

Native-sized spider silk proteins synthesized *in planta* via intein-based multimerization

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Abstract The synthesis of native-sized proteins is a pre-requisite for exploiting the potential of spider silk as a bio-based material. The unique properties of spider silk, such as extraordinary tensile strength and elasticity, result from the highly repetitive nature of spider silk protein motifs. The present report describes the combination of spider silk flagelliform protein (FLAG) production in the endoplasmic reticulum of tobacco plant leaf cells with an intein-based post-translational protein fusion technology. The repeated ligation of FLAG monomers resulted in the formation of large multimers. This method avoids the need for highly repetitive transgenes, which may result in a higher genetic and transcriptional stability. Here we

show, for the first time, the production of synthetic, high molecular weight spider silk proteins larger than 250 kDa based on the assembly of protein monomers via intein-mediated *trans*-splicing *in planta*. The resulting multimeric structures form microfibers, thereby demonstrating their great potential as a biomaterial.

Keywords Spider silk · Flagelliform protein · Intein · Protein *trans*-splicing · Microfibers · Tobacco

Introduction

Spiders have evolved to produce a variety of protein-based silk materials, which are characterized by remarkable levels of toughness, tensile strength and elasticity (Craig 2004; Vollrath and Knight 2001). Capture spiral (or flagelliform) silk is composed exclusively of the FLAG protein. It can be stretched to at least double its length before rupture, thereby providing the high level of elasticity required to assure prey capture (Vollrath and Edmonds 1989; Köhler and Vollrath 1995). Therefore, flagelliform silk perfectly dissipates the kinetic impact energy of flying prey and withstands the massive impact caused by the relative high velocity of prey insects (Römer and Scheibel 2008). The elastic nature of the FLAG protein is based on the presence of particular GPGGX repeats. The protein is also characterized by helical GGX repeats. The non-repetitive spacer elements provide the necessary intra-molecular alignment of crystalline

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and other regions, which is required to present a surface able to interact with other silk components (Hayashi et al. 1999; Hayashi and Lewis 2000). The size of spider silk proteins is assumed to be a key determinant of the fiber's mechanical properties, as all native spider silks characterized to date contain proteins larger than 250 kDa (Ayoub et al. 2007; Spöner et al. 2005). Two additional essential characteristics of large spider silk proteins are the high frequency of motifs conducive for inter- and intra-chain interactions and the small number of chain end defects. Recombinant spider silk proteins of native size would extend the use of the structures derived thereof, i.e., in medicine for the enhancement of axonal regeneration and for the production of artificial skin, as demonstrated for native silk fibers (Radtke et al. 2011; Wendt et al. 2011). The availability of suitable cDNA sequences is crucial for the production of spider silk proteins in any heterologous expression system. To date, only two spider silk sequences are completely known: the FLAG protein of *Nephila clavipes* and a major ampullary silk protein of *Latrodectus hesperus* (Hayashi and Lewis 2000; Ayoub et al. 2007; Ayoub and Hayashi 2008). In the present study, we used the FLAG protein of *Nephila clavipes* to examine a plant expression approach.

In former studies, the heterologous production of high molecular weight spider silk proteins has been performed in *E. coli*. The material produced with this technology could be spun out from hexafluoroisopropanol-solubilized native-sized spider silk proteins. However, its production requires the host to generate a rapid and high supply of glycyl-t-RNA and optimized synthesis of glycine (Xia et al. 2010). Therefore, less rapidly growing organisms, such as transgenic plants, have been selected as a promising alternative production system to overcome the limitations associated with t-RNA and amino acid synthesis. Recently, plant-based protein expression via *Agrobacterium*-mediated plant transformation resulted in the expression of spider silk proteins and spider silk-ELP fusion proteins of approximately 100 kDa in size (Scheller et al. 2001; Scheller et al. 2004).

We aimed to develop a solution for the expression of native-sized spider silk proteins and high molecular weight repetitive proteins in general. Protein-splicing by inteins has been demonstrated as a tool to assemble protein subunits *in planta* (Yang et al. 2003). Inteins are intervening protein elements that are autocatalytically excised from precursor molecules and covalently

ligate the flanking protein sequences (exteins) via a process termed protein splicing (Perler 1998). Inteins catalyze *cis*-splicing reactions but are also able to catalyze *trans*-splicing events between non-covalently linked protein fragments (Saleh and Perler 2006). Here we demonstrated that multimers of the FLAG spider silk protein (of at least native size) could be produced in plant systems and that the products can be efficiently purified and characterized. We conclude that posttranslational intein-mediated fusion of precursor molecules is a suitable solution to circumvent the problems of gene and mRNA instability that are encountered in the synthesis of highly repetitive recombinant proteins.

Materials and methods

Design of synthetic constructs

The synthetic *InteinC::Flag::InteinN* (*IntC::Flag::IntN*) gene constructs [*IntC::Flag(c-myc)::IntN*, *IntC::Flag(His)::IntN*] and an *IntC::60×ELP::IntN* construct were generated by GENEART AG, Munich, Germany. The FLAG gene motifs were selected from public *Nephila clavipes* cDNAs (GenBank accession nos. AF027972 and AF027973). For protein detection, either a c-myc (Munro and Pelham 1986) or a His tag was incorporated. The N- and C-terminal intein sequences and short extein stretches were those present in the *Synechocystis* sp. gene *DnaB* (UniProtKB/Swiss-Prot accession no. Q55418). Additionally, a flexible triple GGGGS linker was inserted as described previously (Kempe et al. 2009). For the *IntC::60×ELP::IntN* construct, a specific ELP sequence (VPGXG)₆₀ was designed, in which X represents a random amino acid other than proline. The number of VPGXG pentapeptides was selected based on previous experiments using ELPylated proteins (Conley et al. 2009; Scheller et al. 2004).

All constructs described in this paper were verified by DNA sequencing.

PCR site-directed mutagenesis

To exchange the protein splicing key residues, site-directed mutagenesis was performed with the *IntC::Flag(c-myc)::IntN* construct using a QuikChange II-E Site-Directed Mutagenesis kit (Agilent Technologies,

Santa Clara, CA, USA). Two Ala residues were introduced to replace *DnaB* (Asn⁺¹⁵⁴) and the following amino acid residue of the c-terminal extein (Ser⁺¹) using the primers IntCNSAA-FII (5'-CGATA TTATCGTGCACGCCGCCATTGAGCAAGATGG) and IntCNSAA-RII (5'-CCATCTTGCTCAATGGCG GCGTGCACGATAATATCG). A third Ala was introduced to replace the N-terminal amino acid of *DnaB* intein (Cys⁺¹) using the primers CAIntN-F (5'-AGG ATCTCAACAGAGAGTCCGGTGCTATTTCTGG TGATTCTC and CAIntN-R (5'-GAGAATCACCA GAAATAGCACCGACTCTCTGTTGAGATCCTC). The altered sequences are underlined.

Construction of expression cassettes

The different sequences of the *IntC::Flag::IntN* and *IntC::60× ELP::IntN* constructs were individually introduced into the *NcoI* and *BamHI* restriction sites of a pRTRA15 vector (Artsaenko et al. 1995) to incorporate the *CaMV35S* promoter, a *LeB4* legumin signal peptide, a KDEL coding sequence and a *CaMV35S* terminator. For the transformation of tobacco plants, the expression cassettes were excised using the *HindIII* enzyme and transferred into the pCB301-based binary vector, pCB301-Kan (Xiang et al. 1999; Gahrtz and Conrad 2009).

Transient expression in *Nicotiana benthamiana*

For transient assays, intact leaves of *N. benthamiana* plants were infiltrated with the *Agrobacterium tumefaciens* strain C58C1 (harboring the vector pGV2260) (Deblaere et al. 1985) as previously described (Gahrtz and Conrad 2009; Kapila et al. 1997). The plant leaves were co-infiltrated with HcPro, a suppressor of gene silencing that has been found to significantly increase the expression levels of recombinant protein in plant cells (Conley et al. 2009; Sudarshana et al. 2006). Infiltrated plants were grown in the greenhouse. The maximum levels of product accumulation in leaves were observed after 5–6 days as determined by Western blot analysis.

Stable transformation of *Nicotiana tabacum*

The binary vectors were introduced into *Agrobacterium* by electroporation and introduced into *Nicotiana tabacum* cv. Samsun NN (SNN) leaf discs by agroinfiltration according to a previously described protocol

(Floss and Conrad 2010; Horsch et al. 1985). The transgenic plants were cultured on MS agar containing 50 mg/L kanamycin and analyzed by Western blot using either an anti-c-myc or anti-His monoclonal antibody. High expressing T₀-tobacco plants were selected and further propagated.

Production of rabbit sera

The *InteinN*-encoding sequence from the *Synechocystis* sp. gene *DnaB* (UniProtKB/Swiss-Prot accession no. Q55418) was introduced into the vector pET23a (Novagen, Merck KGaA, Darmstadt, Germany), resulting in an expression vector, designated as IntN-pET23a. The recombinant protein was produced in *E. coli* BL21 according to the standard protocol and purified by Ni-NTA affinity chromatography. Rabbits were immunized with 1 mg antigen and complete Freund's adjuvants (Difco, USA), and animals were boosted twice with 500 µg antigen and incomplete Freund's adjuvants. Sera were collected 1 week after the last immunization, enriched by ammonium sulphate precipitation and used in appropriate dilutions for Western blot analysis.

SDS Page and Western Blotting

Leaf material was powdered in liquid nitrogen and suspended in 72 mM Tris, 10 % v/v glycerine, 2 % w/v SDS, 5 % w/v 2-mercaptoethanol and 0.0025 mM bromophenol blue, pH 6.8. The homogenate was incubated for 10 min at 95 °C and cleared by centrifugation (30 min, 4 °C, 36,000g). The concentration of the total soluble protein was determined using the Bradford assay (Bio-Rad, Munich, Germany). A 40 µg sample of extracted protein was separated by 3–10 % SDS-PAGE at reducing conditions and electroblotted onto a nitrocellulose membrane (Whatman GmbH, GE Healthcare, Dassel, Germany) using 25 mM Tris, 0.1 % w/v SDS, 192 mM glycine and 20 % v/v methanol. For detection of the transgenic product, the membranes were blocked for 2 h in 5 % w/v fat-free dry milk in 180 mM NaCl and 20 mM Tris, pH 7.8. The membranes were probed with either the anti-c-myc or the anti-His monoclonal antibody and subsequently incubated with an anti-mouse peroxidase conjugate. Signals were detected by enhanced chemiluminescence (Amersham ECL Plus TM, GE Healthcare UK Ltd., UK).

Heterologous protein expression in *E. coli*

The mutated *intc::flag(c-myc)::intn* sequence was introduced into the *Bam*HI-*Nco*I restriction sites of a pET16b plasmid and expressed in the cytosol of the *E. coli* strain BL21 (Novagen, Merck KGaA, Darmstadt, Germany). An extract of induced cells was denatured at 95 °C, separated by 12 % SDS-PAGE, electroblotted, incubated first with an anti-c-myc monoclonal antibody and then with an anti-mouse peroxidase conjugate. Protein expression was detected by enhanced chemiluminescence (Amersham ECL Plus™, GE Healthcare UK Ltd., UK).

Purification of His-tagged spider silk protein

Frozen leaf material was ground in liquid nitrogen and extracted with 50 mM phosphate buffer pH 6.8 containing 0.05 % v/v Triton X-100. After extraction, 1.32 g ascorbic acid per 100 g leaf was added. After 2 h of shaking at 4 °C, the extracts were cleared by centrifugation followed by filtration through a fine gauze. The subsequent protein purification via affinity chromatography was based on Ni-NTA agarose as described (QIAGEN, Hilden, Germany). No imidazole was included in the binding step, and 50 mM was used for the washing step. The proteins were eluted by flushing first with 100 mM and then with 200 mM imidazole. Diafiltration was performed using a 150-kDa filter (Pierce^R Concentrator, Thermo Scientific, Rockford, USA) and centrifugation at 5,000×g followed by re-dilution in Millipore water; the process was repeated five times. The protein product was then lyophilized.

SEM

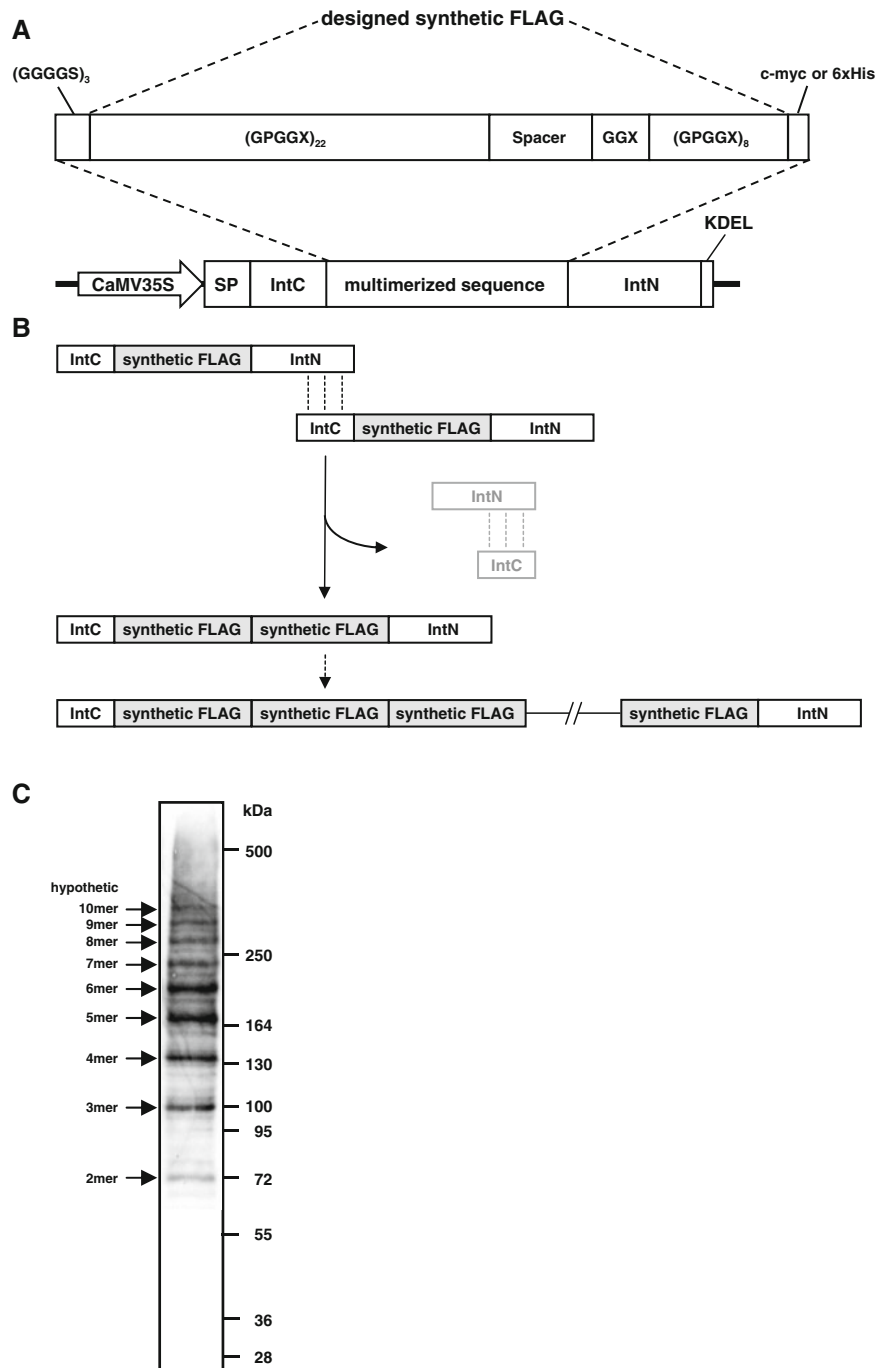
Purified, diafiltrated and desalted proteins were lyophilized and fixed to a standard SEM holder. The probe was exposed to 4 % w/v OsO₄ vapor for 2 h and then coated with a ~5 nm carbon layer. The SEM experiments were performed using a Quanta™ FEG 3D electron microscope (FEI, Hillsboro, USA) in a high vacuum using the secondary electron detection mode. The image was recorded in a low acceleration voltage at 5 kV. A magnification of 8,000× was adjusted at a working distance of 10.1 mm. Secondary electrons were detected by an Everhart–Thornley detector at a sample tilt angle of 0°.

Results

A plant expression vector was designed encoding a fusion protein of the structure “IntC-FLAG-IntN” (Fig. 1A). The synthetic FLAG sequence is able to multimerize due to an autocatalytical intein-mediated *trans*-splicing reaction. FLAG typically contains the GPGGX and GGX motifs and a non-repetitive spacer element. At the 5′ end of the FLAG-coding sequence, a flexible 3× GGGGS linker was added to improve the intein-based protein fusion *in planta* (Kempe et al. 2009). A tag suitable for both the detection of the protein via Western blot analyses and affinity purification (c-myc or 6× His) was fused to the C-terminus of the synthetic FLAG protein (Fig. 1A, the sequences are provided in the Supplementary Figure S1A, S1B). The localization of the expressed proteins in the endoplasmic reticulum (ER) was assured by an N-terminal LeB4 signal peptide. A KDEL signal at the C-terminus caused ER retention. This expression cassette was introduced into a vector with the CaMV35S promoter and stop signals for both transcription and translation (Artsaenko et al. 1995). The expression cassette was inserted into a pCB301-based binary vector, pCB301-Kan (Gahrtz and Conrad 2009). The resulting vector was transformed in agrobacteria and both transient and stable expression experiments were performed using *N. benthamiana* and *N. tabacum*, respectively.

The post-translational protein splicing triggered by the terminal inteins was expected to generate FLAG multimers of various distinct sizes, reflecting the varying degrees of multimerization. Indeed, such band patterns were detected in Western blot experiments using protein extracts of transgenic tobacco plant leaves (Fig. 1C). Strikingly, the molecular weights of several bands were significantly higher than 250 kDa. Similar profiles were generated regardless of whether the construct contained the c-myc or the His-tag coding sequences (Figs. 1C, S3). The post-translational protein splicing reaction triggered by the terminal inteins resulted in the introduction of a new extein element (FLAG) and the removal of the flanking N- and C-intein parts (Fig. 1B). This was expected to generate ~54 kDa FLAG dimers, which include each one of the flanking intein sequences at the N and C termini (Supplementary Figure S1C). However, the molecular weight of each of the putative multimers appeared to be higher than expected (Fig. 1C).

Fig. 1 Design of the expression cassette and the principle of intein-mediated assembly of FLAG multimers. **A** Schematic overview of the expression cassette. Abbreviations: *CaMV35S*, cauliflower mosaic virus 35S constitutive promoter; *SP*, legumin B4 signal peptide; *KDEL*, ER retention sequence; *IntN/IntC*, C- and N-terminal intein sequences of the *Synechocystis* sp. DnaB gene; *GPGGX_n*; *GGX/spacer*, motifs in wild type FLAG sequences; *(GGGS)₃*, flexible spacer; *c-myc-tag* (detection) or *6xHis-tag* (purification). **B** Multimerization of synthetic FLAG monomers via intein-mediated protein splicing. **C** Western blot analysis of extracts of tobacco leaves overexpressing the *IntC-FLAG(c-myc)-IntN* protein. Plant leaf extracts were separated by 3–13 % SDS-PAGE and electroblotted, and distinct FLAG multimers were detected by enhanced chemiluminescence (ECL) based on the presence of the c-myc tag



We believe that this is most likely attributed to the specific structural properties of the FLAG protein. In particular, the GPGGX motif readily forms β -turns, which are associated with a substantial level of conformational variability in the case of elastin-like polypeptides (ELPs) (Ohgo et al. 2006). It is well known that the

glycosylation status of a protein strongly influences its migration behavior in SDS-PAGE gels. Hence, we performed an in silico N- and O-glycosylation site analyses (www.cbs.dtu.dk/services/NetNGlyc; www.cbs.dtu.dk/services/NetOGlyc) and identified several potential o-glycosylation sites within the FLAG

sequence. Furthermore, a recombinant, IntC-FLAG-IntN protein (including the plant signal peptide; Supplementary Figure S1D) was produced in *E. coli* to prevent glycosylation and to minimize the effects of posttranslational protein modification on the electrophoretic properties. In fact, we were able to produce a recombinant protein of the expected molecular weight (39.3 kDa; Supplementary Figure S2).

A control experiment was performed to prove that intein-mediated *trans*-splicing fosters the assembly of high molecular FLAG proteins. Key residues located at the extein-intein border that are known to be essential for rearrangement during splicing were altered by site-directed mutagenesis. The C-terminal amino acid of the *DnaB* Intein (Asn¹⁵⁴), the following amino acid of the C-terminal extein stretch (Ser⁺¹) and the N-terminal amino acid (Cys⁺¹) were replaced by alanine residues (Fig. 2A, Supplemental Figure S1E). The mutated expression cassette was introduced into the shuttle vector, and the transgenic tobacco plants were then produced and examined by Western blot analysis. The independent transgenic tobacco lines failed to produce multimerized, synthetic FLAG protein. These results clearly demonstrated that a *trans*-splicing mechanism is responsible for multimerization of the IntC-FLAG-IntN protein. Strikingly, the FLAG monomer differed from the theoretical molecular weight of 36.7 kDa (Fig. 2B). This observation is in accordance with the size-deviation detected in the case of FLAG-multimers (Fig. 1C).

FLAG multimers, transiently expressed in *N. benthamiana* leaves, were affinity-purified using Ni-NTA agarose. The purified spider silk multimers were dialyzed against water and concentrated by diafiltration in which the fraction larger than 90 kDa was enriched by applying a cut-off of 150 kDa, as demonstrated by Western blot analysis based on the His-tag (Fig. 3A). The diafiltrated proteins were subsequently lyophilized and the weight was quantified. 1.8 mg spider silk multimers were thus isolated from 50 g leaf material. The material was further analyzed via Scanning Electron Microscopy (SEM). The SEM image shows fibrillae with drop-shaped ends up to 500 μm in length and diameters ranging from 1 to 2 μm (Fig. 3B). The appearance of these fibers demonstrates the self-organizing capacity of FLAG intein multimers.

After successfully applying the intein-technique for large spider silk protein production, we aimed to

demonstrate that other interesting repetitive proteins could be multimerized by a *trans*-splicing mechanism. We selected elastin-like peptides (ELPs) as they provide a suitable feedstock for designing materials for nanotechnology (Arias et al. 2006), for the expression enhancement of fusion proteins in plants and for the purification via Inverse Transition Cycling (ITC) (Floss and Conrad 2010). An expression cassette for the production of multimers of 60 \times ELP was designed (Fig. 4A, Supplementary Figure S1F). The sequence of the monomers is VPGXG, in which X represents any amino acid other than proline (Scheller et al. 2004). This sequence was introduced into a shuttle vector similar as described for the synthetic FLAG proteins. The vector provides both stable constitutive expression in plant leaves through CaMV35S promoter-control and ER retention of the protein. Transient expression experiments in *N. benthamiana* leaves were performed, and the products were analyzed by Western blot experiments using a specific anti-InteinN antibody produced from rabbits. We were able to detect distinct bands covering a range from 47 kDa (monomer) to 250 kDa (Fig. 4B). Thus, intein-based multimerization represents a feasible method for the production of multimerized ELPs.

Discussion

Here we have demonstrated that high molecular weight spider silk multimers of native size can be produced *in planta* using intein-mediated protein splicing. The multimerization leads to very high molecular weight, plant-expressed spider silk proteins. The extraordinary size of spider silk proteins is thought to be a key determinant of the spider silk fiber's excellent mechanical properties (Ayoub et al. 2007; Sponner et al. 2005). The specificity of the splicing reaction was verified by the site-specific mutagenesis of key amino acids, which prevents the multimerization of FLAG proteins in several independent transgenic tobacco lines.

In *E. coli*, native sized spider silk proteins were only produced by the specific design of host strains to generate a large supply of glycyl-t-RNA and optimized glycine synthesis (Xia et al. 2010). We believe that the general approach that we developed will allow for the production of a broad range of different repetitive multimerized proteins of high value. We demonstrated

Fig. 2 Mutagenesis of key residues for *trans*-splicing prevents FLAG-multimerization. **A** Three conserved residues that are essential for the intein-mediated *trans*-splicing reaction were changed to alanines (in red), extein stretches were labeled in gray. **B** FLAG proteins from tobacco leaf extracts from twelve independent transgenic T₀ plants transformed with mutated intein key residues were separated by 10 % SDS-PAGE. Only FLAG monomers and no multimeric proteins were identified by Western blot analysis. *c+*, Western blot standard

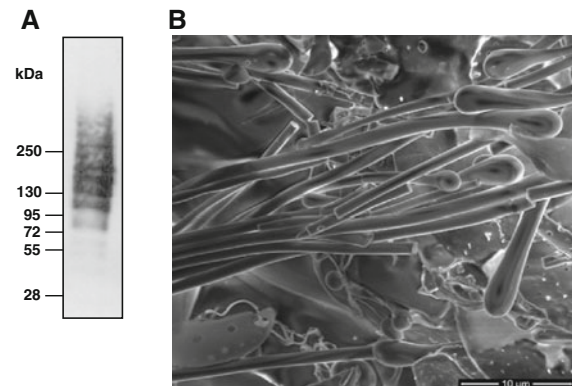
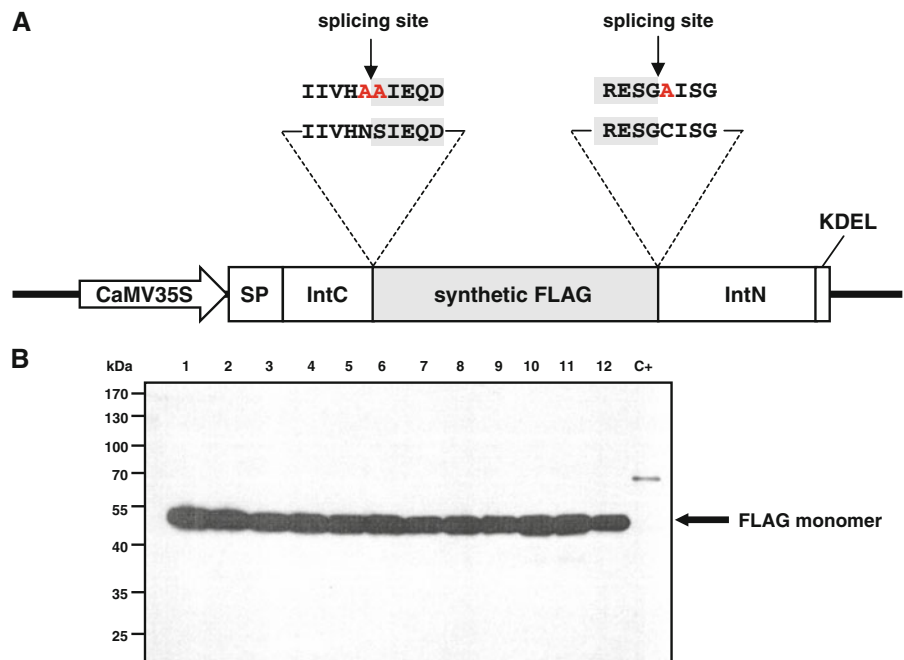


Fig. 3 Purification and characterization of FLAG multimers. **A** Transiently expressed FLAG intein multimers were purified from plant extracts via His-tag affinity chromatography, concentrated and diafiltrated against water, applying a cut-off of 150 kDa. The proteins were separated by 3–10 % SDS-PAGE and visualized by Western blotting using a His-tag antibody. The resulting protein product sizes ranged from >72 to >250 kDa. **B** SEM image of *in planta*-produced FLAG multimers. Fibrillae with a diameter ranging from approximately 1–2 μm and a length up to 500 μm are visible

this by the successful posttranslational multimerization of 60× ELP, a highly repetitive artificial protein designed from human elastin sequences (Floss and Conrad 2010; Urry et al. 1991). The great potential of repetitive proteins, such as spider silk proteins and

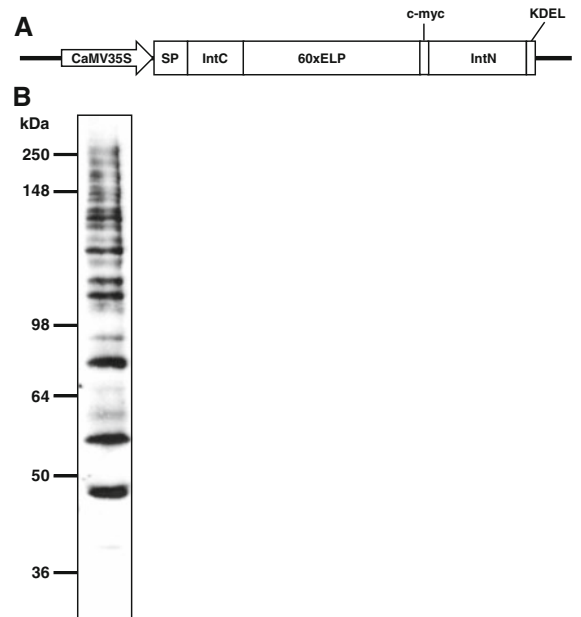


Fig. 4 Heterologous production of c-myc-tagged ELP multimers in stably transformed tobacco leaves. **A** Expression vector used for the production of 60× ELP multimerized by intein-based assembly (for sequence see Supplementary Figure S1). **B** Western blot analysis of extracts of *N. benthamiana* leaves transiently overexpressing the *IntC::60× ELP::IntN* protein. Plant leaf extracts were separated by 3–13 % SDS-PAGE and electroblotted, and distinct FLAG multimers were detected via ECL using a rabbit anti-InteinN antibody

ELPs, to form nanospheres, hydrogels or different fibers has been discussed in detail (Römer and Scheibel 2008; Vendrely et al. 2008). *In planta* expression offers the possibility to produce a plethora of heterogeneous artificial multimers via the simple procedure of intercrossing a pair of distinct transgenic plant lines. In this way, composite materials such as spider silk and ELP can be synthesized. Spider silk-ELP fusion proteins have been demonstrated to enhance the viability of human chondrocytes and to prevent the dedifferentiation of these cells (Scheller et al. 2004). By intercrossing several pairs of transgenic lines expressing two different repetitive proteins at different expression levels, varieties of mixed proteins could be designed and synthesized.

However, an extended application of the approach reported here requires further detailed analysis of several technical aspects. For example, it must be examined whether multimerization by intein-based protein splicing in plants also causes cyclization. Here, specific antibodies against InteinN and InteinC may demonstrate the absence of these intein parts, thus supporting the presence of circles. Although our analysis is not comprehensive, the detection of ELP monomers using anti InteinN antibodies indicate the presence of linear monomers (Fig. 4B) because circular products are expected to contain no inteins as a result of protein *trans*-splicing.

Intermolecular hybrids could be detected by crossing lines expressing various repetitive proteins labeled with different tags. The use of ELPylation for simpler purification via ITC (at best membrane-based ITC; (Phan and Conrad 2011) requires detailed analyses of both the size and extension of the ELP portion. Furthermore, the mechanical analyses of the material produced by intein-based protein splicing should be extended to electrospinning, production of layers and nanospheres, detailed nanomechanical analyses, such as nanointendation and single molecule force microscopy. Scale-up and the optimization of the purification procedure will in future provide sufficient amounts of spider silk multimers as an essential base of these investigations. The data revealed by such experiments would help to fully explore the potential of this technique for nanotechnology and medicine.

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