

Full Length Research Paper

An endoplasmic reticulum (ER)-directed fusion protein comprising a bacterial subtilisin domain and the human cytokine interleukin 6 is efficiently cleaved *in planta*

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A major limitation of plant bioreactors is the lack of suitable and cost-effective purification methods for the extraction of pharmaceutical-grade proteins. In contrast to that, there are numerous established purification systems for heterologous proteins, expressed in *Escherichia coli*, which are used for the commercial production of therapeutic proteins. Therefore, we wanted to adapt the BioRad Profinity eXact™ one-step protein purification system (originally designed for microbial expression platforms) to purify recombinant proteins in crude plant extracts. This system based on the prodomain of microbial subtilase as fusion partner and a column-bound subtilisin protease. The engineered protease captures and cleaves the fusion protein, retaining the tag and releasing the native protein into the eluate. The subtilase tag was fused to human interleukin 6 (IL6) and transiently expressed in *Nicotiana benthamiana* leaves using the MagnICON system. The fusion protein was expressed at lower levels than native IL6, suggesting it is expressed less efficiently and/or has a lower stability. However, free IL6 was also detected in the extract and was unaffected by the addition of protease inhibitors during extraction, suggesting that the fusion protein is cleaved *in planta* by endogenous proteases. Purification of the recombinant protein using the Profinity eXact™ system reduced the yield still further. The inefficient production of tagged IL6, coupled with the extensive losses during purification, indicate that the Profinity eXact™ system is not suitable for the extraction of IL6 from crude plant extracts.

Key words: Tobacco, transient expression, endoplasmic reticulum, Profinity protein purification, partial cleavage.

INTRODUCTION

The production of pharmaceutical proteins in plants is attractive because of the anticipated lower costs, greater scalability and increased safety compared to traditional microbial and mammalian bioreactors (Davies, 2010).

Many recombinant therapeutic proteins have been expressed in transgenic plants to demonstrate proof-of-concept and there have been significant improvements in yields (Sharma and Sharma, 2009). However, downstream processing costs remain a major constraint for the commercial development of plant-derived pharmaceuticals (Wilken and Nikolov, 2012). For example, tobacco leaves contain more than 2500 compounds, including many alkaloids, phenolics and terpenoids (Nugroho and Verpoorte, 2002), which interfere with downstream processing (Wilken and Nikolov, 2012). The removal of such undesirable

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Abbreviations: IL6, Interleukin 6; ELISA, enzyme-linked immunosorbent assay.

Table 1. Primers used in this investigation.

| Primer | Sequence | Fragment length |
|--------------------|--|----------------------|
| 29-Bsal-NtIL6ER-fw | 5'-ttttGGTCTCACATGGCTTCCTCCAATTCATTCTC-3' | 1110 bp ² |
| NtIL6ER-Bsal-rv | 5'-TTTTGGTCTCTAAGCTTAAAGCTCATCCTTCTCAGAC-3' | 1116 bp ³ |
| Subi-Spacer2-fw | 5'-CTTTTCAAAGCTCTTACAAGTCCAGTCCGCCAGGTGAAGATTC-3' | |
| Subi-Spacer2-rv | 5'-ACCTGGCGGAACTGGACTTGTAAAGAGCTTTGAAAAGTTTATCTTC-3' | |
| Subi-fw | 5'-CGTAGATGCTGCTAGTGCTACAC-3' | 530 bp ² |
| NtIL6-rv | 5'-GTGTAGTCATGTCTTGAACC-3' | 536 bp ³ |
| TMV-fw | 5'-GATCCGGACGTCGAAGTTTCGAAGG-3' | 1409 bp ¹ |
| TMV-rv | 5'-CTTGACTCTAGCTAGAGCGGCCGCTGG-3' | 1723 bp ² |
| | | 1726 bp ³ |

¹pICH18711; ²pICH29912-SubiIL6ER; ³pICH29912-Subi-s-IL6ER.

substances can account for more than 80% of the overall costs of production (Conley et al., 2011; Wilken and Nikolov, 2012). The development of a cost-effective system for the purification of recombinant proteins from crude plant extracts is therefore a significant challenge in the deployment of plant bioreactors for large-scale protein production (Schillberg et al., 2005). The typical approach used in experimental plant bioreactors is to express fusion proteins carrying affinity tags, such as polyhistidine or StrepII, for subsequent affinity purification (Valdez-Ortiz et al., 2005; Witte et al., 2004). However, because fusion tags can affect the structure, activity and immunogenicity of recombinant pharmaceutical proteins, they must be removed using appropriate proteases.

To facilitate the separation of the target protein from the protease and the cleaved fusion partner, BioRad has developed the Profinity eXact™ purification system (Gallagher et al., 2009; Ruan et al., 2004; Ruan et al., 2008). The N-terminus of the recombinant protein is fused to the subtilisin BPN' prodomain (prosignal sequence, 75 amino acids) and the corresponding subtilisin protease is immobilized on a chromatography column. When the correct buffer is used, the engineered protease captures and cleaves the fusion protein, retaining the tag and releasing the native protein into the eluate. This system has already been used for heterologous proteins expressed in *Escherichia coli* (Christian et al., 2011; Motta et al., 2011).

We attempted to adapt the system to work with proteins transiently expressed in plants with the MagnICON system (Gleba et al., 2007) using human interleukin 6 (IL6) as the target protein and recorded a partial cleavage of the fusion protein *in planta*.

MATERIALS AND METHODS

Construction of plant expression vectors

The sequence encoding the subtilisin BPN' prodomain (228 bp, 75 amino acids), which is the basis of the Profinity eXact™ system (Bio-Rad, Hercules, USA), was codon optimized for tobacco

(<http://www.kazusa.or.jp/codon>). The second intron from the *StSL1* gene [accession no. X04753 M25351] (Vancanneyt et al., 1990) was inserted between bases 38 and 39 of the subtilisin fragment generating a cassette 417 bp in length. This modified prodomain was synthesized by the DNA Cloning Service (Hamburg, Germany) and inserted into the IL6 coding sequence between the ER-targeting signal and mature protein in vector pLH-IL6ER (Nausch et al., 2012). The final binary vector was named pLH-Subi-IL6ER.

We used transient expression vectors based on *tobacco mosaic virus* (TMV) provided by Nomad Bioscience, Halle/Saale, Germany. These are derivatives of ¹pICH18711, which has been optimized for high yields (Marillonnet et al., 2004). The ¹pICH18711 vector is similar to pICH29912 except the green fluorescent protein (GFP) coding region which has been inserted into the Bsal cloning site of pICH29912.

The coding region of Subi-IL6ER was amplified from pLH-Subi-IL6ER to incorporate flanking Bsal restriction sites using primers 29-Bsal-NtIL6ER-fw and NtIL6ER-Bsal-rv (Table 1). The Subi-s-IL6ER coding region (where, s = spacer) was generated in two steps. First, two independent fragments were amplified from pLH-Subi-IL6ER using primers 29-Bsal-NtIL6ER-fw with Subi-Spacer2-rv (516-bp product) and Subi-Spacer2-fw with NtIL6ER-Bsal-rv (600-bp product) (Table 1). The Subi-Spacer2 primers correspond to the junction of the BPN'-prodomain and the mature IL6 protein but include additional threonine and serine codons as a spacer element. In the second polymerase chain reaction (PCR), the two first-round products were used as the template with primers 29-Bsal-NtIL6ER-fw and NtIL6ER-Bsal-rv, resulting in the final product Subi-s-IL6ER (1116 bp). PCR products were inserted into the Bsal site of the corresponding TMV vectors (Engler et al., 2008) and verified by sequencing using primers TMV-fw and TMV-rv (Table 1).

The transient expression vectors were introduced into *Agrobacterium tumefaciens* strain ICF 320, a disarmed, auxotrophic derivative (Δ cysKa, Δ cysKb, Δ thiG) of strain C58 (Bendandi et al., 2010).

Transient expression in tobacco

We evaluated transient expression in *Nicotiana benthamiana* and two commercial cultivars of *Nicotiana tabacum*, namely Geudertheimer and Virginia. Six-to-nine-week-old tobacco plants were agroinfiltrated as described by Giritich et al. (2006). The bacteria were inoculated into a 5-ml starter culture containing 50 μ g/ml rifampicin and 50 μ g/ml kanamycin, and were incubated overnight at 28°C and 220 rpm. The overnight culture was adjusted to an OD_{600nm} of ~ 1.5 and 10 ml of the adjusted culture centrifuged for 10 min, 4500 x g, 4°C and the pellet resuspended in 1000 ml

infiltration buffer (10 mM 2-(*n*-morpholino)-ethanesulfonic acid (MES) pH 5.5, 10 mM MgCl₂) corresponding to a 1:100 dilution.

Enzyme-linked immunosorbent assay (ELISA), western blot and protein purification

Leaf samples (150 mg) were homogenized in liquid nitrogen and resuspended in 250 µl cold protein extraction buffer (50 mM HEPES pH 7.2 supplemented either with 2 mM phenylmethylsulfonyl fluoride (PMSF) or 5 µg/ml aprotinin). Samples were centrifuged for 10 min in a cooled bench-top centrifuge and the protein concentration in the supernatant was measured according to the Bradford (1976) method using Pierce reagent with bovine serum albumin (BSA) as the standard (Thermo Scientific, Bonn, Germany).

Recombinant IL6 was quantified using a commercial IL6 ELISA Kit (Human IL-6 Ready-SET-Go ELISA; catalog no. 88-7066-86, eBioscience, Frankfurt, Germany) according to the manufacturer's instructions. Briefly, 96-well plates were coated with a mouse anti-human IL6-specific antibody at a final concentration of 1 µg/ml at room temperature overnight. Following five washes with phosphate buffered saline (PBS) containing 0.05% Tween-20, the plates were incubated with 100 µl diluted leaf extract at room temperature for 2 h. After another wash, the plates were incubated with the corresponding biotinylated detection antibody at room temperature for 1 h, washed again and incubated with streptavidin conjugated to horseradish peroxidase at room temperature for 30 min. Finally, the plate was incubated with tetramethylbenzidine (TMB) at room temperature for 15 min in the dark. The reaction was stopped with 250 mM sulfuric acid. Extinction was measured at 450 nm in a Synergy HT multidetection reader (Bio-Tek, Bad Friedrichshall, Germany).

For western blot analysis, total soluble protein was extracted from leaf samples as described above and 10 to 100 µg of total soluble protein was resuspended in 1x sample buffer containing 10% glycerol, 150 mM Tris (pH 6.8), 3% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol and 2.5% bromophenol blue. The samples were heated to 95°C for 5 min and separated under denaturing conditions by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to a 0.45-µm polyvinylidene fluoride (PVDF) membrane (VWR; Darmstadt, Germany). The proteins were transferred at a constant 2 mA/cm² at room temperature for 2 h in a Bio-Rad Trans-Blot semi-dry transfer cell using 50 mM Tris, 40 mM glycine, 0.01% SDS and 20% methanol as the transfer buffer (pH 8.5). The membrane was blocked using PBS containing 0.05% Tween-20 and 5% nonfat milk powder at room temperature overnight and then probed at room temperature for 2 h with a mouse monoclonal anti-human IL6 antibody (catalog no. MA1-35475; ABR BioReagent, USA) or a rabbit monoclonal anti-human-IL6 antibody (catalog no. AB1421; Millipore, Schwalbach, Germany), each at 1:1000 dilution in PBS. After three washes, the membrane was probed at room temperature for 2 h with a horseradish peroxidase (HRP) -conjugated secondary antibody, either goat anti-mouse (catalog no. 715-035-151; Dianzova, Hamburg, Germany) or donkey anti-rabbit (catalog no. NA934V; GE Healthcare, Munich, Germany), each at 1:10,000 dilution in PBS. The signal was detected by electrochemiluminescence (ECL) and the membrane was exposed to Kodak Biomax light X-ray film (VWR; Darmstadt, Germany) for 1 min before it was developed and fixed.

Recombinant IL6 was extracted from crude leaf extracts using the Profinity eXactTM protein purification system according to the manufacturer's recommendations (BioRad, Munich, Germany). Briefly, the columns were washed twice with pre-cooled wash buffer (100 mM sodium phosphate, pH 7.2) and centrifuged at 1000 x g for 30 s. We then incubated 600 µl of the extract for 1 h at 4°C or

for 30 min at room temperature with gentle shaking. After another centrifugation step the column was washed twice with 500 µl pre-cooled wash buffer. The recombinant protein was eluted by incubating the column with 500 µl elution buffer (100 mM sodium fluoride, 100 mM sodium phosphate, pH 7.2) for 1 h at room temperature or overnight at 4°C with gentle shaking. The eluate was collected by centrifugation and the column was stripped with 100 mM phosphoric acid.

Statistical methods

Statistical comparisons were calculated with the program SPSS and carried out using the *F*-test analysis of variance (ANOVA) including the Bonferroni post-hoc test with $p \leq 0.05$ (two-sided) considered significant.

RESULTS

Construction of plasmids

In order to test the Profinity eXactTM protein purification system we designed two constructs (Figure 1) which were introduced into the MagniCON vector pICH29912 for transient expression in tobacco. In the first construct (²pICH29912-Subi-IL6ER), the BPN' prodomain was inserted directly between the IL6 native coding sequence and the ER-targeting signal, allowing tag-free purification of IL6. In the second construct (³pICH29912-Subi-s-IL6ER), we inserted additional triplets for threonine and serine between the BPN' prodomain and the sequence of the mature protein, since the first two amino acids of the target protein can affect the efficiency of the corresponding protease. The threonine-serine linker is recommended by the manufacturers of the Profinity eXactTM protein purification system, although these two amino acids remain on the purified protein after cleavage. We also tested the native IL6 sequence without tags using vector pICH29912-IL6ER (Nausch et al., 2012).

Transient expression of IL6ER, Subi-IL6ER and Subi-s-IL6ER

The three vectors were compared head to head in the three tobacco hosts. As shown in Figure 2a, the highest recombinant protein yields were achieved in *N. benthamiana* and the performance of the two *N. tabacum* cultivars was much less remarkable. We therefore focused on *N. benthamiana* for further experiments. The presence of the fusion tag reduced the yield of IL6 significantly in all three hosts. In *N. benthamiana*, the yield of IL6ER was up to 7% total soluble protein (TSP), whereas the yield of Subi-IL6ER and Subi-s-IL6ER reached a maximum of 1% TSP (Figure 2a; Table 2).

In western blots of crude plant extracts, Subi-IL6ER and Subi-s-IL6ER were represented as 33-kDa bands, suggesting that both fusion proteins were glycosylated (Figure 2b). However, additional 25 and 28 kDa bands

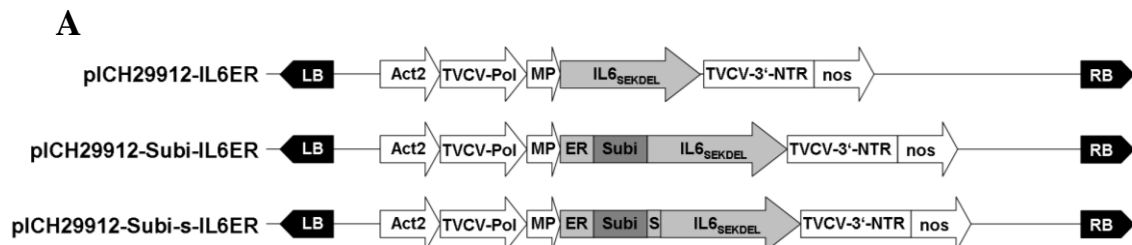


Figure 1. A. Schematic representations of the T-DNA constructs used to express recombinant IL6 using MagnICON vectors. LB, Left border; RB, right border; IL6, synthetic human *IL6* gene codon optimized for tobacco; ER, human IL6 native ER targeting signal; SEKDEL, ER retention motif; Subi, BPN'-Profinity eXact™ tag; Act2, actin promoter; TVCV-Pol, RNA-dependent RNA polymerase from *Turnip vein clearing virus* (TVCV); MP, *tobacco mosaic virus* (TMV) movement protein; TVCV-3'-NTR, TVCV-3' untranslated region; nos, *A. tumefaciens* nopaline synthetase gene terminator. **B.** Protein and codon optimized nucleotide sequence of the BPN' prodomain and human IL6. The ER-targeting peptide of IL6 includes three triplets downstream the initiator codon ATG that ensure recognition. The sequence of the BPN' Profinity eXact™ tag is shaded, the *StSL1* intron is underlined and the Thr-Ser-spacer is double underlined.

were observed, corresponding to the predicted molecular weight of *N*-glycosylated and *N*/*O*-glycosylated IL6 without a fusion tag (Simpson et al., 1997). No bands were observed in western blots of control tobacco leaf extracts.

The BPN' domain and the corresponding protease were derived from a microbial subtilisin protease, which belongs to the serine protease family. We therefore added the broad-spectrum serine protease inhibitors PMSF and aprotinin to the extract during purification, but the yield of free IL6 was unaffected suggesting the free molecule was generated by endogenous proteases *in planta* (data not shown).

Purification of recombinant IL6ER using the Profinity eXact™ system

Although the yields of intact prodomain-IL6 complexes were comparatively low (Figure 2b), we processed the crude extracts from *N. benthamiana* leaves harvested 10 days post infiltration (dpi) using the Profinity eXact™ purification system. The amount of recombinant protein in each crude extract and the different fractions was determined by IL6-ELISA, which did not distinguish between the intact fusion proteins and IL6, which has already been cleaved from the tag, because the detection antibody recognized a conserved domain on the IL6 moiety. For both fusion proteins, nearly 75% of the total recombinant protein was found in the flow through and first washes, showing that the fusion proteins did not bind the protease immobilized on the Profinity column (Figure 3a and Table 3). Only small amounts of recombinant protein were found in the eluate after cleavage (Table 3). Approximately 25% of the Subi-IL6ER fusion protein was recovered by stripping the column (which removes the subtilisin tag from the immobilized protease) suggesting that direct linkage results in inefficient cleavage, whereas minimal amounts of the Subi-s-IL6ER variant were recovered by stripping, suggesting that the two additional

amino acids were important for accurate and efficient cleavage (Table 3). The different fractions were also analyzed by western blot, apart from the stripped fraction which is incompatible with electrophoresis due to the presence of phosphoric acid (this was diluted out in the ELISA procedure). As shown in Figure 3b, the size of IL6 recovered before protease induction corresponded to the pure IL6 protein without tag. The serine-threonine linker also appeared to increase the efficiency of cleavage that already occurs *in planta*. However, neither PMSF nor aprotinin increased the amount of bound fusion protein (Table 3). Prolonged incubation of the crude leaf extracts at 4°C or room temperature also had no significant effect (data not shown).

DISCUSSION

The aim of this investigation was to determine whether the BPN' prodomain of subtilisin (which is the basis of the Profinity™ eXact™ purification system) was suitable for the isolation of recombinant proteins from plants. We created two MagnICON transient expression constructs to incorporate this domain (Subi-IL6ER and Subi-s-IL6ER) and tested them in *N. benthamiana* leaves. We found that the yield of both fusion proteins was approximately eight-fold lower than that of the IL6 protein without a tag. This potentially reflected the less efficient expression of the fusion constructs, given that the Subi-IL6ER and Subi-s-IL6ER inserts both exceed 1 kb in length, which can be a trigger for vector instability and inefficient viral replication during transient expression (Ahlquist et al., 2005; Avesani et al., 2007; Castro et al., 2005; Gleba et al., 2007). Alternatively, the lower yields could reflect protein instability, given the presence of free IL6 in the crude extracts prior to the on-column induced cleavage. This suggests that the fusion protein undergoes cleavage *in planta* or during extraction, in the absence of the column-bound protease, which is

B

M A S S N S F S T S A F G P V A F S L G
 Subi-IL6ER ATGGCTTCCTCCAATTCATTCTACTTCGGCCTTTGGTCCAGTTGCATTCAGCCTTGGG
 Subi-s-IL6ER ATGGCTTCCTCCAATTCATTCTACTTCGGCCTTTGGTCCAGTTGCATTCAGCCTTGGG

L L L V L P A A F P A M G G K S N G E K
 Subi-IL6ER TTGCTCTTAGTGTTCCTGCTGCATTTCTGCGATGGGTGTTAAAAGTAATGGTGAAAAA
 Subi-s-IL6ER TTGCTCTTAGTGTTCCTGCTGCATTTCTGCGATGGGTGTTAAAAGTAATGGTGAAAAA

K Y I V-
 Subi-IL6ER AAATACATTGTAGGTAAGTTTCTGCTTCTACCTTTGATATATATATAATAATTATCATT
 Subi-s-IL6ER AAATACATTGTAGGTAAGTTTCTGCTTCTACCTTTGATATATATATAATAATTATCATT

ATTAGTAGTAATATAATATTTCAAATATTTTTTCAAATAAAAAGAAATGTAGTATATAGC
 Subi-IL6ER ATTAGTAGTAATATAATATTTCAAATATTTTTTCAAATAAAAAGAAATGTAGTATATAGC
 Subi-s-IL6ER ATTAGTAGTAATATAATATTTCAAATATTTTTTCAAATAAAAAGAAATGTAGTATATAGC

AATTGCTTTTCTGTAGTTTATAAGTGTATATTTTAATTTATAACTTTTCTAATATATG
 Subi-IL6ER AATTGCTTTTCTGTAGTTTATAAGTGTATATTTTAATTTATAACTTTTCTAATATATG
 Subi-s-IL6ER AATTGCTTTTCTGTAGTTTATAAGTGTATATTTTAATTTATAACTTTTCTAATATATG

-V G F K Q G F K S C A K K E
 Subi-IL6ER ACCAAAAATTTGTTGATGTGCAGGTTTCAAACAAGGTTTTAAAAGTTGCGTAAAAAGAA
 Subi-s-IL6ER ACCAAAAATTTGTTGATGTGCAGGTTTCAAACAAGGTTTTAAAAGTTGCGTAAAAAGAA

D V I S E K G G K L Q K C F K Y V D A A
 Subi-IL6ER GATGTTATTAGTGAAAAAGGTGGTAAACTTCAAAAATGCTTCAAAATACGTAGATGCTGCT
 Subi-s-IL6ER GATGTTATTAGTGAAAAAGGTGGTAAACTTCAAAAATGCTTCAAAATACGTAGATGCTGCT

S A T L N E K A V E E L K K D P S V A Y
 Subi-IL6ER AGTGCTACACTTAATGAAAAAGCTGTTGAAGAACTTAAAAAGATCCGAGTGTAGCTTAC
 Subi-s-IL6ER AGTGCTACACTTAATGAAAAAGCTGTTGAAGAACTTAAAAAGATCCGAGTGTAGCTTAC

V E E D K L F K A L T S P V P P G E D S
 Subi-IL6ER GTTGAAGAAGATAAACTTTTCAAAGCTCTT-----CCAGTTCCGCCAGGTGAAGATTCT
 Subi-s-IL6ER GTTGAAGAAGATAAACTTTTCAAAGCTCTTACAAGTCCAGTTCGCCAGGTGAAGATTCT

K D V A A P H R Q P L T S S E R I D K Q
 Subi-IL6ER AAGGACGTAGCTGCGCCACACAGACAACCATTAACCTCTAGTGAGAGAATTGATAAGCAG
 Subi-s-IL6ER AAGGACGTAGCTGCGCCACACAGACAACCATTAACCTCTAGTGAGAGAATTGATAAGCAG

I R Y I L D G I S A L R K E T C N K S N
 Subi-IL6ER ATACGATACATTCTCGATGGAATCTCTGCTCTGAGGAAGGAGACTTGTAAACAAATCTAAT
 Subi-s-IL6ER ATACGATACATTCTCGATGGAATCTCTGCTCTGAGGAAGGAGACTTGTAAACAAATCTAAT

M C E S S K E A L A E N N L N L P K M A
 Subi-IL6ER ATGTGCGAATCATCTAAGGAAGCTCTAGCCGAGAACAATCTTAATCTGCCTAAGATGGCT
 Subi-s-IL6ER ATGTGCGAATCATCTAAGGAAGCTCTAGCCGAGAACAATCTTAATCTGCCTAAGATGGCT

E K D G C F Q S G F N E E T C L V K I I
 Subi-IL6ER GAGAAGGATGGATGCTTCCAGTCTGGCTTTAATGAAGAGACATGCTTGTAAAGATCATT
 Subi-s-IL6ER GAGAAGGATGGATGCTTCCAGTCTGGCTTTAATGAAGAGACATGCTTGTAAAGATCATT

T G L L E F E V Y L E Y L Q N R F E S S
 Subi-IL6ER ACTGGGTTATTGGAATTTGAAGTGTATCTCGAGTATCTCAGAACAGGTTTGAATCATCT
 Subi-s-IL6ER ACTGGGTTATTGGAATTTGAAGTGTATCTCGAGTATCTCAGAACAGGTTTGAATCATCT

E E Q A R A V Q M S T K V L I Q F L Q K
 Subi-IL6ER GAGGAACAAGCTCGTGTGTTTCAGATGAGTACTAAAGTCTTATACAATTCCTTCAGAAG
 Subi-s-IL6ER GAGGAACAAGCTCGTGTGTTTCAGATGAGTACTAAAGTCTTATACAATTCCTTCAGAAG

K A K N L D A I T T P D P T T N A S L L
 Subi-IL6ER AAGGCTAAGAATCTTGATGCCATTACGACTCCTGATCCAACAACCAACGCCCTCTCTTCTT
 Subi-s-IL6ER AAGGCTAAGAATCTTGATGCCATTACGACTCCTGATCCAACAACCAACGCCCTCTCTTCTT

T K L Q A Q N Q W L Q D M T T H L I L R
 Subi-IL6ER ACTAAGTTGCAAGCTCAGAATCAATGGTTGCAAGACATGACTACACATCTTATTCTTAGA
 Subi-s-IL6ER ACTAAGTTGCAAGCTCAGAATCAATGGTTGCAAGACATGACTACACATCTTATTCTTAGA

S F K E F L Q S S L R A L R Q M
 Subi-IL6ER AGCTTCAAGGAATTTCTTCAATCTAGTCTTAGAGCTCTTAGACAAATG
 Subi-s-IL6ER AGCTTCAAGGAATTTCTTCAATCTAGTCTTAGAGCTCTTAGACAAATG

S E K D E L *
 Subi-IL6ER TCTGAGAAGGATGAGCTTTAA

Figure 1. Continued.

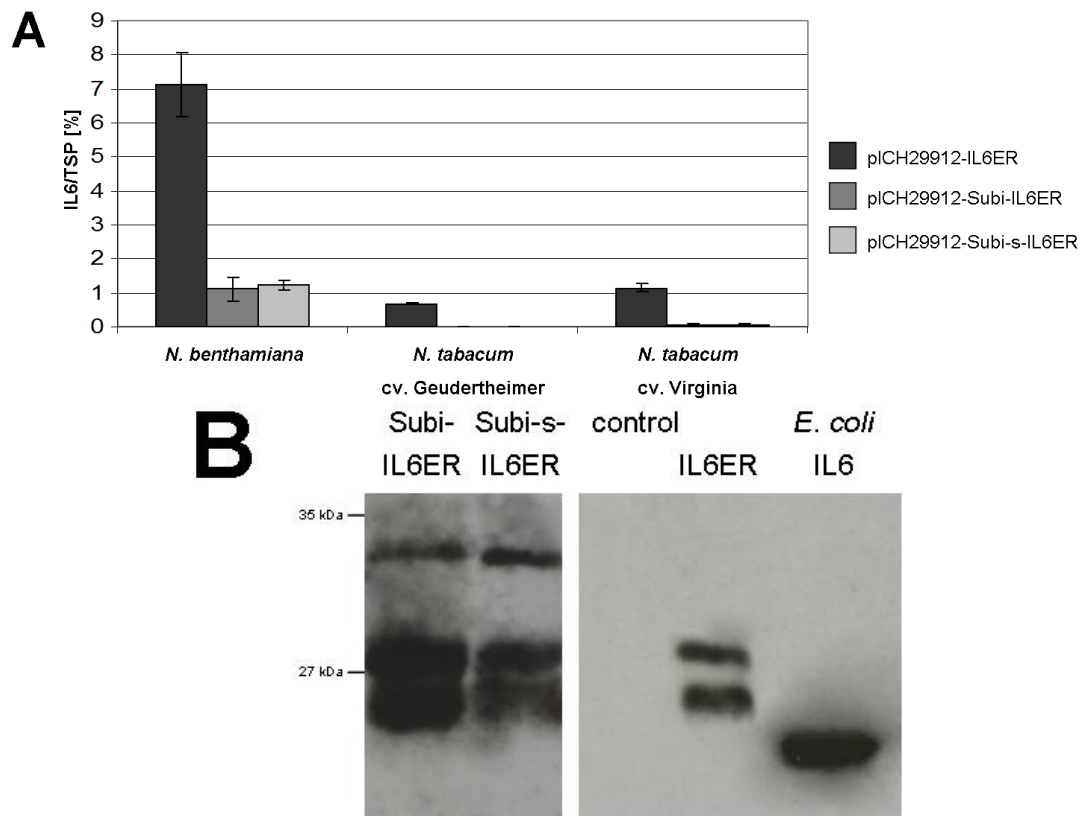


Figure 2. A. Expression levels of pICH29912-IL6ER, pICH29912-Subi-IL6ER and pICH29912-Subi-s-IL6ER in leaf extracts from *N. benthamiana*, *N. tabacum* cv. Geudertheimer and cv. Virginia sampled 10 days post infiltration. The quantity of recombinant protein was determined by ≥ 2 independent ELISAs. B. Western blot analysis of 10 μ g leaf samples, 10 days post infiltration with *A. tumefaciens* strain ICF320 carrying vectors pICH29912-IL6ER, pICH29912-Subi-IL6ER and pCH29912-Subi-s-IL6ER. Untreated leaves were used as a negative control and recombinant IL6 derived from *E. coli* was used as a positive control.

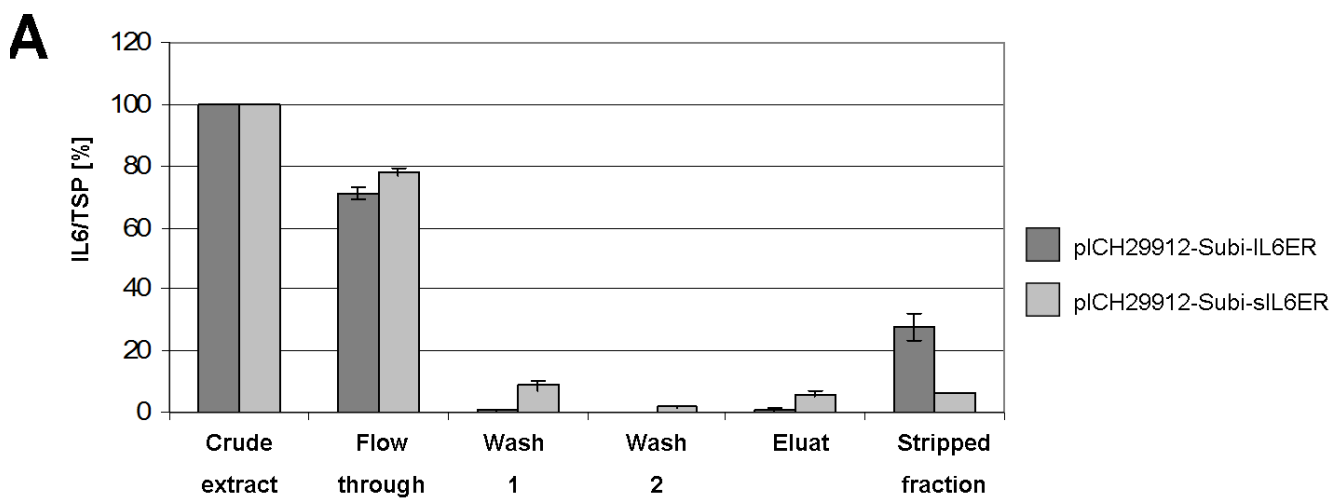


Figure 3. Application of crude extracts from *N. benthamiana* leaves (harvested 10 days post infiltration with pICH29912-Subi-IL6ER and pICH29912-Subi-s-IL6ER) to the Profinity eXact™ purification system. A. The quantity of IL6 in each fraction was determined by ≥ 2 independent ELISAs and is presented as a proportion relative to the absolute amount of IL6 applied to the column. B. Western blot analysis of the different purification fractions, using untreated leaves as a negative control. The stripped column fraction could not be analyzed because the phosphoric acid interferes with electrophoresis.

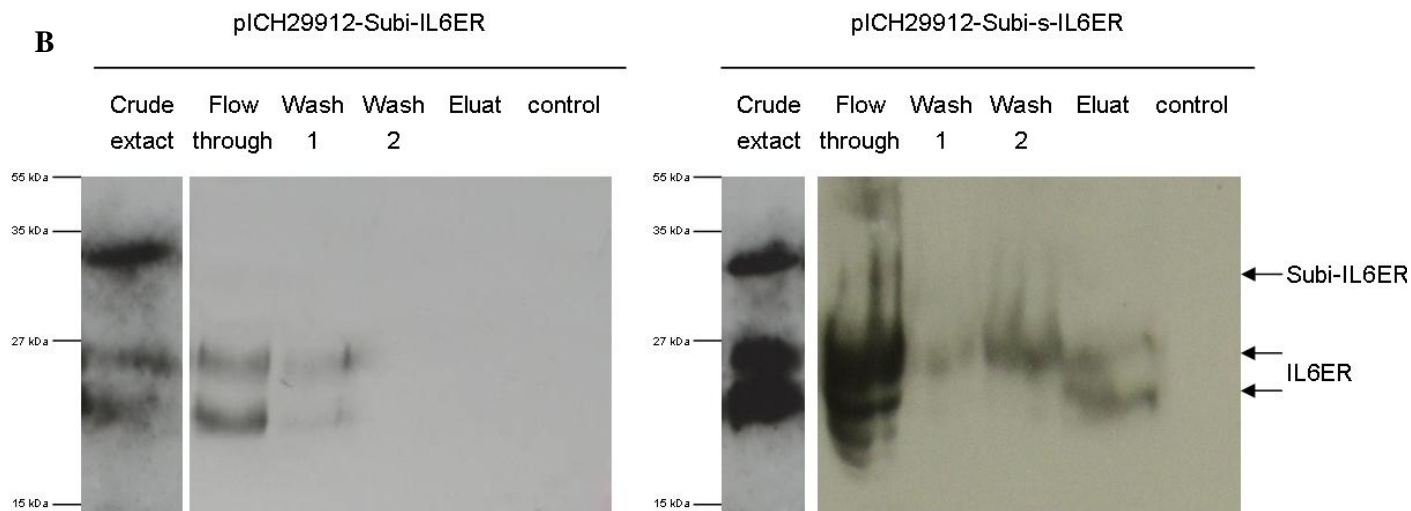


Figure 3. Continued.

Table 2. Expression of recombinant IL6 fusion proteins in tobacco leaf samples harvested 10 days post infiltration with *A. tumefaciens* strain ICF320 carrying the vectors pICH29912-IL6ER, pICH29912-Subi-IL6ER and pICH29912-Subi-s-IL6ER.

| Cultivar | IL6ER | Subi-IL6ER | Subi-s-IL6ER |
|-------------------------------------|----------------------|-----------------------|-----------------------|
| <i>N. benthamiana</i> | 7.121 (\pm 0.928) | 1.117% (\pm 0.345) | 1.221 (\pm 0.137) |
| <i>N. tabacum</i> cv. Geudertheimer | 0.652 (\pm 0.025) | 0.004% (\pm 0.002) | 0.002 (\pm 0.0003) |
| <i>N. tabacum</i> cv. Virginia | 1.139 (\pm 0.114) | 0.070% (\pm 0.040) | 0.061 (\pm 0.029) |

The yield, determined by ≥ 2 independent ELISAs, is presented as a percentage of TSP with the standard deviation in parentheses.

Table 3. Purification of recombinant IL6 fusion proteins from crude *N. benthamiana* leaf extracts harvested 10 days post infiltration with *A. tumefaciens* strain ICF320 carrying the vectors pICH29912-IL6ER, pICH29912-Subi-IL6ER and pICH29912-Subi-s-IL6ER.

| Fraction | Subi-IL6ER | Subi-s-IL6ER | Subi-s-IL6ER + PMSF | Subi-s-IL6ER + aprotinin |
|-------------------|----------------------|----------------------|------------------------|-----------------------------|
| Crude extract | 100% | 100% | 100% | 100% |
| Flow through | 71.07% (\pm 1.96) | 77.95% (\pm 1.26) | 76.15% (\pm 1.27) | 75.53% (\pm 1.44) |
| Wash 1 | 0.35% (\pm 0.24) | 8.64% (\pm 1.65) | 8.60% (\pm 0.30) | 5.76% (\pm 0.21) |
| Wash 2 | 0.08% (\pm 0.06) | 1.64% (\pm 0.22) | 1.46% (\pm 0.13) | 1.20% (\pm 0.28) |
| Eluat | 0.77% (\pm 0.47) | 5.90% (\pm 1.10) | 7.68% (\pm 1.25) | 7.90% (\pm 1.60) |
| Stripped fraction | 27.73% (\pm 4.21) | 6.32% (\pm 0.25) | 6.21% (\pm 0.71) | 6.69% (\pm 0.33) |

The recombinant proteins were purified using the Profinity eXact™ system. The quantity of IL6 in each fraction was determined by ≥ 2 independent ELISAs and is presented as a relative amount compared to the total applied to the column, with the standard deviation in parentheses. PMSF and aprotinin are broad-spectrum protease inhibitors, which were added to the extraction buffer.

claimed by the manufacturers to be the only protease capable of releasing the fusion tag. We found that adding protease inhibitors to the extraction buffer had no impact on the amount of free IL6 in the extracts, which demonstrated that cleavage occurs *in planta* and may involve endogenous proteases. Hehle et al. (2011) also found that a cocktail of protease inhibitors had little effect on the degradation pattern of heterologous proteins

targeted to the secretory pathway, concluding that the proteins are already degraded in the secretory pathway *in planta*.

The manufacturers also advise that a two-residue linker should be placed between the fusion tag and target protein to increase the efficiency of cleavage. Speculating that such a linker might also increase the efficiency of cleavage *in planta*, we compared crude

extracts from plants expressing Subi-IL6ER and Subi-s-IL6ER, but found that the amount of free IL6 was the same in each case. However, the elimination of the linker did increase the amount of fusion protein remaining bound to the column when cleavage was induced, confirming that the linker is required for efficient cleavage *in vitro*.

The *in planta* cleavage might be carried out by one of several endogenous subtilisin-like proteases that are not present in bacteria but are found in plants (Goulet et al., 2012). Partial trimming has been widely reported for antibodies expressed in tobacco leaves (Goulet et al., 2012; Ma et al., 1994; Sharp and Doran, 2001; Villani et al., 2009). Hence, the degradation of recombinant proteins in plants is considered to be a general phenomenon (Benchabane et al., 2008; Doran, 2006). However, without testing other target proteins we cannot exclude the possibility that the specific instability of our IL6 fusion proteins (which leads to the release of the native protein *in planta*) may be unique to this protein/tag combination. It will also be necessary to express the same fusion proteins in *E. coli* to test the manufacturer's claim that there is no cleavage *in vivo* because of the specificity of the column-bound protease. All our expression constructs encoded proteins targeted to the endoplasmic reticulum (ER), which is generally regarded as a protective environment for proteins because there is little proteolytic activity, as borne out by the higher yields achieved when targeting proteins to the ER compared to other compartments (Benchabane et al., 2008; Doran 2006; Vitale and Pedrazzini 2005). However, the degradation of recombinant proteins specifically in the ER/Golgi system has been reported (Pagny et al., 2003; Sharp and Doran 2001) and together with our observations this may reflect the presence of subtilisin-like proteases that are targeted to the secretory pathway (Goulet et al., 2012).

However, degradation could also take place in the cytoplasm, because misfolded proteins are sent there following recognition by the ER-associated protein degradation pathway (ERAD) (Hirsch et al., 2004; Romisch 2005). Some ER-targeted proteins are also known to be transported to lytic vacuoles for degradation (Tamura et al., 2004). We would need to investigate the subcellular localization of our Subi-IL6ER and Subi-s-IL6ER fusion proteins to be sure which of these degradation pathways is involved.

Conclusion

The use of subtilisin as a fusion partner in the Profinity eXact™ protein purification system is an efficient method for the purification of recombinant proteins expressed in bacteria. Using IL6 as a test case, we found that the system is unsuitable in its current form for use with crude plant extracts due to: (i) the apparent presence of an endogenous protease with the same specificity as the on-

column enzyme provided with the commercial system and (ii) lower sensitivity to the inclusion of a dipeptide linker. Therefore, a significant amount of the native protein is released from its fusion protein *in planta* and does not bind to the column. It is likely that principles of the system can be used in plants as long as a protease/tag combination can be identified with no matching endogenous activity. Alternatively, it may be possible to coexpress protease inhibitors with the target proteins to inhibit the protease *in planta* as previously described (Komarnytsky et al., 2006; Rivard et al., 2006).

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