Production of spider silk proteins in tobacco and potato

Jürgen Scheller[1], Karl-Heinz Gührs[2], Frank Grosse[2], and Udo Conrad[1]*

Spider dragline silk is a proteinaceous fiber with remarkable mechanical properties that make it attractive for technical applications. Unfortunately, the material cannot be obtained in large quantities from spiders. We have therefore generated transgenic tobacco and potato plants that express remarkable amounts of recombinant *Nephila clavipes* dragline proteins. Using a gene synthesis approach, the recombinant proteins exhibit homologies of >90% compared to their native models. Here, we demonstrate the accumulation of recombinant silk proteins, which are encoded by synthetic genes of 420–3,600 base pairs, up to a level of at least 2% of total soluble protein in the endoplasmic reticulum (ER) of tobacco and potato leaves and potato tubers, respectively. Using the present expression system, spider silk proteins up to 100 kDa could be detected in plant tissues. When produced in plants, the recombinant spidroins exhibit extreme heat stability—a property that is used to purify the spidroins by a simple and efficient procedure.

The dragline silk of the spider *Nephila clavipes* has a high tensile strength that is comparable to that of the synthetic superfiber Kevlar, but it additionally shows high elasticity[1]. Such biomaterials could therefore be useful for industrial and medical purposes. The modular nature of spider silk proteins has led to several attempts to design synthetic genes (for review see Hinman *et al.*[2] and Gosline *et al.*[3]) and to express them in microorganisms. Because spider silk proteins (also called spidroins) consist largely of glycine and alanine, an extensive pool of these amino acids has to be provided if spidroins are to be produced by fast-growing microorganisms such as yeast or bacteria. Another difficulty in bacterial production is genetic instability due to recombination, resulting from the highly repetitive genes encoding the repetitively composed spidroins.

To overcome these and other limitations, we have chosen plants for the production of synthetic spidroins. Plants have been successfully used for the production of different transgenic products[4]. In several cases, stable accumulation of functional proteins to high levels has been achieved by retention in the ER. Here we show that synthetic spidroins can be efficiently and stably produced in transgenic tobacco and potato plants by a similar retention approach. Furthermore, the extreme heat stability of these plant-produced synthetic spider silk proteins has been used for the development of simple purification procedures.

Results and discussion

Strategy. Basic synthetic sequences were used for the construction of a set of genes coding for spider silk proteins of different molecular masses. Here, the synthetic spider silk genes were designed to match the known *N. clavipes* MaSp1 (spidroin1) complementary DNA (cDNA) as exactly as possible. However, different synthetic fusion genes could be constructed using this approach. In future, the modular structure of the synthetic genes could be combined with other sequences to produce spider silk-like proteins with new properties. A set of spidroin gene constructs coding for proteins of different sizes was used to test the plant expression system and to provide a panel of spider silk proteins for the optimization of downstream processing and purification. Eighteen oligodeoxyribonucleotides (see Fig. 1) were synthesized and assembled into six short gene fragments. Using these fragments synthetic spidroin genes were constructed, matching to a considerable extent the natural spidroin sequences (Fig. 2A). These genes coding for proteins of different size were used to test the plant expression system and to provide a panel of spider silk proteins for the optimization of downstream processing, purification, and future spinning experiments. These units were inserted in pUC19-derived plasmids and cloned in *Escherichia coli*. Fragments were digested with specific restriction endonucleases and subsequently used in different ligation steps to construct larger and larger segments of the synthetic spidroin1 gene. In the final step, a synthetic 1.8 kilobase homolog of the *N. clavipes* MaSp1 (spidroin1) cDNA, lacking the nonrepetitive part at the 3′ terminus, was generated. The fibroin fragment used for the construction of the gene encoding the fusion protein FA2 is a synthetic homolog of a segment of the gene of the fibroin heavy chain of *Bombyx mori*, the caterpillar of the silkworm moth. Fibroin is the major silk protein from *B. mori*, and the combination with spider silk elements will allow us to explore whether silks with new properties can be engineered. FA2 was constructed from 14 oligodeoxyribonucleotides using an approach similar to that described above (data not shown).

Synthetic spider silk genes (Fig. 2B) were cloned into an expression vector allowing ubiquitous expression in transgenic plants under the control of the cauliflower mosaic virus (CaMV) 35S promoter. A signal peptide at the N terminus of the spider silk protein, together with the KDEL signal at its C terminus, provide ER retention of the transgenic proteins in plant cells[5]. A c-myc tag[6] was fused to the C terminus of the silk protein for detection of transgenic products by western blot. Spider silk genes were assembled from the basic modules (corresponding molecular weights from 12.9 kDa to 99.8 kDa; see Table 1 and Experimental Protocol). A synthetic hybrid protein (FA2, 19.5 kDa), consisting of sequences of *N. clavipes* dragline silk and *B. mori* fibroin, respectively, was included in this investigation. The expression cassettes were then cloned into the plant transformation vectors pGSGLUC1 (ref. 7) or pBIN19 (ref. 8). The resulting expression plas-

[1]*Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben, Corrensstraβe 3, D-06466 Gatersleben, Germany.* [2]*Institut für Molekulare Biotechnologie e. V., PF 100 813, D-07708 Jena, Germany.***Corresponding author (conradu@ipk-gatersleben.de).*

A

No. of oligo	Sequence in 5′ to 3′ direction
1	TATGAGCGCTCCCGGGCAGGGT
2	AGCTTTTAGGTACCAATATTAATCTGGCCGGCTCCACC
3	TATGGTCTGGGG
4	GGCCAGGGTGCTGGCCAA
5	GGTGCAGGAGCWGCWGCWGCWGCTGCAGGTGGA
6	GCCGGCCAGATTAATATTGGTACCTAAA
7	CTGCCCGGGAGCGCTCA
8	ACCACCATAACCTCC
9	AGCACCCTGGCCCCCCAG
10	TGCAGCWGCWGCWGCWGCTCCTGCACCTTGGCC
11	TATGAGATCTGGCCAAGGAGGT
12	TTGGCCAGATCTCA
13	AGTCAGGGTGCTGGTCGTGGAGGCCAA
14	TCCACGACCAGCACCCTGACTCCCCAG
15	AGTCAGGGCGCTGGTCGTGGGGGACTGGGTGGCCAA
16	ACCCAGTCCCCCACGACCAGCGCCCTGACTCCCCAG
17	CTGGGAGGGCAGGGAGCGGGCCAA
18	CGCTCCCTGCCCTCCCAGACCTCC

B

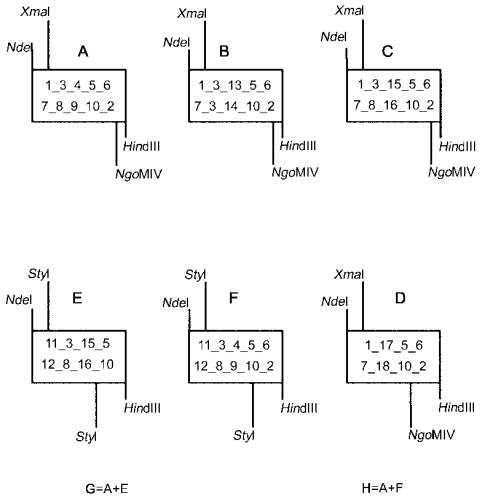

Figure 1. Basic elements for synthetic spider silk genes. (A) Sequences of the oligonucleotides used in construction of synthetic spider silk genes. (B) Schematic representation of the assembly of the gene cassettes from synthetic oligodeoxyribonucleotides.

mids were transformed into *Agrobacterium tumefaciens*. After leaf disk transformation of tobacco and potato, between 30 and 156 kanamycin-resistant plants were obtained, transferred to soil, and tested for transgene expression.

Accumulation of synthetic spider silk proteins in transgenic plants. Between 31% and 69% of the kanamycin-resistant plants transformed with the different constructs accumulated high amounts of recombinant spider silk proteins to >0.5% of the total soluble protein (Table 2). All first- and second-generation transgenic plants showed normal growth and morphology. These results demonstrate the general feasibility of the approach. Protein amounts were determined by comparing c-myc tagged recombinant silk proteins bands on western blots with defined standards of recombinant antibody molecules carrying the same tag. The best producer plants accumulated spider silk proteins to >2% of total soluble protein as demonstrated in Figure 3. The accumulation level of transgenic silk protein did not depend on size. The proteins are stable in leaves and tubers, as indicated by clear-cut bands in western blot analysis (Fig. 3). Production of the same recombinant spider silk proteins in *E. coli* yielded a significant fraction of c-myc tagged smaller peptides on western blot (data not shown). Similar findings for *E. coli* expression of related silk genes have been reported by others[9]. In contrast, the ER serves as an optimal compartment for stable accumulation of spider silk proteins in transgenic plants, as already shown for other transgenic proteins[5,10]. These results indicate that different crop plants could serve as inexpensive bioreactors for the large-scale production of spider silk proteins.

Table 1. Grouping of basic cassettes (as illustrated in Fig. 1) into recombinant silk genes included in this study

Encoded protein	Arrangement of expressed gene cassettes in silk genes
SD1	G_D_B_C
SM12	G_D_C_B_C_B_B_G_D_B_C
SO1	H_B_C_B_C_G_D_C_G_D_C_B_C_B_B_G_D_B_C
SO1SO1	H_B_C_B_C_G_D_C_G_D_C_B_C_B_B_G_D_B_C_ H_B_C_B_C_G_D_C_G_D_C_B_C_B_B_G_D_B_C
FA2	G_D_C_B_C_B_B_Fibroin

Solubility and heat stability of synthetic spider silk proteins produced in transgenic plants. For use in the fabrication of biomaterials and medicine, the recombinant spider silk proteins must be extracted and solubilized using simple and reproducible methods that are amenable to scaleup. We extracted transgenic plant material with 50 mM Tris, 100 mM NaCl, 10 mM $MgSO_4$, pH 8 (soluble extracts), and in parallel with sodium dodecyl sulfate (SDS) sample buffer (see Experimental Protocol). Comparable amounts of the synthetic spider silk proteins could be extracted with both buffers (see first two lanes in Fig. 4A). Large amounts of other tobacco leaf proteins were also extracted with the first buffer, as shown by Coomassie blue staining (Fig. 4B, lane 1). Soluble extracts were then heated at 95°C for 10 min and cleared by centrifugation. Supernatants were tested by western blot for synthetic spider silk proteins. As shown in Figure 4A, lane 3, spider silk proteins of different size are still detectable in the soluble fraction in amounts comparable to the extracts before heating. In agreement with other reports[11], we have found that the recombinant silk proteins are hardly detectable in crude extracts by protein staining techniques. Only minor quantities of such leaf proteins could still be detected after the heating procedure (Fig. 4B, lane 2). According to the results of protein assays, >90% of the soluble leaf proteins were denatured and precipitated by this heat treatment. Additionally, the recombinant spider silk proteins were acid soluble whereas acidifi-

Table 2. Number of kanamycin-resistant and producer plants compared

	Tobacco		Potato	
Synthetic spidroin construct	Σ producer plants[a]	Σ kan^R plants[b]	Σ producer plants[a]	Σ kan^R plants[b]
FA2	12	30	59	156
SD1	14	37	61	151
SM12	20	55	30	52
SO1	31	55	17	54
SO1SO1	38	55	33	55

[a]Producer plants, plants accumulating >0.5% recombinant spider silk protein.
[b]kan^R plants are kanamycin resistant.

A

```
SO1          GQGGYGGLGGQGAGQGGYGGLGGQGAGQGAG-AAAAAAGGAGQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGL   78
             ||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Spidroin1    GQGGYGGLGGQGAGQGGYGGLGGQGAGQGAGAAAAAAAGGAGQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGL   79

SO1          GSQGAGRGGLGGQGAG-AAAAAAGGAGQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGLGSQGAGRGGLGGQGA  156
             ||||||||||||||| |||||||||||||||||| ||||||| ||||||||||||||||||||||||||||||||||
Spidroin1    GSQGAGRGGLGGQGAGAAAAAAAGGAGQGGYGGLGNQGAGRGGQ--GAAAAAAGGAGQGGYGGLGSQGAGRGGLGGQGA  156

SO1          GAAAAAAGGAGQGGYGGLGGQGAGQGGYGGLGSQGAGRGGLGGQGAG-AAAAAAGGAGQGGLGGQGAGQGAGAAAAAAG  234
             |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
Spidroin1    GAAAAAAGGAGQGGYGGLGGQGAGQGGYGGLGSQGAGRGGLGGQGAGAAAAAAAGGAGQGGLGGQGAGQGAGASAAAAG  235

SO1          GAGQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGLGGQGAGQGGYGGLGSQGAGRGGLGGQGAGAAAAAAGGAG  313
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||  ||||||||
Spidroin1    GAGQGGYGGLGSQGAGRGGEGAGAAAAAAGGAGQGGYGGLGGQGAGQGGYGGLGSQGAGRGGLGGQGAG--AAAAGGAG  312

SO1          QGGLGGQGAGQGAGAAAAAAGGAGQGGYGGLGSQGAGRGGLGGQGAGA-AAAAAGGAGQGGYGGLGSQGAGRGGQGAGA  391
             |||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
Spidroin1    QGGLGGQGAGQGAGAAAAAAGGAGQGGYGGLGSQGAGRGGLGGQGAGAVAAAAAGGAGQGGYGGLGSQGAGRGGQGAGA  391

SO1          AAAAAGGAGQGGYGGLGSQGAGRGGLGGQGAG-AAAAAAGGAGQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYG  468
             |||||||||| |||||| |||||||||||||| |||||||||||||||||| ||||||||||   ||||||||||||||
Spidroin1    AAAAAGGAGQRGYGGLGNQGAGRGGLGGQGAGAAAAAAAGGAGQGGYGGLGNQGAGRGGQGA--AAAAGGAGQGGYG  466

SO1          GLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGLGGQGAGQGGYGGLGSQGAGRGGLGGQGAGAAAAAAGGAGQGGLGGQ  546
             |||||||||||||||||||| ||||    |   |||||||||||||||| |||||||||||||||||||||||||||||
Spidroin1    GLGSQGAGRGGQGAGAAAAAAVGAGQE--GIRGQGAGQGGYGGLGSQGSGRGGLGGQGAGAAAAAAGGAGQGGLGGQ  541

SO1          GAGQGAGAAAAAAGGAGQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGLGSQGAGRGGLGGQGAGAAAAAAGG  624
             |||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Spidroin1    GAGQGAGAAAAAAGGVRQGGYGGLGSQGAGRGGQGAGAAAAAAGGAGQGGYGGLGGQGVGRGGLGGQGAG--AAAAGG  618
```

B

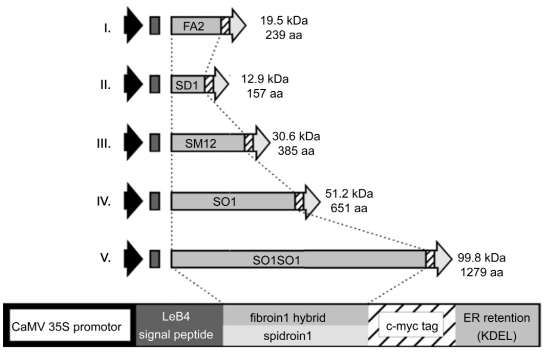

Figure 2. Synthetic spidroins and plant expression cassettes. (A) Alignment of the synthetic spidroin1 sequence SO1 with the repetitive portion of the known spidroin1 sequence. (B) Schematic representation of plant expression cassettes for the synthetic genes FA2, SD1, SM12, SO1, and SO1SO1. A synthetic spidroin or fibroin–spidroin 1 hybrid is inserted between the LeB4 signal peptide and a c-myc tag.

cation caused further precipitation of the majority of plant proteins (Fig. 4B, lane 3). Fractional ammonium sulfate precipitation was applied for further enrichment and purification of the spider silk proteins (Fig. 4A). As expected, the ammonium sulfate concentration needed to precipitate the silk proteins depends on their size. Whereas the large protein SO1SO1 (99.8 kDa) precipitated at 20% saturated ammonium sulfate, the smaller protein SD1 (12.9 kDa) required 50% saturation of the ammonium sulfate solution. By combining these methods, heat treatment, acidification, and salt precipitation, we were able to achieve a remarkable enrichment of spider silk protein (Fig. 4B, lanes 1–7). In addition, the identity of the spider silk protein and the correct cleavage of the signal peptide was verified by N-terminal sequencing. These facile methods of purification could be scaled up in future experiments.

Conclusions. Biomaterials from renewable sources could serve as alternatives for limited petrol-based basic materials. Prerequisites of their widespread application are sufficient availability as well as the existence of suitable processing technologies. Spider silk proteins could be employed as raw material for the production of fibers, foils, and overlays useful in both technology and medicine. Flexible and lightweight textiles could be developed from such materials. Wound closure systems and scaffolds for tissue engineering could be further applications.

Plant production of spider silk proteins has not been reported previously. Using plants as bioreactors should substantially increase the level of production of spider silk proteins. The plant system is superior to the bacterial expression system in that it offers an efficient and cheap production (roughly calculated as 10–50% of production cost in bacterial fermenter) that can be scaled up very easily. Simple purification procedures can produce highly enriched protein solutions. In the present study, recombinant spider silk proteins were successfully formed in tobacco leaves and potato tubers. Expression studies could be extended to other crop plants as well as to other plant organs, such as seeds, providing higher protein amounts.

Another barrier to the application of such materials is the present lack of appropriate techniques to convert the raw material into manufacturable intermediate products. The provision of enough recombinant spider silk protein, a major prerequisite for spinning experiments, should now be feasible. This will allow investigations into the mechanisms underlying the controlled liquid-to-solid phase transition of concentrated silk protein solutions, and thus open new fields of application. Large-scale production of spider silk proteins in plants will create new opportunities to utilize such materials in everyday products.

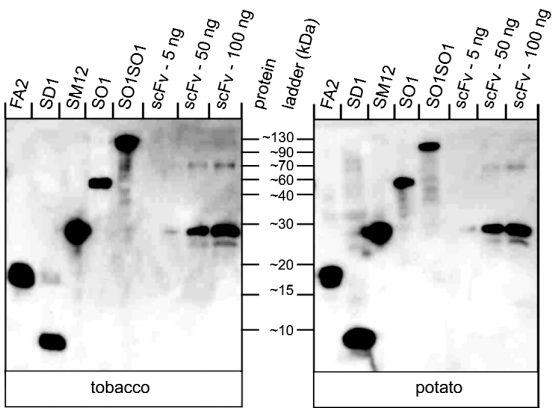

Figure 3. Detection of synthetic spider silk proteins in transgenic tobacco and potato plant organs. Plant material was ground in liquid nitrogen and extracted as described in the Experimental Protocol section. Tobacco: FA2, 5 µg leaf protein (~2% recombinant protein); SD1, 5 µg leaf protein (~1% recombinant protein); SM12, 2,5 µg leaf protein (~2% recombinant protein); SO1, 10 µg leaf protein (~1% recombinant protein); SO1SO1, 5 µg leaf protein (~2% recombinant protein). Potato: FA2, 5 µg leaf protein (~1% recombinant protein); SD1, 40 µg leaf protein (~0.5% recombinant protein); SM12, 5 µg leaf protein (~2% recombinant protein); SO1, 5 µg leaf protein (~1.5% recombinant protein); SO1SO1, 5 µg leaf protein (~2% recombinant protein).

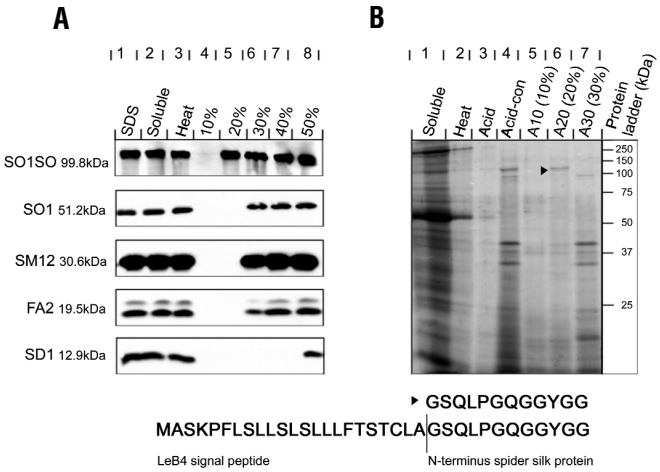

Figure 4. Solubility, heat stability, and acid solubility of plant-produced synthetic silk proteins from tobacco leaves. (A) Western blot analysis. Lane 1, 10 µg leaf protein extracted in SDS sample buffer; lane 2, 10 µg leaf protein extracted in raw extract buffer; lane 3, cleared supernatant of 10 µg protein after heat treatment. Lanes 4–8, different precipitates after heat treatment and $(NH_4)_2SO_4$ precipitation of an original 10 µg quantity of protein. (B) Purification of the recombinant spider silk protein SO1SO1 by heating, acidification, and salt fractionation from transgenic plant material. Samples were analyzed by 10% SDS–polyacrylamide gel electrophoresis under reducing conditions and stained with Coomassie blue. Lane 1, 40 µg leaf protein extracted in raw extract buffer; lane 2, cleared supernatant of original 40 µg protein after heat treatment; lane 3, cleared supernatant of original 40 µg protein after heat treatment and acidification; lane 4, 10 times concentrate of sample of lane 4; lanes 5–7, different $(NH_4)_2SO_4$ precipitates; lane 5, A10 precipitate; lane 6, A20 precipitate containing the purified recombinant protein SO1SO1; lane 7, A30 precipitate. Arrow indicates where the spider silk protein was blotted to polyvinylidene fluoride (PVDF) membrane and N-terminal sequenced. For comparison, the N-terminal sequence of the construct (signal peptide and fused spider silk protein) is shown, demonstrating correct cleavage of the signal peptide and the identity of the transgenic spider silk protein.

Experimental protocol

Assembly of the synthetic spider silk genes. Eighteen oligodeoxyribonucleotides (Fig. 1) were synthesized using phosphoramidite chemistry in a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). All oligonucleotides except No. 1, 2, and 11 were chemically phosphorylated at the end of the synthesis. The oligodeoxyribonucleotides were annealed and ligated to yield six cassettes (Fig. 1). These cassettes, which have *Nde*I- and *Hin*dIII-compatible ends, were inserted into pUC19 and cloned in *E. coli* TG1. In the protein derived from the spidroin1 cDNA, glycine-rich segments and oligoalanine stretches alternate. However, at four sites within the protein, expected oligoalanine runs are missing, yielding two types of longer glycine-rich segments. To construct these segments, small *Sty*I fragments were isolated from plasmids carrying cassette E and cassette F. These fragments were inserted in the unique *Sty*I site of the plasmid-containing cassette A, creating plasmids carrying cassettes G (from cassettes A and E) and H (from cassettes A and F), respectively. Acceptor plasmids for the uptake of silk gene cassettes were created by digestion of the plasmids carrying the cassettes B, D, G, and H with *Xma*I and *Hin*dIII. Controlled elongation of the silk genes was achieved by ligating the respective donor fragments, generated by digestion of the cassette-containing plasmids with *Ngo*MIV and *Hin*dIII, with the compatible ends of the aforementioned acceptor plasmids. Multimers of spider silk gene elements could be achieved by repeating this construction procedure (Fig. 1B). Using this methodology, four plasmid-encoded protein cassettes, each starting with a long glycine-rich segment, were obtained, which we regard as basic modules forming the silk protein. These modules, as well as smaller units, can be easily combined into proteins of different composition, enabling investigation of possible structure and function relationships. Recombinant silk proteins included in the present study are summarized in Table 1.

Construction of the chimeric spider silk genes and transformation of tobacco and potato. The synthetic spider silk genes were inserted into pRTRA7/3 (ref. 12) (after removal of ScFv coding sequence). First a *Bam*HI site was mutated by cloning a PCR product (synthesized with the 5′-primer 5′-ATAAGAATGCGGCCGCAGAACAAAAACTCATCTCAGAAGAG-GATCTGAATGGTTCCAAAGACGAACTCTAGC-3′ and 3′-primer 5′-ACTGTCATGCCATCCGTAAGATGC-3′) into *Not*I and *Sca*I sites of pRTRA7/3, leading to pRTRA7/3mut. SD1 and FA2 were excised from the plasmid p9905-SD1 and p9905-FA2 with the restriction enzymes *Sma*I and *Nae*I. Prehybridized oligonucleotides (5′-primer 5′-ATTCATTGACGCG-GATCCCAGTTA-3′ and 3′-primer 5′-TAACTGGGATCCGCGTCAAT-GAATATA-3′; 5′-primer 5′-TGATAGTTTAGCGGCCGCTTGTCC-3′ and 3′-primer 5′-GGACAAGCGGCCGCTAAACTATCAATA-3′) were ligated to the ends of the SD1 and FA2 fragments. The ligation products were amplified by PCR using the 5′-primer 5′-ATTCATTGACGCGGATCCCAGTTA-3′ and 3′-primer 5′-GGACAAGCGGCCGCTAAACTATCAATA-3′ and cloned into the *Bam*HI and *Not*I sites of pRTRA7/3mut, creating the pRTRA7/3-SD1 and pRTRA7/3-FA2. To remove a *Sma*I site for the subsequent cloning of SM12 and SO1, the pRTRA7/3mut was digested with *Cfr*9I and the residual sticky ends were filled by T4 DNA polymerase and religated. The introduction of a *Sma*I and a *Nae*I site into the resulting vector pRTRA7/3(*Bam*HI + *Sma*I-mut) was achieved by cloning a PCR product (primers: 5′-primer 5′-CGCGGATCCCAGTTACCCGGGATGTCCCGAG-GTTCCAAGACAAGG-3′ and 3′-primer 5′-ATAGTTTAGCGGC-CGCTTGTCCGCCGGCGGGTACTCTCGATGTTGCATACA-3′) into the *Bam*HI and *Not*I site of pRTRA7/3(*Bam*HI + *Sma*I-mut). SM12 and SO1 were cloned into *Sma*I and *Nae*I sites of the resulting pRTRA7/3-trunc. The plasmids were named pRTRA7/3-SM12 and pRTRA-7/3-SO1. pRTRA7/3 SO1SO1 was constructed by cloning a *Sma*I- and *Nae*I-cleaved SO1 gene into the *Nae*I site of pRTRA7/3-SO1. The pRTRA7/3-spidroin derivatives were digested with *Hin*dIII and the fragments containing the cassette CaMV 35S promoter/legumin signal/synthetic spider silk gene/c-myc tag/KDEL/CaMV 35S terminator was cloned into pBin19 (ref. 7) and pGSGLUC1 (ref. 6) vectors restricted with *Hin*dIII. Binary vectors were transferred into *A. tumefaciens* strain 2260 by electroporation. Tobacco (*Nicotiana tobaccum* cv. SNN) and potato (*Solanum tuberosum* cv. Solaria, Desi) leaf disks were transformed according to Zambrisky *et al.*[13], using *Agrobacterium*-mediated gene transfer. Transgenic plants were selected on medium containing 100 mg/L kanamycin. Regenerated plants containing the transgene were selected for further investigation after western blot analysis with anti-c-myc monoclonal antibody[5].

Analysis of the synthetic spider silk proteins in transgenic tobacco and potato leaves and potato tubers. Total soluble protein was extracted from leaves and tubers of transgenic tobacco and potato plants by homogenization in SDS sample buffer (625 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.0025 mM bromophenol blue). The homogenate was heated at 100°C for 10 min and then centrifuged for 10 min at 20°C and 16,000 *g*. Protein concentrations in the supernatants were determined by Bradford assay (Bio-Rad, Hercules, CA). Western blot analysis was carried out according to the method described by Conrad *et al*[14]. Apparent molecular weights of proteins were estimated by comparison with the Benchmark prestained protein marker (Gibco BRL, Rockville, MD). Western blots were quantified by comparing the intensity of the bands with a defined standard (purified recombinant antibodies with fused c-myc tag)[5].

Purification strategy for the synthetic spider silk proteins from tobacco and potato leaves. Soluble proteins were extracted from 10 g ground leaf material of transgenic tobacco and potato plants with raw extract buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgSO_4$). The extract was stirred for 30 min at 37°C and then cleared by centrifugation at 16,000 *g* for 15 min. The supernatant was incubated at 95°C for 10 min and then chilled on ice for 20 min. The precipitate was removed by centrifugation at 16,000 *g* for 15 min. The supernatant (95°C supernatant) was divided into five fractions and solid $(NH_4)_2SO_4$ was added to 10%, 20%, 30%, 40%, and 50% saturation. After incubation overnight at 20°C, the precipitate was removed by centrifugation at 16,000 *g* and 20°C for 30 min and redissolved in G-buffer (0.1 M sodium phosphate, 0.01 M Tris-HCl, 6 M guanidine HCl, pH 6.5). Alternatively, the heat supernatant for SO1SO1 was adjusted to pH 2 with HCl. After incubation overnight at 4°C the precipitate was removed by centrifugation at 16,000 *g* and 4°C for 30 min. Solid $(NH_4)_2SO_4$ was added to 10% saturation to the supernatant (A10 precipitate). The precipitate was removed by centrifugation and the supernatant was adjusted to 20% saturation by addition of solid $(NH_4)_2SO_4$ (A20 precipitate). The precipitate was removed by centrifugation, and the supernatant was adjusted to 30% saturation by addition of solid $(NH_4)_2SO_4$ (A30 precipitate). The precipitates were redissolved in G-buffer. N-terminal sequence was determined by automated Edman sequencing using a Procise machine (Applied Biosystems).

Acknowledgments

The authors thank K. Heinemann (Rudolstadt) for initial ideas and discussions, and I. Tiroke, I. Pfort, and C. Helmold for excellent technical assistance. This study was supported by the Bundesministerium für Landwirtschaft und Forsten represented by Fachagentur für Nachwachsende Rohstoffe (98NR050).

Received 12 October 2000; accepted 30 March 2001

1. Tirrell, D.A. Putting a new spin on spider silk. *Science* **271**, 39–40 (1996).
2. Hinman, M.B., Jones, J.A. & Lewis R.V. Synthetic spider silk: a modular fibre. *TIBTECH* **18**, 374–379 (2000).
3. Gosline, J.M., Guerette, P.A., Ortlepp, C.S. & Savage, K.N. The mechanical design of spider silks: from fibroin sequence to mechanical function. *J. Exp. Biol.* **23**, 3295–3303 (1999).
4. Hood, E.E. & Jilka, J.M. Plant-based production of xenogenic proteins. *Curr. Opin. Biotechnol.* **10**, 382–386 (2000).
5. Fiedler, U., Phillips, J., Artsaenko, O. & Conrad, U. Optimization of scFv production in transgenic plants. *Immunotechnology* **3**, 205–216 (1997).
6. Munro, S. & Pelham, H.R. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899–907 (1987).
7. Saito, K., Keneko, H., Yyamazaki, M., Yoshida, M. & Murakoshi, I. Stable transfer and expression of chimeric genes in licorice (*Glycyrrhiza uralensis*) using an Ri plasmid binary vector. *Plant Cell Rep.* **6**, 718–721 (1990).
8. Bevan, M. Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acid Res.* **12**, 8711–8721 (1984).
9. Fahnestock, S.R. & Irwin, S.L. Synthetic spider dragline silk proteins and their production in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **47**, 23–32 (1997).
10. Wandelt, C.I. *et al.* Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *Plant J.* **2**, 181–192 (1992).
11. Prince, J.T., McGrath, K.P., DiGirolamo, C.M. & Kaplan, D.L. Construction, cloning, and expression of synthetic genes encoding spider dragline silk. *Biochemistry* **34**, 10879–10885 (1995).
12. Artsaenko, O. *et al.* Expression of a single-chain Fv antibody against abscisic acid creates a wilty phenotype in transgenic tobacco. *Plant J.* **8**, 745–750 (1995).
13. Zambriski, P. *et al.* Ti-plasmid vector for introduction of DNA into plant cells without altering their normal regeneration capacity. *EMBO J.* **2**, 2143–2150 (1983).
14. Conrad, U., Fiedler, U., Artsaenko, O. & Phillips, J. Single-chain Fv antibodies expressed in plants. In *Methods in biotechnology—recombinant proteins from plants: production and isolation of clinically useful compounds.* (eds Cunningham, C. & Porter, A.) 103–127 (Humana Press, Totowa, NJ; 1997).