Transgenic Res (2007) 16:239–249
DOI 10.1007/s11248-006-9026-2

ORIGINAL PAPER

Elastin-like polypeptide fusions enhance the accumulation of recombinant proteins in tobacco leaves

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Received: 13 April 2006 / Accepted: 27 June 2006 / Published online: 15 November 2006

Abstract The production of recombinant proteins in plants is an active area of research and many different high-value proteins have now been produced in plants. Tobacco leaves have many advantages for recombinant protein production particularly since they allow field production without seeds, flowers or pollen and therefore provide for contained production. Despite these biosafety advantages recombinant protein accumulation in leaves still needs to be improved. Elastin-like polypeptides are repeats of the amino acids ''VPGXG'' that undergo a temperature dependant phase transition and have utility in the purification of recombinant proteins but can also enhance the accumulation of recombinant proteins they are fused to. We have used a 11.3 kDa elastin-like polypeptide as a fusion partner for three different target proteins, human interleukin-10, murine interleukin-4 and the native major ampullate spidroin protein 2 gene from the spider *Nephila clavipes*. In both transient analyses and stable transformants the concentrations of the fusion proteins were at least an order of magnitude higher for all of the fusion proteins when compared to the target protein alone. Therefore, fusions with a small ELP tag can be used to significantly enhance the accumulation of a range of different recombinant proteins in plant leaves.

Keywords ELP · Fusion protein · Molecular farming · Recombinant protein

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Introduction

Transgenic plants have considerable potential for the production of recombinant proteins, particularly because they can reduce costs and offer virtually unlimited scalability (Giddings et al. 2000). Many different crop platforms have been used to produce recombinant proteins (Twyman 2004), but because of biosafety concerns the use of food crops is meeting with increasing criticism (Fox 2004; Macaulay 2003; Nature Biotechnology 2004; New Scientist 2005). Tobacco leaves are an attractive alternative to the use of food crops since tobacco is a non-food crop, which minimizes regulatory barriers associated with plant recombinant protein production by eliminating the risk of entry into the food chain (Menassa et al. 2001). The leaves are harvested before flowering, significantly reducing the potential for gene leakage into the environment through pollen or seed dispersal. Unlike seeds or tubers, tobacco leaves are perishable and will not persist in the

environment. While tobacco is inherently biosafe the accumulation of some recombinant proteins in tobacco leaves can be low (Rymerson et al. 2002), often several orders of magnitude below the 1% of total soluble protein economic threshold proposed by Kusnadi et al. (1997). Therefore, improvements in recombinant protein accumulation in tobacco leaves are necessary. The reasons why a specific recombinant protein does not accumulate are numerous and many efforts have been made in tobacco and other species to optimize transcription, translation, and intra-cellular targeting (Richter et al. 2000; Sojikul et al. 2003) but none so far offer the magnitude of increase really needed. Chloroplasts are a potential solution and can be engineered to produce very large amounts of recombinant protein (Staub et al. 2000), but issues related to post-translational processing and assembly of complex proteins are significant so the system cannot be used with all possible protein candidates. There are already effective methods of improving recombinant protein accumulation in seeds (De Jaeger et al. 2002; Scheller et al. 2006), but seeds present significant biosafety challenges even in non-food platforms. Given that no complete platform is yet available, methods to improve leaf-based recombinant protein production are worthy of exploration.

Most common fusion proteins in use today were originally conceived as aids to isolation and purification, but some were also shown to enhance recombinant protein accumulation. Fusion tags have been used in various fermentation based production systems in order to enhance protein solubility (maltose-binding protein (MBP) and N-utilizing substance A (NusA)), facilitate detection (c-myc tag) and purification (poly-histidine, glutathione-*S*-transferase (GST)) (Terpe 2003). Early attempts to use a thioredoxin fusion to increase accumulation of recombinant antibodies in tobacco leaves were not successful, but GST-antibody fusions did accumulate to slightly higher levels than controls (Spiegel et al. 1999). Hondred et al. (1999) showed that ubiquitin-GUS C-terminal fusions accumulated in transgenic tobacco leaves at concentrations over four times higher than controls, but many biopharmaceuticals are secreted proteins and would not necessarily lend themselves to a ubiquitin based fusion system. Mainieri et al. (2004) showed that fusions with domains from zein, a seed storage protein from corn, formed protein bodies in the ER and more interestingly accumulated to seven times higher concentrations than the target protein with a KDEL ER localization signal. Recently Obregon et al. (2005) reported that a fusion between the HIV-1 antigen and human p24-IgA increased accumulation 13-fold relative to the antigen alone. While it is clear that certain fusions can improve accumulation, exactly how the carrier increases passenger protein accumulation in plants is not completely understood. In bacteria the carrier protein may act as a stabilizing agent, forcing the fusion protein through the chaperone pathway in the context of the carrier, which promotes proper folding and stability of the target (Douette et al. 2005). Similarly when zeolin, a fusion between two seed storage proteins, is expressed in tobacco leaves, the aromatic/hydrophobic amino acids in the γ-zein carrier interact with the chaperone BiP, which imparts stability and enhances accumulation of the fusion (Mainieri et al. 2004).

Elastin-like polypeptides are synthetic proteins made from pentapeptide repeats of the amino acids 'VPGXG' that occur in all mammalian elastin proteins (Raju and Anwar 1987). In its native state elastin co-occurs with collagen and is the major matrix protein of large arteries, lung tissue, intestines and skin where it imparts extensibility and elastic recoil on the tissue. Aside from interest in elastin as a biomaterial, elastin-like polypeptides (ELP) can be used as thermally responsive tags for the temperature based non-chromatographic separation of recombinant proteins (Meyer and Chilkoti 1999). Recently, Scheller et al. (2006) showed that a 100 mer ELP tag increased the accumulation of a scFv passenger protein by up to 40 times in tobacco seeds. While large ELP tags have utility in the purification of proteins and a positive impact on accumulation they can also make up a very large proportion of the total recombinant protein and as such reduce yield of target protein. We used a small 27 mer (~11.3 kDa) ELP-tag with three different target proteins in an effort to minimize tag size and to determine the range of possible

target proteins that could be used with ELP. The target proteins were: human interleukin-10 (IL10; Menassa et al. 2001), murine interleukin-4 (IL4; Ma et al. 2004) and the native major ampullate spidroin protein 2 (MaSp2; Menassa et al. 2004) gene from the spider *Nephila clavipes*. IL10 and IL4 are potential therapeutic agents and spider silk is a high strength biofibre with many potential industrial and medical applications. Although we have already shown that these target proteins can be produced in plants and that the two cytokines are biologically active, the concentration of all three proteins in tobacco leaves can be rather low. So our interest was to determine whether ELPs solubility and stability could be used to improve plant recombinant protein accumulation, instead of its use as a purification tool. In this study we demonstrate that our ELP tag dramatically improves the accumulation of the three very different target proteins in tobacco leaves. It is also possible that our results could be part of a more generalized system to improve recombinant protein accumulation in leaves.

Materials and methods

Design and construction of the ELP-tag

The complete synthetic ELP sequence was first codon-optimized to remove cryptic splice sites and rare codons. The resulting sequence was then used to design eight overlapping oligomers, which also included 5′ *Bam*HI and 3′ *Eco*RI restriction enzyme sites (Table 1). The Roche Expand Long Template PCR System was used to sequentially assemble the eight oligomers into a single nucleotide sequence. Reactions contained 100 pM of each oligomer, 350 μM dNTP's, and 1 × PCR buffer containing 1.75 mM $MgCl_2$, and sterile water to bring the reaction volume to 50 μl. Oligomers were denatured at 94°C for 1 min. The temperature was then ramped down to 60°C over 35 min, and held for an additional five minutes. Five units of Expand DNA polymerase (Roche) was then added to the reaction. Temperature was ramped up to 68°C over 5 min. Extension was continued at 68°C for an additional 2 h. This reaction with oligomers 1 and 2 resulted in the formation of the central 114 base pair double-stranded template, which was gel purified using standard methods. Oligomers 3 and 4 were added onto this template using PCR and 200–250 ng of template DNA from the previous step. This reaction resulted in a new 237 base pair DNA fragment. The final PCR reaction was conducted adding the other four oligomers to this central template and the reaction was subjected to gel electrophoresis. A 400–500 bp fragment was excised from the gel, purified and then cloned into pBS KS+. The fragment was fully sequenced using an ABI 3100 capillary DNA Sequencer and the 420 bp shown to code for a 27 mer of the VPGVG sequence, which consisted of 17

Table 1 The 5′ to 3′ sequence of oligomers used to synthesize the elastin like polypeptide tag

Oligo	5′ to 3′ Primer sequence
1	GAGTACCTGGCGTGGGTGTACCTGGTGTTGGTGTCCCAGGAGTGGGAGTTCCTGGAGTTG-GAGTCCCTGGAGTCGGAG
2	CTCCGGGTACACCAGGCACGCCTACACCGGGTACACCCACTCCTGGCACTCCGACTCC-AGGGACTCCAACTCCAGG
3	CCAGGAGTCGGAGTCCCCGGAGTAGGAGTTCCAGGGGTGGGAGTTCCAGGAGTAGGAG-TACCTGGCGTGGGTGTACCTGGT
4	AGGGACACCGACTCCGGGCACCCCCACACCTGGAACCCCTACGCCGGGCACTCCAACTCC-GGGTACACCAGGCACGCCTACAC
5	GTACCGGGTGTCGGAGTGCCTGGCGTAGGGGTTCCGGGAGTGGGTGTCCCAGGTGTCGGCGTA-CCAGGAGTCGGAGTCCCC
6	CGGGGACCCCCACACCAGGTACTCCAACACCCGGCACGCCGACTCCTGGAACCCCTACACCA-GGGACACCGACTCCGGG
7	GGCGAATTCATGGTTCCAGGGGTAGGTGTCCCTGGTGTCGGTGTACCGGGTGTCGGAGTGC
8	GCCGGATCCTTAGCCGACACCAGGCACCCCTACTCCGGGGACCCCCACACCAG

pentamer repeats linked by the sequence VPG and flanked by 10 more pentamer repeats. The tag had a predicted molecular weight of 11.3 kDa.

Design and construction of plant expression vectors

In order to assemble the IL10 and IL4 expression constructs the TEV protease site was first added to the 5′ end, and a KDEL and *Eco*RI restriction enzyme site were added to the 3′ end of the synthetic ELP sequence using PCR. The IL4 and IL10 coding sequences were then amplified with a 5′ primer that included 20 bp of the 3′ end of the Pr1 b (Matsuoka et al. 1987) secretory signal and a 3′ primer that included the 21 bp of the TEV protease recognition sequence (Smith and Kohorn 1991). The tCUP translational enhancer (Wu et al. 2001) and Pr1 b signal peptide sequences were amplified with primers that incorporated a *Bam*HI restriction enzyme site at the 5′ end and 20 bp of the 5′ end of either the IL4 or the IL10 coding sequence at the 3′ end. To produce the IL10 and IL4 control constructs with a HIS-tag, fragments were amplified with a 3′ primer that incorporated the HIS tag, KDEL and an *Eco*RI restriction enzyme site. The components of the expression constructs were sequentially assembled using homology overlap extension, with the Expand Long Template PCR System (Roche) (Horton et al. 1989). MaSp2-ELP was constructed by adding the TEV protease cleavage site sequence to the 3′ end of MaSp2 by PCR (Menassa et al. 2004). Homology overlap extension and a 52°C annealing temperature was used to add ELP-KDEL-*Eco*RI to the 3′ end of the MaSp2-TEV fragment. The MaSp2 control construct was used by Menassa et al. 2004 and had no HIS-tag. Each fragment was then digested with *Bam*HI and *Eco*RI and cloned into pBS KS+ for sequencing and the pCaMterX binary vector for plant transformation. The genes were under the control of CaMV35S promoter with duplicated enhancers (35S × 2; Kay et al. 1987) and nopaline synthase (*nos*) terminator. The expression constructs were transformed by electroporation into the *Agrobacterium tumefaciens* strain EHA105.

ELP expression in Escherichia coli

A protein expression construct was made for bacterial expression of ELP for antibody production. ELP was amplified with primers that incorporated a 5′ *Nco*I site and the amplification product digested with *Nco*I and *Eco*RI and then cloned into pET30 b, in frame with the start site and a N-terminal HIS tag. It was then transformed by electroporation into *E. coli* strain BL21 (DE3). Expression of recombinant protein was conducted according to manufacturers instructions (pET System Manual, Novagen) and extracts subjected to immobilized metal affinity chromatography using the chelating Sepharose Fast Flow system (Amersham Biosciences) to separate the HIS-ELP from the remaining bacterial proteins in the extract. Purification was conducted using a gravity flow column as per supplier instructions. The HIS-ELP protein bound to the Ni^{2+}-sepharose was eluted using 250 mM imidazole and then used for the generation of polyclonal antibodies. All animal experiments were conducted by the Canadian Food Inspection Agency in accordance with approved animal care protocols.

Agrobacterium-mediated transient expression assays

Transient assays were performed using tobacco plants that were 10–16 weeks old and the various *Agrobacterium* lines containing each of the expression constructs. The *Agrobacteria* were prepared for leaf infiltration as described in Kapila et al. (1997). Syringe injections were conducted using a method similar to that described in Yang et al. (2000). Briefly, Infiltrations were conducted using a 1 ml plastic syringe to inject the *Agrobacterium* suspension into the abaxial surface of the leaf just under the epidermis. Three or four leaf panels each from a different leaf from the same plant were injected with each construct. Following infiltration the tobacco plants were maintained in a controlled environment chamber at 20°C, with 16 h daylength for 4 days and the individual infiltrated panels were collected and analysed separately. The average of the 3 or 4 leaf panels were then used to represent the concentration of a given recombinant protein.

Stable transformation of tobacco with the six expression constructs

The six plant expression constructs (pIL4-HIS, pIL4-ELP, pIL10-HIS, pIL10-ELP, MaSp2 and MaSp2-ELP) were introduced into the low alkaloid tobacco (*Nicotiana tabacum*) cultivar ''81V9'' (Menassa et al. 2001) using *Agrobacterium*-mediated transformation of leaf discs. Primary transformants were grown in a greenhouse and the leaves harvested when the plants had 10–15 leaves. Three representative leaves (young, medium and mature leaves) were collected from each plant and stored at –75°C for later analyses. Subsamples from the individual leaves were taken and the average of the three leaves used to represent the concentration of recombinant protein in the whole plant.

Plant protein extraction

Leaf tissue was weighed and homogenized in 4 volumes of extraction buffer (phosphate buffered saline, pH7.4, 0.05% Tween 20 (v/v), 2% (w/v) polyvinylpolypyrrolidone, 100 mM ascorbic acid, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin). The extract was clarified by centrifugation at 13,000 × *g* for 15 min at 4°C. Total soluble protein concentrations were determined by dye binding.

Enzyme linked immunosorbent assay (ELISA) for quantification of IL4 and IL10

The levels of IL4 and IL10 proteins present in protein extracts and purified protein fractions were quantified by comparing several protein sample dilutions to known standard concentrations of recombinant IL10 and IL4 using an ELISA. The ELISA assay specifically detects IL4 and IL10 using purified monoclonal rat anti-IL4 or IL10 capture antibodies, purified monoclonal rat biotinylated anti-IL4 or IL10 detection antibodies and streptavidin-HRP conjugate (BD PharMingen). ELISAs were performed according to the cytokine ELISA protocol provided with the antibodies by the manufacturer (BD PharMingen).

Immunoblot analysis/Western blot analysis

Proteins from one-dimensional PAGE gels were transferred to a Sequi-Blot^{TM} PVDF (Bio-Rad) or nitrocellulose membrane in a semi-dry electroblotting apparatus (Bio-Rad) using transfer buffer (48 mM Tris/39 mM glycine) containing 20% methanol (v/v). Membranes were stained after protein transfer with 0.02% Ponceau-S (w/v) in 1% acetic acid solution (v/v) to visualize transferred proteins and standards. Membranes were blocked with 10% (w/v) non-fat milk powder in Tris Buffered Saline pH 7.5 (TBS) overnight. Membranes were then probed with appropriate primary antibodies diluted in 3% BSA (w/v); biotinylated polyclonal goat anti-IL4 (1:500, R&D Systems), biotinylated polyclonal goat anti-IL10 (1:1000, R&D Systems), mouse anti-HIS (1:5000, Sigma), rabbit anti-MaSp2 (1:1000, Nexia) or rabbit antiserum containing anti-ELP (1:5000, see antibody production section), for 1 h at room temp. After membranes were washed twice with TBS, secondary antibodies were applied diluted in 5% (w/v) non-fat milk powder/TBS; biotinylated primary antibodies were incubated with either Streptavidin-HRP (1:3000, Genezyme) or rat anti-goat IgG-conjugated with HRP (1:5000, Sigma), primary anti-HIS antibody was incubated with goat anti-mouse IgG-conjugated with HRP (1:10,000, Sigma), and the MaSp2 and ELP antibodies were followed by incubation with goat anti-rabbit IgG-conjugated with HRP (1:4000, Sigma). Blots were washed in TBS four times and detected using ECL detection reagents (Amersham Biosciences) as per the suppliers protocol.

Results and discussion

Construction of the ELP tag and production of anti-ELP antibodies

The temperature at which a given ELP protein will desolvate is directly related to its size, such that large proteins have lower desolvation temperatures. Since most studies that have been conducted with ELP were focused on protein

purification and they used large ELP tags, in the range of 30–65 kDa, so that the transition temperatures would be between 30 and 39°C (Stiborva et al. 2003; Kostal et al. 2003; Shimazu et al. 2003). However, Meyer and Chilkoti (1999) reported that yields of recombinant protein in *Escherichia coli* were inversely proportional to the size of the ELP tag. This may help to explain the results of Guda et al. (2000) who expressed an synthetic ELP gene with 121 repeats (51.7 kDa) in tobacco chloroplasts and found that ELP protein did not accumulate to very high levels. They speculated that the low levels of ELP accumulation were likely a consequence of the unusually large number of the amino acids glycine and proline required to synthesize such a repetitive protein leading either to tRNA starvation or a shortage of specific amino acids. Since low accumulation was already an issue with our three target proteins and the intent of the fusion was the enhancement of protein accumulation we chose a small tag so as not to create a disadvantage right at the outset. The tag was engineered using oligonucleotides and a synthetic DNA sequence that minimized the use of rare codons and that was kept variable to avoid sequence repetition. The result was a 420 bp sequence, with start and stop codons, that coded for a 27 mer 11.3 kDa ELP peptide.

Expression of the ELP peptide with a HIS-tag in *E. coli*, followed by SDS-PAGE and Western analysis of protein extracts using a HIS tag specific antibody, showed a band similar in size to what we expected (Fig. 1) so it was reasonable to conclude that the complete peptide was being synthesized. Recombinant ELP was purified from a total protein extract from induced bacterial cells using Immobilized Metal Affinity Chromatography and, following elution with imidazole two fractions were used for antibody production in rabbits. The resulting polyclonal serum was highly specific to ELP.

We then constructed plant expression vectors for each of the proteins we were interested in: IL10, IL4 and MaSp2. In the IL4 and IL10 vectors the target gene was fused to HIS, in another version the IL4, IL10 genes were fused to ELP. In

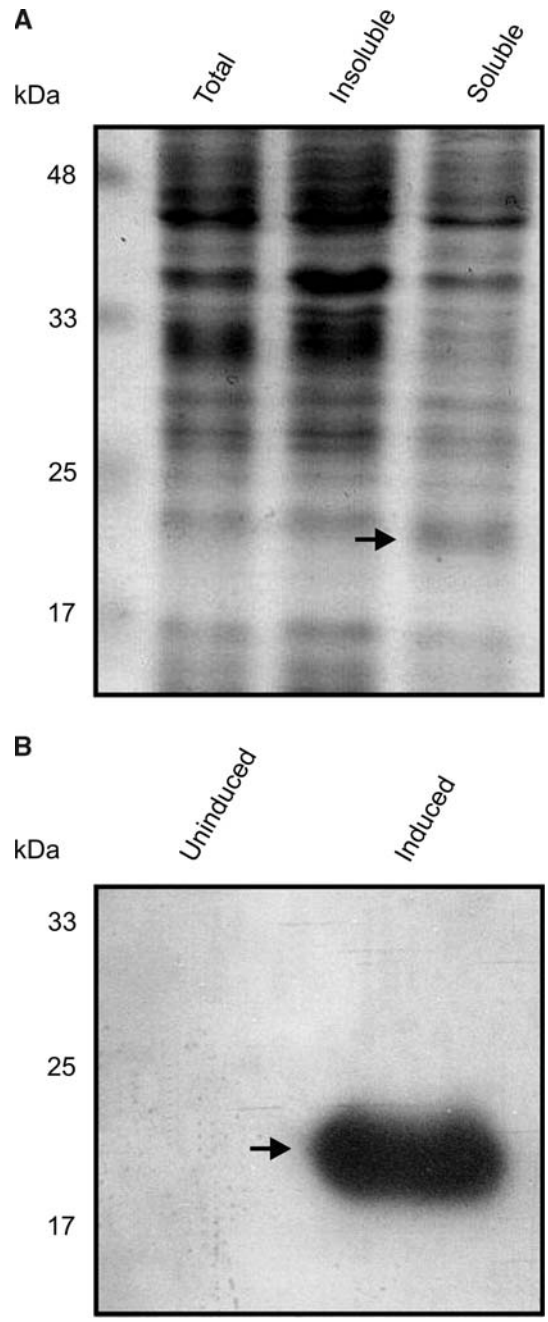

Fig. 1 Expression of HIS-ELP in *E. coli*. (**A**) SDS-PAGE analysis and Coomassie staining of an uninduced total protein extract (lane 1), induced total insoluble protein (lane 2) and induced total soluble protein (lane 3). (**B**) Western analysis using an anti-hexahistidine antibody of total protein extracts from *E. coli* before (lane 1) and after (lane 2) induction with IPTG. Arrows indicate the position of the recombinant ELP

both the HIS and ELP vectors the target protein was separated from the tag by TEV protease site to allow the ELP and HIS tags to be separated from the target protein upon purification. A earlier version of a MaSp2 vector that had no HIS tag was used for the MaSp2-ELP comparison. All six vectors were used in both transient expression analysis and to create stable transgenic plants (Fig. 2).

Transient plant expression of the fusion constructs

The temporal decay in transgene expression in transient assays has led to the general conclusion that expression is independent of the position effects normally observed with stable integration of transgenes into plant genomes (Kapila et al. 1997; Janssen and Gardner 1990). Transient analysis is therefore a good indicator of the effect that an ELP tag has on recombinant protein accumulation, without being confounded by variability in gene expression. ELISA analysis of leaf tissue from transient assays showed that the IL4-ELP combination accumulated 19-times more recombinant protein than IL4-HIS (Fig. 3A). ELISA analysis also showed that IL10-ELP protein concentrations were approximately 15 times higher than IL10-HIS (Fig. 3B). The concentration of MaSp2 spider silk protein was estimated by Western blot using dilutions of known concentrations of MaSp2 protein and the concentration of MaSp2-ELP was found to be approximately 100-fold higher than MaSp2 alone (Fig. 4). Since the ELP fusion proteins were quantified relative to unfused standard proteins, the amount of total fusion protein is actually under estimated. For IL4-ELP, IL10-ELP and MaSp2-ELP fusions, the ELP tag makes up 37, 33 and 17% of each fusion protein. Transient assays clearly supported our hypothesis that an ELP fusion tag can be used with a range of protein targets to substantially increase their accumulation.

Stable transgenic plants expressing the fusion proteins

Although production systems for recombinant protein production in plants that rely on transient expression have been developed, the majority of the efforts to date have focused on systems that rely on stable transformation. In addition, the results of transient experiments while instructive do not always correlate with those involving stable transformations (Maximova et al. 1998). In that context it is important to examine the impact of ELP carrier proteins on recombinant protein accumulation in stable transgenic plants as well. To further examine the effect of the ELP fusion on protein accumulation in leaf tissue from plants transformed with the IL4 and IL10 constructs, an ELISA analysis was used. From a population of 13 IL4-HIS and 17 IL4-ELP primary transgenic plants, the five from each group with the highest IL4 protein levels were compared. As expected the individual primary transformants varied in terms of recombinant protein concentration and so the mean of the top five transformants used to demonstrate the difference between the HIS and ELP fusions. IL4 protein levels in the five best IL4-ELP plants were 85-fold higher than the concentration of IL4 in the top five IL4-HIS transgenic plants (Fig. 5A). Similarly, from a population of six IL10-HIS and 22 IL10-ELP transgenic plants, the best five IL10-ELP plants had IL10 concentrations that were 90-fold higher than the IL10-HIS plants (Fig. 5B). MaSp2 protein concentration from stable MaSp2-ELP transgenic plants was estimated to be 60 times higher than MaSp2 alone (Fig. 5C). A hexahistidine tag and a large 90 mer (36 kDa) ELP tag, each with three different carrier proteins were expressed in *E. coli* and compared by Trabbic-Carlson et al. (2004) who found the two tags gave similar recombinant protein yields. Scheller et al. (2004) expressed a 100 mer ELP-spider silk fusion in tobacco leaves, although they did not compare yields to controls with no ELP, the accumulation of spider silk was within the same range or perhaps slightly higher (0.5–4%) than had been reported previously (1–2%) for synthetic spider silk genes not fused to ELP (Scheller et al. 2001). Conversely, our small ELP tag appears to have a highly significant impact on protein accumulation in tobacco leaves, consistent with earlier observations in bacteria where yields of ELP-thioredoxin fusions were about 60% higher for a 30 mer tag when compared to a 180 mer

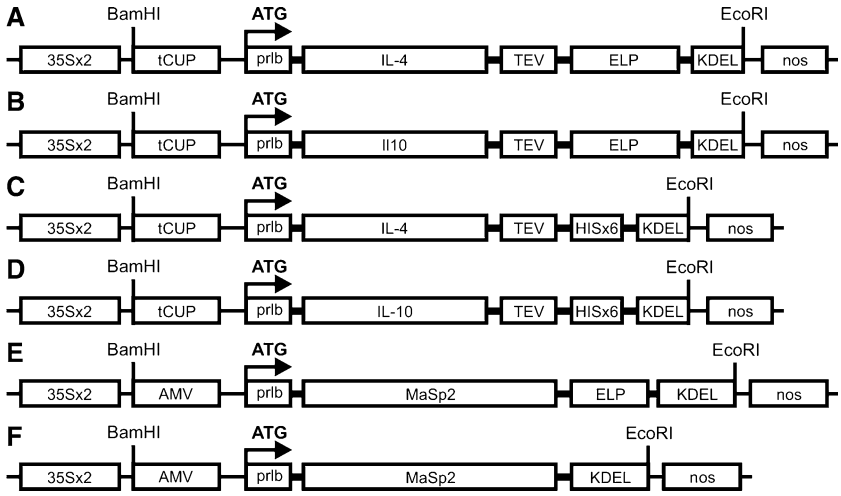

Fig. 2 Plant expression constructs for murine interleukin-4 (IL4), human interleukin-10 (IL10) or major ampullate spidroin protein (MaSp2) with or without a 11.3 kDA elastin like polypeptide tag

(Meyer and Chilkoti 1999). In addition to its impact on accumulation, smaller tags make up a lesser portion of the fusion protein mass and as such can have a positive impact on yield simply because the target protein makes up a larger proportion of the polypeptide (Trabbic-Carlson et al. 2004).

ELP is relatively rich in hydrophobic amino acids and it is possible that BiP, the major ER chaperone, is interacting with our ELP tag and preventing non-specific aggregation of the newly synthesized fusion proteins in a manner similar to that observed with zeolin (Mainieri et al. 2004). The second component in our system was ER localization and we used a KDEL ER localization signal to keep the target protein in the ER. Since we already knew from our previous work with IL10 that a KDEL increases IL10 accumulation more that 60 times (Menassa et al. 2001) and now that ELP increases it another 90 times, it may be that the KDEL and ELP act synergistically to enhance accumulation. Further experiments with ELP fused to target proteins without a KDEL will

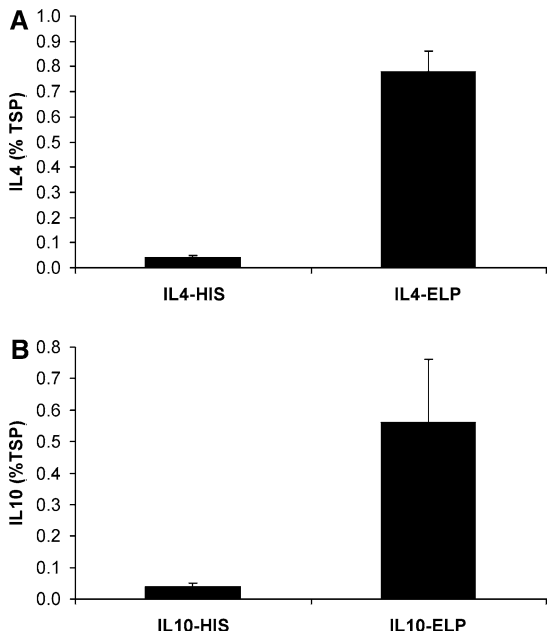

Fig. 3 Accumulation of murine interleukin-4 (**A**, IL4), human interleukin-10 (**B**, IL10) with either a hexahistidine tag (IL4-HIS, IL10-HIS) or a 11.3 kDA elastin like polypeptide tag (IL4-ELP, IL10-ELP) following transient expression by agroinfiltration

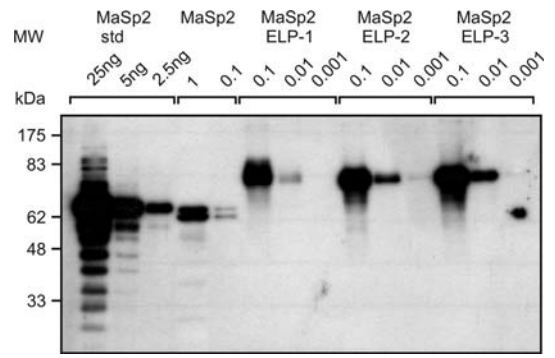

Fig. 4 Western analysis showing accumulation of the major ampullate spidroin protein with a 11.3 kDa elastin like polypeptide tag (MaSp2-ELP) or without a tag (MaSp2) following transient expression by agroinfiltration. Samples were standardized to 20 μg of total soluble protein and numbers above each lane indicate dilution factors used for each replicate

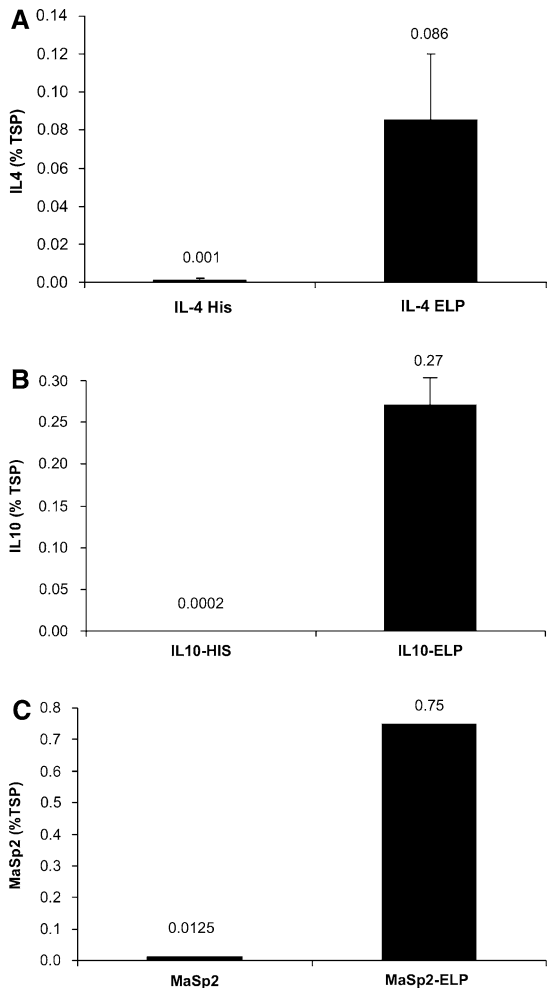

Fig. 5 Accumulation of murine interleukin-4 (**A**, IL4), human interleukin-10 (**B**, IL10) or major ampullate spidroin protein (**C**, MaSp2) with or without the 11.3 kDA elastin like polypeptide tag in stable transgenic plants. For IL10 and IL4 the five transgenic plants with the highest levels of recombinant protein accumulation were used and for MaSp2 it was the top two plants

be needed to confirm that possibility. However, both our transient and stable expression results suggest that the small ELP tag confers increased stability to a wide range of proteins and may have general application in the improvement of recombinant protein accumulation in tobacco leaves. Our results confirm the results of Scheller et al. (2006) and demonstrate that ELP tags can significantly enhance recombinant cytokine and spider silk protein accumulation in leaves.

Protein fidelity

Plants are higher eukaryotes and have been shown to have a wide range of post-translational capabilities including biosynthesis, folding, and assembly of multimeric proteins via disulfide bridges (Ma et al. 1995; Merle et al. 2002). IL4-HIS and IL-10-HIS were both purified using IMAC and their size approximated using Western analysis and IL4 and IL10 specific antibodies. A single band of predicted size was observed with IL10-HIS (Fig. 7A) but two bands were seen with IL4-HIS (Fig. 6B). IL4 and IL10-specific antibodies were used to purify both IL4-ELP and IL10-ELP proteins (Figs. 6A, 7B). In this analysis the IL4-ELP protein also separated into two distinct bands of approximately 27 and 30 kDa in size, and IL10-ELP was a single 31.5 kDa band. Given that recombinant human and equine IL4, like murine IL4 also have two glycosylation sites and were reported to be heterogenous, then the size heterogeneity we observed may be the result of differential glycosylation of murine IL4 (Le et al. 1988; Magnuson et al. 1998; Dohmann et al. 2000). However, further experiments will be required to confirm that the plant IL4 is glycosylated. When Western analysis was conducted with the same proteins and the ELP specific antibody the same results were obtained demonstrating that the ELP tag was intact (data not shown).

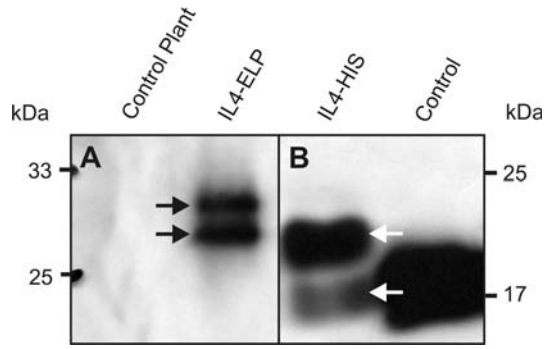

Fig. 6 Accumulation of murine interleukin-4 (A, IL4), human interleukin-10 (B, IL10) or major ampullate spidroin protein (C, MaSp2) with or without the 11.3 kDA elastin like polypeptide tag in stable transgenic plants. For IL10 and IL4 the five transgenic plants with the highest levels of recombinant protein accumulation were used and for MaSp2 it was the top two plants

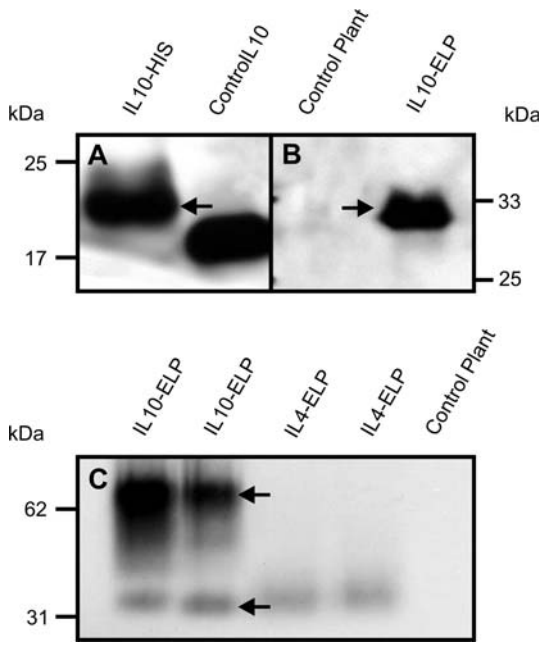

Fig. 7 Western analysis of (**A**) IMAC purified crude protein from IL10-HIS agroinfiltrations IL10-HIS (lane 1) and recombinant IL10 control protein (lane 2). (**B**) Immunoprecipitate from untransformed 81V9 control plants (lane 1) and transgenic IL10-ELP plants (lane 2). (**C**) Native gels with crude protein extracts from IL10-ELP agroinfiltrations (lanes 1, 2), IL4-ELP (lanes 3,4) or 81V9 control (lane 5) analyzed using an ELP specific antibody and showing monomeric and dimeric IL10-ELP and monomeric IL4-ELP. The arrow heads mark the position of the IL10 monomeric and dimeric proteins

Interleukin-10 is biologically active only as a non-covalently associated homodimer. When IL10-ELP protein extracts were separated by native PAGE and analyzed by immunoblot with ELP-specific antibodies, two protein bands were observed, corresponding to dimeric and monomeric IL10-ELP, respectively (Fig. 7C). Therefore, the ELP tag is not restricting IL10 dimer formation.

In summary it is clear that the 11.3 kDa ELP tag, in conjunction with an ER retention signal, was instrumental in increasing the accumulation of three quite different target proteins in tobacco leaves. The application of these results will help to improve the utility of tobacco leaves for the production of recombinant proteins. However, the tag still needs to be optimized. For example, it remains to be determined just how small an ELP tag can be used and still enhance protein accumulation. In addition more experiments are needed to demonstrate if there is an interaction between ELP and BiP and to quantify the synergy between ELP and the KDEL.

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