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Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application

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Elastin-like polypeptides (ELPs) are highly biocompatible and exhibit a potentially highly useful property: that of a thermally responsive reversible phase transition. These characteristics make ELPs attractive for drug delivery, appealing as materials for tissue repair or engineering, and improve the efficiency with which recombinant proteins can be purified. ELP fusion proteins (referred to as ELPylation) inherit the reversible phase transition property. ELPylation technology recently has been extended to plant cells, and a number of plant-based expression systems have been evaluated for the production of ELPylated proteins. Here, we discuss recent developments in ELP technology and the substantial potential of ELPs for the deployment of transgenic plants as bioreactors to synthesize both biopharmaceuticals and industrial proteins.

From elastins to elastin-like polypeptides (ELPs) and ELPylation

Elastin is a structural extracellular matrix protein that is present in all vertebrate connective tissue. Its functions include the provision of elasticity and resilience to tissues, such as large elastic blood vessels (aorta), elastic ligaments, lung and skin, which are subjected to repetitive and reversible deformation [1,2]. Its ability to withstand severe deformation without rupture makes it a particularly interesting target for tissue repair and cell culture applications. The soluble precursor of elastin, tropoelastin, which is deposited into the extracellular space, is highly cross-linked through the action of lysyl oxidase. This is actually the property that confers the prominent physical features of the natural elastin protein [3]. A high level of homology exists between the genes that encode human, chicken, rat and cattle tropoelastin [4], the sequence of which is characterized by two major domains: the first is hydrophilic and contains many cross-linked Lys and Ala residues, whereas the second is hydrophobic and rich in nonpolar residues, in particular Val, Pro, Ala and Gly, characteristically present as tetra-, penta- and hexa-repeats, such as Val-Pro-Gly-Gly, Val-Pro-Gly-Val-Gly and Ala-Pro-Gly-Val-Gly-Val. These repeats are responsible principally for the elasticity of the protein [5]. The physical properties of these repetitive motifs were initially

studied using synthetic gene products [6–8]. Derivatives of such molecules, for example, poly(Val-Pro-Gly-Xaa-Gly) (where Xaa represents one random amino acid except Pro) have been designated as ELPs, and the process of functionalizing target proteins by their genetic C- or N-terminal fusion to an ELP is now termed ELPylation.

The temperature-dependent, reversible self-aggregation (termed inverse phase transition) of ELPs occurs within a 2–3 °C range and can be monitored spectrophotometrically by measuring the turbidity of the protein solution. Below the transition temperature (Tt), ELPs are monomeric and soluble, whereas, at temperatures higher than Tt, they aggregate and become insoluble [7–9] (Figure 1a). One possible explanation for this process is the development of closer associations between single β-spiral strands of ELP and the subsequent formation of interstrand β-sheet structures that mediate close association or aggregation of different ELP chains [10] (Figure 1a). Aggregation and insolubility is reversed completely when the temperature decreases below Tt, which itself is determined by a combination of buffer composition, salt concentration, ELP concentration, chain length, the degree of ionization of any functional side chains and the guest residue Xaa, and the polarity arrangement along the molecule [11–14]. Thus, for example, Tt is decreased as the ELP chain length or the salt concentration is raised, or ELP concentration and guest residue composition is reduced [13,15].

The following sections summarize recent progress to use ELPylation as a tool to purify efficiently recombinant proteins. Properties of ELPs in terms of biocompatibility and their applications in medicine and nanobiotechnology will be reviewed thereafter. Finally, we conclude that ELPylation now emerges as a general principle to increase massively the yield of recombinant proteins in plant-based expression systems.

ELPylation as a tool for recombinant protein purification

The current state-of-the-art procedure for the purification of recombinant proteins uses affinity chromatography, a technology that relies on reversible immobilization of the target protein. This is usually achieved by fusing the target protein to either a carrier protein, such as maltose-binding protein, Fc region of immunoglobulin, glutathione S-transferase or thioredoxin, or to short peptides such as oligohistidine or the FLAG-peptide. The temperature-dependent, reversible
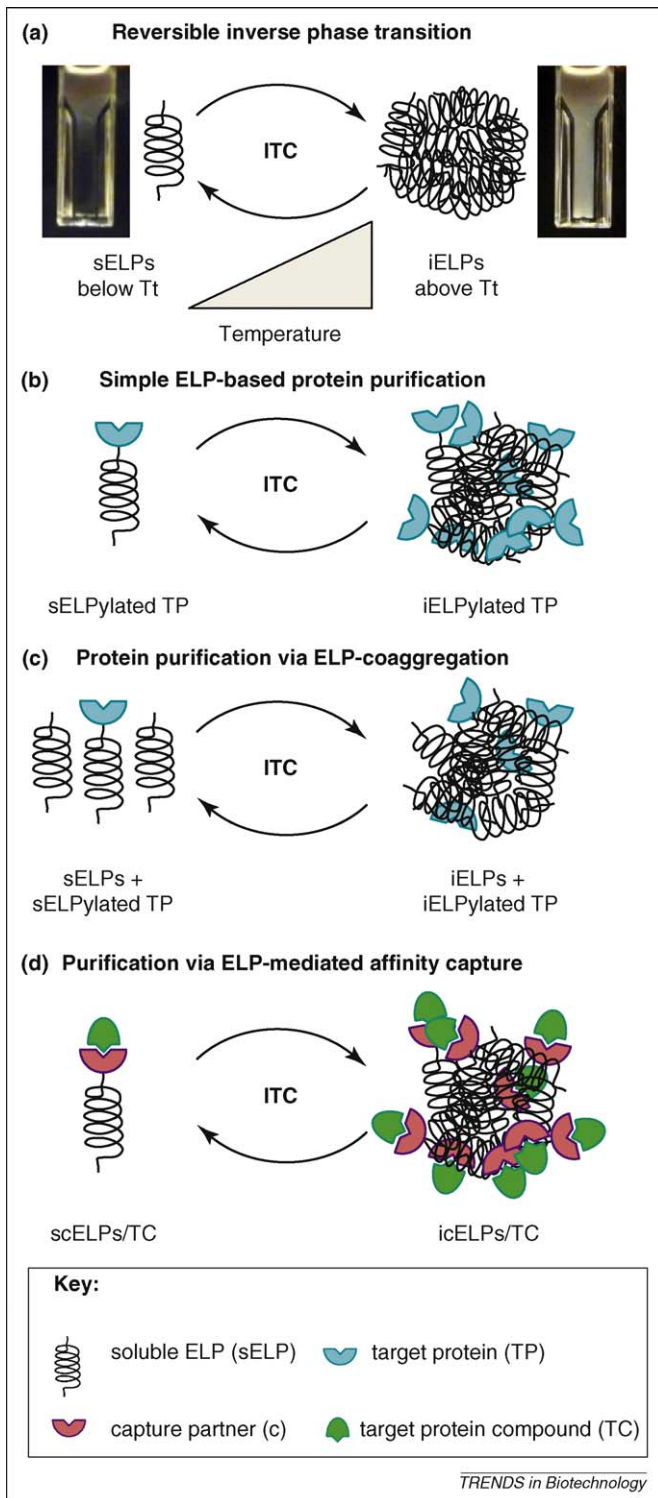
Figure 1. Purification of ELPs by ITC. **(a)** Reversible inverse phase transition. sELPs remain in solution at a temperature below their Tt, but become increasingly insoluble above Tt. The insoluble ELP aggregates (iELPs) can be precipitated by centrifugation, thereby allowing their separation from other proteins present. The iELP-containing precipitate can then be re-solubilized in buffer at a temperature below Tt. This procedure can be used to purify recombinant ELP fusion proteins. The increased turbidity of an ELPylated TP is monitored spectrophotometrically as illustrated by the cuvettes shown: on the left, the cuvette contains soluble ELP at room temperature and there is no turbidity of the protein solutions; on the right: protein solution turbidity is induced as the cuvette now contains aggregated ELP at 40 °C. **(b)** Simple ELP-based protein purification. A soluble ELPylated target protein (sELPylated TP) becomes reversibly insoluble at temperatures higher than Tt (insoluble ELPylated TP; iELPylated TP). **(c)** Protein purification via ELP co-aggregation. An excess of free sELPs enhances the co-aggregation of trace quantities of sELPylated TP at temperatures higher than Tt. **(d)** Purification via

aggregation/precipitation behavior of ELPs provides a novel means to avoid this cost-intensive affinity chromatography (Figure 1a). Because it side-steps the need for affinity chromatography, the method is simplified, less expensive and readily upgradable to a preparative mode. The first application of this strategy was used to purify heterologously expressed ELPs from *Escherichia coli* cells [16]. Following their ELPylation, the thermally stimulated purification of recombinant ELP fusion proteins by inverse transition cycling (ITC) was achieved [17] (Figure 1b). Here, aggregation of ELPylated target proteins (TP) was achieved by raising the temperature above Tt and ELPylated TPs were precipitated by subsequent centrifugation. The resulting supernatant, which contained the host proteins, was removed and the remaining recombinant ELP–TP-containing pellet was resuspended in low-salt buffer at a temperature below Tt. This strategy leads to an almost complete removal of host proteins and the complete recovery and purification of the ELP–TPs. In this example, the synthetic ELP component of the fusion protein was an oligomerized series of Val-Pro-Gly-Xaa-Gly pentapeptides, where Xaa possessed a 5:2:3 ratio of Val:Ala:Gly, which was engineered to produce a specific Tt. These ELPylated fusion proteins were expressed in *E. coli*. Following fusion, it became apparent that the identity of the fusion partner also influenced Tt, because it was found that the hydrophobic tendamistat protein lowered Tt, whereas the hydrophilic thioredoxin protein slightly raised it. However, ELPylation did not affect the biological activity of either of these proteins [16]. Recently, it has been shown that positioning the ELP component at the C-terminus, but not at the N-terminus of the TP, can enhance expression levels, which leads to an improved yield of purified protein [18].

Cleavage of the ELP part from the TP can be achieved by treatment with specific proteases, such as factor X or thrombin. However, the addition of such proteases could raise cost issues for large scale protein production or might interfere with the structure of the target protein, such as minimal nonspecific cleavage of the protease within the ELP fusion partner. An alternative strategy exploits intein technology, designed to induce self-cleavage, which has been combined with ELP technology to purify a number of native recombinant TPs from *E. coli* [19]. This method requires the introduction of an intein coding sequence between the sequence that encodes the TP and the ELP component, which results in TP–intein–ELP. First of all, the triple fusion (TP–intein–ELP) is purified by ITC, and subsequently intein self-cleavage is induced by pH/temperature-shift or dithiothreitol treatment to release the TP from the intein–ELP fragment. The temperature-based precipitation step specifically affects the ELP component, therefore, the intein–ELP fragment can be removed efficiently by a second round of ITC [19,20]. In a scaled-up form of this procedure, the separation of the aggregated ELP fusion proteins can be achieved by continuous centrifugation or tangential-flow microfiltration

EMAC. EMAC allows target purification without ELPylation. As shown here, ELPs are fused to a capture protein (cELP), which binds specifically and reversibly to the TP. Soluble cELPs bind to the TP compound (scELPs/TC), which can then be aggregated differentially at temperatures higher than Tt to form insoluble cELPs–TC complexes (icELPs–TC).

[21]. Unfortunately, high concentrations of sodium chloride, commonly used to lower Tt, are corrosive in the context of industrial synthesis. However, ammonium sulfate might represent a suitable substitute for sodium chloride because the concentration of this salt that is sufficient to achieve an equivalent efficiency of ELP precipitation is fourfold lower than for sodium chloride. The use of salt, rather than heat, as inducer of precipitation is advantageous for cost reasons, and furthermore, allows for a gentler purification procedure because lower transition temperatures can be applied [22]. Even though ammonium sulfate triggers the phase transition at a lower molar concentration, it should be noted that ammonium sulfate precipitates more contaminant proteins than sodium chloride does. Therefore, quite often more rounds of ITC are necessary to obtain pure ELP fusion proteins when ammonium sulfate is used in the precipitation procedure. A very low concentration of ELPylated molecules has been purified by ITC following the addition of an excess of free ELP, as the co-aggregation between the ELP and the ELPylated target is highly specific [23,24] (Figure 1c). As a further development, ELP-mediated affinity capture (EMAC) procedures, which avoid the need of ELPylation, have since been developed (Figure 1d). Here, ELP is genetically or chemically modified so that it can bind specifically and reversibly to the TP [25–33]. This procedure also eliminates the need for post-purification enzymatic or chemical cleavage of the ELP, and has the potential to allow a re-use of the ELP-capture molecule.

The advent of ELPs in biotechnology has not merely added an additional for the purification of recombinant proteins, but has revealed a conceptually new principle for protein purification, which makes conventional affinity-chromatography-based protein purification methods almost dispensable. An outstanding issue is whether ELPs might not only be helpful in biotechnology, but could also aid drug application in medicine or give rise to new purposes in nanobiotechnology as outlined below.

The exploitation of ELPs in medicine and nanobiotechnology

To assess potential biological responses of ELPs, poly(Gly-Val-Gly-Val-Pro) has been applied to a number of tissue, tissue-fluid and blood tests, as approved by the relevant United States regulatory body [34]. Both *in vitro* and *in vivo* studies of poly(Val-Pro-Ala-Val-Gly) have failed to reveal any evidence of cytotoxicity, nonspecific depression of cellular respiration in macrophages, or general inflammatory response [35]. The high level of biocompatibility might reflect the chemical similarity of the ELPs to native elastin, which makes them immunologically invisible and therefore attractive for biomedical applications [36–38]. One of these applications is the use of ELPs as a scaffold for tissue growth, which is necessary for tissue repair and *de novo* tissue engineering.

ELPs that have been genetically engineered to resemble the ocular surface extracellular matrix have proven useful as a substratum for the *in vitro* culture of ocular surface cells [39]. ELP-containing polymers have been used to reversibly adhere mammalian cells to culture dishes via ELP–ELP aggregation. Here, one ELP protein was hydrophobically bound to the culture dish, whereas the second ELP was modified with an RGD-motif to facilitate, on the one hand, the binding to the immobilized ELP protein and, on the other hand, to the respective mammalian cell. Recovery of the cell sheet embedded into the extracellular matrix was achieved by lowering the temperature to 20 °C. This application is particularly interesting for tissue engineering [40]. Another application is the use of an ELP hydrogel as a matrix to grow human adipose-derived adult stem cells, which show signs of chondrogenesis without addition of differentiation supplements [41].

In vivo, ELPs have the potential to act as homing devices for pharmaceuticals and as carriers for thermally inducible therapeutics [42]. The effectiveness of a pharmaceutical treatment, as well as the severity of any side effects, is largely dependent on the precision with which the drug can be targeted to the site that requires treatment. This is a particularly significant issue in chemotherapy, where the cytotoxic agents used inevitably cause damage to non-target tissue. Using the ELPylation strategy, the active compound can be directed to precipitate specifically within the target tissue by the imposition of localized hyperthermia [43,44]. Thus, micro-sized ELP aggregates could be made to adhere to tumour vasculature by ensuring that the tumor, but not any of the surrounding tissues, is heated locally. When the temperature is restored to normal levels, the aggregated ELP particles re-solubilize, which increases the local plasma levels of the drug, thereby driving a further flow of ELP particles across the tumor-associated vasculature [45]. The assembly of ELPs into nano- and microparticles to form a phase-separated state above their Tt [46] is also of relevance for drug delivery [47]. Self-assembled poly(Val-Pro-Ala-Val-Gly) particles have been exploited as a means to obtain the controlled release of the drug dexamethasone phosphate (DMP). Once formed, these structures remain stable at room and at body temperature, and can be used to encapsulate DMP, and allow a sustained release of the drug over ~30 days [48]. To generate nano-scale bioresponsive peptide-based particles of defined morphology, which can encapsulate drugs, electrospraying has been used, such as in the preparation of ELP particles that contain the chemotherapy agent doxorubicin [49]. The ELPylation of soluble tumor necrosis factor receptors (sTNFRs) has been used as a strategy for the delivery of anti-TNF drugs. The ELPylated sTNFRII has significantly reduced *in vitro* bioactivity [50]. However, TNF-mediated effects in cultured astrocytes and microglia or dorsal root ganglion explants have been attenuated by ELPylated sTNFRII, consistent with the anti-TNF activity of unfused sTNFRII [51]. As aggregated ELPs have demonstrated a sevenfold longer perineural half-life than soluble ELPs, the thermally stimulated aggregation of ELPylated therapeutics has been suggested as a means to create a perineural drug depot for long-term delivery to an inflamed nerve [52].

Further medical applications of ELPylation include the engineering of a cell-penetrating peptide fused with a cell-cycle-inhibitory peptide derived from the p21 protein [53]. The engineered ELPylated protein aggregates at temperatures >39 °C, and in this form, inhibits cell proliferation. It might therefore be used as a thermally responsive carrier
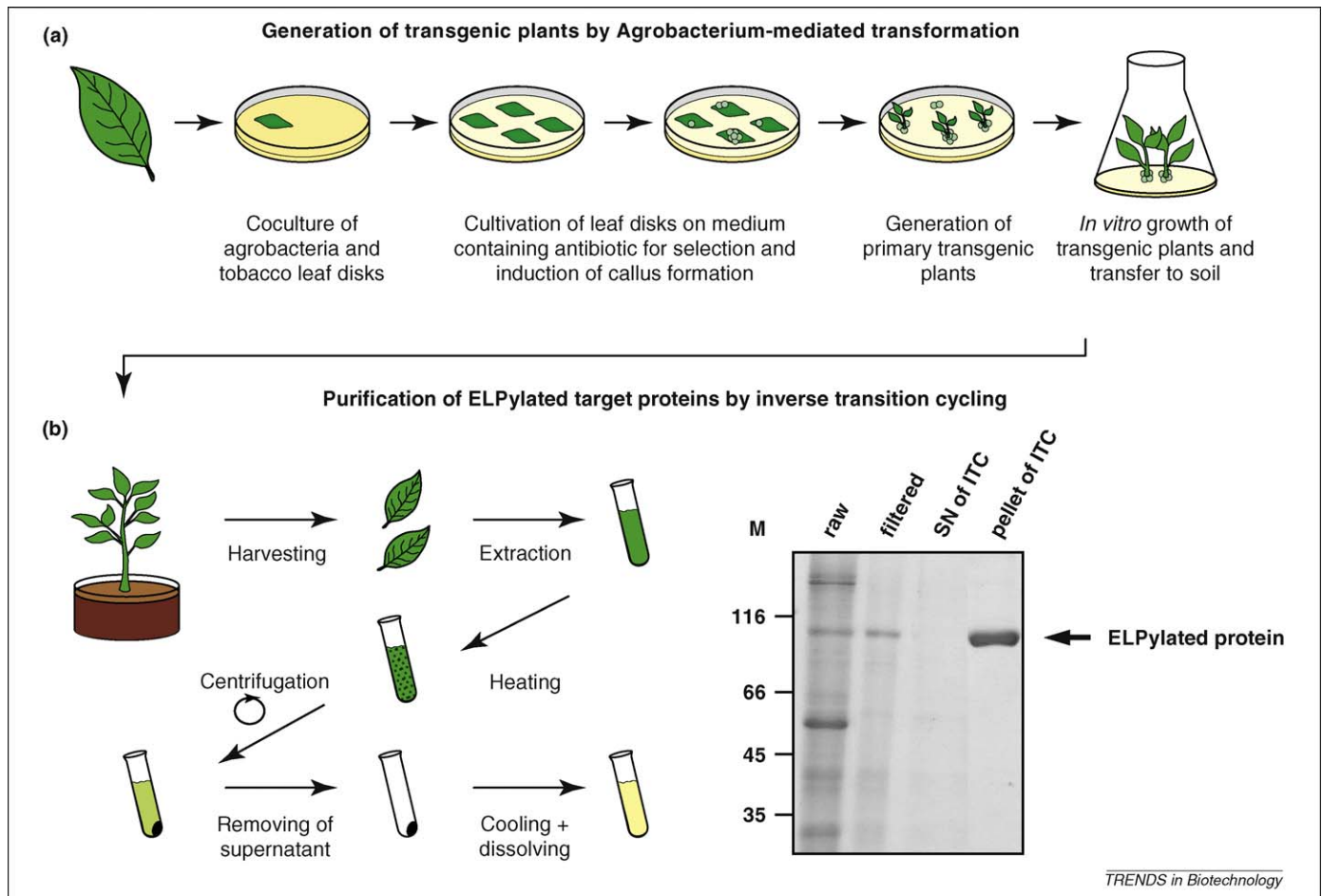
Box 1. Heterologous protein expression in plants

The two major building blocks of current plant biotechnology were put in place in the latter years of the 20th century (for reviews, see Refs. [88,89]). These were, firstly the establishment that the Gram-negative soil bacterium *Agrobacterium tumefaciens* has the capacity for horizontal gene transfer, and secondly, that it is possible to regenerate adult plants from isolated cells. Since the first successful experiments with genetically modified plant cells, a number of plant-based expression systems have been established. These include the use of stably transformed plants that carry the transgene(s) within the nucleus or within the plastid genome, and transient systems based on a viral or plasmid vector. Recombinant proteins produced in whole plants can be secreted into the endomembrane system of the plant or externally into a culture medium via the roots (rhizosecretion). Transformed plant cell cultures also have been generated that cannot develop into adult plants, but synthesize recombinant proteins in a manner analogous to transformed bacterial or mammalian cells. Expression systems based on whole plants increasingly can take advantage of the availability of tissue-specific promoters, a case in point being the seed-based expression of recombinant antibodies and vaccines. Antibodies deposited in the seed appear to remain stable and functional even after long-term storage of the seed at room temperature. For transient expression, a deconstructed viral expression system based on tobacco mosaic virus has been applied successfully in a variety of situations. Accumulation levels of recombinant proteins are influenced by the site within the plant cell where the expressed proteins are retained, and the ER has been shown to provide the most suitable environment for the completion of the posttranslational modifications, such as glycosylation, which are necessary to establish functionality. Thus, retention of the recombinant protein within the ER is held to be the optimal strategy for recombinant protein production in leaves, seeds and tubers.

to target solid tumors via localized hyperthermia [54]. Similarly, a simple ELPylated form of the cell-penetrating Tat peptide inhibits *in vitro* cell adhesion, spreading, invasion and migration of ovarian cancer cells, whereas *in vivo*, it exhibits anti-metastatic activity in an ovarian cancer metastasis model [55]. When the L21 lytic peptide, which has been shown to exhibit anticancer activity, is fused to the C-terminus of ELPylated Tat, the resulting fusion protein is toxic to cancer cells *in vitro*, and thus it has been suggested that this synthetic product has potential for the thermal targeting of tumors [56].

In another application, the self-assembly of ELPs into capsules and aggregates might be relevant as a bridge between biomaterials and nanodevices [57]. ELPs could be cross-linked to form matrices with mechanical properties comparable to those of native elastin [58]. With a better understanding of the 3D architecture and structural transformation of ELP aggregates, it should be possible to tailor ELPs to suit specific biotechnological applications. A pioneering example that demonstrates what might be possible is the SELP polypeptide (silk-elastin-like protein copolymer), which combines the physical and mechanical features of silkworm silk, and the chemical properties of ELP. SELPs form temperature- and pH-responsive hydrogels [59] and can be spun into fibers [60,61] that possess good tensile strength and a high level of deformability [62]. A second example is the production of an ELP layer in which nanopores can be formed in response to changes in pH [63]. Given the temperature-responsiveness of ELPs, it should also be possible to build switchable nanostructures that are designed to open or close nanopores simply by manipulating the temperature.

ELPs and fusion proteins therefore have been shown to be highly biocompatible, which launches ELPs into biomedical applications, such as tissue engineering or hyperthermia-induced concentration of ELPylated drugs *in vivo*. Thus, direct administration of functional ELP fusion proteins for therapeutic interventions, such as ELP-Tat or sTNFRII-ELP, might be a generally feasible strategy.

Heterologous gene expression in plants: a new application for ELPylation

The successful development of transgenesis in plants has promoted the idea of their use for the production of recombinant proteins ([64], Box 1). In resource-poor areas with a low level of technological infrastructure, using plants to produce recombinant proteins is probably more realistic than the current alternatives of bacterial, yeast, insect and mammalian cells [65–67]. Candidate proteins are, for example, neutralizing and infection-preventing recombinant antibodies against HIV as components of microbicides, or recombinant vaccines against avian influenza.

(Gly-Val-Gly-Val-Pro)$_{121}$ was purified from the cytosol of transgenic tobacco leaves as early as 1995, although only very low levels of product were recovered {0.01–0.05% of total soluble protein (TSP), equivalent to ~0.5 µg/g fresh leaf [68,69]}. The relatively low protein accumulation relied on cytosolic localization [70]. The expression of the transgene in the chloroplast rather than in the nucleus did not increase the yield of recombinant protein [71]. In 2004, the ELPylation strategy was transferred to plants, as exemplified by the transgenic expression of ELPylated spider silk in the endoplasmic reticulum (ER) of tobacco and potato [72]. When the synthetic spider silk protein SO1, which corresponds to the golden silk spider *Nephila clavipes* MaSp1 protein [73], was fused to (Val-Pro-Gly-Xaa-Gly)$_{100}$, recombinant ELPylated SO1 represented up to 4% of TSP, equivalent to twice the level of the non-ELPylated SO1 [73]. A laboratory-scale ITC-based purification process was able to recover 80 mg ELPylated SO1 per kilogram of tobacco leaf material [72] (Figure 2b). The biocompatibility of the transgenic protein was demonstrated by the successful growth of anchorage-dependent mammalian cells on SO1–ELP-coated culture plates [72]. In addition, layers and foils of SO1–ELP prepared by spin-coating and casting were more elastic and harder than the semi-rigid to rigid thermoplastic polymers polyethylenterephthalate (PET) or polyetherimide (PEI), which currently are used for industrial applications such as PET bottles for soft drinks, food packaging and thermal insulation, and, moreover, were comparable in properties to those of sericine-free *Bombyx mori* silk. Thus, ELPylated SO1 has been suggested as a promising starting point for the development of novel layers and membranes [74,75]. MaSp2, the natural *N. clavipes* silk protein, has been ELPylated with (Val-Pro-Gly-Xaa-Gly)$_{27}$ and expressed in tobacco. The expression level of non-ELPylated MaSp2 was 0.0125% TSP, and that of the ELPylated form 0.75% TSP [76]. The substantial improvement in the recoverable yield of recombinant protein was facilitated by C-terminal

Figure 2. Generation of transgenic plants and purification of ELPylated target proteins using ITC. **(a)** First, tobacco plants are transformed by *Agrobacterium*-mediated leaf disc transformation. **(b)** Leaves from the resulting transgenic tobacco plants are harvested, ground in liquid nitrogen, and a crude plant extract is prepared by suspending the powdered leaf material in buffer before centrifugation and filtration. The crude extract is then supplemented with sodium chloride and the temperature is raised above Tt. Aggregated ELPylated TPs are precipitated by centrifugation, the supernatant is removed, and the pellet resuspended in low-salt buffer below Tt. The ITC process can be repeated in order to further concentrate the ELPylated TPs. The representative result shown here illustrates the ITC-mediated purification of mini-sgp130-ELP (adapted from Ref. [83]). Proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. M, molecular mass markers (kDa). Other lanes are: raw, raw extract of ground leaf material; filtered, centrifuged and filtered raw leaf extract; SN of ITC, supernatant after the first round of ITC; pellet of ITC, resuspended proteins after ITC.

ELPylation [77–82]. A systematic analysis has shown that only protein targeting to, and retention in, the ER leads to the enhancement of ELP-tagged TP accumulation [70]. Notably, recombinant ELP fusion proteins in leaves and seeds are deposited in a novel type of protein body surrounded by a double membrane, thought to represent the host's means of excluding heterologous proteins from normal physiological turnover. These protein bodies appear to originate from the ER as small punctuate structures [68,78]. Deposition of ELPs in protein bodies might have enabled the tremendous increase of ELP–TP accumulation in transgenic plants compared to that of TP alone [70,80]. In the future, it will be interesting to see whether increased accumulation of ELPylated proteins in transgenic plants can also be achieved in the field, and is not restricted to controllable environmental conditions in the green house.

The ELPylation of a single-chain Fv antibody fragment (scFv) resulted in a 40-fold increase in scFv accumulation, with levels approaching 25% of total soluble seed protein in transgenic tobacco [82]. The ELPylated scFv remained stable and functional even when mature seeds were stored for a long period at room temperature. The binding affinities of ELPylated scFvs with their respective antigen were not reduced by ELPylation [82]. A fusion between (Val-Pro-Gly-Val-Gly)$_{28}$ and an scFv targeting the foot and mouth disease virus coat protein VP1 was expressed in tobacco, and a tobacco etch virus protease recognition sequence was inserted between the scFv and the ELP to remove the ELP part. The maximum accumulation of transgenic protein achieved was 0.8% TSP, and the affinity of the scFv was not compromised by ELPylation [81]. The transfer of intein–ELP technology to plants has yet to be accomplished. We have tested ELP–intein fusion proteins in transgenic plants and experienced a high degree of *in planta* intein cleavage, which resulted in a major decrease of the final yield (J. Scheller and U. Conrad, unpublished observations). However, this problem might be overcome by the use of tailor-made inteins or with the application of a fast transient transfection system (Box 1). Two ELPylated [(Val-Pro-Gly-Xaa-Gly)$_{100}$] antibodies with neutralizing capacity against HIV-1 have been expressed in tobacco leaves and seeds [78–80]. Once again, ELPylation resulted in a more efficient accumulation of recombinant protein (of the heavy and light chain of the antibodies) [78,80]. Independently of the purification method used (ITC or protein-A affinity chromatography), ELPylation did not

affect the binding affinity, but a slightly negative effect on HIV neutralization capacity after ELPylation to the light chain was shown [80]. Furthermore, it had no effect on the folding, assembly or posttranslational modification of the antibodies [77]. The N-glycans of the naked antibodies, but not of the ELPylated antibodies expressed in tobacco seeds, were however, extensively trimmed, which might have been caused by the deposition of these proteins in special protein bodies within the plant cell [80]. At this point, we cannot exclude steric hindrance by attached ELP upon glycan transferase.

A number of other pharmaceutically interesting proteins have been heterologously expressed as ELPylation products in plants. For example, the pioneering ELPylated plant-expressed, non-antibody-based recombinant protein is mini-sgp130-(Val-Pro-Gly-Xaa-Gly)$_{100}$ [83] (Figure 2a and b). Mini-sgp130-ELP is the cytokine-binding portion of the soluble receptor protein gp130 (sgp130), which is a natural inhibitor of interleukin 6 (IL-6) *trans*-signaling mediated by the soluble IL-6 receptor [84]. Recombinant full-length sgp130 expressed in CHO cells prevents the development of rheumatoid arthritis, inflammatory bowel disease and inflammation-associated colon cancer in mice [85,86]. Similarly, the tobacco-derived recombinant ELPylated mini-sgp130 inhibits IL-6 *trans*-signaling mediated by the soluble IL-6 receptor, but not classical IL-6 signaling mediated by the membrane-bound IL-6 receptor [83]. An improved level of accumulation of both IL-10 and IL-4 has been obtained by their fusion with (Val-Pro-Gly-Val-Gly)$_{27}$, but the biological activity of these recombinant cytokines in terms of their receptor-binding or immune-suppression/stimulation has not been reported [76]. The production of human erythropoietin (EPO) for therapeutic purposes currently is carried out using mammalian cell culture. While ELPylation with (Val-Pro-Gly-Val-Gly)$_{28}$ does not influence the biological activity of the recombinant protein when expressed in tobacco, it also does not significantly increase its level of accumulation *in planta*. This failure is ascribed to a possible toxicity of the EPO–ELP protein in the plant [87].

A recent investigation of ELPylation technology has shown that ELP size, orientation and peptide sequence/composition all play a role in determining the extent to which a range of recombinant proteins is accumulated, and that ELP size has an impact on the efficiency of recombinant protein recovery from plant extracts using

Table 1. ELPs

Recombinant protein	Plant expression system	Accumulation level	Purification	Biological functionality	References
(Gly-Val-Gly-Val-Pro)$_{121}$	Tobacco suspension cultures	N. D.	Not done	N. D.	[68]
(Gly-Val-Gly-Val-Pro)$_{121}$	Tobacco leaves	0.01–0.05% TSP	ITC	N. D.	[69]
(Gly-Val-Gly-Val-Pro)$_{121}$	Tobacco chloroplasts	N. D.	Not done	N. D.	[71]
SO1-(Val-Pro-Gly-Xaa-Gly)$_{100}$	Tobacco leaves	0.5–4% TSP	ITC (80 mg/kg FW)	Growth of anchorage-dependent mammalian cells	[72]
scFv-(Val-Pro-Gly-Xaa-Gly)$_{100}$	Tobacco seeds	25% TSP	Not done	Comparable binding affinities to unfused scFv	[82]
Mini-sgp130-(Val-Pro-Gly-Xaa-Gly)$_{100}$	Tobacco leaves	N. D.	ITC (141 µg/g FW)	Inhibition of sIL-6R-mediated trans-signaling	[83]
MaSp2-(Val-Pro-Gly-Val-Gly)$_{27}$	Tobacco leaves	0.25% TSP; 0.75% TSP	Not done	N. D.	[76]
mIL4-(Val-Pro-Gly-Val-Gly)$_{27}$	Tobacco leaves	0.75% TSP; 0.086% TSP	Not done	N. D.	[76]
hIL10-(Val-Pro-Gly-Val-Gly)$_{27}$	Tobacco leaves	0.55% TSP (transient); 0.27% TSP (stable)	Not done	N. D.	[76]
mAb 2F5-ELP	Tobacco leaves	0.2-0.6% TSP	ITC	Comparable binding parameters to CHO-derived non-fused 2F5	[78,79]
scFv-(Val-Pro-Gly-Val-Gly)$_{28}$	Tobacco leaves	0.08-0.1% TSP; 0.8% TSP	ITC (1.5 mg from 660 g FW)	Binding to corresponding antigen	[81]
GFP-(Val-Pro-Gly-Val-Gly)$_n$	Tobacco leaves	0.2-0.5%TSP, 21% TSP (n=10 and p19)	ITC	-	[70,77]
IL10-(Val-Pro-Gly-Val-Gly)$_n$	Tobacco leaves	0.2-0.5% TSP, 4.5% TSP (n=5 and p19)	ITC	N. D.	[77]
EPO-(Val-Pro-Gly-Val-Gly)$_n$	Tobacco leaves	<0.2% TSP	ITC	Binding to human EPO receptor	[70,77]
mAb 2G12-ELP	Tobacco leaves and seeds	0.1-1% TSP	ITC	HIV-1 neutralization	[80]

TSP, total soluble protein; N. D., not determined; p19, gene silencing suppressor; FW, fresh weight.

ITC purification [77]. Shorter (n=5–40) (Val-Pro-Gly-Val-Gly)$_n$ oligomers appear to be more effective in terms of protein accumulation than longer ones (n=80–160), although the longer ELPs were more easily purified by ITC. The authors have suggested a chain length of (Val-Pro-Gly-Val-Gly)$_{30}$ as an optimal compromise, which how-ever, might not be ideal for other guest residues (Xaa) at the fourth position of ELPs. A random rearrangement of the ELP-containing amino acids Val-Pro-Gly-Val-Gly is associated with a significant loss in protein yield, which shows that the peptide sequence and composition (which determine the stereochemistry of the ELP) are important determinants of the efficiency with which recombinant ELP fusion proteins are accumulated *in planta* [77].

In summary, over the past 14 years, four major findings/technological advances have had an impact on the production of ELPs and ELPylation in plants. Firstly, it has become clear that ELPs and ELPylated proteins are expressed freely in transgenic plants (Table 1); secondly, ELPylation generally increases the level of accumulation of recombinant protein, provided that the protein can be targeted to, and then retained in the ER; thirdly, ELPs can be efficiently and specifically purified from plant tissue using ITC; and, finally, plant-made ELPylated recombinant proteins retain the expected bioactivity and appropriate glycosylation patterns.

Conclusions and further perspectives

The development of ELPylation in the 1990s has sparked a revolution in laboratory-scale recombinant protein expression and purification. The attractiveness of ELPylation is based on a thermally stimulated inverse phase transition, which can be exploited for the non-chromatographic purification of recombinant proteins. EMAC or combination of ELPylation and intein technology is designed to allow the production of ELP-free TPs. The excellent biocompatibility of ELPs and the ability to control their aggregation *in vitro* and *in vivo* opens the way to a series of advanced medical applications, such as hyperthermia-induced localized extravascular drug accumulation, drug depot formation, and the design of novel materials suitable for tissue repair or engineering. The medical use of recombinant proteins produced by plants is, as yet, an immature technology. However, a number of clinical studies currently are under way that are seeking to test the feasibility of using plant-made

pharmaceuticals. The transfer of ELPylation to transgenic plants has already shown to improve the efficiency of recombinant protein accumulation, and the products, which can be readily recovered by ITC, appear to retain their expected functionality. Thus, ELPylation has substantial potential for the large-scale and cost-competitive production of recombinant proteins as outlined in Box 2.

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