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PLANT TISSUE CULTURE

Co-expressing *Turnip Crinkle Virus*-coat protein with the serine protease α-thrombin precursor (pFIIa) in *Nicotiana benthamiana* Domin

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Abstract

The serine protease α-thrombin (FIIa) plays a fundamental role in blood clotting. In the present report, a FIIa precursor (pFIIa) was expressed in *Nicotiana benthamiana* Domin. The expression construct featured the Kozak consensus sequence and the 2S2 *Arabidopsis thaliana* (L.) Heynh. signal peptide to direct the protein into the secretory pathway (sec-pFIIa). A version carrying the KDEL endoplasmic reticulum (ER) retention signal (pFIIa-ER) was also constructed. Transient expression of pFIIa in *N. benthamiana* leaves was achieved by *Agrobacterium tumefaciens* infiltration. The influence of post-transcriptional gene silencing (PTGS) was analyzed by co-infiltrating with an *A. tumefaciens* strain carrying the construct for the *Turnip Crinkle Virus*-coat protein (TCV-CP) known for interfering with PTGS. Reverse transcription polymerase chain reaction and Western blot analyses confirmed the presence of the corresponding messenger RNA and the recombinant pFIIa protein in plant extracts. A positive effect of the addition of the PTGS inhibitor was demonstrated. The accumulation of sec-pFIIa and pFIIa-ER was estimated to be 6 μg g^{-1} fresh weight (FW) (0.07% (*w/w*) total protein concentration; TPC) and 17 μg g^{-1} FW (0.21% (*w/w*) TPC), respectively. Furthermore, stably transformed callus and suspension cultures were obtained. The recombinant protein was detected only in the biomass of the pFIIa-ER cell suspension line at a concentration of 0.25 μg mL^{-1} (0.017% (*w/w*) of total soluble protein). This appears to be the first report describing the expression of a precursor of FIIa in plants.

Keywords Alpha-thrombin · Plant-made recombinant protein · *Agrobacterium tumefaciens* infiltration · Post-transcriptional gene silencing · Plant cell suspension cultures

Introduction

Blood clotting is a complex mechanism that results from interactions between blood, vessels, platelets, and coagulation factors. The blood-clotting cascade is triggered by any injury, ultimately resulting in the conversion of prothrombin into the proteolytic enzyme α-thrombin (FIIa) as one of its last steps. Then FIIa activates fibrinogen to fibrin, participates in the activation of platelets and other blood coagulation factors (V, VIII, IX, and XIII), and plays a role in the activation of factor C in the presence of thrombomodulin. Platelets aggregate on the fibrin clot, collagen, and endothelial cells from the injured vessels, initiating sealing that prevents bleeding (DiBella *et al.* 1995; Heemskerk *et al.* 2002; Adams and Huntington 2016). If any of the mentioned steps fail, the consequence is a hemorrhage.

The pharmaceutical industry is attracted by the production of proteins involved in the blood coagulation mechanism (Moura *et al.* 2011; Casademunt *et al.* 2012; Rech *et al.* 2014; Santagostino *et al.* 2014). Particularly, the production of recombinant FIIa, or any of its precursors, is considered of interest for the manufacture of hemostatic sealants. Prethrombin-2, corresponding to amino acids Thr_{272} to Glu_{579} of the human prothrombin, is the smallest single-chain precursor of FIIa. The difference between

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prethrombin-2 and FIIa is a cleavage at the position Arg_{320}-Ile_{321}, followed by the release of a 13 amino acid peptide from the N-terminus. Recombinant prethrombin-2 was already expressed in *Escherichia coli* (Choi *et al.* 1989; So *et al.* 1992; DiBella *et al.* 1995; Soejima *et al.* 2001; Osadská *et al.* 2014). However, that recombinant protein aggregates into inclusion bodies and has to be solubilized and refolded, hindering the recovery of an active, properly folded enzyme. A recombinant FIIa was also expressed in yeast, but the product was not identical to human FIIa (Holly and Foster, 1996). Finally, other precursors of FIIa were expressed in mammalian cell cultures (Le Bonniec *et al.* 1992; Russo *et al.* 1997; Oates *et al.* 2001; Yonemura *et al.* 2004).

There is an interest in obtaining a reliable source of FIIa utilizing plants as biofactories for recombinant proteins. One of the remarkable traits of plants is that their protein synthesis machinery produces mammal proteins with often only slight differences from the original source. Additionally, plants can perform complex post-translational modifications, such as the formation of disulfide bridges and folding, plant-made proteins do not aggregate as inclusion bodies, and the final product does not carry any risk of contamination with prions, oncogenes, toxins, or human pathogens (Twyman *et al.* 2012; Fischer *et al.* 2013; Sabalza *et al.* 2014; Sack *et al.* 2015).

When it comes to production, plant systems have the advantage of an easy and economical scale-up. Additionally, in the case of confined production (greenhouses or *in vitro* cultures), the process can be conducted in environmentally controlled conditions, following good manufacturing practices and good laboratory practices, if required (Sharma and Sharma 2009; Fischer *et al.* 2012; Twyman *et al.* 2012; Merlin *et al.* 2014).

As for the drawbacks, plant-made proteins may have glycosylation modifications (Lerouge *et al.* 1998; Gomord *et al.* 2010, Batra and Rathore 2016), and the yields of the recombinant proteins are usually low (Ullrich *et al.* 2015). Subcellular targeting can enhance protein stability, thus increasing the accumulation of heterologous proteins in plants (Ferraro *et al.* 2008; Nelson *et al.* 2012). Endoplasmic reticulum (ER)-retention *via* the addition of lysine-aspartic acid-glutamic acid-leucine (KDEL) to the C-terminus is often used to minimize foreign protein degradation since the ER provides a relatively protected environment, with a high concentration of molecular chaperones and a low presence of proteases (Fischer *et al.* 2004; Laguia Becher *et al.* 2010). Meanwhile, the secretion of foreign proteins into the medium of plant cell cultures, where metabolites and contaminant proteins are generally absent, makes for an easier and cheaper protein recovery (Conrad and Fiedler 1998; Doran 2006).

Another factor that influences the yield of recombinant proteins in plants is gene silencing, which can take place at the transcriptional or translational level. Post-transcriptional gene silencing (PTGS) is a defensive response that recognizes foreign RNA, triggering its degradation (Stam *et al.* 1997; Vaucheret *et al.* 2001). Some plant viruses have evolved strategies to elude this defense mechanism by expressing proteins that suppress PTGS. *Turnip crinkle virus* (TCV)-coat protein (CP), not only has a structural role but it also functions as a strong suppressor of PTGS (Qu *et al.* 2003).

The aim of the present work was to clone and evaluate the expression of two versions of a FIIa precursor (pFIIa), sec-pFIIa (secretory), and pFIIa-ER (ER-retained) in *Nicotiana benthamiana* Domin. The influence of PTGS on recombinant protein production in a transient expression system by co-expressing TCV-CP was evaluated. Additionally, pFIIa expression was analyzed in stably transformed callus and cell suspension cultures.

Materials and Methods

Construction of plant expression vectors Dr. David G. Ross from the University of British Columbia (Vancouver, Canada) kindly provided the human prothrombin coding region (accession no. MN_000506). Two synthetic sequences, comprised of amino acids Thr_{285} to Glu_{579} of the human prothrombin (pFIIa), were designed. First, a secretory version (sec-pFIIa) was constructed by adding the signal peptide 2S2 from the seed storage protein of *Arabidopsis thaliana* (L.) Heynh. to the N-terminus of the pFIIa-coding sequence (Krebbers *et al.* 1988). Secondly, the endoplasmic reticulum-retention version (pFIIa-ER) was obtained by adding the ER-retention motif KDEL to the C-terminus of sec-pFIIa. In both versions (sec-pFIIa and pFIIa-ER) the Kozak consensus sequence (accatgg) was placed before the 2S2 signal and a six histidine tag (6xHis) was added to the C-terminus (Fig. 1*A*).

The sec-pFIIa and pFIIa-ER transgenes were amplified by a polymerase chain reaction (PCR) using the following primers: forward (Fw) 5′-aaccatggcaaacaagctcttcctc-3′ (underlined is an incorporated 5' *Nco*I restriction site) and reverse (Rv) sec-pFIIa 5′-ctcgag-tcattaatgatgatgatgatgctctccaaactgatc-3′ for the secretory version, or Rv pFIIa-ER 5'ctcgag-tcattatagctcatctttatgatgatgatgatgctctccaaactgatc-3′ for the ER-retention version (underlined is an incorporated 3' *Xho*I restriction site). The PCR products were cloned into the pGEM®-T Easy vector (Promega®, Madison, WI). To confirm the correct synthesis of the genes, both inserts were sequenced using specific primers for the promoters T7 and Sp6. The sec-pFIIa and pFIIa-ER inserts were digested with *Nco*I and *Xho*I enzymes and cloned into the entry vector pENTR4 (Invitrogen™, Carlsbad, CA), previously digested with the same enzymes. The resulting plasmids, pENTR4:sec-pFIIa and pENTR4:pFIIa-ER, were each recombined with the plant binary expression vectors pK7WG2 (pK) and p35SGATFH (p35S) (Karimi *et al.* 2002; Zanetti *et al.* 2005) using the

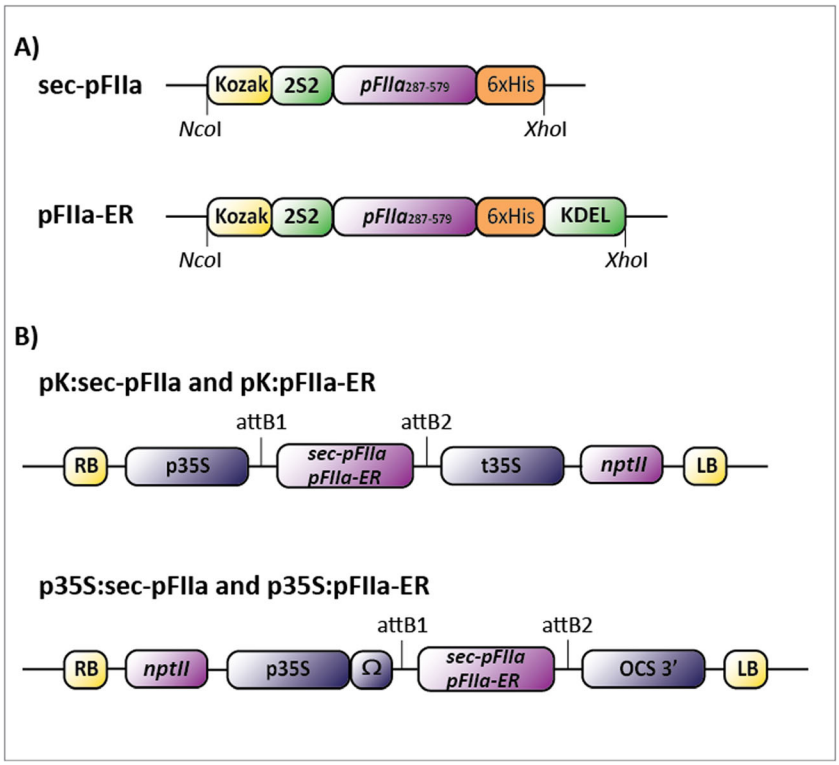

Figure 1. Schematic representation of the constructions used to express the α-thrombin precursor *pFIIa* in *Nicotiana benthamiana* Domin. (***A***) Characteristics of the secretory (*sec-*) and endoplasmic reticulum (*ER*) retained pFIIa transgenes. *Kozak*, Kozak consensus sequence; *2S2*, signal peptide from the seed storage protein of *Arabidopsis thaliana* (L.) Heynh.; *6xHis*, six histidine tag; *KDEL*, ER-retrieval motif; *NcoI* and *XhoI*, restriction enzyme sites. (***B***) Expression vector transfer-DNA (T-DNA) regions. *RB* and *LB*, right and left borders of the T-DNA; *p35S* and *t35S*, *Cauliflower Mosaic Virus* 35S promoter and terminator; *Ω*, *Tobacco Mosaic Virus* 5′ leader sequence; *OCS 3′*, *Agrobacterium tumefaciens* octopine synthase terminator; *nptII*, neomycin phosphotransferase II (kanamycin resistance gene); *attB1* and *attB2*, recombination sites.

Gateway® LR Clonase™ II Enzyme Mix (Invitrogen™). The pK vector contained the *Cauliflower Mosaic Virus* 35S promoter and terminator (p35S and t35S); meanwhile, the p35S vector contained the same promoter (p35S) with the translational enhancer sequence Ω from *Tobacco Mosaic Virus* and the *Agrobacterium tumefaciens* octopine synthase terminator (OCS 3′; Fig. 1*B*). In both cases, neomycin phosphotransferase II (*nptII*) was the selectable marker gene that confers resistance against kanamycin. In the pK vector, the *nptII* gene was under the transcriptional regulation of *A. tumefaciens* nopaline synthase (nos) promoter and terminator (Hellens *et al.* 2000). In the p35S vector, the *nptII* gene was under the control of p35S and t35S (Hajdukiewicz *et al.* 1994). The resulting four final plant expression vectors were checked by PCR and restriction endonuclease mapping (data not shown).

***Agrobacterium tumefaciens* infiltration** The obtained plant expression vectors pK:sec-pFIIa, pK:pFIIa-ER, p35S:sec-pFIIa, and p35S:pFIIa-ER were separately introduced into *A. tumefaciens* strain EHA101 by electroporation. Fresh single colonies of recombinant *A. tumefaciens* clones were inoculated in 10 mL of Luria-Bertani (LB, Bertani 1951) medium autoclaved for 20 min at 0.1 MPa of pressure (Arcano, LS-B75L; Ningbo, China). After the sterilization procedure, the medium was supplemented with 20 μg mL^{-1} rifampicin, 50 μg mL^{-1} kanamycin, and 100 μg mL^{-1} spectinomycin (for pK vectors) or 50 μg mL^{-1} chloramphenicol (for p35S vectors). The antibiotics were filter-sterilized by Millipore MILLEX-GV® 0.22 μm Filter unit (Biopore, Buenos Aires, Argentina). *Agrobacterium tumefaciens* cultures were incubated overnight at 28 °C and 210 rpm in an environmental shaking incubator (Labnet®, 311DS; Edison, NJ). Cultures were centrifuged at 4000×*g* for 15 min and the pellets were resuspended in an infiltration buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.5, 10 mM $MgCl_2$, 100 μM acetosyringone) to a final optical density at 600 nm (OD_{600nm}) of 0.8 (Tatineni *et al.* 2012).

For co-expression experiments, suspensions containing a pFIIa construction were mixed with an equal volume of *A. tumefaciens* ($OD_{600nm} = 0.8$) carrying the PTGS inhibitor TCV-CP (Qu *et al.* 2003). As a negative control, a wild-type (WT) *A. tumefaciens* culture was used. The *A. tumefaciens* suspensions were kept at 24 ± 2 °C for 2 h after being used for plant transient expression. *Nicotiana benthamiana* plants were grown from seeds in pots filled with soil and were maintained at 22 to 24 °C under a 16-h photoperiod using

fluorescent daylight lamps (Narva T8 LT 18 W/760–010 daylight, Schwandorf, Germany) with an irradiance intensity of 13.5 μmol m^{-2} s^{-1} in a growth room. Six-week-old-plants with six to eight leaves were gently infiltrated with the *A. tumefaciens* suspensions by inoculation with a 1-mL syringe without a needle into the abaxial side of the lamina (Leuzinger *et al.* 2013). The *A. tumefaciens*-infiltrated plants were maintained in a growth room as described before. A minimum of three plants per experimental group was infiltrated (three leaves per plant). For sampling, three leaves per experimental group (one leaf per plant) were harvested at 3 and 5 d post-infiltration (dpi), frozen in liquid nitrogen, and stored at − 80 °C until use. *Agrobacterium tumefaciens* infiltrations, and their corresponding Western blot analyses (see below) were repeated at least three times.

Protein extraction and Western blot analysis Frozen *A. tumefaciens*-infiltrated leaves were ground in liquid nitrogen to a fine powder using a mortar and pestle and extracted with three volumes of cold Laemmli (1970) buffer (0.5 M tris(hydroxymethyl)aminomethane (Tris)-HCl pH 6.5, 4% (*w/v*) sodium dodecyl sulfate (SDS), 20% (*v/v*) glycerol, 10% (*v/v*) 2-mercaptoethanol (Bio-Rad®, Hercules, CA), and 0.1% (*w/v*) bromophenol blue). Protein extracts were centrifuged at 17,968×*g* for 20 min at 4 °C and total protein concentration (TPC) in the supernatant was measured using the reducing agent and detergent compatible (RC-DC) protein assay (Bio-Rad®) with bovine serum albumin (BSA) as a standard.

Protein samples were boiled for 5 min and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with a 12% separating gel and a 5% stacking gel and run in a Mini-Protean® Tetra cell (Bio-Rad®) at 150 V for 2 h. The gels were transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore™, Billerica, MA) in a Mini Trans-Blot® cell (Bio-Rad®) at 80 V for 1 h. The membranes were blocked overnight at 4 °C with 0.5% (*w/v*) casein in Tris-buffered saline, pH 7.5 containing 0.05% (*v/v*) Tween® 20 (TBST) and then probed for 1 h at 24 ± 2 °C with sheep polyclonal anti-human thrombin antibody (PAHT-S; Haematologic Technologies Inc., Essex Junction, VT) diluted 1:1000 in TBST with 0.5% (*w/v*) casein. After three washes in TBST, the membranes were probed for 1 h at 24 ± 2 °C with anti-sheep IgG-peroxidase antibody produced in donkey (A3415; Sigma-Aldrich®), diluted 1:15,000 in TBST with 0.05% (*w/v*) casein. Immunoreactive complexes were detected by chemiluminescence using Clarity™ Western enhanced chemiluminescence (ECL) substrate (Bio-Rad®) and the membrane was exposed to Kodak® BioMax® Light Film (Carestream, Rochester, NY) for 3 min before it was developed and fixed. The accumulation of pFIIa in *N. benthamiana, A. tumefaciens*-infiltrated leaves was estimated by comparing plant protein samples with a serial dilution of a known concentration of purified human thrombin (hT; T1063; Sigma-Aldrich®) electrophoresed on the same polyacrylamide gel. The band intensity of hT detected by Western blot was estimated with the Gel-Pro® analyzer software (Media Cybernetics, Rockville, MD) and used as a standard to build a calibration curve, as previously described by Del L Yácono *et al.* (2012) and Albarracín *et al.* (2015). The pFIIa band intensity detected by Western blot was also estimated using the Gel-Pro® analyzer software and compared with the calibration curve obtained by the band intensity of hT. This analysis allowed an estimation of the concentration of pFIIa expressed in the *A. tumefaciens*-infiltrated leaves.

Relative quantification by real-time PCR Frozen *A. tumefaciens*-infiltrated leaves (100 mg) were ground to a fine powder in a 1.5-mL microcentrifuge tube using a plastic pestle. Total RNA was extracted using RNeasy® Plant Mini Kit (Qiagen®, Valencia, CA) according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometric analysis. For the complementary DNA (cDNA) synthesis, 2 μg of total RNA was reverse-transcribed using Invitrogen™ Superscript® IV First Strand Synthesis System (Thermo Fisher Scientific®, Waltham, MA) and random primers. Transcript quantification was performed on the ABI 7500 real-time PCR system (Applied Biosystems™, Foster City, CA). The pFIIa specific primers (Fw pFIIa 5′-tacaagcctgatgaagggaaac-3′ and Rv pFIIa 5′-tgagacgatgcccatttgatac-3′) were designed with the software PrimerQuest Tool (Integrated DNA Technologies, Skokie, IL). The *N. benthamiana* 60S ribosomal protein L23 was used as the endogenous reference gene (Liu *et al.* 2012). Polymerase chain reaction amplification efficiency (E) was estimated for each primer set by standard curve measuring tenfold serial dilutions of 100 ng of pool cDNA. The slopes from the regression line plot of cycle threshold (CT) value *vs.* log of input cDNA were related with PCR amplification efficiency by the formula (− 1 ÷ slope).

Real-time PCR was carried out in a final volume of 25 μL using 2.5 μL of 1:15 diluted cDNA (5 ng), 12.5 μL Power SYBR® Green PCR Master Mix 2X (Applied Biosystems™), and 2.5 μL of each primer set. The final concentrations of primers used were: 150 nM Fw pFIIa–300 nM Rv pFIIa and 150 nM Fw L23–150 nM Rv L23. The amplification program consisted of a DNA polymerase activation step of 10 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C with a 1 min annealing and an extension step at 60 °C. Three independent *A. tumefaciens*-infiltrated leaf samples of each construct combination (pK:sec-pFIIa, pK:pFIIa-ER, p35S:sec-pFIIa, and p35S:pFIIa-ER ± TCV-CP) were used with three technical replicates each.

The relative quantification (RQ) of gene expression was performed using the comparative CT (ΔΔCT) method in which the amount of pFIIa, normalized to an endogenous reference (L23) and relative to a calibrator (WT), was given by the formula $2^{-\Delta\Delta CT}$ (Schmittgen and Livak 2008). Relative quantification for the three biological samples and the corresponding technical replicates was calculated using the SDS software V. 1.3 (Applied Biosystems™). The error bars displayed the calculated maximum (RQMax) and minimum (RQMin) expression levels that represented the standard error of the mean expression level (RQ value). Collectively, the upper and lower limits defined the region of expression within which the true expression level value was likely to occur. Prism 5.0 software (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. Data were compared by one-way analysis of variance (ANOVA) followed by the Bonferroni's multiple comparison tests. A P value < 0.05 was considered as statistically significant.

Callus cultures *N. benthamiana* plants were *A. tumefaciens*-infiltrated with the p35S:sec-pFIIa and p35S:pFIIa-ER plasmids as previously described. After 3 d, *A. tumefaciens*-infiltrated leaves were washed in running tap water, dipped in diluted commercial bleach (55 g Cl L^{-1}, Ayudin®, Buenos Aires, Argentina) at 3.5% (*v/v*) sodium hypochlorite final concentration for 10 min and rinsed four times in sterilized distilled water to eliminate bacterial cells. Sterilized leaf explants (1 cm^2) were placed in Petri dishes containing solid Murashige and Skoog (MS, Murashige and Skoog 1962) medium supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar (A296, PhytoTechnology Laboratories®, Shawnee Mission, KS), and 2 mg L^{-1} naphthaleneacetic acid (NAA) and 0.2 mg L^{-1} kinetin (KIN) as plant growth regulators. The pH was adjusted to 5.7 to 5.8 with 1 M KOH prior to autoclaving as described before. The medium also contained the filter-sterilized antibiotics 50 mg L^{-1} kanamycin and 500 mg L^{-1} carbenicillin added after autoclaving. The explants were incubated in a growth room in the same conditions described before. After 2 wk., the explants started to show hyperplasia and after 4 wk. calluses were produced. Calluses were separated and transferred to identical fresh medium without carbenicillin. Each callus was transferred every 3 wk. to fresh medium (López *et al.* 2010, Alvarez *et al.* 1993). After 6 mo of culture, independent callus lines showing kanamycin resistance were established.

Molecular analysis of transgenic callus lines Genomic DNA was isolated from putative transgenic callus lines. Briefly, 200 mg of callus was ground in a 1.5-mL microcentrifuge tube using a plastic pestle and extracted with 350 μL of DNA extraction buffer (50 mM Tris-HCl pH 8, 10 mm ethylenediaminetetraacetic acid (EDTA; Genbiotech SRL, Buenos Aires, Argentina), 100 mM NaCl, 1% (*w/v*) SDS, and 10 mM 2-mercaptoethanol). After 10 min at 65 °C, 300 μL of 3 M potassium acetate pH 5.5 was added and incubated on ice for 20 min. The samples were centrifuged at 17,968×*g* for 10 min at 4 °C. The supernatant was collected, mixed with one volume of isopropanol, and centrifuged at 17,968×*g* for 15 min. The pellet was washed with 80% (*v/v*) ethanol and resuspended in 40 μL of dH_2O. The insertion of the pFIIa transgene into the callus genome was confirmed by PCR screening using the following primers: Fw 5′-caccatggcaaacaagctcttcctc-3′ and Rv 5′-atgatgatgatgatgctctccaaactgatc-3′.

Transformation efficiency (%) was calculated as: (the total number of PCR positive calluses) ÷ (total number of kanamycin resistant calluses) × 100.

Finally, for the calluses that exhibited the correct DNA pFIIa transgene fragment of 973 bp, the presence of the recombinant protein was analyzed by Western blot as described before. For callus protein extraction, 300 mg of fresh callus tissue was weighed into a 1.5-mL microcentrifuge tube and extracted with 300 μL of cold Laemmli buffer using a plastic pestle. Protein extracts were centrifuged at 17,968×*g* for 20 min at 4 °C.

Cell suspension cultures Fresh friable calluses from the lines that expressed higher amounts of pFIIa were transferred to a 225-mL Erlenmeyer flasks containing 50 mL of MS medium with 2 mg L^{-1} NAA and 0.2 mg L^{-1} KIN as plant growth regulators and shaken at 100 rpm on an orbital shaker (Mod Bm023, Biomint, Buenos Aires, Argentina) in a growth room as described before. Every 3 wk. during a 3-mo period, an inoculum of 5% (*w/v*) was transferred to fresh medium and maintained in the same culture conditions (Lopez *et al.* 2010). Based on the characteristics of cell growth (homogeneous growth with small cell clusters, optimal biomass yield, and lack of oxidation) and the presence of the recombinant protein pFIIa, cell suspension cultures were selected to be scaled up in a stirred-tank bioreactor (Minifors; Infors HT, Bottmingen, Switzerland). An inoculum size of 2% (*w/v*) of 10-d-old cell suspensions was transferred to a 2-L vessel containing 1.5 L of the culture medium with the same composition used before. A marine propeller provided mechanical agitation (100 rpm) and a porous metal sparger supplied a bubble aeration system. The process was performed at 24 ± 2 °C, 0.1 gas volume flow per unit of liquid volume min^{-1} (VVM) set point aeration, and a starting oxygen mass transfer coefficient (kLa) value of 42.7 h^{-1}. The O_2 relative partial pressure (OxyFerm 225; Hamilton, Reno, NV) and pH (Mettler Toledo, Columbus, OH) were monitored in line; O_2-electrode calibration was made with pure N_2. The oxygen uptake rate (OUR) was estimated at 4.57 mmol O_2 $(L\ h)^{-1}$. The whole process was monitored by the Iris Explorer software version 5.2 (Polyhedron Software & Services LTD., Standlake, United Kingdom). Samples (10 mL per duplicate) were harvested at 0, 2, 4, 6,

8, 10, 13, and 15 d and filtered using 0.45 μm filter discs with a low protein binding Durapore (PVDF) membrane (Merck Millipore™, Sao Paulo, Brazil) to separate the biomass from the culture medium. The plant cells were weighed for fresh weight (FW) assessment, which was used as a measure of cell growth. The whole experiment was performed twice.

Protein extraction and enzyme-linked immunosorbent assay (ELISA) Samples of cell suspensions were filtered to separate the biomass from the culture medium. Filtered cells (300 mg) were powdered in a 1.5-mL microcentrifuge tube in the presence of liquid nitrogen with a plastic pestle and extracted with 300 μL of cold Tris-buffered saline (TBS). The homogenized material was placed on ice for 15 min and centrifuged at 17,968×*g* for 20 min at 4 °C. Total soluble protein (TSP) concentration (in the biomass and the culture medium) was determined according to Bradford (1976) using BSA as the standard protein. Quantification of pFIIa was performed by ELISA sandwich using commercial antibodies. Briefly, 96-well Maxisorp® immuno plates (Nunc; Roskilde, Denmark) were coated with 100 μL per well of mouse monoclonal anti-human prothrombin antibody (AHP-5013; Haematologic Technologies Inc.) diluted to 2 μg mL^{-1} in carbonate buffer (50 mM pH 9.6) and incubated overnight at 4 °C. Plates were blocked with 250 μL per well of TBS containing 1% (*w/v*) BSA for 2 h at 24 ± 2 °C. For each plate, a standard curve was prepared with 0, 0.16, 0.31, 0.62, 1.25, 2.5, and 5 μg mL of purified human prothrombin (HCP-0010; Haematologic Technologies Inc.) diluted in TBS with 0.25% (*w/v*) BSA. Plates were incubated with 100 μL per well of samples and standards for 2 h at 24 ± 2 °C. After five washes with TBST, the plates were probed for 1 h at 24 ± 2 °C with sheep polyclonal anti-human prothrombin antibody conjugated to horseradish peroxidase (P9115-16A; United States Biological, Salem, MA) diluted 1:1000 in TBS with 0.25% (*w/v*) BSA. The washes with TBST were repeated and the plates were incubated with a 3,3′,5,5′-Tetramethyl-benzidine liquid substrate for ELISA (T0440; Sigma-Aldrich®). The reaction was stopped by the addition of 100 μL of 1 N HCl and the absorbance was measured at 450 nm with a microplate reader (μQuant; BioTek Instruments Inc., Winooski, VT). Each sample and standard was assayed in triplicate and the concentrations were interpolated in the linear portion of the standard curve.

All chemical, standards, and solvents were purchased from Sigma-Aldrich® (Saint Louis, MO).

Results and Discussion

***Agrobacterium*-mediated transient expression** Transformed *A. tumefaciens* clones were used to infiltrate leaves of *N. benthamiana*. The *A. tumefaciens*-infiltration experiment was carried out independently three times. The expression of pFIIa was confirmed by Western blot analysis of leaf protein extracts using a polyclonal antibody against human thrombin. As shown in Fig. 2*A* and *B*, pFIIa was visualized as a faint single band of approximately 35 kDa only at 3 dpi, with the exception of pK:pFIIa-ER, where it could not be detected (Fig. 2*A*). Transient expression usually peaks at 2–3 d after *A. tumefaciens* infiltration and then declines quickly as a result of PTGS (Voinnet *et al.* 2003).

In order to enhance pFIIa expression, co-infiltration with *A. tumefaciens* containing the silencing suppressor TCV-CP

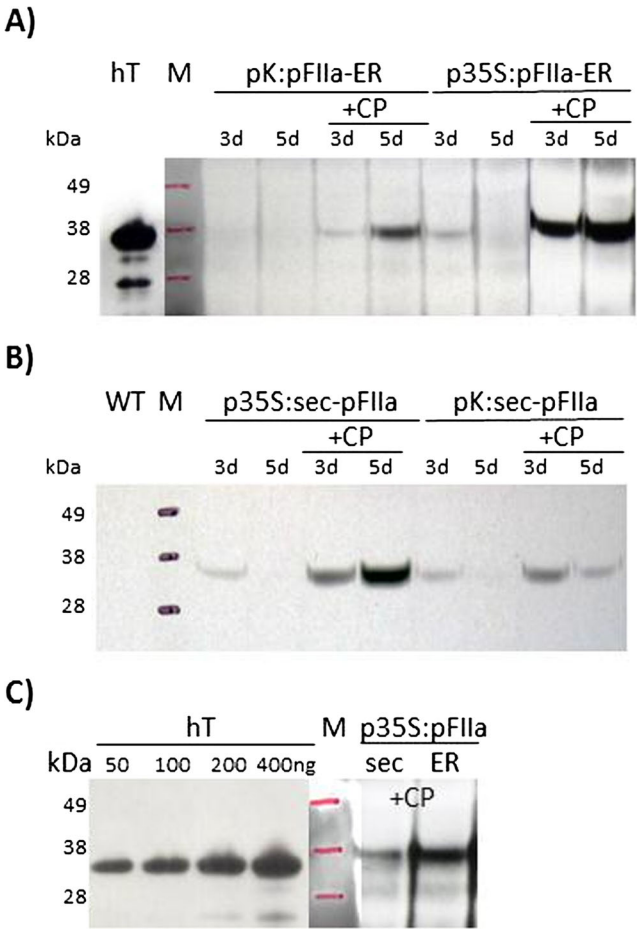

Figure 2. Representative Western blot analysis of the α-thrombin precursor *pFIIa* transient expression in *Nicotiana benthamiana* Domin leaves. (***A***) Endoplasmic reticulum (*ER*)-retained *pFIIa* version; (***B***) secreted *pFIIa* version. (***C***) Semi-quantification of pFIIa co-expressed with *Turnip Crinkle Virus*-coat protein (*TCV-CP*). Total proteins were extracted from *Agrobacterium tumefaciens*-infiltrated leaves with Laemmli buffer and 15 μg was electrophoresed in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane, and probed with a sheep anti-human thrombin polyclonal antibody. *d*, days post-*A. tumefaciens* infiltration; *+CP*, leaves co-*A. tumefaciens*-infiltrated with TCV-CP; *WT*, wild-type *N. benthamiana*; *hT*, human thrombin. The pFIIa accumulation was calculated by densitometry analysis on immunoblots using the GelPro analyzer software. Calibration curve was made with 50, 100, 200, and 400 ng human thrombin. The Western blot results presented are representative of three independent experiments.

was tested. The Western blot analysis showed that pFIIa expression increased and was maintained up to 5 dpi (Fig. 2*A* and *B, lanes + CP*). A higher protein accumulation in *A. tumefaciens*-infiltrated leaves was achieved when pFIIa expression was driven by p35S vector (Fig. 2*A* and *B*). The amount of p35S:pFIIa accumulated in *A. tumefaciens*-infiltrated leaves was estimated by comparing the intensity of the immunoreactive bands corresponding to pFIIa with a standard curve of purified human thrombin (50, 100, 200, and 400 ng; Fig. 2*C*). The expression level of p35S:pFIIa from the secreted and ER-retained versions was estimated to be 6 and 17 μg g^{-1} FW, which correspond to 0.07 and 0.21% TPC, respectively, at 5 dpi in the presence of TCV-CP (Fig. 2*C*). These results indicated that the addition of the KDEL tetra-peptide to the C-terminus of sec-pFIIa and co-expression with TCV-CP had a positive effect on pFIIa accumulation in *A. tumefaciens*-infiltrated leaves. As it has been previously reported, TCV-CP seems to act as a strong silencing suppressor, preventing PTGS that might have been triggered by recombinant pFIIa production (Qu *et al.* 2003).

Relative quantification of pFIIa expression by real-time PCR The ΔΔCT method was used to assess and compare pFIIa expression levels in *A. tumefaciens*-infiltrated leaves. The internal reference gene used was the 60S ribosomal protein gene L23. To ensure the comparability of transcripts, all real-time PCR reactions were performed with equal quantities of cDNA (5 ng). Polymerase chain reaction amplification efficiencies of pFIIa and L23 primer sets were 110 and 105%, respectively. In all cases, when comparing the same construction with or without the silencing suppressor TCV-CP, a significant increase in pFIIa expression levels in the presence of the TCV-CP was observed. This result showed that the silencing suppressor TCV-CP could efficiently enhance transient expression of pFIIa in *N. benthamiana, A. tumefaciens*-infiltrated leaves by increasing pFIIa transcript levels (Figs. 2 and 3).

Callus culture Stable transgenic calluses were generated with the constructs that yielded the highest accumulated levels in *A. tumefaciens*-infiltrated *N. benthamiana* leaves (p35S:sec-pFIIa and p35S:pFIIa-ER). Initial attempts to obtain transformed calluses were made by standard leaf disk transformation using the antibiotics cefotaxime and carbenicillin as bacteriostatic agents. However, the effective concentration of antibiotics needed to suppress *A. tumefaciens* growth resulted in explant death during the process of callus formation. Therefore, *A. tumefaciens*-infiltrated *N. benthamiana* leaves were sterilized with 3.5% sodium hypochlorite solution and used as initial explants for callus cultures. Carbenicillin was added to the medium at 500 mg L^{-1}, a concentration that does

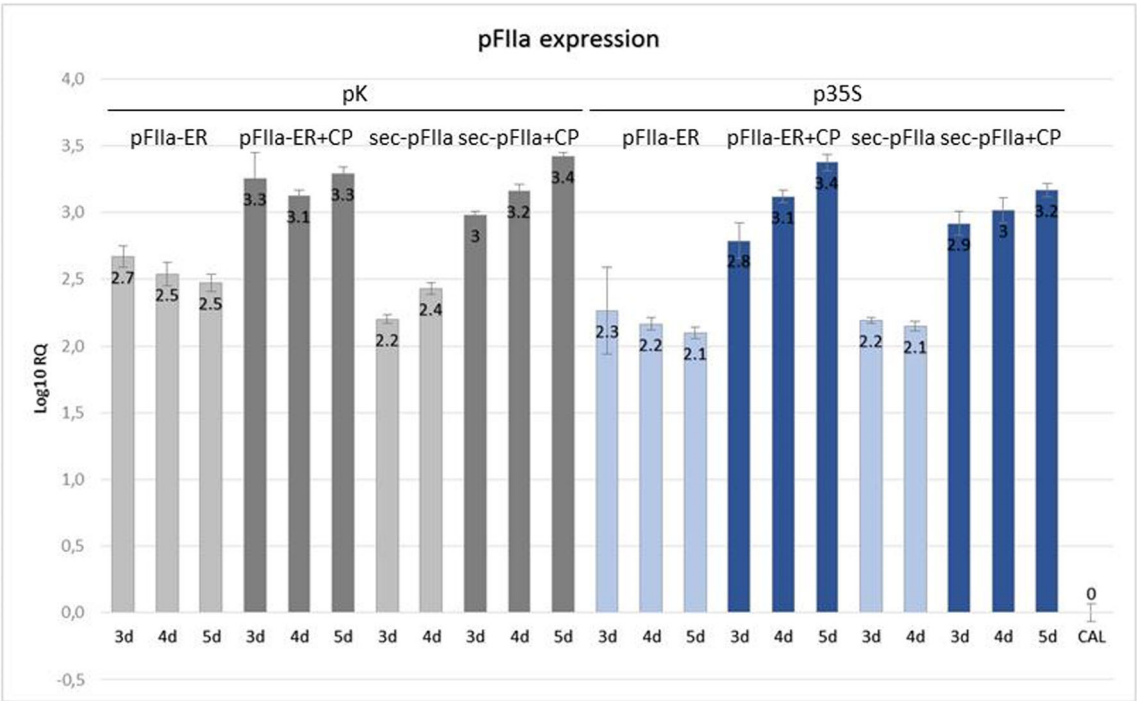

Figure 3. Relative quantification (*RQ*) of the α-thrombin precursor *pFIIa* expression in *Nicotiana benthamiana* Domin using quantitative polymerase chain reaction analysis (qPCR). Results shown as fold change (*log 10 RQ*) relative to the wild-type calibrator (*CAL*). The data *error bars* represent the standard error of the mean expression level. *pK*, *Cauliflower Mosaic Virus* 35S promoter and terminator; *p35S*, *Cauliflower Mosaic Virus* 35S promoter followed by translational enhancer sequence *Ω* from the *Tobacco Mosaic Virus* 5′ leader sequence and terminated by the *Agrobacterium tumefaciens* octopine synthase terminator; *pFIIa-ER*, endoplasmic reticulum (*ER*)-retained pFIIa version; *+CP*, leaves co-*Agrobacterium*-infiltrated with *Turnip Crinkle Virus*-coat protein; *sec-pFIIa*, secretory pFIIa version.

not affect callus formation. This alternative method was effective for eliminating *A. tumefaciens*. The growth of *A. tumefaciens* was not detected even after removing the antibiotic. After 6 mo of culture, a total of 23 s-pFIIa and 21 pFIIa-ER putative transgenic callus lines were established. To verify the insertion of the pFIIa transgene into the callus genome, a PCR-based method was carried out for the fast screening of callus clones (Fig. 4*A*). The transformation efficiency was 52.2 and 66.7% for sec-pFIIa and pFIIa-ER, respectively. In order to select the callus lines for the establishment of cell suspension cultures, the expression of pFIIa was evaluated by Western blot. Figure 4*B* shows a weak signal for pFIIa in transgenic callus lines, with different levels of accumulation. These differences in the expression levels of pFIIa might be due to position effects which maybe conducive to gene silencing (Finnegan and McElroy 1994; Gelvin 2003). This result indicated that the yields obtained in calluses were lower than those attained by *Agrobacterium*-mediated transient expression. Usually, plant cells infected with recombinant *A. tumefaciens* presented multiple copies of transcriptionally active T-DNA molecules that produced large amounts of recombinant protein in a short period of time (Narasimhulu *et al.* 1996).

Cell suspension cultures Seven transgenic callus lines for each version showing high levels of pFIIa accumulation were selected to establish plant cell suspension cultures. Only two (sec-pFIIa) and four (pFIIa-ER) cell lines were successfully adapted to growth in suspension cultures in Erlenmeyer flasks, having only one of each a good performance in the bioreactor (Fig. 5). The other cell lines, when scaled up exhibited different problems, such as micro-callus formation, cell deposit on the vessel walls, and/or slow growth that hampered the formation of dispersed cell suspensions. The two cell lines that had a good performance in the bioreactor were selected to evaluate their pFIIa production. The line pFIIa-ER showed a cell growth curve with a 4-d lag phase, followed by an exponential phase extended up to the 13th day when the stationary phase started (Fig. 5*A*). Line sec-pFIIa had a longer lag phase (8 d), a shorter exponential growth period, and started its stationary phase at the 13th day of culture as well (Fig. 5*A*). In line sec-pFIIa, the final biomass was sevenfold higher (approximately 150 g FW L^{-1}) than the initial biomass, whereas, for line pFIIa-ER, the final biomass was fivefold higher than the initial biomass, corresponding to a growth index (GI) of 6.17 and 3.7, respectively (Table 1). The specific growth rate (μ) was higher in the sec-pFIIa line (0.264 d^{-1}) than in the pFIIa-ER line (0.152 d^{-1}), with a doubling time of 2.63 d and 4.57 d for sec-pFIIa and pFIIa-ER, respectively (Table 1). The recombinant pFIIa protein from these cell lines was quantified by ELISA. The secreted version of pFIIa remained negligible in the biomass, whereas the ER-retained version of pFIIa increased during the exponential phase, reaching a maximum concentration of 0.25 μg mL^{-1} (0.017% TSP) at the 13th day of the culture (Table 1 and Fig. 5*B*). In both cases, the recombinant protein pFIIa was undetectable in the culture medium (data not shown). One possible explanation for the lack of recoverable sec-pFIIa during suspension cultures might be

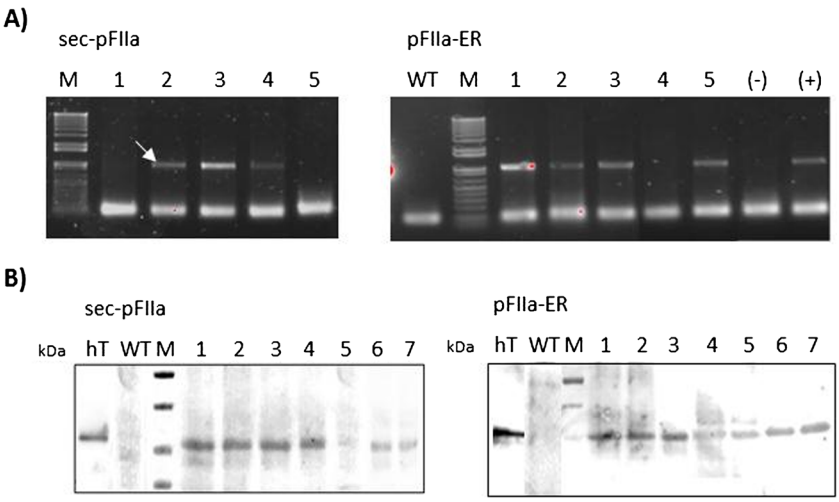

Figure 4. (***A***) Representative polymerase chain reaction (PCR) amplification for the secretory (*sec-*) and endoplasmic reticulum (*−ER*) retrained α-thrombin precursor (*pFIIa*) *sec-pFIIa* and *pFIIa-ER* transgenes from transformed *Nicotiana benthamiana* Domin callus genome. *WT*, wild-type callus; *M*, molecular weight standards; *lanes 1 to 5*, kanamycin resistant callus lines; *(−)*, negative PCR control; *(+)*, positive control (p35S:sec-pFIIa plasmid). The *arrows* indicate the expected 973 bp size of the amplification product. (***B***) Representative Western blot analysis of *sec-pFIIa* and *pFIIa-ER* expression from transgenic callus lines. Total proteins were extracted from callus with Laemmli buffer. The equivalent to 25 mg of fresh callus per *lane* were electrophoresed in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane, and probed with a sheep anti-human thrombin polyclonal antibody. *hT*, human thrombin; *WT*, wild-type *N. benthamiana* callus; *M*, molecular weight standards; *lanes 1–7*, transgenic callus lines.

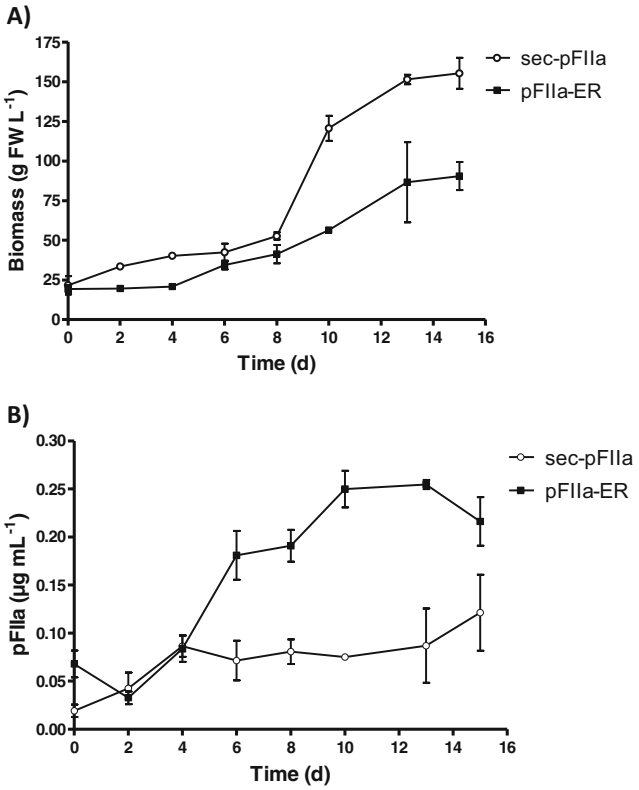

Figure 5. (***A***) Time course of cell growth of stable *Nicotiana benthamiana* Domin suspension culture transformed with secretory (*sec*-) and endoplasmic reticulum (−*ER*) retrained α-thrombin precursor (*pFIIa*) *sec-pFIIa* and *pFIIa-ER* in bioreactor. Biomass was measured as fresh weight (*FW*). Each point represents the mean ± SD value of two replicates. (***B***) Concentration of *pFIIa* in the biomass. Each point represents the mean ± SD value of three replicates. In both cases the time of culture was 15 d. The experiment was repeated once with similar results and only one experiment is shown.

that the secreted protein was degraded by host proteases (Doran 2006). Therefore, ways to increase pFIIa accumulation in the culture medium could be achieved by the addition of protease inhibitors and/or by minimizing the effect of extracellular proteases (Huang *et al.* 2009). From these present results, it was clear that obtaining a suitable line to grow in suspension culture required the screening of a large number of cultures from different callus lines. Additionally, the productivity of the recombinant protein in suspension cultures did not necessarily correlate to the expression level obtained in callus cultures. Callus is a mixture of dedifferentiated cells with different transgene copy numbers and insertion sites (Nocarova and Fischer 2009). If in the present study heterogeneity at the callus stage occurred, then this might account for the variable recombinant protein yields observed in suspension cultures (James and Lee 2006).

Table 1. Growth parameters and α-thrombin precursor *pFIIa* production in stably transformed *Nicotiana benthamiana* Domin cell suspensions

	sec-pFIIa	pFIIa-ER
μ (d^{-1})	0.264	0.152
Time of duplication (d)	2.63	4.57
GI	6.17	3.7
pFIIa (μg mL^{-1})	–	0.25

sec-, secretory pFIIa version; −*ER*, endoplasmic reticulum retrained pFIIa version; *μ*, specific growth rate; *GI*, growth index: (final biomass−initial biomass) ÷ (initial biomass)

The production of pFIIa in stable-transformed suspension cultures was lower than that attained by transient expression in *A. tumefaciens*-infiltrated leaves. In addition to inherent differences between these two expression systems, PTGS reduction by co-expression with TCV-CP demonstratively enhanced transient pFIIa expression levels. Thus, pFIIa yield in suspension cultures could be enhanced by using PTGS silencing suppressors (Sudarshana *et al.* 2006; Boivin *et al.* 2010).

Conclusions

The present study reports for the first time that a precursor of the human α-thrombin was expressed in plants. Transient expression was attained after 3 d post-*A. tumefaciens* infiltration and the yields were higher when co-infiltration with a PTGS inhibitor was performed (0.07 and 0.21% (*w/w*) TPC for the secreted and retained version, respectively). As for the stable expression, pFIIa yields were only detected in the version retained in the ER (0.017% (*w/w*) TSP). In no case was the recombinant protein found in the culture medium. Clearly, transient expression yielded higher amounts of pFIIa compared to the stable transformation in the conditions of this present work. Future experiments will be performed in order to optimize the growth parameters, including the culture conditions (light source and intensity, culture medium composition, plant growth regulators, and stabilizing agents) for increasing pFIIa yields in *in vitro* plant cultures.

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