et al. 2005). Isoelectrofocusing was carried out with protein samples corresponding to about 100 µg of total proteins extracted from leaves of tobacco plastid transformants. Proteins were separated using gel strips forming an immobilized nonlinear pH gradient from 3 to 10 (Immobiline Dry Strip pH 3-10 NL, 18 cm; Amersham Pharmacia Biotech). Strips were rehydrated for 14 h at 22°C with the thiourea/urea lysis buffer as described (Harder et al. 1999), containing 2% v/v Triton X-100, 20 mM dithiothreitol and the proteins extracts. Isoelectric focusing was performed at 22°C in the Multiphor II system (Amersham Pharmacia Biotech) for 1 h at 300 V and 7 h at 3,500 V. Then, the gel strips were equilibrated for 2 × 20 min in 2 × 100 ml of equilibration solution containing 6 M urea, 30% v/v glycerol, 2.5% w/v SDS, 0.15 M bis-Tris, and 0.1 M HCl (Görg et al. 1987; Harder et al.1999). DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4% w/v) was added to the second (Harder et al. 1999). Separation in the second dimension was carried out in polyacrylamide gels (10% w/v acrylamide, 0.33% w/v piperazidine diacrylamide, 0.18 M Trizma base, 0.16 M HCl, 0.07% w/v ammonium persulfate, and 0.035% v/v Temed). Electrophoresis was performed at 10°C in a buffer (pH 8.3) containing 25 mM Trizma base, 200 mM taurine, and 0.1% w/v SDS, for 1 h at 35 V and for 14 h at 110 V. 2D gels were stained with silver nitrate according to Blum et al. (1987). Mass spectrometry analysis was performed on spots excised from two-dimensional PAGE gels and treated with trypsin according to Bally et al. (2008).

Antitrypsin mobility shift assay with elastase

Total soluble proteins were extracted from leaf material of line pAPR23-1 ground in liquid nitrogen using as extraction buffer Tris–HCl 25 mM, NaCl 100 mM, pH 8. After centrifugation, protein quantification was performed on the supernatant according to Bradford (1976) using the Protein Assay Reagent kit from Bio-Rad. Extracts containing 10 µg of proteins were aliquoted in Eppendorf tubes and various amounts of porcine pancreatic elastase (Sigma E1250) were added. Samples were incubated 15 min at room temperature, then stored at −20°C. Laemmli buffer was added, supplemented with 10% v/v β-mercaptoethanol, and the samples were boiled for 5 min before loading and separation by SDS-PAGE (12%), as described by Zbikowska et al. (2002).

Results

Human α1-antitrypsin plastid expression vectors

High-level expression of recombinant proteins has been consistently, but often unpredictably, observed in tobacco chloroplasts using combinations of strong plastid promoters, 5′-unstranslated leader regions and 3′ ends of various origins. We have evaluated in tobacco four different expression cassettes driving a recoded human *A1AT*, adapted to the tobacco plastid codon usage (Fig. 1). A translation start site has been added at the N-terminus of mature A1AT, deleted of its secretion signal sequence, giving a polypeptide of 395 amino acids with a predicted molecular weight of 44 kDa.

The transformation vectors pAPR23 (Genbank EU497666), pAPR36 (Genbank EU497667), pAPR37 (Genbank EU497668) and pAPR52 (Genbank EU497669) allow integration of the transgenes into the large single-copy region of the tobacco

plastid genome, between the *rbcL* and *accD* genes. The antibiotic resistance gene *aadA* is used for selection of the transformants on spectinomycin, and except for pAPR52, the *aadA* expression cassette is identical to that originally described by Svab and Maliga (1993). For vector pAPR52, some plastid expression elements derived from other plant species, corn and soybean (Fig. 1), have been incorporated, in order to minimize the risk (during or after selection) of recombination between introduced and resident sequences. The vectors pAPR23, pAPR37 and pAPR52 encode A1AT under the control of either the tobacco *psbA* promoter (and 5′ UTR), or of the complete tobacco 16S rDNA promoter followed by the ribosome binding site of phage lambda gene 10. These 5′ regulatory elements have consistently allowed high-level expression of many different recombinant proteins in tobacco chloroplasts (Ye et al. 2001; Fernandez-SanMillan et al. 2003; Daniell 2006). In vector pAPR36, a 25 bp region surrounding the nuclear-encoded polymerase transcription start site of the ClpP-53 promoter (Hajdukiewicz et al. 1997) replaces the equivalent region of the 16S rDNA promoter and is followed by the ribosome binding site of the *rbcL* gene. This expression cassette tested previously in our lab on the GUS reporter gene (not published) was expected to lead to lower expression levels of A1AT in chloroplasts, and to enable the isolation of transplastomic lines in case of phytotoxicity.

Generation and analysis of tobacco plastid transformants

Tobacco plastid transformants (cv. PBD6) were generated according to the procedure described by Svab and Maliga (1993). A single round of selection and regeneration was performed. Antibiotic-resistant events were obtained for all genetic constructs and first screened by PCR to check the presence and insertion of the transgenes at the expected location in the tobacco plastid genome (not shown). Selected transgenic plants were then transferred to the greenhouse. A Southern blot analysis was performed with three different probes covering the insertion site (*rbcL*), the selection marker (*aadA*) and the gene of interest (*A1AT*). For each construct, two PCR-positive putative transformants were analyzed (Fig. 2), after DNA restriction with either *HindIII* or *NcoI* as indicated on Fig. 1.

The observed pattern is entirely in agreement with the integration of the transgenes in the tobacco plastome between the *rbcL* and *accD* resident genes. Based on the blot obtained with the *rbcL* probe, the transgenic lines generated with pAPR23 and pAPR52 are homoplasmic. Some residual wild-type plastome at around 13 kb seems to be present in the lines generated with pAPR36 and pAPR37. Another possibility is that this weak signal, which is visible only in highly loaded lanes, corresponds to conserved copies of this plastid region in the nuclear genome of

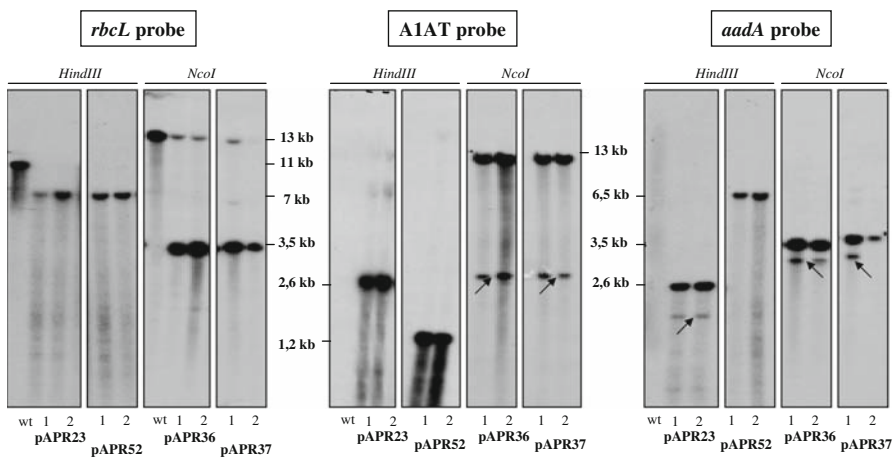

Fig. 2 Southern blot analysis of transgenic tobacco lines. Total DNA from wild-type tobacco (wt) and from two independent lines (1 and 2) generated with vectors pAPR23, pAPR52, pAPR36, and pAPR37. Samples were digested either by *HindIII* or *NcoI* as indicated, and independent membranes incubated with radioactive probes covering the coding regions of *rbcL*, *A1AT* and *aadA* as indicated in Fig. 1. White arrows indicate the position of unexpected bands

tobacco (Ayliffe and Timmis 1992). We have recently reported similar results in the case of tobacco plastid transformants expressing a herbicide tolerance gene (Dufourmantel et al. 2007), a bacterial alkaline phosphatase (Bally et al. 2008) or aprotinin (Tissot et al. 2008). In all cases, the progenies of the characterized lines were uniformly resistant to spectinomycin as expected with a plastid-encoded trait and a homoplasmic state.

For all transgenic lines, major bands migrating at the predicted sizes (Fig. 1) were detected on the DNA blots with probes covering both the selection marker and the gene of interest (Fig. 2). In some cases though, supplementary bands of weaker intensity and of lower molecular weight are present which are indicated by white arrows in Fig. 2. We suspect that these bands represent recombination products, possibly circular, between introduced and resident plastid sequences, as characterized recently in McCabe et al. (2008). In accordance with this hypothesis, there is always a unique hybridizing band for the lines generated with pAPR52, a vector which contains only one tobacco plastid sequence (Fig. 1) versus four in the other vectors. This suggests that the use of heterologous expression elements, for instance derived from the plastid genome of other species, is advantageous. Such elements are potentially fully functional in tobacco. We have for instance not observed any difference in transformation efficacy when using the pAPR52 antibiotic resistance cassette, driven by the corn 16S rDNA promoter, versus the other transformation vectors. Finally, no difference in plant development, growth, pigmentation or fertility was detected for any of the characterized lines, when compared to wild-type tobacco.

Expression of human alpha1-antitrypsin in tobacco leaves

Expression of human A1AT was examined in extracts from mature fully developed leaves of two transgenic lines per construct by western blot analysis (Fig. 3). No signal is observed for wild-type tobacco. For lines generated with pAPR23, pAPR37 and pAPR52, a major band is observed at around 45 kDa, whereas the glycosylated human standard migrates at around 54 kDa. A fainter band at a slightly smaller molecular weight than 45 kDa is visible in all positive transgenic extracts, suggesting that some degradation affects recombinant A1AT either at the NH_2 or COOH terminus. No expression was found for vector pAPR36. The A1AT expression level obtained with the *psbA* (pAPR23) or *Prrn*-g10L (pAPR37 and pAPR52) regulatory elements is very similar. By comparing the signal to the human standard, we estimate that A1AT represents around 2% of total

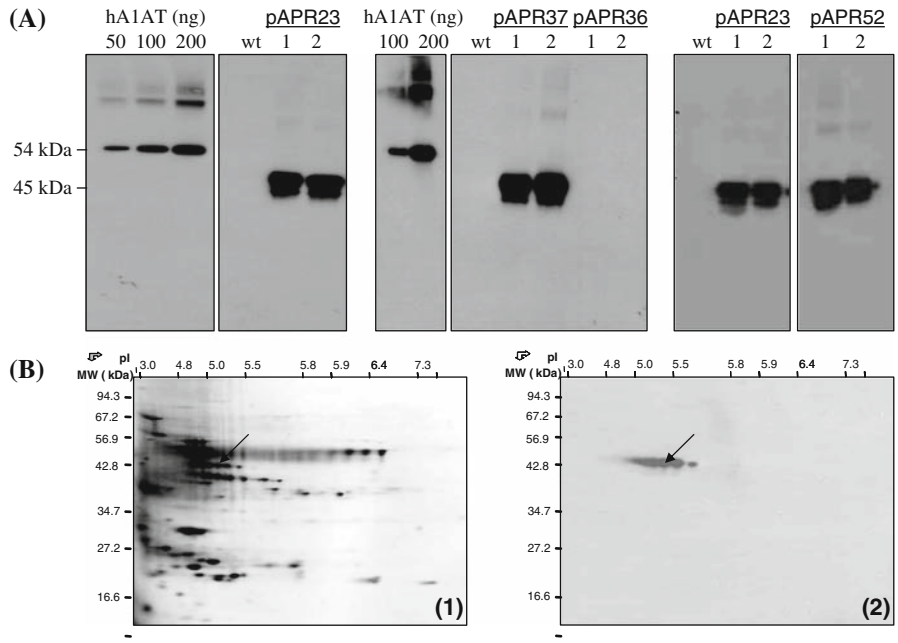

Fig. 3 Expression of A1AT in transgenic tobacco lines. (**a**) Western blot analysis performed on leaf extracts (20 µg proteins) of wild-type (wt) and transgenic tobacco lines. Human standard (hA1AT) (**b**) two dimensional separation of a leaf extract (100 µg proteins) from a pAPR52 line, silver stained (1) or probed on immunoblot (2). White arrows indicate the spots corresponding to recombinant A1AT

soluble proteins in leaves, which translates in our growing and extraction conditions to approximately 400 mg/kg fresh leaf weight.

A leaf extract from a pAPR52 line was separated on 2D gels, stained with silver nitrate (Fig. 3b-1) or probed after transfer with antibodies directed against A1AT (Fig. 3b-2). The result of this analysis shows that recombinant A1AT can be separated into at least four spots of similar intensity differing in their isoelectric point, indicated by the white arrow. These spots were excised, pooled, and mass spectrometry analysis performed on tryptic fragments. Only peptide sequences corresponding to A1AT were obtained, covering 82 amino acids, but this did not allow to clarify the post-translational modification(s) affecting recombinant A1AT.

Biological activity of recombinant alpha1-antitrypsin

Human A1AT belongs to the serpin family of protease inhibitors, and its major physiological function is to trap elastase. The inhibition mechanism involves the formation of a 1:1 covalent complex between A1AT and elastase, which is also associated with some cleavage of the inhibitor by the protease (Dobo and Gettins 2004; Dementiev et al. 2006). The functionality of recombinant A1AT produced in tobacco chloroplasts was examined using a mobility shift assay adapted from Zbikowska et al. (2002).

Leaf protein extracts from line pAPR23-1 were incubated with various amounts of porcine pancreatic elastase (PPE), then separated by SDS-PAGE, and A1AT detected by western blot (Fig. 4a). This analysis shows that the totality of A1AT (band 1) can be displaced by increasing amounts of PPE, either (i) to the higher molecular weight band 3, corresponding to the expected covalent complex between A1AT and PPE, or (ii) to lower molecular weight bands 2, 4 and 5 which correspond to cleaved products of A1AT. This pattern is very similar to that described by Zbikowska et al. (2002) for genuine plasma-derived human A1AT as well as for the recombinant inhibitor in mouse urine, and confirms the finding made in *E. coli* (Courtney et al. 1984, 1985) that unglycosylated A1AT is an active inhibitor.

The fact that the totality of A1AT present in 10 µg of a leaf extract is complexed by a PPE amount below

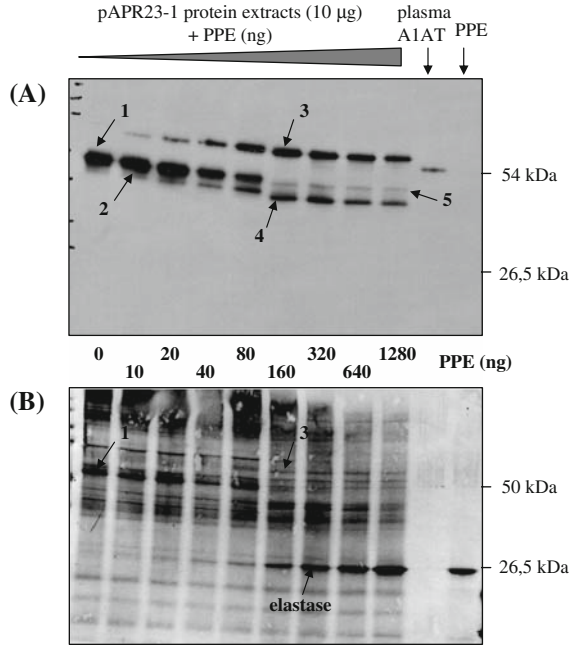

Fig. 4 Mobility shift A1AT bioassay with elastase. (**a**) A1AT detection by western blot analysis, and (**b**) coomassie-blue stained membrane after western blot analysis. Leaf protein extracts (10 µg) from line pAPR23-1 were incubated with increasing amounts of porcine pancreatic elastase (PPE) as indicated, before separation by SDS-PAGE. Arrows indicate the positions of recombinant A1AT (1), A1AT-PPE covalent complex (2), and cleaved products of A1AT (2, 4, 5)

but close to 160 ng is compatible with our previous estimations of A1AT expression level in tobacco leaves for vector pAPR23 at around 2% of total soluble proteins. The membrane, stained with Coomassie blue after the western analysis, is presented in Fig. 4b. It shows that the interaction between A1AT and PPE is rather specific, since most protein bands are not affected, even by exposure to excess protease. The putative recombinant A1AT (band 1) and its complex with PPE (band 3) are indicated on Fig. 4b.

Discussion

We have expressed in tobacco chloroplasts human alpha1-antitrypsin (A1AT), a protease inhibitor of important therapeutic value. Four different expression vectors containing a synthetic gene encoding A1AT have been evaluated. The highest expression level in leaves was estimated at around 2% of total soluble proteins (tsp), which corresponds to approximately

400 mg/kg of leaf fresh weight. This compares very favourably to the levels reported in other expression systems such as yeasts, mammalian cells or plant nuclear transformants (Karnakhova et al. 2006). Compared to the secretion strategy described in rice cell suspensions (Terashima et al. 1999), and according to SDS-PAGE protein separation followed by western blot analysis, the production of A1AT in chloroplasts leads to a much more homogeneous product, and essentially to bioactive molecules able to bind elastase. The fractionation of proteins on 2D gels shows that the situation is in fact more complex, since multiple spots differing slightly in isoelectric point have been detected. We have no clue yet about which post-translational modification is affecting recombinant A1AT, but the same type of heterogeneity was reported recently also for bacterial alkaline phosphatase expressed in tobacco chloroplasts (Bally et al. 2008). Protein truncation at the N or C-terminus which contain charged residues is a possible explanation for A1AT. As an alternative, a very diverse set of post-translational modifications, such as deamidation or phosphorylation, can have an impact on the charge of a polypeptide (Gianazza 1995).

The plastid *psbA* 5′ region and the combination of 16S rDNA promoter coupled to the ribosome binding site of phage lambda gene 10 are the two strongest known and widely used 5′ regulatory elements for recombinant protein expression in chloroplasts (Daniell et al. 2004). In the case of A1AT, the result is similar in term of expression level, around 2% tsp, for these two 5′ elements for vectors pAPR23, pAPR37 and pAPR52. In contrast, A1AT was not detected in tobacco when using less optimal elements as in pAPR36, despite the presence of the strong 16S rDNA short promoter transcribed by the plastid-encoded RNA polymerase. An important point for plastid genetic engineering is the confirmation that promoters and terminators derived from plastid genes of other plant species, such as soybean or corn in pAPR52, are functional in tobacco. The interesting consequence of using heterologous sequences to drive transgene expression in chloroplasts is that it lowers significantly the risk of unwanted recombinations between introduced and resident sequences, which is a requisite for long term stability (Dufourmantel et al. 2006). Based on the Southern blot analysis, this is indeed the case for the lines generated with pAPR52 versus the lines generated with the other vectors, since no unexpected bands are detected. The *aadA* selection cassette of pAPR52 driven by corn plastid sequences will be useful in future experiments and is as effective in our hands as the original marker developed by Svab and Maliga (1993).

Expression in chloroplasts leads to high levels of unglycosylated but active A1AT, as in *E. coli.* Both prokaryotic production systems eliminate (i) the concerns about the potential immunogenicity of alien carbohydrate motifs decorating recombinant A1AT and (ii) provide an enhanced safety profile versus animal-derived A1AT. The plant system presents over the bacterial system the advantages that there is no evidence for misfolded inactive recombinant protein aggregates as inclusion bodies (Kwon et al. 1995), and that there is no limit to the production capacity with the molecular farming approach. There is a strong potential to increase the significantly reduced in vivo half-life of unglycosylated A1AT by chemical conjugation to polyethylene glycol (Cantin et al. 2002). The expression level of 2% tsp in leaves could be further increased in tobacco chloroplasts, since there is no sign of any phytotoxicity induced by the synthesis of this protease inhibitor, unlike aprotinin (Tissot et al. 2008). The main options are to test other synthetic recoded genes and/or other 5′ control elements. In particular, the optimization of the sequence context around the translation start site is known to be a critical factor which can impact on the expression level of the encoded protein over four orders of magnitude (Ye et al. 2001). Significant higher yields are also certainly achievable by fine tuning the growing conditions of the tobacco plants, especially illumination, and the extraction protocol.

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